Analysis of the Dissociated Steroid RU24858 Does Not Exclude a Role for Inducible Genes in the Anti-Inflammatory Actions of Glucocorticoids

Joanna E. Chivers, Wei Gong, Elizabeth M. King, Joachim Seybold, Judith C. Mak, Louise E. Donnelly, Neil S. Holden, and Robert Newton

Department of Cell Biology & Anatomy, Respiratory Research Group, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada. (W.G., E.M.K., N.S.H., R.N.); Department of Thoracic Medicine, National Heart and Lung Institute, Imperial College, Faculty of Medicine, London, United Kingdom (J.E.C., L.E.D.); Department of Internal Medicine/Infectious Diseases, Charite Universitaetskinderklinik Berlin, Humboldt-University, Berlin, Germany (J.S.); and Department of Medicine, Queen Mary Hospital, Hong Kong, China (J.C.M.)

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ABSTRACT

Although repression of inflammatory gene expression makes glucocorticoids powerful anti-inflammatory agents, side effects limit usage and drive the search for improved glucocorticoid receptor (GR) ligands. In A549 pulmonary cells, dexamethasone and the prototypical dissociated ligand RU24858 (Mol Endocrinol 11:1245–1255, 1997) repress interleukin (IL)-1β-induced expression of cyclooxygenase (COX)-2 and IL-8. Although RU24858 is a weaker GR ligand, both glucocorticoids showed similar efficacies on transrepression of nuclear factor (NF)-κB (NF-κB)-dependent transcription, whereas RU24858 yielded less than 12% of the response to dexamethasone on a classic glucocorticoid response element (GRE) reporter (transactivation). Modest NF-κB-dependent transrepression (~40%), along with analysis of IL-8 transcription rate and the accumulation of unspliced nuclear RNA, indicates that transrepression does not fully account for the repression of genes such as IL-8. This was confirmed by the finding that mRNA degradation is increased by both dexamethasone and RU24858. Analysis of IL-1β-induced steady-state mRNA levels for IL-8 and COX-2 show that dexamethasone- and RU24858-dependent repression of these genes is attenuated by inhibitors of transcription and protein synthesis. Because similar effects were observed with respect to COX-2 and IL-8 protein expression, we conclude that glucocorticoid-dependent gene expression is necessary for repression by both glucocorticoids. Despite RU24858 being defective at classic GRE-dependent transactivation, both dexamethasone and RU24858 induced the expression of potentially anti-inflammatory genes and metabolic genes, suggesting the importance of nontraditional glucocorticoid-dependent gene expression. Thus, classic transactivation- and transrepression-based screens for anti-inflammatory “dissociated” GR ligands may be flawed because they may not reflect the effects on real glucocorticoid-inducible genes.

Glucocorticoids suppress the expression and/or release of multiple inflammatory mediators, cytokines, chemokines, adhesion molecules, and other inflammatory proteins, making these agents first-line therapy in the treatment of inflammatory diseases such as asthma (Barnes, 2001; Rhen and Cidlowski, 2005). However, the clinical use of anti-inflammatory glucocorticoids is limited by numerous side effects, including osteoporosis, arterial hypertension, obesity, diabetes, cataracts, skin thinning, and muscle weakness (Barnes, 2001; Rhen and Cidlowski, 2005). Glucocorticoids are believed to act via the glucocorticoid receptor (GR), which in its inactive form is retained in the cytoplasm in a multiprotein complex containing heat shock proteins and immunophilins (Rhen and Cidlowski, 2005). After binding of steroid ligand to GR, a conformational

Abbreviations: GR, glucocorticoid receptor; AMP N, aminopeptidase N; AP-1, activator protein 1; COX, cyclooxygenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GILZ, glucocorticoid-inducible leucine zipper protein; GRE, glucocorticoid response element; IL, interleukin; MET 1X, metallothionein 1X; MAPK, mitogen-activated protein kinase; MKP-1, mitogen-activated protein kinase phosphatase-1; NF-κB, nuclear factor κB; PG, prostaglandin; PGES prostaglandin E synthase; PCR, polymerase chain reaction; RGS2, regulator of G-protein signaling 2; RT-PCR, reverse transcriptase-polymerase chain reaction; SFM, serum-free medium; ELISA, enzyme-linked immunosorbent assay; RU486, mifepristone.
change results in the dissociation of the complex and translocation of GR to the nucleus. Nuclear GR may then dimerize and bind specific DNA sequences, known as glucocorticoid response elements (GREs), to promote transcription (transactivation) of responsive genes (Rhen and Cidlowski, 2005). However, ligand-activated GR may also inhibit, or transpress, transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) via processes that are believed to be independent of GR dimerization and GR DNA binding (Barnes, 2001; Rhen and Cidlowski, 2005). Thus, transgenic (GRdim/dim) mice, which harbor an A458T mutation within the GR dimerization loop that renders GR defective in both DNA binding and classic GRE-dependent transcriptional responses, are still capable of repressing inflammatory gene expression (Reichardt et al., 1998, 2001). Furthermore, such dimerization loop mutations do not prevent glucocorticoid-mediated repression of AP-1 and NF-κB-dependent transcription (Heck et al., 1994; Reichardt et al., 1998, 2001). Because NF-κB and AP-1 are key regulators of many inflammatory genes, these data support the widely held belief that transrepressive mechanisms account for the dominant anti-inflammatory benefits of glucocorticoids (Barnes, 2001). Conversely, the undesirable metabolic effects of GR are often attributed to transactivation, and this concept has led to the search for novel, or disassociated, GR ligands that selectively activate the transrepressive but not the transactivation functions of GR (Heck et al., 1994; Vayssiere et al., 1997; Barnes, 2001; Uings and Farrow, 2005).

The continued analysis of transrepression now suggests that glucocorticoids may differentially affect the interaction with the basal transcriptional machinery, the phosphorylation of RNA polymerase II, the recruitment of coactivators/corepressors, and may involve chromatin remodelling via histone deacetylases to repress inflammatory gene transcription (De Bosscher et al., 2000; Nissen and Yamamoto, 2000; Adeeock et al., 2004; Garside et al., 2004). However, there is also considerable evidence that glucocorticoid-mediated repression of inflammatory genes involves significant post-transcriptional and/or translational mechanisms (Newton, 2000; Stellato, 2004). Thus, the repression of inflammatory genes, including cyclooxygenase (COX)-2, interleukin (IL)-8, and inducible nitric-oxide synthase, involves mechanisms that regulate mRNA stability (Ristimaki et al., 1996; Newton et al., 1998; Chang et al., 2001; Lasa et al., 2001; Korhonen et al., 2002). Furthermore, a requirement for de novo protein synthesis in glucocorticoid-dependent repression has been highlighted in several cases (Ristimaki et al., 1996; Newton et al., 1998; Newton, 2000; Chang et al., 2001; Lasa et al., 2001; Korhonen et al., 2002). In this context, glucocorticoids are known to target p38 mitogen activated protein kinase (MAPK)-dependent mRNA stabilization of inflammatory genes (Lasa et al., 2001). This occurs via the glucocorticoid-dependent induction of mitogen activated protein kinase phosphatase (MKP)-1, which in turn inhibits p38 MAPK and causes mRNA destabilization (Clark and Lasa, 2003).

In addition, a number of other genes that show anti-inflammatory properties are also known to be glucocorticoid-inducible and could contribute to the anti-inflammatory effects of these drugs (Newton, 2000; Clark and Lasa, 2003; Rogatsky et al., 2003). Therefore, the rationale for the use of novel dissociated GR ligands as improved anti-inflammatory compounds with reduced side effect profiles remains equivocal (Newton, 2000; Belvisi et al., 2001; Schacke et al., 2004). Therefore, we have chosen COX-2 and IL-8 as model inflammatory genes to examine the effect of RU24858, a prototypical dissociated glucocorticoid (Vayssiere et al., 1997). In this study, we specifically address the requirement for steroid-inducible gene expression in the repression by both dexamethasone and the dissociated ligand RU24858.

Materials and Methods

Cell Culture. A549 cells and stable reporter lines were grown as described previously (Chivers et al., 2004), and used at confluence after overnight incubation with serum-free media (SFM). Dexamethasone (Sigma, Poole, Dorset, UK) was dissolved in Hanks’ balanced salt solution (Sigma), RU486 (Sigma) was dissolved in ethanol, and RU24858 (a gift from Sanofi-Aventis Pharmaceuticals, Bridgewater, NJ) was dissolved in dimethyl sulfoxide. Drugs were added 1 h before stimulation with IL-1β (R&D Systems, Oxon, UK) unless otherwise stated. Final concentrations of dimethyl sulfoxide and ethanol were never in excess of 0.2% (v/v). This had no effect on GRE-dependent transcription, NF-κB-dependent transcription, or the expression of COX-2 or prostaglandin (PG) E2 release (data not shown).

Analysis of IL-8 and PGE2 Release, COX/Prostaglandin E Synthase Activity, and COX-2 Protein Expression. Culture medium was collected for measurement of IL-8 and PGE2 release by ELISA (R&D Systems, Minneapolis, MN) and radioimmunoassay (Sigma) using commercially available antibodies. For the measurement of combined COX/prostaglandin E synthase (PGES) activity, cells were rinsed with SFM, before incubation at 37°C for 10 min with SFM containing 30 μM arachidonic acid (Sigma). PGE2 release was measured as above and taken as an index of COX/PGES activity. For Western blot analysis of COX-2, total proteins were harvested in reporter lysis buffer (Promega, Madison, WI) supplemented with complete protease inhibitor cocktail (Roche, Mannheim, Germany), before analysis using Santa Cruz Biotechnology antibodies (Santa Cruz, CA) as described previously (Chivers et al., 2004).

Reporter Cell Lines and Luciferase Assay. A549 cells containing the NF-κB-dependent reporter 6xBtkluc, which contains six copies of the consensus NF-κB binding site (GGG ACT TTC C) derived from the human immunodeficiency virus enhancer have been described previously (Chivers et al., 2004). The GRE-dependent reporter, pGL3.neo.TATA.2GRE, contains two copies of a consensus GRE site (TGT ACA GGA TGT TCT) positioned upstream of a minimal β-globin promoter driving a luciferase gene and a separate neomycin gene to confer resistance to G418. A549 cells harboring this reporter have also been described previously (Chivers et al., 2004). Reporter cells were harvested in reporter lysis buffer (Promega) 6 h after treatment, and luciferase activity was determined using a commercial kit (Promega).

RNA Isolation, Northern Blot Analysis, and Nuclear Run on Transcription Assay. Total RNA was isolated using the RNeasy mini kit (QIAGEN Ltd., Crawley, UK). Northern blot analysis and nuclear run-on assays were performed according to standard procedures as described previously (Newton et al., 1998).

Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction. Total RNA (1 μg) was incubated at 70°C for 5 min before reverse transcription in a total reaction volume of 20 μl including 1 mM concentration of each dNTP, 10 ng/μl random hexamers, 0.4 U/μl avian myeloblastosis virus reverse transcriptase, 1× avian myeloblastosis virus buffer, and 1.6 U/μl RNasin (all from Promega). The reaction was incubated at 37°C for 60 min. Reverse transcription were terminated by heating to 90°C for 4 min, and the resulting cDNA was then diluted 1:5 with nuclease-free water.

PCR was carried out by adding 5 μl of diluted cDNA to 20 μl of reaction mixture giving final concentrations of 0.2 mM dNTPs, 1× NH4 buffer [16 mM (NH4)2SO4, 67 mM Tris-HCl, pH 8.8, and 0.1% (v/v) Tween 20], 1.5 mM MgCl2, 0.1 U/μl Taq polymerase, and 5 ng/μl
of both the upstream and downstream PCR primers (Sigma-Genosys, Cambridge, UK). The primer sequences are shown in Table 1. After an initial denaturation at 94°C for 2 min, RT products were amplified for 22 to 30 cycles (dependent on expression level of each gene) of PCR using 30-s denaturation at 94°C, 30-s primer annealing at 58 to 62°C (dependent on primer), 30-s primer extension at 72°C, and a final extension of 72°C for 10 min. Annealing temperatures were the following: COX-2, GAPDH, ROS-2, and GILZ, 58°C; IL-8, 60°C; MKP-1, 59°C; AMP N, 59°C; and MET IX, 62°C. PCR products were then subjected to electrophoresis on 2% agarose gels containing 0.5 μg/ml ethidium bromide and visualized using ultraviolet illumination and the GelWorks documentation and analysis system (GelWorks ID Intermediate, Cambridge, UK). In each case, the number of amplification cycles used was carefully optimized to allow the detection of cDNA within the exponential phase of the reaction (i.e., where starting cDNA concentrations are proportional to the product generated) and thereby enable relative quantification (see Supplemental Data for further details). Representative examples are shown and these provide validation of this approach (Supplemental Fig. S1, A and B).

**Real-Time TaqMan PCR Analysis.** After cDNA synthesis, above, TaqMan PCR was performed using 2.5 μl of cDNA in a reaction volume of 20 μl essentially according to the manufacturer's specification (Applied Biosystems Inc., Foster City, CA) using a premade master mix and an ABI 7900HT instrument (Applied Biosystems). Analysis of COX-2 and GAPDH was carried out using the validated off-the-shelf assays, hs00150153_m1 and 432631E, respectively (Applied Biosystems). IL-8 was amplified using the primers 5'-CTG GCC GTG GCT CCT CGT TTG-3' (forward) and 5'-TTA GCA CTC TTA GCA ACC AAC ACT-3' (reverse) with the 5-carboxyfluorescein/5-carboxytetramethylrhodamine-linked probe 5'-CCT TCC TGA CGT CCC TTT GC-3' (Applied Biosystems) such that one member of each pair was positioned within an exon and the other primer was positioned in the adjoining intron. This ensures that mRNA, which has undergone splicing to remove introns, cannot give rise to an amplification product. Primers spanning the IL-8 (accession number NM_000584.2) exon 1-intron A junction were 5'-CTC TTG GCA CAT CCT CGT AA-3' (forward) and 5'-CTC TTT CTG AAT AAA AAG GAT GTT GTG TAC-3' (reverse). Primers spanning the GAPDH (accession number NM_002046) exon 2-intron B junction were 5'-CCA CAT CGC TCA GAC ACC AT-3' (forward) and 5'-CGC TGA CCT TGA GCT CCT CTT-3' (reverse). Amplicon sizes were 151 and 184 base pairs, respectively. After PCR optimization and validation, 5-carboxyfluorescein/minor groove binder TaqMan probes (Applied Biosystems) for IL-8, 5'-TGA AGG TAA GCA CAT CCT TCT GAC CTA CAG GG-3', and GAPDH, 5'-TCA AGC CGG GAG GCT TGC-3', were designed using Primer Express version 2 software (Applied Biosystems). All samples were analyzed in duplicate. Relative cDNA concentrations were determined from a cDNA standard curve that was analyzed simultaneously with the test samples (see Supplemental Fig. S1, C and D, for an example).

**Analysis of Unspliced Nuclear RNA for IL-8 and GAPDH.** Cells were harvested by scraping on ice before a brief centrifugation. The cell pellet was resuspended in 150 μl of ice-cold 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1.5 mM MgCl₂, and 0.65% Nonidet P-40 supplemented with 20 U RNasin (Promega). After 10 min on ice and a 10-s vortex, the intact nuclei were pelleted at 13,000 rpm in a bench-top centrifuge. The cytoplasmic lysate was removed, and RNA was prepared using the RNAeasy kit (QIAGEN). The nuclear pellet was also processed according to the RNAeasy method, except that the on-column DNase I digestion was extended to 30 min. Preparation of cDNA was as above.

To detect the presence of unspliced RNA intermediates, amplification primers were designed using Primer Express software (Applied Biosystems) such that that one member of each pair was positioned within an exon and the other primer was positioned in the adjoining intron. This ensures that mRNA, which has undergone splicing to remove introns, cannot give rise to an amplification product. Primers spanning the IL-8 (accession number NM_000584.2) exon 1-intron A junction were 5'-CTC TTG GCA CAT CCT CGT AA-3' (forward) and 5'-CTC TTT CTG AAT AAA AAG GAT GTT GTG TAC-3' (reverse). Primers spanning the GAPDH (accession number NM_002046) exon 2-intron B junction were 5'-CCA CAT CGC TCA GAC ACC AT-3' (forward) and 5'-CGC TGA CCT TGA GCT CCT CTT-3' (reverse). Amplicon sizes were 151 and 184 base pairs, respectively. After PCR optimization and validation, 5-carboxyfluorescein/minor groove binder TaqMan probes (Applied Biosystems) for IL-8, 5'-TGA AGG TAA GCA CAT CCT TCT GAC CTA CAG GG-3', and GAPDH, 5'-TCA AGC CGG GAG GCT TGC-3', were designed using Primer Express version 2 software (Applied Biosystems). All samples were analyzed in duplicate. Relative cDNA concentrations were determined from a cDNA standard curve that was analyzed simultaneously with the test samples (see Supplemental Data for further details). Representative examples are shown and these provide validation of this approach (Supplemental Fig. S1, A and B).

**Microarray Analysis.** Total RNA (5 μg) was prepared as above and assayed for quality using RNA LabChips (Agilent Technologies, Palo Alto, CA). RNA was reverse-transcribed to generate cDNA and was subsequently transcribed in vitro to generate biotin-labeled cRNA before fragmentation and hybridization with the GeneChip expression arrays (Human genome U95Av2 and B arrays) as specified by the manufacturer (Affymetrix Inc., Santa Clara, CA). The array was subsequently washed and stained with a streptavidin-phycocerythrin-conjugated antibiotin to visualize hybridized cRNA, and the GeneChip was scanned to quantify gene expression. After global normalization, analysis was performed using the P-FOLD algorithm for Bayesian estimation of -fold changes (Theilhaber et al., 2001).

**Data Presentation and Statistical Analysis.** Densitometry was performed using TotalLab software (Nonlinear Dynamics, Newcastle, UK). All graphical data are presented as means ± S.E. Multiple comparisons were analyzed by analysis of variance with a Bonferroni post test, and paired analyses were by Mann-Whitney U test as appropriate.

**Results**

Dexamethasone and RU24858 Repress PGE₉ Release and Inflammatory Gene Expression. Previous studies have documented the repression of IL-6 and inducible nitric-oxide synthase by RU24858 and have suggested a lower potency, and possibly efficacy, relative to dexamethasone (Vanden Berghe et al., 1999; Korhonen et al., 2002). A549 cells were therefore stimulated with IL-1β (1 ng/ml) to induce the expression of the inflammatory genes, COX-2, and IL-8 after treatment with various concentrations of either dexamethasone or RU24858. Initially, PGE₂ release was measured, and in both cases, this was reduced to near basal levels (95 and 90% repression, respectively), suggesting similar levels of efficacy (data not shown). In contrast, EC50 values for
RU24858 and suggest that the differences in potency with respect to the above outputs are primarily accounted for by the lower binding affinity of RU24858 to GR.

The role of GR in the repression of COX-2 and IL-8 was further confirmed by analysis of the receptor antagonist, RU486. At concentrations of 1 μM and above, RU486 by itself may elicit repression of COX-2, NF-κB-dependent transcription, and IL-8 release (Chivers et al., 2004) (Fig. 2) (and data not shown). Therefore, the lower concentrations of 0.1 μM dexamethasone and 1 μM RU24858 were selected for analysis of antagonism by RU486. In each case, the repression of IL-1β-induced COX-2 and IL-8 mRNA by dexamethasone and RU24858 was progressively reversed by increasing concentrations of RU486 (Supplemental Fig. S3). Similar antagonism was revealed at the level of COX-2 protein expression, COX/PGES activity, and IL-8 release (data not shown).

RU24858 Is a Dissociated Steroid in A549 Cells. To examine the ability of RU24858 to activate classic GRE-dependent transcription, A549 cells harboring a previously characterized 2x GRE luciferase reporter were treated with increasing concentrations of dexamethasone or RU24858 (Chivers et al., 2004) (Fig. 2A). Dexamethasone resulted in greater than 25-fold activation of the reporter (EC50 = 93 nM). By contrast, RU24858 was essentially silent, giving rise to less than 12% of the response to dexamethasone at the maximum test concentrations. The steroid antagonist RU486 produced a concentration-dependent loss of GRE-dependent transcription produced by dexamethasone and failed to alter the already low RU24858 response (Fig. 2B).

Because IL-1β-dependent induction of COX-2 and IL-8 is highly NF-κB-dependent in A549 cells (Catley et al., 2005), we made use of a previously characterized NF-κB-dependent reporter to examine transrepression by RU24858 (Chivers et al., 2004). This luciferase reporter is driven by six classic NF-κB sites and is proven to be NF-κB-dependent (Catley et al., 2005). Reporter activity was partially repressed by dexamethasone to a level that was approximately 60% of the response to IL-1β alone (Fig. 2C). RU24858 also achieved this level of transrepression indicating that both steroids are equally transrepression-competent in this system. Although the relative potency of dexamethasone (EC50 = 2.2 nM) to RU24858 (EC50 = 1090 nM) was maintained, it was noteworthy that the response to dexamethasone showed more than a

![Fig. 1. Inhibition of IL-8, COX-2, and COX/PGES activity by glucocorticoids. A549 cells were treated with concentrations of dexamethasone (●) or RU24858 (○) for 1 h before stimulation with IL-1β (1 ng/ml) or not stimulated (NS). After 24 h, COX/PGES activity (A), COX-2 and GAPDH protein (B), and IL-8 release (C) were assayed by radioimmunoassay, Western blot, and ELISA, respectively. D, after 6 h, cells were harvested for Northern blot analysis of COX-2, IL-8, and GAPDH. All data are n = 5 to 8, * P < 0.05; **, P < 0.01; and *** P < 0.001.]
40-fold enhanced potency relative to that observed on the GRE reporter (i.e., on transactivation). This distinction between the potency for transactivation versus transrepression has been reported previously, but the mechanistic basis for this effect remains unclear (Jonat et al., 1990). Prior addition of RU486 resulted in a loss of transrepression by both dexamethasone and RU24858 at concentrations of up to 0.1 μM RU486 (Fig. 2D). Above this level, RU486 alone showed considerable ability to repress NF-κB-dependent transcription (Fig. 2D). Given that RU486 binds GR with a lower Kᵯ than dexamethasone (Chivers et al., 2004), these effects are likely to be nonspecific.

Although both the 2× GRE and the 6xBtk A549 transcripational reporters were shown to be dexamethasone-sensitive, the repression of IL-1β-induced PGE₂ release was also analyzed to confirm full dexamethasone-responsiveness of each reporter cell line. In each case, the inhibition curves produced by dexamethasone were virtually identical with wild-type A549 cells (Supplemental Fig. S4). Thus, these lines were fully competent for the above analyses.

Transcriptional Repression of Inflammatory Genes.

In terms of inflammatory gene expression, the above data suggest that classic GRE-mediated transcripational responses cannot, in this model, account for the ability of glucocorticoids to repress the expression of COX-2 and IL-8. However, the relatively modest repression of NF-κB-dependent transcription also seems insufficient to explain the full repression that is observed at the RNA and protein levels of these NF-κB-dependent genes. In a previous study, we showed that IL-1β-induced COX-2 transcription rate was also modestly (~40%) repressed by dexamethasone (Newton et al., 1998). We suggest that this is reasonably consistent with the observed repression of the NF-κB reporter. To provide further insight on the role of transcriptional repression, we performed nuclear run on analysis for IL-8 (Fig. 3A). These data, like those observed previously for COX-2, revealed a robust increase in IL-8 transcription after IL-1β treatment. After a 1-h preincubation with dexamethasone (1 μM), there was no obvious effect on IL-1β-induced IL-8 transcription rate at 2 h (Fig. 3A). By 6 h after stimulation, IL-8 transcription had decreased to ~50% of the response to IL-1β alone, and this is consistent with effects observed on the NF-κB reporter (above).

Analysis of Unspliced Nuclear RNA as a Surrogate of Transcription Rate. Various studies have documented the ability to detect unspliced mRNA precursors in the nucleus and have described the use of this approach as a surrogate for the nuclear run on assay (Lipson and Baserga, 1989; Elferink and Reiners, 1996). In pilot studies, we have been able previously to detect IL-1β-dependent induction of nuclear intermediates for COX-2 (data not shown), and such an approach has also been described for COX-2 in mouse fibroblasts (Gilbert et al., 1994). PCR primers were therefore designed that span the IL-8 exon 1-intron A and the GAPDH exon 2-intron B boundaries. The presence of these intermediates was then assessed in IL-8 mRNA prepared from the nuclei of cells that had been treated with IL-1β for 2 and 6 h or not treated (Fig. 3B). Qualitative PCR, using 32 cycles for unspliced IL-8, revealed low to undetectable levels of IL-8 product in untreated cells, but an intense band in IL-1β-treated samples. By comparison, the product for GAPDH seemed to be uniformly present in unstimulated and stimulated samples when analyzed using 34 cycles (Fig. 3B). In each case, product sizes were of the expected sizes, and amplification from samples prepared without reverse transcriptase resulted in either no or barely detectible product. To provide a more quantitative assessment of the relative levels of these products, TaqMan probes were designed that spanned the exon-intron boundary delimited by each set of amplification primers. TaqMan PCR analysis of the samples shown in Fig. 3B is depicted in the Supplemental Data (Supplemental Fig. S5). These data very clearly confirm the high level of IL-8 inducibility and furthermore show that genomic contamination is not a problem to the analysis.

To examine the effect of dexamethasone on the accumulation of nuclear intermediates, A549 cells were treated, as in Fig. 3A, with dexamethasone and then with IL-1β for either 2 or 6 h. After 2 h, the accumulation of unspliced IL-8 mRNA was hugely up-regulated from essentially undetectable levels in unstimulated cells (Fig. 3C). Prior treatment with dexamethasone produced a statistically significant, but rather modest, 28% drop in unspliced nuclear IL-8. By 6 h after stimulation, the level of unspliced IL-8 intermediates had decreased dramatically, presumably due to either reductions in the transcription rate or an increased efficiency of splicing.
However, the repression by dexamethasone was now increased to 60% of the IL-1β-treated value at 6 h (i.e., the level of repression exerted by dexamethasone on events in the nucleus was increased in a time-dependent manner). Taken together with the nuclear run-on data, these data support a modest initial repressive effect of dexamethasone but suggest that by 6 h, this repression may increase to between 50 and 60%. Parallel examination of cytoplasmic IL-8 mRNA revealed a considerably greater level of inhibition at each time point and suggest that events within the nucleus cannot fully account for the inhibition of cytoplasmic IL-8 mRNA by dexamethasone. Finally, analysis of RU24858, not previously possible using nuclear run-on, suggests a lesser inhibitory effect compared with dexamethasone (Fig. 3D).

Steroid-Dependent Repression of Inflammatory Gene mRNA Expression Is Blocked by Actinomycin D. In previous studies, the repression of IL-1β-induced steady-state COX-2 mRNA was profoundly repressed by dexamethasone despite only a 40% reduction in COX-2 transcription rate and even when added 2 h after the IL-1β stimulus (Newton et al., 1998). This repression was in part attributed to destabilization of COX-2 mRNA, and such effects are widely reported (Ristimaki et al., 1996; Newton et al., 1998; Lasa et al., 2001). Therefore, IL-8 mRNA expression was examined after treatment with IL-1β and dexamethasone, which was added 2 h after the IL-1β (Fig. 4A shows the treatment protocol). Unstimulated cells revealed low or undetectable levels of IL-8 mRNA, which were substantially elevated by 2 h of treatment with IL-1β and at time points thereafter (Fig. 4B). Steady-state mRNA levels reached a peak (2 + 3 h) after the addition of IL-1β and decreased thereafter. The addition of actinomycin D, an inhibitor of RNA polymerase II, 2 h after the IL-1β stimulus (t = 0) revealed that the IL-1β-induced IL-8 mRNA was relatively stable, and little decay was observed in these experiments (Fig. 4B). In marked contrast, the addition of dexamethasone initially had no effect on steady-state IL-8 mRNA levels induced by IL-1β, but after 1 h, any further increases in IL-8 mRNA were halted, and mRNA expression decreased over the next 3 h (Fig. 4B). Thus, simple blockade of RNA polymerase II-dependent transcription is insufficient for repression of IL-8 mRNA. This, along with the reporter, nuclear run-on, and nuclear RNA data, strongly implicates a requirement for additional post-transcriptional mechanisms of repression by glucocorticoids. In context of the current study, we wished to know whether the dexamethasone-dependent repression of steady-state IL-8 mRNA was dependent on ongoing gene expression. A549 cells were therefore treated with IL-1β for 2 h before the addition of various combinations of dexamethasone and actinomycin D and harvesting 6 h later (Fig. 4C). As above, IL-1β strongly induced IL-8, and this was not significantly affected by actinomycin D. Again, dexamethasone produced a robust repressive effect, and this

Fig. 3. Effect of dexamethasone on IL-8 transcription rate and accumulation of unspliced RNA intermediates. A, A549 cells were treated with dexamethasone (1 μM) (Dex) for 1 h before stimulation with IL-1β (1 ng/ml). Cells were harvested at the times indicated, and nuclei were prepared for run-off transcription reactions. Radiolabeled transcripts were hybridized to immobilized probes for IL-8 and GAPDH. Representative blots are shown, and after densitometric analysis, data from three such experiments, expressed as a percentage of IL-1β-stimulated value at 6 h (i.e., the level of repression exerted by dexamethasone on events in the nucleus was increased in a time-dependent manner). Taken together with the nuclear run-on data, these data support a modest initial repressive effect of dexamethasone but suggest that by 6 h, this repression may increase to between 50 and 60%. Parallel examination of cytoplasmic IL-8 mRNA revealed a considerably greater level of inhibition at each time point and suggest that events within the nucleus cannot fully account for the inhibition of cytoplasmic IL-8 mRNA by dexamethasone. Finally, analysis of RU24858, not previously possible using nuclear run-on, suggests a lesser inhibitory effect compared with dexamethasone (Fig. 3D).

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Fig. 3. Effect of dexamethasone on IL-8 transcription rate and accumulation of unspliced RNA intermediates. A, A549 cells were treated with dexamethasone (1 μM) (Dex) for 1 h before stimulation with IL-1β (1 ng/ml). Cells were harvested at the times indicated, and nuclei were prepared for run-off transcription reactions. Radiolabeled transcripts were hybridized to immobilized probes for IL-8 and GAPDH. Representative blots are shown, and after densitometric analysis, data from three such experiments, expressed as a percentage of IL-1β-stimulated value at 6 h (i.e., the level of repression exerted by dexamethasone on events in the nucleus was increased in a time-dependent manner). Taken together with the nuclear run-on data, these data support a modest initial repressive effect of dexamethasone but suggest that by 6 h, this repression may increase to between 50 and 60%. Parallel examination of cytoplasmic IL-8 mRNA revealed a considerably greater level of inhibition at each time point and suggest that events within the nucleus cannot fully account for the inhibition of cytoplasmic IL-8 mRNA by dexamethasone. Finally, analysis of RU24858, not previously possible using nuclear run-on, suggests a lesser inhibitory effect compared with dexamethasone (Fig. 3D).
was totally prevented by simultaneous addition of actinomycin D.

To further explore this effect, A549 cells were treated with dexamethasone or RU24858 in the presence or absence of IL-8 and COX-2 was examined by semiquantitative reverse-transcriptase polymerase chain reaction RT-PCR (Fig. 5). In these experiments, IL-1β induced the expression of both IL-8 and COX-2, and this was repressed by the simultaneous addition of dexamethasone (1 μM) or RU24858 (10 μM) (Fig. 5). The addition of either steroid at 2 h after the IL-1β resulted in a similar level of mRNA repression, and this effect was prevented by the presence of actinomycin D (Fig. 5). The addition of actinomycin D alone showed no significant effect on the expression of IL-8 or COX-2. Given the pivotal nature of this experiment, all cDNA samples were subsequently reanalyzed using TaqMan real-time PCR, and essentially identical data were obtained (Supplemental Fig. S6A). In addition, the existence of a feedback process resulting in the loss of steady-state IL-8 mRNA levels from 5 (2 + 3) h onward (Fig. 4B) increases the possibility of confounding factors. Therefore the above experiment was also conducted at a shorter time point (i.e., after only 3 h of steroid/actinomycin D treatment and only 5 h in total). Again, a qualitatively similar result was obtained (Supplemental Fig. S6B) and suggests that inhibition by both dexamethasone and RU24858 requires ongoing transcription at all time points after the addition of steroid.

Effect of Dexamethasone and RU24858 on mRNA Decay. The above data strongly implicate additional cytoplasmic mechanisms of repression to account for the full repression effects of glucocorticoids. To specifically test this hypothesis, classic actinomycin D chase experiments were conducted (Fig. 6). In the previous analyses (Figs. 4 and 5), the addition of actinomycin D at the same time as either dexamethasone or RU24858 resulted in no repression of COX-2 or IL-8 mRNA. Therefore, to examine the possibility of steroid-dependent changes in mRNA stability, A549 cells were first stimulated with IL-1β for 2 h before the addition of dexamethasone or RU24858 (Fig. 6A). To allow time for steroid-dependent gene expression, the classic chase experiment was commenced by the addition of actinomycin D 1 h after the steroids (t = 0). RNA was then harvested, and expression of IL-8, COX-2, and GAPDH monitored in cDNA by TaqMan PCR (Fig. 6B). In each case, the addition of dexamethasone resulted in more rapid initial decay rates of both COX-2 and IL-8 mRNA (Fig. 6B and Table 2). In addition, dexamethasone led to the loss of a greater fraction of the initial starting mRNA compared with IL-1β-treated samples. In each case, RU24858 resulted in an intermediated effect, which for COX-2 was not significantly different from either the dexamethasone or the IL-1β-treated decay rates. In the case of IL-8, the initial decay rate produced by RU24858 was significantly more than for IL-1β alone but was less than that produced by IL-1β plus dexamethasone (Fig. 6B and Table 2).

Steroid-Dependent Repression of Inflammatory Gene mRNA Expression Is Attenuated by Cycloheximide. In complementary experiments, A549 cells were incubated with various combinations of IL-1β, the protein synthesis inhibitor cycloheximide, dexamethasone, and RU24858 before incubation for 4 h and semiquantitative RT-PCR analysis of steady-state mRNA levels (Fig. 7). The addition of steroid had no effect on basal levels of IL-8 and COX-2 because these were essentially undetectable. Alone, cycloheximide had little or no effect on IL-8 but resulted in an increased expression of COX-2. IL-1β resulted in robust increases in IL-8 and COX-2 mRNA, and this was repressed by the simultaneous addition of either dexamethasone or RU24858. This steroid-dependent repression was significantly reduced in the presence of cycloheximide, suggesting a requirement for ongoing protein synthesis.

![Fig. 4. Effect of dexamethasone on steady-state IL-8 mRNA.](image)

A, schematic representation of the experiments in B and C. A549 cells were treated with IL-1β (1 ng/ml) or not stimulated for 2 h before the addition of combinations of dexamethasone (1 μM) and actinomycin D (10 μg/ml) (Act D) and then harvested. B, cells were treated as indicated and harvested at the indicated times (after Dex/Act D addition) for Northern blot analysis of IL-8 and GAPDH. Representative blots are shown. After densitometric analysis, data from four such experiments were expressed as a percentage of the IL-1β plus dexamethasone decay rates. In each case, the addition of dexamethasone or RU24858 resulted in no repression of COX-2 or IL-8 mRNA. Therefore, to examine the possibility of steroid-dependent changes in mRNA stability, A549 cells were first stimulated with IL-1β for 2 h before the addition of dexamethasone or RU24858 (Fig. 6A). To allow time for steroid-dependent gene expression, the classic chase experiment was commenced by the addition of actinomycin D 1 h after the steroids (t = 0). RNA was then harvested, and expression of IL-8, COX-2, and GAPDH monitored in cDNA by TaqMan PCR (Fig. 6B). In each case, the addition of dexamethasone resulted in more rapid initial decay rates of both COX-2 and IL-8 mRNA (Fig. 6B and Table 2). In addition, dexamethasone led to the loss of a greater fraction of the initial starting mRNA compared with IL-1β-treated samples. In each case, RU24858 resulted in an intermediated effect, which for COX-2 was not significantly different from either the dexamethasone or the IL-1β-treated decay rates. In the case of IL-8, the initial decay rate produced by RU24858 was significantly more than for IL-1β alone but was less than that produced by IL-1β plus dexamethasone (Fig. 6B and Table 2).
Evidence for a Requirement of Steroid-Inducible Genes in the Repression of Inflammatory Gene Protein Expression. In previous studies, dexamethasone was reported to block the IL-1β-induced expression of COX-2 protein, even when added substantially after the IL-1β (Newton et al., 1998). To examine the possible role of steroid-inducible genes in the repression of COX-2 and IL-8 protein expression, A549 cells were treated with IL-1β for 6 h before the addition of dexamethasone, RU24858 or actinomycin D (Fig. 8A). The addition of either dexamethasone or RU24858 6 h after the IL-1β stimulus resulted in a marked repression of COX-2 (Fig. 8B). The repression of IL-8 was considerably weaker (only 25–30% repression) (Fig. 8C), presumably because significant quantities of IL-8 protein had already been produced by the time the steroids were added after the IL-1β stimulus. It is noteworthy that the presence of actinomycin D with both dexamethasone and RU24858 at 6 h resulted in a total loss of the inhibitory properties in respect of COX-2 and IL-8 protein expression.

Effects of Dexamethasone and RU24858 on Steroid-Inducible Genes. In the above sections, the repression of inflammatory gene expression by dexamethasone and the dissociated steroid RU24858 is prevented by actinomycin D or cycloheximide. This suggests a requirement for steroid-dependent gene expression but raises a considerable issue in respect of the action of RU24858, which is largely incapable of classic GRE-dependent transcription (Fig. 2A). However, in addition to the simple or classic GRE, GR may induce transcriptional responses via interactions with numerous other transcription factors (Newton, 2000). Because the effects of dissociated steroids on such forms of transactivation are not generally tested, these could, in theory, be important in the current responses. However, because the functional relevance of nonclassic or complex GREs is unclear with respect to glucocorticoid-inducible gene expression, we elected to directly test dexamethasone-inducible genes for possible inducibility by RU24858.

![Image](Image 62x174 to 278x445)

**Fig. 5.** Repression of inflammatory gene mRNA expression by dexamethasone and RU24858 is prevented by actinomycin D. A549 cells were stimulated with IL-1β (1 ng/ml), or not stimulated (NS). Dexamethasone (1 μM) (Dex) or RU24858 (10 μM) was either added simultaneously with the IL-1β (t = -2), or 2 h after the IL-1β (t = 0) in the presence or absence of actinomycin D (10 μg/ml) (Act D) (as shown in Fig. 3B). Cells were then harvested 6 h later (i.e., a total of 8 h after the IL-1β), and RNA was prepared. Semiquantitative RT-PCR analysis for COX-2, IL-8, and GAPDH was performed. Each experiment carried two IL-1β-stimulated samples, and these were averaged and set to 100% for data presentation. Data (n = 8 for COX-2 and 7 for IL-8) were normalized to GAPDH, expressed as a percentage of the averaged IL-1β, and are plotted as mean ± S.E.M. *P < 0.05; **P < 0.01.

**Fig. 6.** Effect of dexamethasone and RU24858 on IL-1β-induced COX-2 and IL-8 mRNA stability. A549 cells were stimulated with IL-1β (1 ng/ml) for 2 h before no further treatment (C) or were treated with dexamethasone (1 μM) (Dex) or RU24858 (10 μM) (F). After a further 1 h (t = 0), actinomycin D (10 μg/ml) (Act D) was added. Cells were harvested for total RNA at 0, 0.25, 0.5, 1, and 2 h after the addition of actinomycin D. A, schematic depicting the above experimental protocol. B, after RNA extraction and cDNA synthesis, real-time TaqMan PCR was used to analyze the expression of COX-2, IL-8, and GAPDH. In each case, relative mRNA levels were obtained by reference to a standard curve generated by serial cDNA dilution. Relative COX-2 and IL-8 mRNA levels were normalized to GAPDH and were expressed as a percentage of the value at t = 0 for each treatment group. Data (n = 7–8) are plotted as means ± S.E.M. Values from data at t = 0, 0.25, 0.5, and 1 h were used to calculate the initial slope for each treatment, and these are to be found in Table 2.

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>COX-2</th>
<th>IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Slope</td>
<td>P</td>
<td>Mean ± S.E.</td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β + dexamethasone</td>
<td>22.4 ± 4.9</td>
<td>NS</td>
</tr>
<tr>
<td>IL-1β + RU24858</td>
<td>22.4 ± 4.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

*P < 0.05; ***P < 0.001.
A prior Affymetrix microarray (Hu95Av2 and B chips) study in A549 cells identified some 300 genes as being dexamethasone-inducible (data not shown). Importantly, a number of these dexamethasone-inducible genes are also potentially anti-inflammatory in effect (Table 3). For example, MKP-1 switches off MAPK signaling (Clark and Lasa, 2003), GILZ represses AP-1-dependent transcription (Mittelstadt and Ashwell, 2001), and regulator of G-protein signaling (RGS)-2 reduces Gq-linked signaling (Heximer, 2004). In addition, the metabolic genes AMP N and MET 1X were also identified as being dexamethasone-inducible.

A549 cells were therefore treated with dexamethasone or RU24858 for various periods of time, and the expression of MKP-1, RGS-2, GILZ, AMP N, and MET 1X was examined by semiquantitative RT-PCR (Fig. 9). In each case, mRNA expression was up-regulated by dexamethasone, and this largely correlated with the array data (Fig. 9 and Table 3). The expression of MKP-1, GILZ, AMP N, and MET 1X, but not RGS2, were also up-regulated by RU24858. Thus, although RU24858 is dissociated by reference to a classic GRE response, this compound can nevertheless induce the expression of glucocorticoid-inducible genes. Because these genes may contribute to both the anti-inflammatory or side effect profile of this steroid, these data provide an explanation for the ability to block the repressive effects of RU24858 with transcriptional and translational blockers.

**Discussion**

The GR is modular, with functional activities localized to specific domains (Rhen and Cidlowski, 2005). However, whereas the ligand binding domain and the DNA binding domains are necessary for both transactivation of classic GRE-dependent transcription and transrepression (e.g., of AP-1), mutations in these domains may differentially affect each function. For example, the GR A458T mutation used to generate the GR<sup>dim/dim</sup> mouse renders the receptor inca-

---

**TABLE 3**

Dexamethasone-inducible genes in A549 cells

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession No.</th>
<th>Affymetrix ID</th>
<th>6 h -Fold (R)</th>
<th>6 h P</th>
<th>18 h -Fold (R)</th>
<th>18 h P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GILZ</td>
<td>AL590423</td>
<td>36629_AT</td>
<td>15.9</td>
<td>0.00224</td>
<td>3.57</td>
<td>0.00047</td>
</tr>
<tr>
<td>MKP-1</td>
<td>X68277</td>
<td>1005_AT</td>
<td>7.7</td>
<td>0.4179</td>
<td>3.73</td>
<td>0.0820</td>
</tr>
<tr>
<td>RGS-2</td>
<td>L13463</td>
<td>37701_AT</td>
<td>6.77</td>
<td>0.0025</td>
<td>3.37</td>
<td>0.0208</td>
</tr>
<tr>
<td>AMP N</td>
<td>M22324</td>
<td>39385_AT</td>
<td>3.59</td>
<td>0.104</td>
<td>5.4</td>
<td>0.00944</td>
</tr>
<tr>
<td>MET-1X</td>
<td>P80297</td>
<td>39120_AT</td>
<td>2.62</td>
<td>0.104</td>
<td>4.68</td>
<td>0.000443</td>
</tr>
</tbody>
</table>
able of classic GRE-dependent transcription, but both anti-
inflammatory activity and the ability to transrepress are
retained (Reichardt et al., 1998, 2001). Likewise, the genera-
tion of dissociated GR ligands that are transrepression-
competent, defective at classic transactivation, yet retain
anti-inflammatory effects seems to support the view that
transrepression and not transactivation accounts for the
main anti-inflammatory properties of glucocorticoids (Heck
et al., 1994; Vayssiere et al., 1997). Likewise, RU24858 is
dissociated in A549 cells and represses IL-1β-induced expres-
sion of COX-2 and IL-8. At first, this result seems to agree
with the above dogma and is supported by the correlation
between the EC_{50} values for inhibition of NF-κB and these
functional outputs. Despite this, the slightly reduced efficacy
of RU24858 on IL-8 and COX-2 expression leaves open the
possibility of a minor repressive role for classic GRE-depen-
dent responses. However, the fact that activation of GRE-
dependent transcription by dexamethasone occurred at a
higher concentration (EC_{50} = 93 nM) than that observed for
repression of COX/PGES (EC_{50} = 3.78), or IL-8 (EC_{50} = 2.55
nM) also argues against a major role for classic GRE-depen-
dent transcription. Importantly, the finding that IL-8 tran-
scription rate, the activation of NF-κB-dependent transcription
and the analysis of IL-8 nuclear mRNA intermediates show
no more than 40 to 60% repression by steroids, plus
similar data in respect of COX-2 transcription rate (Newton
et al., 1998), casts considerable doubt on transcriptional re-
pression being the sole, or even main, mechanism for glu-
ocorticoid-dependent repression. Indeed, we show a signifi-
cant role for post-transcriptional events in mediating the
glucocorticoid-dependent repression of IL-8 and COX-2, and
this is consistent with a substantial body of data document-
ing critical roles for post-transcriptional repression (Newton,
2000; Stellato, 2004).

In considering the contribution of transrepression and
transactivation to the anti-inflammatory properties of glu-
ocorticoids, it is worth remembering that GR dimerization
and activation of classic GRE-dependent transcription is not
the only mechanism of GR-dependent transactivation (New-
ton, 2000). Thus, GR may interact, cross-talk, or even syner-
gize with signal transducer and activator of transcription 3,
signal transducer and activator of transcription 5, CATT
enhancer binding protein family members, and other nuclear
hormone receptors to bring about transcriptional activation
(Kordula and Travis, 1996; Stocklin et al., 1996; Boruk et al.,
1998; Newton, 2000; Savory et al., 2001; Cassuto et al., 2005).
Even interactions between GR and AP-1 components or on
NF-κB reporters may lead to transcriptional activation (Di-
adam et al., 1990; Hofmann and Schmitz, 2002). Such ef-
fects are clearly not mediated via classic GREs and, critically
for the present discussion, are not generally evaluated in the
context of GR mutants, dissociated GR ligands, or the induc-
tion of glucocorticoid-inducible genes. Therefore, there exists
a formal possibility that nonclassic forms of GR transactiva-
tion are important in glucocorticoid-dependent repression of
inflammatory genes. Furthermore, numerous prior studies
have documented that the ability of glucocorticoids to repress
inflammatory gene expression is lost in the presence of either
transcriptional or translational blockers (Ristimaki et al.,
1996; Newton et al., 1998; Chang et al., 2001; Lasa et al.,
2001; Korhonen et al., 2002). Such observations are difficult
to reconcile with models for anti-inflammatory glucocorticoid
action that predominantly invoke classic transrepression of
NF-κB and AP-1. In these schemes, GR is believed, either by
direct interaction or indirectly by recruitment of corepressors
or histone deacetylases, to interfere with transcriptional ac-
tivation of inflammatory genes, and this does not require
steroid-dependent gene expression. In contrast, the dexam-
ethasone- and RU24858-dependent repression of IL-8 and
COX-2 steady-state mRNA and protein synthesis requires
both transcription and translation because repression is both
actinomycin D- and cycloheximide-sensitive. Thus, despite
not activating classic GRE-dependent transcription, our data
clearly suggest that repression by dexamethasone and
RU24858 requires GR-dependent gene expression.

In past years, the existence of steroid-inducible genes, for
example lipocortin 1 (annexin 1), β_{2}-adrenergic receptor, se-
cretery leukocyte protease inhibitor, IL-1 receptor antago-
nist, and others (Newton, 2000), although undoubtedly mak-
ing an anti-inflammatory contribution, was not generally
considered sufficient to explain the repression of inflamma-
tory gene expression. More recently, microarray-based ap-
proaches have documented the existence of numerous glu-
ocorticoid-inducible genes (Rogatsky et al., 2003). Thus,
glucocorticoid-inducible genes, such as MKP-1, RGS-2, and
GILZ, show potentially anti-inflammatory properties and of-
fer a real possibility that substantive anti-inflammatory ef-
fects may be attributed to glucocorticoid-dependent gene in-
duction. To examine this possibility with respect to RU24858,
the expression of MKP-1, RGS-2, and GILZ were examined.

**Fig. 9.** Dexamethasone-inducible genes may be induced by
RU24858. Cells were stimulated with dexamethasone (1
μM) (Dex) or RU24858 (10 μM) for 2, 6, or 18 h. Cells were
harvested, and RNA was prepared for semiquantitative
RT-PCR analysis of the indicated genes. A, representative
ethidium bromide-stained gels showing expression of
MKP-1, RGS2, GILZ, AMP N, MET IX, and GAPDH are
provided. B, After optical density determination (TotalLab
software) data (n = 4) normalized to GAPDH are pre-
sented as -fold induction ± S.E.M.
In this analysis, the expression of MKP-1 and GILZ were both induced by RU24858 and dexamethasone, whereas RGS-2 was not induced. These data confirm the possibility that RU24858 can induce anti-inflammatory glucocorticoid-inducible genes, presumably via mechanisms other than at a classic GRE. Furthermore, this supports the idea that such effects could be important in the repression of inflammatory genes such as COX-2 and IL-8. This statement also finds considerable support from the work of Rogatsky et al. (2003), in which microarray profiling was used to describe the induction of multiple genes by dexamethasone. Critically for the current argument, mutations in either of the two GR activation domains (AF1 and AF2) or the GRdim mutant resulted in loss of responsiveness for different groups but not all dexamethasone-inducible genes (Rogatsky et al., 2003). Thus, different mechanisms of transactivation are responsible for the induction of these different genes, and this is consistent with the possibility that GR ligands differentially activate these functions to induce discrete subsets of glucocorticoid-inducible genes.

Likewise, in vivo analysis of RU24858 revealed all of the main side effects of standard balanced glucocorticoids, suggesting that classic GRE-dependent transactivation does not, by itself, account for these side effects (Belvisi et al., 2001). Our analysis also supports this view as the glucocorticoid-inducible genes MET-1X and AMP N were induced by both dexamethasone and by RU24858. Assuming that other metabolic gene may also be induced, this provides an explanation for the emergence of side effects with RU24858 (Belvisi et al., 2001). In contrast, the nonsteroidal GR ligand ZK 216348 was obtained after functional cell-based screens for GR ligands that did not induce tyrosine amino transferase, yet were still able to repress IL-8 expression (Schacke et al., 2004). This approach resulted in GR ligands that were considerably more ‘dissociated’ with respect to the ability to cause repression of IL-8 and other inflammatory genes but showed considerably reduced metabolic side effects.

In terms of different GR ligands differentially inducing GR-dependent responses, we have documented previously the ability of the antagonist, RU486, to cause repression of PGE2 release via effects predominantly occurring at the level of arachidonic acid release (Chivers et al., 2004). RU486 causes GR translocation yet is silent on both transrepression and transactivation (Chivers et al., 2004); therefore; it is possible that this could represent a nongenomic mechanism of glucocorticoid action, and this may explain the relatively potent effect of RU24858 on the repression of PGE2 release (EC50 = 53.9 nM). In addition, it is now clear that different GR ligands, presumably as a consequence of different conformational changes induced in respect of GR activation domains, are capable of differentially recruiting coactivators and corepressors (Adcock et al., 2004; Garside et al., 2004).

Therefore, the relationship between the ligand, conformational changes produced by the ligand, and the recruitment (or not) of coactivators and repressors will have a profound effect on the transcriptional responses observed in different promoter contexts. Thus, different GR ligands have the potential to produce variable activation or repressional responses, depending on the exact nature of both the promoter and the complement of transcription factors, coactivators, and repressors that are present within the cell.

In conclusion, the repression of IL-8 and COX-2 by dexamethasone and RU24858 is not fully accounted for by transrepression (e.g., of NF-κB), and requires ongoing, steroid-dependent gene expression and post-transcriptional mechanisms of repression. Furthermore, and despite not activating a classic GRE-dependent reporter construct, RU24858 was nearly as effective as dexamethasone at inducing certain glucocorticoid-inducible genes, suggesting that such processes may not involve classic GRE-dependent transcription. Finally, because a number of these dexamethasone- and RU24858-inducible genes are potentially anti-inflammatory, we propose that glucocorticoid-dependent genes play a significant role the anti-inflammatory effects of glucocorticoids.

Acknowledgments

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References


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Address correspondence to: Dr. Robert Newton, Department of Cell Biology and Anatomy, Faculty of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada, T2N 4N1. E-mail: rnewton@ucalgary.ca
Supplemental data

Analysis of the dissociated steroid, RU24858, does not exclude a role for inducible genes in the anti-inflammatory actions of glucocorticoids

JOANNA E. CHIVERS, WEI GONG, ELIZABETH M. KING, JOACHIM SEYBOLD, JUDITH C. MAK, LOUISE E. DONNELLY, NEIL S. HOLDEN, ROBERT NEWTON.

Department of Cell Biology & Anatomy, Respiratory Research Group, Faculty of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada. T2N 4N1 (W.G., E.M.K., N.S.H., R.N.); Department of Thoracic Medicine, National Heart and Lung Institute, Imperial College, Faculty of Medicine, London, SW3 6LY. U.K (J.E.C., L.E.D.); Department of Internal Medicine/Infectious Diseases, Charite Universitätsmedizin Berlin, Humboldt-University, Augustenburger Platz 1, 13353 Berlin, Germany (J.S); Department of Medicine, Queen Mary Hospital, Hong Kong SAR, China (J.C.M.).
Supplemental Experimental Procedures

Immunocytochemistry. Cells grown on coverslips were transferred at 70% confluence to SFM for 24 h. After incubation with steroid for the indicated times, cells were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde before successive incubations in 0.5% NP-40 and 100 mM glycine. Coverslips were blocked in PBS, 0.1% Tween-20, 0.1% bovine serum albumin and 10% human serum prior to incubation for 1 h in 5µg/ml rabbit-anti human GR (PA1-511A, Affinity Bioreagents, Colorado, USA) or rabbit isotype control (Dako, Glostrup, Denmark). After washing with PBS, 0.1% Tween and incubation with biotinylated anti-rabbit immunoglobulins (Dako) for 1 h, cells were incubated with FITC-linked streptavidin (Dako) for 1 h. Nuclei were then stained with 1 µM 4',6'-diamidino-2-phenylinole dihydrochloric hydrate (DAPI) (Sigma) and coverslips mounted on glass slides using Citifluor mounting fluid (Citifluor Ltd, London, UK.) prior to analysis using a Leica TCS 4D confocal microscope (Leica Microsystems, Milton Keynes, UK) equipped with argon, krypton, and ultraviolet lasers. Confocal images were acquired at ×40 magnification using TCS NT software (Leica Microsystems).

Ligand binding. At 80% confluence, cells were transferred to SFM and harvested the following day in cell-dissociation fluid (Sigma) and incubated overnight at 4 °C with increasing concentrations of [3H]-dexamethasone in the presence of 10 µM dexamethasone to define non-specific binding. Free radioligand was removed by rapid filtration of cells glass-fibre filters (GF/B) pre-coated with 0.1% (V/V) polyethylenimine in PBS, using a cell harvester (M-24R Brandel, Maryland, USA.). Filters were combined with Filtron-X scintillant (National Diagnostics, Georgia, USA) and counted in a beta counter (2200CA Tri-carb Liquid Scintillation Analyser, Canberra Packard, Berkshire, UK). Kd and Bmax were determined using saturation
binding isotherms and Scatchard analysis, \([\text{Bound}]/[\text{Free}]\) vs [Bound], where x-intercept = Bmax and gradient = -1/Kd (Fig. 1A) (Prism 4, GraphPad, California, USA). Relative binding affinity was assessed by incubating cells with increasing concentration of unlabelled steroid and 4 nM \(^3\text{H}\)-dexamethasone overnight at 4°C. Bound and free radioligand were separated as above. Specific binding was calculated by subtraction of non-specific from total binding and Cheng-Prusoff analysis was performed to determine Ki, 

\[
Ki = \frac{IC_{50}}{1 + ([\text{Free Count}]/Kd)}
\]

as previously described (Chivers et al., 2004).

**Supplemental Results**

**Linear relationship between starting cDNA concentration and amplification product using semi-quantitative RT-PCR.** Semi-quantitative RT-PCR is only capable of producing meaningful relative concentration data where a linear relationship exists between the target cDNA corresponding to the gene of interest (e.g. IL-8, COX-2 or GAPDH) and the amount of amplification produced that is detected, in this case by visualisation on ethidium bromide-stained agarose gels. In the semi-quantitative RT-PCR methodology used in the current studies, we perform "cycle profiles" in which an average sample, created by mixing a fraction of all the cDNAs in an experiment or batch to be analysed, is subject to PCR amplification for increasing numbers of cycles (e.g. 18, 20, 22, 24, 26, 28, 30). The products from each of these reactions are then analysed by electrophoresis on ethidium bromide-stained agarose gels. In previous studies, we have empirically shown that the cycle numbers just after a band becomes visible on ethidium bromide stained gels represents the exponential phase of amplification (Newton et al., 1997a). In these earlier studies, a combination of southern blotting and dot blotting was used to confirm a linear relationship between starting product and amplification product that extended over a range
of at least 10 to 100 fold (Newton et al., 1997a). In subsequent studies, we showed that this linear relationship also holds true if, instead of using either southern or dot blot analysis, we simply perform optical density measurements on appropriately amplified products that have been run on ethidium bromide-stained agarose gels (Staples et al., 2003).

In the current studies, this approach of generating "cycle profiles" has been used for all the semi-quantitative RT-PCR analysis. Clearly, the number of amplification cycles is critical to the analysis as too many cycles will result in saturation at all cDNA concentrations, whereas too few cycles will be insufficient to allow optical density determination. Therefore, we here present representative data showing a linear relationship between starting cDNA concentration and amplification product for IL-8, COX-2 and GAPDH after amplification for 20, 20 and 22 cycles respectively (Supplemental Fig. 1A & B). In each case $r^2$ values of 0.99, 0.99 and 0.98 were obtained and these confirm the effectiveness of this approach over these concentration ranges.

**Relationship between starting cDNA concentration and amplification product using real-time TaqMan PCR.** TaqMan PCR was carried out according to the manufacturer's recommendations. To confirm the validity of this approach, serial dilutions of cDNA were analyzed. A representative example, in this case, for COX-2 is shown (Supplemental Fig. 1C & D). In this example, 6 10-fold dilutions were made from an IL-1β-stimulated cDNA sample and TaqMan PCR was performed in triplicate on each of these dilutions (Supplemental Fig. 1C). In each case, threshold $C_T$s were calculated by the instrument and these are plotted as means ± SEM for each cDNA dilution (Supplemental Fig. 1D). Supplemental Fig. 1D shows a linear relationship ($r^2 = 0.998$) between $C_T$ value and log cDNA concentration, where the initial undiluted samples is designated a value of 1 (i.e. 0 as a log), that extends over the full range of concentrations tested. These data are representative for all probes used in the current study and therefore confirm the validity of this approach to cDNA quantification.
**Dexamethasone and RU24858 activate and bind the glucocorticoid receptor.** Treatment of A549 cells with either dexamethasone or RU24858 resulted in a pronounced translocation of GR to the nucleus of essentially all cells (Supplemental Fig. 2A).

Saturation binding studies using $[^3]H$dexamethasone demonstrate one-site binding in A549 cells (Chivers et al., 2004). Competitive binding studies were performed to analyse the GR binding affinity of RU24858, which revealed a $K_i$ of $110.0 \pm 24.0$ nM compared to dexamethasone with a $K_i$ $4.9 \pm 1.3$ nM (Supplemental Fig. 2B).

**RU486 antagonizes the repression of COX-2 and IL-8 by dexamethasone and RU24858.** To confirm the involvement of GR in the repression of both COX-2 and IL-8 by dexamethasone and RU24858, A549 cells were stimulated with IL-1$\beta$ in the presence of each glucocorticoid and increasing concentrations of the antagonists, RU486. In each case the repression of both COX-2 and IL-8 mRNA by dexamethasone and RU24858 was progressively lost in the presence of increasing concentrations of RU486 (Supplemental Fig. 3). These data suggest that both glucocorticoids are indeed acting via GR.

**Dexamethasone represses COX/PGES activity and PGE$_2$ production in A549 derived reporter cell lines.** To confirm that the NF-$\kappa$B reporter (6$\kappa$Btk) and 2$\times$GRE reporter A549 cell lines were competent in steroid responsiveness, cells were stimulated with IL-1$\beta$ in the presence of various concentrations of dexamethasone (Supplemental Fig. 4). In each case, COX/PGES activity and PGE$_2$ release were profoundly repressed by dexamethasone and in each case this was indistinguishable between native and transfected cell lines.

**Real-time TaqMan analysis of unspliced nuclear IL-8 and GAPDH nuclear RNA.** Following the qualitative RT-PCR analysis of nuclear RNA samples shown in Fig. 3B, TaqMan PCR was performed to provide a more quantitative assessment of the RNA levels. As nuclear RNA represents a minority of total cellular RNA and this was extracted from the nuclear fraction...
following soft lysis of the cells (Gough, 1988), co-purified genomic DNA represents a greater fraction of the total nucleic acid than is apparent with a whole cell total RNA preparation. Furthermore, the probe and primers to detect unspliced RNA will inherently detect genomic DNA. Therefore all samples are analyzed following reverse transcription reactions both in the presence (RT+) and absence (RT-) of reverse transcriptase to allow assessment of amplification from genomic DNA. In Supplemental Fig. 5, the RT- samples are indicated and these reaction profiles are situated to the right of the RT+ samples. This indicates a predominant signal from cDNA and not genomic DNA. In the case of GAPDH, the presence of contaminating genomic DNA (RT- samples) represents less than 3% of the RT+ sample. In the case of nuclear IL-8, genomic contamination represents less than 0.05% of the signal from IL-1β-treated samples. However, the very low level of unstimulated IL-8 expression resulted in genomic DNA contributing to up to 5% of these signals. In conclusion, the analysis of unspliced GAPDH and IL-1β-stimulated unspliced IL-8 RNA can occur free of worries resulting from contaminating genomic DNA. Although not a problem to the current study, the analysis of unspliced IL-8 nuclear RNA in unstimulated samples may be complicated by the presence of genomic DNA. In all subsequent analyses, any samples showing a genomic contribution of greater than 5% to the GAPDH signal were excluded.

**Steroid-dependent repression of IL-1β-induced COX-2 and IL-8 mRNA induction is blocked by actinomycin D.** In Fig. 5 of the main manuscript, a semi-quantitative RT-PCR analysis is shown in which A549 cells were stimulated with IL-1β for 2 hours prior to further treatment with combinations of dexamethasone, RU24858 and actinomycin D. Following incubation for a further 6 hours, total RNA was prepared, cDNA generated and the samples analyzed for COX-2, IL-8 and GAPDH expression using semi-quantitative RT-PCR. These data indicate that both dexamethasone and RU24858 are able to repress COX-2 and IL-8 mRNA
expression whether added at the same time as the IL-1β stimulus or if added 2 hours after the IL-1β (Fig. 5). Importantly, the ability of dexamethasone and RU24858 to cause mRNA repression is totally prevented by the presence of actinomycin D suggesting that ongoing gene transcription is necessary for the repressive effect of each steroid. Given the key importance of this experiment, we have re-analyzed these cDNA samples using TaqMan real-time PCR (Supplemental Fig. 6A). In each case, COX-2 and IL-8 mRNA expression was significantly repressed by both dexamethasone and RU24858 when added either at the same time as the IL-1β or when added 2 hours after. As was shown using semi-quantitative RT-PCR in the main paper, the addition of actinomycin D totally prevented the ability of either steroid to cause repression of both COX-2 and IL-8 mRNA.

As steady state levels of IL-1β-induced IL-8 and COX-2 mRNA are maximal between 4 and 6 hours post stimulation (Figs. 3 & 4) (Newton et al., 1997a; Newton et al., 1997b; Newton et al., 1998), the analyses presented in Fig. 5, and Supplemental Fig. 6A, reflect a time point (2 + 6 h) at which IL-1β-induced steady state levels of IL-8 and COX-2 mRNA are in fact declining. Thus the observed effect may be due to a combination of the steroid as well as normal feedback control processes. To confirm that these observations were not simply a facet of normal feedback control, this experiment was modified such the cells were harvested after a total of 5 hours (i.e. 2 + 3 hours), i.e. prior to the decline in IL-1β-induced mRNA. As was previously observed, the addition of either dexamethasone or RU24858 resulted in a significant reduction in both COX-2 and IL-8 mRNA levels (Supplemental Fig. 6B). This level of repression was maintained even when the steroids were added 2 hours after the IL-1β and the cells harvested after a further 3 hours. In each case, dexamethasone produced a more robust repression than RU24858 and this is consistent with data elsewhere in this study. Importantly, the addition of actinomycin D totally prevented the ability of either steroid to repress steady state levels of COX-2 and IL-8 mRNA.
Therefore these data support our hypothesis that ongoing transcription is necessary for these steroids to exert their repressive effects and this effect occurs irrespective of the presence of endogenous feedback control processes.

References


Supplemental Fig. 1. Relationship between starting cDNA concentration and amplification product using semi-quantitative RT-PCR and real-time TaqMan PCR. Following reverse transcription to generate cDNA, a representative IL-1β-stimulated sample was subject to serial two fold dilution (1×, 2×, 4×, 8×). PCR amplification was performed using; 20 cycles for IL-8 and COX-2, and 22 cycles for GAPDH using the conditions described in the main manuscript. 

A, Reaction products (10 µl) were run on 1.5% agarose gels and visualised by ethidium bromide staining with UV illumination. 

B, Following densitometry using Totallab software (Non-linear Dynamics), optical densities were plotted against relative cDNA concentration (where the starting sample is designated a value of 1). Linear regression analysis was performed using Prizm 4.0 (Graphpad software). \( r^2 \) values were 0.99, 0.99 and 0.98 for IL-8, COX-2 and GAPDH respectively. 

C, TaqMan PCR was performed in triplicate on 6 serial 10-fold dilutions of an IL-1β-stimulated cDNA sample. A plot of \( \Delta \text{Reaction} \) (i.e. accumulated fluorescence normalized to ROX) against cycle number is shown for these dilutions. The \( C_T \) level is shown is red. 

D, \( C_T \) values for each replicate are plotted as means ± SEM against log relative cDNA concentration. Linear regression analysis using Prizm 4.0 was performed. \( r^2 = 0.998. \)
Supplemental Fig. 2. Activation and relative GR affinities for dexamethasone and RU24858. A, Cells were treated with dexamethasone (1 µM) (Dex) or RU24858 (10 µM) for 1 h prior to probing with a FITC-conjugated GR antibody (green) or an isotype control for the GR antibody prior to imaging by confocal microscopy. Nuclear structure is indicated using by DAPI staining of chromatin (blue). Images are representative of three experiments. B, Competition binding curves showing relative affinity in A549 cells, where dexamethasone (■) and RU24858 (○) compete with 4 nM [³H] dexamethasone to bind GR. Data is presented as mean ± SEM for (n = 5) observations.
Supplemental Fig. 3. Repression of COX-2 and IL-8 mRNA expression by dexamethasone and RU24858 is antagonized by RU486. A549 cells were treated with dexamethasone (Dex) (0.1 µM) (■), or RU24858 (1 µM) in the presence of increasing concentrations of RU486 for 1 h prior to stimulation IL-1β (1 ng/ml) or not stimulated (NS). After 6 h, cells were harvested for northern blot analysis of COX-2, IL-8 or GAPDH as indicated. Blots representative of at least 4 such experiments are shown.

Supplemental Fig. 4. Effect of dexamethasone on wild-type and transfected cell lines. Wild-type A549 (■), 2×GRE A549 reporter (○) and 6κBtk A549 reporter (●) cells were cultured with various concentrations of dexamethasone for 1 h, prior to stimulation with IL-1β (1 ng/ml) or not stimulated (NS). After 24 h PGE2 release (A) and COX/PGES activity (B) was measured. Data (n = 5 - 6), expressed as a percentage of IL-1β, are plotted as means ± SEM.
Supplemental Fig. 5. TaqMan real-time RT-PCR analysis of unspliced IL-8 and GAPDH nuclear RNA. Following the qualitative RT-PCR analysis of nuclear samples in Fig. 3B, TaqMan PCR was performed for unspliced IL-8 and unspliced GAPDH nuclear RNA. The analysis was performed in duplicate from reverse transcription reactions that either contained reverse transcriptase (RT+) or had no reverse transcriptase present (RT-). In each case, the samples were analyzed for unspliced IL-8 nuclear RNA using primers and a probe spanning the IL-8 exon 1-intron A boundary (A), and for GAPDH nuclear RNA using primers and a probe spanning the GAPDH exon 2-intron B boundary (B). Plots of Δreaction against C_T are shown. The positions of reaction profiles corresponding to non-stimulated and IL-1β-treated samples are indicated. These are distinct from the reaction profiles derived from samples processed without reverse transcriptase, which allow assessment of the contribution by co-purified genomic DNA. H2O blanks are also indicated.
Supplemental Fig. 6. Real-time RT-PCR analysis to show that repression of COX-2 and IL-8 mRNA by dexamethasone and RU24858 is prevented by actinomycin D. A549 cells were stimulated with IL-1β (1 ng/ml), or not stimulated (NS). Dexamethasone (1 µM) (Dex), or RU25858 (10 µM), was either added simultaneously with the IL-1β (t = -2), or, 2 hours after the IL-1β (t = 0) in the presence or absence of actinomycin D (10 µg/ml) (Act D) (as shown). Cells were then harvested A, 6 hours later (i.e. a total of 8 h after the IL-1β), or B, 3 hours later (i.e. a total of 5 h after the IL-1β) and RNA was prepared. Real-time PCR analysis for COX-2 and IL-8 and GAPDH was performed using a cDNA dilution series to generate a standard curve of the relationship between log [cDNA] and C_T value. C_T values were converted into relative cDNA concentrations and these were then normalised to GAPDH expression. Each experiment carried two IL-1β-stimulated samples and these were averaged and set to 100% for data presentation. Data (A, n = 10 and B, n = 6) are expressed as a percentage of the averaged IL-1β and are plotted as means ± SEM.