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Original Article

Hypoxia- and dexamethasone-dependent HIF1α-glucocorticoid receptor interaction leads to degradation of glucocorticoid receptor in pituitary adenomas

Chenran Zhang[1,2], Xiaoshu Dong[3], Weiwei Mao[3], Chen Wang[1], Bin Ma[4], Liuhua Hu[5], Ruoping Chen[1]

[1]Department of Pediatric Neurological Disease Centre, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China; [2]Clinical Research Unit, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China; [3]Department of Pediatric Neurosurgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China; [4]Department of Neurosurgery, Qinghai Women's and Children's Hospital, Qinghai, China; [5]Division of Cardiology, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China

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Abstract: Cushing disease has a very high mortality rate and glucocorticoid resistance caused by GR down-regulation is one major reason of mortality. Although HIF1α signaling and GR signaling are involved in the pathogenesis of pituitary adenomas, it's unclear whether and how these two essential pathways could cross-talk with each other. Here, we performed a comprehensive study to investigate the reciprocal effects of HIF1α and GR on each other in AtT20 cell lines and explored the potential therapeutic effect of HIF1α inhibitor in in-vivo mouse model. We find that hypoxia up-regulated the promoter activity, mRNA and protein levels of GR and the induced GR protein was localized in cytosol. On the other hand, GR activation by its agonist DEX increased HIF1α protein through post-transcriptional mechanism. However, hypoxia and DEX show differential synergistic effects on HIF1α and GR. In hypoxia-DEX condition, HIF1α protein was further up-regulated but mainly localized in cytosol while GR was trapped and degraded in cytosol via UPS pathway. Further Co-IP experiments demonstrate that DNA binding domain of GR can interact with PASb domain of HIF1α. In a in-vivo mouse model of Cushing's disease, HIF1α inhibitor reduced HIF1α and GR protein levels, reduced tumor size and lowered the plasma concentrations of ACTH and corticosterone. In summary, we find that a novel HIF1α-GR crosstalk contributes to the pathogenesis of pituitary adenomas and HIF1α inhibitor shows potential therapeutic effects for Cushing's disease.

Keywords: Hypoxia, glucocorticoid receptor, HIF1α, pituitary adenomas, Cushing's disease

Introduction

Pituitary adenoma is a common neuroendocrine intracranial tumor in the general population [1]. Autopsy and brain imaging show an average prevalence of 16.7% in the population [2] and the incidence of pituitary adenoma has been increasing recently due to enhanced power of endocrine test. About two-thirds of pituitary adenomas secrete excessive hormones and 2% to 6% of patients secrete excessive ACTH [3]. Over-secretion of ACTH by pituitary tumors (ACTH tumors) can stimulate the adrenal cortex to synthesize and secrete cortisol, causing Cushing disease [4]. Patients with Cushing's disease will develop a series of disorders such as centripetal obesity, hypertension, diabetes, dyslipidemia, coagulopathy, osteoporosis, and mental disorders, which seriously impair the quality of life and survival of patients. Although clinical trials of new drugs including Gefitinib, an inhibitor of Epidermal growth factor receptor, seliciclib, an inhibitor of Cyclin-dependent kinase and Osilodrostat, an 11β-hydroxylase inhibitor, are currently undergoing, there are no effective drugs directly acting on the pituitary to inhibit tumor growth and ACTH secretion so far [5]. Thus, it's imperative to further study the pathogenesis of Cushing's disease and find new therapeutic targets.

Hypoxia-inducible factor 1 (HIF1) is a heterodimeric transcription factor consisting of two subunits, HIF-1α and HIF-1β [6]. HIF-1α is typically degraded rapidly in normoxic cells. However, hypoxia is a common feature in brain tumors [7] and in hypoxic environment, HIF1α activates the transcription of a broad range of genes involved in angiogenesis, erythropoiesis, cell cycle, metabolism and apoptosis to promote the oncogenesis of tumor cells. Indeed, previous studies show that HIF1α is frequently expressed in pituitary tumor [8, 9] and HIF1α activates its target gene like VEGF-A to promote aggression in pituitary tumor [10, 11].

Glucocorticoid receptor (GR) is a member of the nuclear hormone receptor superfamily of transcription factors and it respond to Glucocorticoid hormones to control cellular proliferation, inflammation and metabolism [12]. Interestingly, GR expression has been found increased in ACTH tumors [13] and GR antagonist shows benefits in patients with Cushing Disease [14, 15]. It suggests that GR overactivation contributes to the pathogenesis of Cushing Disease. However, whether and how could HIF1α signaling crosstalk with GR signaling in pituitary adenomas is unclear. Here, we investigated the reciprocal effects of HIF1α and GR on each other in AtT20 cell line and explored the potential therapeutic effect of HIF1α inhibitor in in-vivo mouse model.

Methods and materials

Cell cultures

AtT20 (ATCC® CCL-89™) from ATCC were maintained in F-12K Medium with 2.5% fetal bovine serum, 15% horse serum, 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C. Chemical hypoxia was induced by treatment with $CoCl_2$ at indicated concentrations. Physical hypoxia was induced by a modular incubator chamber which was flushed with a 94% N_2, 5% CO_2, and 1% O_2 gas mixture. The chamber was then sealed and placed in a 37°C container.

Reagent

Dexamethasone, dimethyl sulfoxide (DMSO) and $CoCl_2$ were from Sigma-Aldrich. HIF1α inhibitor LXY6006 [16] was from Glixx Laboratories Inc. UPS inhibitors MG132 and lactacystin were from Selleck Chemicals.

Over-expression of HIF1α and GR

For over-expression constructs of HIF1α, the full length of HIF1α CDS with C-terminal Myc tag and different truncations with C-terminal Myc tag were cloned into pcDNA 3.1 vector, respectively. For over-expression constructs of GR, the full length of GR CDS with C-terminal HA tag and different truncations with C-terminal HA tag were cloned into pcDNA 3.1 vector, respectively. The constructs were transfected for 48 h with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, US).

Knock-down of GR

The short-interfering RNA (siRNA) against mouse GR and mock siRNA (nonsense control) were purchased from Santa Cruz Technology (Santa Cruz, CA). The siRNA was transfected at the final concentration of 50 nM with Lipofectamine 2000. Cells were first transfected with indicated siRNAs for 12 h, then medium were replaced by new medium containing Dex and incubated for another 96 h.

In-vivo xenografted study

The care of athymic nude mice was in accord with the animal welfare guidelines of Xinhua Hospital, Shanghai Jiaotong University. Female athymic nude mice (6 weeks old, BALB-c/nu/nu strain) were kept in specific pathogen-free condition and were randomly assigned to two groups: HIF1α inhibitor and DMSO group, 7 animals per group. A total of 1×10^6 AtT20 cells were implanted into subcutaneous tissue of bilateral forelimb armpit with a 26-gauge needle/1 ml syringe. Mice received intraperitoneal injection of HIF1α inhibitor (50 mg/kg) or DMSO every week. Tumor size was measured after 4 weeks by measuring the length (L) and width (W) of xenografted tumors with a vernier caliper. Tumor size was calculated as follows: $L\times(W)^2/2$.

Quantitative real-time PCR

Total RNA was extracted from cell line using TRIzol reagent and quantitative real time-PCR was performed as previously described [12]. Following qRT-PCR primers were used. HIF1α:

GTCCCAGCTACGAAGTTACAGC (forward) and CAGTGCAGGATACACAAGGTTT (reverse); GR: AGCTCCCCCTGGTAGAGAC (forward) and GGTGAAGACGCAGAAACCTTG (reverse); actin: GGCTGTATTCCCCTCCATCG (forward) and CCAGTTGGTAACAATGCCATGT (reverse).

Western blot

Proteins were extracted from cultured cells using sodium dodecyl sulfate lysis buffer (2% sodium dodecyl sulfate, 10% glycerol, 0.1 mM dithiothreitol, and 0.2 M Tris-HCl, pH 6.8). Protein samples were resolved by SDS-PAGE and detected with indicated antibodies. Following antibodies were used: HIF1α (H-206, Santa Cruz), GR (M-20, Santa Cruz), Myc (CST, 2276) and HA (CST, 3724), HIAP1 (CST, 3130), SLUG (CST, 9585), Kip2 (CST, 2557).

Co-immunoprecipitation and preparation of cytoplasmic and nuclear fractions

Cells were lysed in RIPA lysis buffer with complete protease inhibitor cocktail and lysates were centrifuged at 15000 g for 20 min at 4°C. The supernatant was incubated indicated antibodies or magnetic beads (Pierce Anti-HA Magnetic Bead or Pierce Anti-myc Magnetic Bead) at 4°C overnight. Then, the protein complexes were collected by incubation with Protein A/G beads or magnetic stand. The collected protein complexes were washed with RIPA buffer for 5 times and eluted by SDS loading buffer. The isolation of cytoplasmic and nuclear fractions was performed using Nuclear/Cytosol Fractionation Kit (Biovision, K266-100) according to the manufacturer's instruction. The samples were analyzed by western blot.

Luciferase activity assay

To construct the promoter luciferase vector, 1500 bp of promoter sequence of GR were amplified by PCR and cloned into pGL410, respectively. For luciferase reporter assay, cells were transiently transfected with pGL410 containing GR promoter using Lipofectamine 2000. After 48 h, reporter gene activity was measured using the dual-luciferase assay-system (Promega). Renilla luciferase activity was used to normalize for transfection efficiency.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. All data were presented as mean ± SD and statistical analysis was performed by two-tailed Student t test for two groups and one way ANOVA with Newman-Keuls post hoc test for more than two groups. Statistically significant differences were defined as $P<0.05$. For all, *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Results

Chemical hypoxia induces GR expression in AtT20 cell

To investigate the potential effects of hypoxia on GR expression in ACTH pituitary tumors, chemical hypoxia was induced by incubating AtT20 cell with $CoCl_2$ (0, 10, 100 uM) for 6 h. The expression of GR and HIF1α was first measured by qPCR and the results show that $CoCl_2$ increased GR mRNA level in a dose-dependent way (**Figure 1A**) but $CoCl_2$ had no effect on HIF1α mRNA level (**Figure 1B**). In addition, luciferase assays showed that $CoCl_2$ enhanced GR promoter activity in a dose-dependent way (**Figure 1C**) but $CoCl_2$ had no effect on HIF1α promoter (**Figure 1D**). These data suggest that $CoCl_2$ induced chemical hypoxia activated GR transcription. Consistently, western blots show that $CoCl_2$ increased GR protein and HIF1α protein in a dose-dependent way (**Figure 1E**). It's important to note that LXY6006, a potent HIF1α inhibitor which suppress HIF1α protein level, could reduce HIF1α proteins and block the $CoCl_2$-induced GR up-regulation shown by qPCR, luciferase and western blot. Taken together, it suggests that chemical hypoxia induced GR expression through HIF1α in AtT20 cell.

Physical hypoxia induces GR expression in AtT20 cell

To further confirm the effects of hypoxia on GR expression in AtT20 cell, physical hypoxia was induced by culturing cells under 1% O_2 for 0, 12, 24 h, respectively. The expression of GR and HIF1α was measured by qPCR and the results show that physical hypoxia increased GR mRNA level in a dose-dependent way (**Figure 2A**) but physical hypoxia had no effect on HIF1α mRNA level (**Figure 2B**). In addition, luciferase assays showed that physical hypoxia enhanced GR promoter activity in a dose-dependent way (**Figure 2C**) but physical hypoxia had no effect on HIF1α promoter (**Figure 2D**). These data suggest that physical hypoxia activated GR transcription. Consistently, western

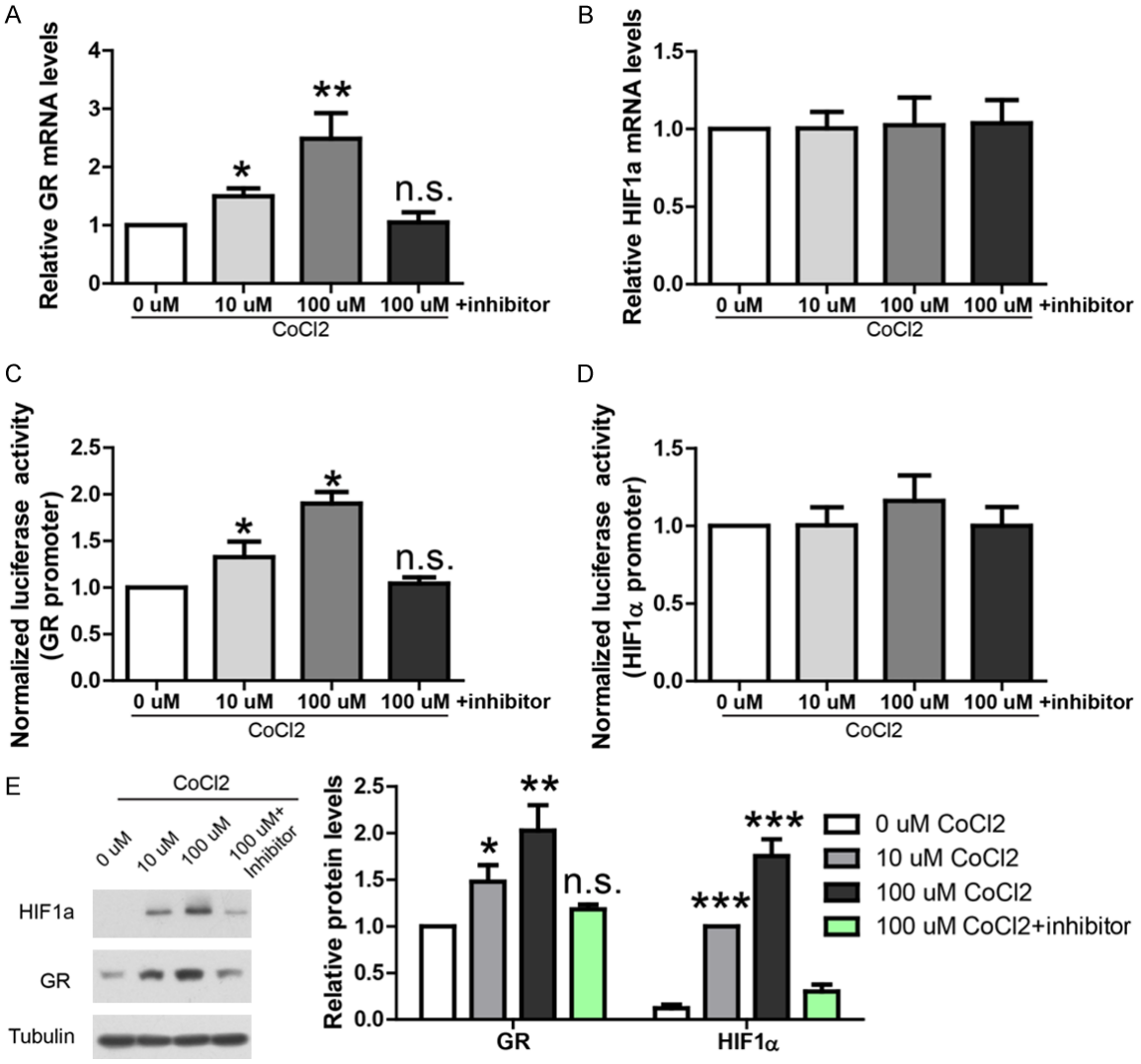

Figure 1. Chemical hypoxia induces GR expression in AtT20 cell. A. qPCR results showing GR mRNA level in AtT20 cell treated with $CoCl_2$ (0, 10, 100 uM) for 6 h. Inhibitor indicates HIF1α inhibitor LXY6006. B. qPCR results showing HIF1α mRNA level in AtT20 cell treated with $CoCl_2$ (0, 10, 100 uM) for 6 h. C. Luciferase assay showing GR promoter activity in AtT20 cell treated with $CoCl_2$ (0, 10, 100 uM) for 6 h. D. Luciferase assay showing HIF1α promoter activity in AtT20 cell treated with $CoCl_2$ (0, 10, 100 uM) for 6 h. E. Western bolts showing GR and HIF1α protein levels in AtT20 cell treated with $CoCl_2$ (0, 10, 100 uM) for 6 h. For all, *P<0.05; **P<0.01; ***P<0.001.

blots show that physical hypoxia increased GR protein and HIF1α protein in a dose-dependent way (**Figure 2E**). Similarly, HIF1α inhibitor LXY6006 reduced HIF1α proteins and blocked 1% O_2-induced GR up-regulation shown by qPCR, luciferase and western blot. Taken together, it confirms that hypoxia induced GR expression through HIF1α in AtT20 cell.

Subcelluar distribution of hypoxia-induced GR protein

GR is a hormone binding receptor in the cytosol and ligand binding could trigger its nuclear translocation to modulate target gene expression. To investigate the subcelluar distribution of GR under hypoxia, chemical (**Figure 3A**) or physical (**Figure 3B**) hypoxia was induced AtT20 cell, and cytoplasmic and nuclear fractions were isolated and analyzed by western blot. The results show that, as expected, hypoxia stabilized HIF1α protein and HIF1α protein was localized in the nucleus under both conditions. In contrast, hypoxia-induced GR was mainly localized in the cytosol without nuclear translocation. The cytoplasmic retention of GR is possibly due to the lack of ligand binding which is

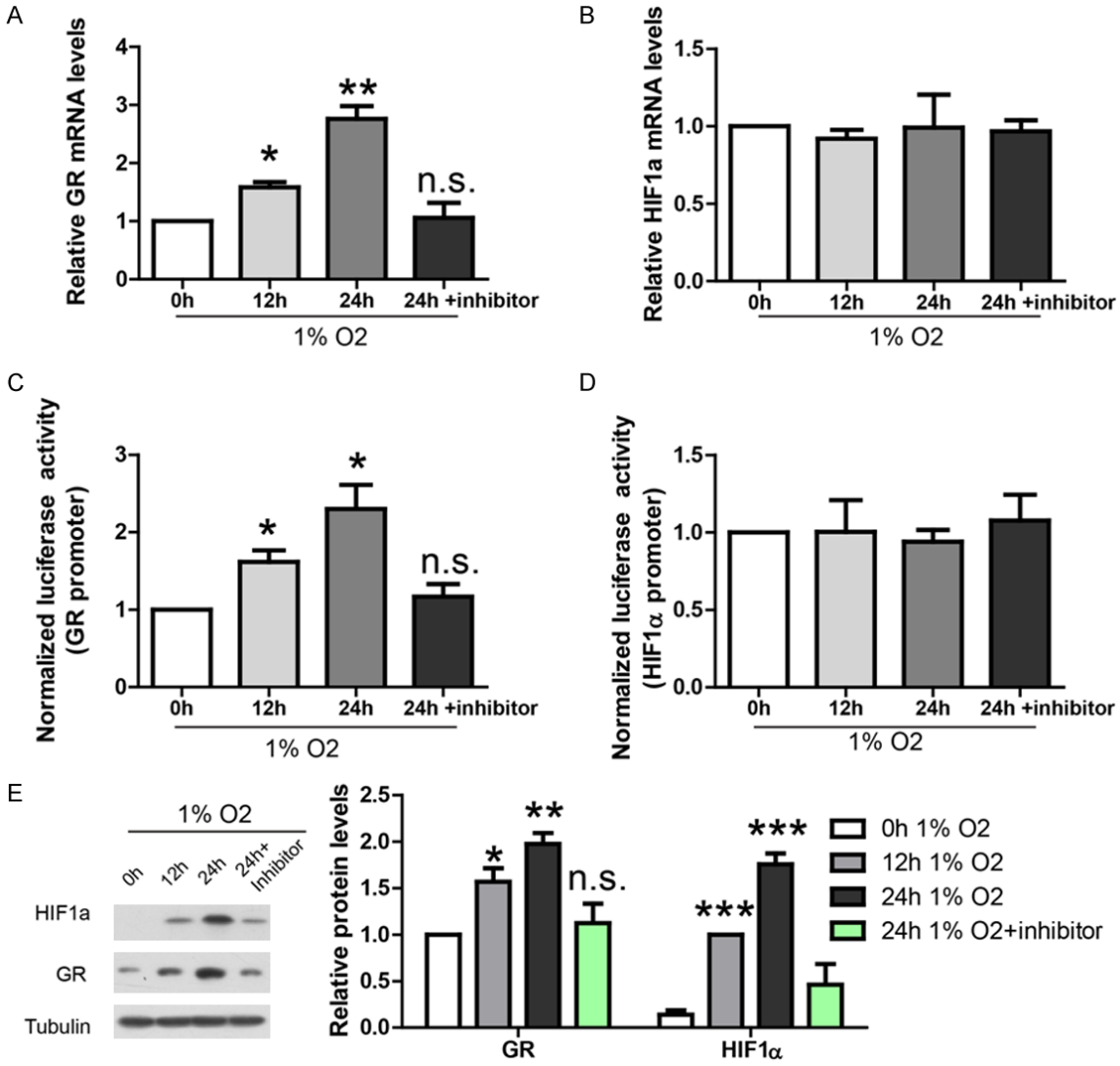

Figure 2. Physical hypoxia induces GR expression in AtT20 cell. A. qPCR results showing GR mRNA level in AtT20 cell treated with 1% O_2 for 0, 12, 24 h. Inhibitor indicates HIF1α inhibitor LXY6006. B. qPCR results showing HIF1α mRNA level in AtT20 cell treated with 1% O_2 for 0, 12, 24 h. C. Luciferase assay showing GR promoter activity in AtT20 cell treated with 1% O_2 for 0, 12, 24 h. D. Luciferase assay showing HIF1α promoter activity in AtT20 cell treated with 1% O_2 for 0, 12, 24 h. E. Western bolts showing GR and HIF1α protein levels in AtT20 cell treated with 1% O_2 for 0, 12, 24 h. For all, *P<0.05; **P<0.01; ***P<0.001.

necessary to trigger the conformational change of GR.

Synergistic effect of DEX and hypoxia on GR

To investigate the potential effects of GR on HIF1α expression in normoxia condition, AtT20 cell was treated with DEX at indicated concentrations (0, 10, 100 nM) to activate GR. Western blots show that DEX increased HIF1α protein in a dose-dependent manner while DEX had no effect on GR protein level (**Figure 4A**). It's important to note that the effect of DEX was dependent on GR as GR knock-down abolished the effect of DEX. Meanwhile, qPCR shows that DEX had no effect on HIF1α mRNA (**Figure 4B**), suggesting that DEX activated GR regulated HIF1α at post-transcriptional level. In addition, subcelluar distribution assay shows that DEX-induced HIF1α was localized in the nucleus and DEX also induced nuclear translocation of GR in a dose-dependent manner (**Figure 4C**). These data suggest that, in normoxia condition, DEX increased HIF1α protein

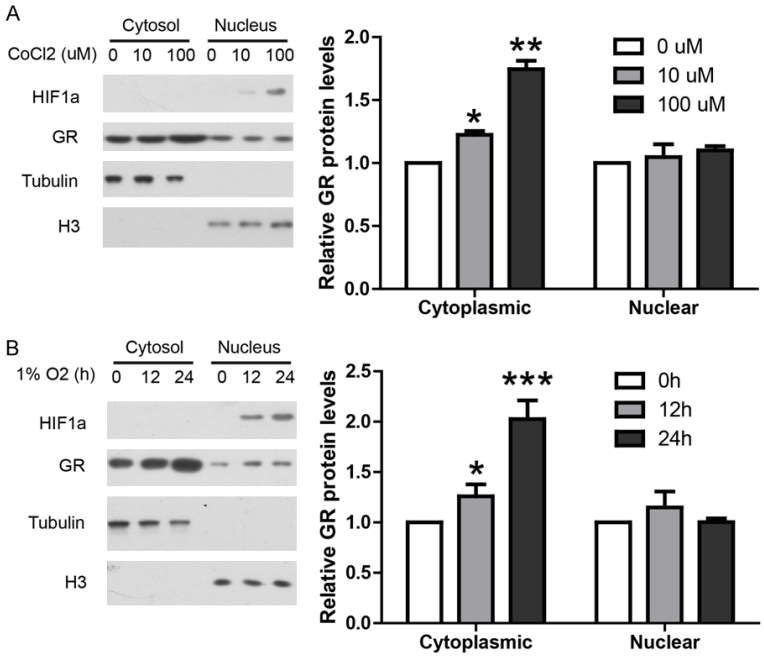

Figure 3. Subcelluar distribution of hypoxia-induced GR protein. A. Representative western blots and quantification showing the subcellular distribution of GR and HIF1α in AtT20 cell treated with $CoCl_2$ (0, 10, 100 uM). B. Representative western blots and quantification showing the subcellular distribution of GR and HIF1α in AtT20 cell treated with 1% O_2 for 0, 12, 24 h. For all, *P<0.05; **P<0.01; ***P<0.001.

level in the nucleus and DEX induced nuclear translocation of GR.

To investigate the synergistic effect of DEX and hypoxia in AtT20 cell, physical hypoxia was induced and cells were treated with DEX at indicated concentrations. Western blots show that hypoxia induced HIF1α protein level and co-treatment with DEX further increased HIF1α protein level. In contrast, hypoxia induced GR protein level and co-treatment with DEX resulted in decrease of GR protein (**Figure 4D**). The effect of DEX on GR protein under hypoxia was dependent on HIF1α as HIF1α inhibitor LXY6006 abolished the effect of DEX. Meanwhile, qPCR shows that DEX had no effect on GR or HIF1α mRNA under hypoxia (**Figure 4E**), suggesting that GR protein reduction took place in the presence of DEX and hypoxia through a post-transcriptional mechanism. In addition, subcelluar distribution assay shows that hypoxia-DEX induced HIF1α protein was localized mainly in the cytosol and hypoxia-DEX induced GR reduction took place in the cytosol (**Figure 4F**).

GR interacts with HIF1α under hypoxia condition

To detect the potential interaction between GR and HIF1α, Co-IP of endogenous HIF1α and GR was performed in AtT20 cell under normoxia, hypoxia or hypoxia-DEX condition. Cell lysates were precipitated with GR antibody and potential interacting HIF1α was detected by western blot (**Figure 5A**). First, the results show that there was no GR-HIF1α interaction in normoxia condition because HIF1α was fully degraded. Second, there was no GR-HIF1α interaction in hypoxia condition because HIF1α was localized in the nucleus while GR was trapped in cytosol due to the lack of ligand binding. Third, there was GR-HIF1α interaction in the presence of hypoxia and DEX. Taken together, the above data support that ligand binding of GR results in conformational change of GR which allows nuclear translocation and the activated GR would be degraded in the cytosol if it is trapped in cytosol and fails to enter nucleus. In the presence of hypoxia and DEX, HIF1α protein is induced but many of the HIF1α proteins are localized in the cytosol. It's likely that the interaction between HIF1α and GR is responsible for the cytoplasmic retention and degradation of GR under hypoxia-DEX condition.

To further confirm HIF1α-GR interaction and identify the interacting domain on each protein, Myc tagged full-length HIF1α was co-expressed with HA tagged GR fragments (GR-1-HA: 263-520aa; GR-2-HA: 1-262aa; GR-3-HA: 520-777aa) in AtT20 cell. Co-IP results show that full-length HIF1α-Myc interacted with GR-1-HA (263-520aa). The 263-520aa domain of GR was further truncated into GR-4-HA (263-400aa) and GR-5-HA (400-520aa). Full-length HIF1α-Myc was co-expressed with GR-4-HA or GR-5-HA in AtT20 cell and Co-IP results show that full-length HIF1α-Myc interacted with GR-5-HA (263-520aa) which is the DNA binding

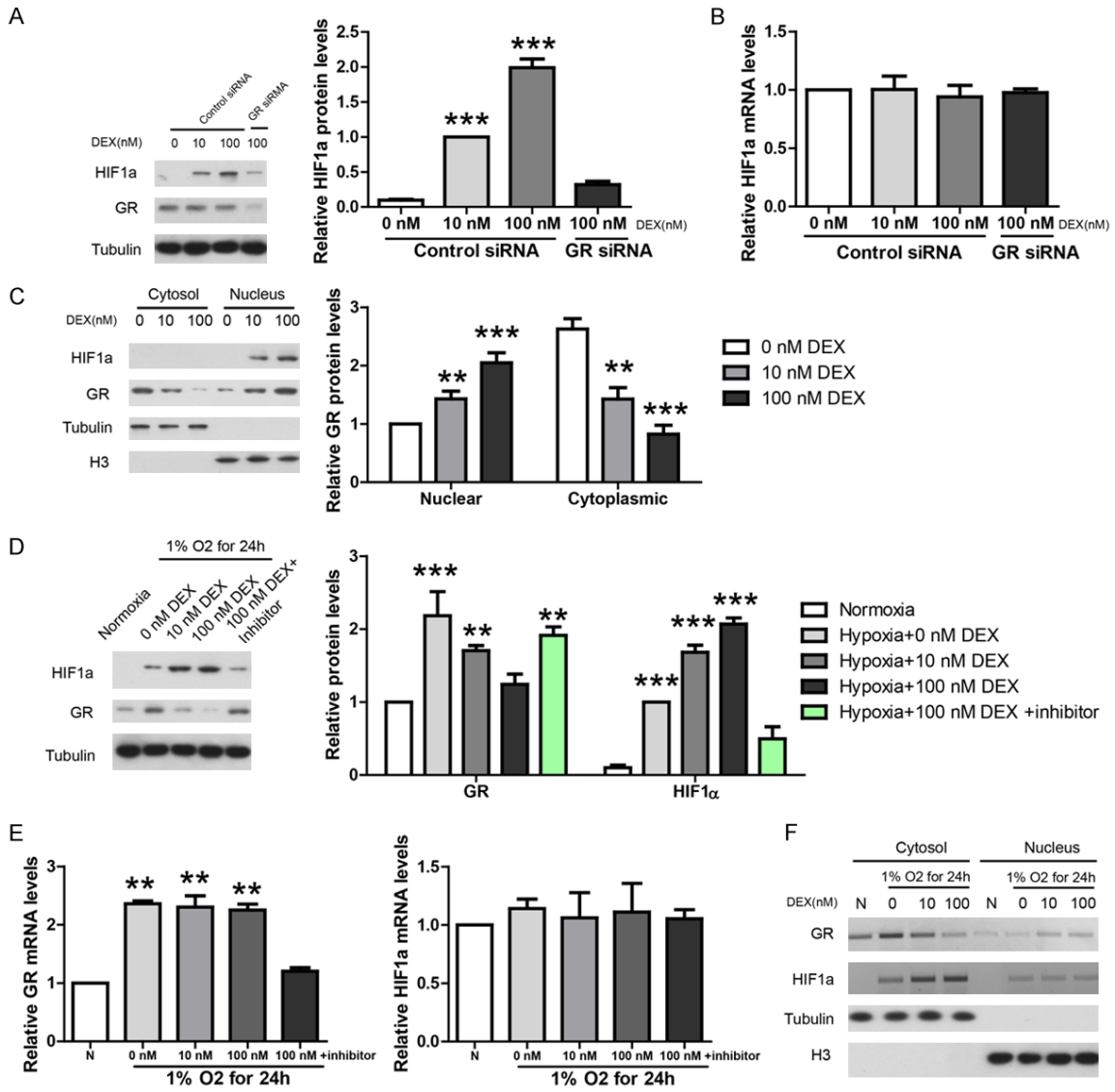

Figure 4. Synergistic effect of DEX and hypoxia on GR. A. Western blots showing GR and HIF1α protein levels in AtT20 cell treated with DEX at indicated concentrations (0, 10, 100 nM) or GR siRNA. B. qPCR showing HIF1α mRNA level in AtT20 cell treated with DEX at indicated concentrations (0, 10, 100 nM) or GR siRNA. C. Representative western blots and quantification showing the subcellular distribution of GR and HIF1α in AtT20 cell treated with DEX at indicated concentrations (0, 10, 100 nM). D. Western blots showing GR and HIF1α protein levels in AtT20 cell treated with DEX (0, 10, 100 nM) in hypoxia condition. Inhibitor indicates HIF1α inhibitor LXY6006. E. qPCR showing GR and HIF1α mRNA levels in AtT20 cell treated with DEX (0, 10, 100 nM) in hypoxia condition. Inhibitor indicates HIF1α inhibitor LXY6006. F. Western blots showing the subcellular distribution of GR and HIF1α in AtT20 cell treated with DEX (0, 10, 100 nM) in hypoxia condition. For all, *P<0.05; **P<0.01; ***P<0.001.

domain of GR (**Figure 5B**). Similarly, HA tagged full-length GR was co-expressed with Myc tagged HIF1α fragments (HIF1α-2-myc: 1-400aa; HIF1α-1-myc: 401-826aa) in AtT20 cell. Co-IP results show that full-length GR-HA interacted with HIF1α-2-myc (1-400aa). The 1-400aa domain of HIF1α was further truncated into HIF1α-3-myc (1-158aa) and HIF1α-4-myc (159-310aa). Full-length GR-HA was co-expressed with HIF1α-3-myc or HIF1α-4-myc in AtT20 cell and Co-IP results show that full-length GR-HA interacted with HIF1α-4-myc (159-310aa) which is the PASb domain of HIF1α (**Figure 5C**). Finally, in AtT20 cell co-expressing HIF1α-4-myc (159-310aa) and GR-5-HA (263-520aa), Co-IP results show the interaction between HIF1α-4-myc (159-310aa) and GR-5-HA (263-520aa) (**Figure 5D**). This indicates that the DNA binding domain of GR and the PASb domain of HIF1α can interact with each other.

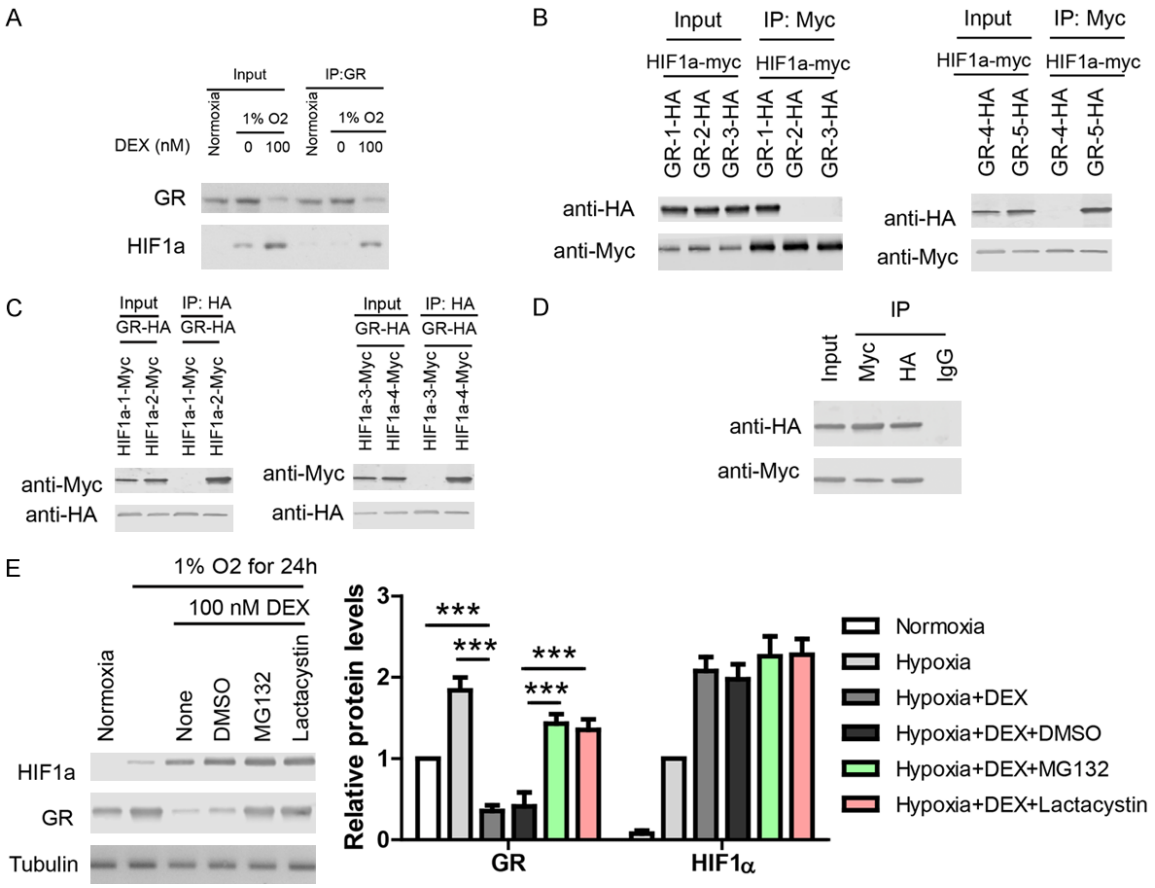

Figure 5. GR interacts with HIF1α under hypoxia condition. A. Representative Co-IP experiment showing the precipitated endogenous HIF1α protein levels by GR antibody in AtT20 cell treated with 1% O_2 or 100 nM DEX. B. Representative Co-IP experiment showing the precipitated HA tagged GR fragments by Myc antibody in AtT20 cell co-expressing full-length HIF1α-Myc and indicated GR fragments. C. Representative Co-IP experiment showing the precipitated Myc tagged HIF1α fragments by HA antibody in AtT20 cell co-expressing full-length GR-HA and indicated HIF1α fragments. D. Representative Co-IP experiment showing reciprocal Co-immunoprecipitation of HA-tagged GR fragment and Myc-tagged HIF1α in AtT20 cell. E. Representative western blots and quantification showing GR and HIF1α protein levels in AtT20 cell treated with 1% O_2, 100 nM DEX, 5 uM MG132 or 5 uM lactacystin. For all, ***P<0.001.

To explore the underlying mechanism of cytoplasmic degradation of GR under hypoxia-DEX condition, AtT20 cell was treated with MG132 and lactacystin, two inhibitors for Ubiquitin/Proteasome System (UPS), in the presence of hypoxia and DEX. Western blots show that both inhibitors could restore GR protein level (**Figure 5E**), suggesting that GR is degraded under hypoxia-DEX condition through UPS pathway.

HIF1α inhibitor shows anti-tumor effects in in-vivo mouse model

To explore the potential therapeutic effect of HIF1α inhibitor LXY6006 in ACTH pituitary tumors, we established subcutaneously xenografted model in nude mice. A total of 14 nude mice were randomly assigned to two groups: HIF1α inhibitor LXY6006 (n=7) and DMSO group (n=7). The mice received intraperitoneal injection of HIF1α inhibitor or DMSO every week. Four weeks after implantation, mice were sacrificed and tumor size of HIF1α inhibitor group was significantly smaller than that of DMSO group (**Figure 6A**). WB was performed in the tumor tissues from in-vivo model and results show that HIF1α inhibitor reduced protein levels of HIF1α and GR (**Figure 6B**). In addition, we also measured protein levels of GR target genes including HIAP1, SLUG and Kip2 [17]. HIAP1 and SLUG regulate apoptosis while Kip2 regulates growth. The results show that

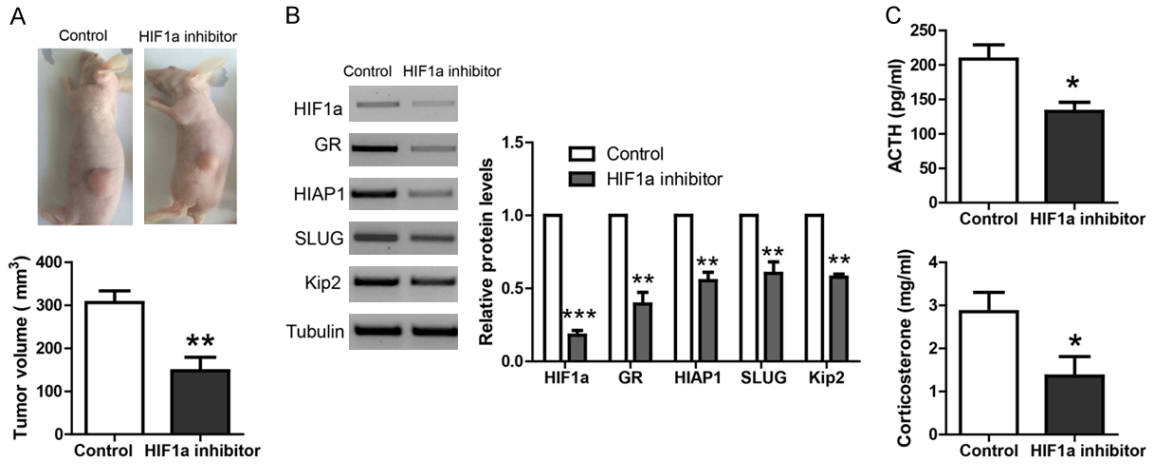

Figure 6. HIF1α inhibitor shows anti-tumor effects in in-vivo mouse model. A. Representative images and quantification of tumor size in xenografted mice receiving intraperitoneal injection of DMSO or HIF1α inhibitor LXY6006. B. Representative western blots and quantification showing indicated protein levels in tumors from xenografted mice receiving intraperitoneal injection of DMSO or HIF1α inhibitor LXY6006. C. Quantification of plasma ACTH and corticosterone levels by ELISA in xenografted mice receiving intraperitoneal injection of DMSO or HIF1α inhibitor LXY6006. For all, *P<0.05; **P<0.01; ***P<0.001.

HIF1α inhibitor also inhibited the expression of GR target genes (**Figure 6B**). The plasma levels of ACTH and corticosterone were also measured by ELISA and the results show that HIF1α inhibitor reduced ACTH and corticosterone (**Figure 6C**) in xenografted mice. Taken together, these results further support the important roles of HIF1α-GR crosstalk in the pathogenesis of pituitary adenomas.

Discussion

Cushing Disease has a very high mortality rate and there are few effective treatments except for surgery. Here, we performed a comprehensive study to investigate the reciprocal effects of HIF1α and GR on each other in AtT20 cell line and explored the potential therapeutic effect of HIF1α inhibitor in in-vivo mouse model. We find that hypoxia up-regulated the promoter activity, mRNA and protein levels of GR in AtT20 cell and the induced GR protein was localized in cytosol. On the other hand, GR activation by its agonist DEX increased HIF1α protein through post-transcriptional mechanism. However, hypoxia and DEX show differential synergistic effects on HIF1α and GR in AtT20 cell. In the presence of hypoxia and DEX, HIF1α protein was further up-regulated but mainly localized in cytosol while GR was trapped and degraded in cytosol via UPS pathway. Further Co-IP experiments demonstrate that DNA binding domain of GR can interact with and PASb domain of HIF1α. Taken together, our data support the following working model. DEX binding of GR resulted in conformational change of GR which allows nuclear translocation and the activated GR would be degraded in the cytosol by UPS if it is trapped in cytosol and fails to enter nucleus. In the presence of hypoxia and DEX, HIF1α protein is induced but many of the HIF1α proteins are localized in the cytosol. The cytoplasmic HIF1α interacts with GR to prevent its nuclear translocation, leading to UPS-mediated degradation of GR.

Our previous study reveals that HIF1α has anti-apoptotic effect and promotes proliferation in AtT20 cell [18]. Here, we further show that HIF1α could crosstalk with GR via protein-protein interaction. The potential interaction-mediated crosstalk between HIF1α and GR has been pointed out by an independent study in COS-7 and Hela cell lines. Here, we show direct evidence by Co-IP experiment and further identified the interacting domains. Although the crosstalk between HIF1α and GR have been discovered in non-cancer context such as human renal proximal tubular epithelial cells in inflamed conditions [19], our study is the first report to elucidate a detailed protein interaction-mediated mechanism in ACTH pituitary adenomas. We notice that many studies find that Dex treatment results in GR degradation, but it's important to note that GR protein levels usually were measured 12-16 h post Dex treat-

ment [20]. In addition, Dex induced GR degradation can be quickly rescued by new protein synthesis after 24-48 h [21]. In our experiments (**Figure 4A**), cells were first transfected with indicated siRNAs for 12 h, then medium were replaced by new medium containing Dex and incubated for another 96 h to achieve GR knockdown. In fact, WB showed a moderate trend of GR reduction in **Figure 4A** (100 nM vs. 0 nM), but it was not very robust likely because that GR level had recovered from new protein synthesis.

It's also important to note that it's common to see the cytoplasmic retention of HIF1α in tumors tissues like prostate cancer [22], colorectal cancer [23, 24], squamous cell head-and-neck cancer [25]. Thus, our data of cytoplasmic accumulation of HIF1α/GR are not artifacts. Instead, these data have important clinical implications as they recapitulate what happens in tumor tissues. In addition, the activity of HIF1α is regulated at multiple levels. For example, the stabilization of HIF1α is controlled by prolyl hydroxylases while the translocation of HIF1α is controlled by microtubule related proteins. Thus, the subcellular localization is essential for the fine tuning of HIF1α function. In this context, we can speculate the biological relevance of accumulated HIF1α/GR in cytosol is that they represent a storage pool which could respond quickly to various stimuli in an energy-efficient way compared to transcription-translation mediated mechanism.

Another novel finding in our study is that LXY6006 could reduce tumor size and lower plasma ACTH and corticosterone levels in in-vivo mouse model. LXY6006 is a synthetic derivative of Manassantin A [26]. Manassantin A is a dineolignan isolated from the herb Saururus cernuus. Manassantin A inhibit NF-κB and nitric oxide production in macrophages, block MAPK activation in mast cells, and inhibit transcription of a wide range of genes in various cell types at micromolar concentrations [26-28]. However, Manassantin A shows inhibitory effect on HIF-1 with IC50 values ranging from 1 to 10 nM in cell- and reporter-based assays [29, 30]. Importantly, Manassantin A inhibits HIF-1 activity by blocking hypoxia-induced nuclear HIF-1α accumulation without altering HIF-1α mRNA level. As a derivative, the potency of LXY6006 is 10 times higher than Manassantin A and LXY6006 may serve as an ideal lead for further drug development. Our results support that HIF1α is a plausible target for pituitary adenomas. Thus, the combination of HIF1α inhbitors such as LXY6006, surgery, radiation and chemotherapy might significantly improve the prognosis of pituitary adenomas.

In summary, we find that HIF1α interacts with GR to prevent its nuclear translocation and results in UPS-mediated degradation of GR in ACTH pituitary tumor. HIF1α inhibitor shows therapeutic effects in a in-vivo mouse model of Cushing's disease.

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Disclosure of conflict of interest

None.

Address correspondence to: Liuhua Hu, Department of Cardiology, Renji Hospital, Shanghai Jiaotong University School of Medicine, No. 160 Pujian Road, Shanghai 200127, China. E-mail: iam565@126.com; Ruoping Chen, Department of Pediatric Neurological Disease Centre, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, No. 1665 Kongjiang Road, Shanghai 200092, China. E-mail: rubinchen@126.com

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