Retinoic acid receptor-related orphan receptor (ROR) α4 is the predominant isoform of the nuclear receptor RORα in the liver and is up-regulated by hypoxia in HepG2 human hepatoma cells

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The retinoic acid receptor-related orphan receptor α (RORα) is critically involved in many physiological functions in several organs. We find that the main RORα isoform in the mouse liver is the RORα4 isoform, in terms of both mRNA and protein levels, while the RORα1 isoform is less abundant. Because hypoxia is a major feature of liver physiology and pathology, we examined the effect of this stress on *Rora* gene expression and RORα transcriptional activity. HepG2 human hepatoma cells were cultured for 24 h under normoxia (20 % O_2) or hypoxia (10, 2, and 0.1 % O_2) and the abundance of the *Rora* transcripts measured by Northern blot and semi-quantitative RT-PCR. Hypoxic HepG2 cells contained more *Rora* mRNA than controls. This was also observed in rat hepatocytes in primary culture. Cobalt chloride and desferrioxamine also increased the amount of *Rora* mRNA in HepG2 cells. It is likely that these treatments increase the amount of the RORα4 protein in HepG2 cells as evidenced by Western blotting in the case of desferrioxamine. Transient transfection experiments indicated that hypoxia, cobalt chloride, and desferrioxamine all stimulate RORα transcriptional activity in HepG2 cells. Hence, we believe that RORα participates in the control of gene transcription in hepatic cells and modulates gene expression in response to hypoxic stress.

Key words: transcription factor, low oxygen tension, hepatic cell.

INTRODUCTION

Nuclear hormone receptors are ligand-regulated transcription factors that modulate the expression of target genes in response to physiological and environmental stimuli (for review, see [1]). Many nuclear hormone receptors have been identified over the past ten years using similarities between their sequences and those of hormone receptors, such as steroid and retinoid receptors. Subsequent investigations have focussed on the functions of these newly identified regulators of gene transcription. One challenge is to identify their target genes. Another is to describe the regulatory pathway(s) that modulate the production and activity of the nuclear receptors, as this is essential for understanding their physiological function. This includes finding their specific ligands and the stimuli that alter their production and/or activity.

The retinoic acid receptor-related orphan receptor α (RORα) (NR1F1 in the unified nomenclature [2]) belongs to the steroid hormone receptor superfamily and is closely related to the retinoic acid receptors [3,4]. RORα is classified as an orphan receptor because no natural ligand has yet been identified. RORα usually binds as a monomer to a ROR response element (RORE) composed of a 6 bp AT-rich sequence 5′ to a half-site core PuGGTCA motif, and activates transcription [4–7]. The *Rora* gene generates four isoforms by a combination of alternative promoter use and exon splicing: RORα1, RORα2, RORα3, and RORα4 (also termed RZRα). These isoforms have common DNA and putative ligand-binding domains, but differ by their amino-terminal sequences, which confer slightly different DNA binding preferences [3,4,8,9]. The wide distribution of the *Rora* transcripts suggests that this receptor functions in several organs, including the brain, heart, liver, testis, and skin [5]. RORα plays a crucial role in the central nervous system. *Staggerer* ($Rora^{sg}/Rora^{sg}$) mice, whose *Rora* gene has a deletion [8], and RORα-deficient mice obtained by homologous recombination, both suffer from severe cerebellar ataxia due to a defect in the development of their Purkinje cells [10–12]. RORα has also been recently shown to be involved in susceptibility to atherosclerosis and hypoalphalipoproteinaemia [13,14], regulation of the inflammatory response [15], myogenesis [16], and bone formation [17]. *Rora* gene expression is also up-regulated during adipocyte differentiation [18]. Lastly, thyroid hormone stimulates *Rora* gene expression in the cerebellum [19], and the Ca^{2+}/calmodulin-dependent kinase IV activates the RORα transcriptional activity [20,21]. This suggests that studying the role of RORα in different organs can provide useful information. However, little is known of the physiological or pathophysiological stimuli that regulate RORα synthesis and transcriptional activity.

We recently established that RORα stimulates the transcription of the gene coding for α-fetoprotein, a plasma protein principally produced by the fetal liver and yolk sac, and also by the adult liver undergoing regeneration or carcinogenesis [22]. This result prompted us to postulate that RORα is involved in liver physiology and/or pathology. This involvement was recently confirmed with the demonstration that, in liver, RORα controls the transcription of the gene encoding apolipoprotein C-III, a glycoprotein that plays a key role in plasma triglyceride metabolism [23].

Decreased tissue oxygenation (hypoxia) is an essential modulator of liver physiology and pathology. The metabolic activity of the liver causes the oxygen tension to drop between the periportal and the perivenous zones as blood passes through the sinusoids. Under physiological conditions, this oxygen tension gradient is a key regulator for the zonal expression of genes coding for

Abbreviations used: ROR, retinoic acid receptor-related orphan receptor; RORE, ROR response element; RT-PCR, reverse transcription-PCR; sg, staggerer.

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metabolic enzymes (for review, see [24]). Various hormones and growth factors, including erythropoietin and vascular endothelial growth factor, are also regulated by oxygen tension (for review, see [25]). Hypoxia appears to be a major factor in several liver diseases, such as cirrhosis and hepatocellular carcinoma [26]. Hypoxia also plays a key role in the development of solid tumours, influencing processes such as genetic instability, cell growth and differentiation, metastasis, and angiogenesis (for review, see [27]).

We have therefore determined those RORα1–4 isoforms that are produced in liver cells, by identifying the mRNA and the protein present. We have also investigated the effect of hypoxia on *Rora* gene expression and RORα transcriptional activity in the hepatic cell, using the HepG2 human hepatoma cell line.

EXPERIMENTAL

Animals

Animal studies were conducted according to French guidelines for the care and use of experimental animals.

The *staggerer* mutation is maintained in a C57BL6/J genetic background in our breeding colony. It was developed from mice kindly provided by Professor Jean Mariani (UMR Neurobiologie des Processus Adaptatifs, Université P. et M. Curie, Paris, France). *Staggerer* ($Rora^{sg}/Rora^{sg}$) and wild-type ($Rora^{+}/Rora^{+}$) mice were obtained by crossing fertile heterozygous ($Rora^{+}/Rora^{sg}$) mice and identifying homozygous offspring by PCR genotyping [28]. Mice were housed at 24 ± 1 °C in a temperature-controlled room with a 12 h light-dark cycle. Water and food (A03 granules, UAR, Epinay-sur-Orge, France) were provided *ad libitum*. Mashed moistened food was provided for *staggerer* mice because their ataxia prevents them from feeding conventionally.

Hepatocytes were prepared from 2-month-old male rats of the Wistar CF strain.

Cell culture and experimental conditions

Human hepatoma HepG2 cells (American Type Culture Collection HB 8065) were cultured as monolayers at 37 °C in a humidified 95 % air/5 % CO_2 incubator. The culture medium used was a 1:1 mixture of Dulbecco's modified Eagle's medium, and Ham-F12 media with Glutamax-I, supplemented with 10 % (v/v) fetal bovine serum, 100 μg/ml gentamycin, and 2.5 μg/ml fungizone (all from Gibco/BRL). HepG2 cells were 70–80 % confluent before use.

HepG2 cells plated on 60-mm-diameter glass dishes were made hypoxic by placing them in stainless steel hypoxia chambers. The chambers were evacuated until the desired oxygen partial pressure, deduced by measuring the pressure with an electronic pressure sensor (SMC digital pressure sensor, Tokyo, Japan), was reached. The chambers were then refilled with a gas mixture containing 5 % CO_2 and 95 % N_2. Final oxygen concentrations of 10, 2, or 0.1 % were used. The chambers were then placed in an incubator at 37 °C for 24 h. Cells were still viable under these conditions (Trypan Blue exclusion, 98 % viability after 24 h at 0.1 % O_2).

When desired, HepG2 cells were incubated for 24 h with 100 μM cobalt chloride or desferrioxamine mesylate (Sigma), and for a further 24 h period after adding 100 μM cobalt chloride or desferrioxamine.

For transfection experiments, the hypoxic condition was 5 % O_2. Hepatocytes were prepared from 2-month-old Wistar CF rats as previously described [29] and cultured under 20 or 10 % O_2 for 24 h.

RNA isolation

Total RNA was isolated from HepG2 cells and mouse tissues by a one-step guanidinium thiocyanate-phenol-chloroform method, based on the protocol of Chomczynski and Sacchi [30], using TRIZOL™ LS reagent (Gibco/BRL) according to the manufacturer's guidelines. RNA was recovered in 0.5 mM EDTA and quantified by spectrophotometry at 260/280 nm. The integrity of each of the RNA samples was assessed by electrophoresis on 1 % (w/v) agarose gels (*28S*:*18S* ratio).

Semi-quantitative RT-PCR analysis

Reverse transcription was performed at 37 °C for 1 h in a total volume of 20 μl of first-strand buffer [250 mM Tris/HCl, 375 mM KCl, 15 mM $MgCl_2$] containing 12.5 ng/μl of the pd(T)12–18 primers (Amersham Bioscience, Orsay, France), 0.5 mM each dNTP (Gibco/BRL), 10 mM dithiothreitol, 200 U M-MLV reverse transcriptase (Gibco/BRL), 20 U RNasin (Promega, Charbonnières, France) and 2 μg of total RNA. The absence of contaminating DNA from each RNA sample was checked by omitting the reverse transcriptase from the RT reaction (RT control). When the reaction was complete, 2 μl of the cDNA solution were amplified in a final volume of 50 μl of PCR buffer [20 mM Tris/HCl, 50 mM KCl, 1.5 mM $MgCl_2$] containing 0.5 μM each primer, 0.2 mM each dNTP (Gibco/BRL), and 1 U *Taq* DNA polymerase (Gibco/BRL). The PCR primers used were: for *Rora* (all transcripts), RORα-5′, 5′-GTCAGCAGCTTCTACCTGGAC-3′, and RORα-3′, 5′-GTGTTGTTCTGAGAGTGAAAGGCACG-3′ (482 bp product); for *β-actin*, β-actin-5′, 5′-TCTACAATGAGCTGCGTGTGG-3′, and β-actin-3′, 5′-GGAACCGCTCATTGCCAATG-3′ (496 bp product). Each of the *Rora1-4* transcripts was analysed using the RORα-3′iso primer, 5′-AACAGTTCTTCTGACGAGGACAGG-3′ for all the isoforms, and the RORα1-5′ primer, 5′-GAGGTATCTCAGTCACGAAG-3′ (183 bp product) for *Rora1*; the RORα2-5′ primer, 5′-CAGTGTATCCTGTCTTCAGG-3′ (274 bp product) for *Rora2*; the RORα3-5′ primer, 5′-ACATAAACTGGGATGGAGCC-3′(234 bp product) for *Rora3*; and the RORα4-5′ primer, 5′-TGTGATCGCAGCGATGAAAG-3′ (170 bp product) for *Rora4*. The RORα isoform-specific PCR primer sequences were deduced from the published sequences of the human and mouse RORα1 and RORα4, and the human RORα2 and RORα3 cDNA (Genbank accession numbers U04897, U53228, L14611, Y08640, U04898, and U04899). The thermal cycle conditions included 1 cycle at 95 °C for 10 min, *n* cycles of (95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 sec), and 1 cycle at 72 °C for 10 min by using an iCycler thermal cycler (Bio-Rad, Marnes La Coquette, France). The number (*n*) of amplification cycles was 17 for *β-actin* and usually 25 for all the *Rora* targets. To ensure the absence of contaminating material in the PCR step, the product of the RT step was replaced by distilled water (PCR control). Amplification products (10 μl of the reaction mixture) were resolved by electrophoresis in a 1 % (w/v) agarose gel (2 % for the specific *Rora1–4* amplification products). The gels were stained with ethidium bromide (0.5 μg/ml) and photographed under UV light. The molecular weight marker used was the 1 kb DNA ladder (Gibco/BRL). Amplification products were then transferred to a nylon membrane (Hybond N^+, Amersham Bioscience) and fixed by exposure to UV light. The RORα, RORα4, and β-actin cDNA fragments used as probes were obtained by PCR amplification of the pCMX-mRORα4 expression plasmid [4] (with the RORα-5′ and RORα-3′ primers for RORα, and with the RORα4-5′ and RORα-3′iso primers for RORα4) and the HepG2 cDNA (with the β-actin-5′ and β-actin-3′ primers), respectively. Probes

were labelled with [α-^{32}P]-dATP using the Prime-It II random labelling kit (Stratagene). The membranes were prehybridized for 2 h at 65 °C in the hybridization buffer [25 mM phosphate buffer, pH 6.8, 1 mM EDTA, pH 8, 7 % (w/v) SDS, 1 % (w/v) BSA Cohn fraction V] and then hybridized with the [^{32}P]-labelled probe (1–2 × 10^6 cpm/ml hybridization buffer) for 16 h at 65 °C. The hybridized membranes were washed twice at room temperature for 15 min each with 2 × SSC, 0.1 % SDS and once for 30 min with 0.1 × SSC, 0.1 % SDS at 60 °C. Radioactive signals were detected by exposing the filters to Biomax Hyperfilms MP (Kodak). The radioactivity in the bands was quantified with an InstantImager (Packard).

Northern blot analysis

Total RNA (10 μg/lane) was fractionated by electrophoresis through 1 % (w/v) agarose gel containing 0.7 M formaldehyde, transferred to a nylon membrane (Hybond N$^+$, Amersham Bioscience) and fixed by exposure to UV light. The integrity of the blotted RNA was evaluated by staining the *28S* and *18S* ribosomal RNA subunits with Methylene Blue. The RORα probe was obtained as described above. The membranes were hybridized in *Expresshyb* buffer (Clontech, Erembodegem, Belgium) following the manufacturer's guidelines, and radioactive signals were detected by exposing the filters to Biomax Hyperfilms MP (Kodak) with screens at −80 °C.

Preparation of nuclear extracts and Western blot analysis

Nuclear extracts from mouse liver were prepared according to Gorski et al. [31], except that 3 g aliquots of minced liver were diluted to 30 ml with homogenization buffer [2 M sucrose, 10 % (v/v) glycerol, 10 mM Hepes, pH 7.6, 15 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 0.3 mM spermine, 1 mM spermidine, 20 μg/ml aprotinin, and 1 μg/ml each of antipain, leupeptin, and pepstatin]. HepG2 cell nuclear extracts were prepared as previously described [32]. Briefly, about 5 × 10^7 cells were removed from plates using trypsin and suspended in 3 ml buffer H [0.4 M sucrose, 11.4 % (v/v) glycerol, 10 mM Hepes, pH 7.6, 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 0.15 mM spermine, 0.5 mM spermidine, 20 μg/ml aprotinin, and 1 μg/ml each of antipain, leupeptin, and pepstatin]. Cells were lysed by adding 6 ml of buffer H containing 0.5 % (v/v) NP40 and using a Dounce homogenizer (pestle A). The suspension was diluted with 9 ml of buffer H. Nuclei were recovered by centrifugation through a 0.5 M sucrose cushion (in buffer H). The nuclei extracts were then prepared as previously described [31]. Human RORα1 and mouse RORα4 proteins were produced *in vitro* from the pCMX-hRORα1 and pCMX-mRORα4 plasmids [4] using the TNT-T7 Quick coupled transcription/translation system (Promega). Aliquots of the protein extracts (25 μg of mouse liver extracts and 50 μg of HepG2 cells extracts) and of the programmed and unprogrammed lysates were electrophoresed by SDS/PAGE (10 % w/v gel) and transferred by semi-dry blotting to a nitrocellulose membrane (Amersham Bioscience) using standard procedures. The molecular weight marker used was the full range rainbow marker (Amersham Bioscience). The membrane was stained with Ponceau Red (Sigma) to confirm equal protein loading and transfer. The membrane was first incubated overnight at 4 °C in blocking solution [PBS, 0.1 % (v/v) Tween 20, 5 % (w/v) non-fat milk powder], and then for 5 h at 24 °C with a polyclonal antibody against RORα (sc-6062, Santa Cruz Biotechnology/Tebu, Le Perray en Yvelines, France) diluted 1:100 in blocking solution. The membrane was treated with a horseradish peroxidase-coupled secondary antibody (Sigma) diluted 1:4000 in blocking solution, and immunocomplexes were detected by enhanced chemiluminescence (ECL® kit, Amersham Bioscience), according to the manufacturer's guidelines.

Reporter plasmids, transient transfection and luciferase assay

The pRORE3-TK-Luc and pTK-Luc reporter plasmids were kindly provided by Professor Bart Staels (Institut Pasteur, Lille, France). Approximately 10^6 exponentially growing HepG2 cells were transfected using the calcium phosphate method [33] with 4 μg of pRORE3-TK-Luc or pTK-Luc reporter plasmid in a 60-mm-diameter dish. The precipitate was incubated with the cells for 4 h, the medium was replaced with fresh medium, and the cells were incubated for a further 16 h. Cells were then incubated under 5 or 20 % O_2, or with 100 μM cobalt chloride or desferrioxamine for 24 h. The cells were lysed in 200 μl of reporter lysis buffer (Promega) per dish. Protein concentrations were determined by the Bradford method [34] with bovine immunoglobulin as the standard (Bio-Rad). Luciferase activity was assayed as described previously [33].

Statistical analysis

Data are expressed as means ± S.E.M. of the number of experiments indicated in every case. Statistical analysis was carried out using the paired version of the Student's *t*-test. Differences were considered to be significant at $P < 0.05$.

RESULTS

RORα4: the main RORα isoform in the liver

We first detected the *Rora* mRNA in liver by RT-PCR with RORα specific primers designed to amplify all four *Rora* transcripts (Figure 1A). *Rora* transcripts were detected in the fetal and adult mouse liver, and in the mouse yolk sac, another tissue of endodermal origin (Figure 1B). We wanted to identify which of the four RORα isoforms generated by the *Rora* gene were present in these tissues. We designed primers for the selective detection of each *Rora1–4* transcript (Figure 1A). The *Rora4* transcript was readily detected in the liver and yolk sac using 25 cycles of PCR amplification (Figure 1B). The same PCR conditions did not reveal any *Rora1* transcript in these tissues, although this transcript was readily detected in the adult mouse brain (Figure 1B). This tissue was used as a positive control since it is known that the RORα1 isoform is present in the cerebellum [9]. However, the *Rora1* transcript was detected in the liver and yolk sac when the number of PCR cycles was increased to 35 (Figure 1B). *Rora2* and *Rora3* transcripts remained undetectable, even after 35 cycles of PCR amplification (data not shown). These data clearly indicate that the *Rora4* transcript is the main transcript in the mouse liver and yolk sac, while the concentration of *Rora1* transcript is much lower.

We analysed protein extracts from the livers of wild-type (*Rora*$^+$/*Rora*$^+$) and *staggerer* (*Rora*^{sg}/*Rora*^{sg}) adult mice by Western blotting to determine whether the *Rora* transcripts in the liver are translated into the corresponding proteins. *Rora*^{sg}/*Rora*^{sg} mice were used as negative controls as their *Rora* gene has a deletion that prevents production of a full length RORα protein. Preliminary Western blot experiments on total protein extracts from mouse liver showed unspecific immunoreactive bands near the expected molecular weight of the RORα proteins. These bands were unspecific because they were detected in both *Rora*$^+$/*Rora*$^+$ and *Rora*^{sg}/*Rora*^{sg} mice (data not shown). We therefore performed the Western blot experiments with protein extracts prepared from purified nuclei from the livers of

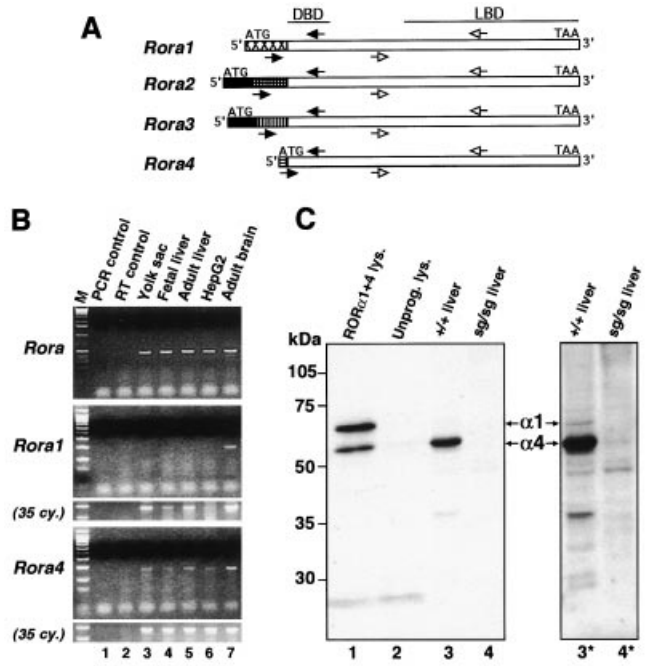

Figure 1 RORα isoforms in the mouse liver and HepG2 human hepatoma cells

(**A**) Location of the PCR primers on the RORα1–4 cDNAs. Similar (black and empty boxes) and distinct (hatched boxes) regions of the four RORα coding sequences, and regions coding for the DNA-binding domains (DBD) and the ligand-binding domains (LBD) are shown. Primers for detecting all of the *Rora* isoforms (RORα-5′ and RORα-3′) are depicted as white arrows. Primers for the specific detection of each of the four isoforms (RORα1-5′, RORα2-5′, RORα3-5′, RORα4-5′, and RORα-3′iso) are depicted as black arrows. (**B**) Total RNA from E17 mouse yolk sac (lanes 3) and liver (lanes 4), 2-month-old mouse liver (lanes 5), HepG2 human hepatoma cells (lanes 6), and 2-month-old mouse brain (lanes 7) were analysed by RT-PCR for the presence of *Rora* (all isoforms) (upper panel), of *Rora1* (middle panels), and of *Rora*4 (lower panels) transcripts. The PCR reactions were performed with 25 or 35 cycles (35 cy.). The PCR control (lanes 1) and a representative RT control (lanes 2) are shown. M, molecular mass markers. (**C**) Aliquots of liver nuclear extracts from 3-month-old wild-type (lanes 3 and 3*) and *staggerer* (lanes 4 and 4*) mice were analysed by Western blotting with an antibody against RORα. Lanes 3* and 4* correspond to a five-fold longer exposure of the ECL® revelation reaction than lanes 3 and 4. A mix of lysates programmed for producing RORα1 and RORα4 proteins (lane 1) and the control unprogrammed lysate (lane 2) were processed simultaneously.

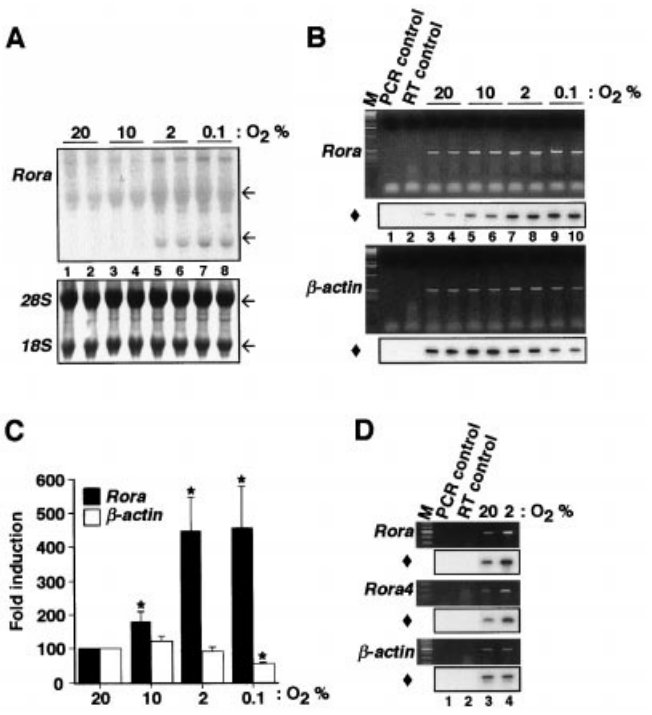

Figure 2 Increased *Rora* transcripts in HepG2 hepatoma cells grown under hypoxia

HepG2 cells were incubated in normoxia (20% O_2) or hypoxia (10, 2, or 0.1% O_2) for 24 h. (**A**) Aliquots of total RNA were analysed for *Rora* mRNA (all isoforms) by Northern blotting (top panel). The Methylene Blue staining of RNA is shown as a control of RNA loading and transfer (bottom panel). The positions of the *18S* and *28S* rRNA are marked with arrows. Two samples are shown for each condition of culture. (**B**) Total RNA was analysed for *Rora* mRNA (all isoforms) by semi-quantitative RT-PCR. *β-actin* mRNA was amplified to confirm the use of equal amounts of cDNA in the PCR step and to allow PCR products to be quantified comparatively. Aliquots of the PCR mixture were electrophoresed and transferred to nitrocellulose filters, which were then hybridized with probes specific for *Rora* or *β-actin*. Ethidium bromide stained gels and their corresponding autoradiograms (◆) of a typical experiment with two samples are shown. The PCR control (lanes 1) and a representative RT control (lanes 2) are shown. M, molecular weight markers. (**C**) The intensities of the radioactive signals corresponding to *Rora* (black columns) and *β-actin* (open columns) amplification products were quantified by InstantImager (Packard). Results are expressed relative to the values at 20% O_2. They are given as means ± S.E.M. of five independent experiments, each carried out in triplicate. Significant differences ($P < 0.05$) from 20% O_2 controls are indicated by ∗. (**D**) Total RNA was analysed for *Rora* mRNA (all isoforms) and *Rora4* mRNA by semi-quantitative RT-PCR as described in (**B**) for the 20% (lanes 3) and 2% (lanes 4) O_2 conditions. A representative experiment out of six is shown.

adult $Rora^{+}/Rora^{+}$ and $Rora^{sg}/Rora^{sg}$ mice. RORα1 and RORα4 proteins produced *in vitro* were used as controls. An immunoreactive band with the same mobility as the *in vitro* produced RORα4 was readily detected in liver nuclear extracts prepared from $Rora^{+}/Rora^{+}$ mice (Figure 1C, lane 3). An immunoreactive band with the same mobility as the *in vitro* produced RORα1 was also detected in liver nuclear extracts of $Rora^{+}/Rora^{+}$ mice when autoradiographic films were exposed for longer (Figure 1C, lane 3*). None of these bands was detected in the liver nuclear extracts from $Rora^{sg}/Rora^{sg}$ mice (Figure 1C, lanes 4 and 4*). Thus RORα4 and RORα1 proteins are present in the nuclei of hepatic cells and the relative concentrations of RORα1 and RORα4 proteins reflect those of the corresponding mRNAs. Hence, our RT-PCR and Western blotting results indicate that RORα4 is the most abundant RORα isoform in the liver. We never detected any specific immunoreactive proteins with a molecular mass corresponding to that of the putative RORα truncated proteins in liver nuclear extracts from $Rora^{sg}/Rora^{sg}$ mice (Figure 1C, lanes 4 and 4* and data not shown).

Stimulation of *Rora* gene expression by hypoxia in HepG2 cells and in adult rat hepatocytes in primary culture

We studied the effect of hypoxia, a stimulus implicated in liver physiology and pathology, on *Rora* gene expression. We chose the HepG2 human hepatoma cell line, a widely used model for studying the effects of oxygen deprivation on gene expression. *Rora* gene expression was detected by measuring the mRNA by RT-PCR. The *Rora4* transcript was more abundant than the *Rora1* transcript in HepG2 cells, as in the mouse liver (Figure 1B). The *Rora2* and *Rora3* transcripts were not detected in HepG2 cells, even after 35 cycles of PCR amplification (data not shown). We examined the effect of hypoxia on *Rora* mRNA amount, by Northern blotting of total RNA samples from HepG2 cells cultured for 24 h under oxygen concentrations of 20, 10, 2, and 0.1% (Figure 2A). There were more *Rora* transcripts in HepG2 cells cultured under 2 or 0.1% O_2 than in

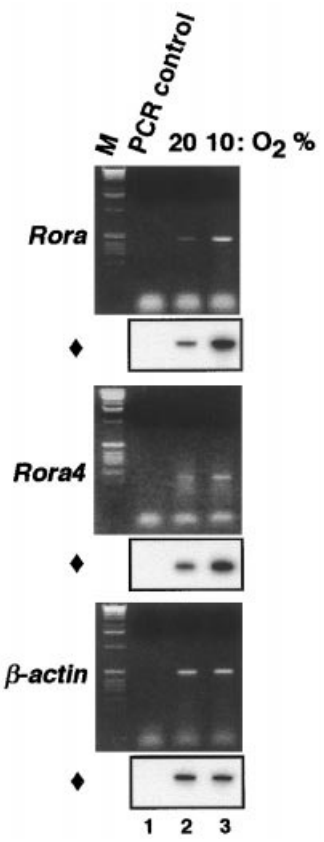

Figure 3 Increased *Rora* transcripts in rat primary hepatocytes grown under hypoxia

Adult rat primary hepatocytes were incubated in normoxia (20% O_2, lanes 2) or hypoxia (10% O_2, lanes 3) for 24 h. Total RNA was analysed for *Rora* mRNA (all isoforms) and *Rora4* mRNA by semi-quantitative RT-PCR. *β-actin* mRNA was amplified to confirm the use of equal amounts of cDNA in the PCR step. Aliquots of the PCR mixture were electrophoresed and transferred to nitrocellulose filters, which were then hybridized with probes specific for *Rora* (all isoforms), *Rora4*, or *β-actin*. Ethidium bromide stained gels and their corresponding autoradiograms (◆) of a typical experiment out of three are shown. The PCR control (lanes 1) is shown. M, molecular weight markers.

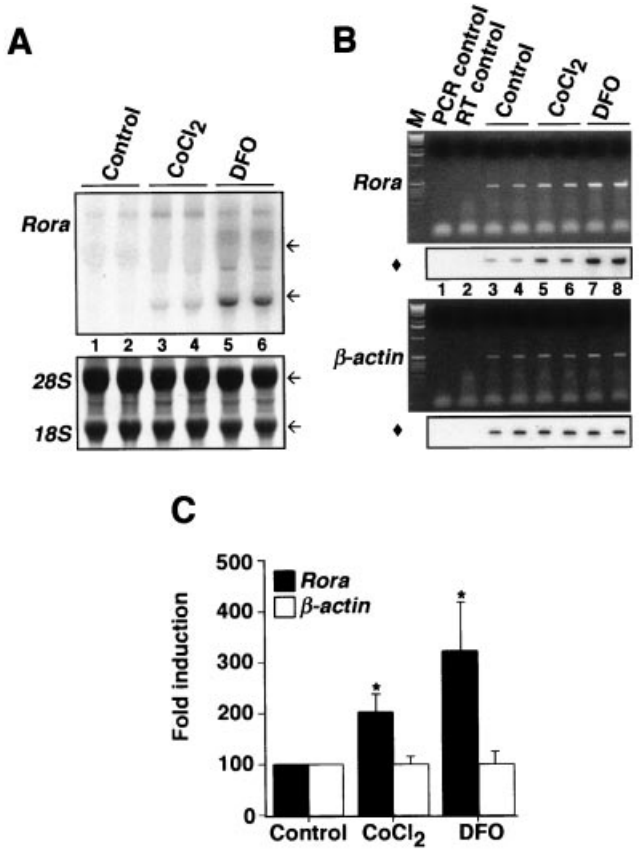

Figure 4 Increased *Rora* transcripts in HepG2 hepatoma cells treated with cobalt chloride and desferrioxamine

HepG2 cells were untreated (Control) or treated with $CoCl_2$ or desferrioxamine (DFO) as described in the Experimental section. (**A**) Aliquots of total RNA were analysed for *Rora* mRNA (all isoforms) by Northern blotting (top panel). The methylene blue staining of RNA is shown as a control of RNA loading and transfer (bottom panel). The positions of the *18S* and *28S* rRNA are marked with arrows. Two samples are shown for each condition of culture. (**B**) Total RNA was analysed for *Rora* mRNA (all isoforms) by semi-quantitative RT-PCR. *β-actin* mRNA was amplified to confirm the use of equal amounts of cDNA in the PCR step and to allow PCR products to be quantified comparatively. Aliquots of the PCR mixture were electrophoresed and transferred to nitrocellulose filters, which were then hybridized with probes specific for *Rora* or *β-actin*. Ethidium bromide stained gels and their corresponding autoradiograms (◆) of a typical experiment with two samples are shown. The PCR control (lanes 1) and a representative RT control (lanes 2) are shown. M, molecular weight markers. (**C**) The intensities of the radioactive signals corresponding to *Rora* (black columns) and *β-actin* (open columns) amplification products were quantified by InstantImager (Packard). Results are expressed relative to the values of the control. They are given as means $\pm$ S.E.M. for four independent experiments, each carried out in triplicate. Significant differences ($P < 0.05$) from the controls are indicated by $*$.

HepG2 cells cultured under 20 % O_2. However, the pattern of the *Rora* transcripts in these Northern blot experiments was too complex for optimal and accurate quantification of the effect of hypoxia on *Rora* gene expression. We therefore developed a semi-quantitative RT-PCR assay. Part of the *β-actin* transcript was amplified to check reverse transcription efficiency and that equal amounts of RNA were used. Preliminary experiments showed that 25 cycles were optimal for the semi-quantitative analysis of *Rora* mRNA and 17 for *β-actin* mRNA (data not shown). We therefore used these conditions to measure the *Rora* mRNA in total RNA from HepG2 cells cultured for 24 h under 20, 10, 2, or 0.1 % O_2 (Figure 2B and C). HepG2 cells incubated under 10 % O_2 had slightly increased *Rora* transcripts (about 1.8 times that of the 20 % O_2 control). Incubation under 2 or 0.1 % O_2 resulted in step-wise increases in *Rora* mRNA (about 4.5 times that of the 20 % O_2 control). Similar results were obtained using the RORα4 specific PCR primers (Figure 2D). In contrast, the abundance of *β-actin* mRNA was not significantly affected by hypoxia, except that incubation under 0.1 % O_2 led to a 40 % decrease in the amount of the *β-actin* mRNA compared to the 20 % O_2 control. The Northern blot and the RT-PCR analyses, therefore, show that in HepG2 hepatoma cells, hypoxia specifically stimulates expression of the *Rora* gene in a manner that depends on the degree of oxygen deprivation. We used adult rat hepatocytes in primary culture to determine if the results obtained from HepG2 hepatoma cells were relevant in a model closer to the *in vivo* situation. The amount of *Rora* transcripts was increased in adult rat hepatocytes incubated for 24 h under 10 % O_2 (Figure 3).

Stimulation of *Rora* gene expression in HepG2 cells by cobalt chloride and desferrioxamine

The metal ion cobalt and the iron chelator desferrioxamine very often mimic the effects of hypoxia [35,36]. Therefore, we tested the effects of these chemicals on *Rora* gene expression. Both the Northern blot and the semi-quantitative RT-PCR studies showed

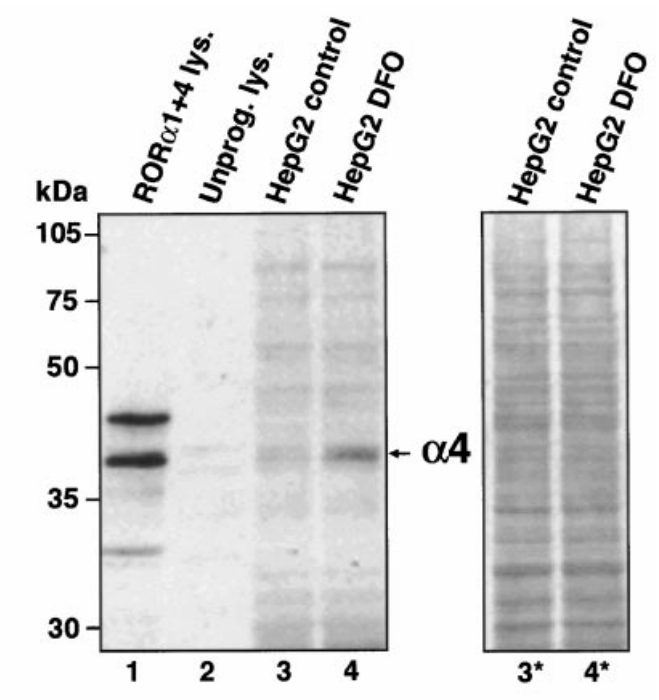

Figure 5 Increased RORα protein in HepG2 hepatoma cells treated with desferrioxamine

Nuclear proteins were prepared from HepG2 cells either untreated (lanes 3 and 3*) or treated with desferrioxamine (lanes 4 and 4*) as described in the Experimental section. Aliquots of the nuclear extracts were analysed by Western blotting with an antibody against RORα (lanes 3 and 4). The Ponceau Red staining of the proteins present in lanes 3 and 4 is shown in the right part of the figure as a control for protein loading and transfer (lanes 3* and 4*). A mix of lysates programmed for producing RORα1 and RORα4 proteins (lane 1) and the control unprogrammed lysate (lane 2) were processed simultaneously.

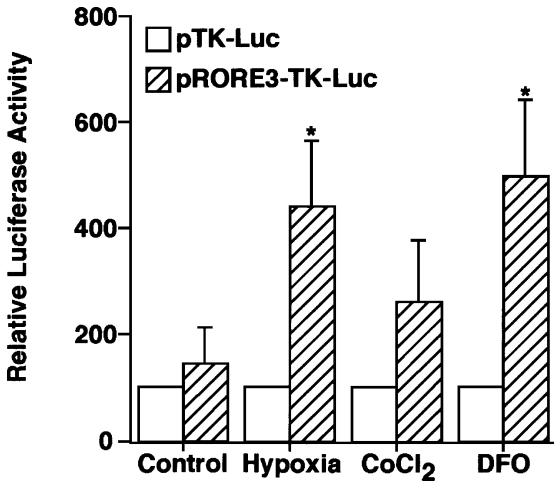

Figure 6 Enhanced RORα transcriptional activity in HepG2 hepatoma cells treated with hypoxia, cobalt chloride, and desferrioxamine

HepG2 cells were transfected with 4 μg of the pRORE3-TK-Luc reporter plasmid, which contains a tandem of 3 ROREs in front of the TK promoter and the luciferase reporter gene, or with 4 μg of the pTK-Luc control plasmid that does not contain the ROREs. Cells were left untreated (Control), treated with 5% O_2 (Hypoxia), or with 100 μM $CoCl_2$ or desferrioxamine (DFO) for 24 h. The luciferase activity of the pRORE3-TK-Luc plasmid (hatched columns) is expressed relative to that of the pTK-Luc control plasmid (open columns) measured under the same conditions. The results are given as means $\pm$ S.E.M. of at least four independent experiments, each carried out in duplicate. Significant differences ($P < 0.05$) from the control are indicated by $*$.

that cobalt chloride or desferrioxamine increased the amount of *Rora* mRNA in HepG2 cells (Figure 4). Thus, cobalt chloride and desferrioxamine, like hypoxia, both influence the amounts of *Rora* gene transcripts in HepG2 cells.

We then used desferrioxamine to determine whether the increase in the abundance of *Rora* mRNA was associated with an increase in RORα protein. Western blot experiments were performed on nuclear extracts from untreated HepG2 cells, and from HepG2 cells incubated with desferrioxamine. Nuclear extracts from HepG2 cells treated with desferrioxamine gave an immunoreactive band, with the same mobility as the *in vitro* produced RORα4, which was more intense than in nuclear extracts from untreated HepG2 cells (Figure 5). This clearly showed that desferrioxamine treatment increased the amount of RORα4 protein in the nuclei of HepG2 cells. And the unchanged apparent molecular mass of the RORα4 protein after desferrioxamine treatment also suggests that there are no or few post-translational modifications, such as changes in phosphorylation in response to such a stress.

Stimulation of RORα transcriptional activity by hypoxia, cobalt chloride, and desferrioxamine

We used transient transfection experiments to analyse the effect of hypoxic stress on RORα transcriptional activity in HepG2 cells. HepG2 cells were transfected with a plasmid bearing the luciferase reporter gene under the control of the herpes simplex virus thymidine kinase promoter alone (pTK-Luc plasmid), or together with three ROREs in tandem (pRORE3-TK-Luc plasmid). The transfected HepG2 cells were incubated for 24 h under normoxic (20% O_2) or hypoxic (5% O_2) conditions. This hypoxic condition (5% O_2) was used because we observed that, under more severe conditions (2 or 0.1% O_2), cumulative stresses induced by transfection and hypoxia led to decreased cell viability. Hypoxia increased the transcriptional activity driven by the DNA sequence containing the three ROREs (Figure 6). Similar results were obtained when HepG2 cells were incubated for 24 h with 100 μM cobalt chloride or desferrioxamine (Figure 6). Thus, hypoxia, cobalt chloride and desferrioxamine all increase RORα transcriptional activity. These data strongly suggest that hypoxia, cobalt chloride and desferrioxamine stimulate the expression of the *Rora* gene, in terms of both mRNA and protein production. These, in turn, increase the RORα transcriptional activity in HepG2 cells.

DISCUSSION

The orphan nuclear receptor RORα appears to be crucial for many physiological processes that occur in tissues such as the cerebellum, adipose tissue, muscle, and bone (for reviews, see [37,38]). The present study was performed to better understand the role of the nuclear orphan receptor RORα in the liver. We find that the *Rora4* transcript is the most abundant *Rora* transcript in the mouse yolk sac and fetal and adult liver, and in HepG2 human hepatoma cells. This is in agreement with studies showing the wide tissue distribution of the *Rora4* transcript [3,9].

It is crucial that any attempt to understand the role of a transcription factor in a given tissue demonstrates that the corresponding protein is present in that tissue. Some transcripts are not translated into protein. The transcript encoding the D-binding protein (DBP) transcription factor, for instance, is detected in several tissues but the DBP protein is present only in

the liver [39]. Our Western blot results show that there is RORα4 protein in the nuclei of the adult mouse liver and HepG2 cells. RORα1 transcript and protein are also present in the mouse liver, but they are less abundant than the RORα4 transcript and protein. Up until now, the RORα1 isoform was known to be specifically produced in the central nervous system [9] but we found that the RORα1 and RORα4 isoforms were both present in an organ other than the brain. In addition, we bring substantial information as to the sub-cellular localization of the RORα proteins. Our results demonstrate that the RORα1 and RORα4 proteins are present, at least, in the nucleus of liver cells under normal conditions. The presence of both RORα1 and RORα4 in the mouse liver strongly suggests that they are involved in the control of liver gene transcription. These results, in conjunction with those obtained by others for the liver [23] and for the intestine [13], indicate that RORα controls gene expression in several organs of endodermal origin, such as the yolk sac, liver and intestine.

The *staggerer* natural mutation is a genomic deletion that results in a stop codon preventing the translation of the putative ligand-binding domain of the RORα protein [8]. The truncated RORα protein can still bind to DNA *in vitro* [7], and behaves as a negative dominant during transfection experiments in muscle cells [16]. The existence of the truncated protein *in vivo* has not yet been addressed. We find no specific immunoreactive band with a higher electrophoretic mobility than the RORα4 protein that might correspond to a putative $ROR\alpha^{sg}$ truncated protein in the liver of $Rora^{sg}/Rora^{sg}$ mice. However, the shorter $Rora^{sg}$ transcript has been detected *in vivo* in the liver (data not shown) and intestine [13] of $Rora^{sg}/Rora^{sg}$ mice. Consequently, our inability to detect the corresponding protein in the liver may reflect the poor translation of the short mRNA and/or the instability of the truncated $ROR\alpha^{sg}$ protein in the *staggerer* mutant.

Hypoxia regulates gene expression in a complex fashion, with control of both transcriptional and post-transcriptional events [25,40]. Hypoxia-inducible factor-1 (HIF-1) is important for the modulation of gene transcription in response to hypoxia (for review, see [25]). Other transcription factors, such as the Sp factors [41], AP-1 [42], p53 [43], and NFκB [44], may also all participate in the regulation of gene transcription by hypoxia. Studies on HNF-4 [45,46], PPARα [47] and RXR [48] suggest that changes in nuclear receptor signalling could be a novel mechanism by which hypoxia controls gene expression. Our present results support this interesting concept; they show that hypoxia stimulates the expression and transcriptional activity of another nuclear receptor: RORα.

The liver plays a major role in adaptative responses to hypoxia, in addition to its essential functions in metabolism, inflammatory response, hormonal control, and blood homoeostasis. The most prominent finding of our study is that hypoxia increases the amount of the *Rora* transcripts in HepG2 human hepatoma cells in a dose-dependent manner. Interestingly, this up-regulation of the *Rora* gene expression was also observed in rat hepatocytes cultured under hypoxic conditions (10 % O_2) which are physiologically relevant. The hypoxic stress also increases the RORα transcriptional activity in HepG2 cells. These results are reinforced by those obtained with cobalt chloride and desferrioxamine, chemicals that can mimic the effects of hypoxia on gene expression [35,36]. We also find an increase in the amount of the RORα4 protein in HepG2 cells treated with desferrioxamine, establishing the effect of such a stress on *Rora* gene expression at the protein level. It is reasonable to think that hypoxia and cobalt chloride also increase the amount of RORα protein, in addition to increasing the amount of *Rora* mRNA. Taken together, our Western blot and transient transfection experiments strongly suggest that hypoxia increases RORα transcriptional activity by, at least partially, up-regulating the amount of RORα protein in the nucleus.

The α-fetoprotein and apolipoprotein C-III genes are the only putative RORα target genes identified in the liver to date. Functional ROREs have been evidenced by electrophoretic mobility shift assays and transient transfection experiments in the transcription regulatory sequences of the genes encoding α-fetoprotein [22] and apolipoprotein C-III [23]. In addition, the expression of apolipoprotein C-III in liver was found to be decreased in the *staggerer* mutant [23]. The identification of new target genes for RORα in liver will help to better define the function of RORα in this organ.

RORα could also be involved in adapting the organism to hypoxic conditions and in disorders in which hypoxia occurs. This may not concern only the liver, but also other organs such as brain or heart, for which ischaemic stroke can have very deleterious consequences. The effects of hypoxia on RORα could also be meaningful in pathological processes such as carcinogenesis. Hypoxia is a major feature of all solid tumours [27]. RORα may well be implicated in oncogenesis since this nuclear receptor controls expression of the genes encoding the N-myc proto-oncogene [49], the cell cycle negative regulator $p21^{Waf-1/Cip-1}$ [50], and interacts directly with the metastasis suppressor Nm23 [51]. Our data showing that RORα responds to hypoxia further strengthen this hypothesis.

In conclusion, we show that hypoxia stimulates *Rora* gene expression and RORα transcriptional activity in cells of hepatic origin, pointing to RORα having a role in the liver under physiological and pathological conditions. The function of this orphan nuclear receptor in the liver must be investigated further, with the identification of new RORα target genes being the next step in this process. The notion that RORα activity is affected by hypoxia may be helpful in this goal. More generally, RORα may also be essential for the adaptation of other organs or cell types to oxygen deprivation and in such processes as angiogenesis and cancerogenesis. This opens the way for further studies on the function of RORα.

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