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Dimethylcelecoxib inhibits mPGES-1 promoter activity by influencing EGR1 and NF-κB

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Abstract

DMC (dimethylcelecoxib = {4-[5-(2,5-dimethylphenyl)-3(trifluoromethyl)- 1H-pyrazol-1 yl]benzenesulfonamide}) is a close derivative of celecoxib, without cyclooxygenase inhibiting properties up to very high concentrations. Nevertheless, after stimulation of various human cell lines with IL-1β/TNFα and simultaneous treatment with DMC PGE_2 synthesis is inhibited [1]. Here we investigated the effect of DMC on mPGES-1 promoter activity, using a reporter-gene assay. Our data demonstrate that DMC inhibits mPGES-1 promoter activity by blocking nuclear EGR1 expression and repressing NF-κB transcriptional activity. Other putative transcription factors, known to regulate mPGES-1 expression, such as SP1 or CREB are not affected by DMC. Over-expression of EGR1 completely prevents the inhibitory effect of DMC on mPGES-1 promoter activity, indicating that the repressing effect of DMC on mPGES-1 expression is mainly dependent on blocking EGR1 expression. mPGES-1, EGR1 and NF-κB are important proteins involved in many pathological conditions such as inflammation and cancer. Therefore, DMC seems to be a promising substance to treat inflammatory and carcinogenic processes, although it does not inhibit cyclooxygenases.

1. Introduction

Prostaglandin E_2 (PGE_2) plays an important role in physiological and pathophysiological processes. It is produced along the enzymatic pathway consisting of phospholipases, cyclooxygenases and PGE synthases. So far three PGE synthases are known: the cytosolic PGE synthase (cPGES), the microsomal PGE synthase-1 (mPGES-1), and the microsomal PGE synthase-2 (mPGES-2). The various PGE synthases vary in their expression levels and in their nature to metabolize PGH_2 from different sources. It is postulated that cPGES is mainly coupled with COX-1, whereas mPGES-1 is coupled with COX-2 [2]. mPGES-2 transforms PGH_2 from both COX enzymes [3]. Furthermore, cPGES and mPGES-2 are expressed constitutively in most tissues whereas mPGES-1 is barely basally expressed, but is inducible by different stimuli and frequently co-expressed with COX-2. Inhibition of PGE_2 production could be achieved by blocking the cyclooxygenases either with unselective or selective COX-inhibitors such as ibuprofen and diclofenac, or celecoxib and etoricoxib, respectively. Due to the fact that long-term treatment with COX-inhibitors is associated with severe side effects like thromboembolic events, liver and skin toxicity, alternatives are sought after to inhibit PG production, especially PGE_2 production. DMC (dimethylcelecoxib = {4-[5-(2,5-dimethylphenyl)-3(trifluoromethyl)- 1H-pyrazol-1 yl]benzenesulfonamide}) is a close derivative of celecoxib, without cyclooxygenase inhibiting properties up to high concentrations. Nevertheless, after stimulation of various human cell lines with IL-1β/TNFα and simultaneous treatment with DMC PGE_2 synthesis is inhibited [1]. Our investigations revealed that DMC prevents mPGES-1 upregulation after IL-1β/TNFα treatment in these cells [1]. mPGES-1 is mainly responsible for the synthesis of PGE_2 after stimulation of cells with various cytokines, growth factors or stress inducible stimuli and play a decisive role in cancer, pain and inflammatory processes. Today,

several groups as well as large pharmaceutical companies are developing mPGES-1 inhibitors. mPGES-1 inhibition is achieved by two methods: 1) PGE_2 production could either be prevented by inhibition of mPGES-1 activity, or 2) by suppression of mPGES-1 transcription and therefore protein expression [4-6]. DMC has been shown to inhibit both mPGES-1 activity and its expression [1]. However, the intracellular signaling pathway controlling mPGES-1 expression by DMC is at present still unknown. The human mPGES-1 gene structure and promoter sequence was first cloned by the group of Jakobsson in 2000 [7]. The mPGES-1 promoter does not contain a TATA box; instead it comprises several putative transcription factor binding sites like two GC boxes, two tandem barbie boxes and a hydrocarbon response element [7]. The GC boxes are binding sites for SP1 as well as EGR1 (early growth response factor-1) and are responsible for basal transcription of the mPGES-1 [8]. In tumor cell lines activation of EGR1 after stimulation with IL-1β, TNFα or LPS is a key event leading to an upregulation of mPGES-1 expression [9]. In human lung carcinoma cells Catley et al. were able to show that IL-1β promotes mPGES-1 expression via NF-κB activation [10]. However, the NF-κB binding site is located on the anti-sense strand of the mPGES-1 promoter; therefore, it is questionable whether NF-κB has a direct or indirect effect on mPGES-1 transcriptional regulation or whether another NF-κB binding site is located further upstream of the mPGES-1 promoter [11]. In addition, a potential CREB binding site is located 5' nearby the NF-κB binding site of the mPGES-1 promoter.

In this study we investigated the effect of DMC on the human mPGES-1 promoter in HeLa cells after stimulation with IL-1β and TNFα. Using different mPGES-1 promoter deletion constructs we characterized the impact of the various transcription factor binding sites on mPGES-1 expression after IL-1β and TNFα stimulation and DMC co-treatment

2. Materials and methods

2.1 Cells and reagents

HeLa (human cervix carcinoma) cells were purchased from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and cultured in RPMI medium 1640. Medium contained high glucose, GlutaMAX, 10% FCS. Medium and FCS were purchased from Invitrogen (Germany) as well as 100 units/ml penicillin G and 100 mg/ml streptomycin. Cells were cultured at 37°C in an atmosphere containing 5% CO_2. DMC was synthesized by WITEGA Laboratorien Berlin-Adlershof GmbH. The identity and purity of Dimethylcelecoxib was determined using ^{1}H NMR and liquid chromatography tandem mass spectrometry (LC/MS–MS) as described previously [12-13] and was >99%. Recombinant human IL-1 beta (IL-1β) and recombinant human tumor necrosis factor alpha (TNFα) were purchased from PeproTech (London, UK).

2.2 Plasmid Constructs

The genomic DNA fragment of the human mPGES-1 promoter (Figure 1A) was amplified by PCR with primers containing a HindIII or XhoI restriction site (5´-tga ctc gag tct cccatc tca aat cct-3`and 5`-tga aag ctt ctc tgg cca gcg cag ctc aa-3`) using genomic DNA of HeLa cells as template and HotStartTaq Plus DNA Polymerase (Qiagen). PCR conditions: 10min 95°C, 3min 95°C, 1min 63°C, 2min 72°C, repeated 30 times, ending with a final 72°C step for 10min. The resulting mPGES-1 promoter PCR product was digested with XhoI and HindIII and cloned into the pGL3-basic vector (Promega) (Figure 1B; construct A). Deletion constructs of this "full length" mPGES-1 promoter were generated by digestion of construct A with SacI and relegation (Figure 1B; construct B) or by digestion of construct A with SacI and

HindIII and subcloning the 450bp fragment into the GL3-basic vector (Figure 1B; construct C).

A 3xκB luc reporter gene (three copies of a NF-κB motif immediately upstream of a β globin TATA box) was used as a NF-κB control vector (kindly provided by Prof. G. Fritz, Mainz, Germany) (Figure 6A). EGR1 overexpression was achieved by transfection of the pCMV-Sport6 EGR1 vector (1µg) purchased from imaGenes (Berlin, Germany).

2.3 Transfection and Luciferase Assay

Transient transfection was performed with TransPass HeLa transfection reagent (New England Biolabs) according to the manufacturer's protocol for adherent cells. HeLa cells (1×10^5) were seeded on Greiner 6 well plates with 1ml of culture medium 1 day before transfection. The cells were transfected with 1.5ng of the distinct firefly luciferase reporter vectors and 1ng of the Renilla luciferase control reporter vector (pRL-TK, Promega) using 7.5µl TransPass HeLa transfection reagent. 1 day after transfection, the cells were stimulated for 24 h with or without TNFα (5ng/ml) + IL-1β (1ng/ml) and simultaneously treated with increasing concentrations of DMC (0.1–30µM) or 0.1% DMSO (dimethyl sulfoxide) (DMC was solved in DMSO). The Luciferase Assay was performed with the Dual Luciferase Assay kit (Promega) and a luminometer (AutoLumat LB953, EG&G Berthold, Bad. Wildbad, Germany) according to the manufacturer's instructions. The activity of firefly luciferase was normalized to the RLU of Renilla luciferase. Data represent the mean +/- S.E.M of at least three independent experiments.

2.4. Western blot assay

HeLa cells were seeded at a density of 1.8 x 10^6 cells per dish in medium containing 10% FCS and incubated for 48 h at 37°C. Cells were then stimulated with or without TNFα (5ng/ml) + IL-1β (1ng/ml) and simultaneously treated with increasing concentrations of DMC (10–30μM) for 1 h. Nuclear extracts for western blot were prepared in the following manner: Cells were gently scrapped and washed in ice-cold phosphate-buffered saline. Cells were aspirated in NP-40 lysis buffer (10mM Tris pH 7.4, 10mM NaCl, 3mM $MgCL_2$, 1mM EDTA, 0.1% NP-40, and Roche complete (Roche Mannheim)) and centrifuge for 10min at 4°C. Pellets were washed with TKM (TKM=Tris-potassium-magnesium) buffer (50mM Tris pH 7.4, 250mM sucrose, 25 mM KCl, 5mM $MgCl_2$, 1mM EDTA and Roche complete), briefly centrifuged and resuspended by sonification in TKM buffer. Immunoblotting was performed as described previously [14]. Briefly, the protein content of lysates was quantified using the Bradford method (Biorad, Germany) and aliquots of 50μg protein were separated onto a 10–15% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Hybond-C, Amersham). Blocked membranes were incubated with the respective primary antibody directed against EGR1 (rabbit polyclonal, EGR1 (H-250); Santa Cruz), SP1 (rabbit polyclonal, SP1 (PEP 2); Santa Cruz) CREB (rabbit monoclonal, CREB (48H2); Cell Signaling) NF-κB (rabbit polyclonal, NF-κB (p65); Cell Signaling). Anti-β-Actin antibody (mouse monoclonal; Sigma) was used as loading control. The blots were incubated with IRDye 680 conjugated goat anti-rabbit IgG(H+L) or IRDye 800CW conjugated goat anti-mouse IgG(H+L) secondary antibodies purchased from LI-COR (Bad Homburg, Germany). Membranes were analyzed on the Odyssey infrared scanner from LI-COR (Bad Homburg, Germany).

2.5 Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)

For EMSA HeLa cells were seeded and treated like described above for western blot assay. Nuclear extracts for EMSA were prepared as following: Cells were gently scrapped and washed in ice-cold phosphate-buffered saline. Then the cells were allowed to swell in ice-cold hypotonic buffer containing 10mM HEPES-KOH, pH 7.9, 10mM KCl, 1.5mM $MgCl_2$, 0.5mM DTT, 1mM PMSF, 1mM Na_3VO4 and Roche complete for 15 min on ice afterwards NP-40 to a final concentration of 0.5% were added. The nuclei were recovered by centrifugation (2500rpm, 5min, 4°C). The pellets were resuspended in high-salt buffer containing 20mM HEPES, pH 7.9, 420mM NaCl, 1.2mM $MgCl_2$, 0.5mM DTT, 0.2mM EDTA, 25% glycerol, 0.5mM PMSF, 1mM Na_3VO_4 and Roche complete, followed by incubation on ice for 20 min. The nuclear extracts were recovered by centrifugation and protein concentration was determined by the method of Bradford (Bio-Rad). The oligonucleotides were synthetic double-stranded oligonucleotides corresponding to the EGR1 binding motifs in the human mPGES-1 promoter (5`-gtg ggg cgg ggc gtg ggc ggt gca-3`), and EGR1 consensus motif (5`-ccc ggc gcg ggg gcg att tcg agt ca-3`) both were 5`-labeled with IRD700. For competition assays the identical unlabeled oligonucleotides were used. The 5x binding buffer consisted of 60mM HEPES, pH 7.9, 20mM Tris-HCl, pH 7.9, 300mM KCl, 3mM DTT, 3mM EDTA, 25mM $MgCl_2$ and 60% glycerol. Binding reactions were conducted with 5µg of nuclear extract, 1µg of poly-(dI-dC) and 250 fmol IRD700 end labeled oligonucleotide probe at 22°C for 30 min in a final volume of 20µl. For supershift assays, an antibody against EGR1 (3µg/reaction) was incubated with the reaction mixture for 30 min at 22°C before the addition of IRD700 labeled oligonucleotide. For competition assays, 100-fold molar excess of mPGES-1 or consensus oligonucleotide was used. Binding complexes were resolved on non-denaturating 4% polyacrylamide gel electrophoresis in a tris borate buffer system, after which the gel was analyzed on an infrared Odyssey scanner.

2.6. Statistics

Data are presented as mean +/- standard error of the mean (S.E.M.). The GraphPad Prism 5 computer software was used for statistical analyses. Statistics were analyzed using univariate analysis of variance (ANOVA) followed by t-tests. p values of < 0.05 were considered statistically significant.

3. Results

3.1. Effect of DMC on the mPGES-1 promoter

Previous data demonstrated that DMC inhibits mPGES-1 expression after stimulation of HeLa cells with IL-1β and TNFα on mRNA as well as on protein level [1]. Here we examined if this inhibition belongs to a repression of the mPGES-1 promoter activity by DMC. Therefore, HeLa cells were transiently transfected with the human mPGES-1 promoter-luciferase construct (Figure 1B; construct A), stimulated with IL-1β/TNFα and co-treated with increasing concentrations of DMC or vehicle. Treatment of HeLa cells with IL-1β/TNFα increased luciferase activity of the transfected mPGES-1 promoter-luciferase construct about threefold. Simultaneous treatment with increasing concentrations of DMC led to a dose-dependent, significant down-regulation of luciferase activity of about 40% (Figure 2 A).

To examine which of the transcription factor binding sites of the mPGES-1 promoter are affected by DMC, leading to the depression of the mPGES-1 promoter activity, we cloned two mPGES-1 promoter deletion constructs, one missing the NF-κB and CREB binding sites (Figure 1B; construct B) and another missing the EGR1 and SP1 binding sites (Figure 1B; construct C). Figure 2B shows that both deletion constructs are less inducible by IL-β/TNFα stimulation than the full-length promoter-luciferase construct. Nevertheless, the activity of both mPGES-1 promoter deletion constructs

could be inhibited by DMC treatment. Interestingly, only the activity of the promoter construct containing the SP1/EGR1 binding sites was significantly inhibited by DMC.

3.2. Effect of DMC on EGR1 and SP1

Next we examined by western blot analysis the effects of DMC on nuclear expression of EGR1 and SP1 protein in HeLa cells after IL-1β/TNFα treatment (Figure 3). Nuclear expression of EGR1 was enhanced about twofold after IL-1β/TNFα treatment and was significantly reduced by co-treatment with increasing concentrations of DMC (Figure 3A). Nuclear expression of SP1 was not affected, neither by treatment of HeLa cells with IL-1β/TNFα nor by co-treatment with DMC (Figure 3B). To verify if changes in nuclear expression of EGR1 have also an effect on its DNA-binding activity, we examined the influence of IL-1β/TNFα treatment and DMC co-treatment on the binding capability of EGR1 to the EGR1 binding site of the mPGES-1 promoter. Therefore, we performed electro mobility shift assays (EMSA) with an oligonucleotide containing the EGR1/SP-1 binding site of the mPGES-1 promoter (mPGES-1 oligo) as well as an oligonucleotide comprising the EGR1 consensus sequence (consensus oligo). Figure 4A demonstrates a typical pattern of the mPGES-1 oligo. With supershift analysis, using an anti-EGR1 antibody, as well as by using the consensus oligo containing the EGR1 binding site, we clearly identified the signal for EGR1-DNA complex. The upper band was identified as SP1-DNA complex whereas other bands are non-specific.

After identification of the EGR1-DNA complex we examined the effect of DMC on the EGR1 DNA binding characteristics (Figure 4B). IL-1β and TNFα treatment lead to an upregulation of EGR1 binding capability, which was diminished by simultaneous treatment with DMC. Competition experiments, using 100-fold molar excess of

unlabeled mPGES-1 oligo or unlabeled consensus oligo, confirmed the specific EGR1-DNA complex. Taken together, we demonstrated that EGR1 expression is diminished by treatment of HeLa cells with DMC which subsequently leads to a reduced DNA binding capability of EGR1 at the mPGES-1 promoter.

3.3. Effect of DMC on CREB and NF-κB

Next we investigated if also other transcription factors with possible binding sites at the mPGES-1 promoter are influenced by DMC. Therefore, we examined the influence of DMC on the nuclear expression levels of CREB and NF-κB by western blot analysis. Nuclear expression of phospho-CREB or CREB protein were neither affected by treatment of HeLa cells with IL-1β and TNFα nor after simultaneous treatment with DMC (Figure 5A). Only nuclear expression of NF-κB increased slightly after IL-1β and TNFα treatment but enhanced further after treatment with increasing concentrations of DMC (Figure 5B). This result was rather unexpected, because our Luciferase Assay, using the mPGES-1 deletion construct C (contains only the NF-κB and CREB binding site), demonstrates that the luciferase activity slightly decreases after DMC treatment (Figure 2). So we investigated the transcriptional activity of NF-κB using a luciferase construct which contains three NF-κB binding sites directly cloned behind a TATA box [15] (Figure 6A). We transfected HeLa cells with the NF-κB-Luc construct and treated them with IL-1β/TNFα and simultaneously with increasing concentrations of DMC. Figure 6B shows that the luciferase activity increased after IL-1β/TNFα treatment but co-treatment with DMC diminished the luciferase activity (Figure 6B). These data let us conclude that in spite of the fact that the nuclear expression of NF-κB increased after DMC treatment, its transcriptional activity decreased.

3.4. Impact of EGR1 overexpression on the mPGES-1 promoter activity

Until now our data demonstrate that out of the four potential transcription factors regulating the human mPGES-1 promoter, only the expression and transcriptional activity of EGR1 and NF-κB are affected by DMC. To make sure that the expression level of EGR1 has a direct impact on the mPGES-1 promoter activity after DMC treatment, we transfected an EGR1 expression plasmid isochronous with construct A, B or C in HeLa cells, treated them with or without IL-1β/TNFα and simultaneously with increasing concentrations of DMC. To ensure that EGR1 is successfully overexpressed we checked EGR1 expression by western blot analysis (Figure 7A). Transfection of HeLa cells with the pCMV-sport6-EGR1 expression vector led to an increase of nuclear EGR1 protein. As shown in Figure 7B the basal activity of the co-transfected mPGES-1 promoter-luciferase construct A was slightly augmented by EGR1 overexpression but its activity could be further increased by treating cells with IL-1β/TNFα. Isochronous treatment with DMC had no effect on the mPGES-1 promoter activity. Also co-transfection of the EGR1 expression vector with construct B prevented the inhibitory effect of DMC on the luciferase activity, whereas EGR1 had no effect on the mPGES-1 promoter construct C that contains only the NF-κB and CREB binding site (Figure 7C and D).

Taken together we were able to show that DMC influences the mPGES-1 expression transcriptionally. Our data indicate that treatment of HeLa cells with DMC leads on the one hand to a decrease of nuclear EGR1 expression that is affiliated with a decrease in its DNA-binding capability. On the other hand, DMC increases nuclear NF-κB expression but this was accompanied with a reduced transcriptional activity of NF-κB. The data obtained with the mPGES-1 promoter deletion constructs indicate

both mechanisms contribute to a reduced mPGES-1 promoter activity after DMC treatment.

4. Discussion

Dimethylcelecoxib was first published by Song et al., who investigated the anti-tumor activity of celecoxib and various celecoxib derivatives with the aim to dissociate between the apoptosis-inducing activity from the COX-2-inhibitory activity of celecoxib [16]. The first evidence showing that DMC is not a COX-2 inhibitor was published by Schönthal [17]. However, we were able to demonstrate that DMC nevertheless inhibits PG production [1]. We ascertained that DMC inhibits on the one hand mPGES-1 activity and, on the other, prevents mPGES-1 expression after stimulation of cells with IL-1β/TNFα [1]. Here we investigated the molecular mechanism contributing to the suppressive effect of DMC on mPGES-1 expression. In general, protein expression was inhibited by transcriptional, post-transcriptional or post-translational mechanisms. Because we demonstrated that DMC inhibits mPGES-1 protein expression as well as mPGES-1 mRNA levels [1] we decided to look for transcriptional mechanisms that may be influenced by DMC. Nevertheless, thus far we cannot exclude that also other mechanisms such as reduced mRNA stabilization or enhanced protein degradation play a role in the suppressive effect of DMC on mPGES-1 expression.

Here we demonstrated that DMC inhibits mPGES-1 transcription by blocking nuclear EGR1 expression and repressing NF-κB transcriptional activity. Other putative transcription factors known to regulate mPGES-1 expression, like SP1 or CREB, are not affected by DMC. Furthermore, overexpression of EGR1 completely prevented the inhibitory effect of DMC on mPGES-1 promoter activity, indicating that the

repressing effect of DMC on mPGES-1 expression is mainly dependent on its effect on EGR1 expression.

EGR1 has been described as a strong regulator of mPGES-1 transcription after stimulation of different cells with IL-1β or TNFα [18-20]. Inhibition of EGR1 expression by DMC could be due to several mechanisms. It is known that EGR1 expression is regulated by EGR1 itself, NF-κB, serum response elements and AP-1 [21-22]. Autoregulation of EGR1 is dependent on phosphorylation and acetylation of histone H3 [22]. Interestingly, also the transcriptional activity of EGR1 on the human mPGES-1 promoter is dependent on acetylation mechanisms mediated by HDAC4 (histone deacetylase 4) which regulates among others the acetylation status of histone H3 [20]. It is still not known if DMC influences HDAC activities; this will be a purpose of future research. The NF-κB element in the EGR1 promoter may be also responsible for the repressive effect of DMC on EGR1 expression because deletion of this element enhances EGR1 promoter activity in some fibroblast cell lines indicating that NF-κB has a repressive effect on EGR1 transcription [21]. As our reporter gene assays showed that also construct C, which contains only the CREB and NF-κB bindings site of the mPGES-1 promoter, is still inducible by IL-1β and TNFα and luciferase activity reduces after DMC treatment, we assume that DMC influences NF-κB signaling. Previously it has been shown that DMC influences NF-κB signaling by inhibition of IKK activity [23], but this mechanism does not seem to play a role in our cell system, because our western blot experiments demonstrate that nuclear expression of NF-κB enhanced after DMC treatment and activation of IKK is one prerequisite for this effect. Therefore, we assume that NF-κB is activated by DMC but has a repressive effect on the mPGES-1 promoter after DMC treatment. This effect is possibly also mainly dependent on EGR1, because treatment of HeLa cells with inhibitors of IKK (BMS-345541and IKK Inhibitor VII) decreases the

Luciferase activity of the mPGES-1 promoter construct A after IL-1β and TNFα treatment, that could be influenced by co-transfection of the EGR-1 expression vector (unpublished data).The transcriptional activity of NF-κB can be regulated by various stimuli and binding of NF-κB to the promoter region can be influenced by different proteins leading to transcriptional activation or repression (due to the enormous flood of NF-κB publications we refer here only to a recently published review which summarizes the regulation mechanisms of NF-κB transcriptional activity [24]). The influence of NF-κB on mPGES-1 and EGR1 after DMC treatment is, as a result of its complexity, the subject of another paper (Deckmann et al. in preparation). From the literature there is also a hint that activation of peroxisome proliferator-activated receptor γ (PPARγ) inhibits IL-1β-induced mPGES-1 expression [25]. Therefore, we checked all PPAR subtypes for their transactivation activity in a ligand-dependent luciferase reporter-gen assay (as described previously [26]) after DMC treatment. Only PPARγ activity increases slightly but significantly after DMC treatment (supplement 1). The inhibitory effect of PPAR agonists on mPGES-1 expression has been thought to be mediated by antagonizing transcriptions factors such as NF-κB [25]. Additionally, PPARγ activation suppresses induction of EGR1 expression [27], so that both interference of DMC activated PPARγ with EGR1 and NF-κB could contribute to the suppressive effect of DMC on mPGES-1 transcription.

Taken together we ascertained that DMC inhibits mPGES-1 transcription in HeLa cells by downregulation of EGR1 a key regulator of mPGES-1 expression. Furthermore, the transactivation activity of NF-κB seems to be affected by DMC as well. Both could be influenced by PPARγ that is activated by DMC. mPGES-1, EGR1 and NF-κB are important proteins involved in many pathological conditions such as

inflammation and cancer. Therefore, DMC seems to be a promising substance to treat inflammatory and carcinogenic processes.

Acknowledgment

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Abbreviations

ANOVA, analyzed using univariate analysis of variance; CREB, cAMP-responsive element-binding protein 1; cPGES cytosolic prostaglandin E synthase; COX-1/2, cyclooxygenase-1/2; DMC, dimethylcelecoxib; DMSO, dimethyl sulfoxide; DTT, dithiotreitol; EDTA, ethylenediaminetetraacetate; EGR1, early growth response 1; EMSA, Electrophoretic Mobility Shift Assay; FCS fetal calf serum; IL-1β, interleukin-1 beta; LC/MS-MS, liguid chromatography tandem mass spectrometry; LPS, Lipopolysaccharide; mPGES-1, microsomal prostaglandin E synthase-1; mPGES-2, microsomal prostaglandin E synthase-2; NF-κB, nuclear factor NF-kappa-B p65; NSAID, non-steroidal anti-inflammatory drug; NP-40, nonidet 40; PBS, phosphate buffered saline; PGE, prostaglandin E; PGH prostaglandin H; PGES, prostaglandin E synthase; PMSF, phenyl-methylsulfonylfluride; SDS-PAGE, sodiumdodecylsulfate-polyacrylamide gel electrophoresis; S.E.M, standard error of the mean; SP1, specificity protein 1; TKM, Tris-potassium-magnesium; TNFα, tumor necrosis factor alpha;

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6. Legend

Figure 1. Schematic diagram of the mPGES-1 promoter

(A) mPGES-1 promoter (B) mPGES-1 luciferase reporter gene constructs: "full length" construct A (-631 to -1bp); construct B (-177 to -1bp), construct C (-631 to -177bp) (C) structural formula of dimethylcelecoxib

Figure 2. Luciferase Assay of mPGES-1 promoter reporter gene constructs in HeLa cells after IL-1β/TNFα stimulation and DMC treatment.

(A) HeLa cells were transiently transfected with the "full length" mPGES-1-luc - construct (construct A), stimulated with IL-1β/TNFα and simultaneously treated with increasing concentrations (0.1-30μM) of DMC. (B) HeLa cells were transiently

transfected with construct A, B or C, stimulated with IL-1β/TNFα and simultaneously treated with 10µM of DMC. Luciferase activity was measured by the use of the Dual Luciferase Assay kit. Renilla luciferase was used as transfection control. All experiments were carried out at least three times; data are the mean ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001

Figure 3. Western blot analysis of EGR-1 and SP1 after DMC treatment

Western blot analysis of (A) EGR1 or (B) SP1 in IL-1β/TNFα stimulated HeLa cells incubated with increasing amounts of DMC. Nuclear extract of HeLa cells were prepared and 50µg protein were separated onto a 12% SDS polyacrylamide gel and transferred to nitrocellulose membranes. EGR1 and SP1 were detected using specific rabbit polyclonal antibodies. The expression of β-Actin was used as loading control. A representative experiment is shown. All experiments were carried out at least three times; data are the mean ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 4. Electrophoretic mobility shift assay (EMSA)

(A) EMSA pattern of an IR700-labeled oligonucleotide containing the two EGR1 binding sites of the mPGES-1 promoter after incubation with nuclear protein extract from HeLa cells treated with IL-1β/TNFα. To specify the EGR1-DNA complex and SP1-DNA complex an EGR1 consensus oligonucleotide was used and anti-EGR1 antibody for supershift analysis (EGR1 SS). (B) EMSA pattern of the IR700-labeled oligonucleotide containing the two EGR1 binding sites of the mPGES-1 promoter after incubation with nuclear protein extract from HeLa cells treated with IL-1β/TNFα and increasing amounts of DMC. Specificity of binding was confirmed using 100-fold molar excess of unlabeled oligonucleotides containing consensus or mPGES-1 EGR1 binding sites. EGR1-DNA complex (EGR1), non-specific protein DNA complex (NS). A representative EMSA of at least three independent experiments is shown.

Figure 5. Western blot analysis of CREB and NF-κB after DMC treatment

(A) Western blot analysis of phospho-CREB and CREB in stimulated HeLa cells incubated with increasing amounts of DMC. (B) Western blot analysis of NF-κB in stimulated HeLa cells incubated with increasing amounts of DMC. Nuclear extractions of HeLa cells were prepared and 50µg protein were separated onto a 12% SDS polyacrylamide gel and transferred to nitrocellulose membranes. phospho-CREB, CREB and NF-κB were detected using rabbit polyclonal antibodies. The expression of β-Actin was used as loading control. A representative experiment out of three is shown. Data are the mean ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 6. NF-κB transcriptional activity after DMC treatment.

(A) Schematic diagram of the luciferase construct including three NF-κB binding sites in front of a TATA box. (NF-κB-Luc) (B) HeLa cells were transiently transfected with the NF-κB-luciferase construct, stimulated with IL-1β/TNFα, and simultaneously treated with increasing concentrations (10µM; 20µM; 30µM) of DMC. Renilla luciferase was used as transfection control. All experiments were carried out at least three times; data are the mean ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 7. Impact of EGR1 overexpression on the mPGES-1 promoter activity

(A) HeLa cells were transiently transfected with a pCMV-sport6 plasmid containing the EGR1-cDNA. Nuclear extracts of HeLa cells were prepared and 50µg protein were separated onto a 12% SDS polyacrylamide gel and transferred to nitrocellulose membranes. EGR1 were detected using a specific rabbit polyclonal antibody. The expression of β-Actin was used as loading control. A representative experiment out of three is shown. (B-D) HeLa cells, transiently transfected with the full length mPGES-1 luciferase construct (construct A), or the two deletions constructs B or C and co-transfected with or without the pCMV-sport6 EGR1 vector, were stimulated with IL-1β/TNFα and simultaneously treated with increasing concentrations (10µM; 20µM; 30µM) of DMC. Luciferase activity was measured by the use of the dual

Luciferase Assay kit. Renilla luciferase was used as transfection control. In all experiments the luciferase activity of IL1β/TNFα stimulated cells were set to 100%. All experiments were carried out at least three times; data are the mean ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001.

*Graphical Abstract

Graphical Abstract

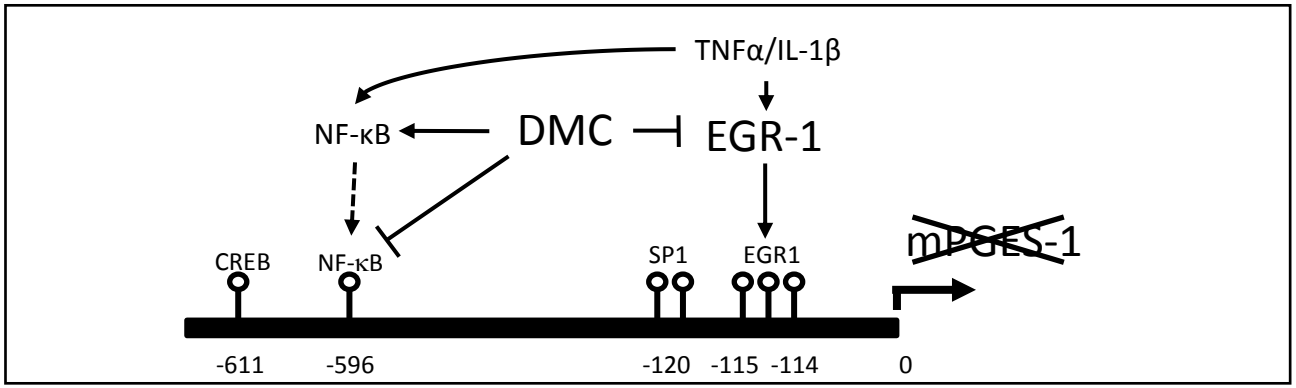
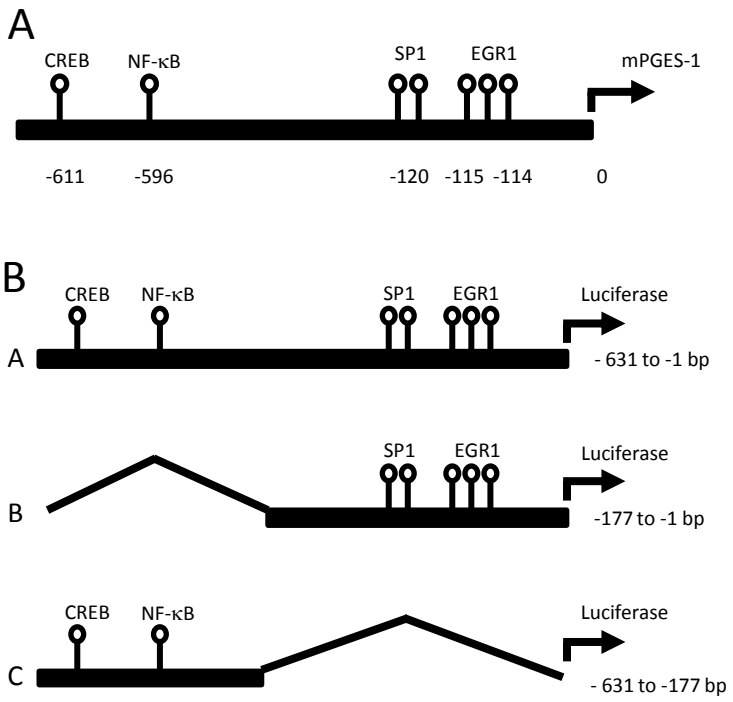
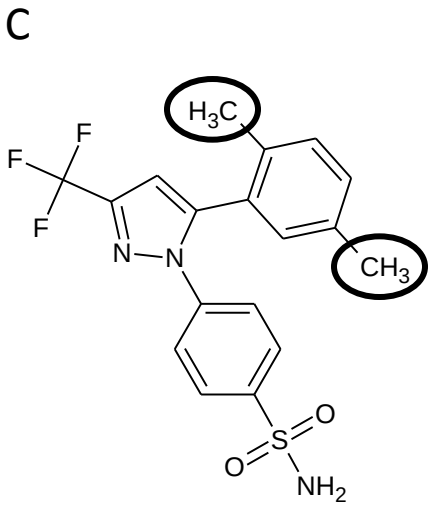


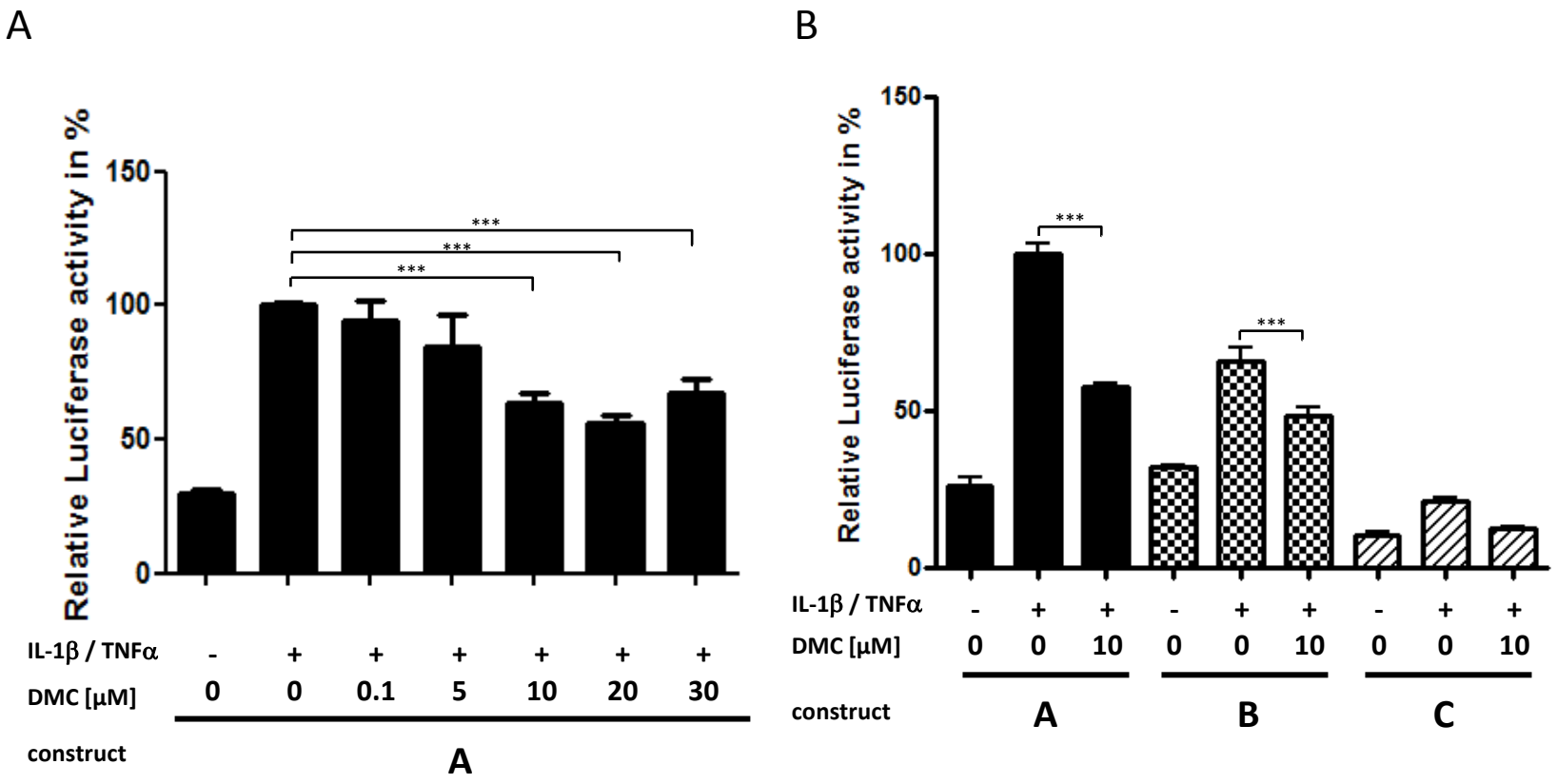
Figure 1





Dimethylcelecoxib (DMC)

Figure 2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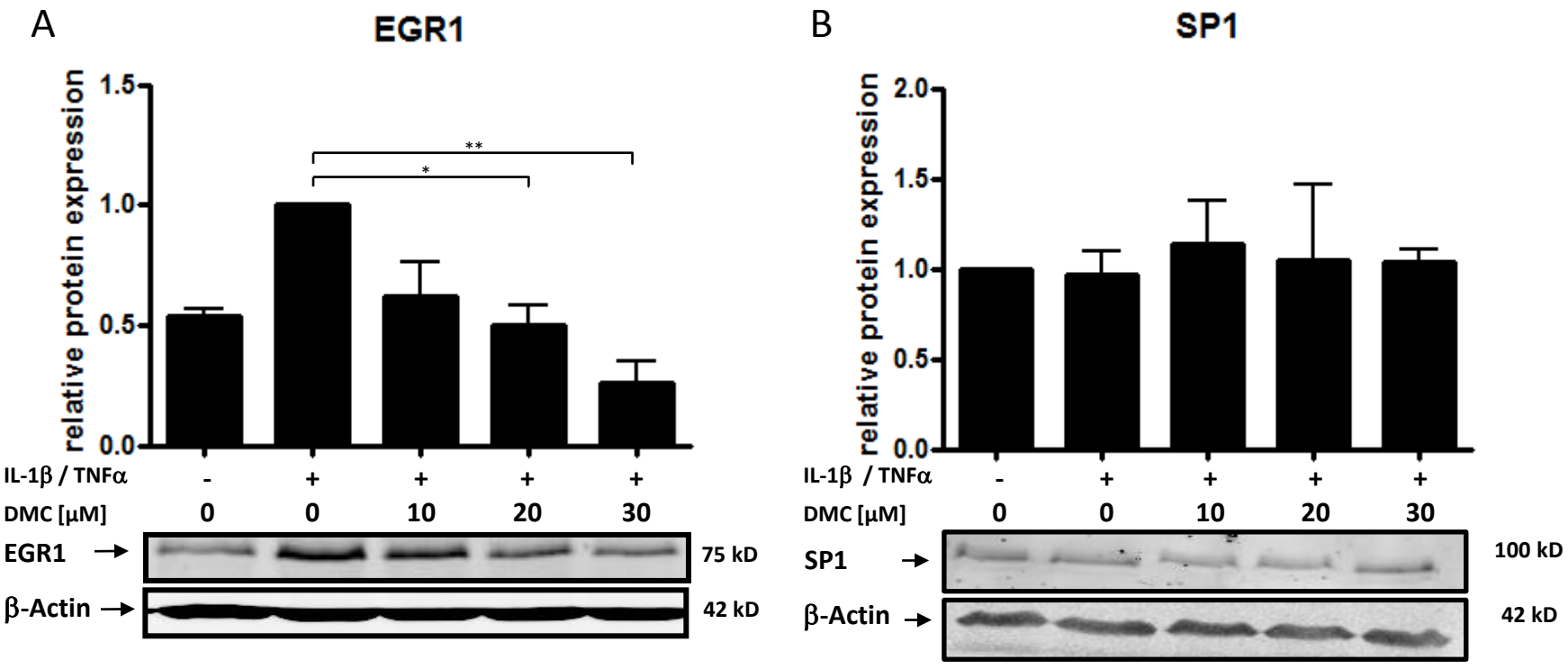
Relative Luciferase activity in %
150
100
50
0
***
***
***
IL-1β / TNFα - + + + + + +
DMC [μM] 0 0 0.1 5 10 20 30
construct
A
B
Relative Luciferase activity in %
150
100
50
0
***
***
IL-1β / TNFα - + + - + + - + +
DMC [μM] 0 0 10 0 0 10 0 0 10
construct
A
B
C

Figure 3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0.5
0.0
**
*
IL-1β / TNFα
-
+
+
+
+
DMC [μM]
0
0
10
20
30
EGR1
75 kD
β-Actin
42 kD
B
SP1
relative protein expression
2.0
1.5
1.0
0.5
0.0
IL-1β / TNFα
-
+
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+
+
DMC [μM]
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0
10
20
30
SP1
100 kD
β-Actin
42 kD

Figure 4

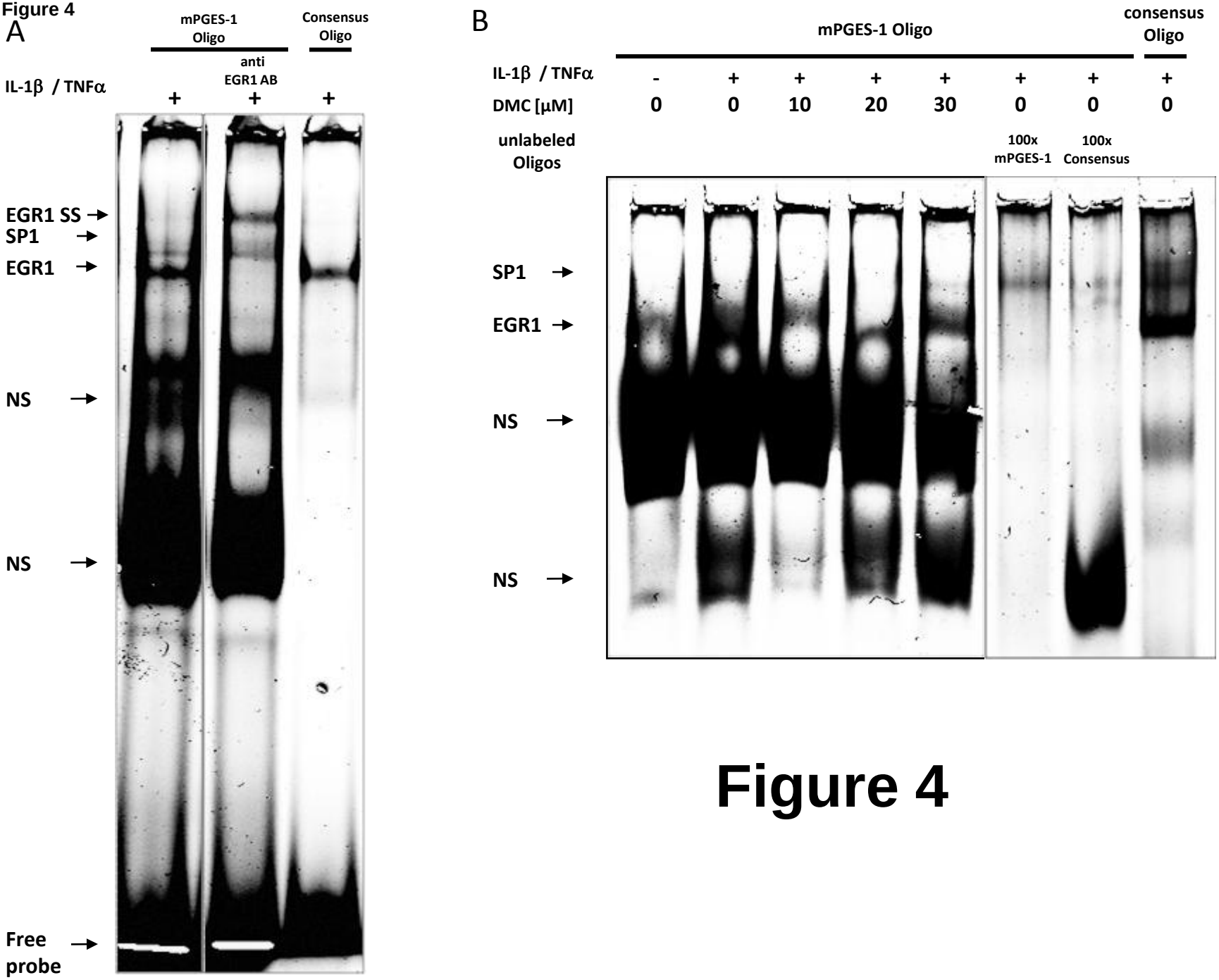
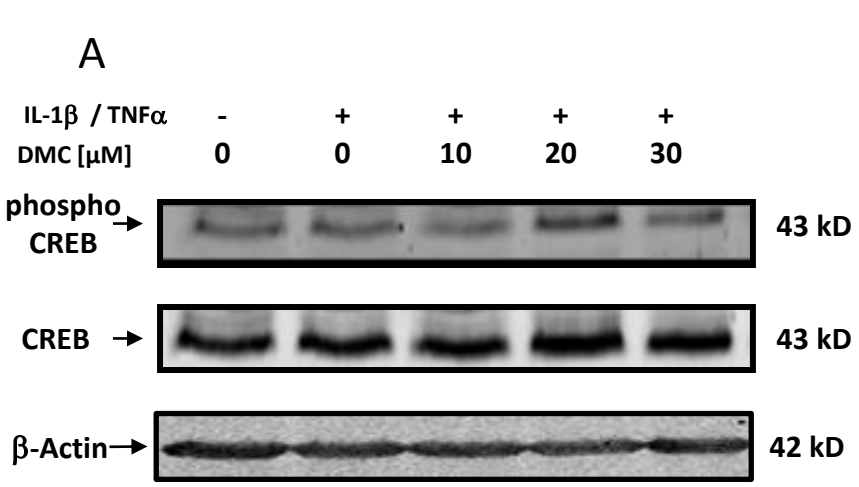


Figure 4

Figure 5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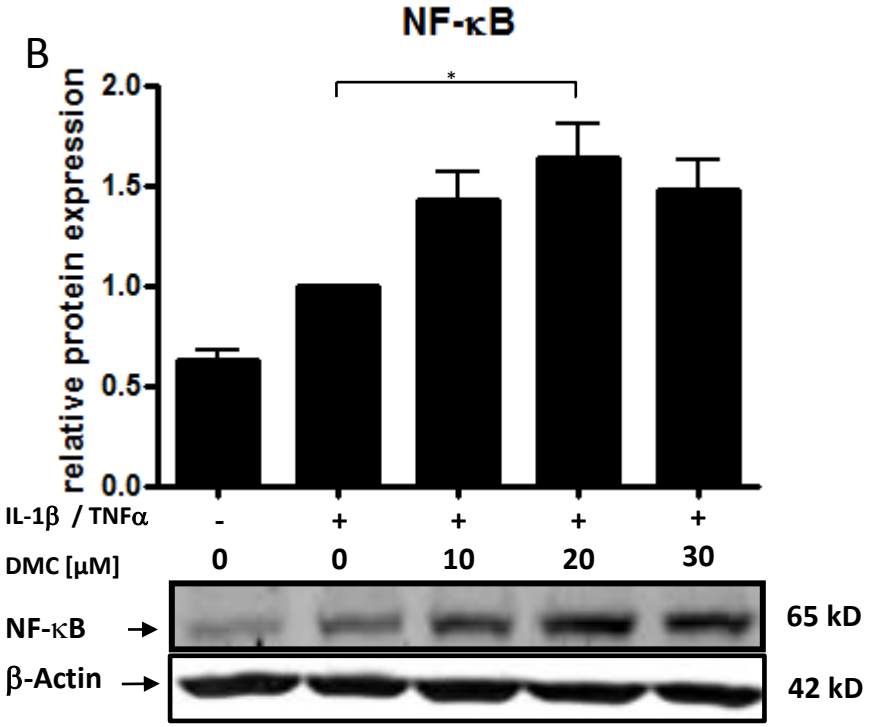
phospho CREB
43 kD
CREB
43 kD
β-Actin
42 kD


B
NF-κB
relative protein expression
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1.5
1.0
0.5
0.0
*
IL-1β / TNFα - + + + +
DMC [μM] 0 0 10 20 30
NF-κB
65 kD
β-Actin
42 kD

Figure 6

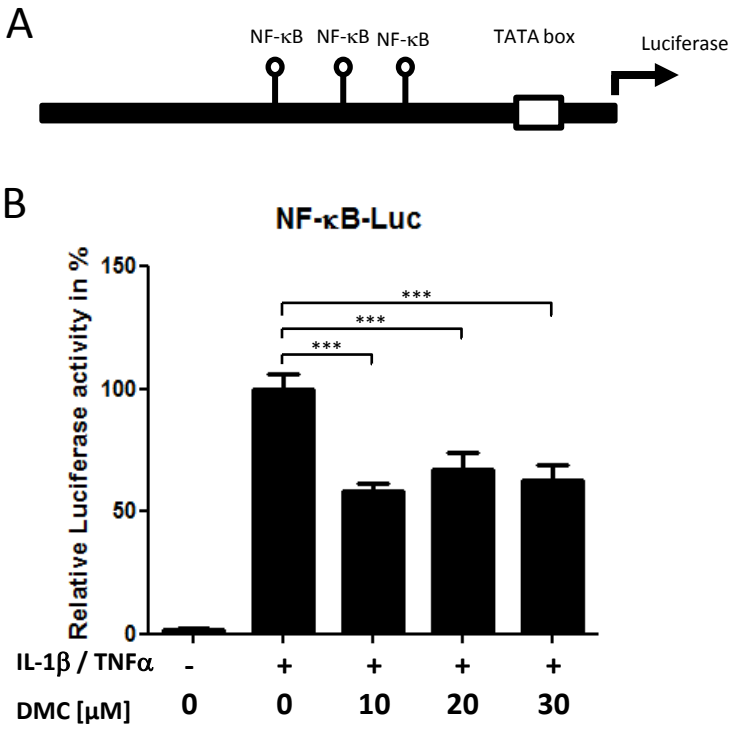
A
NF-κB
NF-κB
NF-κB
TATA box
Luciferase
B
NF-κB-Luc
Relative Luciferase activity in %
150
100
50
0
***
***
***
IL-1β / TNFα
-
+
+
+
+
DMC [μM]
0
0
10
20
30

Figure7

Figure 7