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Dissociated glucocorticoids equipotently inhibit cytokine- and cAMP-induced matrix degrading proteases in rat mesangial cells

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Abstract

Dissociated glucocorticoids are synthetic ligands of the glucocorticoid receptor (GR) and which discriminate between transrepression and transactivation. These compounds were predicted to have large therapeutic benefits when compared to conventional glucocorticoids because of reduced side effects. In this study, we compared the transrepressive properties of different dissociated glucocorticoids on the interleukin-1β (IL-1β)-activated metalloproteinase-9 (MMP-9) and tissue plasminogen activator (tPA) expression in rat mesangial cells (MC). Both proteinases regulate the turnover of extracellular matrix (ECM). We demonstrate that the GR agonist RU 24858, equipotent to dexamethasone (DEX), exhibited strong suppressive effects on the IL-1β-induced MMP-9 and tPA mRNA levels concomitant with an inhibition of corresponding enzyme activities. In contrast, RU 24782 and RU 40066 exhibited weaker inhibitory activities on both proteinases. Mechanistically, the changes in MMP-9 expression level by different RUs were accompanied by an inhibition of cytokine-induced promoter activity indicating that the inhibition occurs on a transcriptional level. In parallel to the reduction in mRNA levels, we observed an attenuation of cytokine-induced DNA binding of nuclear factor kappa B (NF-κB) and reduced contents of the p65 subunit of NF-κB within cell nuclei. Along with these transrepressive activities RU 24858, RU 24782 and RU 40066 displayed similar transactivation potentials as indicated by induction of the glucocorticoid-inducible mouse mammary tumor virus (MMTV) reporter gene and by induced expression level of plasminogen activator inhibitor 1 (PAI-1). Interestingly, the different RUs affected the expression of cAMP-induced tPA and inducible NO synthase with the same potency as the IL-1β-induced protease expression thus indicating that these compounds equipotently modulate cytokine- and cAMP-driven gene expression.

Keywords: Dissociated glucocorticoids; Extracellular matrix; Proteases; Matrix metalloproteinase-9; Tissue plasminogen activator

1. Introduction

Glucocorticoids (GCs) are among the most potent drugs currently used for the treatment of allergic and chronic inflammatory diseases. However, long-term treatment with GCs is associated with numerous side effects, such as diabetes, impaired wound healing, osteoporosis, skin atrophy and muscle wasting. The last decade has provided important insights into the molecular basis of GC-mediated actions on gene expression. GCs exert most of their genomic effects by binding to the cytoplasmic GC receptor (GR) and the binding of the agonist induces the translocation of GR complexes to the cell nucleus (for review, see [1,2]). Within the nucleus, the ligand bound GR can bind as a homodimeric complex to conserved palindromic DNA sequences denominated as glucocorticoid responsive elements (GREs). Binding to this cis-acting promoter element in most cases leads to transcriptional activation of GC-responsive genes (''transactivation''). In contrast to transactivation, most of inhibitory effects exerted by GCs arise from the mutual interaction between the GR and transcriptional activators such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB). This kind of interaction of the GR with other transcriptional activators, which occurs independent from DNA-binding, is termed ''transrepression'' [3,4].

Consequently, the search for a novel class of GCs, the so called ''dissociated GCs'', which display reduced transac-

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tivation properties but still exert a full transrepression activity, have been pursued by pharmaceutical companies as a promising therapeutical approach [5]. Indeed, an in vivo relevance of independent responses of the GR causing either transrepression or transactivation could be demonstrated in a transgenic mouse model of dimerization-deficient GR ("$GR^{dim/dim}$") mice. Administration of GCs in these animals leads to a strong reduction of transactivation properties indicated by the loss of activation of GRE-driven target promoters but almost no changes in the anti-inflammatory response [6,7]. Pharmacologically, the concept of synthetic GCs, which exert dissociated agonistic properties, has been first described for the HOECHST MARION ROUSSEL GR agonists RU 24782, RU 24858 and RU 40066. All of these compounds have shown a high affinity to the GR and in parallel have exerted a strong inhibitory effect on the activity of AP-1 and NF-κB transcription factors [8,9]. However, reports about the simultaneous transactivation potential of these compounds have been quite controversial. Whereas first studies could demonstrate a clear reduction of transactivation properties in vitro and in vivo [8,9], a clear benefit with respect to reduced systemic side effects could not be confirmed by later studies thus indicating that the action profile of dissociated RU compounds is not rigid but may depend on the promoter and cell type used. [10]. Furthermore, the cellular contents of steroid metabolizing enzymes are thought to significantly differ between different cell types, which additionally may contribute to such discrepant observations [11].

In an attempt to better characterize the molecular mechanisms of transrepressive effects exerted by dissociated RU compounds we compared their inhibitory effects on cytokine-induced versus cAMP-induced gene expression. For this purpose, we tested for possible inhibitory effects of RU compounds on two prominent extracellular matrix degrading proteases within glomerular mesangial cells (MC), the matrix metalloproteinase-9 (MMP-9) and the tissue plasminogen activator (tPA). Both proteases play key roles in the pathological remodeling of extracellular matrix (ECM), which accompany inflammatory processes and importantly, both enzymes are potently inhibited by GCs by transcriptional mechanisms [12,13]. Furthermore, the expression of both proteases is strongly induced by pro-inflammatory cytokines, such as IL-1β and tumor-necrosis factor α (TNFα), mainly through an increase in gene transcription. In contrast to MMP-9, the expression of the serine protease tPA in addition to pro-inflammatory cytokines is strongly induced by cAMP elevating agents and cAMP-responsiveness is attributed to a cAMP-response element (CRE) within the tPA promoters of rat, mouse and human tPA genes [13,14]. In this study, we demonstrate that dissociated GCs equally can modulate cytokine- and cAMP-triggered gene expression. We furthermore demonstrate that RU compounds similarly interfere with the cytokine- and cAMP-induced DNA binding of different transcription factors. Besides the strong transrepressive activities the RU agonists RU 24858, RU 24782 and RU 40066 retained their ability to transactivate a mouse mammary tumor virus (MMTV)-driven reporter gene, which further indicates that the dissociating profile of these compounds is strongly cell type specific.

2. Materials and methods

2.1. Reagents

Human recombinant IL-1β was from Cell Concept (Umkirch, Germany). Dexamethasone was purchased from Calbiochem Novabiochem (Bad Soden, Germany). The N^2, 2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate was from Sigma-Aldrich (Deisenhofen, Germany). Antibodies used in this study were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

2.2. Cell culture

Rat glomerular MC were cultured as described previously [15] and grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 5 ng/ml insulin, 100 U/ml penicillin and 100 μg/ml streptomycin. Serum-free pre-incubations were performed in DMEM supplemented with 0.1 mg/ml of fatty acid-free bovine serum albumin (BSA) for 24 h before cytokine treatment. All cell culture media and supplements were purchased from Life-Technologies (Karlsruhe, Germany). Dexamethasone and synthetic GCs whenever used were given in pre-incubations of 30 min before addition of other agents. Determination of cell numbers was done by use of a Neubauer chamber.

2.3. SDS-PAGE zymography

Assessment of gelatinolytic activity of proteins from cellular supernatants was performed as described previously [16]. Proteins with gelatinolytic activity were visualized as areas of lytic activity on an otherwise blue gel. Migration properties of proteins were determined by comparison with that of prestained full range rainbow protein markers (Amersham Pharmacia Biotech, Freiburg, Germany). Photographs of the gels were scanned by an imaging densitometer system from Bio Rad Laboratories (München, Germany).

2.4. Measurements of tPA activity in MC supernatants by ELISA

Quantitative determination of tPA activity in the cellular supernatants was done using the Chromolize tPA-ELISA from Biopool (Umea, Sweden). Confluent MC ((1.0–

1.5) $\times 10^6$ cells) on six-well plates were incubated in DMEM without FCS and stimulated with or without agents for the indicated time periods. After the incubation 100 μl of supernatants were directly subjected to the microtest strip wells of the ELISA. Further measurements were performed following the manufacturer's instructions. The absorbance at 405 nm with a reference wavelength at 495 nm was measured and tPA activity was determined by a calibration curve using human single-chain tPA as a standard.

2.5. cDNA clones and plasmids

A cDNA insert for rat MMP-9 was generated as described recently [16]. A cDNA insert of 0.72 kbp of rat tPA was generated by reverse transcription from mRNA of MC stimulated with IL-1β using internal primers from the complete sequence of the rat tPA mRNA as described recently [17]. A Sma I-digested cDNA insert from pMac-NOS was generated as described previously [18]. A cDNA insert from mouse 18S rRNA was from Ambion (Austin, TX, USA).

A 1.3 kbp fragment of the 5′-flanking region of the rat MMP-9 gene was cloned as described previously [12]. The plasmid pHHluc derived from the mouse mammary tumor virus (MMTV) was used to determine transactivation activity of synthetic GCs [19].

2.6. Northern blot analysis

Total cellular RNA was extracted from MC using the tri-reagent (Sigma). Procedures for RNA hybridization were as described previously [16].

2.7. Western blot analysis

The total cellular level of IκB and PAI-1 were analyzed using total cellular extracts and Western blot analysis of different fractions was performed as described previously [12]. Cytoplasmic levels of IκB were analyzed using 50–100 μg of total protein from cytoplasmatic fractions. Nuclear cell extracts (20–30 μg) were used for detection of nuclear level of p65. Blots were successively probed with anti-p65 and anti-HuR antibodies to ensure equal sample loading.

2.8. Reporter gene assays

Transient transfections of MC were performed using the Effectene reagent (Quiagen, Hilden, Germany). Transfections were performed following the manufacturer's instructions. The transfections were done as triplicates and repeated at least three times to ensure reproducibility of the results. Transfection with pRL-CMV coding for Renilla luciferase was used for control of the transfection efficiency. Luciferase activities were measured with the dual reporter gene system (Promega) using an automated chemoluminescence detector (Berthold, Bad Wildbad, Germany).

2.9. Electrophoretic mobility shift assay (EMSA)

Preparation of crude nuclear extracts from cultured MC and subsequent EMSA was done as described [20]. The sequences of the double-stranded oligonucleotides used for EMSA were derived from the corresponding rat genes and were as follows (coding strands): NF-κB (MMP-9), 5′-TTGCCCCGTGGAATTCCCCCAAAT-3′ (corresponding to a region from −569 to −546) and CRE (tPA), 5′-TGT-GATTCGATGACGTCAGTACGGTGAATA-3′ (corresponding to a region from −195 to −166). For supershift analysis, 2 μl of the antibody was pre-incubated overnight with the nuclear extracts prior to the binding reaction with the labeled oligonucleotides.

2.10. Statistical analysis

Results are expressed as means ± S.D. Statistical analysis was performed using the Student's *t*-test and the analysis of variance (ANOVA) test for significance. The data are presented as relative induction compared to control conditions (*) or compared to IL-1β-stimulated values (#). p-Values ≤ 0.05 (*) (#) and p-values ≤ 0.01(**) (##) were considered significant.

3. Results

3.1. Antagonistic effects of different RU compounds

In rat MC, the expression of IL-1β-induced MMP-9 and tPA is potently inhibited by GC, predominantly on a transcriptional level. Furthermore, the GC-mediated inhibition of both genes is blocked by the GR-antagonist RU 38486 (mifepristone) demonstrating the involvement of a GR dependent mechanism [12,13]. In a first step, we compared the GC-antagonizing properties of different RU compounds, i.e. their capacity to interfere with the dexamethasone (DEX) inhibition of IL-1β-induced MMP-9 expression. MC was stimulated for 24 h with IL-1β (2 nM) in the presence of vehicle (control) or DEX (100 nM) with or without a 10-fold excess of the indicated RU compounds. The content of extracellular MMP-9 was subsequently monitored by zymography using gelatine as a substrate. As shown in Fig. 1 (upper panel), the IL-1β-mediated increase in the MMP-9 content, indicated by a lytic band at 92 kDa is substantially blocked by DEX. Whereas addition of RU 24858 and RU 24782 had no effect on DEX-mediated inhibition of gelatinolytic activity, co-incubation with either RU 43044 or RU 38486 almost totally reversed the DEX-mediated inhibition indicating a full antagonistic potential of the latter compounds. In

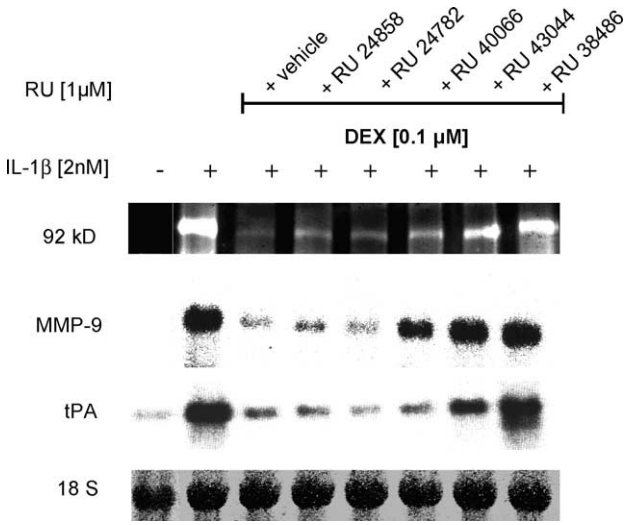

Fig. 1. Antagonistic effects of synthetic GCs on dexamethasone action. Quiescent MC were either left untreated (−), or treated for 24 h with IL-1β (2 nM) in the absence or presence of DEX (0.1 μM) without (“vehicle”) or with 1 μM of the synthetic GC as indicated. Ten microliter of supernatant were subjected to SDS-PAGE zymography to assess the contents of extracellular MMP-9 (upper panel). Migration properties of a lytic band at 92 kDa were determined by a standard molecular weight marker. The zymogram is representative for two independent experiments with similar results. Correspondingly, antagonizing effects on DEX-mediated suppression of IL-1β-induced MMP-9 and tPA mRNA steady-state levels were tested (lower panels). Total cellular RNA (20 μg) was successively hybridized to ^{32}P-labeled cDNA inserts from KS-MMP-9 and from KS-tPA and analyzed by Northern blot analysis. Equivalence of loading was ascertained by a final hybridization to a 18 S RNA probe. The blot is representative for two independent experiments.

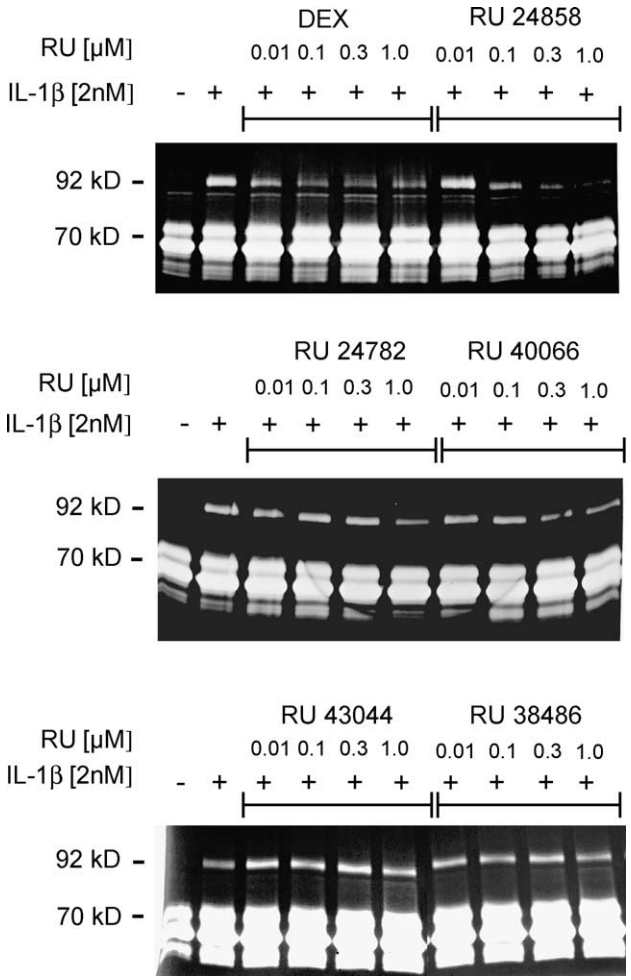

Fig. 2. Dose-dependent modulation of IL-1β-induced MMP-9 activity by synthetic GCs. Quiescent MC were stimulated with either vehicle (−) or IL-1β (2nM) (+) in the presence of the indicated concentrations of synthetic GCs. After 24 h, 10 μl of supernatants were collected for SDS-PAGE zymography The migration properties of lytic bands, corresponding to inactive pro MMP-9 (92 kDa), and the inactive and active forms of MMP-2 (72 and 68 kDa) were determined using standard molecular weight markers. The data shown are representative of three independent experiments giving similar results.

contrast, RU 40066 demonstrated weaker antagonizing effects on DEX-mediated MMP-9 inhibition (Fig. 1, upper panel). The corresponding Northern blot demonstrates similar effects on MMP-9 steady-state mRNA level with RU 43044 and RU 38486 exerting full antagonizing effects on DEX-mediated inhibition whereas RU 40066 only partially reversed the DEX-mediated suppression of cytokine-induced MMP-9 mRNA accumulation (Fig. 1, lower panel). In contrast, the GC-agonists RU 24858 and RU 24782 were unable to alter the DEX-mediated effects on MMP-9 mRNA thus confirming that both compounds possess no antagonistic potential. Similar to MMP-9, the DEX-mediated inhibition of IL-1β-induced steady-state tPA mRNA level was almost completely antagonized by RU 38486 whereas RU 24858, RU 24782 and RU 40066 did not alter the DEX-mediated suppression (Fig. 1). Interestingly, RU 43044, in contrast to exerting a full DEX-antagonizing effect on MMP-9 did not completely reverse the DEX-mediated suppression of IL-1β-induced tPA mRNA level (Fig. 1).

3.2. Inhibition of the lytic content of cytokine-induced MMP-9 by synthetic GCs

Next, we examined the dose-dependent modulation of extracellular MMP-9 content by DEX and different RU compounds by gelatine zymography. As shown in Fig. 2, supernatants of MC after stimulation with IL-1β contain both gelatinases, MMP-2 and MMP-9, which are characterized by their different migration properties in the zymogen gel at 72 and 92 kDa, respectively. In rat MC, MMP-2 is constitutively expressed and not affected by GCs. Therefore, the lytic activities derived from this gelatinase can serve as an internal control for equal loading of the zymography gels. By contrast, DEX and RU 24858 displayed similar inhibitory capacities in reducing the amount of extracellular MMP-9 with a strong inhibition already seen at a concentration of 0.1 μM (Fig. 2, upper panel). RU 24782 and RU 40066 were less effective and moderate inhibition of cytokine-induced MMP-9 was only seen with the highest concentration tested (1 μM) (Fig. 2, middle panel). As a negative control the GR-antagonists RU 43044 and RU 38486 displayed no inhibitory capacity on MMP-9

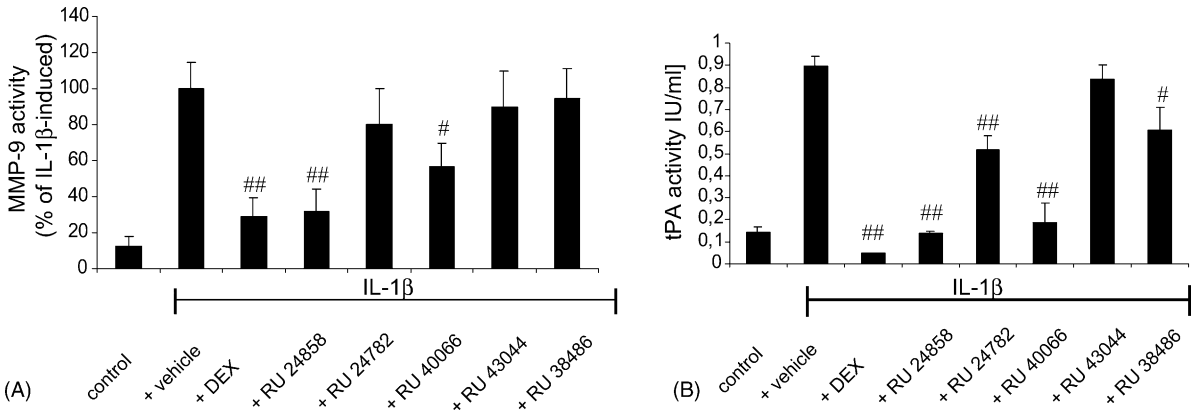

Fig. 3. Modulatory effects of dissociated GCs on the cytokine-induced activitiy of extracellular MMP-9 (A) and tPA (B). Quiescent MC were left untreated (control) or stimulated with IL-1β (2 nM) without (vehicle) or with either DEX (0.3 μM) or the indicated synthetic GC (0.3 μM). Ten microliter of cell culture supernatants were collected and either assayed for gelatinolytic activity (A) or alternatively, for extracellular tPA activity by Chromolize tPA-ELISA (Biopool) (B). Results are depicted as % of IL-1β-induced MMP-9 activity (A) or as absolute tPA activities (B) and are expressed as means ± S.D. ($n = 3$). $p \leq 0.05$ (#), or $p \leq 0.01$ (##) compared to the appropriate IL-1β-stimulated values.

activity but antagonized the DEX-mediated reduction of MMP-9 in a range from 0.3 to 1.0 μM (Fig. 2, lower panel).

3.3. Inhibition of extracellular MMP-9 and tPA activities by RU compounds

In a further experiment, we compared the transrepressive properties of different RU compounds on cytokine-induced MMP-9 and tPA secretion. To this end, DEX and all RU compounds were used in 300 nM concentrations and added 30 min before IL-1β stimulation. The enzyme activities within the conditioned medium of MC withdrawn after 24 h of stimulation were either tested by zymography (MMP-9) or by ELISA (tPA) as described in Section 2. Supernatants of MC under stimulatory conditions contained high level of MMP-9 (100% IL-1β-induced activity), characterized by a lytical band at 92 kDa, which corresponds to the latent form of MMP-9 (Fig. 3A, ''+ vehicle''). The simultaneous incubation of MC with DEX substantially reduced the IL-1β-caused lytic activity of MMP-9 by 72 ± 9%; $p \leq 0.01$; $n = 3$) and a similar inhibitory potential was measured with RU 24858 (70 ± 13% reduction; $p \leq 0.01$; $n = 3$). Whereas RU 40066 caused a moderate inhibition of MMP-9 activity the incubation with RU 24782, RU 43044 and RU 38486 did not significantly change the cytokine-induced MMP-9 content (Fig. 3A). Similar to MMP-9, the extracellular activity of tPA was strongly induced by IL-1β (Fig. 3B, ''+ vehicle'') and the cytokine-triggered increase in tPA activity was completely inhibited by DEX and by RU 24858. Interestingly, RU 40066 exerted a much stronger inhibitory effect on tPA activity (92 ± 8% reduction; $p \leq 0.01$; $n = 3$) than on MMP-9 and also RU 24782 significantly inhibited the IL-1β-dependent increase in tPA activity (48 ± 6% reduction; $p \leq 0.01$; $n = 3$). Surprisingly, whereas RU 38486 had no significant effect on

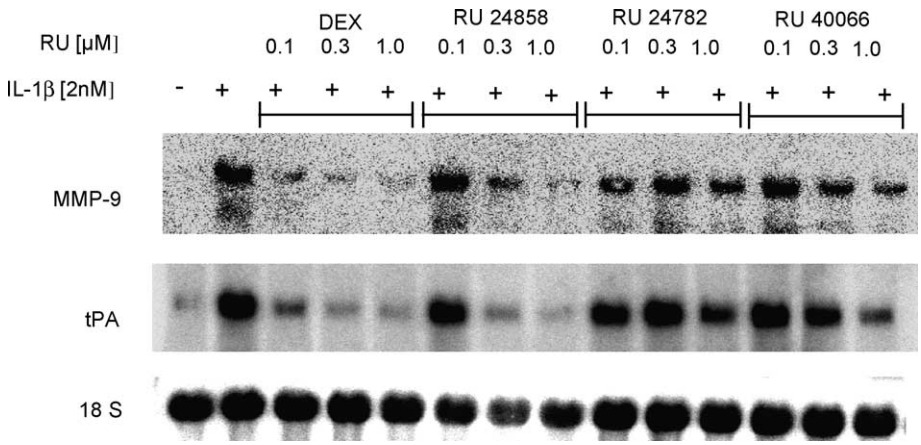

Fig. 4. Dose-dependent inhibition of IL-1β-induced MMP-9 and tPA expression in MC by dissociated GCs. Inhibitory effects on the IL-1β-induced protease mRNA steady-state levels by dexamethasone (DEX) and by the dissociated GCs RU 24858, RU 24782 and RU 40066. Quiescent MC were left untreated (−) or stimulated with IL-1β (2 nM) (+) without or with different concentrations of the GR-agonistic RU compounds as indicated. Total cellular RNA (20 μg) was successively hybridized to a ^{32}P-labeled cDNA insert from KS-MMP-9 or KS-tPA and analyzed by Northern blot analysis as described in Section 2. Equivalent loading of RNA was ascertained by rehybridization to a 18S RNA probe. The blot is representative of three independent experiments giving similar results.

MMP-9 (Fig. 3A) this pure GR antagonist caused a significant reduction of tPA activity (38 ± 8% reduction; $p \leq 0.05$; $n = 3$), thus suggesting that GR antagonists, independent from GR binding, can exert certain inhibitory effects on tPA activity.

3.4. Effects of dissociated GCs on steady-state MMP-9 and tPA mRNA levels

To test whether the inhibitory effects of dissociated GCs on MMP-9 and tPA secretion are caused by a suppression of corresponding gene expressions, we assessed their modulation of MMP-9 and tPA mRNA levels (Fig. 4). In accordance to the reported anti-inflammatory activity of different RUs in vivo the maximal inhibition on cytokine-induced MMP-9 and tPA mRNA by RU 24858 was comparable to DEX, although RU 24858 was less potent than DEX and strongest inhibition was observed in a concentration range from 0.3 to 1.0 μM (Fig. 4) [8,10]. RU 24782 and RU 40066 caused only weak inhibitory effects on MMP-9 and tPA mRNA levels when compared to RU 24858 (Fig. 4). The effects by these RU compounds on tPA activity were somewhat stronger than their inhibitory effects on the corresponding tPA mRNA levels, which may point to additional modes of action on post-transcriptional

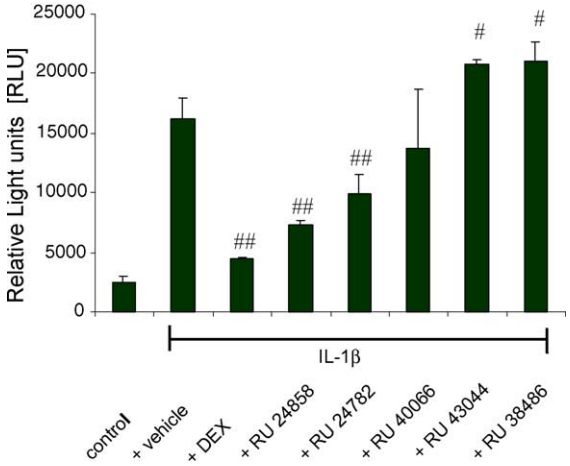

Fig. 5. Effects of synthetic GCs on IL-1β-induced MMP-9 promoter activity. Quiescent MC were transiently cotransfected with 0.4 μg of pGL-MMP-9 (1.3 kbp) and with 0.1 μg of pRL-CMV coding for Renilla luciferase. After overnight transfection, MC were left untreated (control), or treated for 24 h with IL-1β (2 nM) in the absence (''+ vehicle'') or presence of the indicated synthetic GC (0.3 μM) as indicated. Preparation of cells and subsequent measurement of dual luciferase activities was done as described in Section 2. The values for beetle luciferase were related to values for Renilla luciferase and are depicted as relative light units (RLU). Data represent means ± S.D. ($n = 6$). $p \leq 0.05$ (#) or $p \leq 0.01$ (##) compared to IL-1β-stimulated values.

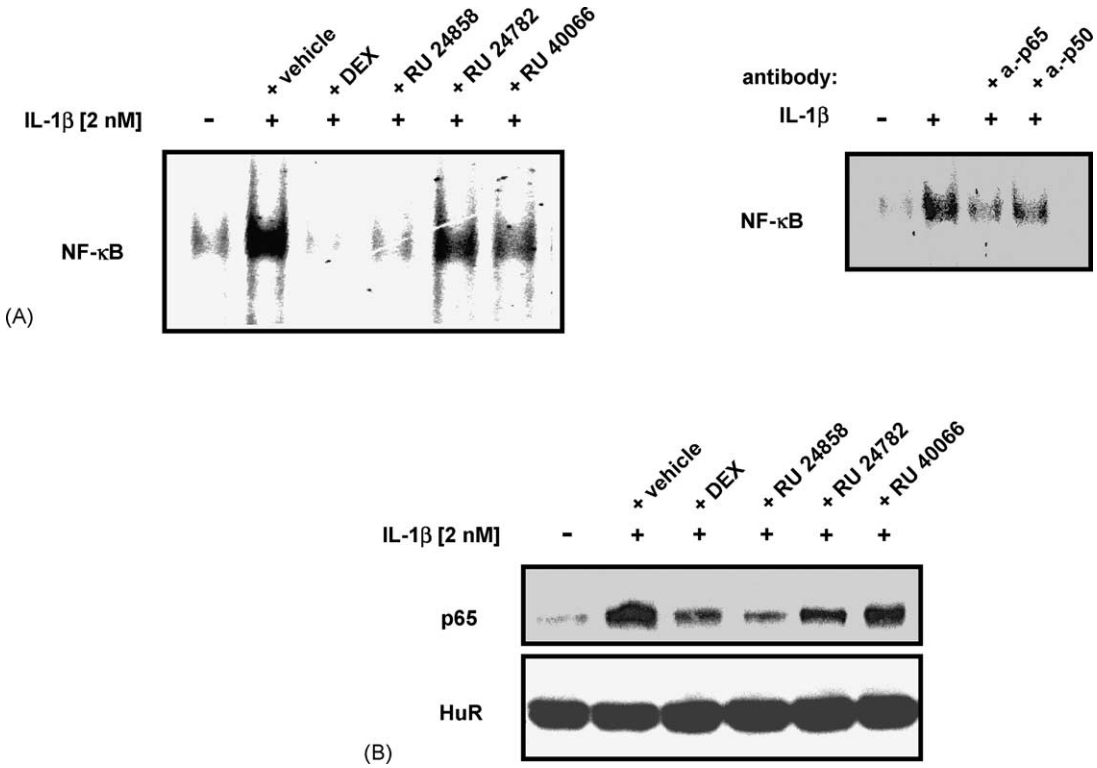

Fig. 6. Inhibition of IL-1β-induced NF-κB by the GR-agonistic RUs is paralleled by a modulation of cytokine-induced nuclear translocation of p65 (A). The DNA binding of NF-κB was analyzed by EMSA using a gene-specific NF-κB oligonucleotide from the rat MMP-9 promoter as described in Section 2. Serum-starved MC were stimulated with either vehicle (−) or with IL-1β (2 nM) in the absence or presence of the indicated GC (0.3 μM) for 1 h before being harvested for nuclear extract preparations. DNA–protein complexes were resolved from unbound DNA by non-denaturating gel electrophoresis as described in Section 2. Supershift analysis identifying p50/p65 heterodimers as the main components of IL-1β-induced complexes (right panel). For supershift analysis the antibodies were pre-incubated overnight at 4 °C before addition of the labeled probe. Panel (B) shows a Western blot analysis of the same nuclear extracts (30 μg) used for EMSA after immunodetection with anti-p65 and anti-HuR-specific antibodies, respectively.

and/or post-translational tPA regulation which is well documented for GCs [21,22]. Furthermore, all RU compounds with the exception of RU 38486 caused a clear increase in PAI-1 expression (Fig. 11), indicating that the modulation of tPA activity by RU compounds is additionally ensured by an increase of the intrinsic tPA inhibitor PAI-1.

3.5. Inhibition of cytokine-induced MMP-9 promoter activity by dissociated GCs

To further evaluate whether the inhibitory effects of RU compounds on IL-1β-induced MMP-9 expression occur on a transcriptional level, we performed reporter gene assays using a 1.3 kbp promoter fragment from the 5′-flanking region of the rat MMP-9 gene [12]. We recently have demonstrated that this part of the promoter is sufficient for the induction of MMP-9 by IL-1β and, furthermore, the cytokine-triggered activation of this promoter fragment is inhibited by DEX [12]. After transient transfection with pGL-MMP-9 1.3 kbp MC were treated for 30 min with either DEX (0.3 μM) or with different RU compounds (0.3 μM) followed by a 24-h stimulation with IL-1β (2 nM). Treatment with DEX caused a strong and significant reduction of cytokine-induced MMP-9 promoter activity (from 6.6- to 1.76-fold induction, $p \leq 0.01$) and a similar reduction of IL-1β-caused luciferase activity was observed with RU 24858 (from 6.6- to 2.9-fold induction, $p \leq 0.01$) (Fig. 5). Consistent with the weak inhibition of IL-1β-induced MMP-9 mRNA steady-state levels and the reduction of the gelatinolytic contents within the conditioned media, RU 24782 caused only a weak reduction of cytokine-activated MMP-9 promoter activity (from 6.6- to 4.2-fold induction, $p \leq 0.01$). In contrast, RU 40066 caused no significant effects on cytokine-induced MMP-9 promoter activity (Fig. 5). Interestingly, the GR-antagonists RU 43044 and RU 38486 caused an additional weak stimulatory effect on the IL-1β-mediated MMP-9 promoter activity. In summary, these data indicate that the transrepressive capacities of dissociated GCs are in close correspondence to their inhibitory effects on MMP-9 promoter activity.

3.6. Inhibition of NF-κB by dissociated GCs

Previous studies have demonstrated that the transrepressive potential exerted by dissociated GCs is mainly due to an interference with the transcriptional activators AP-1 and NF-κB [8,9]. In rat MC, the cytokine-induced expression of MMP-9 critically depends on the activation of both of

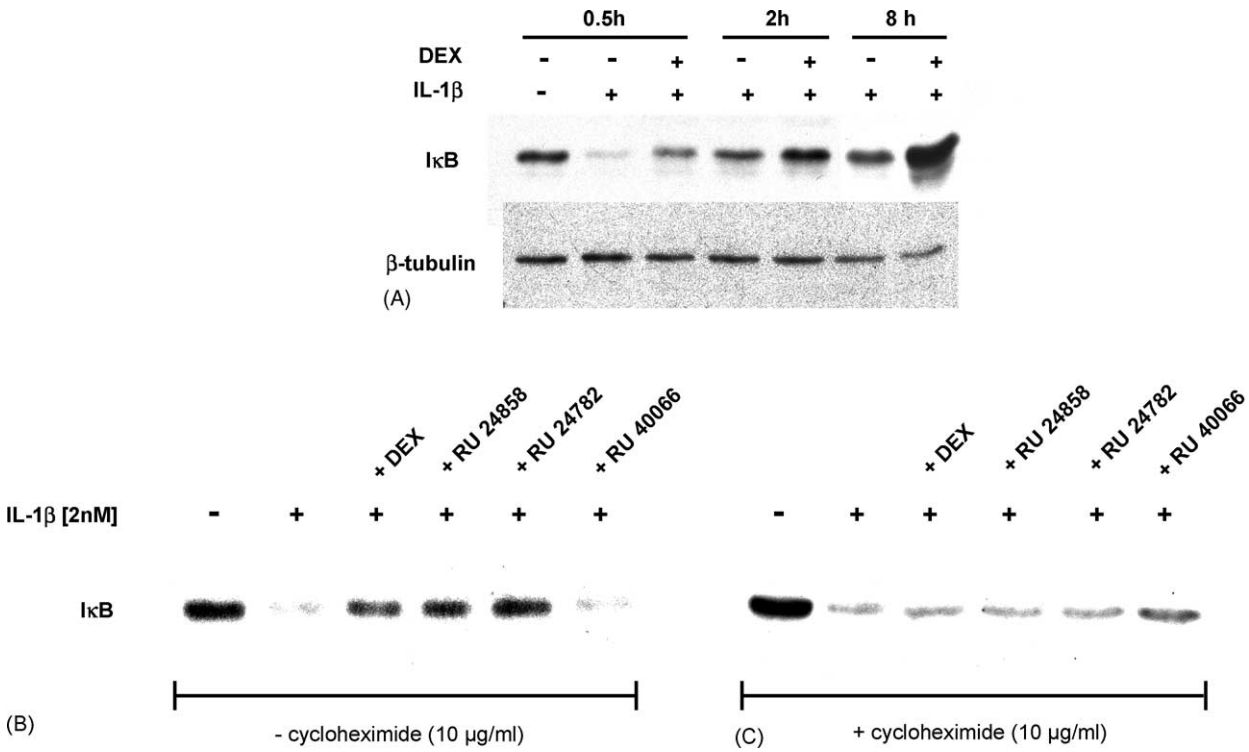

Fig. 7. Inhibition of cytokine-activated NF-κB by GR-agonists is paralleled by an increase in IκB synthesis (A). IL-1β-mediated degradation of cytoplasmic IκB is attenuated in cells simultaneously treated with DEX. Serum-starved MC were either left untreated (−) or treated with 2 nM of IL-1β (+) without (−) or with 0.3 μM DEX (+) for the indicated time periods. 100 μg of protein lysates from cytoplasmic fractions was subjected to SDS-PAGE and immunoblotted using an anti-IκBα-specific polyclonal antibody. To correct for variations in the protein loading, the blots were stripped and reprobed with an anti-β-tubulin antibody. (B) Effects of GR-agonistic RU compounds on IL-1β-mediated degradation of cytoplasmic IκB. MC were left untreated (−) or treated for 30 min with 2 nM of IL-1β (+) either alone or in the presence of the indicated GC (0.3 μM). Conditions for Western blot analysis were as described in (A). (C) Alternatively, serum-starved MC were treated as in (B) but in the presence of the protein synthesis inhibitor cycloheximide (10 μg/ml) which was added 30 min prior to the addition of the indicated synthetic GC. All blots shown in figure are representative for two experiments giving similar results.

these transcription factors [23]. To compare the inhibitory effects of synthetic GCs on NF-κB we performed EMSA using a probe encompassing a MMP-9-specific NF-κB binding-site [23]. Nuclear extracts from unstimulated cells or from MC treated for 30 min with IL-1β (2 μM) without or with DEX (0.3 μM) or different RU compounds (0.3 μM) were assessed for NF-κB binding. As shown in Fig. 6, treatment of MC with IL-1β caused a strong DNA binding of one complex containing p65 and p50 subunits as demonstrated by supershift analysis (Fig. 6A, right panel). MC treated in the presence of 0.3 μM DEX showed a complete inhibition in IL-1β-triggered DNA binding and a similar inhibition of DNA binding was caused by RU 24858 (Fig. 6A). In agreement with their weak inhibitory effects on MMP-9 and tPA expression levels, RU 24782 and RU 40066 caused only a weak suppression of IL-1β-induced DNA binding of NF-κB (Fig. 6A). In addition, the GC-mediated changes in DNA binding were attributable to the reduced nuclear content of p65 (Fig. 6B). As a control the nuclear content of the mRNA-stabilizing factor HuR, which is predominantly located in cell nuclei, remained unchanged thus indicating that alterations in the p65 level are not due to an overall change in the nuclear protein content.

3.7. Dissociated GCs inhibit IL-1β-induced degradation of IκB by increased IκB synthesis

Repressive activity of NF-κB by DEX in some cell types including rat MC, is attributable to the elevated levels of the intrinsic NF-κB inhibitor IκBα [3,12]. Fig. 7A demonstrates that in MC the IL-1β-evoked degradation of IκBα, which is maximal after 30 min of cytokine treatment, is blocked by DEX. Interestingly, the IκB level at the later time points (2 and 8 h) fully reappeared in the presence of DEX. Moreover, after 8 h of DEX treatment the IκB content was even higher than in untreated cells (Fig. 7A). Next, we investigated whether dissociated GCs, similar to DEX, could inhibit the cytokine-induced degradation of IκB. For this purpose, MC were treated with IL-1β alone or in presence of either DEX (0.3 μM) or different RU compounds (0.3 μM) for 30 min the time point when IκBα degradation is maximal (Fig. 7A). To furthermore test whether the inhibitory effects of RUs on IκB degradation depend on protein synthesis, MC were pretreated for 30 min with the protein synthesis inhibitor cycloheximide (10 μg/ml). As shown in Fig. 7B, RU 24858 and RU 24782, similar to DEX, prevented the IL-1β-caused decay of IκB. By contrast, RU 40066 was unable to affect IκB degradation although this GR agonist exerted a clear inhibitory effect on the DNA-binding of NF-κB (see also Fig. 6A). Interestingly, this RU compound consistently caused only a weak inhibition on p65 translocation (Fig. 6B), which suggests that the effects of RU 40066 on NF-κB binding at least partially occur independent of IκB degradation. Alternatively, we cannot exclude that the RU-specific differences in IκB degradation result from different time-courses in the RU-triggered recovery of IκB. Incubation with cycloheximide substantially prevented the GC-dependent upregulation of IκB but interestingly, had no inhibitory effects on the constitutive IκB level (−) (Fig. 7B and C). These data suggest that the effects of RU compounds on NF-κB mainly rely on new IκB synthesis but not on a direct interference with the proteosomal degradation pathway of IκB. To discriminate between RU-dependent IκB degrading and RU-dependent stimulatory effects on IκB synthesis we assessed whether RU compounds could affect the steady-state protein level of IκB (Fig. 8). To this end, MC were treated for 2 h in the absence (vehicle) or presence of either DEX (0.3 μM) or different RU compounds (0.3 μM). Similar to the changes observed in the cytoplasmic IκB level, RU 24858, RU 24782 and DEX had comparable stimulatory effects on the total IκB content which supports the notion that the repressive effects on IL-1β-induced IκB degradation primarily result from an upregulation of IκB synthesis (Fig. 8).

3.8. Dissociated GCs inhibit cAMP-induced tPA mRNA steady-state levels

In contrast to MMP-9, tPA expression is not only triggered by pro-inflammatory cytokines, but additionally by cyclic adenosine 3′,5′-monophosphate (cAMP)-elevat-

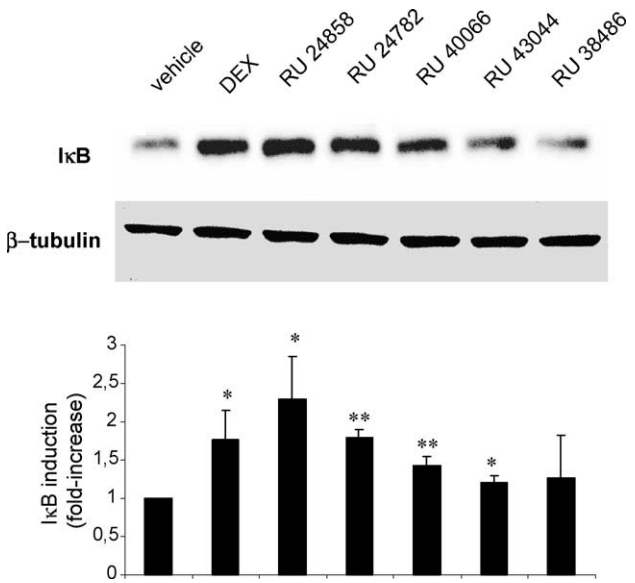

Fig. 8. Levels of total IκBα are modulated by synthetic GCs. The total amount of IκBα in quiescent MC were determined in cells either treated with vehicle, or with DEX (0.3 μM) or with the indicated synthetic GC (0.3 μM) for 2 h. Protein lysates (100 μg) were subjected to SDS-PAGE and immunoblotted using an anti-IκBα specific antibody. To correct for variations in the protein loading the blot was stripped and additionally incubated with a β-tubulin-specific antibody. An analysis of three independent experiments is shown in the lower panel. Data represent means ± S.D. ($n = 6$). $p \leq 0.05$ (*), or $p \leq 0.01$ (**) compared with vehicle.

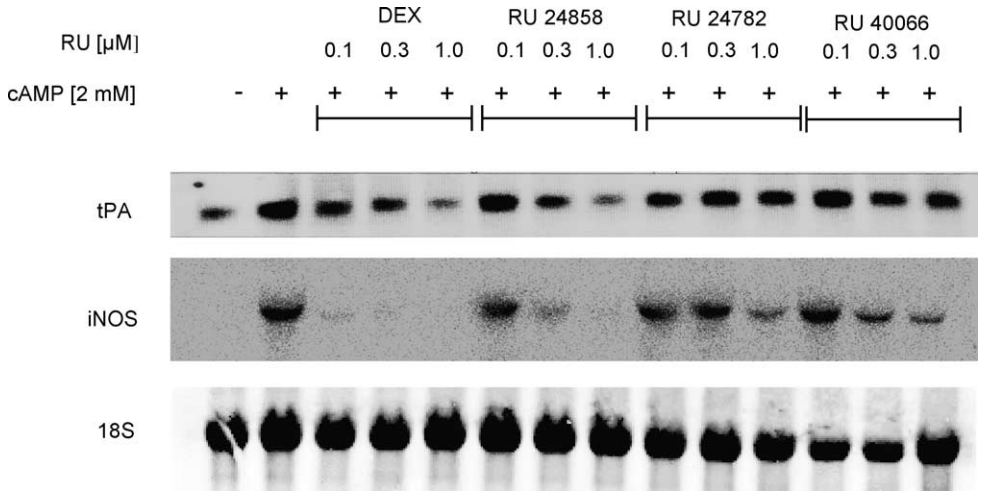

Fig. 9. Dose-dependent inhibition of cAMP-induced tPA and iNOS mRNA steady-state levels in MC by GR-agonistic RUs. Quiescent MC were left untreated (−) or stimulated for 24 h with the membrane permeable cAMP analog db-cAMP (2 mM) (+) without or with different concentrations of the indicated GR-agonist. Total cellular RNA (20 μg) was successively hybridized to a ^{32}P-labeled cDNA insert from KS-tPA or pMac-iNOS and analyzed by Northern blot analysis as described in Section 2. Equivalent loading of RNA was ascertained by rehybridization to a 18S RNA probe. The blot is representative of two independent experiments.

ing agents. We have recently demonstrated that GCs can inhibit cAMP-triggered tPA expression in MC mainly by suppressing the activity of the transcription factor CREB which is critically involved in the upregulation of tPA by cAMP [13]. To test whether different RU compounds similar to conventional GCs can affect the cAMP-triggered increase in tPA mRNA level MC were treated with different concentrations of dissociated GCs before stimulated with the membrane-permeable cAMP analogue dibutyryl-cAMP (db-cAMP). Similar to the inhibitory effects observed for DEX, RU 24858 at the highest concentration of 1 μM totally reversed the cAMP-mediated rise in the steady-state tPA mRNA level (Fig. 9). According to the effects on cytokine-mediated tPA, RU 24782 and RU 40066 were less efficient in inhibiting tPA mRNA levels. Again, RU 24782 exerted the weakest inhibitory potential also on cAMP-induced tPA expression (Fig. 9). In addition, we analyzed for possible modulatory effects of different RU compounds on the expression level of inducible NO synthase (iNOS) which similar to tPA is a gene highly induced by either pro-inflammatory cytokines or cAMP-elevating agents. Furthermore, the expression of both genes is inhibited by GCs [13,24]. Interestingly, the inhibitory effects on cAMP-induced iNOS mRNA levels by different GR agonists were much stronger than the reduction of tPA mRNA levels (Fig. 9).

3.9. Modulated DNA-binding of nuclear factors interacting with CRE by dissociated GCs

We further tested whether the inhibitory effects of dissociated GCs on cAMP-induced tPA expression were paralleled by an inhibition of the DNA-binding of CREB, which, at least in rat MC, is indispensable for cAMP-induced expression of tPA [13]. To this end we tested for possible changes of the cAMP-induced binding affinity to a tPA-specific CRE by different RU compounds [13]. The nuclear extracts derived from MC, which had been exposed to db-cAMP (2 mM) without or with either DEX (0.3 μM) or with different RU compounds (0.3 μM), were assessed for DNA-binding by gel shift assay. A co-incubation for 5 h was chosen in order to ensure maximal cAMP effects on

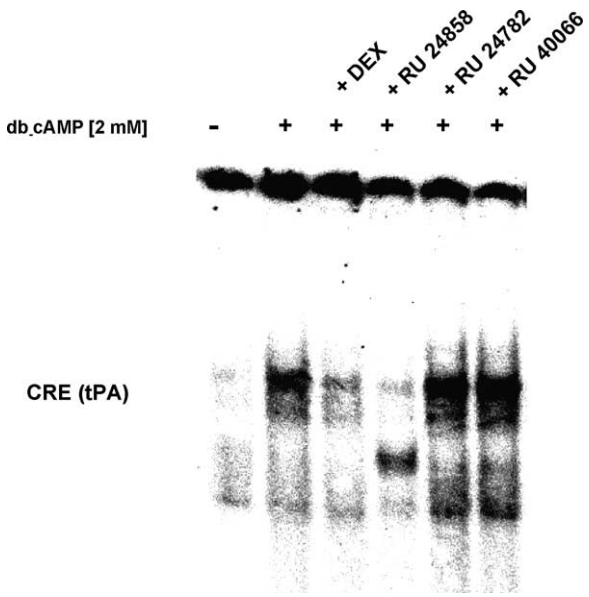

Fig. 10. Modulation of db-cAMP-induced DNA binding to a tPA-specific CRE by different GR-agonists. The sequence of a gene-specific CRE motif encompassing a region from −195 to −166 of the rat tPA promoter was used for EMSA as described in Section 2. Nuclear extracts were prepared from quiescent MC either treated with vehicle (−) or with db-cAMP (2 mM) without or with 0.3 μM of the indicated GC for 5 h before being harvested for nuclear extract preparations. DNA–protein complexes were resolved from unbound DNA by non-denaturating gel electrophoresis. The EMSA shown is representative of three independent experiments giving similar results.

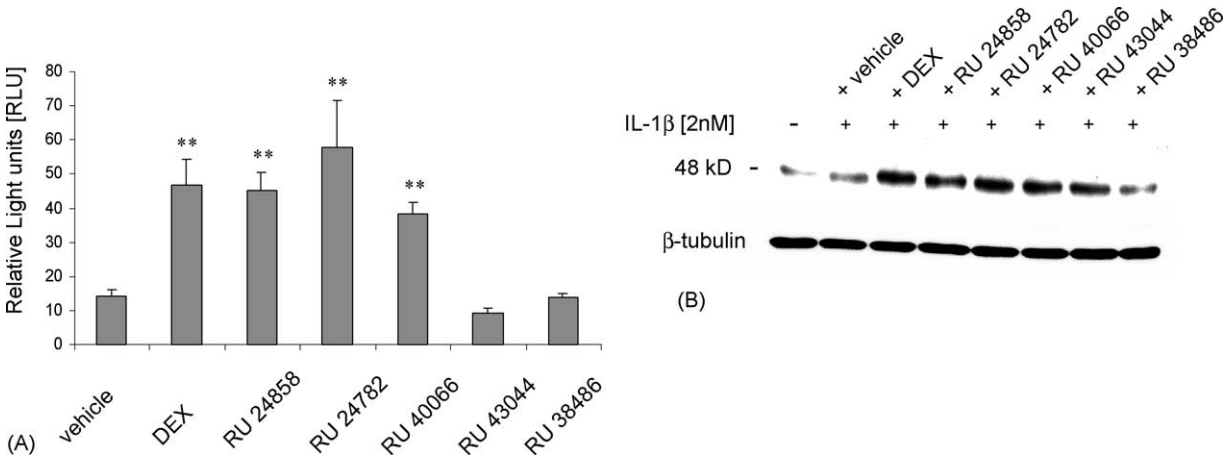

Fig. 11. Transactivation of GRE-driven MMTV-Luc promoter (A) and modulation of cytokine-induced PAI-1 (B) by synthetic GCs. (A) The luciferase activities of a MMTV-based pHHluc reporter gene were determined to estimate the transactivation potential of different synthetic GCs. Quiescent MC were transiently cotransfected with 0.4 μg of pHHluc and with 0.1 μg of pRL-CMV coding for Renilla luciferase. After an overnight transfection, MC were treated for 24 h with vehicle, or with 0.3 μM of the synthetic GC as indicated before being harvested for measuring dual luciferase activities. The values for beetle luciferase were related to values for Renilla luciferase and are depicted as relative luciferase activities. Data represent means ± S.D. ($n = 6$). $p \leq 0.05$ (*) or $p \leq 0.01$ (**) compared to unstimulated controls. (B) Serum-starved MC were either untreated (−) or treated for 24 h with 2 nM of IL-1β (+) without or with 0.3 μM of synthetic GC as indicated. 100 μg of protein lysates was subjected to SDS-PAGE and immunoblotted using an anti-PAI-1-specific polyclonal antibody. To correct for variations in the protein loading, the blots was stripped and re-incubated with an anti-β-tubulin antibody.

CRE binding [13]. Nuclear extracts from untreated MC exhibited a very weak constitutive binding of a main complex, which however, is strongly induced upon treatment of cells with db-cAMP (Fig. 10). This complex mainly consists of CREB and C/EBPβ transcription factors as we have recently shown by supershift analysis [13]. The DNA binding of this inducible complex was strongly reduced by DEX and by RU 24858 (Fig. 10). Interestingly, the treatment of cells with the latter GR agonist induced the DNA binding of an additional faster moving complex, which however, could not be characterized by supershift analysis (data not shown). In contrast to RU 24858, the GR agonists RU 24782 and RU 40066 did not exert any inhibitory effects on DNA binding (Fig. 10). These data implicate that suppressive effects of the dissociated GC RU 24858 on cAMP-induced tPA mRNA level result from an inhibition of the cAMP-triggered DNA-binding of CREB. Whether dissociated GCs in addition to CRE can impair the binding to other tPA promoter binding sites needs further investigations.

3.10. Induction of GRE-driven MMTV promoter by different GCs

To finally evaluate the transactive potential of the different RU compounds in MC we assessed their modulatory effects on the expression of the MMTV-based pHHluc reporter gene. This artificial promoter contains the mouse mammary tumor virus long terminal repeat under the control of several GC responsive elements and therefore is used to measure the transactivatory potential by GCs [19]. Exposure of transiently transfected MC to DEX (0.3 μM) for 5 h exhibited an overall three-fold increase in luciferase activity (3.0 ± 0.5-fold; $n = 3$; $p \leq 0.01$; Fig. 11A). Interestingly, a similar transactivation of MMTV promoter was measured after exposure of MC to RU 24858, RU 24782 and RU 40066 which were used at a concentration of 0.3 μM (Fig. 11A). In contrast, the GR antagonists RU 43044 and RU 38486 displayed no transactivation potential, thus confirming that these GR antagonists exert only a weak transactivation in MC (Fig. 11A).

3.11. Induction of PAI-1 level by different GCs

To further evaluate whether the observed transactivating potential on MMTV-promoter by RU agonists was only a promoter-specific phenomenon, we tested for possible stimulatory effects on PAI-1 expression. The PAI-1 promoter contains a functional GRE and therefore in many cell types is induced by GCs. MC were treated with IL-1β in the presence of either DEX (0.3 μM) or with different RU compounds (each at 0.3 μM) for 24 h, the time point where the amplificatory effect of DEX on cytokine-induced PAI-1 expression has reached a maximal level [13]. Whereas IL-1β itself caused only a moderate increase in basal PAI-1 levels, all synthetic GCs with exception of RU 38486, were able to further amplify the cytokine-induced PAI-1 level (Fig. 11B). This is in a certain contrast to the activation profile of RU compounds observed with the pHHluc reporter gene (Fig. 11A) since RU 43044, similar to RU 38486 was unable to cause a significant increase in MMTV-promoter activity. Therefore, we suggest that the transactivation properties of different synthetic GCs can vary depending on which promoter/enhancer environment is used for the evaluation of their transactivation capacity.

4. Discussion

In a first attempt, we have compared the antagonizing effects of different RU compounds on the DEX-mediated inhibition of cytokine-induced MMP-9. A full recovery of cytokine-induced MMP-9 expression is obtained by the classical GR antagonists RU 43044 and RU 38486 thus confirming that the DEX-mediated suppression of MMP-9 is completely exerted via GR binding [12]. Principally, most of genomic anti-inflammatory activities of GCs are due to a physical interference between the ligated GR and activating transcription factors most importantly, NF-κB and AP-1 [1,2]. MMP-9 similar to most of genes negatively regulated by GCs is under the transcriptional control of both of these transcription factors [23]. Additionally, by means of co-immunoprecipitation we previously could demonstrate that Ets-1 is a further target of negative regulation of MMP-9 expression by GCs [12]. Whether RU compounds can induce a similar physical interaction of the GR with Ets-1 remains to be investigated. In accordance to a recent study demonstrating a NF-κB-mediated inhibition of interleukin-6 expression by different RU agonists we found that RU 24858, RU 24782 and RU 40066 simlar to DEX potently inhibit the cytokine-induced MMP-9 on the protein and the mRNA level [9]. However, in rat MC the inhibitory effects by different GR agonists are paralleled by an interference with the DNA-binding of NF-κB and additionally accompanied by a reduction in the cytokine-induced accumulation of the NF-κB subunit p65 within cell nuclei. Both effects indicate that the GC-dependent inhibition of NF-κB DNA-binding in MC is caused by a sequestration of NF-κB in the cytoplasm thereby impeding its stimulus-dependent translocation into the nucleus. Similar to the inhibitory effects caused by DEX, the inhibited NF-κB binding activity by RU 24858 and RU 24782 is paralleled by an increase in the total IκB level indicating that IκB-dependent NF-κB inhibition is a functional mechanism of the GC-mediated MMP-9 repression. This is in a full agreement to recent studies by Saura et al. which have evidenced that the GC-dependent inhibition of cytokine-induced iNOS in rat MC is mainly attributable to an induction of IκB expression thus inhibiting cytokine-dependent DNA binding of NF-κB [25]. However, contradictory results have been published on the functional relationship between IκB expression levels and NF-κB/DNA binding since in most cell types the GC-dependent inhibition in NF-κB binding via tethering is independent of increased IκB expression (for review, see [26]). By co-immunoprecipitation experiments we have previously demonstrated a physical interaction of p65 with the GR in the nuclear fractions of cytokine plus DEX co-stimulated MC [12]. Similarly, in the same extracts the GR also co-immunoprecipitated with Ets-1 thus demonstrating a soluble interaction between the GR and different cytokine-induced transcription factors [12]. This suggests that independent from IκB upregulation a tethering GR is functionally involved in the DEX-mediated NF-κB inhibition in rat MC. Obviously, both mechanisms are operative in MC supporting the hypothesis that stimulation of IκB is an additional and strongly cell-type specific mechanism of GC-dependent NF-κB inhibition [27]. The observation that RU 40066, although displaying a significant inhibitory activity on NF-κB DNA binding, does not interfere with the cytokine-induced IκB degradation is further evidence that in MC, inhibition of NF-κB occurs independent from increased IκB expression.

Another important finding of this study is that dissociated GCs equipotently can inhibit MMP-9 and tPA expression levels which subsequently leads to a reduction in the respective extracellular protease activities. In contrast to MMP-9, the cytokine-induced tPA expression is thought to be independent form NF-κB promoter binding due to the lack of appropriate binding elements in the upstream promoter region of the rat tPA gene. Nevertheless, RU 24858, similar to DEX and to a lower extent RU 24782 and RU 40066 affected the cytokine-induced expression of tPA. The rat tPA promoter contains several putative regulatory elements which may be negatively targeted by GCs, among them AP-1, which besides NF-κB, is a further well-known target of the dissociated RU compounds [8]. In addition to AP-1, we have recently shown that C/EBPβ together with CREB binds to a composite CRE within the tPA promoter region and thus allow transcriptional activation of tPA by cAMP [13]. We suggest that C/EBP, in addition to cAMP-triggered tPA expression may also be critically involved in the cytokine-dependent expression of tPA. Here, we demonstrate that the cAMP-induced DNA binding to CRE is equipotently affected by DEX and RU 24858 and this capacity parallels the inhibitory effects of both compounds on cytokine-induced NF-κB (see Fig. 6A).

The transcriptional modulation of cAMP-induced tPA expression by GCs can be attributed to a CRE in a promoter region between −185 and −178 within the rat *tPA* gene [13]. Mechanistically, the modulation of CREB-induced expression by GCs in some genes can be explained by a composite GRE, which is occupied by the ligand bound GR, and thus sterically competes with the CREB binding to an adjacant CRE [28,29]. However, since the CRE region of the rat tPA promoter critical for cAMP-induction contains neither a complete GRE nor a GRE half-site, the inhibition via a composite GRE does most probably not account for the observed reduction in CREB binding. Furthermore, the CRE-bound complexes found in the nuclear extracts from DEX-treated MC do not contain immunopositive GR as tested by supershift analysis (our own unpublished observation). Finally, in addition to the direct inhibition of CRE binding competition between GR and CREB for a common transcriptional cofactor, e.g. the CREB binding protein 300 (CBP 300) may be critically involved in the GC-dependent inhibition

of tPA expression [26]. These results indicate that the simultaneous interference with different transcription factors may contribute to the validity of separating transrepression from transactivation within a specific cell type [8,10,11]. Among synthetic GCs which separate transactive and transrepressive properties the compounds RU 24782, RU 24858 and RU 40066 have demonstrated potent inhibitory effects on AP-1 and NF-κB binding activities in vitro paralleled by significantly reduced transactivation activity of GRE-driven reporter genes. Furthermore, these properties were assumed to confer a clearly improved profile when compared to conventional GCs, such as DEX [8,9]. However, the dissociated profile of these compounds observed in vitro so far was not coherent with the results from animal experiments since a clear benefit of these compounds could not be demonstrated in vivo [10]. Reasons for these discrepancies are still speculative although the metabolic conversion of synthetic GCs may additionally determine their transactivation activity in vivo. In this context, a recent in vitro study in accordance to in vivo studies could demonstrate a clear transactivation potential of the dissociated GR agonist RU24858 in CV-1 cells [11]. The latter observations fit to our finding that the transrepressive activities of the GC agonists RU 24858, RU 24782 and RU 40066 are still accompanied by a significant transactivation potential as indicated by activation of a MMTV-based pHHluc reporter gene [19]. The transactivating potential of these dissociated RU compounds is additionally indicated by upregulation of PAI-1 which in many cells is positively regulated by GCs. Therefore, we suggest that differences in promoter responsiveness may contribute to the inconsistent findings in respect to the transactivation capacity of dissociated RU derivatives.

This is in a line with the general assumption that the dissociated profile of a synthetic GC is not rigidly determined by its chemical structure but critically depends on the cell-type and most importantly, on the GC-responsive gene evaluated. In summary, our data highlight the complex roles of regulatory events influencing the validity and also the variabilities within the concept of ‘‘dissociated GCs’’.

Acknowledgements

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