Role of the Glucocorticoid Receptor for Regulation of Hypoxia-dependent Gene Expression*

Glucocorticoids are secreted from the adrenal glands and act as a peripheral effector of the hypothalamic-pituitary-adrenal axis, playing an essential role in stress response and homeostatic regulation. In target cells, however, it remains unknown how glucocorticoids fine-tune the cellular pathways mediating tissue and systemic adaptation. Recently, considerable evidence indicates that adaptation to hypoxic environments is influenced by glucocorticoids and there is cross-talk between hypoxia-dependent signals and glucocorticoid-mediated regulation of gene expression. We therefore investigated the interaction between these important stress-responsive pathways, focusing on the glucocorticoid receptor (GR) and hypoxia-inducible transcription factor HIF-1. Here we show that, under hypoxic conditions, HIF-1-dependent gene expression is further up-regulated by glucocorticoids via the GR. This up-regulation cannot be substituted by the other steroid receptors and is suggested to result from the interaction between the GR and the transactivation domain of HIF-1α. Moreover, our results also indicate that the ligand binding domain of the GR is essential for this interaction, and the critical requirement for GR agonists suggests the importance of the ligand-mediated conformational change of the GR. Because these proteins are shown to colocalize in the distinct compartments of the nucleus, we suggest that these stress-responsive transcription factors have intimate communication in close proximity to each other, thereby enabling the fine-tuning of cellular responses for adaptation.

In man, glucocorticoids are secreted from the adrenal glands and act as a peripheral effector of the hypothalamic-pituitary-adrenal (HPA) axis, playing an essential role not only in energy metabolism but in stress response and homeostatic regulation as well. The central perception of stress, thus, is transmitted to peripheral tissues by glucocorticoids via blood stream, thereby enabling coordinated responses for individual adaptation to environments. However, it remains unknown how glucocorticoids finally integrate cellular pathways in harmonization with tissue and systemic responses (1).

Glucocorticoids elicit hormone action via binding to their cognate receptor glucocorticoid receptor (GR), which is a member of the nuclear receptor superfamily and localizes in the cytoplasm as a latent species. The GR is composed of several functional domains, including AF-1 transactivation domain, DNA binding domain (DBD), nuclear localization signal (NLS), ligand binding domain (LBD), and AF-2. On binding hormone, the GR translocates into the nucleus and modulates gene expression in a variety of ways. The most classical model is that the GR binds as a homodimer to the glucocorticoid response element (GRE) in the promoter region of a target gene and positively regulates its transcription (2). On the other hand, the GR also modulates transcription through interaction with other transcription factors and co-regulators (3–5). For example, the anti-inflammatory action of glucocorticoids is believed to be mediated by the interaction between the GR and proinflammatory transcription factors including AP-1 and NF-κB (6). Of note, the gene targeting approach has revealed that GR DNA binding is shown not to be essential for survival in mice (7). Moreover, we have shown that the GR function is also tightly controlled by cellular redox regulators (8). It is therefore likely that not only direct DNA binding but also modulation of other cellular machinery by the GR may be important for stress response, because cellular stress evokes distinct intracellular signals and alters the gene expression profile via modulation of a battery of transcription factors. Such diversity of mode of GR action, thus, might be one of the molecular bases for rationale interaction between the HPA axis and cellular adaptive responses.

Low oxygen availability, hypoxia, can be encountered not only under pathological but also physiological conditions (9–13). It has been reported that the distribution of oxygen tension shows considerable variation among different tissues, and parts of certain tissues including the liver and brain are exposed to hypoxia even under physiological conditions (13). When exposed to hypoxia, a variety of cellular responses is generated, leading to cell and tissue adaptation via induction of the expression of a number of genes including those for glucose transporters (GLUTs), vascular endothelial growth factor (VEGF), mineralocorticoid receptor (MR), aldosterone, dexamethasone, green fluorescent protein (GFP), adenomomedullin, α-SMA, and ACTH, among others. Among these, the vascular endothelial growth factor (VEGF) and glucose transporters (GLUTs) are known to be an essential component for cell survival under conditions of low oxygen tension. Furthermore, a number of other proteins, such as hypoxia-inducible factor-1α (HIF-1α), are induced by hypoxia to support cell survival and adaptation to hypoxic environments.

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‡The abbreviations used are: HPA axis, hypothalamic-pituitary-adrenal axis; ADM, adrenomedullin; AP, activation function; ALD, aldosterone; DBD, DNA binding domain; DEX, dexamethasone; GFP, green fluorescent protein; GLUT, glucose transporter; GR, glucocorticoid receptor; HIF, hypoxia-inducible factor; HRE, hypoxia response element; LBD, ligand binding domain; MR, mineralocorticoid receptor; NLS, nuclear localization signal; RT-PCR, reverse transcriptase PCR; VEGF, vascular endothelial growth factor.
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(VEGF), and adrenomedullin (ADM). Moreover, hypoxia also enhances gene expression of the hematopoietic hormone erythropoietin in the kidney, enabling humans to adapt systemically at high altitudes via increasing blood levels of hemoglobin. These hypoxic responses are controlled mainly at the level of transcription by hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimer of α and β subunits (Arnt), both of which belong to a family of basic helix-loop-helix PAS (Per/Arnt/Sim) transcription factors. HIF-1 binds to the hypoxia response element (HRE), which was originally identified in the 3′-enhancer region in the erythropoietin gene and later in the promoter region of the genes for VEGF, GLUTs, and ADM as well (14, 15). The recent discovery of the dioxygenases for oxygen sensing has shed light on the mechanism of oxygen-dependent regulation of HIF-1 activity. Under normoxic conditions, critical proline residues within the oxygen-dependent degradation domain of the HIF-1α proteins are hydroxylated by a certain class of proline hydroxylases, and HIF-1α proteins are targeted for ubiquitination and degradation by the proteasome (15–18). This post-translational modification is inhibited under hypoxic conditions, resulting in stabilization of HIF-1α protein levels. In addition, hypoxia induces the function of the transactivation domains of HIF-1α proteins and enhances their ability to interact with transcriptional coactivator proteins (19). Under normoxic conditions, this interaction has been shown to be blocked by the hydroxylation of a conserved asparagine residue within one of the transactivation domains. This asparagine hydroxylation is catalyzed by asparagine hydroxylase, previously identified as FIH, under normoxic conditions and abrogated under hypoxic conditions (20).

Recently, growing evidence indicates that adaptation to hypoxia is also influenced by the activity of the HPA axis and glucocorticoids. For example, blood levels of cortisol are shown to be elevated via increased secretion of adrenocorticotropic at high altitudes or under intrauterine hypoxic conditions (21–23). Moreover, the prophylactic administration of synthetic glucocorticoids dramatically prevents high mountain sickness (24). In rodents, the administration of glucocorticoids significantly reduces brain tissue damage after cerebral ischemia (25), and stress-induced erythropoiesis under hypoxic conditions is influenced by glucocorticoids (26). Thus, there appears to be cross-talk between hypoxia-dependent signals and the HPA axis and glucocorticoid system. However, the underlying molecular mechanisms have not yet been explored, especially at the cellular level. Given this fact, we were prompted to investigate the interaction between these important stress-responsive pathways, focusing on the transcription factors GR and HIF-1. Here we have shown that HIF-1-dependent transcriptional activation is up-regulated by glucocorticoids via the GR and that the LBD of the GR may play a critical role in the functional interaction between these stress-responsive transcription factors.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Dexamethasone (DEX) and aldosterone (ALD) were purchased from Sigma. RU486 and cortivazol were kindly gifted from Roussel Uclaf and Merck, respectively. Monoclonal anti-HIF-1α antibody Ab463 was purchased from Abcam (Cambridge, UK). Polyclonal anti-rabbit GR antibody PAI-512 was from Affinity Bioreagents (La Jolla, CA). Monoclonal anti-α actin antibody was from Sigma. Other chemicals were from Wako Pure Chemical (Osaka, Japan) unless specified otherwise.

Cell Culture—COS7 and HeLa cells were obtained from the RIKEN Cell Bank (Tsukuba Science City, Japan) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics in a humidified atmosphere at 37 °C with 5% CO2 unless specified otherwise. Hypoxic conditions were achieved by incubation of cells in an acryl anaerobic chamber with 1% O2, 5% CO2, and 94% N2.

In all experiments, serum steroids were stripped from fetal calf serum with 0.01% triton-X-405. Volcano mouse genomic DNA was purchased from Invitrogen (Carlsbad, CA) and used as a template.

RNA Isolation and RT-PCR Analysis—Total RNA was extracted by the spin column method using the SV Total RNA Isolation System (Promega). One-step RT-PCR was carried out using 50 ng of total RNA as a template and the Access Quick RT-PCR system (Promega) in a total volume of 50 μl of mixture containing 5 units of avian myeloblastosis virus reverse transcriptase, 2 units of Taq DNA polymerase, 1.5 mM MgSO4, 200 μM dNTPs, and sense and antisense primers at 0.25 μM each. Aliquots of the PCR products were electrophoresed in 2% agarose gels and stained with ethidium bromide. The amount of CDNA, as estimated relatively by the intensity of the amplified β-actin signal, was comparable among the preparations. Experiments in the absence of reverse transcriptase were performed as negative controls. PCR primer pairs for amplification of each gene are as follows: VEGF: 5′-TGCGCTGTCTCAGTAAAAC-3′ (sense) and 5′-TACCCGTTCCTCACCCG-3′ (antisense); GLUT3: 5′-GATGCTGGAGAGGTTAAGGT-3′ (sense) and 5′-ACTACTCACCCAGAGAAGAAT-3′ (antisense); ADM: 5′-AGAAGTTGGATAGGCGGCT-3′ (sense) and 5′-TGGCCTGAAGAGACCGAGAAG-3′ (antisense); β-actin: 5′-CCCTGGCTTCCTTCGCCATTCC-3′ (sense) and 5′-GGATCTTCATGAGGATGAC-3′ (antisense).

Plasmids—The expression plasmids for the wild-type and mutant human GR, pCMX-hGR and pCMX-GR(1–765), were described previously (27). The expression plasmids for human AF-1-deleted mutant GR Δ49–385 and AF-2-deleted Δ550 and the wild-type human MR, pShMR, were kindly gifted by Dr. R. K. Evans (Salk Institute, La Jolla, CA) (28). The expression plasmid for dimerization-deficient human GR mutant A458T was from Dr. A. C. B. Cato (Forschungszentrum, Karlsruhe, Germany) (29). The expression plasmids for the wild-type human HIF-1α, pCMV4-HIF-1α, and the wild-type human Arnt, pCMV4-Arnt and pCMX-GR4-Arnt, were from Dr. Lorenz Poellinger (Karolinska Institute, Stockholm, Sweden) (30). The expression plasmids for the chimeric protein of green fluorescent protein (GFP) and the wild-type human GR and MR, pCMX-GFP-GR (31) and pCMX-GFP-MR (27), respectively, were described previously. The expression plasmids for a chimeric protein of GAL4-DBD and the LBD of the human GR (Glu–489 to Lys–777), pCMX-GAL4-GR-LBD, and GAL4-responsive reporter plasmid pGRE-Luc were kindly gifted by Dr. K. Umesono (University of Kyoto, Kyoto, Japan). To construct the expression plasmid for the chimeric protein of GAL4-DBD and the C-terminal truncated GR LBD, pCMX-GAL4-GR-LBD (489–765), the DNA fragments encoding the amino acids Leu–596 to Ser–765 of human GR was amplified using PCR with the appropriate flanking sequences and inserted into Pep-BonHI-opened pCMX-GR4-LGRBD. The expression plasmid for the chimeric protein of GAL4-DBD and HIP-1α, pCMX-GA4-HIP-1α, was described previously (32). To construct the expression plasmids for the chimeric protein of NLS obtained from SV40 large tumor antigen and GR LBD (499–777) and GR LBD (499–777), pCMX-NLS-GR-LBD (499–777) and pCMX-NLS-GR-LBD (499–775), the DNA fragments encoding the corresponding amino acids of NLS were inserted into pCMX-GR4-LGRBD, resulting in pCMX-NLS. Then the DNA fragment encoding either amino acids 499–777 or 499–765 of the GR LBD was inserted into pCMX-NLS. HIF-1α-responsive reporter plasmid pT81/HRE-Luc contains three tandem copies of the erythropoietin HRE in front of the herpes simplex thymidine kinase promoter and the luciferase gene (30, 32). The glucocorticoid-responsive reporter plasmid pGRE-Luc was described previously (33). All plasmids constructed as described above were verified by sequencing.

Transfection and Reporter Gene Assay—Cells were plated on 6-cm diameter culture dishes to 30–50% confluence, and the medium was replaced with Opti-MEM (Invitrogen). The plasmid mixture was mixed with TransIT-LT1 transfection reagent (Panvera Corp., Madison, WI) and added to the culture. The total amount of plasmids was kept constant by adding an irrelevant plasmid (pGEM7z was used unless otherwise specified). After 6 h of incubation, the medium was replaced with fresh Dulbecco’s modified Eagle’s medium with 2% dextran-coated, charcoal-treated fetal calf serum, and the cells were further cultured in regular medium for 24–48 h. Luciferase activity was determined using a luminometer (Promega), and relative light units were normalized to the protein amount determined with protein assay reagent according to the manufacturer’s instructions (Pierce).

Western Blot Assay—Whole cell extract of HeLa cells was prepared in lysis buffer containing 25 mM Hepes, 100 mM NaCl, 5 mM EDTA, 1 mM orthovanadate, 1 mM sodium orthovanadate, 5 mM dithiothreitol, and 0.5% Triton X-100, pH 7.9, with a proteinase inhibitor mixture on ice for 15 min followed by centrifugation for 20 min at 14,000 rpm. Twenty micrograms of protein of whole cell extract were separated in 8% SDS-polyacrylamide gels and then blotted to nylon membranes. The membranes were blocked in
Glucocorticoids Enhance Hypoxia-inducible Gene Expression—To examine the effect of glucocorticoids on hypoxia-inducible gene expression, we cultured HeLa cells in the presence or absence of 100 nM DEX under normoxic (N, 21% O2) or hypoxic (H, 1% O2) conditions for 12 h, and total RNA was extracted. RT-PCR for VEGF, GLUT-3, ADM, and β-actin were performed as described under “Experimental Procedures.” These PCR products were electrophoresed in 2% agarose gels and stained with ethidium bromide. 

RESULTS

Glucocorticoids Enhance Hypoxia-inducible Gene Expression—To examine the effect of glucocorticoids on hypoxia-inducible gene expression, we cultured HeLa cells in the presence or absence of 100 nM DEX under normoxic or hypoxic conditions (oxygen concentrations were 21 and 1%, respectively). After total RNA isolation, mRNA expression of HIF-1-target genes, VEGF, ADM, and GLUT3 was analyzed using RT-PCR. As shown in Fig. 1A, mRNA expression of β-actin was not altered under these experimental conditions. Under hypoxic conditions, mRNA expression of VEGF, ADM, and GLUT3 was induced. Treatment with DEX did not significantly increase mRNA expression of these HIF-1-target genes at normoxia. Under hypoxic conditions, however, treatment with DEX enhanced hypoxic inducibility of mRNA expression of these HIF-1 target genes by 1.5–3-fold (Fig. 1). We thus were prompted to investigate whether hypoxia-inducible HIF-1 transcriptional activity is modulated in the presence of glucocorticoids.

GR Enhances Transactivational Function of HIF-1α without Alteration in Protein Levels of HIF-1α—We then studied the effect of treatment with DEX on hypoxic induction of HRE-driven reporter gene expression, because native promoters of these target genes contain multiple regulatory elements and usage of this minimal reporter construct should bypass otherwise complicated interaction among those elements. After transfection of the HRE-luciferase reporter plasmid, HeLa cells were cultured in the presence or absence of DEX under normoxic or hypoxic conditions. It has already been shown that HeLa cells contain endogenous GR and HIF-1. Western blot analysis revealed that protein expression of the GR was almost constant under these experimental conditions (Fig. 2A). Protein levels of HIF-1α were up-regulated under hypoxia due to stabilization and escape from degradation (see the Introduction) but were not affected by treatment with DEX (Fig. 2A). On the other hand, treatment with DEX enhanced hypoxic induction of HRE-driven reporter gene expression in a concentration-dependent manner (Fig. 2A), possibly reflecting the results shown in Fig. 1. This issue was again confirmed in cotransfection experiments in which not only the HRE-luciferase reporter but also the GR expression plasmid pCMX-GR was transfected into HeLa cells; hypoxic induction of HRE-luciferase expression was increased in concert with an increasing dosage of the GR expression plasmid in the presence of DEX (Fig. 2B).

This result raised the possibility that glucocorticoids enhance hypoxic induction of HRE-driven gene expression via the GR. Because protein levels of HIF-1α were not affected by treatment with DEX, we tested the effect of DEX and the GR on the transactivation function of HIF-1α. For that purpose, HIF-1α was expressed as a fusion protein with GAL4 DBD (Fig. 3A), and the effect of hypoxia and DEX on GAL4-reporter plasmid was assayed in COS7 cells. When GAL4-HIF-1α was expressed with a GAL4 reporter plasmid, an ~2.5-fold induction of the reporter gene was observed under hypoxic condition (Fig. 3B). This induction response was not influenced either by treatment with DEX or by ectopic expression of the GR expression plasmid (Fig. 3B). However, when both GAL4-HIF-1α and GR were expressed, hypoxic treatment in the presence of DEX resulted in a robust increase in the induction response of the reporter plasmid, indicating that the GR enhances the transactivational function of HIF-1α in a ligand-dependent fashion. When GAL4-Arnt (Fig. 3A) was cotransfected, Arnt-dependent transactivation was not influenced by either cotransfection of the GR expression plasmid or treatment with DEX (Fig. 3B). We next transfected the expression plasmid for a constitutively active transcriptional activator, HIF-1α(1–396)-VP16 (Fig. 3A), and HRE-luciferase and examined the effect of treatment with DEX and coexpression of the GR. HIF-1α(1–396)-VP16 lacks the oxygen-dependent degradation domain of HIF-1α, thereby escaping degradation even under normoxia and docking in the nucleus (37). As expected, this chimeric protein activated reporter gene expression even under normoxic conditions (Fig. 3C). However, neither treatment with DEX nor coexpression of the GR significantly influenced its transactivation function (Fig. 3C), indicating that VP16 cannot be substituted for the

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Cross-talk between GR and HIF-1

transactivation domains of HIF-1α in terms of functional coupling with the GR. Taken together, we may conclude that ligand-bound GR may not affect protein levels of HIF-1α but modulates the transactivational function of HIF-1α and enhances HIF-1-dependent transcription.

HIF-1 Does Not Enhance GR-dependent Transcriptional Activation—To test the effect of hypoxia and HIF-1 on GR-dependent transcription, we cotransfected expression plasmids for HIF-1α, Arnt, GR, and GRE-luciferase into COS7 cells. Under hypoxic conditions, GRE-dependent transcription was slightly enhanced. However, neither HIF-1α nor Arnt further increased GR-driven transcriptional response (Fig. 4).

Ligand and Receptor Specificity—To confirm the requirement for ligand, COS7 cells in which the expression plasmids for the GR, HIF-1α, and Arnt were transfected and treated with DEX, cortivazol, RU486, and ALD. The characteristics of these ligands are the following: DEX and cortivazol are GR agonists and induce GR-dependent transcription (27); RU486, binding the GR with considerable affinity, acts as an antagonist in terms of GR-dependent transcription (38); ALD is an MR agonist, and it has been shown that DEX and ALD bind not only GR but also MR and that both receptors transactivate GRE-driven reporter gene expression (27, 39–42). When subcellular localization of the GR was examined using a GFP-tagged GR expression system, these ligands promoted nuclear translocation of the GR equally, even under hypoxic conditions (Fig. 5A). Inducibility of HRE-luciferase was enhanced by DEX and cortivazol but not by RU486 and ALD (Fig. 5B). Next, COS7 cells were transfected with the expression plasmids for various steroid receptors, instead of the GR expression plasmid, and treated with the cognate ligands under normoxic or hypoxic conditions. Even in the presence of ligand, MR, PR, AR,
or ER did not show enhanced activity of HIF-1-dependent reporter gene expression (data not shown). We further examined the specificity of ligand-receptor coupling concerning the GR and MR, because the LBD of these receptors are highly homologous and DEX and ALD can bind both receptors and activate GRE-dependent transcription (27). Although GFP-MR, as well as GFP-GR, showed nuclear translocation in the presence of DEX or ALD (data not shown), transcriptional enhancement activity for HRE-driven reporter gene was observed exclusively in the combination of DEX and GR (Fig. 5C). Considering that ligand is believed primarily to determine the conformation of the LBD (43), we suggest that a strictly agonist-mediated conformational change of the GR LBD might be essential for this enhancement of HIF-1-dependent transcription.

Ligand Binding Domain Is Prerequisite for Enhancement of HIF-1α Function—We performed a domain analysis of the GR for up-regulation of HIF-1α function. For that purpose, we cotransfected various GR expression plasmids (Fig. 6A) and HRE-luciferase reporter plasmid into HeLa cells. AF-1-deleted GR mutant Δ9–385 showed this up-regulation of reporter gene expression as well as wild-type GR, however, the LBD/AE-2-deleted constitutive transactivator, I550, did not (Fig. 6B). These results indicate that the DBD and/or LBD of the GR is essential, whereas the AF-1 is not. Another mutant GR, A458T (Fig. 6A), clarifies the necessity of these distinct GR activities, because this mutant can normally bind DEX and translocate into the nucleus but cannot either bind DNA or transactivate target gene expression ((29) (Fig. 6C)). Indeed, when A458T was transfected, ligand-dependent enhancement of the hypoxia-dependent up-regulatory effect was completely preserved when compared with that in wild-type GR (Fig. 6C). Taken together, these results indicate that the LBD/AE-2 is essential but that neither DNA binding nor transactivation is necessary.

These results led us to the somewhat surprising conclusion that the up-regulation of transactivational function of HIF-1α is mediated exclusively by the GR with a strict requirement for agonistic ligand and that neither AF-1 nor DBD but only LBD/AE-2 of the GR might be necessary. To confirm this conclusion, we constructed the plasmids in which GR LBD is fused to either the NLS of SV40 large tumor antigen or GAL4 DBD as well as their mutants (Fig. 7A). Expressed SV40-NLS-GR LBD and GAL4-GR LBD almost docked in the nucleus, regardless of hypoxic treatment, in the absence of DEX (data not shown). When these chimeric proteins were expressed with the HRE-luciferase reporter in COS7 cells, HIF-1α transactivational function was not elicited in the absence of DEX. However, the addition of DEX resulted in a robust increase in reporter gene expression under hypoxic conditions (Fig. 7B), indicating that the GR LBD alone is capable of enhancement of HIF-1α trans-
activational function, as long as the LBD binds ligand and is located in the nucleus. It has already been shown that AF-2 deletion in the GR results in a loss of DEX binding activity. For example, GR-(1–765) does not transactivate GRE-luciferase reporter even in the presence of DEX (27). Although SV40NLS-GR LBD-(499–765) and GAL4-GR LBD-(499–765) cannot bind steroid ligand, substantial amounts of these chimeric proteins still localized in the nucleus (data not shown) but could not stimulate the transactivational function of HIF-1α (Fig. 7B). We therefore conclude that the GR may enhance HIF-1-dependent gene expression via an agonist-dependent conformational change of the LBD in the nucleus.

**GR and HIF-1α Colocalize in the Nucleus**—To address the underlying mechanism of GR-HIF-1α coupling, we examined...
the subcellular localization of these transcription factors. After GFP-HIF-1α and GR were coexpressed in COS7 cells, the cells were treated with DEX and placed under hypoxic conditions. As represented in Fig. 8, the GR and HIF-1α almost completely colocalized in the nucleus, forming distinct foci. This was also the case when GAL4-GR LBD was expressed with GFP-HIF-1α, indicating that the GR docked in those foci and not via DNA binding. Although we do not have direct evidence showing protein-protein interaction between the GR and HIF-1α (data not shown), these results strongly indicate their intimate interaction in close proximity to each other in distinct compartments in the nucleus.

**DISCUSSION**

It has been reported that gene expression of VEGF (44), GLUTs (45), and ADM (46) is under strict but composite regulation downstream of a number of signaling pathways. Among other considerations, however, transcriptional regulation by HIF-1α is thought to be important under hypoxic conditions. On the other hand, expression of these genes is under strict control via interaction between the promoter and many transcription factors downstream of the distinct signaling pathways and perception of oxygen tension.

Ligand specificity and the requirement of the GR LBD for enhancement of HIF-1α action strongly indicate the necessity of the ligand-bound conformation of the LBD. Such conformation is believed to be critical for the GR to recruit cofactors including transcriptional coactivators (27, 34, 43). To this end, we first postulated direct protein-protein interaction between the GR LBD and HIF-1α as the underlying biochemical mechanism for glucocorticoid-dependent up-regulation of HIF-1 function. Indeed, a similar mechanism has been proposed in the case of the short heterodimer partner, which consists mainly of the LBD and acts as a coactivator for PPARγ (peroxisome proliferator-activated receptor-γ) (51). However GST pull-down assays have not provided evidence of direct interaction between the GR and HIF-1α (data not shown), and at this moment we do not favor this hypothesis. On the other hand, we have clearly shown colocalization of the GR and HIF-1α. Because GAL4-GR LBD also behaved in a similar manner, it is strongly indicated that such colocalization is not mediated by GR DNA binding. We, therefore, are considering several possibilities. Because our data suggest that the C-terminal half of HIF-1α is a target of the GR, the GR might be recruited to a multiprotein complex involving HIF-1α and an as yet unidentified factor and might influence a particular function of this region of HIF-1α including recruitment of coactivators (52). It is also possible that the GR might squelch the corepressor for HIF-1α and attenuate otherwise suppressed HIF-1α-dependent transcription. Although further studies are clearly needed, the fact that these stress-transmitting transcription factors reside in the same nuclear compartments strongly supports the important role of intracellular GR-HIF-1α communication in adaptive regulation of gene expression.

As described in the Introduction, a number of reports have demonstrated already that the mode of GR action is extremely variable. Indeed, the DNA binding activity of the GR has been shown not to be essential for survival in mice in which the GR is genetically exchanged to the A55T mutant (7). In the present study, we showed that the same GR mutant could bind the agonistic ligand and enhance HIF-1α-dependent transcription when compared with wild-type GR. This is also the case in the repression of NF-κB- or AP-1-dependent transcription (53, 54). Thus the GRE-independent mode of GR action appears to be multiple in terms of transcriptional regulation in gene expression. Moreover, we cannot exclude the possibility that HIF-1α may modulate non GRE-mediated gene expression via the GR. Taken together, thorough identification of such GR action that is not mediated by DNA binding would contribute to understanding the multiple but essential roles of the GR in homeostatic regulation and survival.

Concerning ligand-receptor coupling, both GR and MR are sensitive to both glucocorticoids and mineralocorticoids. The effects of these corticosteroids, therefore, are determined through asymmetric distribution of the GR and MR and 11β-hydroxysteroid dehydrogenase, which selectively metabolizes glucocorticoids into inactive species (39, 40). It is shown that both GR and MR are abundant in areas such as the hippocampus (55). Although 11β-hydroxysteroid dehydrogenase is not expressed, the two receptors differentially mediate the control of ion regulation and transmitter responsiveness in CA1 pyramidal neurons (55, 56). However, the classical model for the mechanism of corticosteroid action cannot account for such differential regulation of GR and MR by these corticosteroids.

In the present study, the enhancement of HIF-1α-dependent transcription is seen not in MR-transfected cells but exclusively in GR-transfected cells. Moreover, neither RU486 nor ALD elicited that effect even in GR-transfected cells. This strict requirement of the GR and its agonists could be the basis for identification of a novel mechanism of differential regulation of the GR and MR, which may be important for understanding the physiological regulation of these receptors. On the other hand, recent reports have suggested that the MR is involved in ALD-induced vasculitis and cardiovascular fibrosis (57). Because glucocorticoids are frequently used for the treatment of inflammatory disorders at pharmacological dosages, we should not ignore these MR-mediated adverse effects of glucocorticoids. The clarification of the molecular mechanism of ligand-based differentiation of GR and MR, therefore, would also contribute to the reduction of glucocorticoid side effects in certain clinical settings.

Finally, our present work has clearly shown the close connection between hypoxic responses and the HPA axis-glucocorticoid-GR system at the cellular level. Interestingly, it has recently been shown that secreted ADM modulates HPA axis activity and cortisol secretion from the adrenal cortex (58). This indicates that the cross-talk between hypoxia-generated signals and the HPA axis-glucocorticoid-GR system may occur at multiple levels via distinct mechanisms, thereby enabling the fine-tuning of adaptive responses.

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