Differential Mechanism of NF-κB Inhibition by Two Glucocorticoid Receptor Modulators in Rheumatoid Arthritis Synovial Fibroblasts

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Objective. To investigate and compare the molecular mechanisms by which 2 glucocorticoid receptor (GR)–activating compounds, dexamethasone (DEX) and Compound A (CpdA), interfere with the NF-κB activation pathway in rheumatoid arthritis (RA) synovial cells.

Methods. Quantitative polymerase chain reaction was performed to detect the tumor necrosis factor (TNFα)--induced cytokine gene expression of interleukin-1β (IL-1β) and to investigate the effects of DEX and CpdA in RA fibroblast-like synoviocytes (FLS) transfected with small interfering RNA (siRNA) against GR (siGR) compared with nontransfected cells. Immunofluorescence analysis was used to detect the subcellular distribution of NF-κB (p65) under the various treatment conditions, and active DNA-bound p65 was measured using a TransAM assay and by chromatin immunoprecipitation analysis of IL-1β. Signaling pathways were studied via Western blotting of siGR-transfected cells, compared with nontreated and nontargeting siRNA–transfected control cells, to detect the regulation of phospho-IKK, IκBα, phospho-p38, phospho-ERK, and phospho-JNK.

Results. Both DEX and CpdA efficiently inhibited IL-1β gene expression in a GR-dependent manner. In addition, CpdA attenuated the TNFα–induced nuclear translocation and DNA binding of p65 in RA FLS, via the attenuation of IKK phosphorylation and subsequent IκBα degradation. CpdA also displayed profound effects on TNFα–induced MAPK activation. The effects of CpdA on TNFα--induced kinase activities occurred independently of the presence of GR. In sharp contrast, DEX did not affect TNFα–induced IKK phosphorylation, IκBα degradation, p65 nuclear translocation, or MAPK activation in RA FLS.

Conclusion. DEX and CpdA display a dissimilar molecular mechanism of interaction with the NF-κB activation pathway ex vivo. A dual pathway, partially dependent and partially independent of GR (nongenomic), may explain the gene-inhibitory effects of CpdA in RA FLS.

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease that is characterized by the formation of an aggressive tumor-like pannus structure, which invades and destroys the cartilage and bone. The pannus is home to many cell types, including blood-borne cells and mesenchymal cells (fibroblast-like synoviocytes [FLS]) (1,2). It is now clear that FLS not only are considered to be structural elements but also actively contribute to the inflammatory and destructive processes in RA via the production of a battery of cytokines, chemokines, and enzymes degrading the joint matrix (3). Moreover, the invasive FLS display morphologic features that are characteristic of a transformed cell, thereby supporting the hypothesis that the activated phenotype of FLS is an intrinsic property of these cells (4–8). Alternatively, it has been hypothesized that RA FLS are bone marrow–derived mesenchymal cells that are abundantly recruited to the RA synovium and are arrested at various stages of differentiation in the inflamed environment. Aberrantly activated NF-κB ap-
pears to play a central role in the differentiation arrest of these cells (9).

NF-κB is a heterodimeric transcription factor, usually composed of a p50 subunit and p65 subunit. A vast number of studies have demonstrated the involvement of NF-κB in the pathogenesis of RA, which is attributed to the role of NF-κB in the transcriptional regulation of genes encoding different proinflammatory mediators (10–15). Furthermore, by showing that activation of NF-κB protects synovial cells against apoptosis, it has been hypothesized by Miagkov and colleagues that this transcription factor might constitute the missing link between inflammation and hyperplasia in the arthritic joint (16,17). In unstimulated cells, NF-κB is kept inactive in the cytoplasm by inhibitory proteins (IκBs). Triggering of the cell with various extracellular stimuli, including tumor necrosis factor α (TNFα), results in activation of different signaling cascades, which converge on and activate the IKK complex. Subsequently, IκBα is phosphorylated and degraded, allowing NF-κB to translocate to the nucleus and to regulate the expression of its target genes (for review, see refs. 18–20).

The transcriptional activity of NF-κB can be further modulated in the nucleus by different kinases, including the MAPKs (21,22). In this respect, previous reports have described the importance of p38 and ERK and the downstream nuclear kinase MSK-1 for the phosphorylation of p65 at Ser276, thereby evoking full-blown NF-κB activity (23–25). In this way, an additional level of regulation is created, providing a basis for the observed crosstalk with other signaling pathways (26).

Ample amounts of evidence support the idea that the antiinflammatory effects of glucocorticoids (GCs) are a result of their interference with the NF-κB signaling pathway (27,28). Indeed, GCs exert their effects via the GC receptor (GR), which, in the absence of a ligand, can be found in the cytoplasm of the cell in complex with chaperone molecules. After diffusion through the cell membrane, GCs bind to their cognate receptor, thereby inducing its conformational change and spurring the activated ligand–receptor complex to translocate to the nucleus. In the nucleus, GR binds as a homodimer to GC-responsive elements, leading to gene activation (transactivation). Alternatively, the activated GR monomer also regulates gene expression in a negative way (transrepression), mainly through negative interference with the activity of proinflammatory transcription factors such as NF-κB (29–31).

It is generally accepted that mainly the latter characteristic of transrepression by GCs forms the basis for their antiinflammatory potential, and thus explains their success as drug targets (27,28). In addition, recent evidence suggests that GR ligands can also induce cellular responses that can occur in a time frame ranging from a few seconds up to 1 hour (32,33). These rapid effects cannot be explained by the well-known genomic effects of GR ligands, which normally take a few hours to days (32,33); yet, at the moment, it is not completely understood which receptor or pathway is responsible for mediating these so-called nongenomic effects (32).

We previously demonstrated that a small, nonsteroidal, plant-derived GR modulator, Compound A (CpdA), stimulates the transrepression activity of GR, but leaves the transactivation function of the receptor unaffected (34,35). Consequently, it was observed that the therapeutic potential of CpdA for the treatment of collagen-induced arthritis in mice was not overshadowed by diabetogenic effects (35). Subsequently, we were interested in unraveling the precise molecular mechanisms by which CpdA interferes with the NF-κB pathway in primary FLS derived from patients with RA. As a reference compound, we used dexamethasone (DEX), a synthetic steroidal GR ligand.

**PATIENTS AND METHODS**

**Isolation and culture of cells.** FLS were obtained from patients with active RA, whose diagnosis met the revised criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (36). The study was approved by the local ethics committee, and informed consent was obtained from all patients. FLS were obtained by enzymatic digestion from RA synovial tissue, and the cells were then cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. To ascertain that non-FLS cells were not contaminating the culture, FLS with a passage number ranging from 4 to a maximum of 8 were used.

**Cytokines and reagents.** Recombinant murine TNFα was produced in our laboratory (29). DEX was purchased from Sigma (St. Louis, MO). CpdA (Alexis Biochemicals, Lausen, Switzerland) was synthesized as described previously (37). Anti-p65 (C20) and anti-IκBα (C21) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies anti–phospho-p38 MAPK (Thr180/Tyr182), anti-p42/p44 (anti–p-ERK; Thr202/Tyr204), anti–phospho-SAPK/JNK (Thr183/Tyr185), and anti–phospho-IκKα/β (Ser180/Ser181) were purchased from Cell Signaling Technology (Beverly, MA). Anti–β-catenin (Ab2982) and antitubulin (clone B-5-1-2) were purchased from Abcam (Cambridge, UK) and Sigma, respectively. Small interfering RNA (siRNA) against GR (siGR; siGENOME SMARTpool human NR3C1) and nontargeting siRNA controls (siCtrl; siCONTROL Pool #1) were both purchased from Dharmacon RNA Technologies (Lafayette, CO).

**Quantitative polymerase chain reaction (PCR).** FLS were seeded in 6-well plates and serum-starved 24 hours prior
NF-κB INHIBITION BY GR MODULATORS IN RA FLS

RESULTS

Effect of CpDA and DEX on NF-κB nuclear translocation, activation, and DNA binding. To establish the effect of CpDA on NF-κB–driven gene expression, we first performed quantitative PCR analysis for the detection of proinflammatory cytokine gene transcription, using IL-1β as an example of a typical NF-κB–regulated gene (16,42). Stimulation of the FLS with TNFα for 6 hours resulted in a significant increase in IL-1β transcription. Pretreatment of the FLS with CpDA (10 μM) repressed the TNFα-induced IL-1β transcription to a similar extent as that after pretreatment with DEX (1 μM) (Figure 1).

To study the impact of both compounds on the NF-κB activation pathway, we performed immunofluorescence analysis for localization of the p65 subunit of NF-κB. In the presence of TNFα (30-minute period of induction), p65 was predominantly localized in the nucleus of the cell. Treatment with DEX did not change the subcellular distribution of p65 after stimulation with TNFα (Figure 2A). In sharp contrast, after stimulation of the FLS with TNFα followed by treatment with Cpda, p65 was either detected mainly in the cytoplasm of the cell or equally distributed over both subcellular compartments (Figure 2A).

To determine whether the hampered cytoplasmic-to-nuclear translocation after CpDA treatment was correlated with the amount of active p65, we per-
formed a TransAM assay to detect active DNA-bound p65 (Figure 2B). After the appropriate inductions, nuclear proteins were extracted from the FLS and analyzed for binding of active p65 to a target oligonucleotide in the ELISA-based format. Stimulation of the cells with TNFα for 1 hour or 2 hours resulted in a significant increase in binding of active p65. Although treatment of TNFα-stimulated FLS with DEX resulted in a small, but significant, decrease in active DNA-bound p65 after 1 hour of TNFα stimulation, this effect of DEX was completely abolished when TNFα remained on the cells for 2 hours. The effect of CpdA on the amount of active p65 DNA binding was more pronounced than the effect of DEX. Although there was a significant CpdA-mediated decrease in the amount of p65 DNA binding after 1 hour of stimulation with TNFα, the effect of CpdA was even more pronounced after 2 hours of TNFα stimulation, as was exemplified by a partial, yet significant, inhibition of p65 DNA binding (Figure 2B).

In addition, recruitment of the p65 subunit of NF-κB or of the GRα isoform onto the IL-1β promoter was analyzed via a ChIP assay, after treatment of the FLS for 30 minutes with TNFα in the absence or presence of DEX or CpdA. TNFα was able to induce an enhanced promoter recruitment of p65, and pretreatment with DEX or CpdA resulted in decreased p65 DNA binding at the IL-1β promoter (Figure 2C), which is in accordance with the results obtained in the TransAM assay after 1 hour of stimulation with TNFα. Importantly, both DEX and CpdA were able to stimulate promoter accumulation of GRα, strongly suggesting the importance of the GR in the transrepression mechanism of both compounds (Figure 2C).

**Effect of CpdA and DEX on the upstream pathways leading to NF-κB activation.** To investigate the molecular basis of the altered subcellular localization and DNA binding of p65 after CpdA treatment, we assessed the IκBα protein levels in the FLS (Figure 3A). After stimulation of the FLS with TNFα for 15 minutes, IκBα protein expression completely disappeared, in contrast to the findings with the ethanol control solvent (compare lane 1 with lane 4 in Figure 3A). Resynthesis of the IκBα protein could be observed after TNFα stimulation for 60 minutes (compare lane 4 with lane 10 in Figure 3A). These results confirm the known mechanism of NF-κB-driven resynthesis of IκBα.

Following treatment of the cells with DEX and stimulation with TNFα, a regulation pattern similar to that found in untreated cells stimulated with TNFα alone was observed; IκBα protein was completely degraded and only reappeared after 1 hour of TNFα stimulation (compare lane 4 with lane 5 and lane 10 with lane 11 in Figure 3A). Remarkably, in the RA FLS, TNFα stimulation in combination with CpdA treatment...
NF-κB INHIBITION BY GR MODULATORS IN RA FLS

Figure 3. Attenuation of NF-κB activation by CpdA via the inhibition of IκBα degradation, in a manner independent of the glucocorticoid receptor (GR), in rheumatoid arthritis FLS. Cells were treated for 1 hour with DEX (1 μM), CpdA (10 μM), or EtOH control solvent, after which a time kinetics experiment was started. TNFα (2,000 IU/ml) was added and left on the FLS for the indicated time periods (15, 30, or 60 minutes). A, Western blot analysis with IκBα and phospho-IKK antibodies was performed on total extracts. Detection of a nonspecific (NS) band or of ERK was used as a loading control. B, Before starting the inductions, cells were treated for 1 hour with cycloheximide (CHX), or its solvent (DMSO), at a concentration of 20 ng/ml. Western blot analysis with an IκBα antibody was performed on total extracts. The detection of β-catenin (β-cat) protein levels was used for verifying CHX functionality. Equal loading was determined by the detection of ERK or tubulin (tub). C, At 30 hours before starting the inductions, FLS were transfected with control small interfering RNA (siCtrl) or small interfering RNA against GR (siGR), as indicated. Western blot analysis with IκBα, phospho-IKK, and GR antibodies was performed on total extracts; p65 was used as a loading control. See Figure 1 for other definitions.

Figure 3A: Western blot analysis shows IκBα degradation with time in samples treated with DEX, CpdA, or EtOH control solvent. IκBα levels decrease over time in samples treated with DEX or CpdA, but not in samples treated with EtOH control solvent.

Figure 3B: Western blot analysis shows the effects of CHX and DMSO on IκBα levels. CHX treatment results in IκBα degradation, while DMSO treatment does not.

Figure 3C: Western blot analysis shows the effects of siGR and siCtrl transfections. siGR treatment results in reduced IκBα levels compared to siCtrl treatment.

The text continues with detailed discussion of the experiments and results, mentioning the inhibition of NF-κB by CpdA and the role of GR in this process.

did not lead to lower IκBα levels (compare lane 4 with lane 6 in Figure 3A).

Since the concentration of IκBα in the cells after TNFα stimulation is the result of an equilibrium between cyclic events of IκBα degradation and IκBα resynthesis after binding of NF-κB to the IκBα promoter (43), we next determined whether these high levels of IκBα protein observed in the FLS after combined TNFα stimulation and CpdA treatment could result from an inhibition of IκBα degradation. CpdA, in contrast to the effects of DEX, indeed hampered the TNFα-induced phosphorylation of IKKα/β (compare lane 5 with lane 6 in Figure 3A).

To exclude the possibility that a fast resynthesis of IκBα might also be partially responsible for the effect of CpdA on IκBα protein levels, we pretreated the cells with cycloheximide (CHX) for 1 hour, before stimulation with TNFα and treatment with DEX or CpdA. The functionality of CHX was confirmed through the detection of β-catenin protein, which is known for its quick turnover rate (44). Higher levels of β-catenin were apparent in control-treated samples in comparison with CHX-treated samples, thereby showing that new protein synthesis was efficiently blocked (compare lane 13 with lane 14 in Figure 3B). Even in the absence of new protein synthesis, IκBα protein levels were much higher in the CpdA-treated TNFα-stimulated FLS compared with the DEX-treated TNFα-stimulated FLS (compare lanes 10 and 11 with lane 12 in Figure 3B). Treatment of the FLS with CpdA and stimulation with TNFα resulted in protein levels similar to the levels observed in the basal state (compare lane 1 with lane 6 in Figure 3B).

Since the effects of CpdA on the IκBα protein levels occurred rapidly and in the absence of new protein synthesis, we investigated whether these effects were GR-dependent. Consequently, the FLS were transfected with siGR or with nontargeting siCtrl (Figure 3C). Expression of the GR was indeed efficiently silenced in siGR-transfected cells (compare lane 4 with lane 6 and lane 10 with lane 12 in Figure 3C). In parallel, we measured the levels of phosphorylated IKKα/β and found that in siGR-transfected cells, the same pattern of regulation could be observed as that in siCtrl-transfected cells (compare lanes 1–6 with lanes 7–12 in Figure 3C). Treatment of the cells with CpdA hampered the TNFα-induced phosphorylation of IKKα/β in both siGR- and siCtrl-transfected cells (compare lane 4 with lane 6 and lane 10 with lane 12 in Figure 3C), an effect that could not be observed after DEX treatment (compare lane 4 with lane 5 and lane 10 with lane 11 in Figure 3C).

Effect of CpdA and DEX on MAPK activation.

MAPK pathways are considered important for fine-tuning the activity of NF-κB in the nucleus. Therefore, we investigated the effect of DEX and CpdA on activation of the MAPKs p38, ERK, and JNK (Figure 4A). Stimulation of the FLS with TNFα for 15 minutes resulted in a significant increase in the amount of phosphorylated p38, ERK, and JNK (compare lane 1 with lane 4 in Figure 4A). Interestingly, in RA FLS,
treatment with CpdA repressed the TNFα-induced phosphorylation of p38, ERK, and JNK almost completely, which is in contrast to the effects of DEX (compare lane 5 with lane 6 in Figure 4A).

In addition, we also investigated the effect of GR knockdown on the TNFα-induced MAPK activation status in the absence or presence of DEX and CpdA (Figure 4B), in the same experiment as depicted in Figure 3C. Intriguingly, similar to the effects on IKKα/β phosphorylation, treatment of the cells with CpdA could also block TNFα-induced MAPK activation, in both siCtrl-transfected and siGR-transfected cells (Figure 4B).

Antiinflammatory, GR-dependent effect of CpdA. We previously demonstrated that the gene-repressive effect of CpdA depends on the presence of functional GR. To underscore the functional necessity of GR in the antiinflammatory action of CpdA in FLS, the FLS were transfected with control small interfering RNA (siCtrl) or small interfering RNA against GR (siGR). At 30 hours thereafter, cells were treated with EtOH control solvent, DEX (1 μM), or CpdA (10 μM) for 1 hour, after which TNFα (2,000 IU/ml) was added for 6 hours. Total RNA was isolated and subjected to reverse transcription–polymerase chain reaction (PCR). A, Silencing of the GR was monitored by quantitative PCR analysis. The GR expression levels in siCtrl-transfected cells were set at 100%, and values in siGR-transfected cells were determined as the percent silencing relative to siCtrl-transfected samples. B, The amount of cDNA for the proinflammatory cytokine IL-1β after siCtrl or siGR transfection was measured by quantitative PCR. Expression levels in each treatment group were normalized to the values in the TNFα-stimulated control samples. Bars show the mean and SD results from triplicate experiments. † = P < 0.05; ‡ = P < 0.01, by one-way analysis of variance. NS = not significant (see Figure 1 for other definitions).
down-regulated by both DEX and CpdA, but in siGR-transfected FLS, both DEX and CpdA failed to exert an efficient repressive effect on this proinflammatory cytokine.

**DISCUSSION**

Since it has been repeatedly reported that GCs exert their beneficial effects, at least in part, via interference with the NF-κB activation pathway (27–31), a pathway that has been firmly linked to the pathogenesis of RA (9–17), we set out to unravel how the dissociative CpdA imposes its negative effect on the NF-κB activation pathway in human FLS. The results described in previous reports and in the present study clearly indicate that CpdA down-regulates the TNFα-induced and NF-κB-driven transcriptional expression of matrix metalloproteinase 1 (MMP-1), MMP-3, TNFα (35), IL-6, IL-8, monocyte chemotactic protein 1 (45), and IL-1β (Figure 1) to a similar extent as that after treatment with DEX in the ex vivo cell system. Despite the fact that the degree of response may vary when primary material isolated from different patients is studied, our findings confirm that both compounds can efficiently down-regulate the expression of TNFα-induced IL-1β expression in RA FLS.

Although the amount of IL-1β in FLS culture supernatants was too low for detection with ELISA, it is interesting to note that this cytokine is transcriptionally regulated by TNFα in these cells, and thus can be used for studying the NF-κB activation pathway in the cells (46,47). Indeed, even though the IL-1β promoter contains response elements for a variety of transcription factors, thereby allowing its regulation by different signaling pathways (48), the central role of the NF-κB pathway in IL-1β gene expression has repeatedly been recognized (42,49). Moreover, in FLS, it has been reported that TNFα-induced IL-1β gene expression can be ablated by expression of the super-repressor IκBα (16). These findings are supported by the results presented herein, which show that after stimulation of FLS with TNFα, an increased amount of p65 was recruited onto the promoter of IL-1β (Figure 2C).

We therefore determined the functional necessity of activated GR for the repression of inflammatory cytokine production by both DEX and CpdA, and used IL-1β as a paradigm. Our results underscore that knock-down of GR abrogates the repressive effects of both compounds on IL-1β expression (Figure 5). However, the molecular mechanisms by which DEX and CpdA affect the NF-κB activation pathway differ drastically.

Although DEX exerted a slight, but significant, effect on active DNA-bound p65 after 30 minutes or 1 hour of TNFα stimulation, as determined by TransAM assay (Figure 2B) and ChIP analysis (Figure 2C), we could not observe such an effect after 2 hours of TNFα administration (Figure 2B). These discrepancies regarding the degree of GC-mediated inhibition of NF-κB binding at different time points, and yet occurring in the same cell, are likely to depend on the repressive mechanisms that prevail in any given situation at any given time (for review, see ref. 50).

The lack of effect on DNA binding of p65 in the nucleus of FLS after 2 hours of TNFα stimulation (Figure 2B) is in concordance with our observations further upstream in the NF-κB activation pathway, showing that treatment of the cells with DEX did not hamper the TNFα-induced phosphorylation of IKK and the subsequent degradation of IκBα (Figure 3A). Our results are consistent with previous findings reported by Han and colleagues (47), who also failed to observe an effect of DEX on the IκBα expression levels and on NF-κB translocation and who subsequently concluded that steroids act independently of NF-κB to reduce inflammatory cytokine production in FLS. However, it is important to note that in recent years, evidence has accumulated to show that IκBα up-regulation does not constitute an exclusive mechanism to explain the cross-coupling between GR and NF-κB (27,28). It was shown, for example, that blockage of novel protein synthesis still allowed for an efficient DEX-mediated cytokine gene repression at the transcriptional level in a murine fibroblast cell line (29). As such, it may be more likely to assume that also in FLS, a nuclear interference between steroid-activated GR and NF-κB may exist, which may, but does not necessarily have to, preclude the DNA binding of p65. Indeed, depending on the time point studied, we observed that treatment of the FLS with DEX could result in a decrease in p65 DNA binding (Figures 2B and C), concomitant with the absence of a cytoplasmic effect, namely, an effect on IκBα protein levels, and with the absence of a p65 redistribution from the nucleus to the cytoplasm.

Interestingly, we found that the interaction of CpdA with the NF-κB pathway in FLS differed drastically from the pattern observed after DEX treatment. In sharp contrast to the effects of DEX, we observed that CpdA could attenuate cytoplasmic-to-nuclear translocation of p65. This observation is also reflected in the amount of activated p65 that could be found in the nucleus of the RA FLS (Figure 2A) and bound on DNA...
after CpdA treatment (Figures 2B and C). Indeed, both the TransAM assay (Figure 2B) and the ChIP analysis (Figure 2C) consistently showed a lower level of NF-κB DNA binding, regardless of the duration of TNFα induction.

We investigated the mechanism of CpdA-mediated p65 cytoplasmic retention in more depth, which revealed that CpdA interferes with the NF-κB pathway in the cytoplasm of the cell. We showed that CpdA attenuates IKK phosphorylation, and thereby hampers IκBα degradation (Figure 3A). This attenuation of degradation seems to be the only reason for the observed higher levels of IκBα, since blocking of new protein synthesis did not lower the IκBα levels after CpdA treatment (Figure 3B). As such, it is quite unlikely that a rapid resynthesis of IκBα is responsible for the higher IκBα levels observed.

Intriguingly, this rapid effect of CpdA on the phosphorylation of IKK seems to occur largely independent of the presence of the GR (Figure 3C). However, the above finding does not exclude an additional cross-coupling between CpdA-activated GR and NF-κB in the nucleus of FLS. This would be in agreement with previous results showing that CpdA, similar to GCs, interferes with the transactivation potential of a nuclear Gal4-p65 fusion protein, thereby ruling out the absolute necessity for cytoplasmic events in the negative interference between GR and NF-κB in L929xA cell lines (34). In line with this reasoning, we found that the gene-repressive effect of CpdA on TNFα-induced IL-1β expression was negatively affected when the levels of GR were knocked down (Figure 5). Moreover, consistent with a definite role for the GR in the gene-repressive effect of CpdA, we found an increased GR recruitment onto the TNFα-stimulated IL-1β promoter in the presence of CpdA (Figure 2C). Our results thus indicate that although the DEX-activated GR may partially interfere with p65 recruitment at the IL-1β promoter, without a subsequent redistribution of p65 into the cytosol, CpdA may also interfere with p65 recruitment via an additional partial interference with the upstream signaling pathways, resulting in a more cytoplasmic phenotype of p65 (Figure 2).

In addition, we observed that the 3 MAPK pathways, i.e., p38, JNK, and ERK, are efficiently activated by TNFα in FLS. Although it has been described that GCs interfere with MAPK signaling pathways, thereby hampering full-blown NF-κB activity, this effect seems to be cell type–dependent (31), and we could not observe such an effect in RA FLS. In contrast to what has been described by Toh and coworkers (51), who demonstrated a clear suppressive effect of DEX on the amounts of phosphorylated p38, JNK, and ERK, our data showed an almost negligible response to DEX on the level of inhibition of MAPK phosphorylation in FLS (Figure 4A). The differential results in these studies of FLS may be due to the fact that we pretreated the cells with DEX for 1 hour, whereas in the former study (51), a 24-hour pretreatment was applied, a strategy that does not exclude a role for secondary effects.

Notwithstanding these differences, investigation of the effect of CpdA on the MAPK pathways in FLS revealed that CpdA treatment resulted in a strikingly more efficient down-regulation of MAPK phosphorylation, and thus of MAPK activation, in comparison with the effects of DEX (Figure 4B). The observation that both DEX and CpdA suppress IL-1β cytokine mRNA expression equally well would suggest either that blockage of MAPK is not a determining factor for mediating efficient cytokine gene repression or, alternatively, that DEX could target still other transcription factors or cofactors present in the IL-1β promoter enhancerosome. Similar to the inhibition of IKK (Figure 3C), we found that the CpdA-mediated inhibition of activated MAPK also stayed invariable following knockdown of GR (Figure 4B), suggesting a dual mechanism of CpdA for interfering with the pathways leading toward NF-κB.

It has been observed, even in the same cell type, that different mechanisms can contribute to the GC-mediated inhibition of NF-κB at different time points (50). However, which mechanism predominates when a longer time period is assessed seems to be highly cell type– and promoter-specific. In this respect, results from our ChIP assay (Figure 2C) and siRNA analyses (Figure 5) suggest that for the repression of IL-1β, observed over a time span of 6 hours, the genomic effects of the GR, i.e., its interference with proinflammatory transcription factors, such as NF-κB, in the nucleus of the cell do seem to outweigh the rapid, GR-independent effects observed herein.

The present report thus describes 2 compounds with completely different structures, both of which exert their antiinflammatory effects in FLS via the same receptor, namely, the GR. However, in sharp contrast to the classic steroid DEX, CpdA is able to additionally interfere with the NF-κB activation pathway ex vivo in the cytoplasm of RA FLS. A dual pathway, partially dependent and partially independent of the GR, may therefore explain the gene-inhibitory effects of CpdA in RA FLS.
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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. De Bosscher had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES


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American College of Rheumatology Office Has Moved

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