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Mutual regulation of hypoxic and retinoic acid related signalling in tubular proximal cells

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ABSTRACT

Hypoxia-inducible factor-1α (HIF-1α) and all-trans retinoic acid (ATRA) afford protection in several experimental models of kidney disease. HIF-1α protein is degraded under normoxia but stabilized by hypoxia, which activates its transcription factor function. ATRA activates another set of transcription factors, the retinoic acid receptors (RAR) α, β and γ, which mediate its effects on target genes. ATRA also up-regulates the expression of RAR α, β and γ at the transcriptional level. Here we demonstrate the presence of mutual regulation of hypoxic and retinoic acid related signalling in tubular proximal cells. In human proximal tubular HK-2 cells we have found that: (i) ATRA treatment induces HIF-1α under normoxic conditions and also synergizes with hypoxia leading to the over-expression of HIF-1α and vascular endothelial growth factor-A, a HIF-1α-regulated renal protector. ATRA-induced HIF-1α expression involved stabilization of HIF-1α mRNA but not of HIF-1α protein. (ii) Expression of HIF-1α is an absolute requirement for the transcriptional up-regulation of RARβ by ATRA. Transfection with HIF-1α siRNA abolished the induction by ATRA of the expression of both RARβ mRNA and protein while treatment with HIF-1α inhibitor YC-1 results in the abolishment of ATRA-induced activity of a retinoic acid-response element (RARE) construct from the RARβ promoter. (iii) Hypoxia up-regulates RARβ through HIF-1α since this effect was inhibited by HIF-1α knockdown. In contrast to ATRA-induced RARβ up-regulation, induction of RARβ expression by ATRA did not involve transcriptional up-regulation as hypoxia did not increase the expression of RARβ mRNA or the activity of the RARE construct. These results suggest the presence of crosstalk between hypoxia/HIF-1α and ATRA/RARβ that may be physiologically and pharmacologically relevant.

1. Introduction

Transcriptional adaptive responses to hypoxia are controlled primarily through hypoxia-inducible factor (HIF). HIF is a heterodimeric transcription factor consisting of a constitutively expressed β subunit and two α subunits, HIF-1α or HIF-2α.

Both α subunits are mainly regulated by oxygen at the protein level. HIF-α is continuously synthesized but under aerobic conditions the HIF-1α subunit undergoes proline hydroxylation at Pro^{402} and Pro^{564} and is then recognized by von Hippel–Lindau (VHL)-dependent ubiquitin ligases and targeted for proteasomal degradation (Bardos and Ashcroft, 2005; Brahimi-Horn et al., 2005). The proline hydroxylases (PHD) are inhibited under hypoxia, allowing accumulation of HIF-1α, which translocates to the nucleus. Together with the β subunit and transcriptional coactivators, HIF-1α binds to hypoxia-responsive elements (HRE) in target genes (Lando et al., 2002). Several transcriptional targets are activated by HIF-1 that synergistically promotes survival under hypoxic conditions. These include enzymes involved in glucose uptake and metabolism, carbonic anhydrase IX, erythropoietin and vascular endothelial growth factor (VEGF) (Semenza, 2002). HIF-1α expression has also been shown to be increased in normoxic conditions by several cytokines and hormones by increasing HIF-1α transcription and/or translation (Zhou and Brüne, 2006) but not HIF-1α protein half-life.

HIF-1α targets several genes encoding nuclear receptors. For instance, HIF-1α reduces the DNA binding activity of PPARα/RXR in cardiomyocytes (Belanger et al., 2007). The activation of Nur77

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by HIF-1α under hypoxic conditions regulates production of propiomelanocortin in von-Hippel-Lindau mutated renal cell carcinoma (Choi et al., 2004). HIF-1α also regulates the glucocorticoid receptor 1 expression in hypoxic human proximal tubular cells HK-2 (Leonard et al., 2005). In addition, the gene encoding human retinoic acid-receptor-related orphan receptor alpha is a target for HIF-1α (Chauvet et al., 2004). To our knowledge, there are no studies addressing the regulation of retinoic acid receptors (RARs) by HIF-1α.

Biologically active retinoids, a family of vitamin A metabolites or analogues, are characterized by their capacity to bind and activate retinoid nuclear receptors, including RARs and/or retinoid X receptors (RXRs), each one having three isotypes, α, β and γ. All-trans retinoic acid (ATRA) is the carboxylic acid form of vitamin A and its major metabolite. The actions of ATRA are generally mediated by binding to RARs, which act as ligand-regulated transcription factors by heterodimerizing with the RXRs and binding to retinoic acid response elements (RARE) located in regulatory regions of target genes (Thacher et al., 2000). The gene regulatory elements of RARα, RARβ and RARγ contain RARE sequences (Balmer and Blomhoff, 2002) and are up-regulated upon treatment with ATRA.

HIF-1α and ATRA have been shown to have excellent preventive and therapeutic effects in various experimental models of kidney disease (Nangaku et al., 2008; Xu et al., 2004). To date, there are only two pieces of evidence suggesting crosstalk between hypoxia and its master regulator HIF-1α and the retinoid system: (i) ATRA-induced RARβ expression and RAR-mediated transcriptional activity in melanoma cell lines are modulated by hypoxia (Demary et al., 2001) and (ii) accumulation of HIF-1α plays a role in ATRA-induced differentiation of myeloid leukemic cells (Zhang et al., 2008).

The present work was undertaken to analyze the effect of ATRA on the expression of HIF-1α and the effect of hypoxia/HIF-1α on RARβ expression in human proximal tubular cells HK-2. These cells exhibit a non-lethal but dysfunctional phenotype under hypoxic conditions, which reflects the epithelial pathobiology of ischemic acute renal failure (Leonard et al., 2003). Our results suggest the presence of crosstalk between hypoxia/HIF-1α and ATRA/RARβ that may be physiologically and pharmacologically relevant.

2. Materials and methods

2.1. Reagents

ATRA, actinomycin D, cycloheximide, desferrioxamine and YC1 were purchased from Sigma Chemical Co (St. Louis, MO). Lipofectamine was acquired from Invitrogen (Carlsbad, CA). Nitrocellulose membrane was obtained from Bio-Rad (Hercules, CA), Enhanced chemiluminescence ECL detection system was purchased from Amersham Biosciences (Airlington, Heights, IL). Antibodies anti-human proteins were obtained from the following sources: monoclonal HIF-1α (Transduction Laboratories, BD Biosciences, Palo Alto, CA); polyclonal HIF-2α (Abcam, Cambridge, UK) anti-rabbit horseradish peroxidase (Calbiochem, San Diego, CA) and RARβ antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Polyclonal β-actin and anti-mouse horseradish peroxidase antibodies were obtained from Sigma.

2.2. Cell culture

Human proximal tubular epithelial cells (HK-2) cells were purchased from American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM-F12 supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin/amphoterycin B and 1% glutamine (Invitrogen, Carlsbad, CA) and 1% Insulin-Transferrin-Selenium (Sigma, St. Louis, MO). Cells were routinely cultured in 95% air, 5% CO_2 (normoxic conditions) at 37 °C. In all experiments, cells were plated at 70–90% confluence and when completely attached, they were treated for different time periods with ATRA (dissolved in ethanol/DMSO (1:1)) under hypoxic (1% oxygen) or normoxic conditions. When necessary, cells were also treated with actinomycin D, desferrioxamine, cycloheximide or YC-1 under the conditions specified in the legends to figures. Hypoxia experiments were performed in an *In vivo*$_{200}$ hypoxia workstation (Ruskinn Technology, West Yorkshire, United Kingdom).

2.3. Single-step real-time quantitative RT-PCR

HK-2 cells (0.5×10^6 cells) were stimulated with 10 μM ATRA, for different time periods. Total cell RNA was isolated with TriReagent (Sigma) according to the instructions of the manufacturer.

Real-time quantitative RT-PCR analysis was performed using SYBR Green PCR master mix, in one-step RT-PCR protocol according to manufacturer's protocol (Applied Biosystems). 45 ng of RNA was used for each sample. The thermal cycling parameters were 30 min at 48 °C for RT and 10 min at 95 °C for polymerase enzyme activation, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primer sequences for HIF-1α mRNA, RARβ mRNA, $VEGF_{165}$ mRNA and the housekeeping gene β-actin were as follows (sequences 5′–3′): HIF-1α sense: GAAAGCGCAAGTCCTCAAAG, antisense TGGGTAGGAGATGGAGATGC; RARβ sense: GGT TTCACTGGCTTGACCAT, antisense AAGGCCGTCTGAGAAAGTCA; VEGF sense: GACAAGAAAATCCCTGTGGGCAAC, antisense GCGAGTCTGTGTTTTTGC; β-actin sense: GGTTTCACTGGCTTGACCAT, antisense CATGTCCCAGTTGGTGAC. All PCR were performed in a PCR system ABI-Prism 7000 SDS (Applied Biosystems). Results of real-time PCR determinations were presented as C_t values, where C_t was defined as the threshold cycle number at which product is first detected by fluorescence. The amount of target was normalized to an endogenous reference, the housekeeping gene for β-actin. ΔC_t was the difference in C_t values derived from the corresponding gene and β-actin gene in each sample assayed. $\Delta\Delta C_t$ represented the difference between paired samples. The *n*-fold differential ratio was expressed as $2^{-\Delta\Delta C_t}$ (Chang et al., 2002). Equal efficiency of all the primers was previously confirmed.

2.4. Protein isolation and Western-blotting

HK-2 cells were stimulated with 10 μM ATRA for different time periods. Cells were washed twice with ice-cold PBS and then harvested, scraped into ice-cold PBS, and then pelleted by centrifugation at 500 × *g* for 5 min at 4 °C. Cells were kept on ice for 30 min in a solution containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and protease inhibitors. Thereafter, the cells were pelleted by centrifugation at 4000 × *g* for 5 min at 4 °C. Proteins from cell lysates were denatured by heating. Then, they were resolved by 10% SDS–PAGE, and blotted onto a nitrocellulose membrane (BioTrace/NT) overnight in 50 mM Tris–HCl, 380 mM glycine, 0.1% SDS, and 20% methanol. Rabbit anti-RARβ (1:1000) or mouse anti-HIF-1α (1:1000) antibodies were added followed by incubation for overnight at 4 °C. After treatment for 1 h at room temperature with the corresponding secondary antiserum (1:4000), the signals were detected with enhanced chemiluminescence reagent using β-actin antibody (1:5000) as loading control.

2.5. Immunofluorescence analysis

Cells were fixed with 4% paraformaldehyde in PBS, 10 min at RT, rinsed, and permeabilized with 0.15% Triton X-100 in PBS (10 min). Cells were then incubated for 30 min with 5% donkey serum in PBS to block nonspecific binding. Afterwards cells were incubated overnight at 4 °C with anti-HIF-α and anti-RARβ antibodies (1:100)