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Budesonide epimer R or dexamethasone selectively inhibit platelet-activating factor-induced or interleukin 1β-induced DNA binding activity of *cis*-acting transcription factors and cyclooxygenase-2 gene expression in human epidermal keratinocytes

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ABSTRACT To further understand the molecular mechanism of glucocorticoid action on gene expression, DNA-binding activities of the *cis*-acting transcription factors activator protein 1 (AP1), AP2, Egr1 (*zif268*), NF-κB, the signal transducers and activators of transcription proteins gamma interferon activation site (GAS), Sis-inducible element, and the TATA binding protein transcription factor II D (TFIID) were examined in human epidermal keratinocytes. The cytokine interleukin 1β (IL-1β) and platelet-activating factor (PAF), both potent mediators of inflammation, were used as triggers for gene expression. Budesonide epimer R (BUDeR) and dexamethasone (DEX) were studied as potential antagonists. BUDeR or DEX before IL-1β- or PAF-mediated gene induction elicited strong inhibition of AP1-, GAS-, and in particular NF-κB-DNA binding ($P < 0.001$, ANOVA). Only small effects were noted on AP2, Egr1 (*zif268*), and Sis-inducible element-DNA binding ($P > 0.05$). No significant effect was noted on the basal transcription factor TFIID recognition of TATA-containing core promoter sequences ($P > 0.68$). To test the hypothesis that changing *cis*-acting transcription factor binding activity may be involved in inflammatory-response related gene transcription, RNA message abundance for human cyclooxygenase (COX)-1 and -2 (E.C.1.14.99.1) was assessed in parallel by using reverse transcription–PCR. Although the COX-1 gene was found to be expressed at constitutively low levels, the TATA-containing COX-2 gene, which contains AP1-like, GAS, and NF-κB DNA-binding sites in its immediate promoter, was found to be strongly induced by IL-1β or PAF ($P < 0.001$). BUDeR and DEX both suppressed COX-2 RNA message generation; however, no correlation was associated with TFIID–DNA binding. These results suggest that on stimulation by mediators of inflammation, although the basal transcription machinery remains intact, modulation of *cis*-activating transcription factor AP1, GAS, and NF-κB-DNA binding by the glucocorticoids BUDeR and DEX play important regulatory roles in the extent of specific promoter activation and hence the expression of key genes involved in the inflammatory response.

Glucocorticosteroids (GCs) have long been used therapeutically as immunosuppressive and anti-inflammatory agents; however, the mechanism of their activity is only recently becoming understood at the genetic level. GCs interact with an intracellular GC receptor, which subsequently translocates to the nucleus as a ligand-activated transcriptional modulator. In turn, the GC receptor regulates the expression of genes such as those encoding cytokines, matrix metalloproteinases, and cell adhesion molecules known to be critical to both inflammation and the immune response (1, 2). Besides a direct "type 1" interaction with a palindromic GC responsive element in GC-sensitive gene promoters (3, 4), functions of the GC receptor also include a "type 2" interaction with chromatin-associated *cis*-acting transcription factors. For example, the GC receptor can physically interact with the Fos and Jun components of dimeric activator protein 1 (AP1) (5, 6), the p65 subunit of NF-κB (7, 8), and the STAT5 family of signal transducers and activators of transcription (9, 10) to modulate the *trans*-activation of gene promoters that contain no GC responsive elements. GCs also alter the nuclear availability of transcription factors as is the case when NF-κB activity is suppressed by induction of the I-κB inhibitory protein, which sequesters the regulatory element NF-κB as an inactive cytoplasmic complex (11, 12).

GCs also moderate the inflammatory response by stimulating transcription from the interleukin 1 (IL-1) decoy receptor gene, which acts as a scavenger for free IL-1β (13, 14) while down-regulating expression of the inducible cyclooxygenase-2 (COX-2) gene when activated by inflammation or cytokines such as IL-1β (15). The potent inflammation-related *cis*-activating transcription factors AP1 (16, 17) and NF-κB (18, 19), as well as COX-2 (20) and matrix metalloproteinase-1 and -9 gene expression (21), are also strongly induced by the inflammatory mediator platelet-activating factor (PAF; refs. 22–26). Although several synthetic GCs have been intensively studied at the gene signaling level (1–12) the molecular specificity and potency of the nonfluorinated GC budesonide epimer R (BUDeR) remains incompletely characterized. BUDeR, which displays a 14-fold higher relative affinity for the GC receptor when compared with dexamethasone (DEX), has a very high ratio between topical and systemic activity (27), making it active directly at the site of inflammation such as in asthma and rhinitis where inhalation therapy is the drug delivery system of choice.

To further understand the genetic mechanism of GC anti-inflammatory activity, here we have compared the effects of BUDeR with those of DEX on IL-1β- or PAF-stimulated gene expression in human epidermal keratinocyte (HEK) cells in primary culture. DNA binding activities for the *cis*-acting tran-

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Abbreviations: AP1, activator protein 1; BUDeR, budesonide epimer R; COX, cyclooxygenase; DEX, dexamethasone; EMSA, electrophoretic mobility shift assay; GAS, gamma interferon activation site; GC, glucocorticoid; HEK, human epidermal keratinocyte; IL-1β, interleukin 1β; KGM, keratinocyte growth medium; NPXT, nuclear protein extract; PAF, platelet-activating factor; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcription–PCR; SIE, Sis-inducible element; STAT, signal transducers and activators of transcription; TFIID, transcription factor II D.

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scription factors AP1 (16, 17), AP2 (28), Egr1 (also known as *zif268*, krox24, tis-8, d2, and NGF1A; refs. 29 and 30); NF-κB (18, 31, 32); the STAT elements SIE (Sis inducible element) and GAS (gamma interferon activation site; refs. 9, 10, and 33); and the TATA binding protein TFIID (transcription factor II D; refs. 34 and 35) were quantitated in HEK nuclear protein extracts (NPXTs) by using electrophoretic mobility shift assay (EMSA). To correlate transcription factor availability with gene expression, RNA message abundance for two genes critically involved in the inflammatory response, the constitutively expressed COX-1 and the highly inducible COX-2 gene (36–40), were measured in parallel by using reverse transcription–PCR (RT-PCR). The TATA-containing COX-2 gene, a key regulator of prostaglandin biosynthesis, and which contains DNA binding sites for AP1-like, AP2, NF-κB, GAS, SIE, Egr1 (*zif268*), and TFIID located between $-1,000$ bp and $+165$ bp of its promoter (36, 39) was found to be strongly induced in HEK cells after IL-1β or PAF stimulation. Our results also suggest that the GCs BUDeR and DEX each down-regulate COX-2 gene expression because of the reduced ability of the *cis*-acting transcription factors AP1, GAS, and NF-κB to interact with their target DNA consensus sequences located in the immediate promoter of the COX-2 gene.

MATERIALS AND METHODS

Reagents. DEX (Sigma D-1756) and the nonhalogenated GC BUDeR (16,17-butylidenebis(oxy)-11,21-di-hydroxypregna-1,4-diene-3,20-dione) were solubilized as 5 μg/μl solutions in 99.9% dimethyl sulfoxide (Sigma D-8779). PAF (1–0-alkyl-2-acetyl-sn-glycero-3-phosphocholine; Sigma P-7568) was prepared as a 5 μg/μl solution in ethanol. To treat HEK cells with these compounds, keratinocyte growth medium (KGM; Clonetics, San Diego) was made 100 nM with respect to either PAF, DEX, or BUDeR. Human recombinant IL-1β and IL-6 (Sigma) were used at 10 ng/ml. The ligands IL-1β, IL-6, DEX, BUDeR, and PAF were solubilized into KGM by 5 min of vigorous vortexing. Untreated KGM then was decanted and replaced with ligand-containing KGM for 3 hr, after which time NPXTs and RNA were simultaneously isolated from the same sample. This 3-hr time point was selected because this time previously was determined to be the interval at which maximal RNA message levels for several inducible early genes, including COX-2, could be strongly detected (refs. 15 and 26; unpublished observations). All other reagents and enzymes were of the highest grades commercially available and were used without further purification.

Normal HEK Cells in Culture. Cultured keratinocytes have been shown to respond productively to a wide variety of cytokines and lipid mediators (refs. 13 and 14; W.J.L. and N.G.B., unpublished observations). Cryopreserved HEK cells, obtained from either neonatal, adult, or pooled donors were grown in KGM (P2→P4; Clonetics CC-3001) supplemented with a serum containing 15 μg/ml bovine pituitary extract, 0.2 ng/ml human epidermal growth factor, 0.5 μg/ml insulin, 0.15 mM gentamycin/amphotericin-B, and 0.5 μg/ml hydrocortisone until 80% confluent. For maximal GC effect, cells were serum-deprived for 24 hr to 0.5% of normal serum levels before the addition of test compounds. Without serum deprivation, only small effects on transcription factor binding were observed with a pattern similar to those observed with serum deprivation. For each experimental condition, normal HEK cells were cultured in triplicate T75 flasks for preparation of NPXTs and total RNA message extraction.

Harvesting of Cells and Preparation of NPXTs. NPXTs were isolated at 4°C in the presence of proteolytic enzyme inhibitors phenylmethylsulfonyl fluoride (PMSF, Sigma P-7626; 1 mM), aprotinin (Sigma A-6279; 0.05 μg/μl), leupeptin (Sigma L-2884; 0.05 μg/μl), and pepstatin A (Sigma P-4265, 0.025 μg/μl). After incubation with test compounds, ligand containing KGM was decanted and replaced with Dulbecco's PBS (GIBCO/BRL 14040–018) containing 1 mM PMSF and 0.05 μg/μl of aprotinin, and this suspension was pelleted and gently resuspended in 500 μl of nuclei preparation buffer (NPB) consisting of 20 mM Hepes (pKa of 7.55 at 20°C), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1% (vol/vol) aprotinin made 0.6% (vol/vol) with Nonidet P-40 (Sigma N-6507) just before use. Five hundred microliters of NPB was required per single T75 culture flask, which contained 2–4 $\times$ 10^6 HEK cells. After centrifugation at 2,600 $\times$ G_{av}, small aliquots of the pellet were resuspended in NPB, stained with 0.1% crystal violet, and displayed nuclei containing darkly stained chromatin, with cellular and cytoplasmic debris enriched in the supernatant fraction. Nuclear pellets were resuspended in an ice-cold nuclei lysis buffer consisting of 20 mM Hepes (pKa of 7.55 at 20°C), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1% (vol/vol) aprotinin, and 10% (vol/vol) glycerol were vortexed intermittently for 30 min on wet ice. Alternatively, a mini-homogenizer (pellet pestle; Kimble 749520) was used for a more thorough pellet resuspension and nuclear lysis. Lysates were microcentrifuged (12,000 $\times$ G_{av}, 10 min), and the supernatants were aliquoted into presterilized 0.5 ml GeneAmp PCR tubes (Perkin–Elmer), supplemented with 0.025 μg/μl of leupeptin, and stored at -81°C. NPXTs were found to be stable for up to 6 months; repeated freeze-thaw cycles of these NPXTs were avoided. Quantitation of NPXTs was performed by using mini-Bradford assays and whole HEK nucleoprotein as a standard (41).

EMSA. NPXTs derived from cultured HEK cells were subjected to EMSA (42, 43). AP1, AP1-like, AP2, Egr1 (*zif268*), NF-KB, the GAS and SIE elements, and TFIID, and their mutant oligonucleotides containing the relevant consensus sequences (Table 1) were obtained from Santa Cruz Biotechnology or were synthesized at the Louisiana State University Medical Center Core Facility as single-stranded DNAs and then annealed by

Table 1. Oligonucleotide consensus sequences used as probes for EMSA

Transcription factor	DNA target sequence
AP1 consensus	5′-CGC TTG ATG ACT CAG CCG GAA-3′
AP1-like consensus	5-AGA GAA ATG CCT TAA GGC ATA-3′
AP1 mutant	5′-CGC TTG ATG ACT TGG CCG GAA-3′
AP2 consensus	5′-GAT CGA ACT GAC CGC CCG CGG CCC GT-3′
AP2 mutant	5′-CTA GCT TGA CTG GCG GGC GCC GGG CA-3′
Egr1(*zif268*) consensus	5′-GGA TCC AGC GGG GGC GAG CGG GGG CGA-3
Egrl(*zif268*) mutant	5′-GGA TCC AGC TAG GGC GAG CTA GGG CGA-3′
NF-κB consensus	5′-AGT TGA GGG GAC TTT CCC AGG C-3′
NF-κB mutant	5′-AGT TGA GGC GAC TTT CCC AGG C-3′
GAS (STAT1) consensus	5′-AAG TAC TTT CAG TTT CAT ATT ACT CTA-3′
GAS (STAT1) mutant	5′-AAG TAC TTT CAG TGG TCT ATT ACT CTA-3′
SIE (STAT3) consensus	5′-GTG CAT TTC CCG TAA ATC TTG TCT ACA-3′
SIE (STAT3) mutant	5′-GTG CAT CCA CCG TAA ATC TTG TCT ACA-3′
TFIID (TBP) consensus	5′-GCA GAG CAT ATA AAA TGA GGT AGG A-3′
TFIID (TBP) mutant	5′-GCA GAG CAG CTA AAA TGA GGT AGG A-3′

heating equimolar amounts in TE buffer (10 mM Tris·HCl/1 mM EDTA, pH 8.0) to 95°C for 4 min followed by slow cooling to room temperature. Oligonucleotides were end-labeled by using γ-^{32}P-ATP (Amersham Redivue, ≈3,000 Ci/mmol) and were reacted with 0–5 μg of the respective NPXTs in a 5-μl volume containing protein–DNA assembly buffers as previously described (43). Poly[d(G-C)] (5 ng/μl; Boehringer 108–782) was used as nonspecific competitor DNA in TFIID gel shifts. Rabbit polyclonal antibody specific for human TFIID used in gel supershift assay was purchased from Santa Cruz (SC-273X).

Isolation of Total RNA from HEK Cells. Total RNA was isolated from control, PAF, IL-1β, IL-6, BUDeR-treated, and DEX-treated HEK cells by using TRIzol reagent (BRL; ref. 44). PMSF (1 mM), leupeptin (0.05 μg/μl), and 1 unit/μl of RNasin (human placenta ribonuclease inhibitor; Promega) were added to the extraction media to inhibit protease and ribonuclease A, B, C, and H activities.

Quantitation of COX-1 and COX-2 RNA Message Levels by RT-PCR Analysis. Total RNA samples of $A_{260}/A_{280} \geq 2.0$ were used as templates for RT into cDNA. Mini cDNA libraries were constructed from total RNA by using RNase H$^-$ Moloney murine leukemia virus–reverse transcriptase and hexamer primers (Superscript II/One-Step RT-PCR system; BRL; detectability: ≈10 molecules of RNA template). Human-specific COX-1 and COX-2 signals were generated with a double hotstart technique that used HotStart 50 reaction tubes (MβP), Amplitaq Gold DNA polymerase (Perkin–Elmer), the COX-1 sense 5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′ and antisense primers 5′-GTGCATCAACACAGGCGCCTCTTC-3′, the COX-2 sense 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ and antisense primers 5′-AGATCATCTCTGCCTGAGTATCTT-3′, yielding 304- and 305-bp primary PCR products, respectively (15, 36). For signal quantitation, primers were singly end-labeled by using [γ-^{32}P]dATP (3,000 Ci/mmol, Amersham redivue) and T4 PNK (Promega) before use in PCRs. As previously described, PCR was performed on the up-ramp of cycling so that at 25 cycles of amplification the log of primary COX-1 and COX-2 PCR products generated was a linear function of the log of cDNA template added (15, 36). End-radiolabeled primary PCR products generated were analyzed on 5% acrylamide/1 × TBE (90 mM Tris, pH 8.4/90 mM boric acid/1 mM EDTA) gels and were dried under vacuum onto 2-mm Whatman filter paper at 80°C for 2 hr and then were autoradiographed onto PhosphorImager screens.

DNA Sequence Analysis, Data Analysis, and Quantitation. DNA sequence analysis identifying putative *cis*-acting DNA regulatory elements lying between −1,000 bp and +165 bp of the human COX-2 gene promoter (Table 2) was performed by using Kodak/IBI/Pustell subsequence analysis (version 2.04), Hitachi Oligo DNA sequence analysis software (version 5.0), and human COX-2 promoter DNA sequence obtained from GenBank accession D28235 (36, 39). Dried gels were exposed to PhosphorImaging storage screens, and the resulting signals were analyzed on a GS-250 Molecular Imager (Bio-Rad Molecular Analyst Software version 1.4.1) or a Fuji FLA2000 Bio-Imaging Analyzer. Relative intensities of EMSA gel-shifted species or COX-1 and COX-2 RNA message levels were quantitated by using the PhosphorImager analysis and data acquisition/statistical analysis packages provided with each instrument. All P values were derived from protected t tests or least-square means from a two-way factorial analysis of variance (ANOVA).

RESULTS

In comparing the relative DNA-binding signal intensities of NPXTs in uninduced HEK cells by using EMSA, AP1, AP2, GAS, NF-κB, and SIE-DNA binding factors, all are relatively abundant whereas Egr1 and TFIID are present at relatively lower levels. Fig. 1 is overexposed to compare the relative DNA-binding activities for the *cis*-acting transcription factors AP1, AP2, Egr1, GAS, NF-κB, SIE, and the basal transcription factor TFIID in control HEK cells compared with those that have been induced with PAF or IL-1β. Both PAF and the cytokine IL-1β were found to strongly activate transcription factor AP1-, GAS-, and NF-κB-DNA binding ($P < 0.001$); much smaller effects were noted with AP2, Egr1, or SIE ($P > 0.05$). No significant effects were observed on TFIID-DNA binding ($P > 0.68$). The largest increases in DNA-transcription factor binding were obtained for AP1, GAS, and NF-κB to 1.9-, 2.1-, and 2.6-fold above mean control levels after PAF and 2.3-, 2-, and 1.9-fold above mean control levels after IL-1β stimulation, respectively.

Because the TATA binding protein TFIID has been difficult to detect when using NPXTs derived from cultured cells, we therefore examined in more detail the effects of PAF or IL-1β on the TFIID-DNA interaction (34, 35, 45). Fig. 2 shows that using NPXT derived from either control, PAF-, or IL-1β-induced HEK cells in the presence of the ^{32}P-labeled TFIID DNA consensus sequence (Table 1), there is a single prominent gel-shifted species unique to the TFIID complex (arrows). However, PAF or IL-1β did not modify TFIID-DNA binding ($P \geq 0.68$, ANOVA; Figs. 1 and 2). No specific TFIID-DNA complexes were observed when either mutant TFIID oligonucleotides were used in EMSA or when a 60-fold excess of unlabeled TFIID consensus was used

Table 2. DNA regulatory elements lying between −1,000 bp and +165 bp of the human COX-2 gene promoter

Element	DNA sequence	Position*
AP1-like	5′-TGCCTTA-3′	−938 to −932 bp
AP1-like	5′-TGACCCG-3′	−842 to −836 bp
GAS (STAT1)	5′-TT[N_5]AA-3′; 5′-TTTCCAAGAAA-3′	−758 to −748 bp
SIE (STAT3)	5′-TT[N_4]AA-3′; 5′-TTCCACAA-3′	−646 to −532 bp
AP1-like	5′-TCACTAA-3′	−566 to −560 bp
NF-κB*d***	5′-GGAGAGGGGATTCCCTGCGCC-3′	−452 to −433 bp
AP2-like	5′-GAACTCGGGGAGGA-3′	−262 to −249 bp
NF-κB*p***	5′-AGAGTGGGGACTACCCCCTCT-3′	−229 to −209 bp
AP2*p***	5′-GCCCCCACCGGG-3′	−145 to −134 bp
SIE (STAT3)	5′-TT[N_4]AA-3′; 5′-TTACGCAA-3′	−133 to −126 bp
SIE (STAT3)	5′-TT[N_4]AA-3′; 5′-TTTTTTAA-3′	−124 to −117 bp
GAS (STAT1)	5′-TT[N_5]AA-3′; 5′-CTTATAAAAAG-3′	−33 to −24 bp
TATA BOX	5′-TTATAAAAA	−32 to −24 bp
TFIID (TBP)	5′-TATA-3′	−31 to −28 bp
TRXN START (↓)	5′-AGCGACC ↓ AATTGTC-3′	−7 to +7 bp
ATG START	5′-ATG-3′	+136 to +138 bp
Egr1(*zif-268*)	5′-GTGCGCGG-3′	+158 to +165 bp

*Relative to the start of transcription (TRXN) at +1 bp; DNA promoter sequence data from GenBank D28235. ***p* = proximal; *d* = distal.

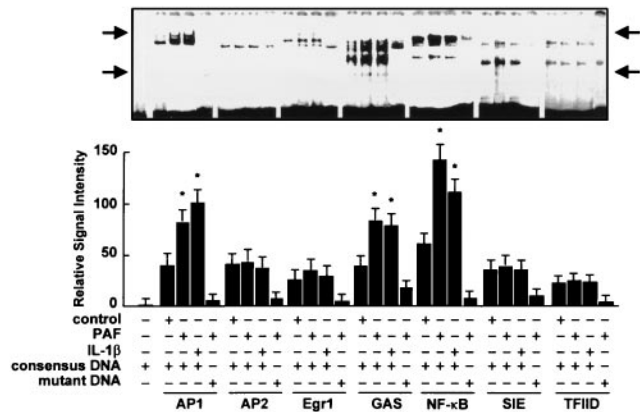

FIG. 1. PAF or IL-1β differentially enhance DNA binding activities of *cis*-acting transcription factors. EMSA using NPXTs from HEK cells reacted with target DNA oligonucleotide consensus or with mutant sequences for the transcription factors AP1, AP2, Egr1 (*zif268*), GAS, NF-κB, SIE, and TFIID described in Table 1. Leftmost lane represents the migration of a typical free ^{32}P end-labeled oligonucleotide with no NPXT. Relative gel shift signal quantitation in the bar graph was for gel-shifted species unique to that transcription factor-DNA binding consensus sequence, minus the signal contributed by the mutant DNA, and located in between the two sets of arrows. NF-κB quantitation includes only the upper (p50-p65) activator complexes whose identity was confirmed by using p50 and p65 antibodies and gel supershift assay (data not shown). Gel is overexposed to show the relative signal intensities among each of the shifted species. Exposure time = 12 hr. $n = 4$; mean ± SD; significance of induced factors over control, *, $P < 0.001$; significance of AP2, Egr1, and SIE over control $P > 0.05$; significance of TFIID over control, $P > 0.68$, ANOVA.

in a "cold" oligonucleotide competition assay. An EMSA supershift, but not a complete disappearance of the TFIID-DNA species, was found with preincubation of a rabbit polyclonal antibody that specifically recognizes human TFIID-TATA binding protein but not other basal transcription factors.

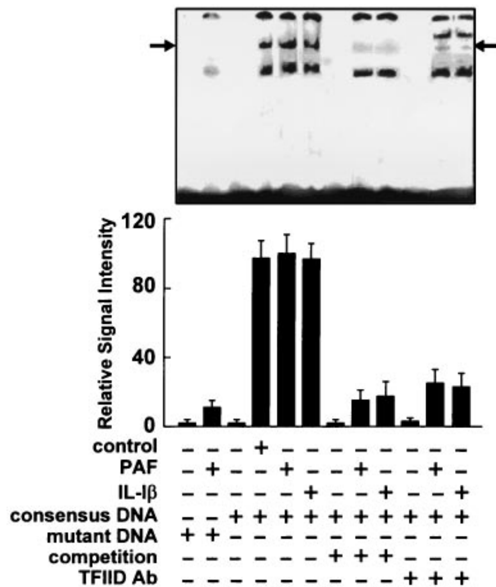

FIG. 2. PAF or IL-1β do not modify TFIID-DNA binding. EMSA using NPXTs derived from HEK cells and the mutant and consensus sequence for the basal transcription factor TFIID. Arrow depicts unique TFIID-shifted complex (34, 45) used for signal quantitation in the bar graph shown below. A 60-fold excess of unlabeled TFIID consensus sequence ("cold" competition assay) almost completely eliminated TFIID gel shift. Rabbit polyclonal antibody specific for human TFIID was used in the gel supershift assay. Exposure time = 36 hr. $n = 4$; mean ± SD; significance of PAF- or IL-1β-induced TFIID factor over control, $P > 0.68$.

Because PAF and IL-1β each strongly stimulated the DNA binding of the three distinct inducible transcription factors AP1, GAS, and NF-κB 1.9- to 2.6-fold, the effects of BUDeR and DEX on AP1-, GAS-, and NF-κB-DNA binding were examined at 10^{-8} M, 10^{-7} M, and 10^{-6} M GC concentration. DNA binding activity for these transcription factors in the presence of BUDeR or DEX are shown after PAF or IL-1β stimulation, respectively, in Fig. 3 *A* and *B*. Generally, BUDeR and DEX each were observed to strongly repress AP1-, GAS-, and NF-κB-DNA binding activities at concentrations of 10^{-7} M ($P < 0.001$). AP2-, Egr1-, and SIE-DNA binding activities also were generally suppressed by the actions of these GCs although to a much smaller extent (data not shown). At 10^{-7} M, PAF induced about 1.9-fold enhancement in transcription factor-DNA binding for AP1 whereas IL-1β induced a 2.3-fold induction of this same DNA binding protein. Conversely, the PAF-mediated induction was 2.6-fold for NF-κB but only 1.9-fold for induction with IL-1β. In each case 10^{-7} M BUDeR or DEX dramatically suppressed the mean value of AP1-, GAS-, or NF-κB-DNA binding and the effects of BUDeR exceeded those of DEX. Again, the binding of TFIID to its target DNA sequence displayed no change when induced with either PAF, IL-1β, or after treatment with the GCs BUDeR or DEX at 10^{-7} M ($P > 0.68$).

Fig. 4 summarizes the effects of PAF, IL-1β, the related cytokine IL-6, DEX, and BUDeR on COX-1 and COX-2 RNA message abundance. Fig. 4*A* shows a schematic diagram of the relative positions of DNA binding sites for AP1-like, AP2, Egr1, GAS, NF-κB, SIE, and TFIID in the human COX-2 promoter (refs. 36 and 39; Table 2). Clustering and positioning of DNA recognition sequences may indicate the relative importance of those *cis*-acting factors on specific promoter activation (46, 47). Results of a typical COX-1 and COX-2 RNA message quantitative RT-PCR analysis are shown in Fig. 4*B*, and the data is summarized in Fig. 4*C*. PAF (100 nM) and IL-1β (10 ng/ml) both induced COX-2 RNA message levels by 6.6- and 4.1-fold, respectively, over control ($P < 0.001$, ANOVA). PAF previously has been shown to be a strong inducer of the COX-2 gene in transfected NG108–15, SH-SY5Y, or NIH 3T3 cells (20), in activated aveolar macrophages (24), and in the brain (25, 26, 48). Fig. 4*D* shows the concentration-response curve for DEX and BUDeR on the down-regulation of the COX-1 and COX-2 genes. Again, as for maximal suppression of AP1-, GAS-, and NF-κB-DNA binding (Fig. 3), the optimal concentrations for COX-2 gene repression were found to be at 10^{-7} M with either BUDeR or DEX. Notably, we found that the COX-2 gene RNA message was not strongly induced in HEK cells by 10 ng/ml IL-6, which was within one SD of untreated controls ($P > 0.05$, ANOVA; Fig. 4).

DISCUSSION

Transcriptional regulation is accomplished, in part, by the interplay between basal and *cis*-acting transcription factors located in class II gene promoters (34, 45). Basal elements such as the TATA-binding protein TFIID appear to be sufficient for RNA polymerase to nucleate initiation complexes that support basal levels of DNA transcription (34, 35). Basal transcription rates may be enhanced by factors of 5–1,000 because of the synergistic interaction of combinations of regulatory *cis*-activator proteins with the basic TFIID-containing basal initiation complex (35, 45, 46). For example, the dimeric Jun/Jun or Fos/Jun AP1 complex stimulates transcription from promoters containing the DNA sequence 5′-TGASTCA-3′ (S=C or G; refs. 16 and 17), however, DNA sequences flanking these core transcription factor recognition sites can further modulate DNA binding (34, 45). A palindromic AP1-like consensus DNA sequence 5′-ATGCCTTAAG GCAT-3′ located in the distal COX-2 promoter (−939 to −926 bp) also was found to bind purified human recombinant Jun/Jun AP1 transcription factor (unpublished observations; Table 1).

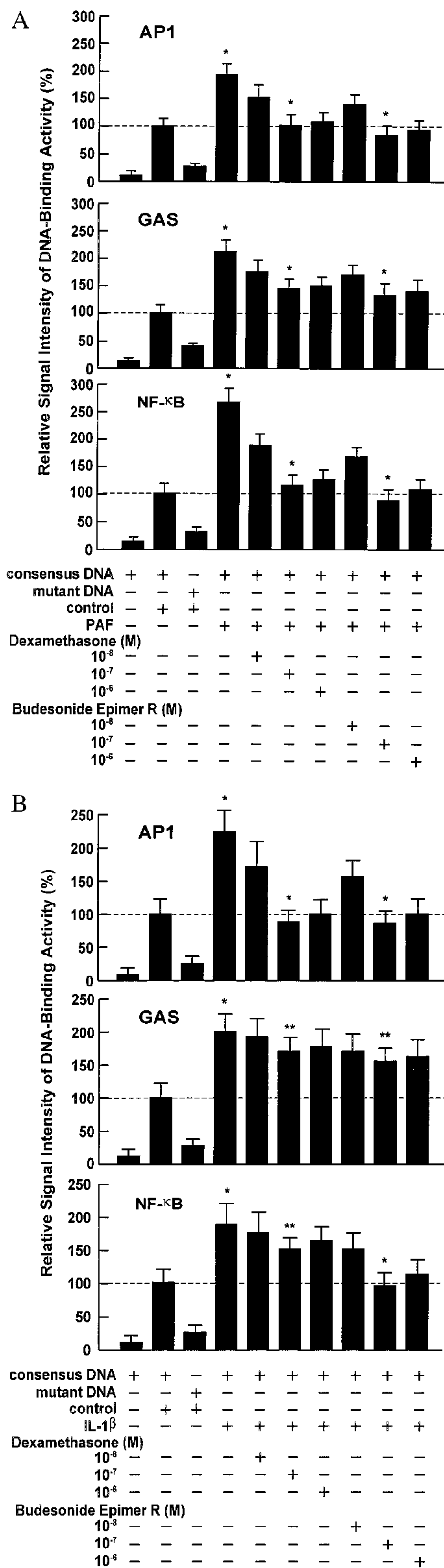

FIG. 3. Dose-dependent inhibition by BUDeR or DEX of DNA binding by *cis*-acting transcription factors AP1, GAS, and NF-κB in HEK cells activated by PAF (*A*) or IL-1β (*B*). Relative signal intensity of DNA binding activity as determined by EMSA using NPXTs derived from HEK cells and reacted with the target oligonucleotide consensus sequences for the transcription factors AP1, GAS, or NF-κB. Leftmost lanes represent the relative signal intensity of a typical free oligonucleotide with no NPXT. Dashed line represents HEK control NPXT and DNA consensus sequences for transcription factors AP1, GAS, or NF-κB (Table 1) equal to 100. Compared with controls, both PAF (*A*) and IL-1β (*B*) elicited a 1.9- to 2.6-fold increase in transcription factor-DNA binding for AP1, GAS, and NF-κB. (M) = molar. $n = 4$; mean ± SD; significance of induced factors over control: *, $P < 0.001$, **, $P < 0.01$, ANOVA. Levels of TFIID-DNA binding remained consistently unchanged ($P \geq 0.68$, ANOVA; Figs. 1 and 2).

Notably both IL-1β (49) and PAF (unpublished observations) but not IL-6 (49) activate the Jun N-terminal kinase JNK1 required for enhancement of AP1 transcriptional activation. Other *cis*-acting elements, including the Zn^{2+}-containing DNA binding protein Egr1 (29), NF-κB, which pre-exists as a latent dimeric activator in the cytoplasm (18, 32), and the SIE and GAS factors, induced by interferons α and γ, respectively (9, 37), have been shown to enhance basal transcription rates depending on their frequency and position relative to the core promoter (10, 35). Importantly, even relatively modest changes in transcription factor-DNA binding elicit significant changes in transcriptional activity (34, 45). For instance, cotransfection of the acetylcholinesterase promoter/luciferase reporter gene in HepG2 and C2 cells with AP1 (Jun/Jun) and AP2 expression vectors has shown a dramatic increase in the transcription rates of genes containing AP1 and AP2 binding sites in their transcriptional regulatory domain (46).

The binding of the nucleoproteins AP1, GAS, and NF-κB onto their target DNA consensus sequence, but not their mutant oligonucleotides, was enhanced after treatment with either PAF or IL-1β 1.9–2.6 times their levels over uninduced controls. Only minor effects were observed after either PAF- or IL-1β-mediated induction of AP2, Egr1, or the STAT element SIE. No effect was observed on the TATA binding protein TFIID to DNA. The DNA binding of AP1, GAS and NF-κB with their DNA target sequences each were markedly diminished during incubation with BUDeR or DEX. In parallel with reduced transcription factor binding, there was decreased COX-2 RNA message abundance. It is of interest that the COX-2 promoter contains multiple copies of AP1-like, GAS and NF-κB transcription factor binding sites (Table 2 and Fig. 4*A*). In contrast, the binding of the general transcription factor TFIID onto its recognition sequence at the COX-2 core promoter was not affected in either PAF-, IL-1β-, DEX-, or BUDeR-treated HEK cells (Figs. 1 and 2). This finding indicates that DNA recognition by *cis*-acting transcription factors may be preferentially targeted by BUDeR or DEX, leaving the basal transcription mechanism that uses TFIID intact. It is also noteworthy that for the *cis*-acting transcription factor NF-κB, BUDeR is a more effective suppressor of DNA binding when compared with DEX (Fig. 3). Despite the lack of a 9α-fluoro atom in BUDeR when compared with DEX, BUDeR binds with a 14-fold higher affinity to the GC receptor as DEX (27). BUDeR, therefore, may have greater anti-inflammatory potential than other synthetic GCs in cases where NF-κB is used in programs of inflammation-stimulated gene transcription.

Finally, strong suppression of transcription factor NF-κB-DNA binding may be a general feature of GC action on transcription signaling (7, 8, 18). NF-κB, with two DNA consensus sequences in the COX-2 promoter (Table 2), was found to be the most strongly induced DNA binding factor in HEK cells after PAF stimulation, and also the most suppressed by the action of either BUDeR or DEX (Fig. 3 *A* and *B*). NF-κB-responsive elements are required for the function of many cytokine promoters (1, 18, 32), and GCs have long been known to inhibit COX (50–52). Mechanistically, the COOH-terminal transactivation domain of the NF-κB p65 subunit may directly contact TATA-binding proteins and thereby influence the activity of the basal transcription machinery (32, 53). We note that the NF-κB distal, NF-κB proximal, and GAS/TFIID DNA-binding sites in the COX-2 promoter, each located ≈200 bp apart, may be aligned into close proximity after nucleosome folding enabling such a direct interaction (Fig. 4*A*). The GCs BUDeR and DEX therefore may inhibit both AP1- and NF-κB-

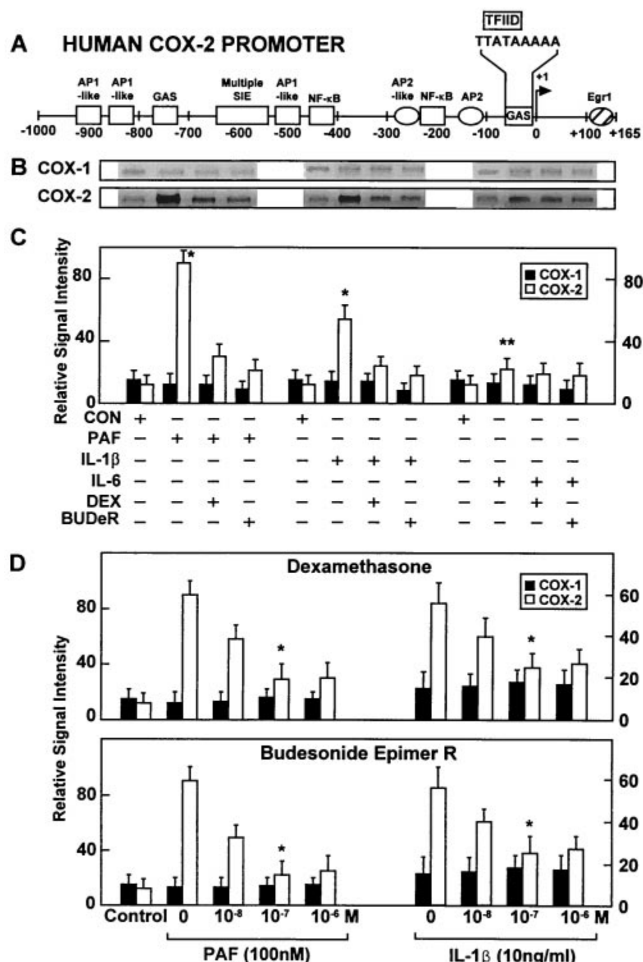

FIG. 4. PAF or IL-1β, but not 1L-6, induces COX-2 gene expression in HEK cells. Dose-dependent inhibition of this induction by BUDeR or DEX. (*A*) Diagram of the human COX-2 promoter showing transcription factor DNA binding consensus sequences from $-1{,}000$ and $+165$ bp relative to the start of transcription ($+1$ bp). DNA binding consensus sequences are described in Tables 1 and 2. Note that the TFIID site doubles as a GAS binding site. (*B*) Representative gel showing COX-1 and COX-2 RNA message abundance after treatment with PAF (100 nM), IL-1β (10 ng/ml), IL-6 (10 ng/ml), or DEX and BUDeR (each at 10^{-7} M). (*C*) RNA message levels quantitated from *B*. $n = 4$; mean $\pm$ SD; significance of induced RNA levels over control, $*$, $P < 0.001$, $**$, $P > 0.05$, ANOVA. (*D*) Dose-dependent inhibition by BUDeR or DEX on PAF- or IL-1β-induced changes on COX-1 and COX-2 RNA message abundance. $n = 4$; mean $\pm$ SD; significance of GC suppression at 10^{-7} M compared with no treatment, $*$, $P < 0.001$, ANOVA.

mediated gene expression via direct "type 2" transcriptional effects that include interference with these factors in recognizing their own DNA-binding consensus sequence via the GC receptor (5–8). The GC receptor also has been shown to interact with a GAS-related STAT5 element to diminish the response of "type 1" GC-sensitive promoters (9, 10). These particularly potent effects of BUDeR and DEX and the activated GC receptor on AP1, GAS, and NF-κB gene activation pathways underscore GC's remarkable effectiveness in deregulating multiple interdependent transcription control elements. The presence, number, and position of AP1, GAS, and NF-κB transcription factor responsive elements in gene promoters may allow one to predict to what extent they participate in the inflammatory response and hence their susceptibility to transcriptional down-regulation elicited by BUDeR, DEX, or other related GC compounds.

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