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Hypoxia-induced regulation of the very low density lipoprotein receptor

Jeanna Perman Sundelin [a,*], Ulf Lidberg [a], Ali Moussavi Nik [b], Peter Carlsson [b], Jan Borén [a]

[a] Department of Molecular and Clinical Medicine, Wallenberg Laboratory, University of Gothenburg, Gothenburg, Sweden
[b] Department of Chemistry and Molecular Biology, University of Gothenburg, Gothenburg, Sweden

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ABSTRACT

The very low density lipoprotein receptor (VLDLr) is highly upregulated during hypoxia in mouse cardiomyocytes and in human and mouse ischemic hearts causing a detrimental lipid accumulation. To know how the gene is regulated is important for future studies. In this study, we have thoroughly mapped the 5′-flanking region of the mouse VLDLr promoter and show that the hypoxia-mediated increase in VLDLr expression is dependent on Hif-1α binding to a hypoxia responsive element (HRE) located at −162 to −158 bp 5′of translation start. We show that classical HRE sites and the previously described PPARγ and Sp1 binding are not involved in the hypoxia-induced regulation of the VLDLr promoter. Using a chromatin immunoprecipitation (ChIP) assay, we show that Hif-1α specifically binds and activates the mouse VLDLr promoter at the previously described non-classical HRE in HL-1 cells. We also show that the same HRE is present and active in response to hypoxia in human cardiomyocytes, however at a different location (−812 bp from translation start). These results conclude that in the hypoxic hearts of mice and men, the VLDLr gene is regulated by a direct binding of Hif-1α to the VLDLr promoter.

1. Introduction

The very low density lipoprotein receptor (VLDLr) is a member of the low density lipoprotein receptor (LDLr) family and is mainly expressed in tissues active in fatty acid metabolism, such as the heart, skeletal muscle and adipose tissue [1,2]. We have recently shown that the VLDLr is significantly upregulated during hypoxic/ischemic conditions in mouse cardiac cells and hearts as well as in human hearts. We found evidence that hypoxia-induced VLDLr expression in mouse cardiomyocytes is dependent on hypoxia-induced factor 1α (Hif-1α), which mediates its effect through a non-classical hypoxia-responsive element (HRE) in the VLDLr promoter [3–6]. However, the exact mechanism for this interaction is yet to be elucidated.

Earlier studies have characterized regulatory elements that are of importance for the VLDLr promoter expression in normal physiology [7,8], such as sterol regulatory elements [7–9], CCAAT/enhancer-binding protein-β (C/EBP-β) [10–12], nuclear factor-Y [13,14], and peroxisome proliferator-activated receptor gamma (PPARγ) [15]. All of these factors have been shown to be important for the VLDLr promoter activity during different conditions and in different cell types. Yet, none of these regulatory elements have been studied regarding the expression pattern of the VLDLr during hypoxic/ischemic conditions.

Knowledge of the true regulation of the VLDLr during hypoxia is important for future studies and development of tissue specific inhibitors. Here, we extend our previous studies and thoroughly map the hypoxia-induced regulation of the VLDLr in mouse and human cardiomyocytes. We aimed to identify if there were additional sites of hypoxia-induced regulation on the VLDLr promoter and to determine if Hif-1α directly affects the VLDLr promoter activity.

2. Materials and methods

2.1. Cell culture and incubation in hypoxia

The HL-1 cardiomyocyte cell line was a generous gift from Dr. Claycomb (Louisiana State University Medical Centre, New Orleans, LA). The cells were cultured as described previously [16], and incubated in supplemented Claycomb media [16] at 21% oxygen (normoxia) or 1% oxygen (hypoxia) for 8 h as described previously [3]. Incubation in hypoxia for 8 h did not alter the viability of HL-1 cells [3]. The human atrial and ventricular cardiomyocytes were purchased from Celprogen (San Pedro, CA) and were cultured according to the manufacturer's instructions.

* Corresponding author. Address: Wallenberg Laboratory, Sahlgrenska University Hospital, 413 45 Gothenburg, Sweden. Fax: +46 31 823762.
E-mail address: jeanna.perman@wlab.gu.se (J.P. Sundelin).

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2.2. Promoter constructs

The pGL3*Vldlr*2590 and the pGL3*Vldlr*2590mut plasmids, containing the proximal 2590 bp of the mouse VLDLr promoter, were generously provided from Takazawa and colleagues [15]. The pGL3*Vldlr*2590 luciferase plasmid was used to make a series of successive 5′ deletion constructs using standard cloning techniques. To make the pGL3*Vldlr*4485 construct, in which the promoter is elongated up to −4485 bp, a mouse DNA fragment was amplified from mouse genomic DNA by PCR. The fragment was then subcloned, sequenced, and finally cloned into the pGL3*Vldlr*2590 construct. We used the same methodology to couple intron one and exon one to the construct creating the pGL3*Vldlr*2590+intron construct, containing the intergenic fragment −29 to +663 subcloned into the construct after the luciferase gene. The mutations of the Sp1 sites were made by exchanging the sequence CCCGCCC at −103 to −97 to CC***AATTG*** and the HRE sequence GCGTG at −162 to −158 to G***GA***T***C*** (bold and italic indicating mutations).

2.3. Transient transfection and reporter gene assay

HL-1 cells were transiently transfected with promoter constructs using Lipofectamine LTX and PLUS reagent (Invitrogen). Briefly, cells were seeded in 12-well plates, transfected with 2.4 μg promoter construct and 0.1 μg pCMV-RL (Renilla construct, Promega). The cells were harvested 48 h after transfection (and incubated in hypoxia where indicated for the final 8 h). The cells were then washed once with ice–cold PBS and harvested in 100 μl lysis buffer, freeze–thawed 3 times and then analyzed using Dual-Luciferase® Reporter Assay System (Promega).

2.4. Nuclear extract preparation and EMSA

Nuclear extract was prepared from HL-1 cells using CelLytic NuCLEAR Extraction Kit (Sigma). Electrophoretic mobility shift assay (EMSA) was performed using LightShift Chemiluminescent EMSA Kit (Sigma), using 5 μg nuclear extract/reaction. Competitors, when used, were added in 200-fold molar excess. For supershift assay, antibodies (2 μg/reaction) were added to the nuclear extract for 20 min; thereafter the oligo was added and the incubation was allowed to proceed for an additional 20 min. The DNA–protein complexes were separated on 6% non-denaturing polyacrylamide gel (Invitrogen) in 0.5× TBE. Oligonucleotides (Sigma) used were: SP1, 5′-GTC TCC TCC CTC CCC GCC CCC ACC TCC TTC-3′; SP1mut, 5′-GTC TCC TCC CTC CAA TTG CCC ACC TCC TTC-3′; SP1 consensus, 5′-ATT CGA TCG GGG CGG GGC GAG C-3′; HRE, 5′-CCC GCT CCT TGC GTG CTC TCC GTG C-3′; and unrelated, 5′-GTC TCC TCC CTC CCC GCC CCC ACC TCC TTC-3′. The antibody used was Sp1 (sc-59 X, St. Cruz).

2.5. Chromatin immunoprecipitation (ChIP) analysis

Chromatin immunoprecipitation was performed using a kit (ChIP A 17-295) from Millipore. Briefly, cells were cultured in 10 cm diameter culture dishes in normoxia or hypoxia for 8 h. Thereafter, cells were crosslinked in 1% formaldehyde in the culture dish, and subsequently harvested and sonicated (10 microns) for 4 cycles (15 s on and 5 min off) on wet ice bath using Sanyo MSE Soniprep 150, resulting in 200–1000 bp long fragments. For HL-1 cardiomyocytes, the DNA was precipitated using Hif-1α antibody (NB100-105, Novus), crosslinking was reversed and the remaining DNA yield was quantified using qPCR, with primers Fw, 5′-GGGGAGTAGAGTGTGCAAGTT-3′ and Rv, 5′-GAGGGGGAAGGAGTGAAGA-3′ flanking the putative HRE located at −162 bp. As a positive control, the mouse erythropoietin (EPO) Hif-1α site [17] was targeted using the primers Fw, 5′-AG GCATCAGATCTGGGAAAC-3′ and Rv, 5′-CAGAGGGGTCAAGAGGTCAG-3′. For human atrial and ventricular cardiomyocytes, the DNA was precipitated using Hif-1α antibody (ab2185, AbCam), crosslinking was reversed and the remaining DNA yield was quantified using qPCR, with primers Fw, 5′-GGTGGCAACAGCAACGATAGT-3′ and Rv, 5′-CAGCCTCCGGCAGAACAC-3′ flanking the putative HRE located at −812 bp. As a positive control, the human EPO Hif-1α site was targeted using the primers Fw, 5′-GTGCAGCAGGTCCAGGTC-3′ and Rv, 5′-AGGGTCGAGAGGTCAGACAG-3′.

2.6. Statistics

Differences between groups were assessed with one-way ANOVA followed by the Dunette or Bonferroni post hoc test to determine statistical significance. P values <0.05 were considered significant and data are shown as mean ± SEM unless otherwise indicated.

3. Results and discussion

3.1. Hypoxia does not regulate VLDLr expression through a classical HRE or the PPARγ binding site

We have previously shown that the VLDLr expression is increased during hypoxia, and to thoroughly map and clarify the molecular mechanism for this hypoxia-induced VLDLr expression we performed reporter gene assays using truncations of the mouse VLDLr promoter coupled to the luciferase gene and measured the luciferase activity relative to the transfection control (*Renilla luciferase*) during normoxia and hypoxia in HL-1 cardiomyocytes. The promotor was truncated from −4485 bp 5′ of translation start. These studies identified key regulatory element(s) important for the hypoxic regulation located within −250 bp 5′ of translation start (Fig. 1A) indicated by a significant increase of the luciferase activity in response to hypoxic treatment. In the present study, none of the longer constructs tested (pGL*Vldlr*4485, pGL*Vldlr*2590 and pGL*Vldlr*866) showed significant increases in luciferase activity response to hypoxia (Fig. 1A), however, the 250 bp long construct, pGL*Vldlr*250, showed an approximate 2,5-fold increase with hypoxic treatment compared to normoxic, supporting our previous data (Supporting Table 1) [3].

One of the most widely studied transcription factors for hypoxic regulation is Hif-1α and when we performed an *in silico* analysis of the mouse VLDLr promoter, we saw that the only potential classical HRE sites within the 4500 bp flanking translation start were located in the first exon and intron (at position 6–10 and position 620–624, respectively). However, we did not observe increased luciferase activity in response to hypoxia when we tested a construct containing pGL*Vldlr*2590 coupled to intron and exon one (pGL*Vldlr*2590+Intron) (Fig. 1B). These findings agree with a study from Benita et al. who showed that the VLDLr is indeed a hypoxia-regulated gene, but assigned it a low Hif score because the promoter lacks a classical HRE site 3′ of translation start [18]. In contrast to our results Shen et al. identified the same HRE in exon 1 and found it to be of importance for hypoxic regulation of the VLDLr promoter in MCF7, HepG2 and HeLa cells [19]. The difference between our findings and theirs underlines the complexity of promoter analysis studies where regulation can be different not only on species level but also on cell type level.

Previous studies have shown PPARγ to regulate the VLDLr promoter [15,20], in addition to other genes involved in lipid metabolism such as CD36, FATP and LPL [21,22]. Forthermore PPARγ has been shown to be regulated by hypoxia [23]. This prompted us to test a role for the PPARγ binding site in hypoxia-induced regulation of the VLDLr promoter. However, we showed that inactivation of the PPARγ binding site located at −2307 bp in the VLDLr promoter (pGL*Vldlr*2590PPARγmut) did not affect the luciferase