

Glucocorticoids promote Von Hippel Lindau (pVHL) degradation and Hif-1 α stabilization

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Abstract: Glucocorticoid (GC) and hypoxic transcriptional responses play a central role in tissue homeostasis and regulate the cellular response to stress and inflammation, highlighting the potential for crosstalk between these two signaling pathways. We present results from an unbiased *in vivo* chemical screen in zebrafish that identifies GCs as novel activators of hypoxia inducible factors (HIFs) in the liver. GCs activated consensus hypoxia response element (HRE) reporters in a glucocorticoid receptor (GR)-dependent manner. Importantly, GCs activated HIF transcriptional responses in a zebrafish mutant line harboring a point mutation in the GR DNA binding domain, suggesting a non-transcriptional route for GR to activate HIF signaling. We noted that GCs increase the transcription of several key regulators of glucose metabolism that contain HREs suggesting a role for GC/HIF crosstalk in regulating glucose homeostasis. Importantly, we show that GCs stabilize HIF protein in intact human liver tissue and isolated hepatocytes. We find that GCs limit the expression of Von Hippel Lindau (pVHL), a negative regulator of HIF and treatment with the c-src inhibitor PP2 rescued this effect, suggesting a role for GCs to promote c-src mediated proteosomal degradation of pVHL. Our data support a model for GCs to stabilize HIF through activation of c-src and subsequent destabilization of pVHL.

Hypoxia Inducible Factor | Glucocorticoid signaling | Metabolism | Liver | Von Hippel Lindau

Introduction:

Glucocorticoids (GCs) are steroid hormones secreted from the adrenal glands that regulate carbohydrate, lipid and protein metabolism. GCs are widely used as anti-inflammatory agents for treating pathological conditions where hypoxia plays a role in disease progression such as rheumatoid arthritis and chronic obstructive pulmonary disease. GCs and hypoxia pathways have a close interplay in physiology and disease (1-3) however, recent studies report conflicting results on the cross-talk between GC action and hypoxia (4, 5). Hypoxia Inducible Factors (HIFs) are oxygen-sensitive transcriptional complexes constituted by α and β subunits, that activate diverse pathways regulating cellular glucose and lipid metabolism and proliferation (6, 7). Under normoxic conditions, the HIF-1 α transcriptional subunit is recognized by prolyl hydroxylases and targeted for degradation via the VHL-mediated ubiquitin proteasome pathway; however, under hypoxic conditions HIF-1 α is stabilized and translocates to the nucleus to exert its transcriptional activity. HIFs play a central role in many disease processes and provide a therapeutic target for treating pathological conditions including: cancer, ischemia, stroke, inflammation and chronic anemia (8-11). Screens to identify agents that stabilize HIFs have identified numerous agents, with the majority acting either via iron chelation or as 2-oxoglutarate analogs (12). *In vitro* HIF-reporter screening methods, although extremely valuable, do not provide physiological information and may overlook tissue-specific activators that require a physiological context.

To identify new regulators of the HIF pathway we developed several HIF-reporter zebrafish lines (13) and completed an unbiased chemical screen. GCs activated HIF-associated transcriptional responses most prominently in the liver. Importantly, we translate these observations to human tissue and show that GCs stabilize HIF in primary human hepatocytes and intact liver slices. Our data support a model where GCs act via a transcriptional independent mechanism, by activating c-src, to repress Von Hippel Lindau (pVHL) expression and stabilize HIF protein under normoxic conditions. Our study identifies a new role for GCs to stabilize HIF and to regulate liver glucose metabolism.

Results:

GCs activate hypoxic-signaling. Prolyl hydroxylase 3 (PHD3) transcription is regulated by HIFs and we used our zebrafish *phd3:eGFP* HIF-reporter line to screen a chemical library (Suppl. Table 1) for HIF-activators. Dimethylxaloylglycine (DMOG), a well-established activator of HIF signaling was used as a positive control (14). Of the 41 initial hits, several GCs activated the reporter to comparable levels as DMOG (60 μ M) (Fig.1a). We confirmed that two GR agonists, betamethasone 17,21-dipropionate (BME) and dexamethasone acetate (DEX), activated the reporter (Fig.1B-C). We noted increased *phd3*-GFP expression mainly in the liver (Fig.1B), suggesting that activation may be due to non-specific stress as result of drug metabolism. To address this potential issue, reporter embryos were treated

Significance

Significance: An *in vivo* chemical screen in zebrafish identified glucocorticoids (GCs) as activators of hypoxia inducible factor transcriptional responses in the liver. This cross-talk is conserved in human liver and requires glucocorticoid receptor signaling but not DNA binding. In human liver cells, GCs down regulate pVHL expression at a post-transcriptional level most likely through c-src mediated proteasomal degradation. Since the liver is an important regulator of blood glucose and HIFs regulate gluconeogenesis/glycogen synthesis, cross-talk between these transcriptional regulators may be essential to control glucose metabolism in the liver. This newly identified, conserved, non-canonical pathway may have wider physiological significance in health and disease.

Reserved for Publication Footnotes

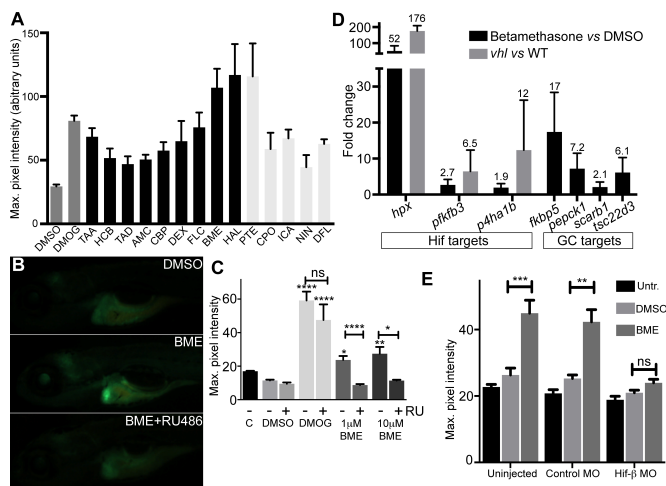


Fig. 1. Identification of Glucocorticoids as HIF activators. (A) Re-test of hits from the Spectrum screen for activators of the HIF response in *phd3:eGFP* zebrafish, where compounds showing an average GFP level of >44 are shown. Averaged max pixel intensity and SEM is shown for between 5-9 embryos. Dark grey: controls, black bars: GC-like compounds, light grey: non-GC hits. TAA:triamcinolone acetonide, HCB:hydrocortisone butyrate, TAD:triamcinolone diacetate, AMC:amcinonide, CBP:clobetasol propionate, DEX:dexamethasone, FLC:fluocinonide, BME:betamethasone, HAL:halcinonide, PTE:ptaeroxilin, CPO:ciclopilox olamine, ICA:icariin, NIN:7-nitroindazole, DFL:8,2-dimethoxyflavone. (B) BME activates *phd3:eGFP* in an RU-486 dependent manner, representative images showing the activation specific to the liver in BME treated embryos, and reduced GFP expression following BME+RU-486 co-treatment. (C) Quantitation of *phd3:eGFP* response. DMSO was added to 1%v/v in all experiments, except Control (c), DMOG to 60μM and RU-486 to 5μM as indicated (n>=9). (D) BME induction of hypoxia regulated genes and GC target genes, where values above bars indicate average fold change (FC) and triplicate samples of 20 embryos were used. For the hypoxia-induced genes FC induction in *vhl* mutants vs wild-type is given as a comparison, where FC is calculated using $\Delta\Delta C_T \pm SEM$. (E) HIF1β-MO abrogates BME activation of *phd3:eGFP*. Max pixel intensity in embryos as grouped by injection showing mean \pm SEM (n=14). Significance was calculated using Kruskal-Wallis One-Way ANOVA followed by Dunns multiple comparison test for treatments against DMSO (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

with GCs in the presence or absence of GR antagonist RU-486 that reduced GFP intensity of BME treated embryos (Fig.1B-C), whilst having minimal effect on DMOG-dependent activation (Fig.1C), demonstrating that GC-augmented *phd3:eGFP* activity is GR-dependent.

To characterize the effects of BME on endogenous hypoxia-associated transcriptional responses, we quantified selected HIF-1 (*pfkfb3*, *p4ha1b* and *hpx*) and GR-responsive genes (*tsr22d3*, *scarb1*, *fbp5*, and *pepck1*) (15-17) by qPCR. *phd3:eGFP* embryos (5dpf) were treated with BME (10μM) for 8h prior to isolating RNA, the optimal time for *phd3*-induction. (Suppl. Data 1). BME induced the transcription of HIF-target genes to variable extents, albeit less than in a *vhl* mutant background, with *hpx* being the most responsive gene (Fig.1D). To determine whether BME activation of *phd3:eGFP* reporter is HIF dependent, we silenced Hif-1β expression, an essential binding partner of both HIF-1α and HIF-2α (18). Hif-1β morphants were incubated with BME from 56hpf until 72hpf and HIF-1β knockdown significantly reduced GFP intensity in the treated embryos compared to control morpholino (MO) injected embryos (Fig.1E), demonstrating that GC activation of *phd3:eGFP* is HIF-dependent.

To confirm that GCs directly activate HIF-transcriptional activity we created a HIF reporter zebrafish expressing tandem copies of a hypoxia response element (HRE) driving eGFP expression, named *Tg(4xhre-tata:eGFP)^{ia21}*. Reporter activity is decreased in Hif-1β morphants and increased by expression of

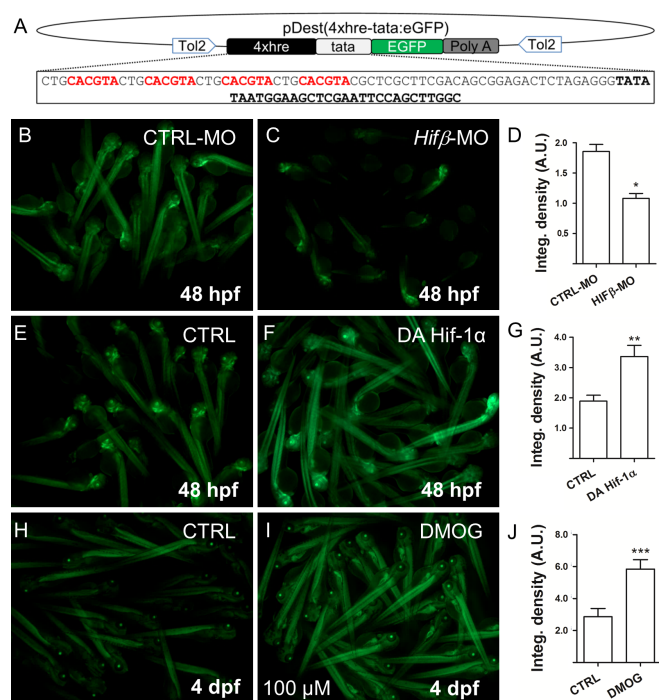


Fig. 2. Generation of 4xhre-tata Hif-reporter lines.(A) Schematic representation of the Tol-2 vector used to generate the *Tg(4xhre-tata:eGFP)^{ia21}* line. The construct consists of a 98 bp fragment encoding 4 HREs (in red) from the murine lactate dehydrogenase followed by a TATA minimal promoter (in bold), eGFP(in green) and the polyA signal. (B,C,D) Representative image of *Tg(4xhre-tata:eGFP)^{ia21}* embryos at 48 hpf injected with a control MO (B) or the HIFβ-MO MO (C). Downregulation of *Hifβ* significantly decreases transgene activity as reported by the integrated density analysis of fluorescence (D). (E,F,G) Hif-1α dominant active (DA) mRNAs injected in *Tg(4xhre-tata:eGFP)^{ia21}* embryos increase transgene expression (F) compared to control-embryos (E). (H,I,J) Treatment from 72 hpf to 96 hpf with 100 μM DMOG significantly increases 4xhre-tata transgene activity (I) with respect to the control embryos (H). (D,G,J) Average values of fluorescence integrated density calculated for treated embryos and controls. Values represent the mean \pm SEM (*** P< 0.001; ** P< 0.01, *P<0.05). (A.U.) arbitrary units.

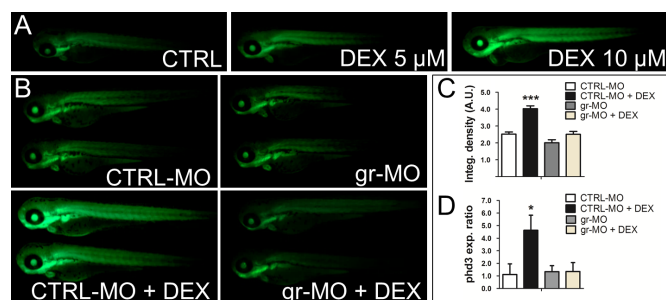


Fig. 3. Cross-talk between the HIF-1 and Glucocorticoid signaling pathways is mediated by the Glucocorticoid Receptor and HREs. (A) 72 hpf *Tg(4xhre-tata:eGFP)^{ia21}* embryos were incubated with different concentrations of DEX. DEX activates the 4xhre-tata transgene. (B) Images of 72 hpf *Tg(4xhre-tata:eGFP)^{ia21}* larvae injected with a glucocorticoid receptor MO (gr-MO) and a control MO (CTRL-MO) alone or combined with DEX (bottom panels). DEX-activation of 4xhre-tata transgene expression is maintained in CTRL-MO injected larvae and ablated in GR-morphants. (C) Histograms showing the average values (\pm SEM) of the fluorescence integrated density in 72hpf *Tg(4xhre-tata:eGFP)^{ia21}* embryos injected with gr-MO and CTRL-MO with or without DEX.A.U. Arbitrary units. (D) Fold changes (\pm SEM) in *phd3* gene expression in 72 hpf embryos injected with gr-MO and CTRL-MO with or without DEX (24 h) compared to non-treated/non-injected controls (set at 1). Target gene mRNA levels were normalized to β -actin. *p<0.05; ***p<0.001.

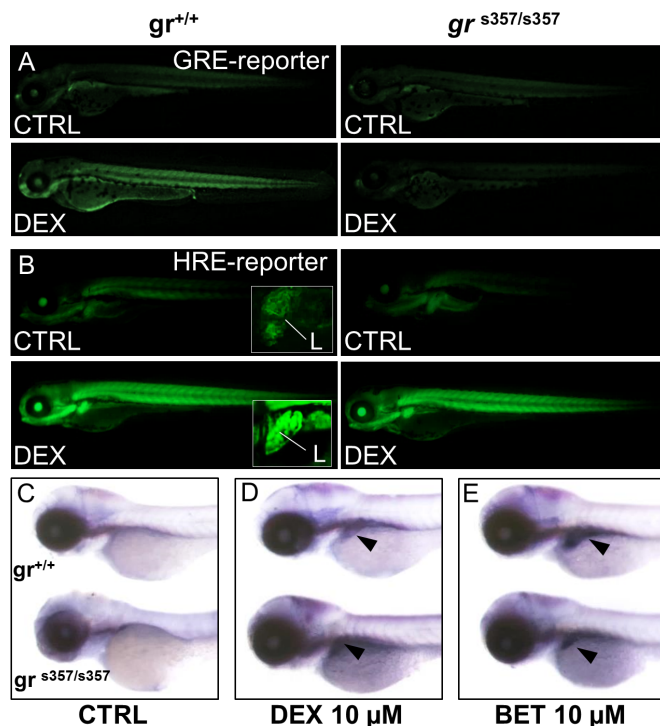


Fig. 4. Glucocorticoid induction of HIF-1 activity is independent of Glucocorticoid Receptor DNA binding. (A) Treatment with 10μM DEX for 24hrs activates wild-type *Tg(9xGRE-HSV:U123:eGFP)^{ia20}* (47) glucocorticoid reporter (GRE-reporter) line, while in *gr^{s357}* mutant background (right panels) DEX is unable to activate GRE reporter. (B) Images of wild type and *gr^{s357}* embryos in *Tg(4xhre-tata:eGFP)^{ia21}* background (HRE-reporter), treated for 24hrs with DEX 10μM. DEX activates the 4xhre-tata transgene in both wild-type and *gr^{s357}* mutant larvae and induces a significant and generalised increase of fluorescence that is particularly evident in the liver (L in inset squares). (C-E) In situ hybridization of *phd3* mRNA antisense probe in wild type and *gr^{s357}* mutants at 80 hpf. DEX (D) or BME (E) activated *phd3* gene expression in the liver (arrowhead).

a dominant active form of *HIF-1α* mRNA or DMOG treatment; confirming the transgene responds to modulators of the HIF-1 signalling pathway (Fig.2, Suppl. Data 2). To examine the influence of GCs, the offspring obtained from a *Tg(4xhre-tata:eGFP)^{ia21}* carrier were exposed to increasing concentrations of DEX for 24h. Treated embryos show a dose-dependent activation of the *4xhre-tata* transgene, confirming that GCs activate HIF-transcriptional responses under normoxic conditions (Fig.3A, Suppl. Data 3A). To independently assess whether GCs affect HIF-1 signaling and the glycolytic pathway, we analysed the expression of 3 known hypoxia dependent regulators of glucose metabolism in zebrafish (19) using WISH; as expected *pfkfb3*, *ldha* and *glut1*, were upregulated after DEX treatment as consequence of HIF-1 activation (Suppl. data 3B).

GC activation of HIF is independent of GR DNA binding. GCs regulate gene transcription through binding and activating cytoplasmic GR, nuclear translocation and direct binding to GC response elements (GRE) (20). To analyze the mechanism underlying GC/HIF crosstalk, we assessed whether DEX-induced effects on HIF-reporter activity were dependent on GR and its DNA binding activity. Single-cell stage *Tg(4xhre-tata:eGFP)^{ia21}* embryos were microinjected with a splice-blocking MO against full length GR (*gr-MO*) (21). Real time PCR analysis of *fbp5* showed significantly reduced expression to confirm *gr-MO* activity (Suppl. Data 3C). Morphants and control *Tg(4xhre-tata:eGFP)^{ia21}* embryos (48 hpf) were incubated with or without DEX (10 μM) for 24h and reporter activity measured. DEX

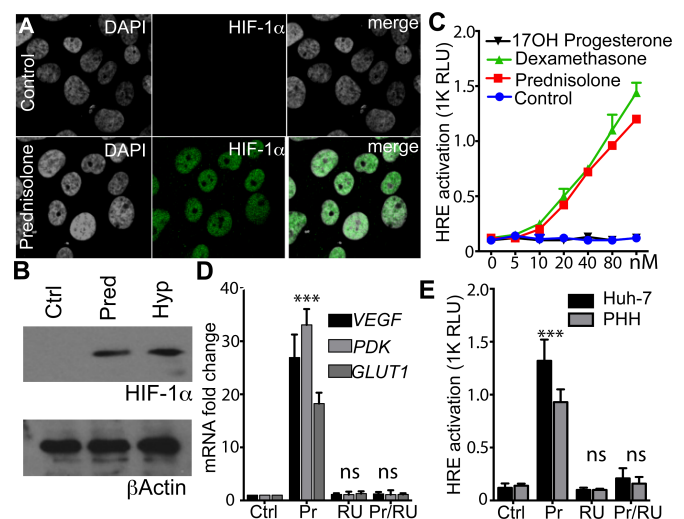


Fig. 5. Glucocorticoids stabilize HIF in human cell lines. (A) Mock or prednisolone (100nM) treated Huh-7 cells were stained for HIF-1α expression (green) and counterstained with DAPI (grey), images show nuclear localization of HIF-1α. (B) HIF-1α expression in hypoxic (3% O₂) and prednisolone (Pred) treated Huh-7 cells. (C) Huh-7 cells were transfected with a HRE luciferase reporter and 24hrs later treated with increasing doses of various steroids (100nM) for 16hrs. GC agonists activated the HRE reporter whilst 17OH did not. (D) Huh-7 cells treated with Pred/RU-486 (100nM) for 24hrs, lysed and RNA purified to quantify HIF-transcriptional targets. Data represents fold change over control sample (see also Suppl. Table 2). (E) Huh-7 cells and primary human hepatocytes (PHH) were transfected with a HRE luciferase reporter and 24hrs later treated with Pred (100nM) and/or RU-486 (100nM) for 16hrs and HRE luciferase activity measured. ***p<0.001; ns not significant (ANOVA). Data is represented as mean ±S.D.

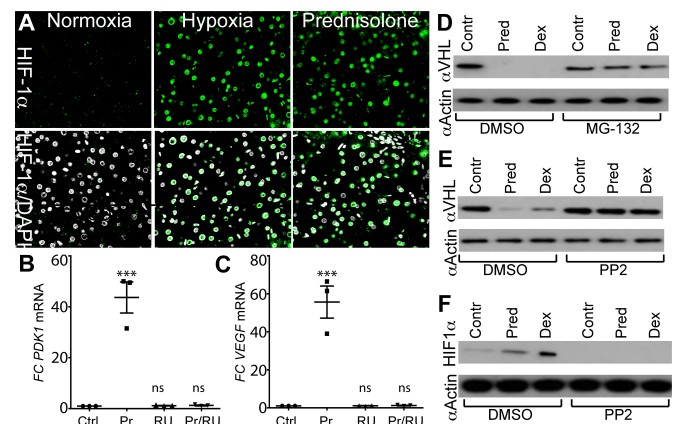


Fig. 6. Glucocorticoids stabilize HIF in human liver slices. (A) HIF-1α expression in human liver slices treated with prednisolone or cultured under low oxygen (3%) for 24hrs and counterstained with DAPI. Both prednisolone and hypoxic treatments induce nuclear HIF-1α expression. Images are representative of 5 donor livers. (B) and (C) HIF-target genes *PDK1* and *VEGF* mRNA levels in human liver slices from 3 donors treated with Pred/RU-486 for 24hrs. (D) VHL expression in Huh-7 cells treated with prednisolone (Pred) or dexamethasone (Dex) in presence or absence of proteasome inhibitor MG-132 at 10μM. (E-F) Treating Huh-7 cells with prednisolone (Pred) or dexamethasone (Dex) in presence or absence of Src inhibitor PP2 at 5μM and probed for VHL (E) or HIF-1α (F). All samples were stained for Actin as a loading control. ***p<0.001; ns not significant (ANOVA) Data is represented as mean ±S.D..

increased *4xhre-tata* activity in controls but had minimal effect on the *gr* morphants (Fig.3B-C). Moreover, HIF activation was independently confirmed by measuring endogenous *phd3* mRNA expression in 72 hpf *gr* morphants and control embryos (Fig.3D).

Finally, GR-dependency of HIF-transcriptional activation was observed in a CRISPR-induced null mutant of GR gene (Suppl. Data 3D).

We also studied the zebrafish *gr^{s357}* mutant (22) that lacks DNA binding function and abrogates GR transcriptional activity (Fig.4A). Interestingly, DEX activated the *Tg(4xhrtata:eGFP)^{ia21}* reporter in a *gr^{s357}* mutant background (Fig.4B), suggesting that a non-transcriptional mechanism underlies GC activation of HIF signaling. To independently assess the role of the GR DNA binding domain in GC/HIF cross-talk, offspring from two *gr^{s357}* homozygous carriers (72 hpf) were incubated with DEX or BME and *phd3* mRNA levels analyzed by WISH (Fig.4C-E). DEX and BME activated *phd3* transcription in both wild type and *gr^{s357}* mutant embryos confirming that HIF-transcriptional activity was preserved.

GCs activate HIF-signalling in human hepatocytes. To verify whether the GC/HIF crosstalk observed in zebrafish is relevant to man, we focused our efforts on human liver-derived cells. Culturing hepatocyte-derived Huh-7 cells under low oxygen stabilized HIF-1 α at comparable levels to prednisolone treatment (Fig.5A-B). To assess transcriptional responses Huh-7 cells were transfected with a *hre-luciferase* (*hre-luc*) reporter and treated with prednisolone and dexamethasone that activated the reporter (Fig.5C). Furthermore, prednisolone induced mRNA levels of three endogenous HIF-target genes (*VEGF*, *PDK*, and *GLUT1*) to a similar degree as low oxygen treatment, thus paralleling the *in vivo* results observed in zebrafish embryos (Fig.5D). Furthermore, GC activation of HRE-activity was sensitive to the GR antagonist RU-486 (Fig.5D), demonstrating GR-dependency. Next we screened a panel of hypoxia-regulated target genes in Huh-7 cells and found that 29/38 HIF-target genes were induced by prednisolone, whereas only 3/38 were induced by cortisone (Suppl. Table 2).

To confirm that the response is not simply a result of using immortalized Huh-7 cells we showed that prednisolone activated the HRE reporter in primary human hepatocytes (Fig.5E). To ascertain whether GCs increase HIF expression and associated transcriptional activity in an authentic liver microenvironment, we treated human liver slices with prednisolone or cultured under low oxygen (3%) for 16h and imaged nuclear HIF-1 α (Fig.6A) and quantified HIF-target gene mRNA levels (Fig.6B-C). Both prednisolone and low oxygen treatments stabilized HIF-1 α (Fig.6A) and increased HIF-target gene expression (Fig.6B-C). Together, these data demonstrate that, as in the zebrafish, GCs can activate HIF-signaling in the human liver.

To determine how GCs activate HIF signaling, we investigated the effect of GCs on pVHL expression, a crucial negative regulator of HIF α protein. We showed that GCs reduce pVHL expression in Huh-7 cells (Fig.6D). This is unlikely to be mediated by a transcriptional mechanism, since VHL mRNA levels were not affected (Suppl. Data 4A). pVHL is degraded by the proteasome (Fig.6D) following post-translational modification by kinases such as c-src and CKII (23). Indeed, we found that inhibiting c-src activity with PP2 (24) rescued the inhibitory effects of GC on VHL expression (Fig.6E), blocked HIF-1 α induction (Fig.6F) and normalized HIF target gene expression (Suppl. Data 4B), suggesting a mechanism for GCs to stabilize HIFs via degrading VHL.

Discussion: The Spectrum chemical library includes compounds that modulate a wide variety of pathways, however, only GCs showed a consistent and robust HIF activation. It is surprising that GCs were not previously identified as HIF-modifiers in cell-based chemical screens. We can only speculate that the apparent liver specificity of the GC effect may have contributed. Thus, *in vivo* chemical screens, providing a variety of cell types in a physiological context, are a useful expansion when compared

to more classical screening approaches, even with “well-trodden” pathways like HIF.

We confirmed that GCs promotes HRE dependent HIF-transcriptional activity in fish and human liver cells. Surprisingly, fish expressing a GR mutant lacking a functional DBD still activated the *phd3* promoter and 4xhre reporter models after treating with DEX. However, complete knockdown of GR and a newly generated truncated GR mutant abrogated the ability of DEX to stabilize HIF. The GR is essential for survival as demonstrated by the report that 90% of GR KO mice die soon after birth (25) and only 10% of GR KO zebrafish survive, with a reduced fitness. Of note, both mouse and zebrafish homozygous mutants of the GR-DBD survive with minor defects (22, 26) suggesting essential roles for GRE-independent GR activities, as exemplified by our study.

Since GCs are reported to activate c-src kinase (27, 28), which is known to phosphorylate and target pVHL for proteosomal degradation (23), we hypothesized a role for GCs to stabilize HIF via c-src-degradation of VHL. Consistent with our results, RU-486 inhibited such non-genomic effects of GR (29). Our *in vitro* studies confirm that GCs destabilize VHL protein expression and this was rescued by co-treating cells with the c-src inhibitor PP2 (Fig.6E). As VHL is a negative regulator of HIF, this results in HIF expression under normoxic conditions.

Although we see activation of *phd3* and 4xhre promoters and other hypoxia response genes by GCs, GCs do not fully replicate a hypoxic response. We find increased expression of many HIF-target targets (Suppl. Table 2), but some exceptions were noted such as Enolase1 and Carbonic anhydrase 9, that were downregulated by prednisolone. A direct interaction between ligand activated GR and HIF proteins cannot be excluded, as Kodama et al (5) have suggested that GR binds the HIF dimer. However, a simpler explanation, is that in addition to stabilising HIF protein, GR binds a subset of promoters to regulate transcription. A complex interplay is suggested by other reports: for example, while HIF upregulates VEGF expression and promotes angiogenesis, GCs are generally angiostatic (30) and negatively regulate VEGF expression (31-33). Similarly, during high altitude sickness, GC and HIF may have apparently opposing effects (34, 35). Therefore, we propose that the interplay we have identified is likely to be tissue and possibly context-specific.

We found that GC agonists increased mRNA levels of the classical HIF target *erythropoietin* both in cell-culture (Suppl. Table 2) and zebrafish embryos and adult zebrafish livers (Suppl. Data 5). Our data is consistent with reports showing a synergistic effect of GCs and HIFs in haematopoiesis (36, 37) and it will be interesting to investigate whether this cooperativity is GR-DBD dependent. GCs received their name because they promote blood glucose levels as well as gluconeogenesis and glycogen storage in the liver and provide an acute response to stress (38). HIF signaling has a profound impact on cellular metabolism, and induces gluconeogenesis and glycogen storage (39, 40). We suggest that GCs modulation of VHL and HIF may contribute to their ability to modulate blood glucose and glycogen storage. In addition, HIF has been shown to impact lipid metabolism in the liver, where its (in)appropriate activation reduces beta oxidation and increases lipid storage capacity leading to steatosis (41, 42). It's worth noting that long term treatment with GCs was reported to increase steatosis (43). Activation of the HIF pathway via crosstalk with the GR may explain how GC excess regulates hepatic fat metabolism and the steatotic phenotype. Our experiments show that GCs predominantly stabilize HIF in the liver, and may only activate a subset of HIF targets, that could be addressed by studying tissue-specific knockout models. Based on the elegant experiments of Rankin et al. (42), we would predict that liver specific deletion of HIF-2 α should protect mice from GC induced steatosis and may reduce the effect of GCs on blood glucose levels.

It is becoming clear that HIF signaling is not only regulated by low oxygen, but by several other inflammatory mediators (44-46). Our data support GC as a further key modulator of HIF signaling to add to this growing list. It is interesting to note that many common GC and HIF gene targets are implicated in regulating hepatic metabolism. This is potentially important, as GCs are secreted in a rhythmic circadian fashion, where circulating GC levels reach a maximum to coincide with the onset of the active phase – morning in humans, evening in mice. Since metabolic processes in the liver are coupled to the circadian clock, our data provide an additional level of control, where GC activation of the HIF pathway helps to ensure that the changing metabolic demands throughout the day are met. Our data suggest a role for GC-driven circadian components in other aspects of HIF signaling.

Material and methods:

Zebrafish Strains: *vhl* mutant and *Tg(phd3:eGFP)^{i144/i144}* (13) fish were maintained in a mixed TL/LWT background. The *Tg(9xGCRES-HSVUI23:eGFP)^{ia20}* line is a GC pathway reporter (47). The zebrafish GR mutant line *gr^{s357}* was incrossed to create homozygotes (22). To obtain wild-type embryos, LWTs were incrossed. To obtain *vhl* mutants *vhl^{hu2117/+}; phd3:eGFP^{i144/i144}*, were incrossed. Embryos were incubated at 28°C in E3 (5mM NaCl, 0.17mM KCl, 0.33mM CaCl₂, 0.33mM MgCl₂, pH7.2) containing methylene blue (Sigma-Aldrich) at 0.0001 %. The generation of *Tg(4xhre-tata:eGFP)^{ia21}* and *Tg(4xhre-tata:mCherry, cmc2:eGFP)^{ia22}* reporters, and GR CRISPR mutation is described in Suppl. Data 6

Drug treatment of embryos: The Spectrum Collection (Microsource Discovery Systems) of 2000 compounds was used, tested compounds and detailed methods can be found in Suppl. Data 6 and Suppl. Table 1.

Hypoxic target gene activation in zebrafish: Three biological replicates of 10 *phd3:EGFP^{i144/i144}* embryos were treated for 8, 12, 24, 36 and 48h starting from 72 hpf. Embryos were treated with 10µM Betamethasone 17,21 dipropionate and 60µM DMOG in 1% DMSO, with untreated and 1% DMSO controls. *vhl^{hu2117/hu2117}; phd3:EGFP^{i144/i144}* mutants were used as positive controls. Biological replicates of 48 hpf, *Tg(4xhre-tata:eGFP)^{ia21}* embryos were incubated with or without 10 µM Dexamethasone (DEX) and/or 3.8 µM 17-AAG (Sigma) for 24h. RNA was isolated using Trizol and quantified using the Nanodrop ND-1000 spectrophotometer. cDNA was reverse transcribed from 0.5 µg RNA using the Superscript III First-Strand Synthesis System (Invitrogen). For the temporal *phd3* expression profile qPCR was performed with optimized primers, using the iCycler iQ system (BioRad). Cycling conditions: 95°C-3min, [95°C-15sec, 60°C-30sec]x40 cycles, 55-95°C in 0.5°C increments 30sec, with β-actin2 as internal control. Primers: see Suppl. Table 3. For hypoxia/glucocorticoid target gene detection qPCR was carried out, using the Applied Biosystems SDS Software v2.4.1 in conjunction with 7900HT Fast Real-Time qPCR System. The cycling conditions: 50°C-2min, 95°C-10min, [95°C-15sec, 60°C-1min]x40 cycles. Primers: see Suppl. Table 3. Cycle threshold (Ct) values were calculated automatically using the software, with ROX reference dye as the passive reference. Fold change was calculated using the ΔΔCT method.

Morpholino and mRNA injections: The translation-blocking morpholino (MO) HIFβ-MO (GGATTAGCTGATGTCATGT-CCGACA) was used as reported (18) while a splice-site targeting MO (gr-MO) was used to silence GR (21). A standard control MO (CTRL-MO, Genetools) was used as a negative control. The MO stock solution (8 mg/ml) was diluted in Danieau's solution, ~2 nl was injected per embryo as previously described (48). Dominant-active *hif-1aa* and *hif-1ab* (kindly provided by P. Elks

Sheffield, U.K.) were synthesized (mMessageMachine; Ambion, Invitrogen) and injected as described in (49).

Image analysis in *Tg(4xhre-tata:eGFP)^{ia21}* embryos: For analysis of *Tg(4xhre-tata:eGFP)^{ia21}* embryos, fluorescence was collected using a Leica M165FC dissecting microscope and a Nikon C2 H600L confocal microscope. PTU-treated larvae were anaesthetised with tricaine and embedded in 1% low melting agarose on a glass slide. Images were analysed with Nikon software. For fluorescence quantification of transgenic embryos, a Leica M165FC microscope and DC500 digital camera were used. All images were acquired using identical parameters and fluorescence-integrated density calculated using ImageJ.

Cell culture, antibodies and treatments: Huh-7 and HEK293 cells were maintained in Dulbecco's Modified Eagle's medium supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamine and 50 units/mL penicillin/streptomycin (Life Technologies, USA). Primary human hepatocytes were isolated as described (50) and maintained in Williams E Medium (Sigma, UK) supplemented with 10% FBS, 5mM HEPES/insulin/L-glutamine (Life Technologies, USA). Cells were grown in a humidified incubator at 37°C, 5% CO₂ and 20% O₂ (normoxia). For hypoxic conditions cells were grown at 37°C in a humidified sealed Galaxy 48R incubator (New Brunswick) at 37°C, 5% CO₂, 92% N₂ and 3% O₂ (hypoxia). Antibodies used: Anti-mouse HIF-1α (Novus Biologicals), Anti-VHL (Cell Signalling #2738), Alexa Fluor goat anti-mouse 488 (Molecular Probes, USA), anti-β-Actin antibody (Sigma Aldrich), Anti-mouse and anti-rabbit HRP-conjugated secondary antibody (GE Healthcare, UK). Prednisolone, Dexamethasone, Cortisone, RU-486 17OH Progesterone, and MG-132 are from Sigma Aldrich (Europe). Src inhibitor PP2 was purchased from Merk Millipore (Europe).

Ex vivo Liver Slices. Liver tissue samples were collected with local NHS research ethics committee approval (Walsall LREC 04/Q2708/40) and with written informed consent. Donor liver tissue surplus to transplantation requirements were collected from the Queen Elizabeth Hospital, Birmingham, UK. Cores were cut from the tissue immediately upon receipt using a Krumdieck Tissue Slicer (Alabama R&D, USA) (51). Briefly, the core was placed into the slicer assembly under aseptic conditions and 240µm sections collected and immediately transferred into Williams E media/1% L-glutamine/ 0.5µM Insulin. An albumin ELISA (Bethyl Laboratories) was used to monitor the viability of the slices. Samples from 5 donors were serum starved followed by incubation under hypoxia (3% O₂) or normoxia (20% O₂) for 24h. Liver slices were treated with prednisolone (100nM) for 24h before fixing for detection of HIF-1α by confocal microscopy.

Confocal microscopy on cells: Cells were grown on 13mm borosilicate glass coverslips at a density of 4x10⁴/well for 24h and serum starved for 5h before treating with prednisolone (100nM). Cells or liver slices were fixed in 3% paraformaldehyde for 25 mins at room temperature (RT), permeabilised with 0.01% TX-100/PBS for 10 mins. Cells were incubated with anti-HIF-1α for 1h at RT, unbound antibody was removed by washing and bound antibody detected with Alexa-488 secondary antibody. Nuclei were counter-stained with 4',6-Diamidino-2-Phenylindole, Dihydrochlorine (DAPI, Life Technologies). Cells were imaged using a Zeiss Meta Head confocal microscope with a 63x water immersion objective.

HRE reporter assay. Cells were transfected with the HRE reporter (a plasmid containing 4 tandem HRE copies; M. Ashcroft, Cambridge University, UK) using Eugene 6 transfection reagent (Promega, USA) as per the manufacturer's guidelines. After 24h, cells were serum starved for 5h, before treating with GC or RU-486, (100nM) for a further 24h. Cells were lysed and reporter activity quantified using a firefly luciferase assay system (Promega, USA) in a luminometer (Berthold Lumat LB 9507).

Western blotting. Cells were harvested in lysis buffer (PBS/1% Triton-X100/0.1% Na deoxycholate/0.1% SDS) containing protease and phosphatase inhibitors (Roche, UK). Lysates were clarified by centrifugation and protein concentration determined using a Bradford Protein Assay Reagent (Pierce, USA). Protein lysates (20 µg) were added to sample running buffer (30% glycerol/6% SDS/0.02% Bromophenol blue/10% 2-β-mercaptoethanol/0.2M Tris-HCl; pH 6.8) and separated by SDS-PAGE. Separated proteins were transferred to PVDF membranes and incubated with primary antibodies, before detection with HRP-conjugated secondary antibody and enhanced chemiluminescence (Geneflow, UK) using a Pxi imaging system (SynGene, USA).

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