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Specific Activation of the Glucocorticoid Receptor and Modulation of Signal Transduction Pathways in Human Lens Epithelial Cells

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Abstract

Purpose—Prolonged use of glucocorticoids (GCs) can lead to cataract formation. Lens GC responses have been difficult to elucidate. A previous study showed the presence of the glucocorticoid receptor (GR) in immortalized and primary human lens epithelial cells (hLECs) and GC-induced changes in gene expression. This study demonstrates specific GR activation and identifies the biological effect of GC-induced changes in gene expression in hLECs.

Methods—HLE B-3 (B-3) and primary cultures of hLECs were transfected with pGRE.Luc and treated with or without dexamethasone (Dex), RU-486, spironolactone, or vehicle. mRNA and protein expression were examined by real-time PCR and Western blot analysis, respectively. Cell proliferation and apoptosis were examined by WST-1 and flow cytometry, respectively.

Results—Dex treatment of B-3 and primary cultures demonstrated specific GR, but not mineralocorticoid receptor (MR), activation and phosphorylation. Pathway analysis revealed GC-induced changes in expression of MAPK regulators. Increased expression of GILZ mRNA and MKP-1 mRNA and protein was observed in immortalized and donor hLECs. This corresponded with a decrease in the phosphorylated forms of RAF, ERK, p38, and AKT, but not in JNK. No net change in LEC proliferation or apoptosis was observed with Dex treatment.

Conclusions—GC treatment of hLECs activates the GR to modulate the expression of MAPK and PI3K/AKT regulators. This is the first demonstration of GC signaling in hLECs. GCs, MAPK, and PI3K/AKT are involved in cell processes implicated in steroid-induced cataractogenesis. The absence of a net change in cell activity with acute steroid treatment is consistent with the possibility that chronic treatment leads to prolonged modulation of these pathways and steroid-induced cataract.

Glucocorticoids (GCs) are steroid hormones involved in physiological processes and are used clinically as immunosup-pressive and anti-inflammatory agents. Their possible role in lens cell function was hypothesized because prolonged GC use lead to the formation of a posterior subcapsular cataract with the finding of nucleated epithelial cells in the posterior region of the

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lens.[1-3] This suggests that GCs alter an aspect of lens cell proliferation, differentiation, or apoptosis. The mechanisms of GC lens action and cataractogenesis are not known.

Classically, in other cell types, GCs exert their effects by binding to a specific intracellular glucocorticoid receptor (GR). GC-binding results in hyperphosphorylation of the hypophosphorylated GR. The GC-GR complex binds to DNA, to modulate gene expression.[4,5] A specific GC response would require the presence of the GR.[6] Intracellular GRs are ubiquitously expressed,[7,8] however, despite past evidence[9-11] the presence of the GR in the mammalian lens epithelium was questioned.[12]

We have reported the unequivocal presence of the classic functional GR in immortalized and primary cultures of human lens epithelial cells (hLECs) able to activate transcription.[13,14] Despite the presence of the GR, a specific lens GC response has been difficult to elucidate.[13,15,16] We recently reported the identification of GC target genes through microarray analysis of hLECs treated acutely (4 and 16 hours) with the synthetic steroid dexamethasone (Dex), but the resultant physiological effect was not identified.[14] To understand the mechanism of cataractogenesis, it is important to demonstrate a lens GC response, which over time, may lead to the steroid-induced cataract observed in patients after prolonged steroid therapy. Since GCs are used clinically for the treatment of many conditions, the understanding of a lens GC response is of high priority.

The GR belongs to a superfamily of nuclear hormone receptors that includes mineralocorticoid receptors (MRs).[17] All members of the family share structural features in the central DNA binding and the C-terminal ligand-binding domains. The N-terminal region is highly variable and distinguishes members of the superfamily.[18] Physiologically, cortisol, the endogenous GC, activates the MR with higher affinity than it does the GR. Once activated, both are able to transactivate glucocorticoid response elements (GREs) of target genes.[19,20] Both the GR and MR have been reported to be expressed in mammalian LECs[9,10]; therefore, it is important to determine whether the lens GC response is due to activation of the GR or MR.

In this study, Dex activated the GR, and not the MR, in HLE B-3 (B-3) cells and primary cultures of hLECs. Dex induced hyperphosphorylation of the GR, further confirming and demonstrating activation. In-depth pathway analysis of previously reported microarray results[14] suggests modulation of mitogenactivated protein kinases (MAPKs) and phosphotidyl inositol-3-kinase/AKT (PI3K/AKT) regulators, which we confirmed by real-time PCR and Western blot. Dex treatment caused no net change in hLEC proliferation or apoptosis. These novel findings demonstrate lens GC signaling for the first time and suggest that the reported crosstalk of GCs, MAPK, and PI3K/AKT[21-25] is involved in the lens GC response.

Materials and Methods

Tissue, Cell Culture, and Treatment

Human lenses were obtained from eye bank donor eyes. The capsule containing epithelial cells was carefully separated from fiber cells. Explants and B-3[26] cells were maintained in phenol red–free MEM containing 20% FBS, 2 mM glutamine, and 50 μg/mL gentamicin and replaced with MEM containing 2% charcoal-stripped serum 16 hours before treatment. Alternatively, whole lenses were placed in serum-free phenol red–free DMEM for 24 hours before treatment, removal of the epithelial layer, and isolation of RNA. Dexamethasone, RU-486, and spironolactone, purchased from Sigma-Aldrich (St. Louis, MO), were dissolved and diluted in 100% ethanol. Ethanol (100%) served as vehicle control and the final concentration never exceeded 0.1%. Cells were treated for 4, 16, or 24 hours. Transfection studies over a period of 72 hours demonstrated GR-mediated transcription as early as 2 hours. Maximum activation was seen after 16 hours of treatment and was sustained over 72 hours. A 4-hour treatment time

was chosen to represent “early” changes in transcription. A 16-hour treatment time was chosen to represent “later” changes in transcription. This research complied with the Declaration of Helsinki and was approved by the University of Medicine and Dentistry of New Jersey (UMDNJ) institutional review board.

Transfections

Plasmid pGRE.Luc (Clontech, Palo Alto, CA) with GRE enhancer elements drives the expression of firefly luciferase. pRL-SV40 (Promega, Madison, WI), which drives expression of *Renilla* luciferase, served to normalize and control for variation in transfection efficiency. B-3 and primary cultures, seeded in triplicate, were cotransfected with the plasmids (Lipofectamine 2000; Invitrogen, Carlsbad, CA), as previously reported.[13] Briefly, transfection medium was changed after 5 hours. Cells were treated 16 hours later with combinations of vehicle, 100 nM Dex, 1 μM RU-486, and/or 1 μM spironolactone for 24 hours. Luciferase activity was measured (Dual Luciferase Reporter Assay System; Promega) on a luminometer (LumiCount; Packard Instrument Company, Downers Grove, IL). Absolute *Renilla* luciferase values were high and reproducible within individual experiments, indicating adequate transfection efficiency. Results reported as ratios of firefly to *Renilla* luciferase, according to the manufacturer’s protocol, were standardized by setting the vehicle control to 1.

Reverse Transcription–Real Time-Polymerase Chain Reaction

Three pairs of donor lenses, carefully dissected from the anterior globe, were incubated in medium with 0% serum and treated as pairs, to more closely resemble in vivo conditions. One lens was treated with 1 μM Dex and the other with vehicle for 4 hours. The epithelial layer was dissected from the fiber cells, and homogenized (RNAzol; Tel-Test, Friendswood, TX). Chloroform was added, and the sample was transferred to a microfuge tube (Phase Lock Gel Heavy; Eppendorf, Westbury, NY) and centrifuged for 20 minutes at 12,000*g* (4°C) before the aqueous phase was transferred to a fresh tube with equal volumes of isopropanol. The sample was stored overnight at −80°C and centrifuged for 20 minutes at 12,000g (4°C). The pellet was washed twice with ethanol, air dried, and resuspended in diethyl pyrocarbonate (DEPC)–treated water. RNA concentration was determined by spectrophotometry, and aliquots were stored at −80°C.

B-3 cells, 10th passage, seeded in triplicate were replaced with medium containing charcoal-stripped serum 16 hours before treatment. Cultures were treated with 1 μM Dex or vehicle for 2, 4, 8, 16, and 32 hours. Total RNA was isolated (RNAzol; Tel-Test) according to the manufacturer’s protocol.

RNA (1 μg) was reverse transcribed using transcription reagents (Applied Biosystems, Inc., [ABI], Foster City, CA) on a PCR system (GeneAmp PCR system 9700; ABI) according to the manufacturer’s protocols. The reaction was incubated at 42°C for 60 minutes and 95°C for 5 minutes and held at 4°C. The primer sequences for DUSP-1/MKP-1and DSIP/GILZ have been published.[14,27] PCR was performed with master mix (LightCycler FastStart DNA Master SYBR Green I kit; Roche Applied Science, Indianapolis, IN), and a thermocycler (LightCycler 1.0; Roche Applied Science), as previously described.[14] Briefly, the reaction was stopped during the log phase and analyzed by crossingpoint analysis. The relative difference (*x*-fold) was defined as $2^{(a-b)}$ where *a* and *b* represent the crossing points of the two samples being compared. All results were normalized to actin. The specificity of PCR products was determined by melting-curve analysis and visualization on 1% or 2% agarose gels stained with ethidium bromide.

Western Blot Analysis

Western blot analysis for total and phosphorylated proteins was performed with whole cell lysates from 10th-passage B-3 cells treated in triplicate and a primary culture of hLECs from a pair of fetal lenses. Cells were scraped into PBS and centrifuged, and the pellet was frozen in a dry ice methanol bath, thawed, and resuspended in lysis buffer (50 mM Tris-HCl; 100 mM NaCl; 0.5% NP40; 0.3 mM Na orthovanadate; 10% glycerol; 1 mM dithiothreitol [DTT]; and protease inhibitors; Mini Complete Tablet, Roche Applied Science). The extract was transferred to a fresh tube, incubated on ice, and centrifuged for 20 minutes at 17,000*g* (4°C), and the supernatant was transferred to a new tube.

Protein concentrations were measured and samples were electrophoretically separated on 12.5% denaturing Criterion Tris-HCl gels (Bio-Rad, Hercules, CA), transferred to nitrocellulose membranes (Bio-Rad), blocked with 5% nonfat-dry milk (or 5% BSA) in TBS-T, and immunoblotted (at the indicated primary antibody dilution) for p-ERK (1:2000), p-p38 (1:300), p-JNK(1:300), p-AKT (1:500; Cell Signaling, Beverly, MA), p-RAF (1:1000; Upstate, Waltham, MA), or MKP-1 (1: 1000; Santa Cruz Biotechnology, Santa Cruz, CA). Phospho-GR antibodies, which recognize phospho-S203 (1:10,000), phospho-S211 (1:1000), and phospho-S226 (1:1000), were a gift from Michael Garabedian (New York University School of Medicine). Blots were reprobed with an antibody to total ERK (1:1000), RAF (1:1000), GR (1:1000; Santa Cruz Biotechnology), p38 (1:300), JNK (1:300), AKT (1:500; Cell Signaling) or α-tubulin (1:3000; Sigma-Aldrich). Appropriate secondary antibody was used at 1:5000 dilution. Five micrograms of total protein extracts were used to analyze α-tubulin expression, 30 μg for phosphorylated and nonphosphorylated GR and α-tubulin expression, and 50 μg for phosphorylated and nonphosphorylated MKP-1, ERK, JNK, p-38, AKT, and α-tubulin expression. The amount of protein loaded remained the same within an experiment. Bands were visualized by chemiluminescence (NEN Life Sciences, Boston, MA) and quantified by densitometry.

Apoptosis and Viability Assays

Apoptosis was measured by annexin V-FITC and PI staining, as previously described.[28] Proliferation was measured using the WST-1 assay (Roche Diagnostics) according to the manufacturer's directions.

Statistics

Experiments were repeated two to three times with each experiment in triplicate. Data were analyzed for significance compared with vehicle by the two-tailed *t*-test assuming unequal variances, and differences at $P < 0.05$ were considered statistically significant.

Results

To verify that the synthetic steroid Dex binds to the GR, and not the MR, B-3 and primary cultures, transfected with pGRE-.Luc and cotreated with combinations of Dex, RU-486, spironolactone, or vehicle, were assayed for luciferase activity. Spironolactone is an MR antagonist and RU-486 is a GR antagonist.[29,30] RU-486, but not spironolactone, inhibited Dex-induced, GRE-mediated luciferase activity in B-3 and primary cultures (Figs. 1A, 1B). This demonstrates that Dex mediated transcription is due to GR, not MR, binding.

The GR is hyperphosphorylated in a hormone-dependent manner. Agonist-dependent phosphorylation was observed at all three serine residues characteristic of GR activation (Fig. 2). Phosphorylation was observed at S211 (Figs. 2A, 2B) as early as 2 hours and as late as 32 hours (data not shown). Phosphorylation at S203, analyzed at 4 and 16 hours, was greater at 4 hours (Fig. 2C) but was still apparent at 16 hours (data not shown). Phosphorylation of S226

was examined at 4 hours of treatment and appeared to have greater basal levels of phosphorylation, compared with S203 and S211, but resulted in hyperphosphorylation with Dex treatment (Fig. 2D). Cotreatment with RU-486 inhibited Dex-induced hyperphosphorylation at all three residues (Fig. 2). Dex treatment of primary cultures for 16 hours induced phosphorylation at S211 that was inhibited by cotreatment with RU-486 (data not shown).

Dexamethasone treatment of LECs did not yield significant changes in cell apoptosis (Figs. 3A, 3B) or proliferation (Fig. 3C); however, we have reported GC-induced changes in global gene expression after 4 and 16 hours of treatment.[14] Although changes in gene expression were identified, analysis of microarray data did not identify a resultant physiological change, consistent with our experimental findings, but instead revealed possible changes in signaling pathways. To further investigate a lens GC response, we analyzed modulated genes with a software tool for biological pathway analysis (Pathway Assist, ver. 2.0; Ariadne Genomics, Rockville, MD), used to investigate interactions among genes and gene regulation networks.[31] This tool was used to identify key relationships between Dex-modulated genes, to identify GC effects on LEC function.

The starting point in pathway analysis is arbitrary and determined by the researcher. The delta sleep-inducing peptidelike immunoreactor (DSIP) was found to be significantly upregulated by 4-, 16-, 24-, and 48-hour GC treatment of HLE B-3 cells and by 4-hour treatment of primary LECs.[14,32] Since it is upregulated to a great degree from 4 to 48 hours, the DSIP protein was chosen as a starting point. The pathways, analyzed by computer (Pathway Assist; Ariadne Genomics), demonstrated that DSIP did not appear to interact with any other Dex-induced genes identified in LECs (Fig. 4). It did, however, interact with RAF-1 and c-Fos/c-Jun, which are upstream and downstream of the MAPK cascade.

The computer program suggested that DSIP is similar to the glucocorticoid-induced leucine zipper (GILZ), and the two have been reported to be homologous.[33] To verify that the modulated gene identified by microarray was homologous to GILZ, alignments were performed using Clustal W multiple sequence alignment program provided in the public domain by the European Bioinformatics Institute, European Molecular Biology Laboratory, Heidelberg, Germany; available at http://www. ebi.ac.uk/clustalw/) to verify that the GenBank mRNA sequence (AL110191.1; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) from a microchip array database (database for the Hum U133A chip; Affymetrix, Santa Clara, CA) a GenBank mRNA sequence for DSIP (BC018148.1), and a GenBank mRNA sequence for GILZ (AB025432) were similar. The mRNA sequences were found to be 100% homologous (data not shown).

GILZ is a regulator of MAPK phosphorylation and subsequent activation.[34,35] Further analysis of MAPK by the pathway analyzer (Pathway Assist; Ariadne Genomics) revealed that as many as 30 of the genes modulated by GCs after 4- or 16-hour treatments in hLECs, including the dual specificity phosphatase (DUSP-1), are involved in the MAPK pathway (data not shown). DUSP-1 is also known as MAPK phosphatase-1 (MKP-1). MKP-1 is a phosphatase that dephosphorylates critical residues in the MAP kinases (ERK, JNK, and p38).[36,37] As expected, pathway analysis of DUSP-1/MKP-1 revealed similar genes identified by analysis of the MAPK pathway (data not shown).

MKP-1 upregulation was reported in B-3 cells treated for 16, 24, and 48 hours with Dex.[14, 32] Pathway analysis suggested that two of the GC-regulated genes in B-3 cells, GILZ and MKP-1, found to be expressed for long periods, decrease phosphorylation of the upstream

kinase RAF-1 and MAPKs (ERK, p38, and JNK) without affecting the total expression levels of the components themselves.

To test this prediction, GILZ and MKP-1 expression was quantitated by RT–real-time-PCR (Fig. 5). In B-3 cells, GILZ expression was increased 4- to 10-fold with Dex treatment, compared with vehicle treatment, over a period of 32 hours (Fig. 5A). Dex treatment induced nearly a fourfold increase in MKP-1 expression over vehicle at 2 hours, which decreased with time, but remained increased by nearly twofold after 32 hours (Fig. 5C). Similar results were observed in donor lenses (Figs. 5B, 5D). Dex treatment induced MKP-1 protein expression over vehicle and RU-486 prevented the Dex induction, in both B-3 (Fig. 6A) and primary hLECs (Fig. 6B).

Phosphorylation of MAPK pathway components was examined in B-3 cells. At 4 hours, a modest but significant ($P < 0.04$) decrease in phospho-RAF-1, but not RAF, expression was observed in extracts of Dex-treated cells compared with vehicle or Dex+RU-486 (data not shown). Although a slight decrease in phosphorylated ERK 1/2 was observed after 4 hours of Dex treatment, the change was not statistically significant ($P < 0.3$; data not shown). At 16 hours, Dex treatment yielded a marked and significant decrease in phosphorylated RAF-1 (Fig. 7A) and phosphorylated ERK 1/2 (Fig. 7B), inhibited by cotreatment with RU-486, without affecting the levels of RAF-1 and ERK 1/2.

A significant decrease in phospho-p38 expression (Fig. 7C), inhibited by RU-486 cotreatment, was observed in Dex-treated extracts at 4 hours, but at 16 hours, phospho-p38 was not observed in any of the treatments (data not shown). There were no changes in p38 expression. No significant changes were observed in phospho-JNK or JNK expression at 4 or 16 hours (Fig. 7D).

It has been suggested that GC-modulated genes are involved in the PI3K/AKT pathway.[14,32] We observed that Dex treatment resulted in a significant decrease in phospho-AKT expression in B-3 cells compared with vehicle or Dex+RU-486 (Fig. 8). There was no change in the levels of AKT protein.

Discussion

In the present study, Dex specifically induced transcriptional activity of the GR, not the MR, and resulted in GR hyperphosphorylation at hormone-dependent residues. Dex modulated the MAPK and PI3K/AKT pathways, thus demonstrating for the first time a novel GC signaling mechanism and an acute GC response in hLECs.

We have reported the expression of the transcriptionally active GR in immortalized and freshly isolated hLECs.[13,14] However, the GR belongs to a superfamily of nuclear receptors that share sequence homology in the ligand and DNA binding domains. In particular, the MR has structural and functional homology with the GR.[38] The GR binds the endogenous mineralocorticoid aldosterone with low affinity, but the MR binds the endogenous GC, cortisol, with greater affinity than does the GR.[38] Both the MR and GR bind to GREs to modulate gene expression. A specific mineralocorticoid response element has not been identified.[39] Receptor specificity, with respect to cortisol binding, can be determined by the expression of the 11β-HSD enzymes. 11β-HSD-1, coexpressed with the GR, converts inactive cortisone to active cortisol. 11β-HSD-2, coexpressed with the MR, converts active cortisol to inactive cortisone limiting the amount of cortisol to the MR.[40] However, receptor specificity cannot be determined by11β-HSD alone, because the human lens epithelium has been reported to express GR, MR, 11β-HSD-1, and 11β-HSD-2.[9,10]

Receptor specificity is also demonstrated by specific agonists and antagonists. The synthetic GR agonist Dex, which with prolonged use can cause a steroid-induced cataract,[36] binds the MR with high affinity, but it does not activate the MR with the same effectiveness as it does the GR.[37,38] Studies with receptor antagonists have demonstrated that spironolactone has high affinity for the MR, but not the GR,[41,42] and RU-486 binds to the GR, but not the MR.[43] Previously, we reported that RU-486 inhibits Dex-induced luciferase activity in transfected immortalized and primary hLEC cultures.[13,14] However, since the MR can bind Dex with high affinity, can transactivate genes through a GRE, and is present in hLECs, it was important to determine whether increased luciferase activity was due to GR binding alone or to GR and MR binding. Our results (Fig. 1) show that RU-486, but not spironolactone, inhibits Dex-induced luciferase activity in immortalized and primary hLEC cultures, demonstrating that Dex-induced changes in gene expression are due to the binding of activated GR, and not MR, to the GREs of target genes.

The hypophosphorylated GR undergoes hyperphosphorylation at multiple serine residues on hormone binding. Seven phosphorylated residues have been identified, mostly serines, clustered in the N-terminal domain.[44,45] Phosphorylation of three specific serine residues—S203, S211, S226—has been reported to be involved in transcriptional regulation,[46] with greater phosphorylation at S203 and S211 on hormone binding.[47-49] RU-486 prevents agonist-dependent phosphorylation.[50] Using specific antibodies, we detected basal phosphorylation, and Dex treatment yielded hyperphosphorylation of all three residues, which was inhibited by cotreatment with RU-486 (Fig. 2). The hormone-dependent phosphorylation of the GR at S203 and S211 in hLECs is similar to what is seen in other cell types, suggesting that the lens GR is functionally activated on hormone treatment.[51]

Posterior subcapsular cataracts are detected in patients after many months to years of steroid treatment.[7,8] Studies have demonstrated that GCs induce changes in LEC morphology and protein expression after prolonged high-dose GC treatment.[15,52] We have been unable to identify changes in LEC proliferation or apoptosis with an acute (15 minutes to 8 days) treatment of 100 nM to 100 μM Dex. Flow cytometry demonstrated that 1 to 50 μM Dex treatment did not change levels of apoptosis after 12 hours of treatment (Figs. 3A, 3B). No changes in hLEC proliferation were observed during the same period (Fig. 3C). Although we could not identify changes in these cellular functions, we were able to identify a transcriptional response as early as 2 to 4 hours, demonstrating an acute GC response in hLECs, despite the lack of detectable physiological changes.[13,14] The elucidation of the lens GC response, which is necessary to determine the mechanism of GC-induced cataractogenesis, requires an understanding of short- and long-term effects of treatment to understand GC action in the lens. Although genes were modulated after an acute (4–16 hours) Dex treatment, they could not be coordinately related to a change in lens physiology, consistent with our findings that Dex did not induce LEC proliferation or apoptosis. Instead, changes in signaling were suggested.[14]

The pathway analyzer (Pathway Assist; Ariadne Genomics)[31] was used to analyze previously reported microarray data from 4 and 16 hour GC treatment to understand the resultant biological effect of Dex-induced changes in LEC gene expression. The predictions are not absolutely precise, but they do identify trends in gene expression and allow for the study of signaling networks as opposed to individual genes. We chose to start the analysis with DSIP because microarray studies revealed that its expression was more than fivefold expression at 4 hours[14] and more than twofold expression as late as 48 hours.[32] Its significant expression over a prolonged period suggests it may have an impact on hLEC function. Pathway analysis suggested that DSIP interacted with components of the MAPK cascade (Fig. 4) and was homologous to GILZ. In other cell types, GILZ is reported to be inducible by Dex and prevent RAF-1 phosphorylation and activation, which subsequently prevents phosphorylation and activation of ERK 1/2.[34]

Although DSIP was chosen as the initial starting point in pathway analysis, other GC modulated genes were examined. The pathway analyzer (Pathway Assist; Ariadne Genomics) revealed that nearly 30 genes identified by microarray were connected to regulation of the MAPK pathway. Of interest was MKP-1, a phosphatase which dephosphorylates critical phosphoserine and phosphotyrosine residues in MAPKs.[53,54] MKP-1 is induced by Dex with simultaneous inactivation of ERK in mast and osteoblast cells.[55,56] Its upregulation is both GR and protein synthesis dependent and makes use of GREs in the promoter region of the MKP-1 gene.[56,57] Significant expression of these genes suggests decreased phosphorylation of MAPK components. Increased expression of GILZ and MKP-1 is expected to result in decreased RAF-1 and MAPK phosphorylation or activation without a change in protein expression because RAF-1, ERK, p38, and JNK mRNA did not change with Dex treatment of hLECs.[14,32]

Predictions were confirmed by real-time PCR and Western blot analysis. A significant increase in the expression of GILZ and MKP-1 mRNA was observed with Dex treatment in B-3 cells and organ cultures (Fig. 5). Increased expression of MKP-1 protein, inhibited by coculture with RU-486, was detected in Dex-treated B-3 cells and primary cell cultures (Fig. 6). Primary cultures were used to examine protein expression because lens organ cultures did not yield sufficient protein amounts and we were limited by the number of donor specimens.

Increased expression of GILZ and MKP-1 suggests that GC treatment of hLECs results in decreased levels of phosphorylated or activated RAF-1 and MAPK without affecting total protein levels. The decrease of phospho-MAPK has been correlated with Dex-induced increases in MKP-1 in other cell types.[58,59] We identified that Dex treatment of B-3 cells resulted in decreased expression of phospho-RAF, phospho-ERK, and phospho-p38, with no change in total protein (Fig. 7). The absence of an effect on p38 at 16 hours may be due to the ethanol vehicle inhibiting the expression of phospho-p38 in all samples. Ethanol has been reported to inhibit the activation of p38 in human mononuclear cells, but the mechanism is not understood.[60]

The early GC response in hLECs appears to be specific and target the MAPKs individually. Dex did not inhibit phospho-JNK expression (Fig. 7D). Dex has been reported to activate or inhibit the MAPKs differentially, depending on the cell type and conditions.[58,59,61,62] It is not uncommon for Dex to inhibit the phosphorylation of one or two of the MAPKs without affecting the third.

Previous reports suggested that GC-modulated genes were involved in the PI3K/AKT pathway in hLECs.[14,32] Increased MKP-1 expression was observed with inhibition of ERK, p85/PI3K, and AKT phosphorylation without affecting total protein expression in hepG2 cells.[63] Increased MKP-1 expression also preceded decreased phospho-ERK and phospho-AKT expression in the MCF-7 cell line.[64] AKT is a downstream target of PI3K, and phosphorylation of AKT is necessary for its activation. We identified a significant decrease in phospho-AKT, without a change in AKT, in Dex-treated hLECs (Fig. 8).

The finding of nucleated epithelial cells in the posterior region of the lens suggests that GCs play a role in LEC proliferation, differentiation, apoptosis, survival, or migration. The PI3K/AKT pathway is involved in cell survival, proliferation, growth, and transformation,[21,22] and the MAPK pathway is involved in cell proliferation, differentiation, motility, survival, and apoptosis.[23] Key components of both pathways are present in the lens.[65] GCs may modulate cellular responses through the MAPK and PI3K/AKT pathways, which crosstalk in many cell types, including LECs.[24,25,65] We observed crosstalk and a redundancy that occurred at several levels of the signaling pathways in the early GC lens response. Specifically, GILZ inhibited RAF phosphorylation, which prevented activation of ERK. However, MKP-1 expression also

resulted in dephosphorylation of ERK. ERK and AKT have been reported to be involved in cell survival. This redundancy and crosstalk between the MAPK and PI3K/AKT pathways may be involved in the mutual suppression or amplification of a function. It has been suggested that these signals are not transmitted in a linear fashion from one point to the next, but through a combination of signals that converge on a final target or cellular function.[65] RAF/ERK can act upstream of PI3K/AKT,[66] but PI3K/AKT can act upstream of RAF/ERK as well.[67] The decrease in both phospho-AKT and phospho-RAF at 4 hours of Dex treatment in hLECs suggests that one pathway may not be upstream of the other (Figs. 6, 7). Instead, the inhibition of both of these pathways may be a redundant effect to inhibit a final cellular target or physiological function, such as inhibition of cell survival.

We have now identified a GC signaling pathway in LECs but have not been able to identify a concomitant physiological response. Our own studies looking at the effect of GCs on hLEC proliferation and apoptosis (Fig. 3) under the conditions shown and over an extended time course (up to 8 days) and dose range (up to 100 μM) showed no significant change. Although we have identified an acute transcriptional GC response, researchers have been able to identify cellular changes in hLECs only after prolonged or chronic high-dosage GC treatment.[1-3,15,32,52] GC treatment alone may not be sufficient to yield a physiological response after an acute treatment. The final physiological response may be due to the background of other effectors, hormones, and growth factors present in the environment after prolonged treatment. It is interesting to note that while activated ERK and AKT play roles in cell survival, activated p38 plays a role in apoptosis. Dex treatment resulted in inhibited ERK, AKT, and p38 activation (Figs. 7, 8). Decreased phospho-p38 expression may be a mechanism to compensate for the inhibition of cell survival. This demonstrates the complexity of the lens GC response and may account for the inability to identify an acute physiological GC response. We predict that long-term GC treatment would lead to more dramatic changes in gene expression, which then can lead to prolonged modulation of these important signaling pathways leading to increased proliferation, decreased differentiation, increased migration, decreased apoptosis, or increased cell survival, all of which can account for the finding of nucleated epithelial cells in the posterior region of the lens. However, implication of these pathways in the formation of a cataract would require examining posterior subcapsular cataract specimens for changes in the MAPK and PI3K/AKT pathways.

Animal models have not been useful thus far in understanding the formation of a steroid cataract in humans. The chick is a common model used to study a lens GC response, but prolonged GC treatment results in a nuclear cataract, which differs from the posterior subcapsular cataract found in humans.[68-70] The chick lens does not contain a GR,[70] which suggests a different mechanism of action. In 1985, a posterior subcapsular cataract was observed in rabbits treated with GC for 72 hours, but since that time, it has not been repeated.[71] Organ cultures of rat lenses treated with high-dose GC for 7 days resulted in migratory LECs to the posterior region of the lens but did not fully result in a posterior subcapsular cataract.[52] Since GR knockout mice die shortly after birth, a lens-specific conditional GR knockout mouse would be needed to study lens GC effects.[72,73] However, it has been suggested that mouse and rat do not express the GRβ isoform, which is hypothesized to regulate the transcriptionally active GRα, and could lead to different results in GR signaling compared with humans.[74,75] Animal models can be used to understand the mechanism of a lens GC response, but it would be important to verify these results in human lenses.

Ideally, these results would have been repeated in human posterior subcapsular cataract specimens, but available specimens are limited. In a study examining 106 cataract specimens, only 2% were posterior subcapsular cataracts, whereas most were cortical, nuclear, or mixed,[76] demonstrating the difficulty in obtaining posterior subcapsular cataractous specimens. The current studies are being repeated in primary hLEC cultures, and preliminary studies have

demonstrated a decrease in phospho-RAF, but not in RAF, expression with Dex treatment, similar to that observed in the B-3 cells. This suggests that the B-3 cells can serve as a model to examine before repeating the results in primary cultures. To understand fully the lens GC response and steroid-induced cataractogenesis, it would be important to study GR, MAPK, and PI3K expression and phosphorylation after acute and prolonged GC treatment of hLECs, specimens of posterior subcapsular cataracts, and in hLECs cotreated with growth factors, such as bFGF, to resemble in vivo conditions.

Although a direct link to posterior subcapsular cataract remains elusive, this is the first demonstration of a lens GC signaling pathway. In relationship to a mechanism of cataractogenesis it is interesting to note that prolonged high-dosage Dex treatment of rat lenses resulted in migration of epithelial cells to the posterior region of the lens and decreased E-cadherin expression.[52] Both the MAPK and PI3K/AKT pathways have been reported to be involved with E-cadherin expression and activity.[77,78]

We have demonstrated that GCs target hLECs directly and specifically activate the GR. We have also demonstrated that GC treatment of hLECs results in increased expression of GILZ and MKP-1, which play a role in regulation of the MAPK and PI3K pathways. Increased GILZ and MKP-1 correlating with decreased phospho-RAF, phospho-ERK, phospho-p38, and phospho-AKT expression demonstrates for the first time an acute GC response and GC signaling pathway. This novel insight is an essential first step in understanding the mechanism of a lens GC response and cataractogenesis, and the foundation for a rational approach to the treatment and prevention of a steroid-induced cataract.

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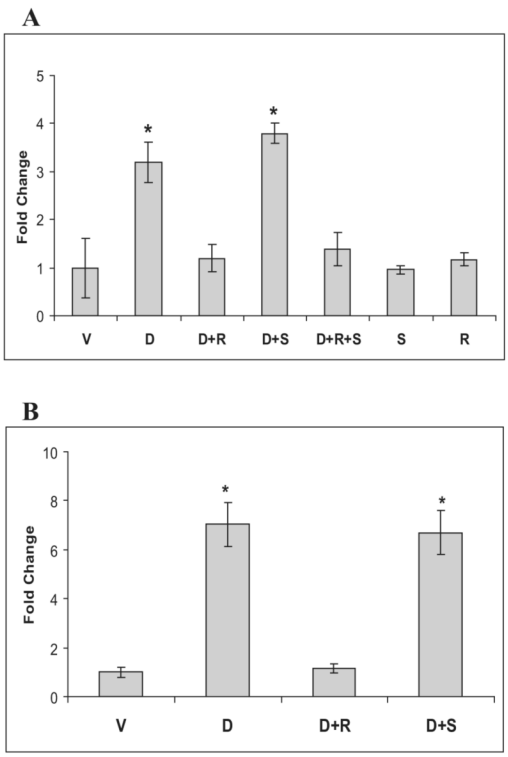

Figure 1.

Dex bound to and activated the GR, but not the MR, to induce transcription. hLECs cotransfected with pGRE.Luc and pRL-SV40 were cotreated with combinations of Dex (D), RU-486 (R), spironolactone (S), and vehicle (V) for 24 hours. Error bars, SE. (**A**) HLE B-3 cells. Treatments yielded values that were significantly different from vehicle (**P* < 0.0003; *n* = 6). (**B**) Primary cultures. Two experiments were performed on cultured cells from two donor pairs. In each experiment, each condition was performed in triplicate, and data were analyzed as *n* = 6. Treatments yielded values that were significantly different from vehicle (**P* < 0.01).

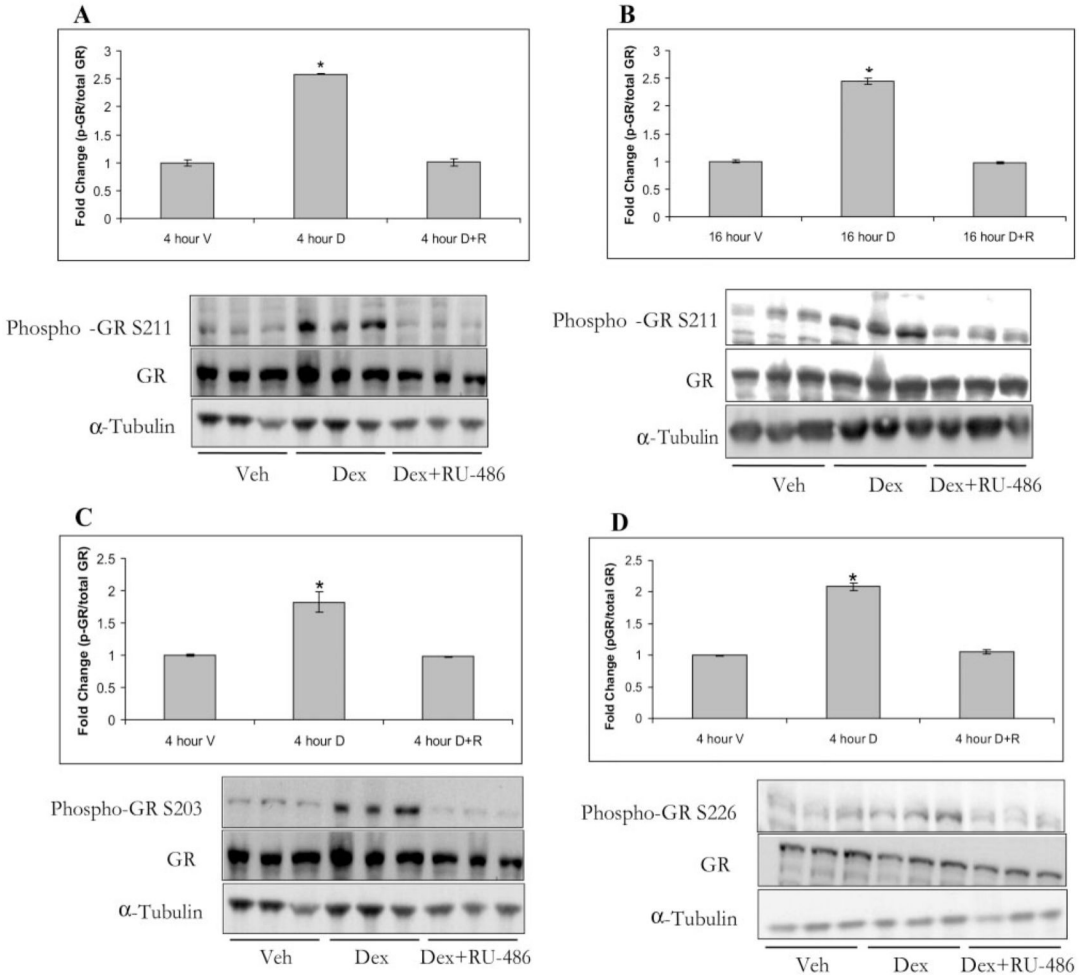

Figure 2.

Phosphorylation of the GR at serine residues was hormone dependent and inhibited by RU-486 in HLE B-3 cells. Cultures were treated with vehicle (V), Dex (D), or Dex+RU486 (D+R) for 4 or 16 hours. Error bars, SE. Phosphorylation of GR at S211 after a (**A**) 4-hour ($n = 9$; $^*P < 0.00004$) or (**B**) 16-hour ($n = 9$; $^*P < 0.0002$) treatment with Dex. (**C**) Phosphorylation of GR at S203 after a 4-hour treatment with Dex ($n = 6$; $^*P < 0.02$). (**D**) Phosphorylation of GR at S226 after a 4-hour treatment with Dex ($n = 9$; $^*P < 0.0004$).

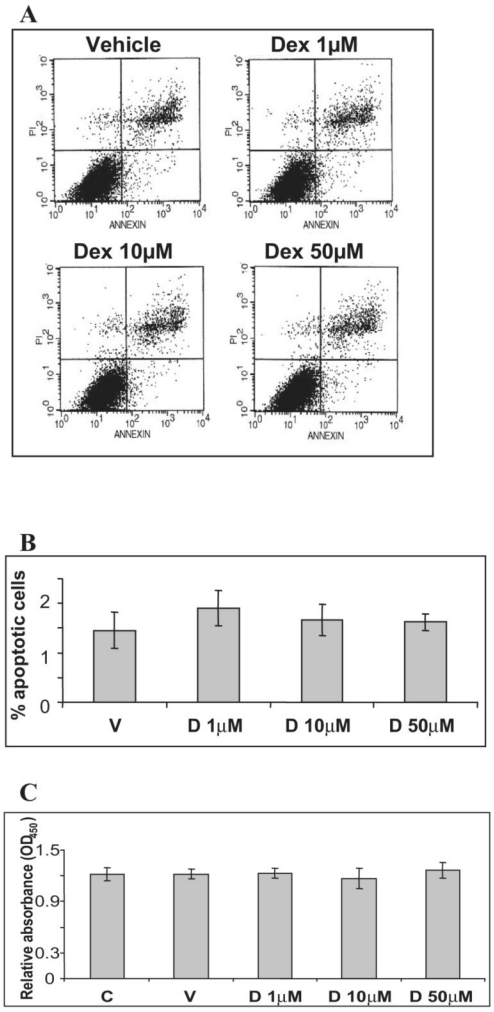

Figure 3.

Dex had no effect on HLE B-3 cell apoptosis or proliferation. Cells treated with vehicle (V) or 1, 10, or 50 μM Dex (D) for 12 hours. (**A**) flow cytometry of vehicle- and Dex-treated cells. Error bars, SD. (**B**) Percentage of early apoptotic (annexin-positive, PI-negative; *bottom right quadrant*) cells in each condition ($n = 3$). (**C**) Relative viability of B-3 cells treated with vehicle or 1, 10 or 50 μM Dex ($n = 3$).

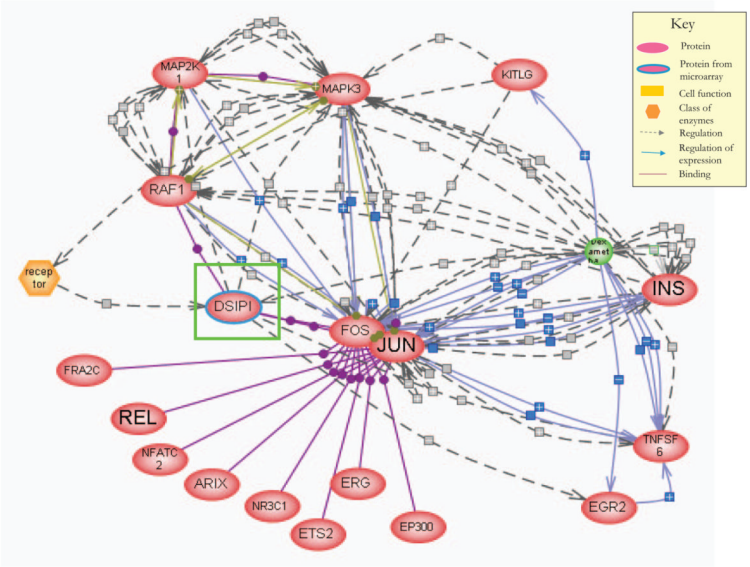

Figure 4.

Pathway analysis of DSIP. Pathway analysis was performed with Pathway Assist 2.0 (Ariadne Genomics, Rockville, MD). Pathway Assist generated a pathway around DSIP and its interactions with other gene products from the microarray and the current literature database. Proteins are depicted as *pink ovals*, whereas proteins identified by microarray are depicted as *pink ovals* with *blue outlines*. *Gray lines*: interaction through regulation.

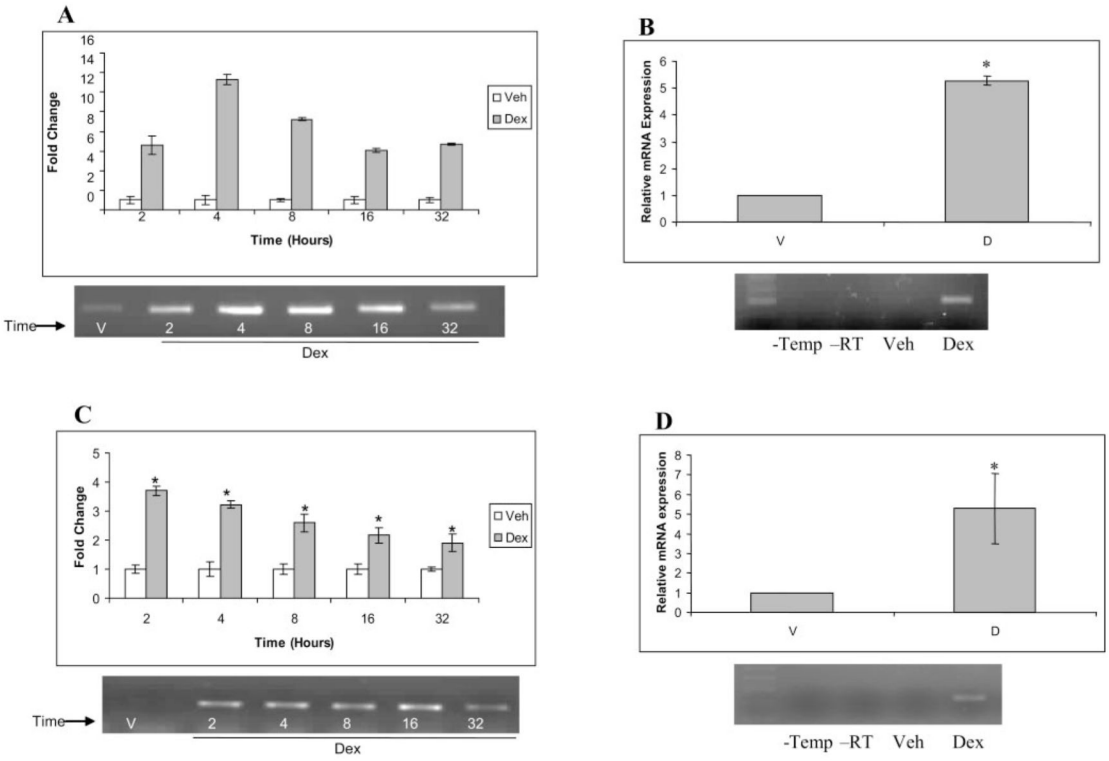

Figure 5.

RT-real time-PCR of GILZ and MKP-1. Error bars, SD. Expression of GILZ (**A**) and MKP-1 (**C**) in HLE B-3 cells treated with Dex or vehicle (Veh; **P* < 0.01; *n* = 3). Experiment was repeated with a second preparation of RNA and yielded similar results. Expression of GILZ (**B**) and MKP-1 (**D**) in three pairs of donor lenses treated for 4 hours (**P* < 0.01; *n* = 3). Vehicle- (32 hours) and Dex-treated samples were electrophoresed on agarose gels.

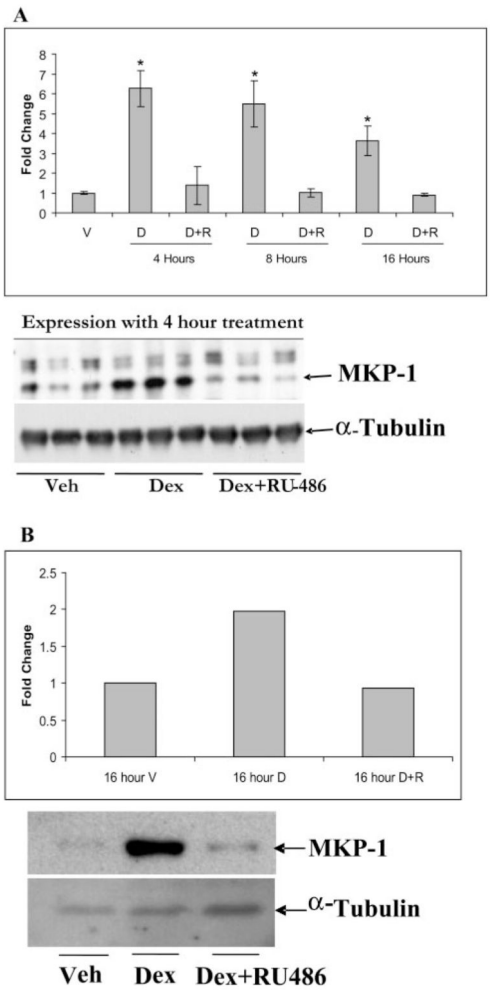

Figure 6.

RU-486 inhibited a Dex-induced increase in MKP-1 protein expression. The cells were treated with vehicle (V), Dex (D), or Dex+RU486 (D+R). (**A**) HLE B-3 cells demonstrated a significant increase in MKP-1 protein expression, inhibited by RU-486. Each time point was examined two to three times, each in triplicate, with similar results. **P* < 0.005; error bars, SD; *n* = 3). (**B**) Explants from a single pair of hLECs treated with 1 μM Dex for 16 hours demonstrated an increase in MKP-1 protein, inhibited by RU-486.

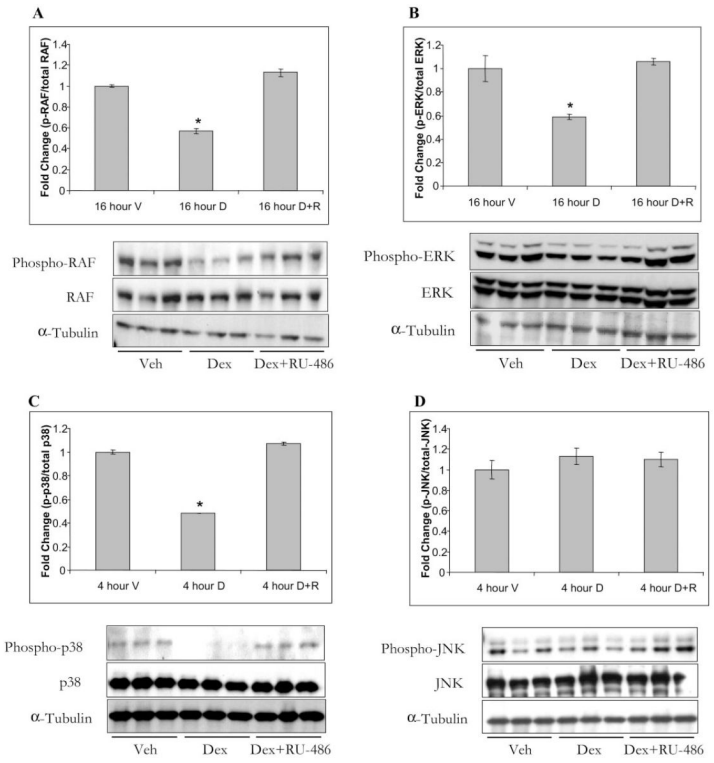

Figure 7.

Dex inhibited RAF-1, ERK, and p-38 phosphorylation in HLE B-3 cells. Cells treated with vehicle (V), Dex (D), or Dex+RU-486 (D+R). Each time point was examined three times in triplicate. Error bars, SE. (**A**) Sixteen-hour Dex treatment demonstrated a significant decrease in phospho-RAF expression, but not RAF (*$P < 0.0005$; $n = 9$). (**B**) Sixteen-hour Dex treatment demonstrated a significant decrease in phospho-ERK expression, but not ERK (*$P < 0.05$; $n = 9$). (**C**) Four-hour Dex treatment demonstrated a significant decrease in phospho-p38 expression, but not p38 (*$P < 0.00008$; $n = 9$). Dex-mediated decreases were inhibited by GR antagonist, RU-486. (**D**) There was no change in phospho-JNK expression with treatment.

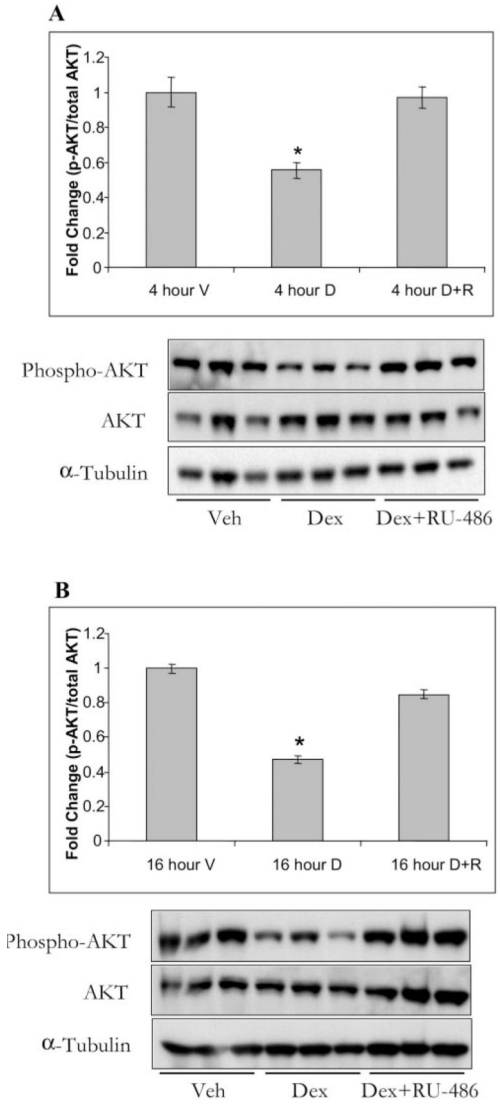

Figure 8.

Dex inhibited AKT phosphorylation in HLE B-3 cells. B-3 cells treated with vehicle (V), Dex (D), or Dex+RU-486 (D+R). Each time point was examined three times in triplicate. Error bars, SE. (**A**) Four-hour (**P* < 0.001; *n* = 9) and (**B**) 16-hour (**P* < 0.0009; *n* = 9) Dex treatment demonstrated a significant decrease in phospho-AKT expression, but not AKT, which was inhibited by RU-486.