A Plant-Derived Ligand Favoring Monomeric Glucocorticoid Receptor Conformation with Impaired Transactivation Potential Attenuates Collagen-Induced Arthritis

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Rheumatoid arthritis (RA) is a debilitating autoimmune disorder affecting ~1% of the Western population and characterized by destruction of articular cartilage and bone. This results in a significant loss of quality of life due to pain and reduced mobility. The prominent destruction of cartilage and bone is orchestrated by the hyperproliferative and invasive nature of fibroblast-like synoviocytes, which are important effector cells in inflammation and matrix degradation in rheumatoid arthritis.

In the present study, we report on a GR-binding, plant-derived compound with marked dissociative properties in rheumatoid arthritis fibroblast-like synoviocytes, which are important effector cells in inflammation and matrix degradation in rheumatoid arthritis. In addition, these findings could be extended in vivo in murine collagen-induced arthritis, in which joint inflammation was markedly inhibited without inducing hyperinsulinemia. Therefore, we conclude that GR monomers are sufficient for inhibition of inflammation in vivo. The Journal of Immunology, 2008, 180: 2608–2615.

The glucocorticoid receptor (GR) is a transcription factor regulating its target genes either positively, through direct binding to the promoter of target genes, or negatively by the interference with the activity of transcription factors involved in proinflammatory gene expression. The well-known adverse effects of glucocorticoids are believed to be mainly caused by their GR-mediated gene-activating properties. Although dimerization of GR is thought to be essential for gene-activating properties, no compound has yet been described which selectively imposes GR monomer formation and interference with other transcription factors. In the present study, we report on a GR-binding, plant-derived compound with marked dissociative properties in rheumatoid arthritis fibroblast-like synoviocytes, which are important effector cells in inflammation and matrix degradation in rheumatoid arthritis.


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of) hyperglycemia and hyperinsulinemia, and remains therefore one of the major drawbacks of long-term therapy, GC induce the transcriptional up-regulation of two rate-limiting enzymes involved in the gluconeogenesis, glucose-6-phosphatase (G6P), and phospho-enol pyruvate carboxykinase (PEPCK). By this specific pathway, the de novo synthesis of glucose is augmented (16, 17). This increase in the glucose concentration results, via the ATP-dependent K + pathway, in the pancreatic β cell, in an immediate secretion of insulin (18, 19), which compensates the hyperglycemic state. Therefore, the characterization and description of new drugs that specifically work through GR showing a selective antagonistic effect on transcription factors, but being devoid of its agonistic effects on GRE-driven genes, remains a major interest in GC research.

Recently, a plant-derived compound, compound A (CpdA), with GR-binding capacities but not belonging to the steroid class of GR-binding ligands, was found to be a selective GR modiﬁer. CpdA, or 2-(4-acetoxyphenyl)-2-chloroethylammonium chloride, is a stable analog of the hydroxypaphenyl aziridine precursor found in the Namibian shrub Salsola tuberculatiformis Botschantzev (20, 21). We previously described the anti-inflammatory effects and the lack of transactivation by CpdA in a fibrosarcoma and a lung carcinoma cell line (22). Additionally, CpdA was able to prevent the onset of paw swelling in a zymosan-induced arthritis model, which is based on the activation of the innate immune system. However, CpdA was administered before the irritant, and measurement of thickness of footpad was performed over a short period of time. Thus, it remained unresolved whether the compound would also be able to modulate an ongoing, formed over a short period of time. Thus, it remained unresolved whether the compound would also be able to modulate an ongoing, more sustained, and Ag-driven inflammatory process. Therefore, CpdA was evaluated in the collagen-induced arthritis (CIA) model by using a therapeutic treatment protocol rather than a preventive treatment regimen. This animal model has been widely studied as a model of RA, largely on the basis of several immunological key similarities with RA. First, susceptibility to both diseases is determined by various investigators in the joint inflammation and destruction occurring in RA pathophysiology (1, 2).

We show in the current article that CpdA, through activation of GR, is able to repress inflammation to a similar extent in vivo, as it does ex vivo. In addition, we present data suggesting that the mechanism of action involves the exclusive activation of monomeric GR moieties. Consequently, our data strongly support the hypothesis that disruption of dimerization can be associated with truly dissociated properties, ex vivo as well as in vivo. To our knowledge, this is the first molecule in which the dissociated action of a selective GR agonist can be linked directly to a selective inhibition of GR dimerization.

Materials and Methods

Cytokines and reagents

Recombinant murine TNF was produced in our laboratory (28). Dexamethasone (DEX) was purchased from Sigma-Aldrich. CpdA was synthesized as described by Louw et al. (21). CpdA was lyophilized and stored at −70°C. Anti-GR (H-300) Ab was purchased from Santa Cruz Biotechnology.

Plasmids

The Flag-tagged GR construct (pEFFlaghGRα) was cloned by inserting a NcoI-XbaI GR fragment, obtained by PCR from pSVhGRα (obtained from Prof. W. Rombaums, Catholic University of Leuven, Leuven, Belgium), into a NcoI-SpeI digested backbone fragment of EF-Flag (a gift from Dr. C. Hill, Cancer Research U.K., London, U.K.). The pEFFlaghGRα plasmid was checked by standard sequencing analysis. The pCMX-GFP-GR construct was obtained from Dr. H. Tanaka (University of Tokyo, Tokyo, Japan).

Immunoprecipitation assay

Flag-tagged GR and GFP-GR or the empty vector, as indicated in the legend of Fig. 2, were transfected in HEK293T cells at equimolar amounts, using the calcium phosphate precipitation method, as described earlier (28). Cells were induced for 1 h with either solvent. 1 μM DEX or 10 μM CpdA, as indicated in Figs. 1–3. After the appropriate inductions, cells were lysed in buffer A (10 mM HEPES (pH 7.5), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.1% Nonidet P-40, and freshly added protease inhibitors Pefabloc and aprotinin). Lysis was allowed to continue for 15 min on ice, followed by two freeze-thaw cycles. A total of 30 μg of the lysate supernatants (as determined by Bradford analysis) was kept aside for input analysis, whereas 100 μg of protein lysate was incubated with 20 μl of M2 Flag beads (prewashed four times with buffer A in the presence of 0.5% BSA; Sigma-Aldrich). After overnight rotation at 4°C, complexes on beads were washed four times with buffer A, supplemented with 150 mM NaCl, and 0.5% Triton X-100. A total of 25 μl of a 2× Laemmli buffer was added to the beads and beads were boiled for 1 min at 95°C. Samples were loaded onto an 8% SDS-PAGE gel together with the control inputs of the total cell lysate.

Isolation and culture of FLS

Synovial tissues were obtained at joint replacement surgery from patients with active RA according to the revised criteria of the American College of Rheumatology (29). FLS were obtained by enzymatic digestion as previously described (30) and grown in DMEM (Invitrogen Life Technologies) with 10% FCS (Invitrogen Life Technologies) in a humidified atmosphere containing 5% CO2. Experiments were performed using FLS with a passage number ranging from 4 to maximally 8. At this stage, the FLS cell culture exists exclusively of FLS, because all possible contaminating non-FLS cells were removed during the subsequent passages (2). The study was approved by the local ethics committee. Informed consent was obtained from all participating patients.

Immunofluorescence analysis

The immunostaining technique was performed essentially as described before (31) by using a 1/200 dilution of the anti-GR Ab (Santa Cruz Biotechnology). Nuclei were visualized by 4,6-diamidino-2-phenylindole (DAPI) staining. Samples were analyzed by using a Zeiss Axioskop immunofluorescence microscope and assessment of intracellular localization of protein signal was performed using coded slides.

Mice

Male 8- to 12-wk-old DBA/1 mice were purchased from Janvier and housed following institutional guidelines. All animal procedures were approved by the institutional animal care and ethics committee.

Induction and analysis of CIA

Chicken collagen type II (CII; Chondrex) was dissolved in 0.1 M acetic acid at a concentration of 2 mg/ml by stirring overnight at 4°C. Male DBA/1 mice were immunized intradermally at the base of the tail with 200 μg of CII emulsified in CFA (Difco). At day 21, a boost with 200 μg of CII emulsified in IFA (Difco) was applied intradermally at the base of the tail. From day 18, mice were monitored daily for clinical symptoms of arthritis.

Once arthritis appeared, mice were randomized in one of the following treatment groups: PBS (200 μl of PBS), CpdA300 (300 μg of CpdA dissolved in 200 μl of PBS). In the acute model of arthritis (zymosan-induced arthritis model), i.p. administration of 300 μg was determined to be highly effective (22). In CIA, a daily dose of 100 μg injected i.p. was found to be not effective in inhibiting the inflammatory response, neither by clinical nor by histopathological analysis (data not shown). In contrast, administration of 1000 μg daily was found to be lethal. The general health condition of naive mice upon daily treatment of 300 μg of CpdA was monitored during 7 days. No gross abnormalities such as scruffy fur, swollen eyes, diminished motility, lack of explorative behavior, or a reduced body weight of >10% (B. Staels and G. Haegeman, unpublished results) were observed, which is in line with previous reports in rats (20). A detailed toxicity study, however, was beyond the scope of this study and remains to be conducted. Each of the three therapies was administered i.p. and both monitoring and treatment was continued for 8
subsequent days, after which the mice were euthanized. The clinical severity of arthritis per paw was graded according to standard evaluation procedures as follows: 0, normal paws; 0.5, edema and erythema in only one digit; 1, slight edema or erythema in at least some digits or slight edema involving the entire paw; 2, moderate or severe edema involving the entire paw; 3, severe edema involving the entire paw together with more than one digit. The total clinical severity score per animal was defined as the sum of the clinical severity of the four different paws. All clinical evaluations were performed by an investigator blinded to the treatment arm.

**Histological evaluation**

Knees were dissected post-mortem, fixed in 10% formalized saline, decalcified, dehydrated, and embedded in paraffin. Sections of 7 µm were made and stained with hematoxylin and safranin O. Serial sections were scored blinded by two investigators. Both the femorotibial joint and the femoropatellar joint were scored on a scale of 0 (no inflammation) to 3 (severe inflamed joint), depending on the number of inflammatory cells in the synovial cavity (exudate) and synovial tissue (infiltrate). Exudate and inflammatory infiltrate were both assigned individual scores. Loss of proteoglycans was scored on a scale of 0–3 ranging from fully stained cartilage to destained cartilage or complete loss of articular cartilage.

Cartilage destruction was scored on a scale of 0–3, ranging from the appearance of dead chondrocytes (empty lacunae) to complete loss of the articular cartilage. Bone erosion was scored on a scale of 0–5 ranging from no damage to a complete disruption of the bone cortex at different places.

**Quantitative RT-PCR analysis of FLS and liver tissue**

Incubation with TNF, where indicated (2000 U/ml), was preceded by inductions with DEX (1 µM) or CpdA (10 µM) for 1 (transrepression) or 24 h (transactivation). After inductions, total RNA was isolated with TRIZol reagent (Invitrogen Life Technologies) according to the manufacturer’s protocol. The mRNA was reverse transcribed by using Moloney murine leukemia virus enzyme (Promega). Gene expression of hypoxanthine phosphoribosyltransferase (HPRT) was used for normalization. For isolation of total RNA from murine liver samples, the tissue specimens were preserved in RNAlater (Ambion) with an RNeasy Minikit (Qiagen), subjected to DNase I treatment (RNase-free DNase set, Qiagen), and were stored at −80°C until analysis. cDNA from total RNA was synthesized. Briefly, 0.5 µg of oligo(dT) primers (Promega) was added to 1 µg of total RNA in 10 µl of diethyl pyrocarbonate water and incubated for 5 min at 65°C. The solution was then quickly transferred to ice and cDNA was prepared with Superscript II reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer’s guidelines. RNasin RNase inhibitor (Promega) was added at 40 U/µl.

Quantitative RT-PCR was performed with the Platinum qPCR Supermix-UDG with ROX (Invitrogen Life Technologies) and premade TaqMan probes (Applied Biosystems) according to the manufacturer’s guidelines. Specificity of the assays was confirmed by melting point analysis. Gene expression of the housekeeping gene ribosomal protein L13a and γ-actin was used for normalization.

**Statistical analysis**

All analyses were performed with the commercially available statistical package SPSS 12.0 and MedCalc (Mariakerke). Appropriate descriptive summary measures were calculated including means, medians, SEs, maximum scores, and areas under the curve. Differences between groups were explored by the one-way ANOVA for normally distributed continuous data and by means of the Kruskal-Wallis statistic and the Mann-Whitney statistic if normality could not be assumed from qq plots and the Kolmogorov-Smirnov statistic. Dichotomous or multinomial variables were evaluated using the Fisher exact test or the chi-square test, respectively. Cells were induced for 1 h with either solvent, 1 µM DEX, or 10 µM CpdA, where appropriate, as indicated in the figure. Upper panel, The coimmunoprecipitated Flag-tagged GR fraction (top band), interacting with the immunoprecipitated Flag-tagged GR fraction (lower band), trapped with M2 Flag beads, as the Western blot was developed using an anti-GR Ab, recognizing simultaneously the differently tagged GR species. Middle panel, The input lysates, detecting both Flag-tagged and GFP-tagged GR using an anti-GR Ab, and by means of the Kruskal-Wallis statistic and the Mann-Whitney statistic if normality could not be assumed from qq plots and the Kolmogorov-Smirnov statistic. Dichotomous or multinomial variables were evaluated using the Fisher exact test or the chi-square test, respectively. Cells were induced for 1 h with either solvent, 1 µM DEX, or 10 µM CpdA, where appropriate, as indicated in the figure. Upper panel, The coimmunoprecipitated Flag-tagged GR fraction (top band), interacting with the immunoprecipitated Flag-tagged GR fraction (lower band), trapped with M2 Flag beads, as the Western blot was developed using an anti-GR Ab, recognizing simultaneously the differently tagged GR species. Middle panel, The input lysates, detecting both Flag-tagged and GFP-tagged GR using an anti-GR Ab, and by means of the Kruskal-Wallis statistic and the Mann-Whitney statistic if normality could not be assumed from qq plots and the Kolmogorov-Smirnov statistic. Dichotomous or multinomial variables were evaluated using the Fisher exact test or the chi-square test, respectively. Cells were induced for 1 h with either solvent, 1 µM DEX, or 10 µM CpdA, where appropriate, as indicated in the figure. Upper panel, The coimmunoprecipitated Flag-tagged GR fraction (top band), interacting with the immunoprecipitated Flag-tagged GR fraction (lower band), trapped with M2 Flag beads, as the Western blot was developed using an anti-GR Ab, recognizing simultaneously the differently tagged GR species. Middle panel, The input lysates, detecting both Flag-tagged and GFP-tagged GR using an anti-GR Ab, and by means of the Kruskal-Wallis statistic and the Mann-Whitney statistic if normality could not be assumed from qq plots and the Kolmogorov-Smirnov statistic. Dichotomous or multinomial variables were evaluated using the Fisher exact test or the chi-square test, respectively.
FIGURE 3. CpdA induces nuclear translocation of GR in FLS. Cells were serum-starved for 24 h, after which they were treated with solvent, 1 μM DEX, or 10 μM CpdA for 1 h. After fixation, followed by Ab staining to detect endogenous GR protein, indirect immunofluorescence analysis was performed using an anti-rabbit Ab coupled to the fluorophore Alexa 488. DAPI staining was used for visualization of the cell nuclei. One representative experiment of three is shown.

FIGURE 4. Treatment with CpdA inhibits further development of arthritis. After onset of arthritis, mice were randomized in either a PBS, CpdA300, or DEX treatment protocol. Therapy was administered daily and i.p. and disease severity was monitored daily. Dots and bars represent mean ± SEM. (CpdA300 vs PBS, p < 0.001 on day 8).

by means of the χ² statistic. Exact inference was calculated when appropriate.

Results
CpdA displays potent anti-inflammatory properties in RA FLS, and leaves transactivation of GRE-driven genes unaffected

The potential of CpdA to inhibit inflammatory cytokine gene expression, via GR, has only been demonstrated using cell lines (22). Therefore, a further evaluation of CpdA in a physiologically relevant ex vivo model was desirable. FLS derived from RA patients are known to produce several inflammatory mediators and matrix-degrading proteases, such as matrix metalloproteinases (MMPs), which contribute significantly to inflammation and tissue destruction, characteristic for RA. Therefore, we evaluated whether CpdA would also be effective in inhibiting proinflammatory gene expression in a physiologically relevant ex vivo model, using FLS cells derived from RA patients. Fig. 1a shows that CpdA is equally potent as DEX to dramatically lower the transcription of TNF-α, MMP1, and MMP3 genes in TNF-induced FLS to basal, noninduced levels of transcription. In view of the well-known, dual activity of GR (activation of GRE-driven genes through direct DNA binding vs transrepression of NF-κB-driven genes through protein-protein interactions), we investigated whether this clear inhibition of inflammatory gene expression acts parallel to the GR-mediated transactivation. Therefore, GRE-driven gene transcription was analyzed in CpdA- vs DEX-treated FLS. Fig. 1b shows that, in sharp contrast to DEX, CpdA does not induce transactivation of GRE-driven genes, such as human placental alkaline phosphatase (hPAP), tyrosine aminotransferase (TAT) or Procollagen C-endopeptidase enhancer 2 (PCOLCE2) in FLS, when measured by quantitative PCR.

CpdA inhibits dimerization of GR

To explain the differential effects of CpdA as compared with the synthetic hormonal ligand, DEX, on gene expression at a molecular level, we hypothesized that CpdA, in contrast to DEX, might be capable of stimulating solely the monomeric form of GR. This would be in line with the data of Reichardt et al. (10), which have been obtained using GRdim mice. They could show that a dimerization-defective GR in vivo still allows transrepression of NF-κB despite a clear deficiency in mediating GRE-driven gene expression. To test this hypothesis for CpdA, we transfected two differently tagged GR species, Flag-tagged GR and GFP-tagged GR, at equimolar amounts into GR-negative HEK293T cells. After performing the appropriate inductions, we immunoprecipitated one type of GR with Flag beads and asked whether we could coimmunoprecipitate the other species of GR in a ligand-dependent manner (Fig. 2). We also verified that upon overexpression of HEK cells with GR, this protein functionally behaves similarly as in physiological conditions: cytoplasmic in noninduced conditions and translocated to the nucleus upon CpdA and DEX treatment (data not shown). In the noninduced state, in which GR is mostly cytoplasmic, GR molecules could be detected in a dimeric (or even multimeric, as we cannot exclude complex formation of multiple GR moieties) complex. As expected, saturating amounts of DEX increased, albeit only slightly, the level of dimerization. Interestingly, CpdA induction substantially diminishes the level of dimerization in a dose-responsive manner (Fig. 2). Similar results were obtained when GFP-GR was immunoprecipitated and when Flag-GR was detected by Western analysis (data not shown), confirming the validity of the assay. We therefore conclude that CpdA induction, as opposed to DEX induction, induces preferentially GR monomers, thereby not preventing its ability to induce nuclear translocation of the GR monomers.

Table I. Overview of pooled clinical data derived from four individual representative experimentsa

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Score Day Onset Arthritis (Severity Score) Mean ± SEM</th>
<th>Time to Onset (Days) Mean ± SEM</th>
<th>Score Day 8 of Arthritis (Severity Score) Mean ± SEM</th>
<th>AUC (Severity) Median 95% CI p Value</th>
<th>Paw Swelling Day 8 of Arthritis (mm) Median 95% CI p Value</th>
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<tbody>
<tr>
<td>PBS</td>
<td>1.73 ± 0.20 REF</td>
<td>26.2 ± 1.0 REF</td>
<td>5.85 ± 0.45 REF</td>
<td>29.75 ± 4.23 REF</td>
<td>1.0 ± 0.2 REF</td>
</tr>
<tr>
<td>CpdA300</td>
<td>1.53 ± 0.30 NS</td>
<td>27.7 ± 1.3 NS</td>
<td>1.46 ± 0.43 &lt;0.0001</td>
<td>1.00 ± 0.17 REF</td>
<td>0.0 ± 0.0–0.2 REF</td>
</tr>
<tr>
<td>DEX</td>
<td>1.70 ± 0.30 NS</td>
<td>25.9 ± 0.7 NS</td>
<td>0.06 ± 0.06 &lt;0.0001</td>
<td>0.00 ± 0.04 REF</td>
<td>0.0 ± 0.0–0.0 REF</td>
</tr>
<tr>
<td>Global</td>
<td>0.59b ± 0.30 NS</td>
<td>0.75b ± 0.06 NS</td>
<td>&lt;0.0001b</td>
<td>&lt;0.0001b</td>
<td>&lt;0.0001b</td>
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| a AUC, Area under the curve; CI, confidence interval; REF, reference; NS, nonsignificant.  
| b P test.      |                                                      |                                 |                                                     |                                                        |
| c Kruskall-Wallis test. |                                              |                                 |                                                     |                                                        |
CpdA markedly attenuates histopathology in CIA

After clinical assessment for 8 days, mice were sacrificed and knee joints were histologically examined to determine whether the amelioration of clinical disease activity was accompanied with reduced histopathological signs of inflammation and tissue damage. Analysis of sections derived from vehicle-treated animals revealed that 67% of the joints exhibited at least some signs of pathology, most frequently inflammatory symptoms, graded as influx of inflammatory cells in synovium (infiltrate) and joint cavity (exudate). Consequently, the majority of joints showed evidence of depletion of proteoglycans, destruction of articular cartilage, and bone erosions, often resulting in substantial loss of bone architecture (Fig. 5a). Only 33% of the joints from the PBS-treated control mice were found to be normal in all assessed parameters. In this analysis, DEX treatment was accompanied with normal histopathologic features in all animals. In sharp contrast to the control group, 79% of the joints from mice treated with 300 μg of CpdA demonstrated normal histopathological features (p = 0.020; χ² test, Fig. 5c), which is representatively depicted in Fig. 5b. The effects were most apparent with regard to the reduction in the degree of inflammation in the synovium and joint cavity but also on bone erosion, proteoglycan depletion, and cartilage destruction (data not shown).

In conclusion, the histopathological data indicate that treatment with CpdA conferred to a marked reduction in synovial inflammation and cartilage and bone destruction compared with the joints of PBS-treated mice.

CpdA does not transactivate GRE-driven genes in CIA

We previously described the absence of hyperglycemia in naive mice after 8 days of CpdA administration, while naive C57BL/6 mice i.p. injected with DEX developed hyperglycemia (22). Indeed, GC are known to induce gene transcription of key gluconeogenic enzymes in the liver, thereby promoting new synthesis of glucose. However, in certain strains of mice, detecting a clear effect of GC on the regulation of blood glucose levels has not been trivial because of the compensatory actions of insulin. In fact, the blood serum insulin level was found to be a more reliable indicator of GR effects on glucose metabolism (17). Therefore, serum levels of insulin were assessed. In line with the findings by Opferk et al. (17), DEX treatment was indeed associated with a profound increase in serum insulin levels compared with PBS (Fig. 6), whereas the blood glucose levels in DBA-1 mice were hardly affected (data not shown). By contrast, CpdA did not induce hyperinsulinemia in CpdA-treated CIA mice (Fig. 6). Consequently, in line with the observation that the dimerization of GR is selectively disrupted after CpdA induction, we further investigated whether...
this absence of hyperinsulinemia could be explained by the absence of induction of gluconeogenic enzymes in the liver. The mRNA levels of G6P and PEPCK, two key gluconeogenic enzymes, harboring both a GRE element in their gene promoter, were shown not to be up-regulated in CpdA-treated mice. In sharp contrast, DEX-treated mice showed up-regulated levels of G6P (median 0.80 vs 1.44, \( p = 0.048 \)), and to a lesser extent of PEPCK, as measured by quantitative RT-PCR analysis (Fig. 7). The differences in maximal responses to DEX between G6P and PEPCK genes are most likely due to differences in the promoter build-up of these GC-responsive genes.

Discussion

The present study describes several novel features of GR biology. We describe that the plant-derived GR agonist CpdA has marked dissociated properties ex vivo and in vivo and is able to attenuate ongoing joint inflammation in CIA. Most intriguingly, the effects of CpdA were found to be mediated by interference with GR dimerization, however, without affecting its nuclear translocation, providing a novel drug-induced mechanism of GR modulation. Consequently, CpdA did not induce hyperinsulinemia because of its inability to up-regulate gluconeogenic enzymes, which is in sharp contrast with the effect of corticosteroids.

In the early 1990s, it became clear that the anti-inflammatory mechanism by activated GR relies on its interference with transcription factors that drive proinflammatory cytokines, such as NF-\( \kappa \)B and AP-1 (by transrepression or tethering) (5, 33), whereas an important side effect, namely hyperglycemia and a generalized insulin resistance leading to diabetes mellitus, rather evolves from a direct transcriptional effect of activated GR on gluconeogenic enzymes, i.e., by transactivation (34). This insight initiated a new wave of pharmacological research directed toward the development of so-called dissociated ligands, favoring only GR-mediated transrepression of NF-\( \kappa \)B- or AP-1-driven gene expression (35–38). Surprisingly, not all steroidal ligands that had dissociated properties in vitro also displayed these characteristics in vivo, especially with regard to the side-effect profile (35).

We recently described the anti-inflammatory action by CpdA in fibrosarcoma and lung carcinoma cell lines. The molecular action mechanism of CpdA involves the interference with both NF-\( \kappa \)B transactivation and DNA binding on NF-\( \kappa \)B-responsive elements (22). In the current manuscript, we investigated the efficacy of CpdA in a therapeutic treatment protocol, in CIA, a murine model resembling human RA (23–27). CpdA proved to be a very potent anti-inflammatory compound in CIA, even after repeated administration, as judged by the disappearance of clinical signs of arthritis, which was not significantly different to the clinical outcome observed in DEX-treated mice. The anti-inflammatory potential was also reflected in the clearly better outcome on knee joint histology compared with PBS-treated mice. Our ex vivo experiments in RA FLS also confirmed the ability of CpdA to repress an ongoing inflammatory reaction as measured by the inhibition of the TNF-induced production of various proinflammatory molecules. In our earlier data using cell lines, we did not observe a stimulating effect of CpdA on the transactivation properties of GR. Here, we also describe the absence of induction of GRE-driven gene expression in FLS with CpdA and we evaluated the effects on gluconeogenesis, one of the most frequently occurring side effects of GC during the course of CIA treatment. Our in vivo analysis of G6P and PEPCK GRE-driven gene expression from liver isolated from arthritic mice demonstrated a clear lack of induction by CpdA in contrast to DEX. This resulted in a significantly lower concentration of insulin in CpdA-treated compared with DEX-treated mice. This finding further supports the idea that the risk of diabetes development is clearly lower with CpdA than with DEX treatment.

Our data clearly show that CpdA has a dissociated mode of action in different, physiologically relevant model systems. We suggest that this differential effect might result from the lack of induction of dimeric GR moieties with CpdA, as demonstrated by means of a coimmunoprecipitation assay, using differently tagged GR species. In the absence of ligand, GR is kept inactive in the cytoplasm of the cell, in a complex with other proteins such as immunophilins and heat shock proteins (39). Upon overexpression, we observed already a substantial amount of GR dimers (or multimers) in the cytoplasm in the uninduced state. Although it has been assumed for a long time that dimerization of the GR only occurs after binding to DNA in the nucleus, recent data seem to contradict this hypothesis. In fact, Savory et al. (40) actually demonstrated directly that GR dimerization already occurs in the cytoplasm of mammalian cells, thus independently of DNA binding. This observation is not completely unexpected, however, as even unliganded GR exists in various stoichiometric GR recep-tosome complexes, which may strongly depend on local GR protein concentrations and/or expression conditions (41). Moreover, GR dimerization in solution was found not to be exclusively ligand-dependent but also apparent when GR was dissociated from its chaperoning proteins through incubation with salt (40). Therefore, upon changing the cellular ratio between receptor and heat shock proteins in the favor of GR, the equilibrium probably shifts to dimer (or multimer) formation between unliganded GR molecules, which explains our results. Upon induction with its ligand DEX, a vast amount of GR dimers (or multimers) is formed, which translocate into the nucleus. Control experiments verified that upon DEX treatment, both Flag-GR and GFP-GR proteins fully translocate into the nucleus (data not shown). Savory et al. (40) showed before that steroid treatment induces the association of GR in solution into at least a receptor dimer, through an interface within a 35-aa region of the receptor hinge. In contrast, upon CpdA induction, the pre-existing GR dimers (or multimers) are actively disrupted to monomers, a process occurring in a dose-dependent manner. Experiments using dimerization-defective GR knockin
mice (11, 42) strongly supported the notion that, at the gene-regulatory level, GC-activated GR essentially displays a dual mode of action. Our data are in concordance with this hypothesis and even more, we demonstrate for the first time directly that a ligand for GR is able to selectively impose GR monomer formation, a phenomenon which may well be the molecular basis for the observed GR-mediated dissociative effects. Our current results thus suggest that a possible differential conformation of GR, induced by CpdA as compared with classical GC, does not support dimer formation. Crystalization studies will be very informative to firmly support this hypothesis.

Interestingly, although CpdA-bound GR is preferentially monomeric, CpdA was equally potent as DEX in inducing the translocation of GR to the nucleus, as was clearly apparent from the immunofluorescence studies in primary FL5 cultures. Our data thus also support the hypothesis that dimerization itself is not a prerequisite for nuclear translocation to occur, although we cannot distinguish between disruption of dimers/multimers occurring either just before or after the translocation of GR into the nucleus. More correctly, we can conclude that dimerization is not essential for the observed net nuclear retention of GR, as it is known that the GR protein, both in absence and presence of hormone, actually shuttles between the nucleocytoplasmic compartments (43–45). A combination of kinetic analysis, live tracking and performing fluorescence resonance energy transfer on differently tagged GR moieties upon different inductions may shed more light on this matter.

Altogether, our data strongly suggest that the use of CpdA is associated with truly dissociated properties at the gene regulatory level in vivo. We have provided molecular evidence that inhibition of GR dimerization by CpdA may well be responsible for the completely dissociated character of CpdA. In addition, we show that dimerization of GR is not an absolute prerequisite for the receptor to reside or remain in the nucleus. Therefore, our data highlight not only the potential of selective GR modulators, but also the ability of a GR ligand to favor a monomeric GR conformation with impaired transactivation properties.

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Disclosures

K. De Bosscher, W. Van den Bergh, and G. Haegeman own a patent on the therapeutic use of CpdA.

References


