

# PGE2 Upregulates IL-8 Via P38 MAPK-Dependent Dual-Activation of CHOP and C/EBP- $\beta$ in Human Astrocytomas

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**Abstract:** We previously showed that in low- as well as in high-grade astrocytomas IL-8 overexpression is triggered by prostaglandin E2 (PGE2) through the upregulation of the transcription factors CCAAT/enhancer-binding protein- $\beta$  (C/EBP- $\beta$ ) and C/EBP homologous protein (CHOP). Here we investigated the signal transduction pathways and the molecular mechanisms underlying the PGE2-dependent IL-8 gene expression in astrocytomas. Low- and high-grade PGE2-treated astrocytoma cells were transfected with wild-type and mutated IL-8 promoter constructs in the presence of various signal transduction pathway inhibitors, and cotransfected with transcription factor overexpressing plasmids or small-interfering RNAs. p38MAPK, C/EBP- $\beta$ , and CHOP phosphorylation was analyzed by Western blotting. Electrophoretic mobility shift assay and chromatin immunoprecipitation evaluated the *in vitro* and *in vivo* binding of CHOP and C/EBP- $\beta$  to IL-8 promoter. The results obtained allowed us to find out the signaling pathways triggered by PGE2 and responsible for the activation of the transcription factors involved in the overproduction of IL-8 by astrocytoma. Therefore, it can be argued that the inhibition of the PGE2 downstream pathways may represent a novel therapeutic approach for the treatment of patients with astrocytoma.

**Keywords:** Astrocytoma, IL-8, PGE2, p38MAPK.

## INTRODUCTION

It is well recognized that the biology of glioma tumor cell invasiveness and malignancy is a complex mechanism in which several well orchestrated signaling pathways are involved [1]. Along with a variety of humoral and microenvironmental factors contributing to the gliomagenesis, including growth factors, cytokines, acidosis and hypoxia [2, 3], the involvement of interleukin (IL)-8 has been extensively studied. IL-8 is a powerful chemoattractant of leukocytes, that mediates the neovascularisation by recruiting and activating endothelial cells, and stimulates the proliferation and motility of several cancer cells [4]. In particular, IL-8 has been reported to have direct growth-stimulating effects on gliomas [5]. Secretion of IL-8 is tightly controlled in normal cells [6], while aberrant expression of this chemokine has been observed in a variety of cancers, including glioblastoma, and implicated in the pathogenesis of a more invasive phenotype in breast, ovarian, pancreatic and other tumors [7]. The expression of IL-8 gene has been shown to be inducible in response to several stimuli including tumor necrosis factor (TNF)- $\alpha$ , IL-1, substance P, IL-17, Fas

ligand, TNF-related apoptosis-inducing ligand (TRAIL), irradiation and hypoxia [8-14]. Previously, we showed a causal effect of PGE2 in inducing in low- and high-grade glioma cells the mRNA overexpression of IL-8 and the transcription factors CHOP and C/EBP- $\beta$  [15]. Further studies by us elucidated that PGE2 induces IL-8 activation through site specific demethylation and histone H3 hyperacetylation at the promoter region of IL-8 gene in human astrocytoma [16]. These data, indicating that the production of IL-8 is related to PGE2 synthesis, prompted us to investigate the details of the signal transduction pathways triggered by PGE2 and responsible for IL-8 overproduction. The present study characterizes the molecular activation sequence leading to PGE2-induced IL-8 release in low- and high-grade human astrocytoma cells. Here we present evidence that PGE2 activates p38 mitogen-activated protein kinase (MAPK), which in turn phosphorylates CCAAT/enhancer-binding protein- $\beta$  (C/EBP- $\beta$ ) and C/EBP homologous protein (CHOP). These two transcription factors are involved in transmitting the PGE2 signal to the IL-8 gene, leading to an increase in the levels of secreted IL-8 in astrocytoma cells.

## MATERIAL AND METHODS

### Cells and Culture Conditions

Human 1321N1 (derived from grade II astrocytoma) and A-172 (derived from grade IV glioblastoma) cell

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lines (American Type Culture Collection, Rockville, MD, U.S.A.) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Milano, Italy) supplemented with 100 units/ml penicillin (EuroClone, Milan, Italy), 100 µg/ml streptomycin (EuroClone, Milan, Italy), and 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich) in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. Human primary cultures were established from fresh, surgically excised astrocytoma and glioblastoma. Surgical specimens were washed in phosphate-buffered saline (PBS; Sigma-Aldrich, Milano, Italy) disaggregated into small pieces, and distributed in a cell culture flask coated with AmnioMax medium (Invitrogen, Carlsbad, Calif). After cell outgrowth, tumour pieces were removed, and cells were covered with AmnioMax medium. Primary cultured cells of passages 1–3 were used for the experiments.

### Measurements of IL-8 Levels from Cell Supernatants

Untreated or PGE2-treated cells were cultured in triplicate in DMEM containing 10% FBS medium for 48 h. Cell-free culture supernatants were analyzed for IL-8 protein content using a commercial enzyme immunoassay kit (Bio Source International, Nivelles, Belgium).

### Western Blotting Analysis

Total cells extracts were analyzed by immunoblotting as reported previously (18). The membranes were probed with anti-phospho-p38MAPK (Thr180 and Tyr182) (Cell Signaling Technology, Milan, Italy), anti-phospho-ATF-2 (Thr71) (Cell Signaling Technology, Milan, Italy), anti-phospho-C/EBP-β (Thr235) (Cell Signaling Technology, Milan, Italy), anti-phospho-serine (Sigma-Aldrich, Milan, Italy), anti-CHOP (Santa Cruz Biotechnology, Santa Cruz, CA), anti-c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA), anti-C/EBP-β (Santa Cruz Biotechnology, Santa Cruz, CA), anti-RelA (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-NFκB1 (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were detected by autoradiography using the SuperSignal West Pico chemiluminescent substrate system (Pierce).

### Plasmid Construction

The constructs wtIL-8, IL-8-97, IL-8-97/mC/EBPβ, IL-8-97/mNF-κB, and IL-8/AP-1 were previously described [17]. The mutant IL-8 promoter construct IL-8/ΔCHOP/m(C/EBPβ-NFκB) with contains only the AP-

1 site was generated by PCR using forward primer 5'-AAggtaccTGA CT CAGGTTT GCCCTG and reverse primer 5'-TTctcgagTTATGGAGTGCTCCGGTG. The PCR product was cloned as a KpnI/XhoI fragment into PGL2 basic vector (Promega). The IL-8 promoter sequence from -249/+15, generated by PCR using forward primer 5'-TGggtaccCACCAAATTGTGGAGCTTC and reverse primer 5'-TTctcgagTTATGGAGTGCTCCGGTG, was cloned as a KpnI/XhoI fragment into PGL2 basic vector (Promega). This construct was used as the template for mutagenesis. Constructs containing a mutated C/EBP binding site (IL-8/mC/EBPβ) and a mutated NFκB binding site (IL-8/mNFκB) in the IL-8 promoter sequence from -249/+15 were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The C/EBP binding site was mutated by substituting the nucleotide sequence <sup>90</sup>TGCAA<sup>-86</sup> with nucleotides CTAGT using mutagenic primers: 5'-CTGAGGGGATGGGCCATCAGTCTAGTATCGTGGAATTCCTC and 3'-GAGGAAATCCACGATACTAGACTGATGGCCCATCCCCTCAG. The NFκB binding site was mutated by substituting the sequence <sup>74</sup>TCCTC<sup>-71</sup> with GAAGT using mutagenic primers: 5'GTTGCAAATCGTGGAATTGAAGTTGACATAATGAAAGATG and 3'-CATCTTTTCATTATGTCAACTTCAATTCACGATTTGCAAC. The constructs were sequenced before utilization (CEQ 2000; Beckman, Fullerton, CA).

### Transient Transfections and RNA Silencing

Cells were transiently transfected with 1 µg of IL-8 constructs using FuGENE-6 Transfection Reagent (Roche Diagnostics). Twenty-four hours after transfection, the cells were treated with 10<sup>-5</sup> M PGE2 (Sigma, Milan, Italy). After an additional 6 h, the cells were harvested, and protein extracts were prepared for the luciferase activity that was normalized for β-galactosidase produced by co-transfected plasmid pnlS-LAC. In some experiments, the following inhibitors were added 1 h before PGE2 treatment: **caffeic acid phenethyl ester** (CAPE; 10 µM; Calbiochem); **SB203580** (SB; 1 µM; Calbiochem); **SP600125** (SP; 20 µM; Calbiochem); **PD98059** (PD; 20 µM; New England Biolabs Inc.); **SQ22536** (SQ; 100 µM; Sigma-Aldrich); **LY294002** (LY; 20 µM; Calbiochem); **H89** (H89; 5 µM; Calbiochem); **U0126** (U0126; 1 µM; Calbiochem); **bisindolylmaleimide I** (BIM; 5 µM; Calbiochem). Where indicated, cells transfected with wt IL-8 promoter were cotransfected with CHOP, c-Jun, C/EBP-β, RelA, or NFκB1 expression vectors (0.2 µg) or doublestranded siRNAs (0.5 µg).

## Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts, prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce), were subjected to electrophoretic mobility shift assay using the LightShift Chemiluminescent kit (Pierce). For supershift assays, nuclear extracts were incubated with 2  $\mu$ g of anti-CHOP, anti-c-Jun, anti-C/EBP- $\beta$ , anti-RelA, or anti-NFKB1 antibodies (Santa Cruz Biotechnology) at room temperature (for 20 min) prior to probe addition. The probes used for EMSAs were as follows: **probe A**, 5'-GATAAGGAACAAATAGGAAGTGTGATG GTCAAGTTTGCCTG-3' (containing only the CHOP active site and a mutation of the c-Jun site); **probe B**, 5'-GATAAGGAACAAATAGGAAGCAGTATGACTCAG GTTTGCCCTG-3' (containing only the c-Jun active site and a mutation of the CHOP site); **probe C**, 5'-TCAGTTGCAAATCGTGGAAATTCCTCTCTGACATAA TGAAAAGAT-3' (containing the C/EBP- $\beta$  and the NF- $\kappa$ B active sites). Bound complexes were separated on 8% polyacrylamide gels, blotted onto membrane, and visualized by autoradiography (Hyperfilm, Fuji, Naples, Italy).

## Chromatin Immunoprecipitation (ChIP) Assay

The ChIP enzymatic assay (Active Motif) was carried out, and the sheared chromatin samples were used for immunoprecipitation with 2  $\mu$ g of anti-CHOP, anti-c-Jun, anti-RelA, anti-C/EBP- $\beta$ , or anti-NFKB1 antibodies (Santa Cruz Biotechnology) overnight at 4°C. Immunocomplexes were subjected to cross-link reversal, extracted, and precipitated. The eluted DNA and the aliquots of chromatin prior to immunoprecipitation (input) were amplified by PCR. Two primers were used to detect the DNA segment located at -168/+11 of IL-8 promoter (forward, 5'-CGTCATACT CCGTATTTGATAAGGA-3'; reverse, 5'-TGCCTTATG GAGTGCTCCGGTG-3'). The PCR conditions were as follows: 95 °C for 4 min and 38 cycles of 94°C for 50 s, 65°C for 50 s, and 72°C for 1 min. PCR products were separated by 2% agarose gel containing ethidium bromide.

## RESULTS

### Inhibition of p38MAPK Blocks PGE2-Induced IL-8 Activation in Astrocytoma Cells

The effects of various inhibitors of specific signal transduction pathways involved in IL-8 upregulation were evaluated in untreated or PGE2-treated grade II (Figure 1A) and IV (Figure 1B) astrocytic glioma cell

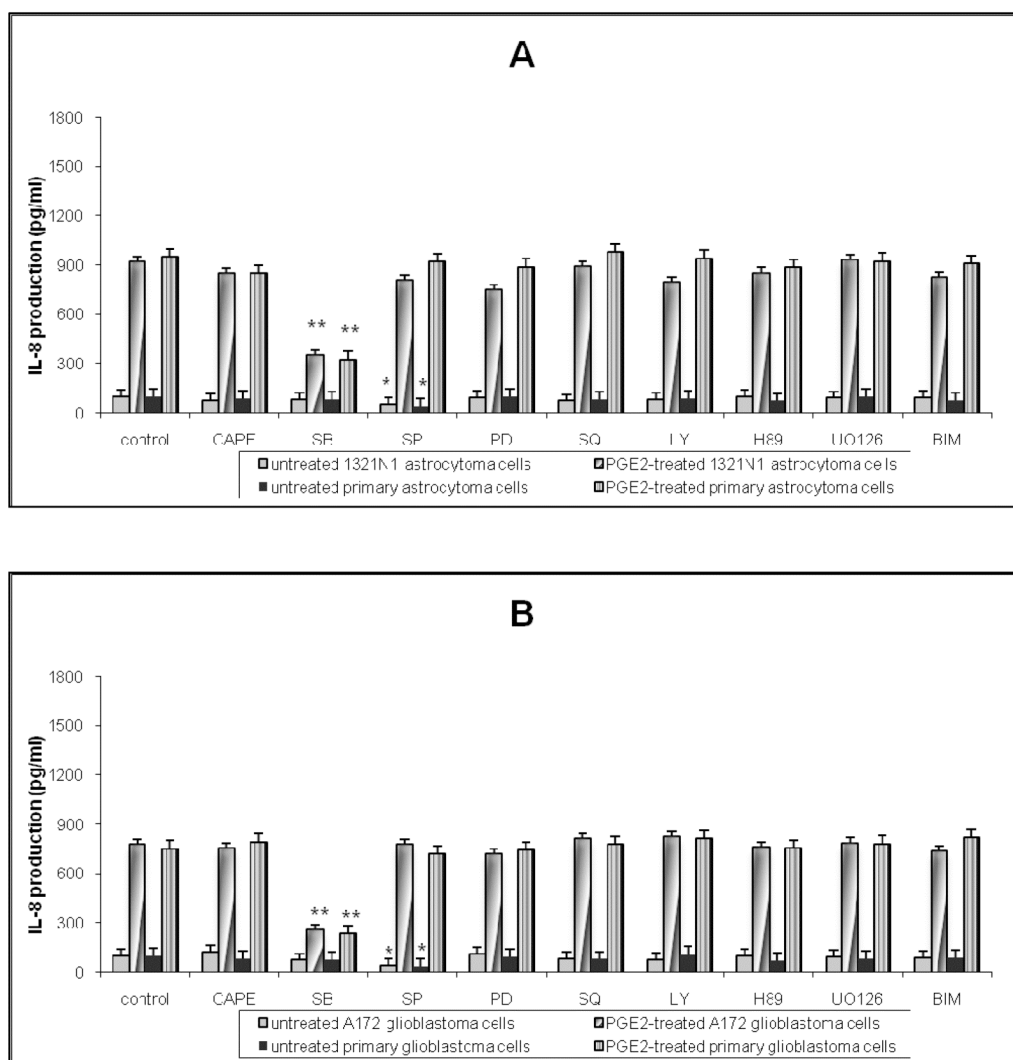
lines and primary cells. SP600125, a JNK inhibitor, was able to reduce the basal level of IL-8 protein synthesis, whereas SB203580, a p38MAPK inhibitor, significantly inhibited the PGE2-induced IL-8 production. PGE2 phosphorylates CHOP and C/EBP- $\beta$  through p38MAPK activation (Figure 2A) shows that PGE2 induced the phosphorylation of p38MAPK starting up at 30 min after treatment and persisting up to 240 min, beyond that of its substrate ATF-2 (Figure 2B). SB203580 prevented the phosphorylation either of p38MAPK or ATF-2 induced by PGE2. Similar results were observed in the A172 glioblastoma cell line and primary cell cultures of low- and high-grade astrocytoma (see Supplemental Figure 1). Then, we questioned whether CHOP and C/EBP- $\beta$  might be effector proteins of p38MAPK [19, 20]. Immunoprecipitation with anti-CHOP antibody and western blots with anti-phosphoserine antibody revealed that PGE2 induced the phosphorylation of this transcription factor (Figure 3A). The phosphorylation of C/EBP- $\beta$  following PGE2 challenge was shown through western blots with anti-phospho C/EBP- $\beta$  antibody (Figure 3B). SB203580 markedly inhibited the PGE2-induced phosphorylation of both transcription factors. Similar results were obtained with the other astrocytic tumoral cells (see Supplemental Figure 2).

### Analysis of IL-8 Promoter Regions Responsive to PGE2

Wt and mutated constructs of IL-8 (Figure 4A) were transfected in untreated and PGE2-treated 1321N1 cells, and the luciferase reporter activities were measured. The results show that wt IL-8, IL-8-97, IL-8-97/mNF- $\kappa$ B, IL-8/mC/EBP, IL-8/mNF $\kappa$ B and IL-8/AP-1 constructs were activated by PGE2. The wt IL-8 and the IL-8/mNF $\kappa$ B constructs were considerably more activated (Figure 4B). When mutations were present in both the binding sites of C/EBP- $\beta$  and CHOP, PGE2-mediated activation of IL-8 promoter was abolished (Figure 4B and Supplemental Figure 3).

### Activation of IL-8 Promoter by PGE2 is Dependent on CHOP and C/EBP- $\beta$ Overexpression

Afterwards, untreated or PGE2-treated 1321N1 cells were co-transfected with wt IL-8 promoter reporter and vectors overexpressing CHOP, c-Jun, C/EBP- $\beta$ , RelA, and NF $\kappa$ B1. As shown in Figure 5A, c-Jun overexpression increased the basal levels of IL-8 promoter, whereas ectopic C/EBP- $\beta$  or CHOP overexpression significantly up-regulated the activation of IL-8 promoter upon PGE2 stimulus. The combined



**Figure 1:** PGE2 induces IL-8 production through p38MAPK in astrocytoma cells. Grade II (section **A**) and grade IV (section **B**) astrocytoma cells were pretreated for 1 h with the following inhibitors: the NF- $\kappa$ B inhibitor caffeic acid phenethyl ester (CAPE; 10  $\mu$ M), the p38MAPK inhibitor SB203580 (SB; 1  $\mu$ M), the JNK inhibitor SP600125 (SP; 20  $\mu$ M), the MEK1 inhibitor PD98059 (PD; 20  $\mu$ M), the adenylyl cyclase inhibitor SQ22536 (SQ; 100  $\mu$ M); the PI3K inhibitor LY294002 (LY; 20  $\mu$ M); the PKA inhibitor H89 (H89; 5  $\mu$ M); the MEK1/2 inhibitor U0126 (U0126; 1  $\mu$ M); the PKC inhibitor bisindolylmaleimide I (BIM; 5  $\mu$ M). Subsequently, cells were treated with  $10^{-5}$ M PGE2. After 24 h of incubation, IL-8 protein was measured in cell supernatants by enzyme linked immunosorbent assay (ELISA). Data are depicted as the mean  $\pm$  S.D. (error bars) of five independent experiments.

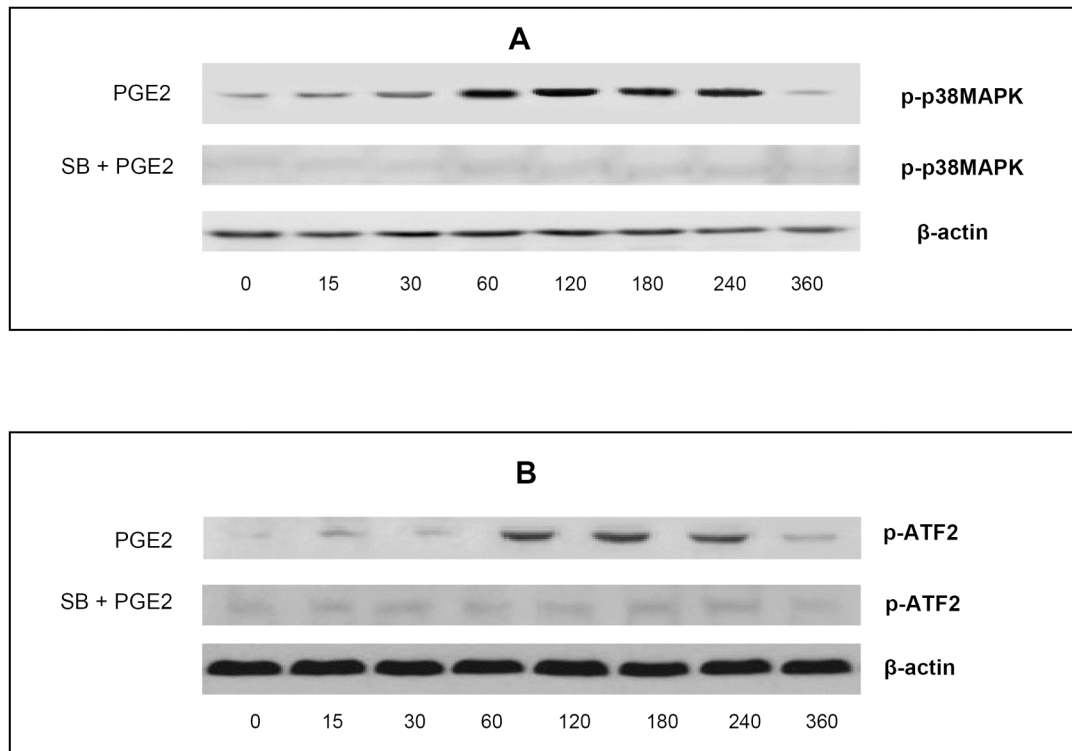
\*\* $p < 0.01$ , SB+PGE2-treated versus PGE2-treated cells (control); \*  $p < 0.05$ , SP-treated versus untreated cells (control), based on Student's two-tailed t test.

overexpression of C/EBP- $\beta$  and CHOP activated IL-8 promoter with a greater extent. RelA and NF $\kappa$ B1 overexpression did not significantly modify either the basal or the PGE2-induced IL-8 activation. The PGE2-induced amounts of IL-8 mRNA (Figure 5B) and protein (Figure 5C and Supplemental Figure 4) showed the same trend of activation. Using specific synthetic siRNAs, we achieved 90, 85, 95, 80, and 90% knockdown of CHOP, c-Jun, C/EBP- $\beta$ , RelA and NF $\kappa$ B1, respectively (Figure 6A). The single silencing of CHOP, C/EBP- $\beta$ , c-Jun, RelA, and NF $\kappa$ B1 did not significantly affect PGE2-induced IL-8 promoter activation (Figure 6B), mRNA synthesis (Figure 6C),

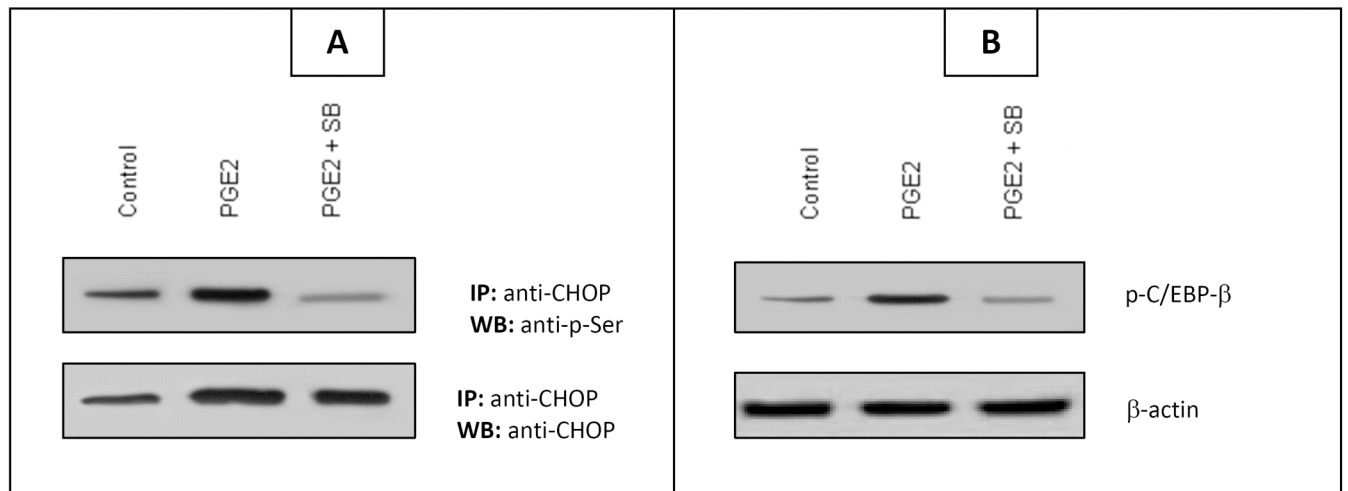
and protein production (Figure 6D), whereas inhibition of the expression of both CHOP and C/EBP- $\beta$  completely suppressed IL-8 response to PGE2. c-Jun down-regulation significantly reduced the basal values of IL-8 gene expression as well as mRNA and protein levels. Supplemental Figure 5 shows a similar pattern in A172 and primary astrocytoma cells.

#### ***In Vitro DNA-Protein Complex Formation in Untreated and PGE2-Treated 1321N1 Cells***

Nuclear extracts from untreated or PGE2-treated 1321N1 cells were analyzed by electrophoretic mobility shift assay and supershift assays. Three double-



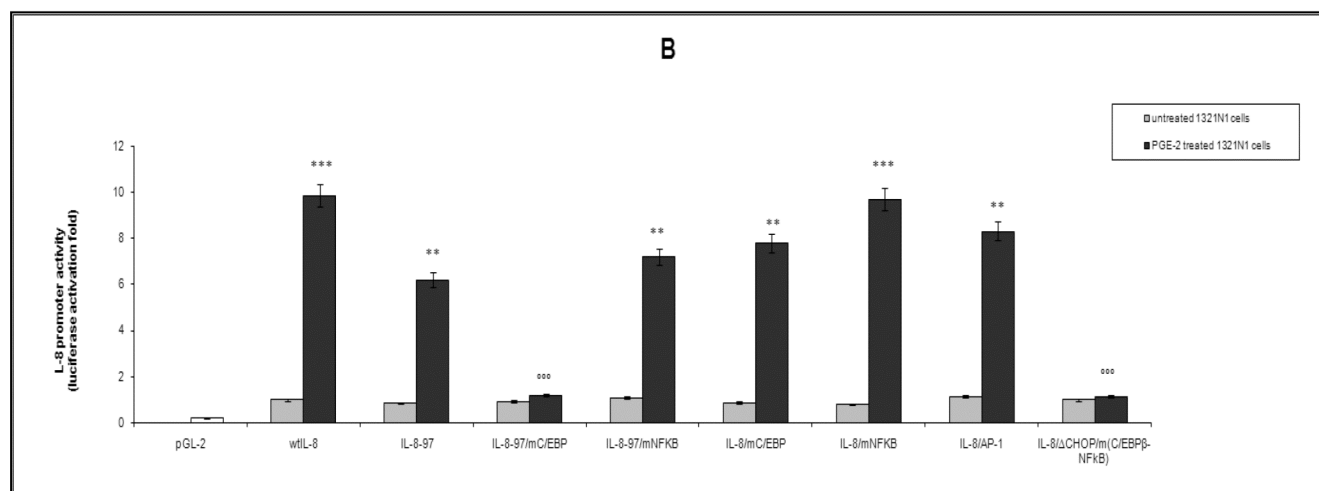
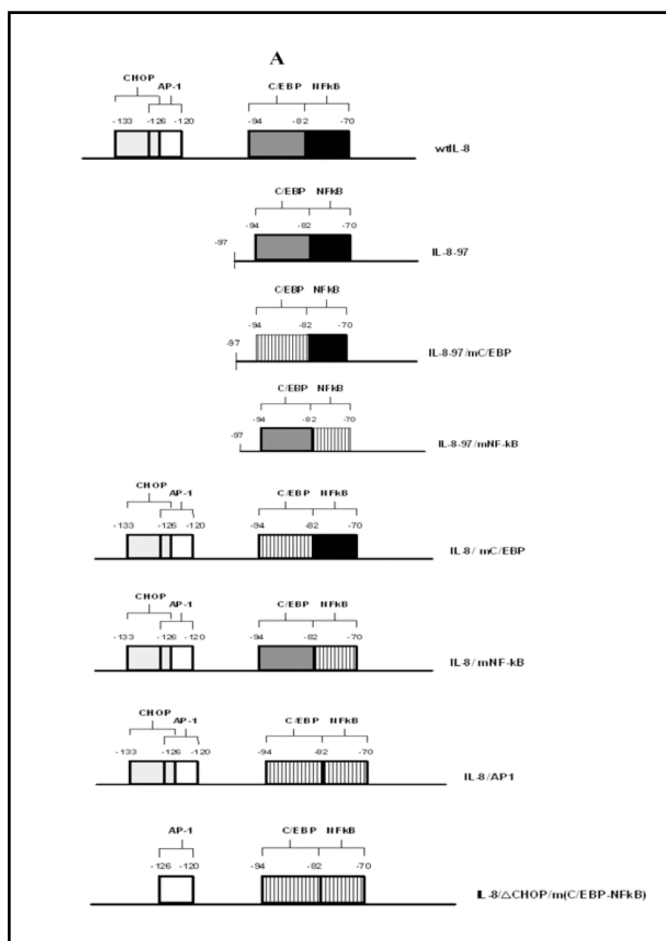
**Figure 2:** PGE2 induces the phosphorylation of p38MAPK in astrocytoma cells. 1321N1 cells were treated with  $10^{-5}$ M PGE2 for the indicated times. Where indicated, cells were pre-treated for 1 h with the specific inhibitor of p38MAPK, SB203580 (SB) (1  $\mu$ M). Total extracts were loaded onto gels (60  $\mu$ g of protein/lane) and subjected to SDS-PAGE and immunoblotting using polyclonal antibodies that recognize the phosphorylated forms of p38MAPK (**A**) and ATF-2 (**B**).



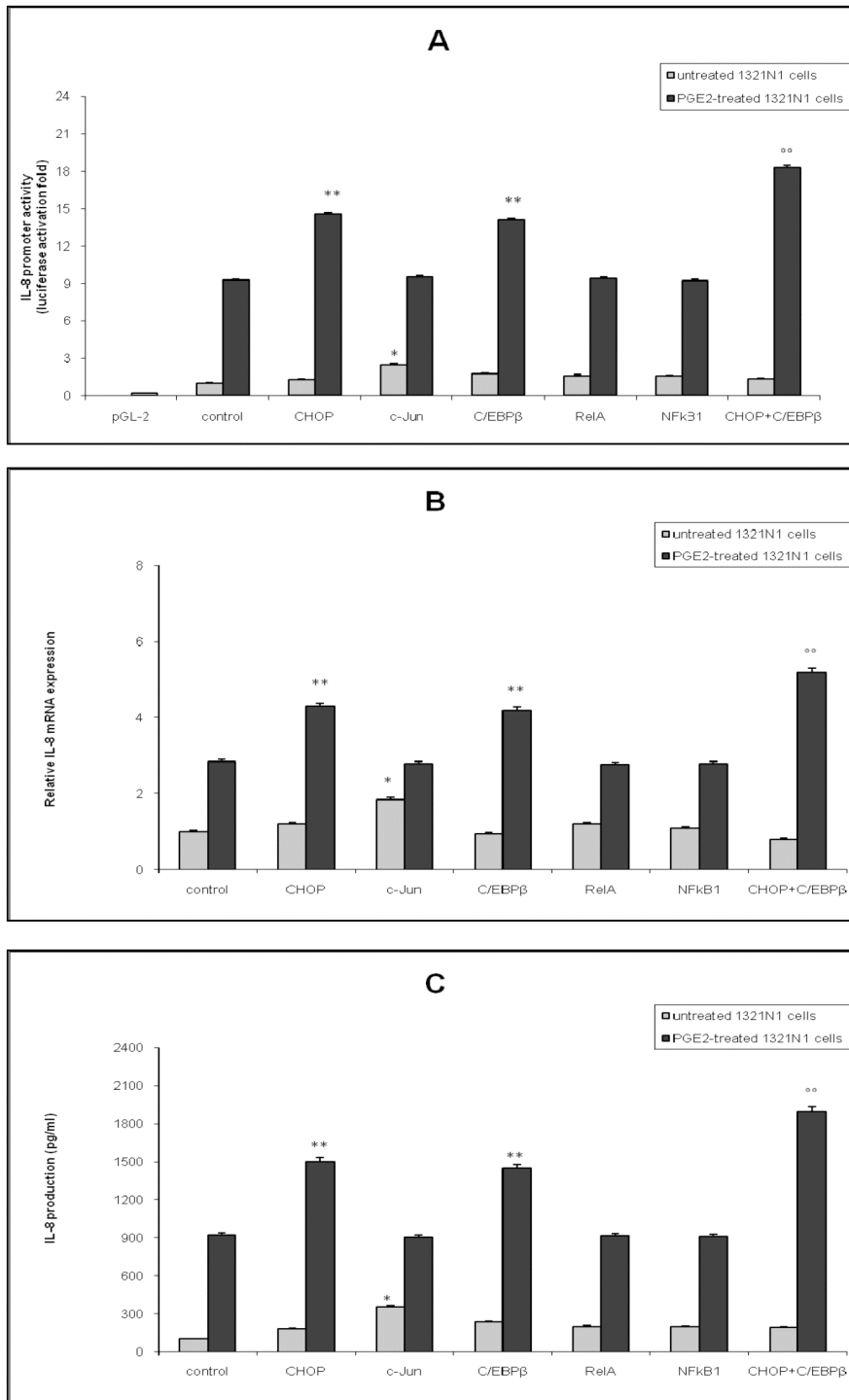
**Figure 3:** PGE2 phosphorylates CHOP and C/EBP- $\beta$  through p38MAPK in astrocytoma cells. 1321N1 astrocytoma cells were treated with  $10^{-5}$ M PGE2 for 120 min and, where indicated, were pretreated for 1 h with the specific inhibitor of p38MAPK, SB203580 (SB) (1  $\mu$ M). (**A**) CHOP was immunoprecipitated with anti-CHOP antibody. Western blotting was performed using anti-phosphoserine or anti-CHOP antibodies. (**B**) Total extracts were loaded onto gels (60  $\mu$ g of protein/lane) and subjected to SDS-PAGE and immunoblotting using polyclonal antibodies that recognize phospho-C/EBP- $\beta$ .  $\beta$ -actin was used as internal control.

stranded oligonucleotides were used: i) probe A, solely encompassing CHOP binding site; ii) probe B, solely encompassing c-Jun motif; iii) probe C, comprising the adjacent C/EBP- $\beta$ , RelA and NFkB1 consensus

sequences. A stronger nucleoprotein complex was generated with lysates from PGE2-treated cells after incubation with the probe A (Figure 7A, lane c) as compared with lysates from untreated cells (Figure 7A,

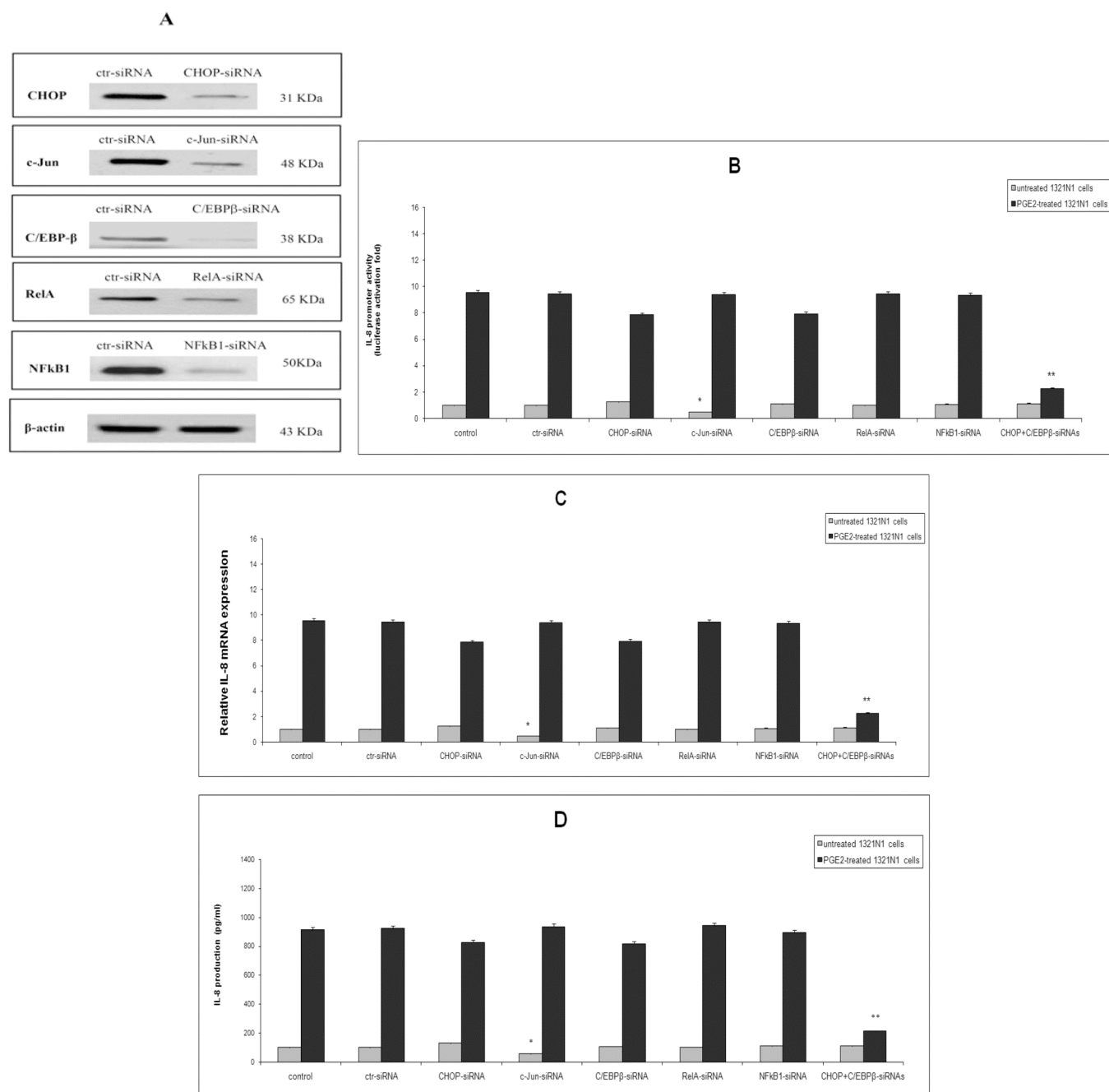


**Figure 4:** PGE2-induced *IL-8* promoter activation through CHOP and C/EBP-β binding sites. **(A)** The *IL-8* promoter mutants used are shown: *black, dark grey, light gray* and *white* boxes, wild-type sites; *striped* boxes, mutated sites. **(B)** 1321N1 astrocytoma cells were transfected with the empty pGL-2 vector, 1 μg wt *IL-8* promoter, *IL-8-97*, *IL-8-97/mC/EBP*, *IL-8-97/mNFkB*, *IL-8/mC/EBP*, *IL-8/mNFkB*, *IL-8/AP-1*, and *IL-8/ΔCHOP/m(C/EBP-NFkB)* promoters and subsequently treated with 10<sup>-5</sup>M PGE2 for 6 h. The graph shows the luciferase activity in cells treated with PGE2 (*black boxes*) in comparison with untreated cells (*gray boxes*). Data are expressed as means ± S.D. of results in five independent experiments. β-galactosidase levels were determined for transfection efficiency. Significant \*\**p* < 0.01 and \*\*\**p* < 0.001, PGE2-treated versus untreated cells, °°°*p* < 0.001 PGE2-treated cells transfected with mutated constructs versus PGE2-treated cells transfected with wtIL-8 plasmid, based on Student's two-tailed *t* test.



**Figure 5:** Effects of the transcription factor overexpression on IL-8 promoter activation following PGE2 treatment. 1321N1 astrocytoma cells were transiently cotransfected with 1  $\mu$ g wt IL-8 construct (control), and the vectors overexpressing CHOP, c-Jun, C/EBP- $\beta$ , RelA, and NF $\kappa$ B1 (0.2  $\mu$ g), or a combination of CHOP and C/EBP- $\beta$  as indicated. After transfection, cells were treated with  $10^{-5}$  M PGE2 for 6 h. **(A)** The IL-8 promoter expression was measured as -fold induction.  $\beta$ -galactosidase levels were determined for transfection efficiency. A representative experiment of five independent experiments is shown. **(B)** The levels of IL-8 mRNA were determined by quantitative Real Time-PCR. The mRNA expression was quantified as its ratio to  $\beta$ -actin. Data are the mean  $\pm$  SD of three independent experiments. **(C)** IL-8 protein was measured in cell supernatants by enzyme linked immunosorbent assay. Data are depicted as the mean  $\pm$  S.D. of five independent experiments.

\* $p < 0.05$ , wtIL-8-transfected cells overexpressing c-Jun versus wtIL-8-transfected cells (control); \*\*  $p < 0.01$ , wtIL-8-transfected and PGE2-treated cells overexpressing CHOP or C/EBP- $\beta$  versus wtIL-8-transfected and PGE2-treated cells (control);  $^{\circ\circ}$   $p < 0.01$ , wtIL-8-transfected and PGE2-treated cells overexpressing both CHOP and C/EBP- $\beta$  versus wtIL-8-transfected and PGE2-treated cells overexpressing CHOP or C/EBP- $\beta$  alone. Error bars correspond to the S.D.

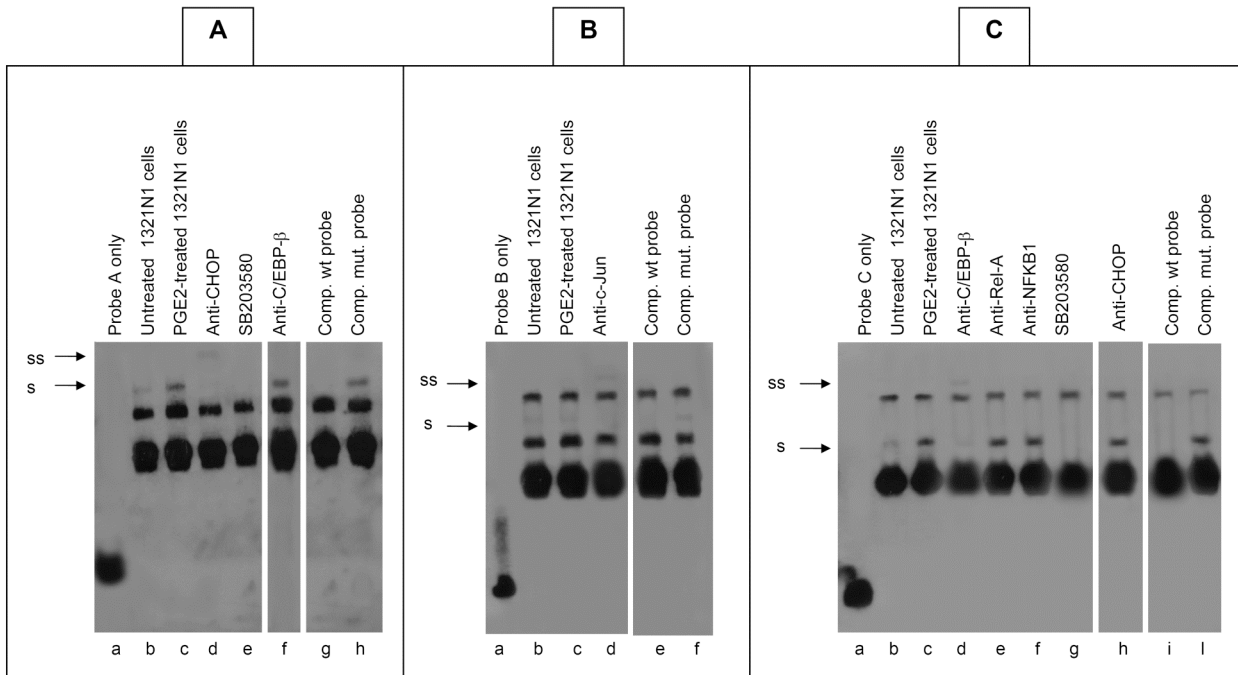


**Figure 6:** Double silencing of CHOP and C/EBP- $\beta$  suppresses the PGE2-induced IL-8 transcriptional activation. **(A)** Representative immunoblots of CHOP, c-Jun, C/EBP- $\beta$ , RelA, and NF $\kappa$ B1 proteins in 1321N1 astrocytoma cells transfected with negative control (ctr) and specific siRNAs and treated with  $10^{-5}$  M PGE2. **(B-D)** 1321N1 astrocytoma cells were transfected with 1  $\mu$ g wtIL-8 promoter construct (control) and, where indicated, cotransfected with siRNAs targeting CHOP, c-Jun, C/EBP- $\beta$ , RelA, and NF $\kappa$ B1, a combination of CHOP and C/EBP- $\beta$ , or an unspecific siRNA. Forty-eight hours after transfection, cells were treated with  $10^{-5}$  M PGE2. **(B)** The IL-8 promoter expression was measured as -fold induction.  $\beta$ -galactosidase levels were determined for transfection efficiency. A representative experiment of five independent experiments is shown. **(C)** The levels of IL-8 mRNA were determined by quantitative Real Time-PCR. The mRNA expression was quantified as its ratio to  $\beta$ -actin. Data are the mean  $\pm$  SD of three independent experiments. **(D)** IL-8 protein was measured in cell supernatants by enzyme linked immunosorbent assay. Data are depicted as the mean  $\pm$  S.D. (error bars) of five independent experiments.

\* $p < 0.05$ , wtIL-8- and c-Jun siRNA-cotransfected cells versus wtIL-8-transfected cells (control); \*\* $p < 0.01$ , wtIL-8 transfected- and double CHOP and C/EBP $\beta$  siRNA-cotransfected cells versus wtIL-8 transfected-cells (control), based on two-tailed Student's  $t$  test.

lane b). The antibody against CHOP (Figure 7A, lane d), but not that against C/EBP- $\beta$  (Figure 7A, lane f),

supershifted the nucleoprotein complex. The PGE2-induced DNA-CHOP complex was almost completely



**Figure 7:** *In vitro* binding of the transcription factors to IL-8 promoter regions. **(A)** Biotin-labeled oligonucleotide probe A was incubated with nuclear extracts from untreated (*lane b*), PGE2-stimulated (*lane c*), and SB203580-pretreated and PGE2-stimulated (*lane e*) 1321N1 astrocytoma cells. In supershift analysis, 2  $\mu$ g antibody against CHOP (*lane d*) and against C/EBP- $\beta$  (*lane f*) was incubated at room temperature (for 20 min) with the nuclear extracts prior to probe addition. For competition assays a 100-fold excess of unlabeled wt or mutant probes was incubated with nuclear extracts at room temperature for 10 min before the addition of labeled probe (*lanes g* and *h*). **(B)** Biotin-labeled oligonucleotide probe B was incubated with nuclear extracts from untreated (*lane b*) and PGE2-treated (*lane c*) 1321N1 astrocytoma cells. In supershift analysis, the antibody against c-Jun (2  $\mu$ g) (*lane d*) was incubated at room temperature (for 20 min) with the nuclear extracts prior to probe addition. Competition assays were performed as in **(A)** (*lanes e* and *f*). **(C)** Biotin-labeled oligonucleotide probe C was incubated with nuclear extracts from untreated (*lane b*), PGE2-treated (*lane c*), and SB203580- and PGE2-treated (*lane g*) 1321N1 cells. In supershift analyses, the antibodies against C/EBP- $\beta$  (*lane d*), RelA (*lane e*), NFkB1 (*lane f*) and CHOP (*lane h*) (2  $\mu$ g) were incubated at room temperature (for 20 min) with the nuclear extracts prior to probe addition. Competition assays were performed as in **(A)** (*lanes i* and *l*). Arrows indicate the locations of shifted (*s*) and supershifted (*ss*) DNA. Shift assays were repeated five times with similar results.

abolished by SB203580 (Figure 7A, lane e). A weak DNA-protein complex was formed after incubation with probe B independently from PGE2 treatment (Figure 7B, lanes b and c). This complex was super-shifted by the anti-c-Jun antibody (Figure 7B, lane d). Finally, a very weak complex was formed when probe C was added to the nuclear extracts obtained from untreated 1321N1 cells (Figure 7C, lane b), whereas a strong DNA-protein complex was formed after PGE2 treatment (Figure 7C, lane c). This complex was super-shifted by the anti-C/EBP- $\beta$  antibody (Figure 7C, lane d), but not by anti-RelA, anti-NFKB1, and anti-CHOP antibodies (Figure 7C, lanes e, f, and h, respectively). The binding was suppressed by the treatment with SB203580 (Figure 7C, lane g).

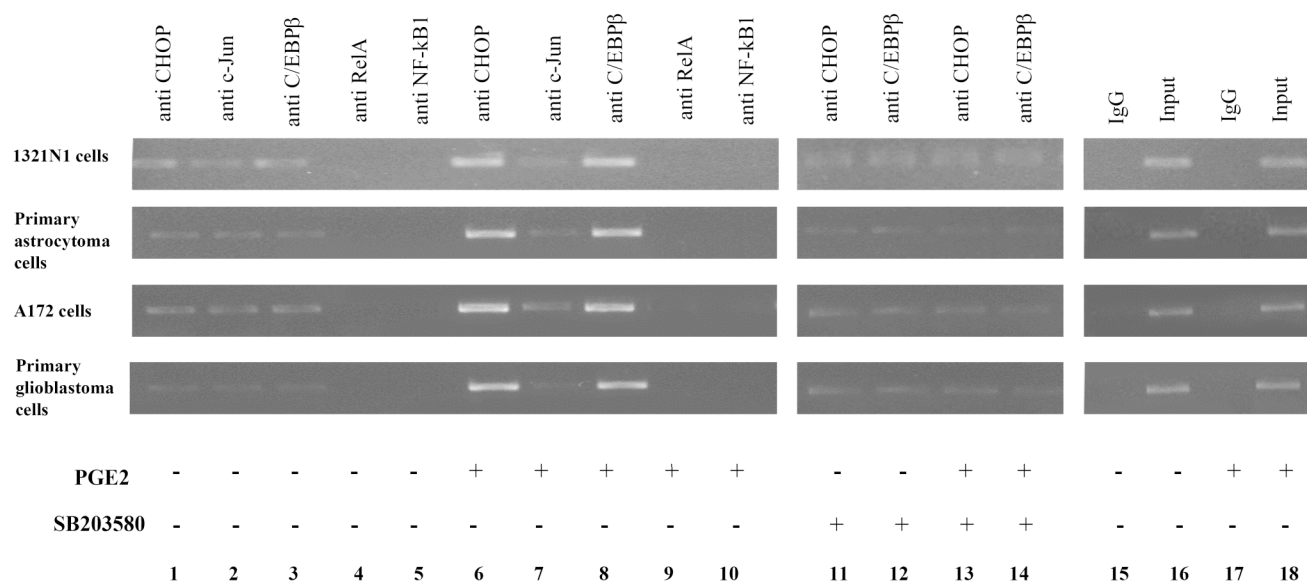
#### **PGE2-Induced *In Vivo* Binding of Transcription Factors to IL-8 Promoter**

Finally, ChIP assay shows that in PGE2-treated cells CHOP and C/EBP- $\beta$  markedly interacted on IL-8

promoter (Figure 8, lanes 6 and 8, respectively), whereas in untreated cells the binding was very weak (Figure 8, lanes 1 and 3). Inhibiting p38MAPK prevented the PGE2-induced recruitment either of CHOP or C/EBP- $\beta$  to the IL-8 promoter (Figure 8, lanes 13, 14.). The weak binding of c-Jun to the IL-8 promoter in untreated cells (Figure 8, lane 2) was not modified by PGE2 (Figure 8, lane 7). No binding of RelA and NFkB1 was detected either in untreated (Figure 8, lanes 4 and 5, respectively) or PGE2-treated cells (Figure 8, lanes 9 and 10, respectively).

#### **DISCUSSION**

In gliomas, it has been suggested that IL-8 increases tumorigenicity *via* its motogenic, mitogenic, and angiogenic properties [21]. Despite the interest in regulating IL-8 expression [22], the factors and the molecular mechanisms underlying IL-8 production by glioma remain still somewhat elusive. Previously, we



**Figure 8:** PGE2-induced *in vivo* binding of CHOP and C/EBP- $\beta$  to the *IL-8* promoter Aliquots of ChIPs immunoprecipitated with CHOP, c-Jun, C/EBP- $\beta$ , ReIA, and NFkB1 from untreated, PGE2-treated and SB203580-treated glioma cells were subjected to PCR analysis using primer pairs spanning the *IL-8* promoter from -168 to +11 bp. Lanes 16 and 18 are "input" lanes where no immunoprecipitation was performed prior to PCR (positive control). Lanes 15 and 17 contained IgG antibody for immunoprecipitation (negative control). A representative agarose gel of five independent experiments is shown for each cell line.

have highlighted in astrocytic glioma a cause/effect relationship between PGE2 and IL-8 induction in astrocytoma [15]. Here, we analyzed the molecular events leading to IL-8 production upon PGE2 stimulus and highlighted the decisive involvement of p38MAPK activation (Figures 1 and 2) that led to the phosphorylation of C/EBP- $\beta$  and CHOP (Figure 3) and the subsequent binding to their consensus sites on *IL-8* promoter (Figures 7 and 8). These data have proved that the activation of these transcription factors is a crucial step in the PGE2-induced *IL-8* gene overexpression. The importance of p38MAPK in the regulation of *IL-8* was extensively demonstrated in different cell types [23, 24], but up to date in astrocytoma cells the link between p38MAPK activation and *IL-8* gene upregulation was shown only once [25]. Moreover, although it is well known that PGE2 induces p38MAPK activation [26-29], no data were available about the role of p38MAPK as an intracellular signal-transducing molecule in the PGE2-induced *IL-8* overexpression in astrocytoma. It is interesting to note that PGE2-induced phosphorylation and activation of p38MAPK is sufficient alone to increase the expression of *IL-8* in astrocytomas, differently to what reported in leukemia T cells in which the production of *IL-8* upon PGE2 treatment only partially was mediated by p38MAPK, being PKC more determinant [29]. Here we showed that the events linking PGE2 and *IL-8* synthesis in astrocytomas point towards CHOP and

C/EBP- $\beta$  as downstream signal transducers activated upon p38MAPK phosphorylation. Any involvement of c-Jun and NF-kB was excluded. The results also indicate that c-Jun contributes to the basal *IL-8* promoter activity, as its overexpression (Figure 5) and its downregulation (Figure 6) affected the constitutive levels of *IL-8*, whereas PGE2-induced *IL-8* release stayed unchanged. EMSA and ChIP assays definitively showed that c-Jun was constitutively present on its consensus site and that the binding was not modified in the presence of PGE2 (Figures 7 and 8). These findings are in agreement with several studies [17, 20, 29, 30-34] showing that in other cell systems and under different proinflammatory stimuli, including PGE2, *IL-8* secretion was not mediated by the JNK pathway. The transcription factor NF-kB appeared to be not involved in *IL-8* production by astrocytoma, despite previous studies showed that in other cell systems it is essential for the TNF- $\alpha$ - and *Pseudomonas aeruginosa*-induced *IL-8* production [6, 29, 35, 36]. The data reported here clearly suggest that both CHOP and C/EBP- $\beta$  are potent mediators of the PGE2-induced *IL-8* activation in astrocytoma, as their phosphorylated forms tightly bound to *IL-8* promoter and activated the gene transcription. The present research corroborates the positive role for CHOP in the induction of *IL-8*, yet shown by us in Jurkat cells [17, 29] and by others in other cell systems [37]. This is of interest, considering that originally CHOP was studied as an inhibitory factor

blocking the binding of C/EBPs to DNA [38-39], whereas here we showed that in astrocytoma CHOP cooperates with C/EBP- $\beta$  in inducing IL-8 transcription under PGE2 stimulus. CHOP interacted directly with IL-8 promoter (Figures 7 and 8) through the binding to the same consensus site GTGTGATG previously identified by us on IL-8 promoter of T cells [17]. The binding was functional in activating IL-8 expression and was suppressed by SB203580. Although C/EBP- $\beta$  was also reported to induce IL-8 expression [40, 41] and hypothesized to play a role in tumor progression of gliomas through the upregulation of IL-8 [15, 42-44], the simultaneous activation and involvement of CHOP and C/EBP- $\beta$  as transcription factors is a rare event. To this regard, it is interesting to note that in PGE2-treated astrocytoma cells p38MAPK phosphorylated both CHOP and C/EBP- $\beta$  leading to their binding to IL-8 promoter (Figures 3, 7 and 8). Both CHOP and C/EBP- $\beta$  were able alone to induce IL-8 activation (Figure 5) and only when both were missing (Figure 6) or not active (Figure 1) the IL-8 response resulted impaired.

The determinant role of C/EBP- $\beta$  and CHOP was confirmed by transfection experiments with mutated constructs of IL-8 promoter lacking both C/EBP- $\beta$  and CHOP elements (Figure 4). On the basis of these data and our previous results [15], showing that both CHOP and C/EBP- $\beta$  were overexpressed after PGE-stimulation, it may be conceivable that in astrocytoma cells the PGE-induced IL-8 activation is mediated either by the phosphorylation or by the overexpression of these transcription factors. This is particularly relevant considering the alteration of IL-8 activation/disregulation in tumors and may have therapeutic implications. Inhibition of C/EBP- $\beta$  is actually proposed in the treatment of patients affected by astrocytomas [42], owing to its ability to bind and enhance IL-8 promoter expression in several activated astrocytoma cells [45]. The findings reported here, showing that under PGE2 stimulus IL-8 gene is activated not only by C/EBP- $\beta$ , but also by CHOP alone, raise the question that additional therapeutic strategies might be taken into account, being CHOP one of the candidate alternative pathways [15]. Indeed, the majority of published works base their studies on the idea that the minimal IL-8 promoter contains response elements for NF- $\kappa$ B, AP-1, and C/EBP- $\beta$  [6, 46] neglecting CHOP-RE which is instead of particular interest in neoplastic diseases characterized by high levels of PGE2 [15, 17].

In conclusion, this study revealed that both CHOP and C/EBP- $\beta$  activated by p38MAPK, independently on each other, up-regulate the expression of IL-8 in

astrocytoma cells upon PGE2 treatment, even though they are more effective together than alone. The elucidation of these mechanisms may lead to the identification of novel strategies to fight brain cancer as astrocytoma targeting the inhibition of PGE2 synthesis or the downstream pathways that lead to the production of IL-8.

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## CONFLICT OF INTEREST

The authors report no conflicts of interest related to this study.

## SUPPLEMENTAL MATERIALS

The supplemental figures can be downloaded from the journal website along with the article.

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