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FAST TRACK

Regulation of HMG-CoA Reductase Expression by Hypoxia

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Abstract The ability to maintain O_2 homeostasis is essential to the survival of all invertebrate and vertebrate species. The transcriptional factor, hypoxia inducible factor 1 (HIF-1), is the principal regulator of oxygen homeostasis. Under hypoxic condition HIF-1 induces the transcription of several hypoxia-responsive genes by binding to hypoxia-response elements (HRE) in their promoters. In recent years it has been demonstrated that hypoxia could be related to metabolic variations such as hyper-cholesterolemia in mouse models. On the basis of this observation, the present study was performed to verify the involvement of HIF-1, and in particular the effect of chemical and environmental induction of HIF-1α (the oxygen sensitive isoform) accumulation in 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMG-CoAR, the key and rate limiting enzyme of cholesterol biosynthetic pathway) regulation. Our results show that HIF-1α accumulation is able to increase level and activity of HMG-CoAR by stimulating its transcription. The raised transcription of the reductase could be related to an induction by HIF-1α even if a parallel action of SREBP-2 actively translocated to nucleus by the increased level of SCAP cannot be excluded. J. Cell. Biochem. 104: 701–709, 2008.

Key words: HepG2; HIF-1α; HMG-CoAR; hypoxia; SCAP.

The ability to maintain O_2 homeostasis is essential to the survival of all invertebrate and vertebrate species. Physiological systems have been evolved to ensure the optimal oxygenation of all cells in every metazoan species [Hirota and Semenza, 2006]. At the molecular level, oxygen homeostasis is achieved by acute adaptive responses which involve change in the activity of pre-existing proteins and chronic responses involving changes in gene expression that lead to new protein synthesis. The transcriptional factor, hypoxia inducible factor 1 (HIF-1), is the master regulator of oxygen homeostasis [Semenza, 2006]. HIF-1 is a heterodimer consisting of two subunits, the oxygen-sensitive HIF-1α and the constitutively expressed HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator, ARNT) [Wang et al., 1995].

In normoxia, the von Lippel-Lindau tumour suppressor (pVHL), which is the recognition component of an E3 ubiquitin ligase complex, targets HIF-1α [Patiar and Harris, 2006], leading to its ubiquitination and consequent proteasomal degradation [Lisztwan et al., 1999].

HIF-1α has an oxygen-dependent degradation domain (ODDD) which when deleted renders the molecule stable even in normoxic cells [Huang et al., 1998]. It has been shown

Abbreviations used: ARNT, aryl hydrocarbon receptor nuclear translocator; CBP, CREB-binding protein; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; FFA, free fatty acids; FIH-1, factor inhibiting HIF-1; HI, intermittent hypoxia; HIF-1, hypoxia inducible factor 1; HMG-CoAR, 3-hydroxy 3-methylglutaryl coenzyme A reductase; HRE, hypoxia response elements; Insig, insulin induced gene; MVA, mevalonate; ODDD, oxygen-dependent degradation domain; pVHL, von Lippel-Lindau tumor suppressor; SCAP, SREBP cleavage activating protein; SREBP, sterol regulatory element binding protein; VEGF, vascular endothelial growth factor.

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that the interaction of pVHL with HIF-1α is regulated by the hydroxylation of 402- and 564-proline residues, conserved either in HIF-1α and HIF-1β and located within the ODDD [Patiar and Harris, 2006]. In hypoxia, the proline residues within the ODDD are not hydroxylated and thus HIF-1α protein level increases. On the other hand, another regulatory protein exists, the factor inhibiting HIF-1 (FIH-1), which along with pVHL is able to inhibit HIF-1 transactivation domain function [Mahon et al., 2001].

Stabilised HIF-1α is translocated to the nucleus where it dimerises with nuclear HIF-1β (nHIF-1β) and it associates with co-activators such as CREB-binding protein (CBP)/p300 [Yamashita et al., 2001]. The HIF-1 heterodimer induces the transcription of several hypoxia-responsive genes, such as the proangiogenic vascular endothelial growth factor (VEGF), by binding to hypoxia-response elements (HRE) in their promoters [Forsythe et al., 1996]. Beside VEGF and angiogenesis involved proteins, HIF-1 regulates the transcription of several genes implicated in biological processes such as cell proliferation, survival, apoptosis, glucose metabolism, and pH regulation [Semenza, 2001; Harris, 2002]. In recent years it has been demonstrated that intermittent hypoxia (HI) is related to metabolic variations such as hyper-cholesterolemia and hyper-triglyceridemia in mouse models [Li et al., 2005b]. Other studies have hypothesised that HIF-1 could modulate metabolic syndrome up-regulating some proteins deeply involved in cholesterol metabolism [Li et al., 2005a]. On the other hand, it has recently been reported that Chinese Hamster Ovary cells (CHO-7) under hypoxic condition show a higher 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR) degradation [Nguyen et al., 2007].

HMG-CoAR, is the key enzyme of the cholesterol biosynthetic pathway that catalyses the reduction of HMG-CoA to mevalonate (MVA). HMG-CoAR is embedded in endoplasmic reticulum (ER), its catalytic domain projects into cytosol and is rapidly regulated by a phosphorylation/dephosphorylation mechanism [Gil et al., 1985; Liscum et al., 1985].

The long-term regulation of HMG-CoAR, operated by the control of synthesis and degradation, is guaranteed by a network of modulatory proteins: sterol regulatory element binding protein (SREBPs), SREBP cleavage activated protein (SCAP), and insulin induced gene (Insigs) [Goldstein et al., 2006].

In HMG-CoAR transcription the family of SREBPs is involved. These membrane-bound transcription factors are activated in sterol deprived cells, by the translocation from ER to the Golgi apparatus, where they are cleaved by specific proteases with the release of a soluble fragment into cytosol [Goldstein et al., 2006]. Processed forms of SREBPs migrate to the nucleus and enhance transcription of genes encoding HMG-CoAR and other enzymes known to be required for cholesterol and free fatty acids (FFA) synthesis [Horton et al., 2002]. Translocation of SREBPs from ER to Golgi requires the escort protein SCAP [DeBose-Boyd et al., 1999; Nohturfft et al., 1999; Rawson et al., 1999].

HMG-CoAR degradation is mediated by the ER membrane proteins Insig-1 and Insig-2 [Sever et al., 2003].

On the basis of all these very intriguing observations though not sufficient to provide the whole picture of the molecular mechanisms involved, the present study was performed to verify the involvement of HIF-1 and in particular the effect of chemical and environmental induction of HIF-1α accumulation in HMG-CoAR regulation. For this purpose, HepG2 cell line one system "in vitro" was used where the experimental condition could be well set. Actually, in this cell model HIF-1α accumulation was stimulated both chemically, by using $CoCl_2$ treatment, and physiologically maintaining cells under 2% O_2 atmosphere.

Our results show that HIF-1α accumulation is related to increased level and activity of HMG-CoAR and probably stimulate its transcription.

This should constitute a starting point for further studies connecting oxygen sensing and HMG-CoAR which exerts an important role in several biological processes.

MATERIALS AND METHODS

Materials

All materials used were obtained from commercial sources and of the highest quality available. All materials with no specified source are obtained from Sigma–Aldrich (St. Louis, MO).

Cell Culture

HepG2 cells were used as experimental models. Cells were routinely grown in air

containing 5% CO_2 in RPMI-1640 medium, containing 10% (v/v) foetal calf serum, L-glutamine (2 mM), gentamicin (0.1 mg/ml) and penicillin (100 U/ml). Cells were passaged every 3 days and media changed every 2 days.

To increase chemically HIF-1α protein level, HepG2 cells were grown to ~70% confluence in six wells plates, then stimulated with 100 μM $CoCl_2$. To make cells hypoxic, culture dishes were placed in airtight modular incubator chambers (Forma Scientific, Mountain View, CA), infused for 20 min with 2% pO_2, and incubated at 37°C for 20 h.

Ten micromolar YC-1 [(5′-hydroxymethyl-2′-furyl)-1-benzylindazole], HIF-1 inhibitor [Sun et al., 2007; Yeh et al., 2007; Zhao et al., 2008], was added to culture medium 5 min prior to $CoCl_2$ stimulation.

Lysate Preparation

After $CoCl_2$ treatment in the presence or absence of YC-1, cells were washed twice in phosphate buffer salina (PBS), harvested with trypsin (1%, v/v) and pelleted by centrifugation. Pellets were resuspended in PBS containing 0.1 M sucrose, 0.05 M KCl, 0.04 M KH_2PO_4, 30 mM EDTA, pH 7.4, and sonicated. The protein concentration was determined by the Lowry method [Lowry et al., 1951]. The samples were subjected to HMG-CoAR activity and level analysis and Western Blot as described below.

Cytosol and Membrane Preparation

Cells were harvested with trypsin (1%, v/v), resuspended in PBS and sonicated. Proteins were solubilised in PBS containing 0.1 M sucrose, 0.05 M KCl, 0.04 M KH_2PO_4, 30 mM EDTA, pH 7.4. The samples were centrifuged for 60 min at 14,000 rpm. The supernatant fraction represented the cytosolic fraction and the pellet represented purified membranes. The protein concentration was determined by the Lowry method [Lowry et al., 1951]. The samples were subjected to $CoCl_2$ stimulation for 2 h and then HMG-CoAR activity analysis and Western Blot were assessed as described below.

Nuclear Extract Preparation

Cells were harvested with trypsin (1%, v/v), washed twice with PBS and centrifuged for 5 min at 1,000*g*. Then pellets were resuspended in a hypotonic buffer containing 10 mM Hepes, 10 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM EDTA, 5% Glycerol, protease inhibitor cocktail, pH 7.8 for 15 min at 4°C. The samples were solubilised with 10% Nonidet-40 and centrifuged for 30 s at 14,000 rpm. The pellet was resuspended in a hypertonic buffer containing 50 mM Hepes, 400 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 10% Glycerol, protease inhibitor cocktail, pH 7.8. The samples were stirred for 15–20 min at 4°C. The samples were centrifuged for 5 min at 14,000 rpm; the supernatant fraction represented nuclear extract. The protein concentration was determined by the Lowry method [Lowry et al., 1951]. The samples were subjected to Western Blot as described below.

Western Blot Analysis

Samples, obtained by different preparations as described previously, were solubilized in 0.25 mM Tris–HCl, (pH = 6.8) containing 20% (w/v) SDS, protease inhibitor cocktail; then they were boiled for 2 min. Total protein were quantified using Lowry [Lowry et al., 1951]. Solubilized proteins (10 μg) were resolved by SDS–PAGE at 100 V for 1 h and then electrophoretically transferred to nitrocellulose for 80 min at 100 V and 4°C. The nitrocellulose was treated with 3% (w/v) bovine serum albumin (BSA) in Tris Buffered Saline (20 mM Trizma base, 137 mM NaCl, 0.1%, v/v, Tween-20, pH = 7.6) and then probed over night with anti-HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA), anti HMG-CoAR (Upstate, Lake Placid, NY), anti-SREBP-2 N-terminal (Santa Cruz), anti-SCAP (Santa Cruz). The nitrocellulose was stripped by Restore Western Blot Stripping Buffer (Pierce Chemical, Rockford, IL) for 10 min at room temperature and then probed with anti-actin (Santa Cruz). Antibody reaction was visualized with chemiolumionescence Western Blotting detection reagent (GE Healthcare, Little Chalfont, UK).

HMG-CoAR Activity Analysis

To evaluate HMG-CoAR activity it has been used a radioisotopic method, following the production of [^{14}C]-mevalonate from 3-[^{14}C]-hydroxymethylglutaryl coenzyme A (3-[^{14}C]-HMG-CoA) as previously described [Pallottini et al., 2005] on lysates obtained by HepG2 cells as described. HMG-CoAR activity was expressed as picomoles of [^{14}C]-mevalonate formed per minute per milligram of total protein (pmol/min/mg prot). To control the possibility of

potential effects of $CoCl_2$ on HMG-CoAR activity 100 μg of isolated membrane were treated for 2 h with 100 μM $CoCl_2$ than the activity of HMG-CoAR was measured as described by Pallottini et al. [2005].

"In Vitro" HMG-CoAR Degradation

The HepG2 cells were washed twice in PBS, harvested with trypsin (1%, v/v) and pelleted by centrifugation; then suspended in ice-cold 10 mM Tris–HCl pH 7.4, 150 mM sucrose and then incubated at 37°C. Fifty microgram lysate was used for each reaction. The protein concentration was determined by the Lowry method [Lowry et al., 1951].

At given time, the incubation was terminated by addition of an equal volume of sample buffer (final concentration 0.125 M Tris–HCl, pH 7.8 containing 10% SDS, protease inhibitor cocktail) and samples were boiled for 5 min and subjected to Western Blot analysis..

HMG-CoAR Promoter Activity Analysis

HepG2 cells (~70% confluence) were transiently transfected with the plasmid containing the promoter of HMG-CoAR (a kind gift by Prof. Demoulin, Universitè Catholique de Louvain, Belgium), linked to the gene of luciferase (pHMG-CoAR-luciferase), using lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmid was purified for transfection using the plasmid preparation kit according to the manufacturer's instructions (NucleoSpin Plasmid, Macherey-Nagel, GmbH and Co., Germany). A luciferase dose–response curve showed that the maximum effect was observed when 0.250 μg/ml DNA was transfected in HepG2 cells (data not shown). Six hours after transfection, the medium was changed and 24 h later cells were treated with $CoCl_2$ in the presence or absence of YC-1. The cell lysis procedure and the subsequent measurement of the luciferase gene expression were performed using the luciferase kit (Promega, Milan, Italy) according to the manufacturer's instructions, with a Wallac Berthold luminometer apparatus (Perkin-Elmer, Italy).

Statistical Analysis

All the images were analysed by ImageJ program as arbitrary units. The statistical analysis by ANOVA followed by Dunnett post-test; all the statistical analysis have been done using GraphPad Instat3.

RESULTS

HIF-1 is known to be a mediator of the hypoxia induced responses in several physiological and pathological situations playing a key role in the adaptation of the cell. Among the metabolic modifications observed in hypoxic conditions, changes in cholesterol metabolism have been reported [Mukodani et al., 1990]. The lack of full information on the mechanisms involved, suggested a study, of the effect of hypoxia on the activity and level of the key enzyme of cholesterologenesis, the HMG-CoAR, in one system "in vitro" where the experimental conditions could be well set.

First, the HepG2 cells were incubated in hypoxic chamber at 2% O_2 atmosphere and after 24 h the HIF-1α and HMG-CoAR levels were measured on the total lysate by western blotting (Fig. 1a,b). In this condition a high increase of HIF-1α along with a parallel increase of HMG-CoAR levels is detectable, while it is completely blocked in presence of YC-1, a HIF-1 inhibitor.

Thus, to study the mechanism underlying such HMG-CoAR behaviour, a chemical hypoxia was used subjecting HepG2 cells to $CoCl_2$ treatment. This compound elicits a durable HIF-1α accumulation even in a normoxic environment blocking the PHD effects.

In such experimental condition (Fig. 2) a time-dependent enhanced protein level of HIF-1α was observable in the total lysate (panel a) as well as in the nuclear extracts (panel b), indicating, beside a block of HIF-1α degradation, also a translocation to the nucleus. In the presence of YC-1 (Fig. 3, panel a) the HIF-1α increment was not observable, and the level HMG-CoAR followed the same trend (Fig. 3, panel b).

In the HepG2 cells treated with $CoCl_2$ the activity of HMG-CoAR was measured. As shown in Figure 4, the increase in the activity of the enzyme (panel a) was detectable just after 2 h of treatment. To verify whether or not such an increase was independent from a direct effect of $CoCl_2$ on the enzyme, the activity was analysed on the isolated membranes which were treated for 2 h with this compound. As observable in Figure 4 (panel b), the $CoCl_2$ treatment does not exert any direct effect on the HMG-CoAR activity.

Whether the increased level of the reductase was dependent on a reduced degradation rate of

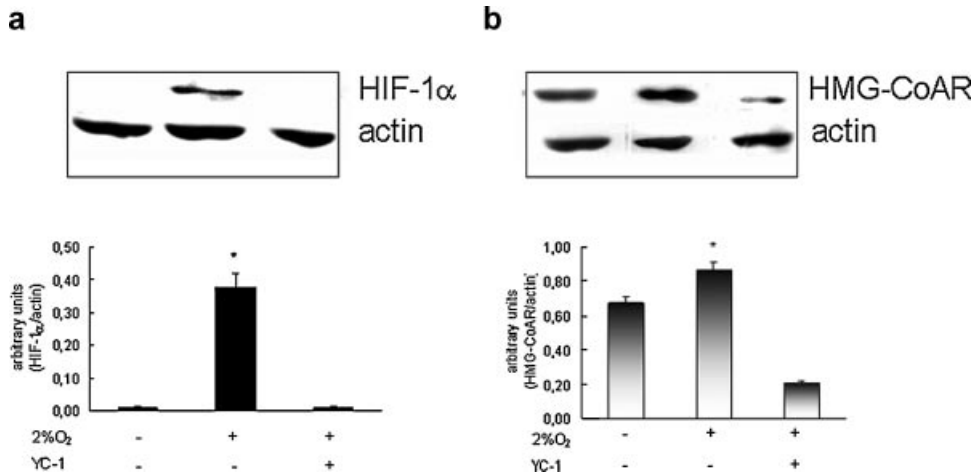

Fig. 1. HIF-1α and HMG-CoAR levels in HepG2 cells under O_2 atmosphere for 20 h. **Panel a** illustrates a typical Western blotting showing HIF-1α level with the densitometric analysis (means ± SD) obtained from four different experiments. **Panel b** illustrates a typical Western blotting showing HMG-CoAR level with the densitometric analysis (means ± SD) obtained from four different experiments. The experiments were performed in HepG2 cells under hypoxic conditions. Ten microgram proteins were resolved by SDS–PAGE and subsequently transferred electrophoretically onto nitrocellulose then probed with primary antibodies as described in the main text. *$P < 0.001$ as from a Dunnett test with the respect to the samples $-2\%O_2$, YC-1.

the enzyme was also checked. As reported in Figure 5 no significant difference between the "in vitro" degradation of HMG-CoAR in $CoCl_2$ treated and untreated cells has been observed.

To consider the HMG-CoAR codifying gene as a target of HIF-1α, the promoter has to present the binding sequence HRE (i.e., 5′-RCGTG-3′). The finding of that sequence on the promoter (Fig. 6, panel a) allowed us to test the effect of 16 and 24 h treatment on the transcription in cell transfected with the pHMG-CoAR-luciferase. At 16 h of $CoCl_2$ treatment a significant increase of the promoter transcription is detectable, that is not observable in presence of YC-1 inhibitor (Fig. 6, panel b).

To clarify the regulatory mechanism underlying the effect of hypoxia on the reductase, the level of SREBP-2 and SCAP, respectively

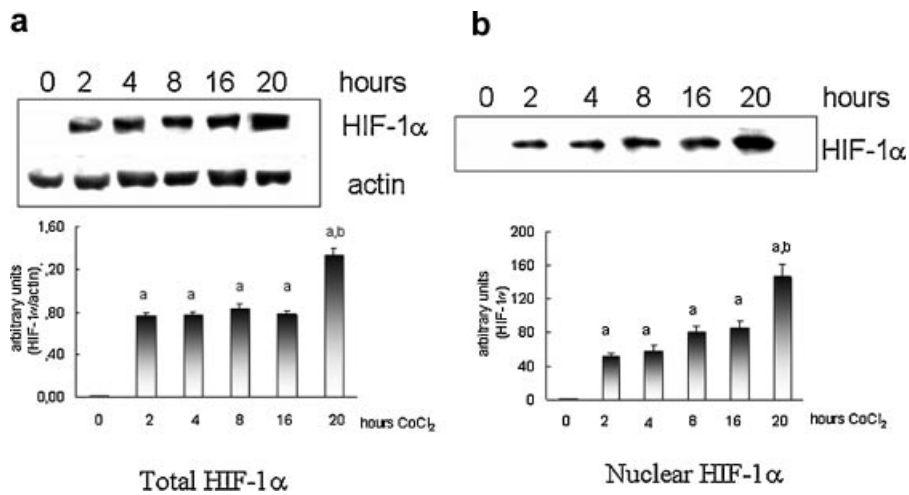

Fig. 2. HIF-1α level both in whole cells and in nuclei of HepG2 subjected to chemical hypoxia by $CoCl_2$ administration. Typical Western blotting and the densitometric analysis of HIF-1α level of total lysate (**panel a**) and nuclei extracts (**panel b**) of HepG2 cells treated for the indicated times with $CoCl_2$. The data are obtained (mean ± SD) from four different experiments performed as described in the Material and Method Section. a: $P < 0.001$ as from a Dunnett test with the respect to 0 h 100 μM $CoCl_2$ treatment; (b) $P < 0.001$ from a Dunnett test with the respect to the other times of $CoCl_2$ treatment.

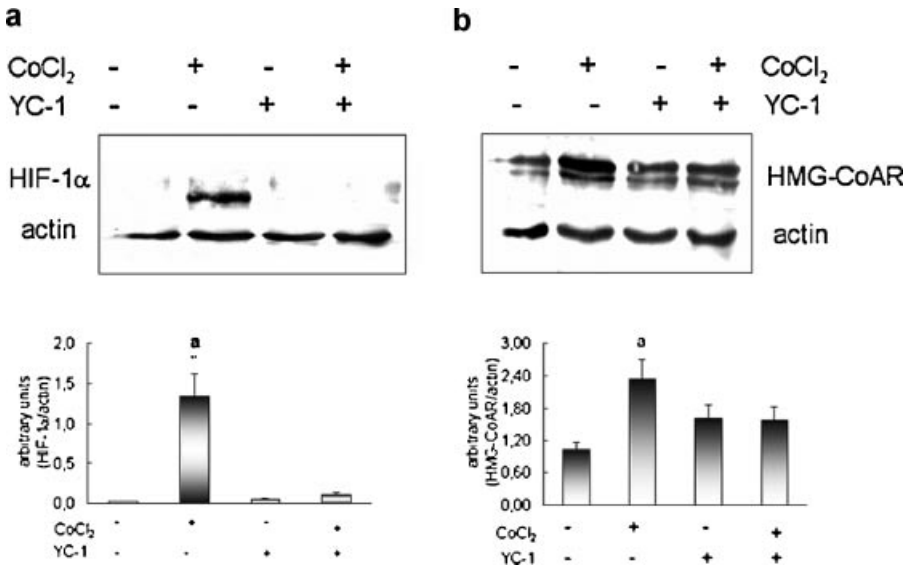

Fig. 3. HIF-1α and HMG-CoAR levels in HepG2 cell line under chemical hypoxia. **Panel a** illustrates a typical Western blotting showing HIF-1α level with the densitometric analysis obtained from four different experiments. **Panel b** illustrates a typical Western blotting showing HMG-CoAR level with the densitometric analysis (mean ± SD) obtained from four different experiments. The experiments were performed in HepG2 cells treated with 100 μM $CoCl_2$ for 20 h in presence and in absence of 10 μM YC-1. For methodological details see the main text. a: $P < 0.001$ as from a Dunnett test with the respect to the samples—$CoCl_2$, YC-1.

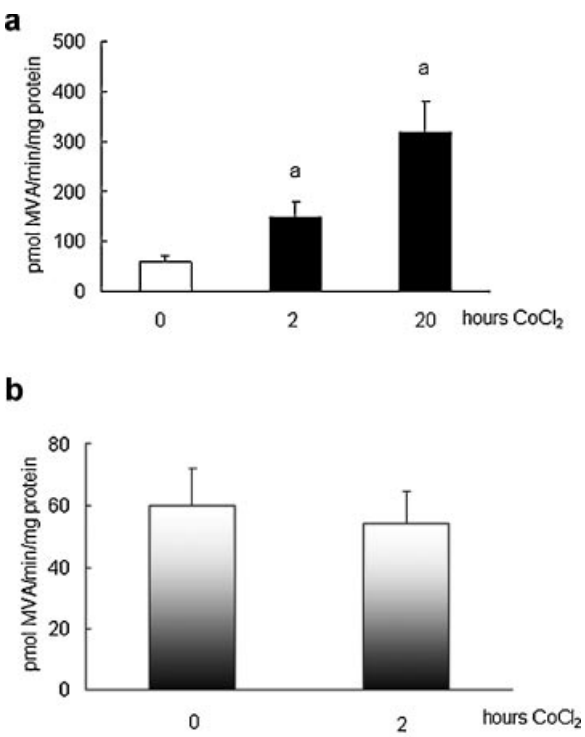

Fig. 4. HMG-CoAR activity. **Panel a** shows HMG-CoAR activity in HepG2 cells treated with 100 μM $CoCl_2$ for 2 and 20 h. **Panel b** shows the HMG-CoAR activity in isolated membranes obtained by HepG2 cells and treated with 100 μM $CoCl_2$ for 2 h. The data are the mean ± SD of four different experiments, for methodological details see the main text. a: $P < 0.001$ as from a Dunnett test with the respect to 0 h treatment.

known as transcription and escort factors, were measured in the cells treated with $CoCl_2$ in presence and in absence of YC-1. As shown in Figure 7 nSREBP-2 levels do not change; on the contrary, SCAP levels (Fig. 8) result increased

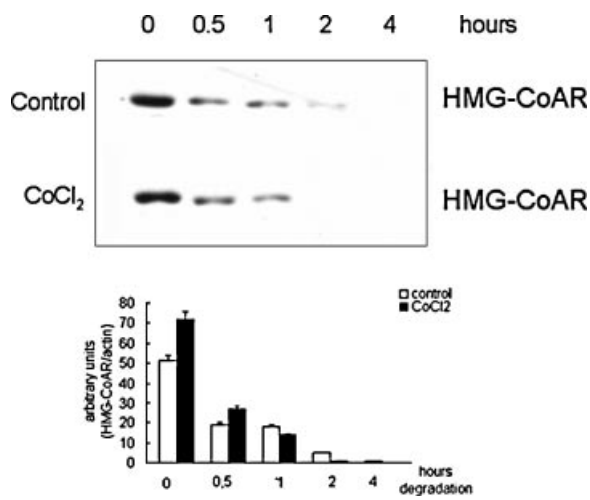

Fig. 5. HMG-CoAR degradation in HepG2 cells under chemical hypoxia. The figure illustrates HMG-CoAR levels and the relative densitometric analysis (mean ± SD) obtained by four different experiments. HepG2 cells were treated with 100 μM $CoCl_2$ for 20 h, then cell total lysates were incubated in appropriate buffer for the indicated times and next subjected to SDS–PAGE (for methodological details see the main text).

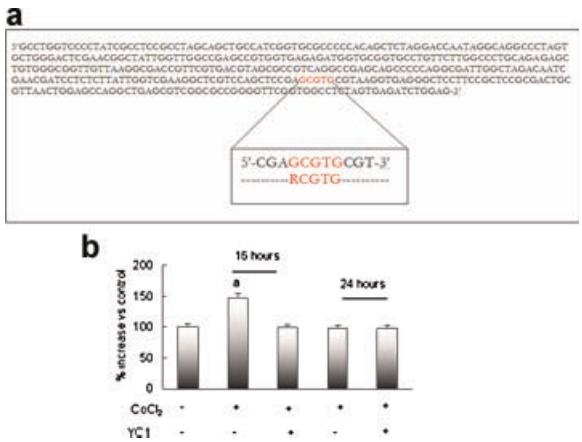

Fig. 6. HMG-CoAR transcription in HepG2 cells under chemical hypoxia. **Panel a** represents the HMG-CoAR human promoter, in red the HRE sequence. **Panel b** illustrates HMG-CoAR transcription expressed as percent versus control of the arbitrary units obtained from the activity of luciferase linked to HMG-CoAR promoter. The experiments were performed in HepG2 cells treated for 16 and 24 h with 100 μM $CoCl_2$ in presence and in absence of YC-1. For methodological details see the main text. a: $P < 0.05$ as from a Dunnett test with the respect to the samples—$CoCl_2$, YC-1. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

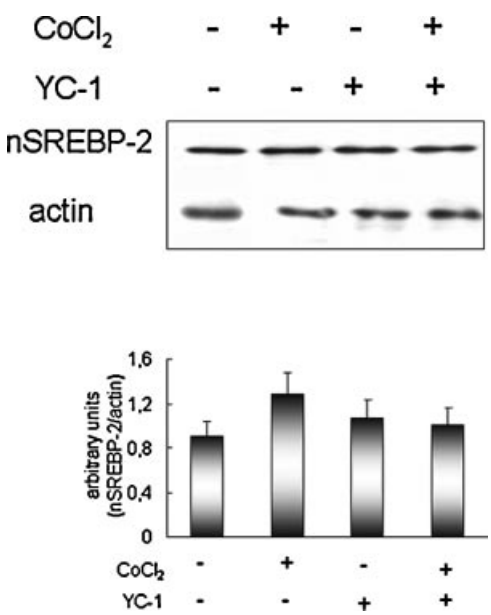

Fig. 7. Nuclear SREBP-2 level in HepG2 cells under chemical hypoxia. The figure illustrates a typical Western blotting and the densitometric analysis (main ± SD) obtained from four different experiments. Cells were treated with 100 μM $CoCl_2$ in presence and in absence of 10 μM YC-1, then cells were lysated and subjected to SDS–PAGE. For methodological details see the main text.

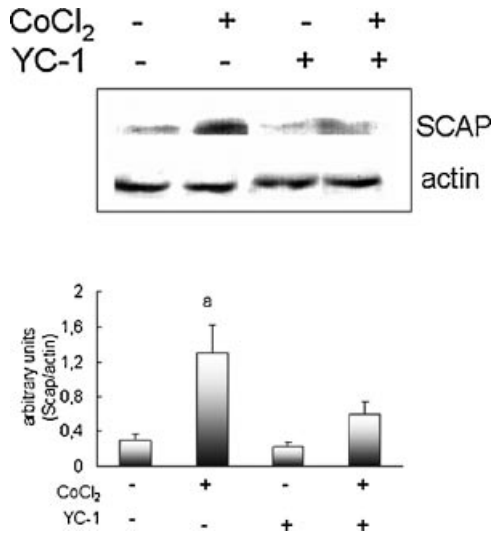

Fig. 8. SCAP level in HepG2 cells under chemical hypoxia. The figure illustrates a typical Western blot and the densitometric analysis (main ± SD) obtained from four different experiments. Cells were treated with 100 μM $CoCl_2$ in presence and in absence of 10 μM YC-1, then cells were lysated and subjected to SDS–PAGE. For methodological details see the main text. a: $P < 0.001$ as from a Dunnett test with the respect to the samples—$CoCl_2$, YC-1.

after treatment of the cells with $CoCl_2$; the increase does not occur in presence of YC-1.

DISCUSSION

In aerobic organisms the lack of oxygen induces several physiological responses which contribute to the survival of the cells in the hypoxic conditions.

HIF-1 plays a key role in the adaptation of cells to hypoxia by activating the transcription genes involved in several biological processes [Patiar and Harris, 2006]. Some of which require metabolic modifications and among these changes in the cholesterol metabolism have been recorded [Mukodani et al., 1990; Li et al., 2005b]. HMG-CoAR is the key enzyme of the cholesterol biosynthetic pathway responsible for the synthesis of sterols and non-sterol isoprenoid compounds. Because the chemical hypoxia, maintains high HIF-1α levels, even in normoxic conditions, allows for the study the role of the transcription factor in HMG-CoAR regulation. The increase of HIF-1α is related to a rise of HMG-CoAR and probably is involved in an enhanced transcription of the enzyme with no effect on HMG-CoAR degradation. The raised transcription of the reductase could be ascribed to a direct induction by HIF-1α even if

the increased level of SCAP suggests that an action of the actively translocated SREBP-2 cannot be excluded.

All the results indicate the need for HMG-CoAR activity in the HepG2 cells to survive and proliferate also in hypoxic condition.

These features seem to contrast previous data by Mukodani et al. [1990] and Li et al. [2005a,b] which showed in other cellular models, fibroblasts and "in vivo" mouse liver, respectively, that hypoxia modifies lipid metabolism without affecting cholesterol synthesis.

On the other hand Nguyen et al. [2007] have recently described a link between synthesis of cholesterol and oxygen sensing in animal cells. In their model the link is provided both by hypoxia-induced accumulation of lanosterols and HIF-1α mediated induction of Insigs. Convergences of these signals should lead to rapid degradation of HMG-CoAR, so limiting cholesterol synthesis.

In contrast, it has been assessed that the lack of oxygen in fission yeast *Saccharomyces pombe* induces a drop in cholesterol synthesis which in turn activates SREBP-mediated transcription of target genes [well reviewed in Robichon and Dugail, 2007]; moreover in a renal model that mimics an hypoxic status, an increase of HMG-CoAR at mRNA and protein level has been observed [Zager et al., 2007].

In our model of HepG2 cells, the reduction of the oxygen level works by increasing HMG-CoAR activity.

Whether or not this difference arises from the nature and the origin of the cell is hard to define at present but the possibility is intriguing and should be looked into in more detail. In fact, proving this hypothesis would be an important step to understanding some differences among experimental models, that is cells derived from pathological or physiological situations, and would also provide useful suggestions for therapeutic efforts.

So, the data obtained in this work should constitute a starting point for further studies addressed to the comprehension of the complicated relationship between hypoxia and cholesterol biosynthetic pathway regulation in different biological systems.

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