EUROPEAN JOURNAL OF CANCER 45 (2009) 2882–2892

available at www.sciencedirect.com

journal homepage: www.ejconline.com

Drug-metabolising enzymes are down-regulated by hypoxia in differentiated human hepatoma HepaRG cells: HIF-1α involvement in *CYP3A4* repression

Claire Legendre[a,b,c], *Tamaki Hori*[d], *Pascal Loyer*[a,b,c], *Caroline Aninat*[a,b,c], *Seiichi Ishida*[d], *Denise Glaise*[a,b,c], *Catherine Lucas-Clerc*[e], *Karim Boudjema*[a,b,c,f], *Christiane Guguen-Guillouzo*[a,b,c], *Anne Corlu*[a,b,c,*], *Fabrice Morel*[a,b,c,*]

[a]*INSERM U522, Hôpital Pontchaillou, Rennes F-35033, France*
[b]*EA-MDC, Université de Rennes 1, Faculté de Médecine, Rennes F-35043, France*
[c]*IFR 140, Rennes F-35043, France*
[d]*National Institute of Health Science, Division of Pharmacology, Biological Safety Research Center, Setagaya-ku, Tokyo 158-8501, Japan*
[e]*Laboratoire de Biochimie et d'Enzymologie, Hôpital Pontchaillou, Rennes F-35033, France*
[f]*Département de Chirurgie Hépatobiliaire et Digestive, Hôpital Pontchaillou, Rennes F-35033, France*

ARTICLE INFO

Article history:
Received 13 May 2009
Received in revised form 10 July 2009
Accepted 17 July 2009
Available online 18 August 2009

Keywords:
Hypoxia
HIF-1α
Cytochrome P450
CYP3A4
Hepatocellular carcinoma
HepaRG
Chemoresistance

ABSTRACT

Weak blood irrigation within solid tumours including hepatocellular carcinomas (HCCs) plays an important role in resistance to anticancer drugs by decreasing accessibility of cytotoxic agents to tumour cells. Reduced oxygen levels, or hypoxia, also contribute to drug resistance because many anticancer drugs require molecular oxygen to be cytotoxic. Our aim was to develop a new *in vitro* model mimicking hypoxic cells within HCCs in order to further explore the molecular responses to hypoxia, including regulation of drug-metabolising enzymes (DMEs) expression. For this purpose, we used the highly differentiated human hepatoma HepaRG cells cultured under either normoxic or hypoxic (24 h at 1% O_2) conditions. Gene and protein expressions were investigated by quantitative PCR and immunoblotting, respectively. We showed that HepaRG cells adapt to prolonged moderate hypoxia by a switch from aerobic to anaerobic glycolysis and a repression of critical genes involved in amino acid, lipid and ethanol metabolisms. Importantly, expression of several DMEs (particularly cytochromes P450 (CYPs) and phase II enzymes) and xenosensors (CAR, PXR and AhR) was down-regulated and CYPs activities (using testosterone and paclitaxel as substrates) were decreased during hypoxia. In addition, a new role for HIF-1α in the repression of *CYP3A4* is demonstrated in cells treated with chemical inducers of HIF-1α, cobalt chloride or desferrioxamine, and by transfecting untreated HepaRG cells with HIF-1α expression vector. In conclusion, HepaRG cells cultured under hypoxia might mimic metabolic changes occurring within poorly irrigated differentiated HCCs. Furthermore, hypoxia down-regulates hepatic DMEs, a phenomenon that might compromise chemotherapy effectiveness in HCC treatment. Thus, HepaRG cells might represent a new *in vitro* model to test anticancer agents in hypoxic versus normoxic conditions.

* *Corresponding authors:* Address: INSERM U522/EA-MDC, Hôpital Pontchaillou, 35033 Rennes, France (A. Corlu, F. Morel). Tel.: +33 299547403; fax: +33 299540137 (A. Corlu), tel.: +33 299547401; fax: +33 299540137 (F. Morel).
E-mail addresses: anne.corlu@inserm.fr (A. Corlu), fabrice.morel@inserm.fr (F. Morel).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2009.07.010

1. Introduction

Molecular oxygen (O_2) is required for aerobic metabolism to maintain intracellular bioenergetics and to serve as an electron acceptor in many organic and inorganic reactions. Reduced O_2 levels, or hypoxia, occur in a variety of pathological situations, including solid tumours.[1] Many molecular and cellular responses to hypoxia are mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimer consisting of a constitutively expressed subunit (HIF-1β) and an oxygen-regulated subunit (HIF-1α).[2] Under hypoxia, HIF-1 complex binds to hypoxia responsive elements (HRE) within the promoters of several genes involved in cell proliferation, differentiation and survival, cell migration, angiogenesis and energetic metabolism.[3] In most cases, HIF-1 activity is associated with activation of gene transcription. However, studies have also demonstrated a direct role of HIF-1α in gene down-regulation.[4–6]

High levels of HIF-1α in various solid tumours correlate with chemotherapy failure and mortality, while loss of HIF-1α activity has dramatic negative effects on vascularisation and tumour growth in xenografts.[7] Inhibition of either HIF-1α or its target genes might lead to selective killing of tumour cells over normal cells and represents a promising new approach to cancer therapy.[8] In most cases, the lack of efficacy of chemotherapy is a consequence of insufficient vascularisation within solid tumours that restricted delivery of many cytotoxic drugs to hypoxic regions. Hypoxia also contributes to drug resistance because some chemotherapeutic drugs require oxygen to generate free radicals that contribute to toxicity.[9] Moreover, hypoxia might modulate expression of enzymes directly involved in metabolism of chemotherapeutic drugs, thereby limiting the toxic effects of these drugs on cancer cells.

Thus, there is an increasingly need to develop new *in vitro* models to better understand response to hypoxia, including chemoresistance mechanisms, particularly in the case of hepatocellular carcinomas (HCCs) which represent the sixth most common neoplasm in the world, with more than half a million new cases annually. HCCs are inherently resistant tumours and are highly refractory to cytotoxic chemotherapy.[10] HCCs are known to overexpress some multidrug resistance genes such as drug transporters,[11] however the mechanisms of resistance are not fully understood. It is noteworthy that hypoxia plays a major role in HCC tumour biology and consequently in chemoresistance mechanisms. Although human hepatic *in vitro* models have been used in order to better understand the effects of hypoxia[12–16] or to test compounds that target HIF-1α,[17–20] all the previous studies were performed with poorly differentiated hepatoma cell lines (HepG2, Hep3B, HuH7,...) which express low levels, if any, of liver-specific markers and drug-metabolising enzymes (DMEs). Our aim was to investigate the physiological and molecular responses to hypoxia in a highly differentiated human hepatoma cell line. Furthermore, since DMEs and transporters play an important role in chemoresistance, a particular attention has been paid to the effect of hypoxia on their expression. For these reasons, we used HepaRG cells, which have been isolated from an Edmondson grade I differentiated tumour and have the ability to differentiate towards hepatocyte-like and biliary epithelial-like cells at confluence.[21,22] HepaRG cells express many specific hepatic functions, including DMEs and transporters at levels comparable to those measured in primary human hepatocyte cultures,[23–25] making them a unique model system for pharmacological and toxicological studies. Our results demonstrate that hypoxia is not deleterious for HepaRG cells, which adapt to hypoxia by modulating aerobic energetic metabolism. Interestingly, we also demonstrate that hypoxia down-regulates several DMEs, a property which might contribute to drug resistance in HCCs.

2. Materials and methods

2.1. Chemicals

Cobalt chloride ($CoCl_2$), desferrioxamine (DFX), testosterone, 6β-hydroxytestosterone, paclitaxel, dimethyl sulphoxide (DMSO), acetaminophen, *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) and insulin were purchased from Sigma Aldrich (St. Quentin Fallavier, France). Williams' E medium was purchased from Eurobio Laboratories (Paris, France). Foetal calf serum (FCS) was obtained from Perbio Science (France). Penicillin and streptomycin were obtained from Invitrogen (France). Hydrocortisone hemisuccinate was obtained from Upjohn Pharmacia.

2.2. HepaRG cell culture and hypoxia procedure

HepaRG cells[†] were cultured as previously described.[22,23] Briefly, HepaRG cells were seeded at a density of 2.6×10^4 cells/cm^2 in Williams' E medium supplemented with 10% FCS (v/v), 100 units/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL insulin, 2 mM glutamine and 5×10^{-5} M hydrocortisone hemisuccinate and incubated in 5% CO_2 and 95% air at 37 °C. After 2 weeks, the medium was supplemented with 2% DMSO (v/v) for two more weeks. Hypoxic conditions (1% O_2) were created with a three-gas incubator MiniGalaxy A (RS Biotech, Irvine, UK) by injection of N_2 (1% O_2/94% N_2/5% CO_2 atmosphere, 37 °C).

2.3. Transaminases, pO_2, pH and metabolite measurements

pH, pO_2 (oxygen partial pressure), lactate and glucose were measured in culture medium with a blood gas analyser GEM Premier 3000 (Instrumentation laboratory, France). Levels of aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) were quantified in culture medium by using a serum multiple analyser Olympus AU2700.

[†] HepaRG cells can be purchased from Biopredic International (Rennes, France).

2.4. Caspase 3 activity assay

After cell lysis, 50 μg of proteins was incubated with 80 μM Ac-DEVD-AMC in caspase activity buffer (20 mM PIPES pH 7.2, 100 mM NaCl, 1 mM EDTA, 0.1% KCl w/v, 10% sucrose w/v and 10 mM DTT) for 1 h at 37 °C. Caspase-mediated cleavage of Ac-DEVD-AMC was measured by spectrofluorimetry (Septra Max Gemini, Molecular Devices) at the excitation/emission wavelength pair of 380/440 nm.

2.5. Isolation of RNA, reverse transcription and real-time quantitative PCR

Total RNA was extracted from HepaRG cells with the SV total RNA isolation system (Promega, Madison, WI). RNAs were reverse-transcribed into complementary DNA using a High-capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed by using the SYBR Green PCR Master Mix (Applied Biosystems) and the ABI Prism 7000 (Applied Biosystems) as previously described.[23] Supplementary Table 1 shows primer pairs used for each transcript. The relative quantification of the steady-state mRNA levels was calculated after normalisation of the total amount of cDNA tested by an active reference, 18S RNA.

2.6. Western blotting

After cell lysis, 50 μg of proteins was resolved on 7.5% or 12.5% SDS–PAGE, transferred to PVDF membranes (GE-Healthcare, Buckinghamshire, UK) and analysed using chemiluminescence detection as previously described.[26] The following antibodies were used: mouse anti-human HIF-1α (610958, clone 54, BD Transduction Laboratories), rabbit anti-human cytochrome P450 3A4 (CYP3A4) (AB1254, Chemicon) and mouse anti-human Heat Shock Cognate 70 (HSC70) (B-6, sc-7298, Santa Cruz Biotechnology).

2.7. DNA microarray analysis

Transcript profiling was performed by using Affymetrix GeneChip Human Genome HG-U95A containing ~12,600 sequences (representing ~11,500 unique GenBank accession numbers) (Affymetrix). Total RNA was extracted from HepaRG cells with the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) and converted to biotin-labelled cRNA for hybridisation according to the Affymetrix manufacturer's instructions. GeneChips were scanned in a GeneChip 3000 scanner. Ratios were generated automatically by the Microarray Suite Expression Analysis software (MAS version 5.0, Affymetrix) which analyses the relative abundance of each gene from the average difference of fluorescent intensities. We defined up- or down-regulation as ratios greater than twofold between treated and untreated groups. The DNA microarray hybridisation and analysis of each sample were done in duplicate.

2.8. Testosterone and paclitaxel metabolism

HepaRG cells were incubated with testosterone (200 μM) or paclitaxel (20 μM) in phenol red-free medium deprived of both FCS and DMSO during 2 h or 4 h, respectively. 6β-Hydroxylation of testosterone was estimated by high-performance liquid chromatography analysis as previously described.[23] Analysis of paclitaxel 6α-hydroxylation has been performed by Xenoblis® (Saint Grégoire, France). Briefly, 6α-hydroxypaclitaxel concentrations in culture medium were determined using a Waters 942 HPLC system with a Nucleodur C18 column, 125 × 3.0 mm, particle diameter 3 μm. 6α-Hydroxypaclitaxel was detected at 230 nm.

2.9. Transfection and luciferase assay

pGL3-CYP3A4(–1100/+11) was provided by Dr. JM Pascussi (INSERM, Montpellier, France). p(HA)HIF1α and p(HA)HIF1α(401Δ603) encoding a full length HIF-1α and a deleted form of HIF-1α (deletion of the C-terminal region between amino acids 401 and 603), respectively, were provided by Dr. Huang (Harvard Medical School, Boston, USA).[27] HepaRG cells were transiently electroporated using a MP-100 Microporator™ (Labtech, France). Briefly, 1×10^5 HepaRG cells were resuspended in 10 μl of Microporation Buffer (Labtech) with 500 ng of pGL3-reporter and 50 ng of pRL-SV40 vectors for luciferase assay or with 500 ng of HIF-1α expression vectors. HepaRG cells were electroporated at 1500 V for 20 ms and then plated at a density of 2×10^5 cells/cm^2. Firefly (pGL3-vector) and Renilla (pRL-vector) luciferase activities were measured with a luminometer (Centro XS3 LB960, Berthold Technologies) using the Dual-luciferase Reporter Assay System (Promega). For HIF-1α overexpression, 2×10^5 HepaRG cells/cm^2 were plated and cultured for 48 h prior to RNA and protein extractions.

2.10. ChIP assay

Chromatin immunoprecipitation was performed using a ChIP Assay Kit (Upstate Biotechnology, Millipore). Sheared HepaRG genomic *DNA* was subjected to immunoprecipitation with an anti-HIF-1α antibody (NB100-134SS, Novus biological). Rabbit IgG (sc-2027, Santa Cruz biotechnology) was used as a negative control for immunoprecipitation and *DNA* extract prior to immunoprecipitation (Input) as a sheared *DNA* control. Supplementary Table 1 shows primer pairs used for ChIP assay. PCR conditions were 38 (*VEGF*) and 30 (*CYP3A4*) cycles for 94 °C for 30 s; 55 °C for 30 s and 72 °C for 60 s.

2.11. Statistical analysis

The results were expressed as mean ± standard deviation (SD). Statistical analysis was done with Student's *t*-test. The differences with $p \leqslant 0.05$ were considered statistically significant.

3. Results

3.1. Effects of hypoxia on HepaRG cell viability

No obvious changes in cellular morphology were observed after 24 h of hypoxia followed or not by 24 h of reoxygenation compared to normoxic condition (Fig. 1A, panels A–C). By contrast, cellular morphology of HepaRG cells treated with 10 mM acetaminophen during 18 h under normoxic condition, used

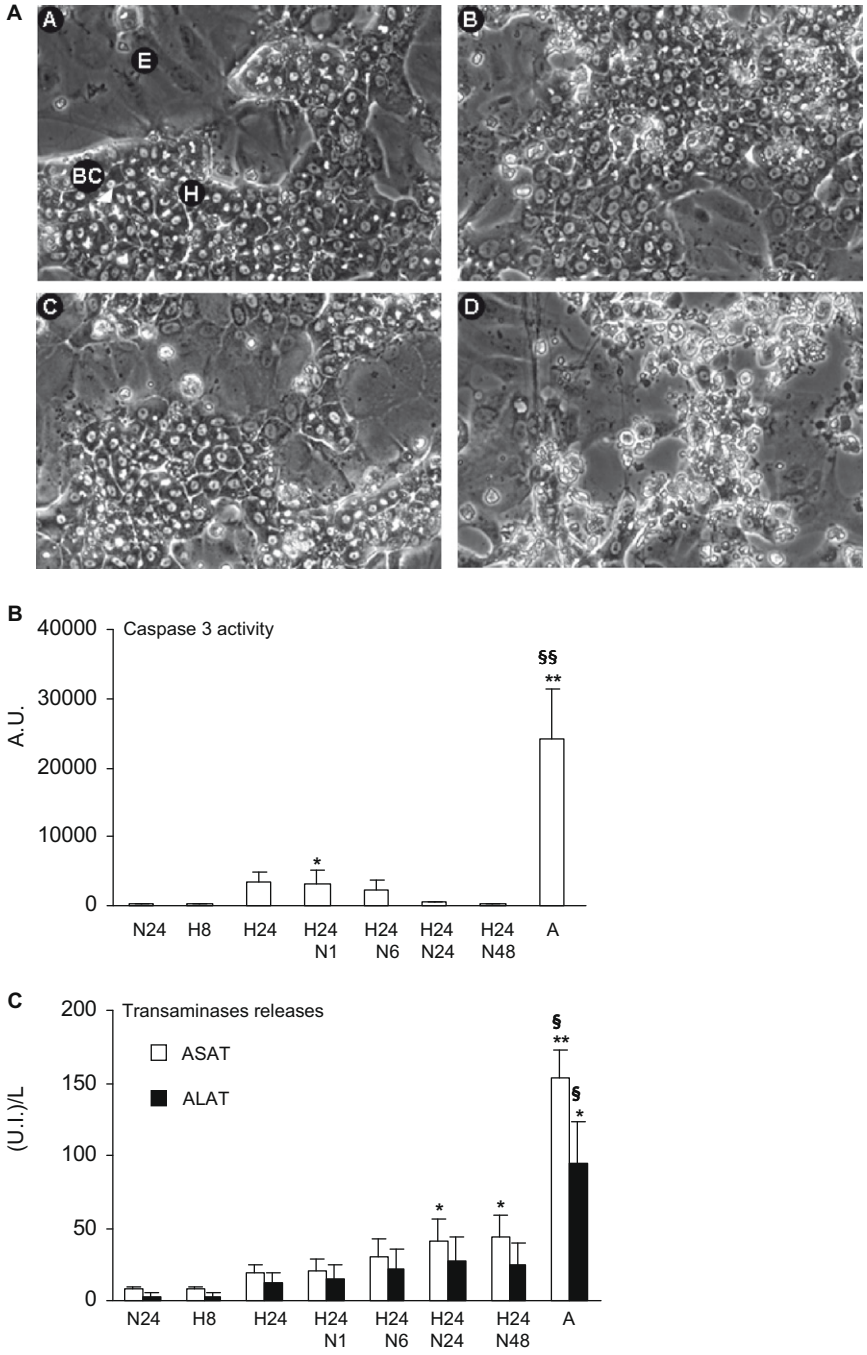

Fig. 1 – Effects of hypoxia on HepaRG cell viability. (A) Phase-contrast microscopy of HepaRG cells in normoxic condition (A), under hypoxia during 24 h (B), under hypoxia during 24 h followed by 24 h of reoxygenation (C) or treated with 10 mM acetaminophen during 18 h under normoxic condition (D). H, hepatocyte-like cells; E, epithelial-like cells; BC, bile canaliculus. Original magnification 40×. (B) Caspase 3 activity in HepaRG cells cultured at the indicated times (hours) of normoxia (N), hypoxia (H), hypoxia followed by different times (hours) of reoxygenation (HN) or treated with 10 mM acetaminophen during 18 h (A). (C) Levels of aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) in the same conditions. Results are expressed as mean ± standard deviation (SD) of three experiments. Student's *t*-test was performed by comparison of hypoxia followed or not by reoxygenation or acetaminophen treatment with normoxic condition (*, $p \leqslant 0.05$; **, $p \leqslant 0.01$); and acetaminophen treatment with hypoxia (§, $p \leqslant 0.05$; §§, $p \leqslant 0.01$).

as positive control for cell death, showed massive cellular damages (Fig. 1A, panel D). Similarly, a slight increase in caspase 3 activity was observed after 24 h of hypoxia, compared to normoxia, but remained much lower than the activity measured in HepaRG cells treated with 10 mM acetaminophen for 18 h (Fig. 1B). Moderate cytotoxicity effect was evidenced by ASAT release in the culture medium of cells reoxygenated after hypoxia. ASAT and ALAT release, however, remained

much lower than that observed after exposure of HepaRG cells to acetaminophen (Fig. 1C).

3.2. Molecular and physiological response of HepaRG cells to hypoxia

When HepaRG cells were cultured under hypoxic conditions, we observed the accumulation of HIF-1α and activation of its downstream target, VEGF-A. The switch back to normoxia caused a rapid degradation of HIF-1α and the decrease in VEGF-A levels (Fig. 2A). The pO_2 value, at 145 mm Hg in culture medium under normoxic condition, dropped to 50 mm Hg during the first hour of hypoxia and then remained stable until return to normoxia (Fig. 2B). During hypoxia, a decrease in pH and glucose concentration in culture medium was concomitant with an increase in lactate production (Fig. 2B). After hypoxia, lactate production decreased while pH and glucose concentration remained low and stable at least until 48 h after reoxygenation.

To better understand the molecular mechanisms that underlie hypoxia effects, we conducted a DNA microarray screen with an Affymetrix HG-U95A GeneChip to identify genes whose expression was modulated in HepaRG cells maintained under hypoxia. Initial analysis with Ingenuity Pathway Analysis software (Ingenuity Systems) revealed some canonical pathways containing groups of down- or up-regulated genes involved in aerobic energetic metabolic pathways. These gene profiling analyses were validated using qPCR on a set of three independent experiments (Fig. 3).

Already known targets of HIF-1α such as glucose transporter (GLUT1), glycolytic enzymes with aldolase C (ALDOC) and phosphoglycerate kinase 1 (PGK1), pyruvate dehydrogenase kinase 1 (PDK1) which reduces oxidative phosphorylation[28,29] and carbonic anhydrases (CA9 and 12) which participate in intracellular pH regulation[30] were strongly increased in HepaRG cells cultured under hypoxia. In parallel, we demonstrated for the first time, that hypoxia dramatically down-regulated critical genes encoding enzymes involved in different aerobic energetic metabolisms. Tryptophan 2,3-dioxygenase (TDO2), monoamine oxidase A (MAOA) and DOPA acid decarboxylase (DDC), involved in amino acid metabolism, were repressed. Acetyl-CoA acetyltransferase 2 (ACAA2) which is involved in fatty acid mitochondrial β-oxidation to produce acetyl-CoA and cholesterol 7-alpha-monooxygenase (CYP7A1) involved in cholesterol degradation into 7-alpha-hydroxycholesterol, a bile acid precursor, were down-regulated. Alcohol dehydrogenase 1C (ADH1C) catalysing ethanol oxidation into acetaldehyde, an acetyl-CoA precursor, was also strongly repressed. These modulations were reversible since initial mRNA levels were restored after 48 h of reoxygenation. Finally, expression levels of genes encoding pro-inflammatory cytokines (i.e. tumour necrosis factor-α, interleukin-6 and interleukin-1β) and proteins involved in inflammatory response (i.e. C-reactive protein, ceruloplasmin and serum amyloid A) were unchanged between the different conditions (data not shown). Measurement of C-reactive protein levels in the culture medium showed no differences between normoxia and hypoxia followed or not by reoxygenation in HepaRG cells (data not shown).

3.3. Hypoxia down-regulates DMEs expression

Gene profiling and qPCR analyses of HepaRG cells cultured under hypoxic condition demonstrated a down-regulation of genes encoding several DMEs such as phase I enzymes (CYP3A4, CYP1A2, CYP2E1 and CYP2C9) and phase II enzymes (glutathione transferases A1/2 (GSTA1/2), UDP-glucuronosyltransferase 1A1 (UGT1A1) and sulphotransferase 2A1 (SULT2A1)) when compared to normoxia (Fig. 4). By contrast, MRP3 and MDR1 (phase III ABC transporters) expressions were not or only slightly affected during hypoxia and/or reoxygenation (Fig. 4). The xenobiotic-activated transcription factors (xenosensors regulating DMEs expression) including aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR), were also repressed under hypoxic conditions (Fig. 4). All these down-regulations were reversible since initial levels of mRNA were restored after 48 h of reoxygenation.

3.4. Hypoxia down-regulates CYP3A4 protein and represses CYP activities

As demonstrated in Fig. 5, CYP3A4 mRNA repression that occurs during hypoxia was correlated with a decrease in the corresponding protein levels. Detoxication capacities of HepaRG cells maintained under hypoxic conditions were also reduced when compared to normoxic conditions. Indeed, hypoxia decreased CYP3A4-dependent testosterone 6β-hydroxylation and the formation of 6α-hydroxypaclitaxel, the major metabolite of the anticancer drug paclitaxel.

3.5. HIF-1α mediates the down-regulation of CYP3A4 expression

Since HIF1-α plays a key role in hypoxia response and has been involved in the down-regulation of certain genes, we investigated the potential role of its stabilisation in the repression of *CYP3A4*, the most abundant liver CYP isoform involved in the metabolism of a large number of drugs. For this purpose, HepaRG cells were treated with different doses of two chemical stabilisers of HIF-1α, $CoCl_2$ and DFX, during 24 h or 48 h (Fig. 6). As expected, $CoCl_2$ and DFX stabilised HIF-1α. Interestingly, $CoCl_2$ and DFX treatments lead to a dose-dependent decrease in CYP3A4 mRNA and protein levels after 24 and 48 h, respectively.

We further demonstrated the involvement of HIF-1α in the down-regulation of *CYP3A4* by enforcing expression of a wild type HIF-1α [p(HA)HIF1α] or a highly stabilised form of mutated HIF-1α [p(HA)HIF1α(401Δ603)] in transfected HepaRG cells under normoxia (Fig. 7A). By using MatInspector 7.4 (http://www.genomatix.com), we identified a putative hypoxia responsive element (HRE) in the *CYP3A4* gene 5′-flanking region (–674/–661). Thus, the ability of DFX to down-regulate *CYP3A4* transcriptional activity was investigated by transient transfection of a construct consisting of the firefly reporter luciferase gene controlled by *CYP3A4* gene 5′-flanking region (–1100/+11) [pGL3-CYP3A4(–1100/+11)]. A 24-h treatment with 125 μM DFX of HepaRG cells transfected with pGL3-CYP3A4(–1100/+11) induced HIF-1α stabilisation, VEGF-A mRNA induction and CYP3A4 mRNA

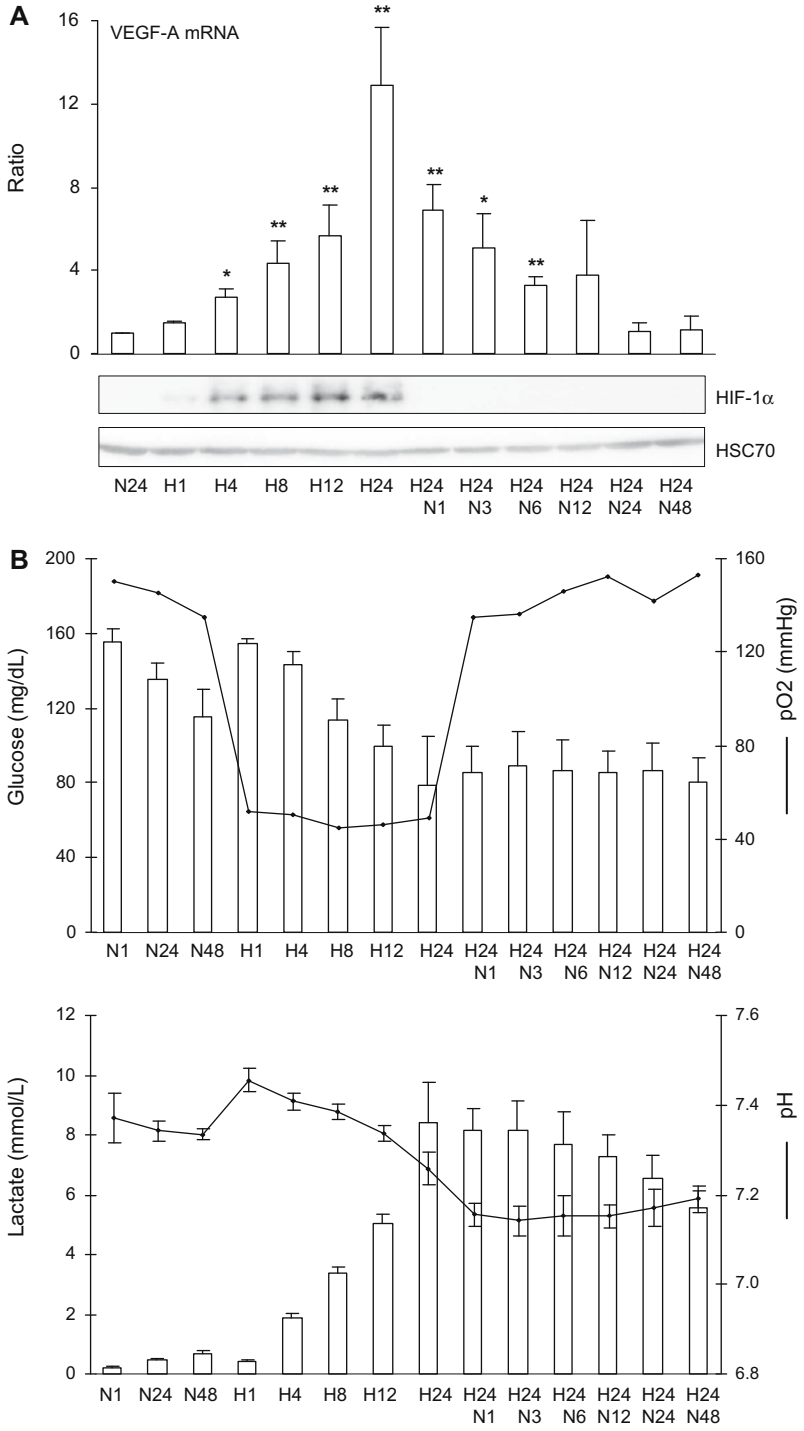

Fig. 2 – Molecular and physiological responses of HepaRG cells to hypoxia. HepaRG cells cultured at the indicated times (hours) of normoxia (N), hypoxia (H) or hypoxia followed by different times (hours) of reoxygenation (HN). (A) Expression of VEGF-A mRNA, HIF-1α and HSC70 (loading control) proteins in HepaRG cells at the indicated times of normoxia, hypoxia or hypoxia followed by reoxygenation. Results are expressed as mean ± SD of three experiments and relative to normoxic condition, given the arbitrary value of 1. Student's *t*-test was performed by comparison of hypoxia or hypoxia followed by reoxygenation and normoxic condition (*, $p \leqslant 0.05$; **, $p \leqslant 0.01$). (B) pO_2 (mmHg), pH and concentrations of glucose (mg/dL) and lactate (mmol/L) were measured in the same conditions. Results are the mean of six values ± SD except for pO_2 values.

repression but had no effect on transcriptional activity of the *CYP3A4* 5′-flanking fragment (–1100/+11) when compared to untreated cells (Fig. 7B). In addition, ChIP experiments using HIF-1α antibody demonstrated binding of HIF-1α to *VEGF-A* promoter but not to *CYP3A4* (–674/–661) HRE motif when HepaRG cells were subjected to hypoxia conditions (1% O_2) during 24 h or treated with 125 μM DFX during 24 h (Fig. 7C).

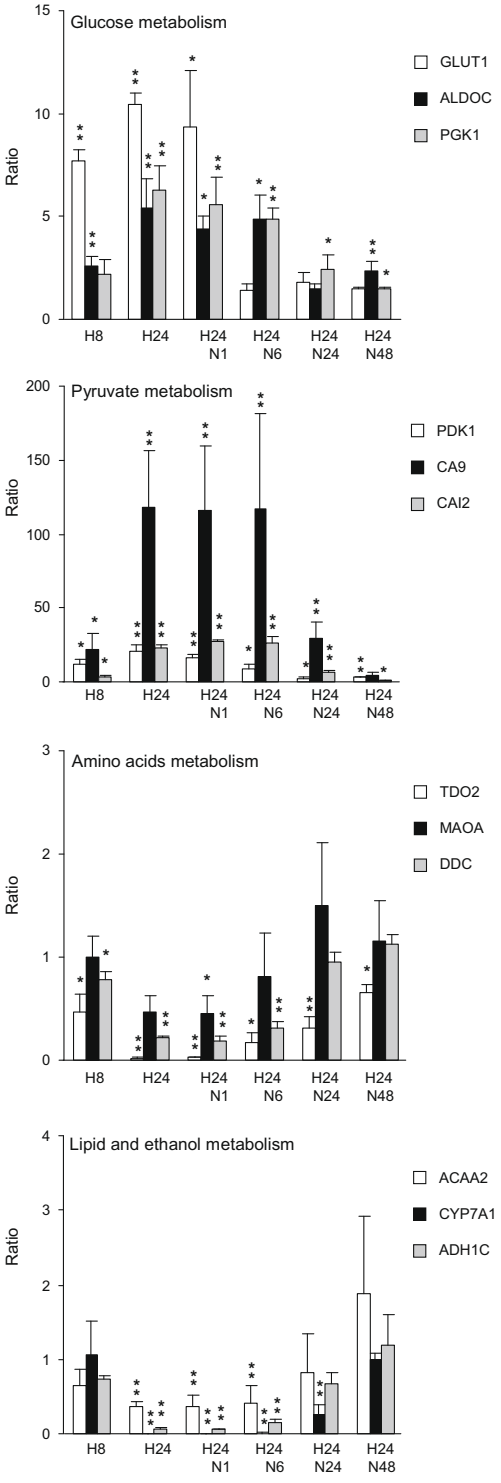

Fig. 3 – Modulation of aerobic energetic metabolic pathways by hypoxia. Expression of mRNAs encoding GLUT1, ALDOC, PGK1, PDK1, CA9, CA12, TDO2, MAOA, DDC, ACAA2, CYP7A1 and ADH1C in HepaRG cells at the indicated times (hours) of hypoxia (H) or hypoxia followed by reoxygenation (HN). Results are expressed as mean ± SD of three experiments and relative to normoxic condition, given the arbitrary value of 1. Student's *t*-test was performed by comparison of hypoxia or hypoxia followed by reoxygenation and normoxic condition (*, $p \leqslant 0.05$; **, $p \leqslant 0.01$).

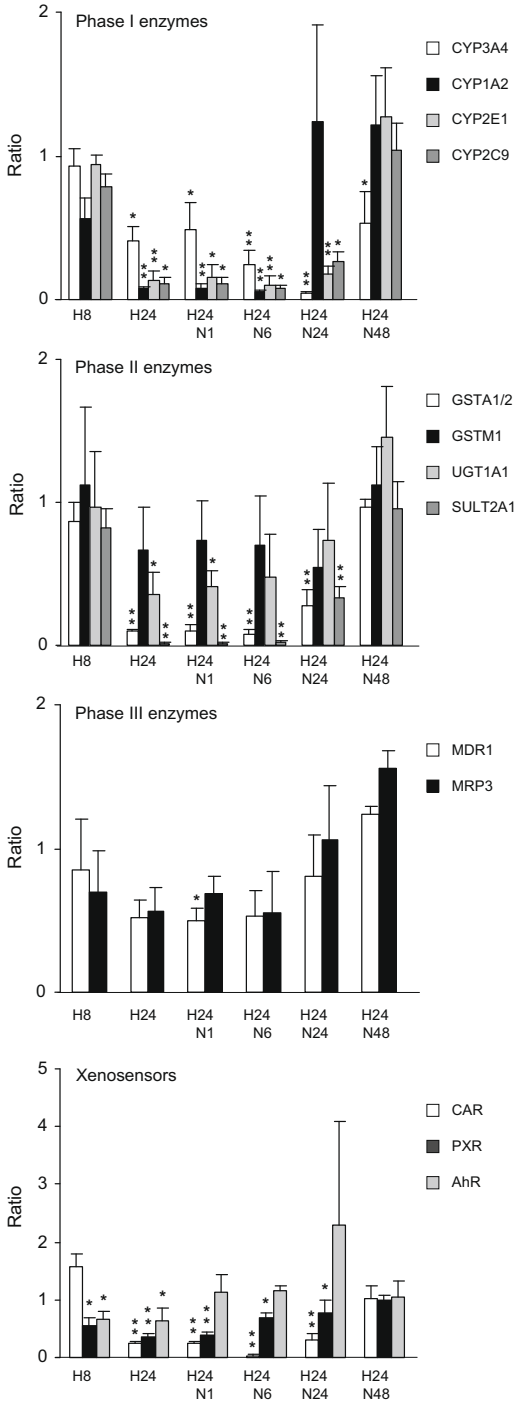

Fig. 4 – Effect of hypoxia on drug metabolism enzymes, ABC transporters and xenosensors expression. Expression of mRNAs encoding phase I enzymes (CYP3A4, CYP1A2, CYP2E1 and CYP2C9), phase II enzymes (GSTA1/2, GSTM1, UGT1A1 and SULT2A1), phase III proteins (MDR1 and MRP3) and xenosensors (CAR, PXR and AhR) in HepaRG cells at the indicated times (hours) of hypoxia (H) or hypoxia followed by reoxygenation (HN). Results are expressed as mean ± SD of three experiments and relative to normoxic condition, given the arbitrary value of 1. Student's *t*-test was performed by comparison of hypoxia or hypoxia followed by reoxygenation and normoxic condition (*, $p \leqslant 0.05$; **, $p \leqslant 0.01$).

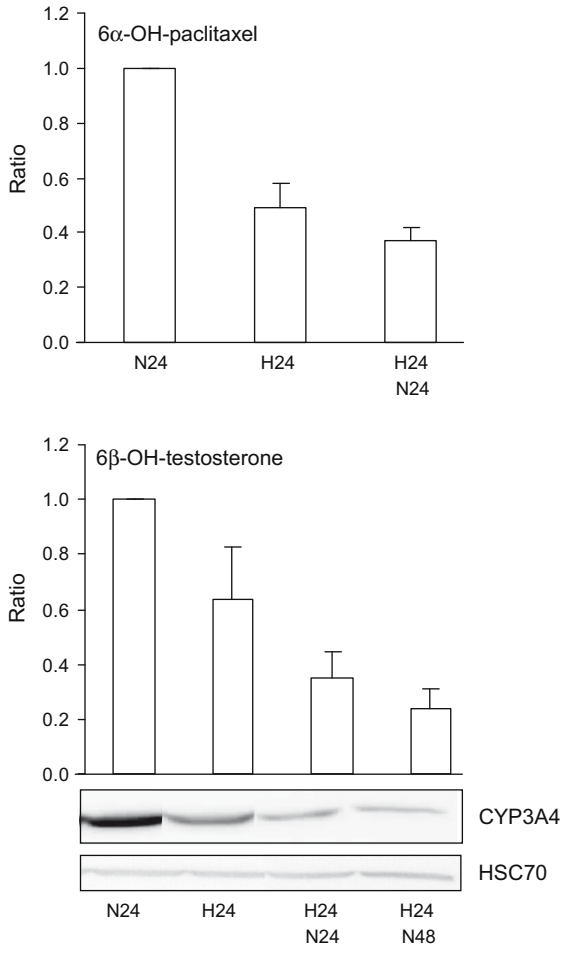

Fig. 5 – Effect of hypoxia on CYP3A4 protein and CYP activities. Expression of CYP3A4 and HSC70 (loading control) proteins in HepaRG cells at the indicated times of normoxia (N), hypoxia (H) or hypoxia followed by reoxygenation (HN). Western blot data represent one of the three independent experiments with similar results. CYP activities were measured by the production of 6β-hydroxytestosterone (CYP3A4) and 6α-hydroxypaclitaxel (CYP2C8 and CYP3A4). Results are the mean of six values ± SD and are expressed relative to normoxic condition, given the arbitrary value of 1. CYP3A4 activities (6β-hydroxylation of testosterone) and 6α-hydroxypaclitaxel concentrations ranged between 260 and 1850 pmol/mg protein/min and between 0.107 and 0.384 μM, respectively.

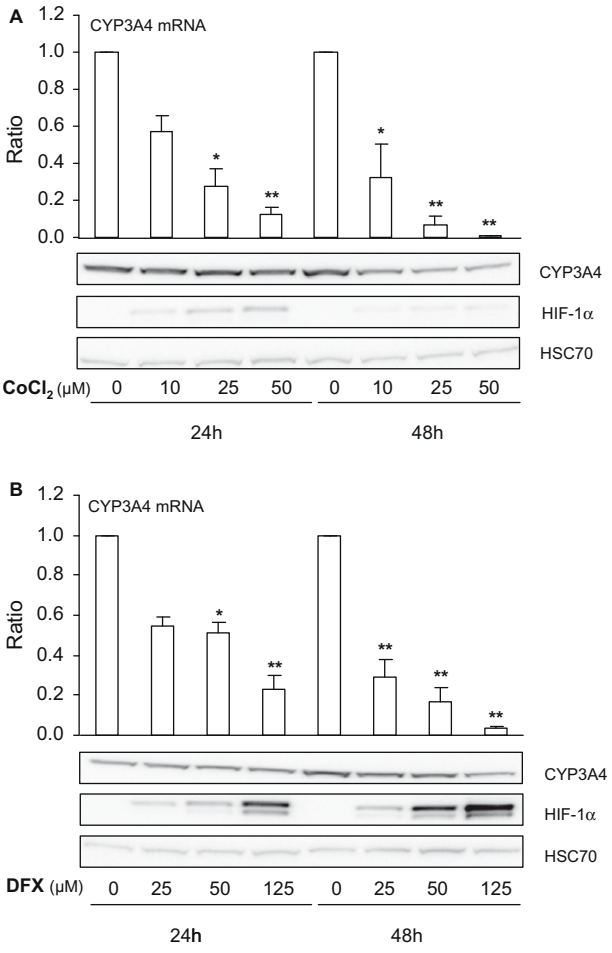

Fig. 6 – Effects of cobalt chloride ($CoCl_2$) and desferrioxamine (DFX) on CYP3A4 mRNA and protein levels. Expression of CYP3A4 mRNA as well as CYP3A4, HIF-1α and HSC70 (loading control) proteins in HepaRG cells cultured in the absence or the presence of different concentrations of $CoCl_2$ (A) or DFX (B). Results are expressed as mean ± SD of three experiments and relative to untreated cells, given the arbitrary value of 1. Student's *t*-test was performed by comparison of $CoCl_2$ or DFX-treated cells and untreated cells (*, $p \leqslant 0.05$; **, $p \leqslant 0.01$). Western blot data represent one of the two independent experiments with similar results.

4. Discussion

Hypoxic environment, frequently encountered in solid tumours,[1] is associated with invasive/aggressive phenotype, therapeutic resistance and correlated to poor prognosis and mortality.[31] Inside solid tumours, hypoxic cells are characterised by a switch from aerobic to anaerobic glycolysis, involving increased glucose cell uptake, increased conversion of glucose to pyruvate and a concomitant decrease in mitochondrial metabolism (oxidative phosphorylation) with an accumulation of acidic products (acidosis).[32] In this study, we showed that differentiated human hepatoma HepaRG cells cultured under hypoxic conditions reproduce the same behaviour. We also demonstrated that prolonged moderate hypoxia (1% O_2, 24 h), although not deleterious for HepaRG cells, dramatically down-regulated critical genes involved in other aerobic cellular metabolic pathways such as amino acid, fatty acid, cholesterol/bile acid and ethanol metabolisms. These repressions of aerobic energetic metabolism during hypoxia are reversible since reoxygenation allowed to restore the expression of most genes at levels comparable to those observed in HepaRG cells cultured in normoxic conditions. It is noteworthy that most of these genes encode enzymes using molecular oxygen as the electron acceptor for the oxidation of their substrates and/or enzymes involved in the synthesis of acetyl-CoA necessary for ATP production during oxidative phosphorylation in mitochondria through the

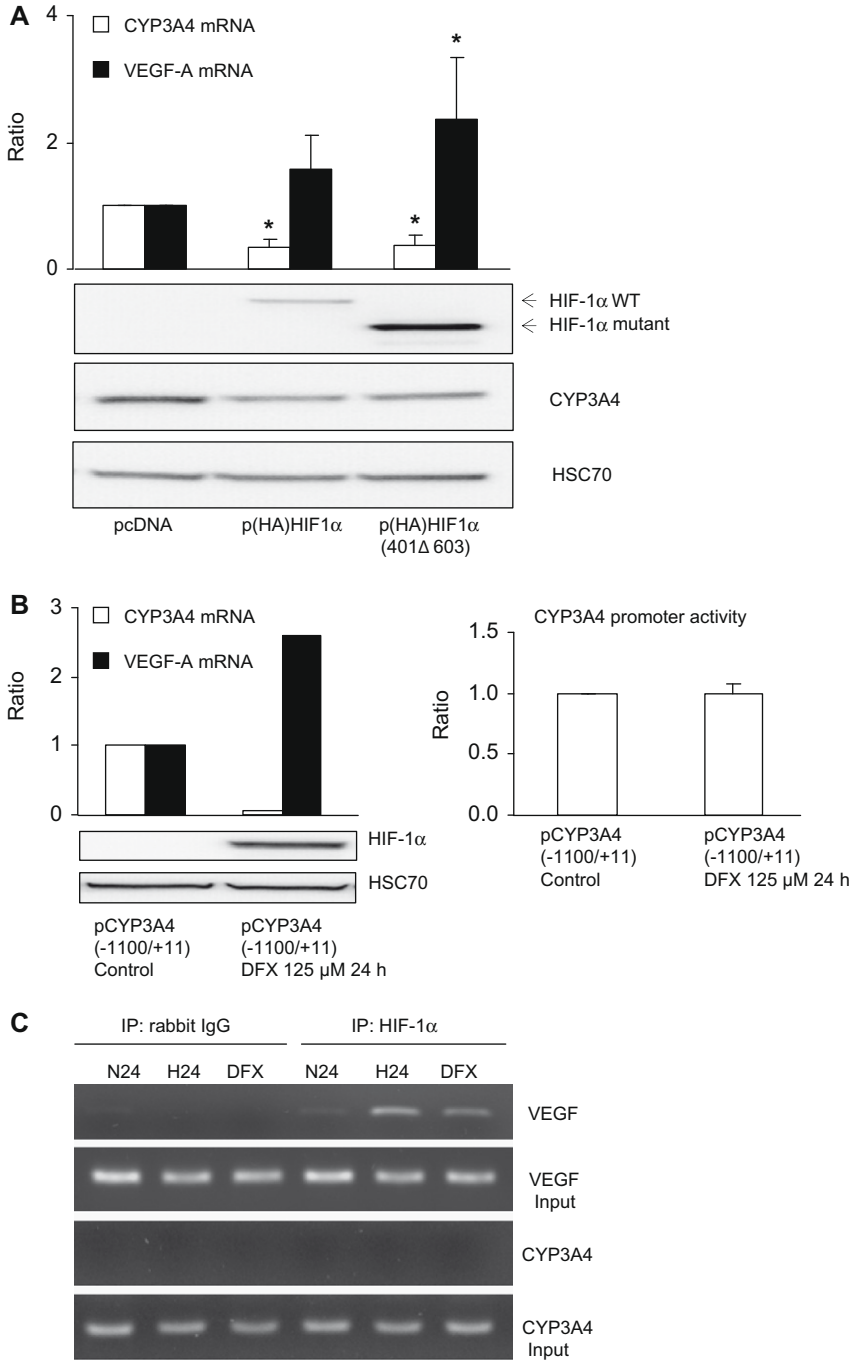

Fig. 7 – Role of HIF-1α in the down-regulation of *CYP3A4* expression. (A) Expression of VEGF-A and CYP3A4 mRNAs as well as HIF-1α and HSC70 (loading control) proteins in HepaRG cells transfected with pcDNA3.1, p(HA)HIF1α or p(HA)HIF1α (401Δ603) constructs. Results are the mean ± SD of three independent experiments and are expressed relative to cells transfected with pcDNA3.1, given the arbitrary value of 1. Student's *t*-test was performed by comparison of HepaRG cells transfected with p(HA)HIF1α or p(HA)HIF1α(401Δ603) and HepaRG cells transfected with pcDNA3.1 (*, $p \leqslant 0.05$). (B) Expression of VEGF-A and CYP3A4 mRNAs as well as HIF-1α and HSC70 proteins in HepaRG cells transfected with pCYP3A4(–1100/+11) and treated or not with 125 μM DFX during 24 h. Comparison of luciferase activities driven by pCYP3A4(–1100/+11) construct between control and DFX-treated HepaRG cells. Results are the mean ± SD of three independent experiments and are expressed relative to cells transfected with pCYP3A4(–1100/+11) without treatment, given the arbitrary value of 1. (C) ChIP assay in HepaRG cells cultured at the indicated times of normoxia (N) or hypoxia (H) or treated with 125 μM DFX during 24 h (DFX). Genomic DNA and proteins from HepaRG cells were immunoprecipitated with rabbit IgG or a HIF-1α antibody. After immunoprecipitation, *VEGF-A* or *CYP3A4* HRE-containing sequences were amplified by PCR. Input: DNA not immunoprecipitated.

tricarboxylic acid cycle. These observations suggest that prolonged moderate hypoxia results in a selective and reversible suppression of aerobic energetic metabolic activities in HepaRG cells that permits to use the limited supply of oxygen and ATP for essential cell functions required for survival. This metabolic adaptation is considered as a hallmark of hypoxia tolerance and the most potent defence against hypoxia.[33]

It is known that hypoxia found in solid tumour abrogates the effect of drugs and is responsible of the poor response to chemotherapy.[34] Here, we evidenced that a 24-h hypoxia down-regulates several DMEs as well as transcription factors CAR, PXR and AhR involved in their regulation. Inflammation is known to down-regulate the expression and activity of several CYPs, leading to a decrease of drug metabolism, an elevation of plasma drug levels and toxic effects.[35] Although the association of inflammation and hypoxia has been demonstrated previously in different cancers,[36] there is no inflammatory response in HepaRG cells cultured under hypoxic conditions. Thus, repression of DMEs expression during hypoxia was independent of an inflammatory process in HepaRG cells.

In order to further investigate mechanism(s) involved in CYP down-regulation by hypoxia, we analysed the potential role of HIF-1α on repression of *CYP3A4* which is known to be responsible for the metabolism of a large number of anticancer drugs (sorafenib, cyclophosphamide, tamoxifen, paclitaxel, etc.). We showed that treatment by $CoCl_2$ and DFX, two HIF-1α stabilisers, and transfection with HIF-1α expression vectors lead to *CYP3A4* down-regulation, evidencing that HIF-1α plays a central role in *CYP3A4* repression. Previous studies have demonstrated a direct binding of HIF-1α to a HRE site in α-fetoprotein, carbamoyl-phosphate synthetase 2 and hepcidin genes leading to the down-regulation of their expression.[4–6] Although a putative HRE sequence is present in the 5′-flanking region of *CYP3A4* gene, luciferase reporter and ChIP assays excluded the involvement of this HRE sequence in the negative control of *CYP3A4* transcription. These results suggest that HIF-1α might regulate *CYP3A4* expression by an indirect mechanism or by interacting with a *DNA* regulatory sequence localised somewhere else in *CYP3A4* gene. Since previous studies have demonstrated post-transcriptional regulation by hypoxia and/or HIF-1α,[37–40] another hypothesis might be that repression of CYP3A4 mRNA levels results from a decreased transcript stability.

Our results are of particular interest since hypoxia plays a key role in chemoresistance of solid tumours. Indeed, vasculature disorders restrict drug delivery, most chemotherapeutic agents are dependent on cellular oxygenation for maximal efficacy/cytotoxicity and extracellular pH decrease (acidosis) modulates cellular drug accumulation/toxicity.[9,41] Furthermore, we demonstrated that hypoxia down-regulates hepatic DMEs, a cellular response that might compromise chemotherapy effects and/or increase toxicity of drugs which are metabolised by these DMEs. Indeed, when HepaRG cells were treated with paclitaxel, an anticancer drug widely used for treatment of malignant tumours and metabolised by CYP2C8 and CYP3A4,[42] we showed that, under hypoxic conditions, paclitaxel metabolism to 6α-hydroxypaclitaxel was lower compared to that in normoxia.

In conclusion, the differentiated HepaRG cells cultured under hypoxic conditions represent a new strategy to study the predictive assessment of anticancer agents in hypoxic versus normoxic conditions. Finally, since HepaRG cells express several hepatic markers at levels comparable to primary human hepatocytes and hypoxia is encountered in other situations such as cirrhosis, acute alcohol intake, liver zonation or surgery, this cell line also represents a model of liver parenchymal cells to study response(s) to oxygen impairment.

Conflict of interest statement

None declared.

Acknowledgements

The authors are grateful to C. Ribault for technical help, Xenoblis® and Dr. F. Bree (Saint Grégoire, France) for analysis of paclitaxel metabolism and Pr. Y. Mallédant and Pr. A. Guillouzo for helpful discussions. The authors also thank the ImPACcell® and Vectorisation plateforms from Biogenouest®. This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM), the Centre National de la Recherche Scientifique (CNRS), the Conseil Régional de Bretagne, the Association pour la Recherche contre le Cancer (ARC), the INSERM/Japan Society for the Promotion of Science (JSPS) cooperation programme, the GIS thérapie cellulaire de Rennes, the «Prométhée» association, the Program for the Promotion of Fundamental Studies in Health Sciences from National Institute of Biomedical Innovation and Astellas Pharma Inc. Claire Legendre was supported by the Conseil Régional de Bretagne and the Ligue Nationale contre le Cancer.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2009.07.010.

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