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Hypoxia-induced regulation of the very low density lipoprotein receptor

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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.

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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

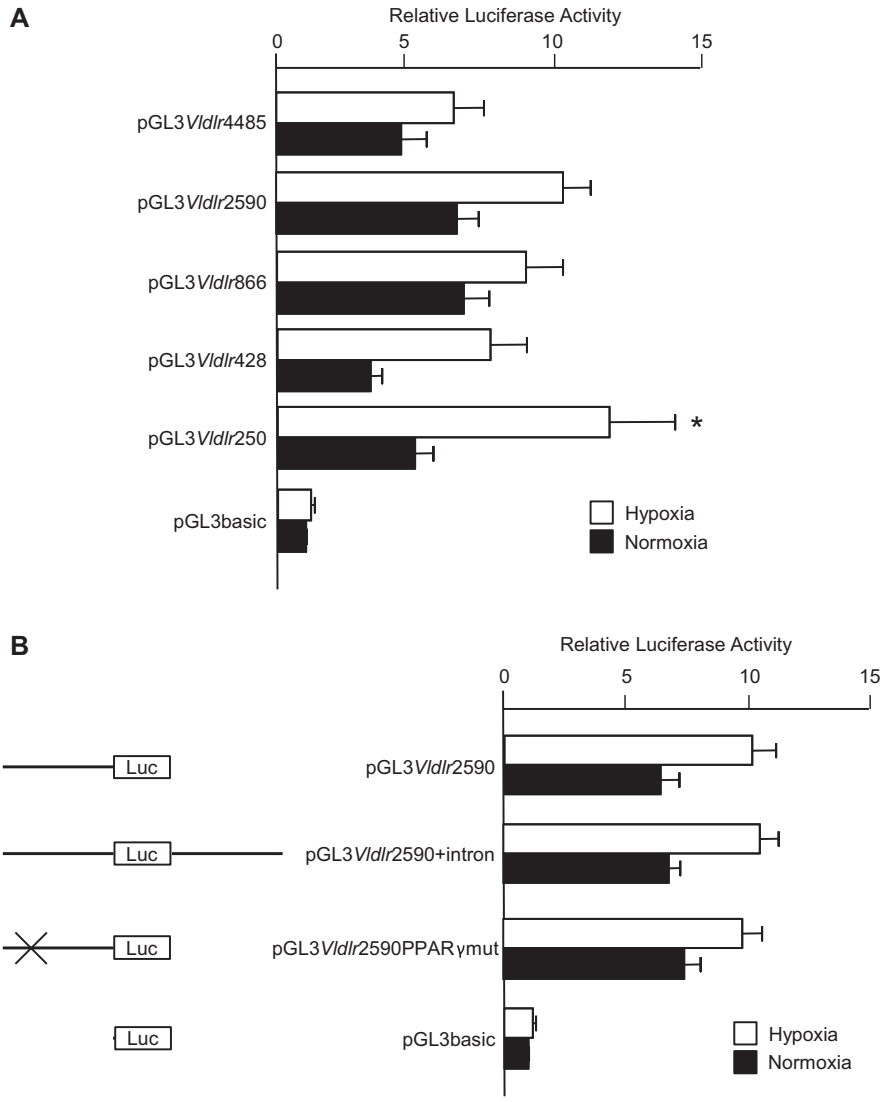

Fig. 1. Hypoxia does not increase activity of the VLDLr promoter through constructs longer than 250 bp, a classical HRE or the PPARγ binding site. (A) Luciferase activity of HL-1 cells transfected with promoter construct pGLVldlr4485 and truncated forms of this construct and incubated in normoxia or hypoxia for 8 h ($n = 25$). (B) Luciferase activity of HL-1 cells transfected with promoter constructs pGLVldlr2590, pGLVldlr2590+intron (which contains the first intron and exon attached to the 5′ end of pGLVldlr2590) and pGLVldlr2590mut (which contains a mutated, non-functional PPARγ binding site) and incubated in normoxia or hypoxia for 8 h ($n = 25$). Data are mean ± SEM, $^{*}P < 0.05$ vs. normoxia.

activity in either hypoxia or normoxia (Fig. 1B). Still, one needs to hold in mind that our findings do not rule out a role for PPARγ in regulating the VLDLr during normoxic conditions. Taken together these results show that in our system the hypoxic response of the VLDLr is regulated by an element(s) located within 250 bp of transcription start and that in contrast to previous studies neither PPARγ nor the HRE located 3′ of transcription start is involved in the regulation.

3.2. Effects of Sp1 binding sites on the VLDLr promoter activity

The transcription factor specificity protein 1 (Sp1) has been shown to participate in hypoxic gene regulation [24–26] and to co-interact with Hif-1α [27]. Sp1 is involved in regulation of many genes where activation of translation requires a well-defined Sp1 binding site [28]. Sp1 has been shown to exert its regulating abilities through many different ways, by itself and also in interaction with other transcription factors. One interaction is where Sp1 and Sp3 bind to the same site, however in different rations under different conditions and thereby regulating the gene transcription. This has been shown to occur during hypoxia where the ratio of binding is changed in favor of Sp1 [24]. Also, Sp1 can interact with MAZ to regulate transcription [29,30]. Because an Sp1 site in the mouse VLDLr promoter located at −105 bp 5′ of translation start has been described previously [1], we investigated if Sp1 binding could play a role in the hypoxia-induced regulation of the VLDLr. We performed an EMSA and showed that Sp1 bound to the VLDLr promoter under normoxic and hypoxic conditions but with no detectable increase in binding in hypoxia (Fig. 2A). Even though the Sp1 binding is not increased during hypoxia the site can still be active through interactions and co-binding. To test the specificity, the binding was inhibited by incubation with an excess of unlabeled oligonucleotides containing the VLDLr Sp1 site or the Sp1 consensus sequence (Fig. 2B, lanes 3 and 5) but not with a nonspecific probe or the mutated Sp1 oligonucleotide (Fig. 2B, lanes 4 and 6). To confirm that the protein-DNA interaction was Sp1, we also performed a supershift assay. We detected a supershift just above the migrating probe-protein complex and also as a weakening of the signal from the probe-protein complex in both normoxia and hypoxia (Fig. 2C, lanes 4 and 5), indicating that the antibody binds

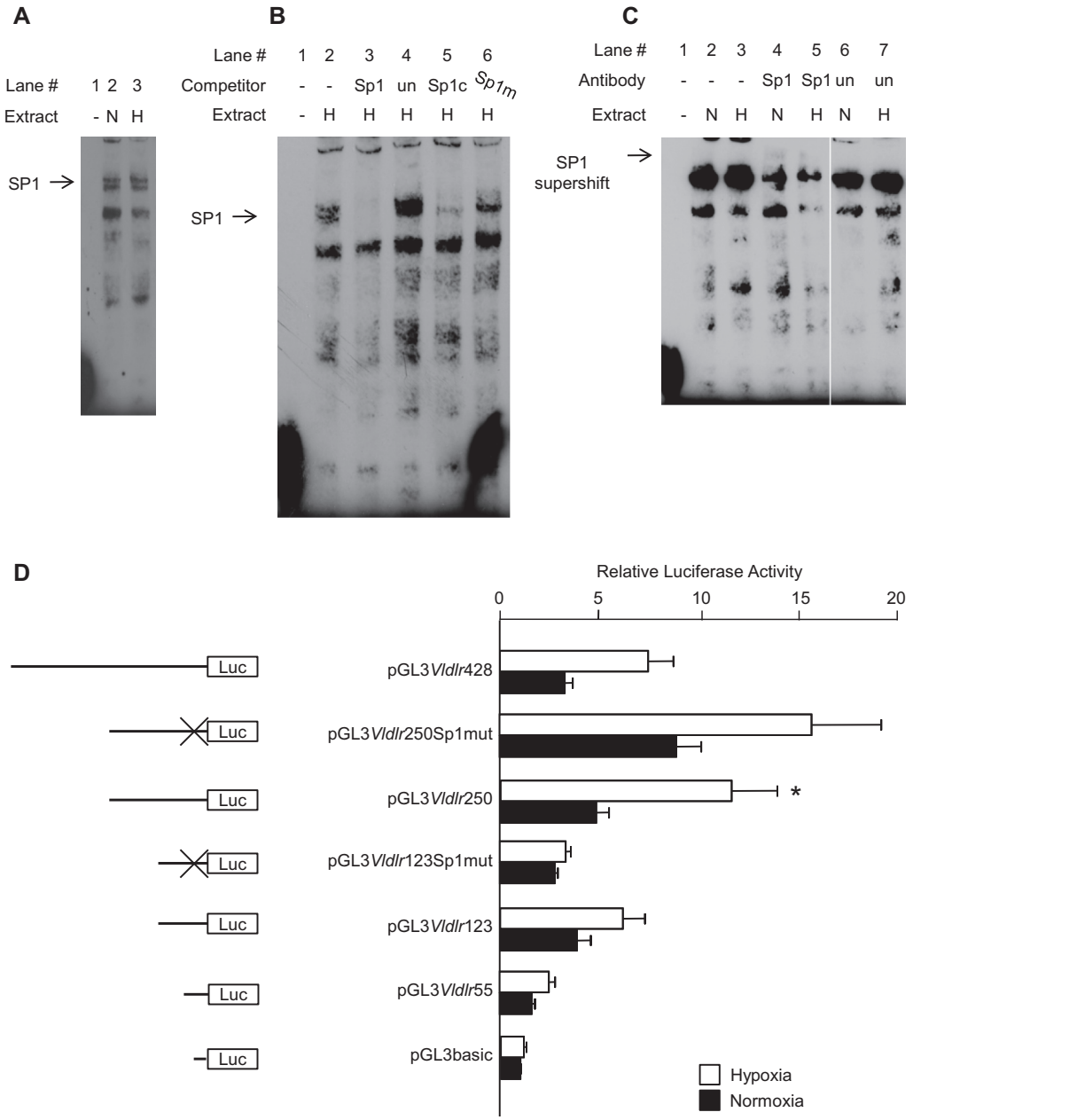

Fig. 2. Sp1 binds to the VLDLr promoter but does not mediate hypoxia-induced regulation. (A) Gel shift assay of nuclear extract from normoxic and hypoxic HL-1 cells. N (normoxia), H (hypoxia). (B) Gel shift assay of nuclear extract from hypoxic HL-1 cells with addition of Sp1 (SP1 consensus oligo), un (control oligo), Sp1c (Sp1 consensus sequence from known Sp1 regulated gene) or Sp1m (oligo with mutated Sp1 site). (C) Gel shift assay of nuclear extract from normoxic and hypoxic HL-1 cells incubated with Sp1 or non-specific (un) antibodies. (D) Luciferase activity of HL-1 cells transfected with luciferase constructs containing truncated and SP1 mutated forms of the VLDLr promoter, and incubated in normoxia or hypoxia for 8 h ($n = 25$). Data are mean ± SEM, $^*P < 0.05$ vs. normoxia.

to Sp1 and interferes with the probe-protein dimerization. No supershift was seen when using a nonspecific IgG antibody (Fig. 2C, lanes 6 and 7). We also showed that inactivation of the Sp1 binding site in the pGL3*Vldlr*250, pGL3*Vldlr*180 and pGL3*Vldlr*123 constructs had no effect on the luciferase activity in a reporter gene assay (Fig. 2D, Supporting Fig. 1). Together, these results indicate that Sp1 binds to the VLDLr promoter during normoxia and hypoxia but that it is not an important regulator for the hypoxia-induced increase in VLDLr in HL-1 cardiomyocytes.

3.3. Hif-1α binds to a non-classical HRE in the VLDLr promoter in mice and humans

A truncated 180 bp version (pGL3*Vldlr*180) of the pGL3*Vldlr*250 construct used for the Sp1 studies was found to have a significantly higher hypoxic response compared to pGL3*Vldlr*250 (fold change; 4.5 ± 1.5 vs. 2.51 ± 0.28) indicating important regulatory element(s) within 180 bp of promoter start. In search for other alternative binding sites we identified a non-classical HRE located between −162 and −158 bp from transcription start. Classical HREs are highly conserved across species with the consensus sequence ACGTG. However, there are variants that contain the core segment RCGTG but with variations in their 5′ and 3′ flanking nucleotides [18]. The non-classical HRE that we identified has the consensus sequence TGCGTG (Fig. 3B), which has previously been described as a putative Hif-1α binding sequence [18]. We showed that mutation of the *non-classical* HRE between −162 and −158 bp within the mouse pGL3*Vldlr*180 construct completely abolished the hypoxia-induced luciferase activity in a reporter gene assay (Fig. 3A and [3]). We also controlled that further mutation of the

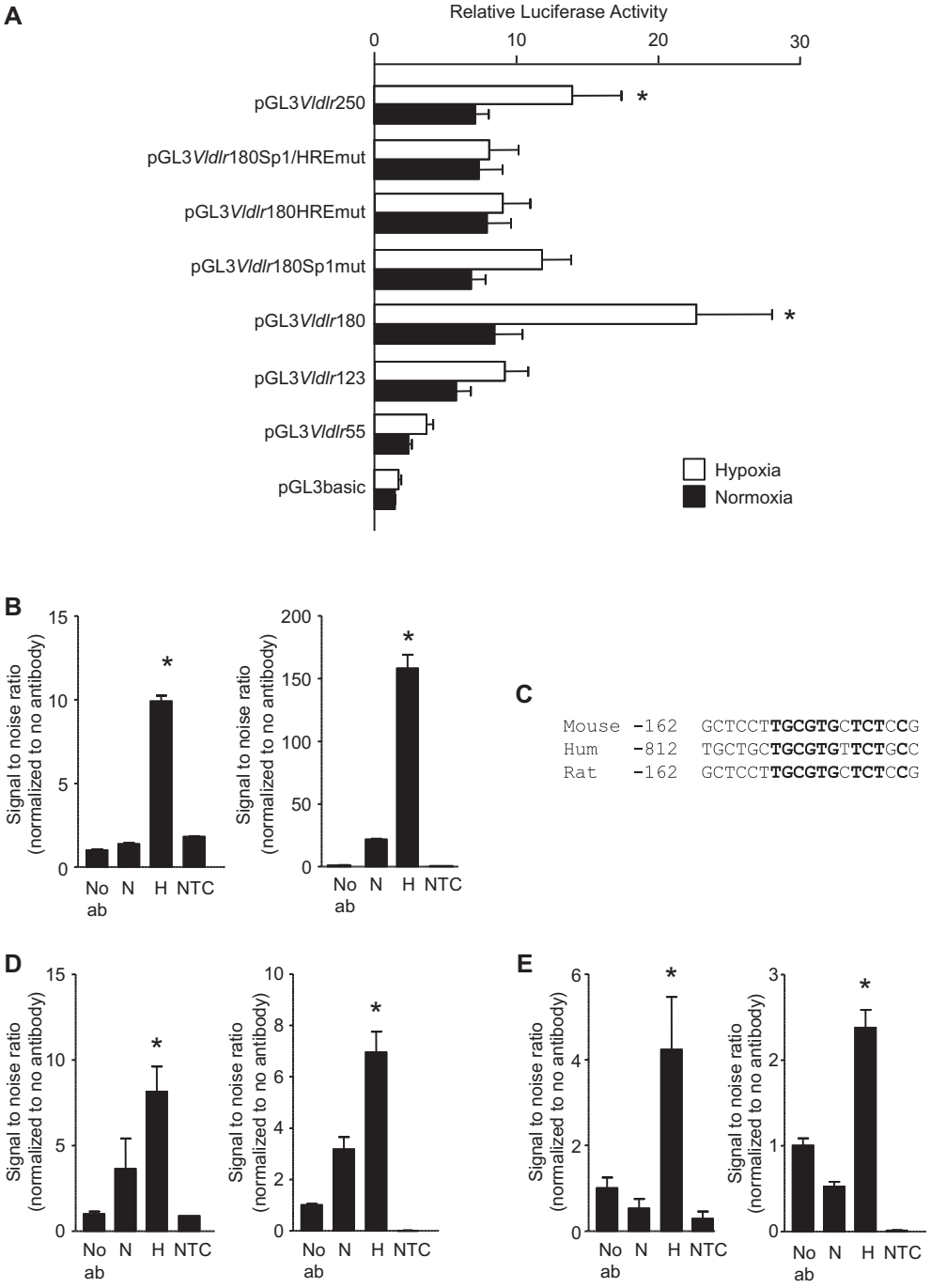

Fig. 3. Hif-1α binds to a non-classical HRE in the VLDLr promoter in mice and humans. (A) The shorter pGLVldlr180 construct have a stronger hypoxic response to hypoxia compared to the pGLVldlr250 construct. Mutation of the Sp1 site does not block the hypoxia-induced increase of VLDLr promoter activity as seen in Fig. 2B. Combined mutation of the SP1 and HRE does not further block the VLDLr promoter activity indicating lack of joint effects between the two regulatory elements. Luciferase activity of HL-1 cells transfected with luciferase constructs containing truncated and SP1 mutated forms of the VLDLr promoter, and incubated in normoxia or hypoxia for 8 h ($n = 25$). (B) Quantification of ChIP assay of HL-1 cells incubated in normoxia and hypoxia with Hif-1α antibody and primers directed against (left) the HRE at −162 bp in the VLDLr promoter and (right) the HRE site in the Hif-1α-regulated EPO gene as a positive control ($n = 3$). (C) Alignment of conserved HRE binding sites in the mouse, rat and human VLDLr promoter. (D and E) Quantification of ChIP assay of (D) human atrial cardiomyocytes and (E) human ventricular cardiomyocytes incubated in normoxia and hypoxia with Hif-1α antibody and primers directed against (left) the HRE at −812 bp in the VLDLr promoter and (right) the Hif-1α-regulated EPO gene as a positive control ($n = 3$). No ab, no antibody; N, Normoxia; H, hypoxia; NTC, no template control. Data as mean ± SEM, $^{*}P < 0.05$ vs. normoxia.

identified Sp1 site did not alter the results seen with only HRE mutation indicating lack of interactions between these two sides in this study (Fig. 3A). To verify the binding to the HRE, we performed a ChIP assay that showed direct binding of Hif-1α to this non-classical HRE and that this binding was significantly increased in hypoxic conditions (Fig. 3B, left panel) using the known Hif-1α responsive target gene EPO as a control (Fig. 3B, right panel).

Our previous work showed that the hypoxic conditions increased the expression of the VLDLr not only in mouse hearts and mouse cardiomyocytes but also in human cardiac biopsies taken from ischemic hearts compared with non-ischemic hearts [3]. We therefore performed an *in silico* analysis of the human VLDLr promoter to see if the same HRE located in the mouse promoter was also present in the human promoter. Indeed, we

identified a sequence −812 bp of translation start in the human gene that was identical to that of the non-classical HRE in the mouse promoter (Fig. 3C). To determine if Hif-1α also binds to this human HRE, we performed a ChIP analysis using human atrial and ventricular cultured cardiomyocytes. We showed that Hif-1α bound to this HRE and that the binding was significantly increased in hypoxic conditions in both cell types (Fig. 3D and E left panels). As in the mouse ChIP analysis, EPO was used as a control gene (Fig. 3D and E right panels). Interestingly, Kreuter et al. reported that the region between −760 bp and −1040 bp (which binds the C/EBP-β) in the human VLDLr promoter is most likely to contain regulatory elements governing the VLDLr gene expression in standard conditions [13]. Together this shows that the increased VLDLr expression during hypoxia in mouse and human cardiomyocytes is regulated by Hif-1α specifically binding to a non-classical HRE in the VLDLr promoter. To know how the VLDLr is regulated during low oxygen pressure could be of great value for future studies. Since we previously show that mice lacking the VLDLr have an improved post myocardial infarction (MI) survival and that the expression of the receptor is increased in ischemic hearts in both mice and men, the VLDLr could be implicated in post MI survival in man. Knowledge of the true gene regulation of the VLDLr during hypoxia/ischemia in humans could be an important tool for future studies and drug development.

In conclusion, in this paper we show that the VLDLr gene expression during hypoxic conditions is significantly upregulated by a direct binding of Hif-1α to the non-classical HRE site in the VLDLr promoter. We also show that the same site is translocated but conserved and active in both mouse and human.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.066.

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