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Potentiation of Glucocorticoid Activity in Hypoxia through Induction of the Glucocorticoid Receptor

Martin O. Leonard, Catherine Godson, Hugh R. Brady, and Cormac T. Taylor

Tissue hypoxia is intimately associated with chronic inflammatory disease and may signal to the resolution of inflammatory processes. Glucocorticoid signaling through the glucocorticoid receptor (GR) represents a clinically important endogenous anti-inflammatory pathway. Microarray analysis reveals that the GR is transcriptionally up-regulated by hypoxia in human renal proximal tubular epithelial cells. Hypoxic up-regulation of the GR was confirmed at the level of promoter activity, mRNA, and protein expression. Furthermore, functional potentiation of glucocorticoid activity in hypoxia was observed as an enhancement of dexamethasone-induced glucocorticoid response element promoter activity and enhanced dexamethasone-mediated inhibition of IL-1β-stimulated IL-8 expression and hypoxia-induced vascular endothelial growth factor expression. Knockdown of enhanced GR gene expression in hypoxia using specific GR small inhibitory RNA (siRNA) resulted in an attenuation of the enhanced glucocorticoid sensitivity. A role for the hypoxia-inducible transcription factor, HIF-1α, in the regulation of GR expression and the associated potentiation of glucocorticoid activity in hypoxia was also demonstrated. These results reveal a novel signaling aspect responsible for the incorporation of hypoxic and glucocorticoid stimuli, which we hypothesize to be an important co-operative pathway for the control of gene expression observed in complex tissue microenvironments in inflamed states. The Journal of Immunology, 2005, 174: 2250–2257.

Glucocorticoids are steroid hormones secreted from the adrenal cortex as part of the hypothalamic pituitary adrenal axis and are critical to physiological function due to their regulatory effects on carbohydrate, lipid and protein metabolism. These hormones are released in response to diverse stimuli and play essential roles in regulating the stress response, endocrine homeostasis, vascular tone, CNS function, proliferation, and apoptosis (1). They also have major regulatory roles in inflammation and the immune response and are one of the most widely used pharmacological agents in medicine. They are used extensively for their anti-inflammatory properties in the treatment of inflammatory and autoimmune disease (2, 3). While much is known regarding the cellular and molecular mechanisms through which glucocorticoids exert their effects, it remains unclear how they integrate their physiological and pharmacological effects in complex systemic and tissue environments.

The actions of glucocorticoids are mediated through an intracellular receptor, the glucocorticoid receptor (GR, NR3C1), which is a member of the nuclear receptor family of ligand dependent transcription factors (4). Ligand binding in the cytoplasm causes a dissociation of the GR from its chaperone complex, allowing it to become hyperphosphorylated and translocated to the nucleus. The GR can then homodimerize and act directly as a trans-acting factor binding to two palindromic glucocorticoid response element (GRE) half sites (5′-TGTTCCTT-3′) within the regulatory regions of glucocorticoid responsive genes (1), including those involved in endocrine homeostasis and metabolism, such as TAT and PEPCK, and also inflammation, including the IL-1R antagonist and IκB (5, 6). As well as transactivation, activated GR can inhibit gene expression through a mechanism known as trans-repression where the GR binds directly to transcription factors such as AP-1 and NF-κB, thus inhibiting their transactivating potential for the induction of gene expression (7). Trans-repression has been suggested as the main mechanism through which glucocorticoids exert their anti-inflammatory effects (8).

Hypoxia occurs when the demand for oxygen to maintain normal cellular ATP requirements outweighs the vascular supply and has been documented as an integral part of many pathological states, including ischemic disease, chronic inflammatory disease, and tumor progression (9, 10). The transcription factor complex hypoxia-inducible factor 1 (HIF-1) primarily mediates the adaptive response to hypoxia. Under low oxygen tension, prolyl hydroxylase-dependent degradation of the regulatory subunit of HIF-1, HIF-1α is inhibited. This results in nuclear accumulation of HIF-1, it’s binding to the hypoxia response element (HRE) (5′-RCGTG-3′), and the subsequent transactivation of genes involved in glycolysis, angiogenesis, and vasodilatation, including phosphofructokinase, adrenomedullin, and vascular endothelial growth factor (VEGF) (10). We have also previously reported that prolonged hypoxia may play a role in the resolution of the inflammatory response (11).

The interplay between hypoxia and glucocorticoid-mediated cellular responses in physiology and disease has become increasingly appreciated over recent years. Release of glucocorticoids in response to atmospheric hypoxia associated with high altitude in humans has been extensively documented and prophylactic treatment with glucocorticoids has been used to attenuate the associated mountain sickness (12). Stress-induced erythropoiesis as a result of hypoxia exposure involves glucocorticoid regulation of erythroid progenitor expansion and terminal differentiation arrest.

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3 Abbreviations used in this paper: GR, glucocorticoid receptor; GRE, glucocorticoid response element; HIF-1, hypoxia-inducible factor 1; WT, wild type; DM, double mutant; VEGF, vascular endothelial growth factor; RT, room temperature; 11β-HSD, 11β-hydroxysteroid dehydrogenase; siRNA, small inhibitory RNA.
(13, 14). Mice carrying a dimerization deficient mutation of the GR exhibit normal erythropoiesis under normoxic conditions but fail to respond to hypoxia to increase erythrocyte count and hemoglobin content in peripheral blood (13). Glucocorticoids have been observed to stimulate erythropoiesis indirectly through up-regulation of erythropoietin in the kidney (15). They have also been used in the treatment of many pathological states in which hypoxia plays a major role in the perpetuation of disease, including inflammatory bowel disease and rheumatoid arthritis (2, 3) Additionally, it has been observed that glucocorticoids protect against experimental cerebral and hepatic ischemia/reperfusion injury (16, 17).

Therefore, the integration of hypoxic- and glucocorticoid-mediated signals is potentially important in various tissue environments. In the context of the global genomic effects hypoxia and glucocorticoids exert on many different cell types and the relative degree of each stimulus in regulating these genomic responses, it is vital to elucidate the precise cross-talk between these pathways in these complex tissue microenvironments. With this in mind, we report for the first time the functional potentiation of glucocorticoid activity in hypoxia, mediated through an up-regulation of the GR.

Materials and Methods

Materials

IL-1β was purchased from R&D Systems, and the Abs to the GR and β-actin were purchased from Santa Cruz Biotechnology. All other chemicals were from Sigma-Aldrich unless otherwise specified.

Cell culture

Human proximal tubular epithelial cells (HK-2; American Tissue Type Culture Collection; Ref. 18) were maintained in DMEM/F-12 containing 5 g/ml transferrin, 5 ng/ml selenium, 36 ng/ml hydrocortisone, 4 pg/ml triiodo-l-thyronine, 10 ng/ml epidermal growth factor, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine (Sigma-Aldrich). Cells were maintained at 37°C in a humidified atmosphere under conditions with a balance of 95% N2/5% CO2. Cells were cultured to precipitated using 0.5 ml of isopropanol. After centrifugation at 12,000 g for 10 min at RT before centrifugation at 12,000 g for 15 min at 4°C. The upper aqueous phase was removed to a separate tube, and RNA was reduced medium (1:25 dilution without hydrocortisone) and subsequent 10 min at 4°C, the pellet was washed with 75% ethanol and resus- pended in 50 μl of TE buffer. RNA was quantified as absorbance at 260 nm. cDNA was synthesized from total RNA using Superscript Choice kit (Invitrogen Life Technologies). Sample preparation and microarray anal- ysis were conducted as previously described (19). Microarray Suite 5.0 software (Affymetrix) was used to analyze the relative abundance of each gene compared with normoxia control, and results were expressed as fold change over control. RT-PCR analysis was conducted with primers specific for the GR (forward, 5'-CTACTAGGCGGCTTGAAAGAGC-3'; reverse, 5'-GCAAGGGTCGGGCCTCTAT-3') (Sigma-Genosys) using a standard real-time PCR amplification as described previously (20). The amplification protocol included an initial 2-min denaturation at 94°C, fol- lowed by 25 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 1 min, with a final extension of 72°C for 5 min. This protocol was also used for amplification of 18S RNA (17 cycles) using specific primers (forward, 5'- GTGGAGCGATTTGTCTGGTT-3'; reverse, 5'-GGTGAGCGCAGCAGCT AGCAGT-3') as a control for equal sample loading. PCR products were analyzed using a 2% agarose gel in 0.5× TAE buffer, stained with ethidium bromide and visualized under UV illumination (Uvitech GDS8000; UVitech).

Western blot analysis

Whole cell extracts were prepared in RIPA lysis buffer (20 mMol/L Tris-HCl, pH 7.4, 50 mMol/L NaCl, 5 mMol/L ethylene diaminetetraacetic acid, 1% Nonidet P-40, 0.1% SDS, 5 mMol/L NaF, 1 mMol/L PMSF, 1 mMol/L Na3VO4, 1 μmol/L leupeptin, and 0.3 μmol/L aprotinin). Protein content was quantified and normalized using the Bradford method (Bio-Rad) and electrophoresed on 10% SDS PAGE gels. Expression levels for the GR and β-actin were measured using specific Abs by Western blot analysis as previously described (21).

Transient transfection of cells

Cells were transfected with 2 μg of a GRE promoter-luciferase reporter construct containing three tandem copies of a GRE enhancer element (BD Biosciences). Cells were also transfected with 2 μg of a GR promoter-reporter construct containing ~1045 bp to +19 bp upstream sequence relative to the human GR exon 1C transcription start site (GR1C) (kind gift from Dr. W. Vedeckis, Louisiana State University Medical Center, New Orleans, LA (22). Overexpression of HIF-1α was conducted through transfection with wild-type HIF-1α (HIF-1α WT) and HIF-1α double mutant (HIF-1α DM), where prolines 564 and 402 were mutated to valine. These residues are responsible for oxygen-dependent degradation of HIF-1α through a mechanism involving specific hydroxylation. pDNA3.1 was used as the vector control. These constructs were kind gifts from Dr. T. Hagen (University of Nottingham, Nottingham, U.K.). Transfection was conducted using Fugene 6 transfection reagent (Roche Applied Science) according to the manufacturer’s guidelines. Following transfection for 24 h and subsequent experimental procedures, cells were washed with 2 ml of 1× PBS (ice-cold) and lysed in 200 μl of 1× lysis buffer (Promega) as described previously (23). Luciferase activity was quantified using a luciferin substrate (Promega) and luminometry (Junior LB 9590; Berthold Technologies). All readings were normalized to protein using the Bradford method (Bio-Rad).

Small inhibitory RNA (siRNA) studies

Cells were transfected with four pooled siRNA duplexes (0–100 nm) di- rected against separate GR mRNA target sequences or with four pooled siRNA duplexes directed against nonspecific mRNA sequences (SMART- Pool) (Dharmacon) for 24 h before experimental protocol. Transfection was conducted using Lipofectamine 2000 (Invitrogen Life Technologies) and protocols specific for siRNA transfection according to the manufac- turer’s instructions. In cotransfection experiments, cells were transfected with 2 μg of GRE at the same time as siRNA using Lipofectamine 2000 transfection protocols.

ELISA

Cell culture medium was analyzed for IL-8 and VEGF expression using the DuoSet ELISA system (R&D Systems). Briefly, wells of a 96-well plate (Corning B.V. Life Sciences) were coated with primary capture Ab (2 μg/ml) in PBS overnight at 4°C. Wells were washed three times with PBS (ice-cold) and lysed in 200 μl of wash buffer (0.05% Tween 20 in PBS; pH 7.4) and 250 μl of blocking buffer (PBS containing 1% BSA, 5% sucrose, and 0.05% Na3VO4) was added for 1 h at RT. After removal of blocking buffer, 100 μl of sample or known standard (0–2000 pg/ml) was added and incubated for 2 h at RT. Wells were then washed three times before addition of 100 μl of 1/2000 biotin- ylated secondary detection Ab made up in reagent diluent (0.05% Tween 20 in TBS (PBS); pH 7.4) and incubated for 2 h at RT. Wells were washed three times and 100 μl of streptavidin-HRP (1/2500 in reagent diluent) was then added to each well and incubated at RT for 20 min. Following three washes, 100 μl of substrate solution containing a 1:1 mixture of H2O2 and tetramethylbenzidine (Sigma-Aldrich) was added. The plate was allowed to develop in the dark for 15 min, and the reaction was stopped with the addition of 50 μl of 1 M H2SO4 (Sigma-Aldrich). OD was determined by spectrophotometry at 450 nm with 570 nm as reference filter, and protein levels were determined by extrapolation from the standard curve.

DNA binding assay

To investigate HIF-1 binding to the consensus HRE sequence we used an ELISA based approach according to manufacturer’s instructions (TransAm; Active Motif). Briefly, after the experimental protocol, nuclear cell extracts were prepared in normoxia or hypoxia and incubated in 96- well plates precoated with an oligonucleotide containing the HRE consensus sequence, 5'-TACGTGCT-3'. Following capture of the transcription fac- tor by the oligonucleotide, a primary Ab to HIF-1 was added. After washing, a HRP conjugated secondary Ab was then added followed by substrate solution. OD was determined by spectrophotometry at 450 nm.
with 570 nm and the intensity of the absorbance signal was proportional to HIF-1 DNA binding.

Statistical analysis
All data is presented as mean ± SEM for n independent experiments. Statistical significance was evaluated using one way ANOVA conducted using InStat software package (GraphPad).

Results
Hyoxia causes an up-regulation of the GR
In hypoxic tissue there is a shift in the cellular transcriptional phenotype toward an adaptive gene expression profile, which aids cell and tissue survival in a lowered oxygen environment (10). We have previously demonstrated that hypoxia causes global transcriptomic alterations in gene transcription. Of the genes tightly regulated in an oxygen-dependent manner was the gene for the GR (19). Here we demonstrate that exposure of human proximal tubular epithelial (HK-2) cells to hypoxia (1% atmospheric oxygen, 20 torr) caused a time-dependent up-regulation of the GR evaluated by microarray analysis (Fig. 1A). This increase in GR expression is oxygen dependent as reoxygenation for 6 h after 36 h of hypoxia attenuated the hypoxia-induced increase. These results were confirmed using semiquantitative RT-PCR analysis (Fig. 1B). Hypoxia also caused a time-dependent increase in GRα protein expression up to 48 h, which returned to near normoxic levels after 24 h of reoxygenation (Fig. 1C). We also observed a time-dependent increase in GR promoter reporter luciferase activity (Fig. 1D). Collectively, these data demonstrate a transcriptionally driven increase in GR protein expression in response to hypoxia in proximal tubular epithelial cells.

Hyoxia causes a potentiation of glucocorticoid-dependent activity
Glucocorticoids affect many systems mainly through ligand-dependent activation of the GR and subsequent transactivation of genes, which contain regulatory GREs. Because we have observed an increase in GR expression with hypoxic exposure and GR expression levels are tightly correlated with glucocorticoid-dependent activity (24), we set out to investigate whether glucocorticoid-dependent GRE transactivating activity is enhanced in hypoxia. Cells were transfected with a GRE-dependent promoter-reporter construct and exposed to hypoxia for 20 h. Treatment with dexamethasone for a further 24 h in hypoxia resulted in a potentiation of stimulated GRE luciferase activity in hypoxia compared with normoxia with the maximum potentiation observed with the higher dose of dexamethasone used (10 nM; Fig. 2).

Glucocorticoids can also act to modulate gene expression through a mechanism termed trans-repression. This mechanism involves direct binding of a ligand activated GR monomer to transcription factors such as NF-κB and AP-1. This prevents these transcription factors’ transactivation potential and inhibits the activation of genes such as IL-8 in response to the proinflammatory signal IL-1β (25). Pre-exposure of cells to hypoxia caused a potentiation of dexamethasone-mediated inhibition of IL-1β-stimulated IL-8 (Fig. 3A). This was observed to be statistically significant at all doses of dexamethasone used with an IC50 for dexamethasone in hypoxia of 0.4 nM compared with 7.5 nM in normoxia. To eliminate the possibility of hypoxia-induced alterations in the IL-1β signaling cascade being responsible for the increased sensitivity to inhibition by dexamethasone, we exposed cells in normoxia or hypoxia to varying concentrations of IL-1β. After 24 h exposure there were no significant alterations in IL-1β-stimulated IL-8 release in hypoxia compared with normoxia (Fig. 3B). It is interesting to note that the concentration of dexamethasone used to inhibit IL-1β-stimulated IL-8 in hypoxia, 31.6% at

FIGURE 1. Hypoxia stimulates an up-regulation of the GR. HK-2 cells were incubated in hypoxia (hyp) at 1% atmospheric O2 (20 torr) for various time points (0–48 h). Cells were also maintained at 21% atmospheric O2 (147 torr) for normoxic (norm) and reoxygenation (reox) (6 h) treatments. Affymetrix microarray analysis (A) and RT-PCR analysis (B) revealed an up-regulation of the GR at the mRNA level. Microarray results were expressed as fold over normoxic (0 h) controls (pooled n = 4). RT-PCR results are representative of three individual experiments. Protein expression for the GRα was also increased in hypoxia (C) as assessed using SDS-PAGE and Western blot analysis. Again results are representative of blots from three individual experiments. Exposure of cells transfected with a GR promoter-luciferase reporter construct to hypoxia revealed a significant increase in activity (D) compared with empty construct (pXp1). Results are expressed as fold over basal pXp1 relative luciferase unit values ± SEM at normoxia (n = 6), hypoxia at 24 h (n = 3), and hypoxia at 48 h (n = 6). *, p < 0.01.
together, these results give further evidence that the potentiated parable to the changes observed in IL-8 production. However, al-
dexamethasone and IL-1β which the Western blot was conducted, just before treatment with reduced, entirely reflective of the levels to which GR protein levels were

ditions (1% O2, 20 torr) for a further 20 h. Cells were then treated with dexamethasone (Dex) (0–10 nM) for 24 h before cell lysis and analysis of luciferase activity using luminometry. Results were expressed as mean fold over basal nondexamethasone-treated luciferase activity in normoxia or hypoxia respectively ± SEM for seven independent experiments (*, p < 0.05).

0.1 nM, did not produce a significant induction of GRE activity, 1.3-fold (Fig. 2). This would indicate that dexamethasone is more potent at inhibiting IL-1β-stimulated IL-8 than at inducing GRE activity and is consistent with previous reports that glucocorticoid-stimulated GR activity is more potent in transrepression than in transactivation mechanisms (5). Therefore, these results are consistent with the hypothesis that enhanced glucocorticoid activity observed in hypoxia is mediated through an increase in the expression of the GR.

Knockdown of GR expression results in an attenuation of the potentiated glucocorticoid activity in hypoxia

Having demonstrated that hypoxia causes an increase in the expression of the GR and that hypoxia predisposes to enhanced sensitivity to glucocorticoid-dependent activity, we next set out to determine whether the increase in GR expression is responsible for the enhanced activity observed in hypoxia. To address this, cells were transfected with siRNA (50 and 100 nM) specific to the GR before experimental protocols. Western blot analysis revealed an ~50% reduction in GRα expression in hypoxia (Fig. 4A). There were no alterations in control β-actin expression. We next went on to examine the effect of inhibition of GR expression on dexamethasone-stimulated glucocorticoid activity. Using GR siRNA (50 nM) we demonstrated a significant reduction of the hypoxia-potentiated, dexamethasone-stimulated GRE promoter-reporter activity (Fig. 4B). Similarly we observed that treatment with GR siRNA caused a significant attenuation of the hypoxia-stimulated potentiation of dexamethasone-mediated inhibition of IL-1β-stimulated IL-8 production (Fig. 4C) compared with nonspecific siRNA control cells. The level to which we observed an attenuation of the potentiated dexamethasone-mediated inhibition of IL-1β-induced IL-8 production in hypoxia by GR siRNA was not entirely reflective of the levels to which GR protein levels were reduced, ~50%. This may be accounted for by the time point at which the Western blot was conducted, just before treatment with dexamethasone and IL-1β. Analysis of GR protein levels at this later time point, 18 h after treatment with dexamethasone and IL-1β, may reveal a lesser attenuation by the GR siRNA more comparable to the changes observed in IL-8 production. However, altogether, these results give further evidence that the potentiated glucocorticoid activity observed under hypoxic conditions is due to the observed increase in GR expression level.

Overexpression of HIF-1α causes an up-regulation of the GR and potentiates glucocorticoid-dependent activity

Having established potentiated glucocorticoid activity in hypoxia as a functional consequence of increased GR expression, we next wanted to delineate the precise signaling events responsible for enhanced GR expression. The transcription factor HIF-1α is a master regulator of transcriptional responses to hypoxia. We have previously demonstrated that overexpression of this transcription factor under normoxic conditions results in an up-regulation of GR mRNA (19). In this study, we demonstrate that overexpression of HIF-1α WT or HIF-1α DM caused an increase in GR protein expression above normoxic and hypoxic vector control levels (Fig. 5A). To investigate whether this increase in GR protein expression

FIGURE 2. Hypoxia pre-exposure results in a potentiation of dexamethasone-stimulated GRE promoter-reporter activity. HK-2 cells were transfected with a GRE promoter luciferase-reporter construct for 24 h before incubation under normoxic (21% O2, 147 torr) or hypoxic conditions (1% O2, 20 torr) for a further 20 h. Cells were then treated with dexamethasone (Dex) (0–10 nM) for 24 h before cell lysis and analysis of luciferase activity using luminometry. Results were expressed as mean fold over basal nondexamethasone-treated luciferase activity in normoxia or hypoxia respectively ± SEM for seven independent experiments (*, p < 0.05).

FIGURE 3. Hypoxia pre-exposure causes a potentiation of dexamethasone-mediated inhibition of IL-1β-stimulated IL-8 expression. HK-2 cells were incubated under normoxic (21% O2, 147 torr) or hypoxic conditions (1% O2, 20 torr) for 20 h. A. Cells were then exposed to various concentrations of dexamethasone (Dex) (0–10 μM) for 2 h before stimulation with IL-1β (2 ng/ml) for a further 18 h. Cell culture supernatant was then removed and analyzed for IL-8 release using ELISA. Results are expressed as mean percentage of nondexamethasone-treated, IL-1β-stimulated IL-8 release ± SEM for four independent experiments (*, p < 0.001). B. Cells were also treated with various concentrations of IL-1β (0–10 ng/ml) for a further 18 h in normoxia or hypoxia. Results are expressed as mean fold over basal non-IL-1β-treated IL-8 release ± SEM for three independent experiments.
translates as a potentiation of glucocorticoid activity, we cotransfected a GRE promoter-reporter construct with these HIF-1α overexpression vectors. Hypoxia alone caused a potentiation of GRE activity compared with the normoxic vector control as previously demonstrated. Overexpression of both HIF-1α constructs resulted in no significant alteration in GRE activity under normoxic conditions (Fig. 5B). However, under hypoxic conditions HIF-1α overexpression caused a significant alteration in GRE activity compared with vector control (Fig. 5B). These results indicate a functional role for HIF-1α in the regulation of GR expression and the associated increase in glucocorticoid activity under hypoxic conditions.

Hypoxia preincubation causes a potentiation of dexamethasone-mediated inhibition of VEGF expression but not HIF-1 DNA binding

VEGF is one of the most potent and specific angiogenic factors acting to promote angiogenesis in hypoxic tissue environments (26). Glucocorticoids have been demonstrated along with their anti-inflammatory properties to be angiostatic. One of the mechanisms suggested for this anti-angiogenic property is through the inhibition of VEGF production (27). Therefore, we investigated whether hypoxia could alter the sensitivity of glucocorticoids to inhibit VEGF expression. Cells were exposed to normoxic or short (1-h) and long (24-h) hypoxic preconditions. Cells were then treated with dexamethasone (Dex) (10 nM) for a further 24 h before cell lysis and analysis of luciferase activity using luminometry. Results were expressed as mean fold over basal luciferase activity in normoxia or hypoxia ± SEM for four independent experiments (*, p < 0.05).
B

FIGURE 6. Hypoxia pre-exposure potentiates dexamethasone-mediated inhibition of hypoxia-induced VEGF expression but not HIF-1 DNA binding. HK-2 cells were maintained under normoxic or hypoxic conditions for 1 and 24 h. Cell culture supernatant was removed and replaced with fresh medium. A. Cells were then treated with dexamethasone (0–0.1 μM) for a further 18 h in normoxia or hypoxia. Cell culture supernatant was removed and analyzed for VEGF release using ELISA. Results are expressed as mean percentage of nondexamethasone-stimulated VEGF release in normoxia or hypoxia ± SEM for three independent experiments (∗, p < 0.01 compared with hypoxia 1 h). B. Cells were also treated with dexamethasone for 2 h in normoxia or hypoxia. Nuclear cell lysates were analyzed for binding of HIF-1 to a consensus HIF-1 DNA binding sequence using an ELISA based system as a possible mechanism for the glucocorticoid-mediated inhibition of VEGF release. After 2 h of hypoxic exposure, cells preincubated for 1 h or 24 h caused a 4.82 ± 0.53- and a 5.17 ± 0.61-fold over normoxic HIF-1 DNA binding, respectively. After cell pre-exposure to normoxia or hypoxia for 1 or 24 h, treatment with dexamethasone for 2 h did not significantly alter HIF-1 DNA binding (Fig. 6B) indicating that the mechanism of potentiated glucocorticoid inhibition of hypoxia-stimulated VEGF production is not through alterations in HIF-1 DNA binding.

Discussion
It has become increasingly apparent that glucocorticoid and hypoxic regulatory responses are closely linked in complex physiologic and pathophysiologic tissue microenvironments. Although much work has focused on characterization of these responses, little work has concentrated on determining the precise molecular and signaling interplay between these two important stimuli. In this present study we report for the first time that hypoxia increases sensitivity to GR agonists, which we demonstrate to be mediated through an up-regulation of the GR.

It has long been established that the level of GR expression can alter the sensitivity of cells to glucocorticoids (28). Transgenic expression of antisense mRNA specific to the GR in mice resulted in a 50–70% reduction in functional GR expression with a concomitant 2- to 4-fold decrease in dexamethasone-stimulated glucocorticoid-inducible promoter activity (29). We have demonstrated that increased GR levels correlate with a potentiation of glucocorticoid-stimulated activity in hypoxia and that reduction of GR using siRNA attenuates this potentiated activity. Although levels of the GR can influence glucocorticoid sensitivity, other factors potentially altered in hypoxia can also alter glucocorticoid-dependent activity.

The 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes are an important prereceptor control mechanism for the activity of glucocorticoids in a tissue-specific manner. They catalyze the interconversion of active glucocorticoids with their inert 11-ketone forms through oxidation and reduction of the 11β-hydroxyl group. There are two main enzymes: 11β-HSD type I, which acts mainly to reduce and activate glucocorticoids, and 11β-HSD type II, which acts to oxidize and inactivate glucocorticoids (30). Levels of these enzymes are tissue specific and confer differential glucocorticoid sensitivity. Chemical hypoxia using antimycin A has previously been observed to reduce 11β-HSD type II expression at the transcriptional level (31). However, we have demonstrated that mRNA levels for the 11β-HSD enzymes are not altered by hypoxia up to 36 h revealed using microarray analysis (data not shown). To discount the possibility that activity of these enzymes independent of their expression level could be responsible for the observations in our study we used specific inhibitors of these enzymes. Pretreatment of cells with enoxolone and carbenoxolone failed to alter the potentiation of glucocorticoid-stimulated GRE activity and inhibition of IL-8 production in hypoxia (data not shown). Together with the fact that dexamethasone does not need to be reduced by these enzymes for activation (32) these data indicate that the 11β-HSD enzymes play no role in influencing glucocorticoid sensitivity in hypoxia.

Glucocorticoids themselves can reduce GR expression level through decreased transcription and increased protein turnover (33). In our present study, however, there is no evidence to suggest that basal glucocorticoid activity in normoxic conditions is higher than that in hypoxia explaining the increased GR expression in hypoxia and thus increased glucocorticoid sensitivity. Quite the contrary, basal levels of GRE activity in hypoxia are higher than those observed under normoxic conditions (data not shown), which we hypothesize is due to the hypoxia-induced increase in GR expression.

In the absence of ligand the GR is held in a multiprotein heterocomplex containing hsp90, hsp70, hop, hsp40, and p23. The GR must be held in this hsp90 chaperone complex for ligand binding and activation to occur (34). Disruption of hsp90 function using the inhibitor geldanamycin prevents GR activation by ligand (35). It is interesting to note that we have observed a hypoxia-dependent inhibition of hsp90, hsp70, and hsp40 expression (unpublished data), suggesting a putative disruption of heterocomplex formation and inhibition of GR activation. ATP levels, which are reduced by hypoxia (10) are also necessary for functional complex assembly (36). Because the most likely alterations in heterocomplex formation to occur under hypoxic conditions would interrupt complex assembly and GR activation it is unclear whether alterations in complex formation could account for an increased sensitivity to glucocorticoids under hypoxic conditions. Many other factors can contribute to alterations in glucocorticoid sensitivity
such as protein kinase A activation (37) among others but the clearest explanation for increased glucocorticoid sensitivity in hypoxia based on our current study is that hypoxia increase the level of GR expression thus increasing the glucocorticoid sensitivity within the cell.

The GR is classified as a housekeeping gene, whose expression is controlled by a GC-rich promoter lacking a consensus TATA or CAAT box (38). Multiple DNA footprint analysis studies of the GR promoter have characterized many transcription factors responsible for maintaining basal activity, including SP1, AP-2, Ku70, Ku80, and Yin Yang 1 (39–41). Therefore, despite the GR being classed as a housekeeping gene, it is open to alternate regulation through alteration in the activity of these and other transcription factors. We have demonstrated in our study that hypoxia up-regulates the expression of the GR at the transcriptional level. The most widely described transcription factor complex responsible for driving hypoxia-mediated transcriptional alterations is HIF-1. In this present study we have observed that overexpression of HIF-1α results in the induction of the GR at the protein level under both normoxic and hypoxic conditions. This observation together with previous data demonstrating HIF-1α-mediated induction of GR mRNA (19) indicate that this transcription factor is responsible at least in part for the hypoxic induction of the GR. Further evidence for the involvement of HIF-1 in the induction of GR expression is revealed by the observation that contained within the 1064 bp of DNA sequence used in the GR promoter-reporter construct up-regulated in hypoxia are five putative HIF-1 binding sites (5'-RCGTG-3'). Therefore, we can infer that hypoxia-driven GR up-regulation occurs through a mechanism likely involving HIF-1 binding to one or multiple HRE sites facilitating enhancement of GR transcription. Although HIF-1α overexpression was sufficient to induce GR expression in normoxia and hypoxia, a contributory role for other transcription factors including SP1/SP3 and AP-2, in the regulation of GR transcription in hypoxia cannot be discounted. We also cannot rule out a co-operative role for nontranscriptional mechanisms such as alterations in mRNA stability and protein turnover as contributing to the up-regulation of the GR in hypoxia.

Having observed that overexpression of HIF-1α increases GR levels, we next went on to investigate the effect of HIF-1α on GR-dependent activity. Neither HIF-1α WT nor HIF-1α DM caused any significant alteration in GRE activity in normoxia as compared with vector control indicating that under normoxic conditions HIF-1α-dependent up-regulation of the GR is not sufficient for enhanced GRE activity. However the overexpression of both HIF-1α constructs resulted in a significant increase in GRE activity under hypoxic conditions as compared with vector control. These results reveal that although hypoxia and HIF-1α can up-regulate the GR, the involvement of other hypoxia-dependent signaling cues necessary to initiate or facilitate GRE-dependent transcriptional activity are likely involved.

The up-regulation of the GR by hypoxia and the subsequent increased sensitivity to glucocorticoid ligand has major implications not only for the regulation of glucocorticoid-dependent responses but also for the regulation of hypoxia-dependent responses and gene expression. We have demonstrated a potentiation of glucocorticoid-mediated inhibition of hypoxia-induced VEGF expression when cells were pre-exposed to hypoxia for 24 h compared with a 1-h control. This result would indicate a potential feedback mechanism for the control of hypoxia-induced VEGF expression through a hypoxia- and HIF-1-mediated up-regulation of the GR. However we observed no differential regulation of HIF-1 DNA binding by dexamethasone upon hypoxia pre-exposure. This would indicate that the mechanism of glucocorticoid-mediated inhibition of VEGF expression is not likely due to alterations in HIF-1-mediated VEGF expression in hypoxia. This mechanism cannot be totally excluded though, as alterations in transactivation potential have been observed without alterations in transcription factor DNA binding (42). However, alternate mechanisms for the inhibition of VEGF expression by glucocorticoids have been observed through a process involving decreased mRNA stability (43). Ultimately, we postulate that alterations in GR expression in hypoxia not only alter GR-dependent gene expression but also influence hypoxia-dependent gene expression. This cross-talk between glucocorticoid and hypoxia-dependent signaling cascades has been demonstrated in other studies. One study revealed that ligand-dependent activation of the GR enhances hypoxia-dependent gene expression and HRE activity (44). Another study has demonstrated inhibition of HIF-1-dependent activity by glucocorticoids (45). Therefore, we can conclude that the interaction between glucocorticoid- and hypoxia-dependent signaling cascades is significant as well as varied and reflects the complexity and context-specific alterations associated with their differential activation in complex tissue microenvironments.

Finally, our present data demonstrate an increased sensitivity to glucocorticoids after hypoxia exposure, which we postulate to be mediated through a hypoxia- and HIF-1-dependent up-regulation of the GR. We have previously demonstrated that hypoxia can act as a stimulus for resolution of inflammatory conditions through alterations in transcription factor modification (11). In the context of our current findings we can suggest that hypoxia can also act as a stimulus for the resolution of inflammatory disease conditions through the induction of the GR and the subsequent increased sensitivity to anti-inflammatory properties of glucocorticoids.

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References


