Adipose differentiation-related protein is not involved in hypoxia inducible factor-1-induced lipid accumulation under hypoxia

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Received December 29, 2014; Accepted September 25, 2015

DOI: 10.3892/mmr.2015.4488

Abstract. Increasing evidence has showed that hypoxia inducible factor-1 (HIF1) has an important role in hypoxia-induced lipid accumulation, a common feature of solid tumors; however, its role remains to be fully elucidated. Adipose differentiation-related protein (ADRP), a structural protein of lipid droplets, is found to be upregulated under hypoxic conditions. In the present study, an MCF7 breast cancer cell line was used to study the role of ADRP in hypoxia-induced lipid accumulation. It was demonstrated that hypoxia induced the gene expression of ADRP in a HIF1-dependent manner. Increases in the mRNA and protein levels of ADRP was accompanied by increased HIF1A activity. In addition, a significant decrease in the mRNA and protein levels of ADRP were detected in presence of siRNA targeting HIF1A. Using a dual-luciferase reporting experiment and chromatin immuno-precipitation assay, the present study demonstrated that ADRP is a direct target gene of HIF1, and identified a functional hypoxia response element localized 33 bp upstream of the transcriptional start site of the ADRP gene. Furthermore, the present study demonstrated the role of ADRP in low density lipoprotein (LDL) and very-LDL uptake-induced lipid accumulation under hypoxia. The knockdown of ADRP did not reduce HIF1-induced lipid accumulation under hypoxia. Together, these results showed that ADRP may be not involved in HIF1-induced lipid accumulation.

Introduction

Hypoxia inducible factor-1 (HIF1) has been identified for ~20 years (1) and is necessary to reprogram cellular metabolism, which is essential during physiological and pathological processes (2). HIF-1 regulates the transcription of hundreds of genes, which are involved in energy metabolism (3). The role of HIF1 in glucose metabolism has been well addressed. Previous investigations have suggested that HIF1 is also important in lipid metabolism, including regulating lipid uptake and trafficking, fatty acid metabolism, sterol metabolism, triacylglycerol synthesis and phospholipid metabolism, lipid droplet formation and lipid signaling (4-6).

Adipose differentiation-related protein (ADRP) is a structural protein in lipid droplets, which regulates lipid accumulation. Overexpression of the ADRP gene stimulates lipid accumulation in mouse fibroblasts (7) and in human macrophages (8), whereas knockdown of the ADRP gene reduces the level of triglycerides and esterified cholesterol in THP-1 macrophages (8). In addition, in a mouse model, inactivation of ADRP gene results in a significant reduction in lipid droplet accumulation following overnight incubation with oxidized low density lipoprotein (oxLDL) (9). These results suggest that ADRP facilitates lipid accumulation.

It has been reported that the expression of ADRP increases under hypoxic conditions (10-12). However, whether ADRP is a direct target gene of HIF1 is unknown. In a previous study, lipid accumulation was observed in human breast cancer and it was identified that enhancement of LDL and VLDL uptake induced by HIF1 under hypoxia contributes to lipid accumulation in the breast cancer cell line MCF7 (13). However, whether ADRP is also involved in lipid accumulation and the uptake of LDL and VLDL under hypoxia in breast cancer, remains

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Key words: adipose differentiation-related protein, hypoxia inducible factor-1, lipid accumulation, hypoxia
to be elucidated. In the present study, a dual luciferase assay and chromatin immunoprecipitation (ChIP) experiment was performed to determine the presence of a functional hypoxia response element (HRE) in the ADRP gene promoter and determine whether ADRP is a direct target gene of HIF1. The results of our previous study showed that hypoxia promoted LDL and very-low-density lipoprotein (VLDL) uptake, in an HIF1-dependent manner, thus the present study investigated the role of ADRP in LDL and VLDL uptake and lipid accumulation under hypoxia.

Materials and methods

Cell culture, hypoxic exposure, DFO, LDL and VLDL treatment. MCF7, a breast cancer cell line used for investigating the role of hypoxia in lipid accumulation in a previous study (13), was purchased from the Cell Center of the Institute of Basic Medical Science, Chinese Academy of Medical Sciences (Beijing, China). MCF7 cells (1x10^6) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.). Cells incubated in normoxic cells were maintained at 37°C in a 5% CO2 and 95% air incubator. The cells exposed to hypoxia were placed in a three gas incubator (YCP-50S; Huaxi Electronic Technologies, Changsha, China) with 5% CO2, 94% N2 and 1% O2 at 37°C. The cells reached 70% confluence prior to treatment with DFO, LDL or VLDL. For DFO treatment, 100 µM DFO (Sigma-Aldrich, St. Louis, MO, USA) was added to the culture medium for 24 h. For LDL treatment, the culture medium was replaced with fresh medium (10% FBS) with, or without, LDL at a final concentration of 200 µg/ml. For VLDL treatment, the culture medium was replaced with fresh medium (10% FBS), with or without, VLDL at a final concentration of 100 µg/ml. The cells were either normoxic or hypoxic incubators, cultured for 24 h, and then fixed with formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and recollected for analysis. Human LDL and VLDL were purchased from Peking Union Bio Co., Ltd. (Beijing, China).

Plasmid construction. The promoter region of the ADFP gene was amplified from human genomic DNA using polymerase chain reaction (PCR) and cloned into a luciferase reporter vector (pGL3-Basic; Promega Corporation, Madison, WI, USA). Briefly, human genomic DNA was extracted using a Quick Genomic DNA Extraction kit (Guangzhou Dongsheng Biotech Co., Ltd., Guangzhou, China) according to manufacturer's instructions. A total of 50 ng genomic DNA was then used as a template to amplify the promoter region of the ADFP gene in 20 µl reaction system containing 1 µl of 10 µM primers (Sangon Biotech Co., Ltd., Shanghai, China), 1 µl of 25 µM dNTP mixture (Beijing TransGen Biotech Co., Ltd., Beijing, China) and 1 µl DNA polymerase (Beijing TransGen Biotech Co., Ltd.). The following primer sequences were used: ADFP forward 5'-agaagctCATGCCCTGGCTATTTAAGTGT-3' and reverse 5'-ccctcagCTCATGCGGTAATCCCGAAG-3'. The PCR reaction was performed in a PCR Thermocycler (Thermo Fisher Scientific, Inc.) with the following reaction conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min. As a positive control, the nucleotide sequence containing the identified HRE of the erythropoietin (EPO) gene (14) was also cloned into the pGL3-promoter.

Western blotting. Total protein was extracted from the tissue samples or cells using lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China), and subsequently quantified using the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of 20 µg protein was separated by 10% SDS-PAGE (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and transferred onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc.) and blocked with 1% bovine serum albumin (Sigma-Aldrich), the membrane was incubated with primary antibodies overnight at 4°C, then washed with Tris-buffered saline with 0.1% Tween 20 buffer (TBST; Beyotime Institute of Biotechnology) at room temperature, prior to incubation with secondary antibody for 1 h at room temperature. Following washing with TBST buffer, the signals were detected using an Electrochemiluminescence kit (EMD Millipore, Billerica, MA, USA) and analyzed with ImageJ version 1.41o (National Institutes of Health, Bethesda, MD, USA). The antibodies used in the present study included: Mouse anti-human HIF-1α monoclonal antibody (dilution 1:500; cat. no. sc-13515; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-human β-actin monoclonal antibody (dilution 1:1000; cat. no. sc-1616-R; Santa Cruz Biotechnology, Inc.), rabbit anti-human ADRP polyclonal antibody (dilution 1:500; cat. no. ab78920; Abcam, Cambridge, MA, USA), and horseradish peroxidase-conjugated goat anti-mouse (dilution 1:10,000; cat. no. sc-205; Santa Cruz Biotechnology, Inc.) and goat anti-rabbit (dilution 1:10,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) secondary antibody.

Transient transfection and luciferase assay. The MCF7 cells were plated at a density of 1x10^5 cells into 24-well plates to reach ~50-70% confluence the following day. The cells were co-transfected with the pGL3-basic-based construct and pRL-TK plasmid DNAs using Lipofectamine Plus (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature. 1 µg) of the lysates were detected using an Electrochemiluminescence system (Promega Corporation). The cells were lysed and reporter gene expression was assessed using a dual-luciferase reporter assay system (Promega Corporation). The cells were lysed and reporter gene expression was assessed using a Dual Luciferase® Reporter Assay system (Promega Corporation) according to manufacturer's instructions. Briefly, the cells were lysed with passive lysis buffer (Promega Corporation) at room temperature, the firefly and Renilla luciferase activities of the lysates were determined, and the firefly luciferase activity levels were normalized to that of Renilla luciferase.

RNA isolation and PCR analysis. The cells were dissolved in TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and total RNA was extracted, according to the manufacturer's protocol. Total RNA (1 µg) was converted into 1 µg cDNA using an M-MLV reverse-transcription system (Invitrogen; Thermo Fisher Scientific, Inc.) in the presence of oligo (dT)18 (Beijing TransGen Biotech Co., Ltd.). Reverse transcription-quantitative (RT-q)PCR was performed using an ABI-7500 Fast Sequence
Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR® Green PCR mix (Beijing TransGen Biotech Co., Ltd.). The reaction system contained 10 µl 2X SYBR® Green PCR Master mix (Beijing TransGen Biotech Co., Ltd.), 4 pmol of each primer (Sangon Biotech Co., Ltd.) and 0.2 µl RT reaction product. The samples were set in triplicate. The thermocycling parameters were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec, and a detection step at 72°C for 30 sec. The specific gene primers were as follows: HIF-1α forward, 5'-AGG TGG ATA TGT CTG GTTTG-3'; HIF-1α reverse, 5'-AAG GAC ACA TTC TGTT TTG-3'; ADRP forward, 5'-GCC TAG ACA GGA TTG AGG AGAG-3'; and ADRP reverse, 5'-CTC CTG CCC CTT TGTT CTTTG-3'. The relative abundance of the HIF-1α and ADRP transcript was quantified using the comparative Cq method (15), with β-actin as an internal control.

**ChIP assay.** The MCF7 cells were plated (2x10^5) into a 15 cm plate and grown to ~70% confluence. Then the cells were exposed to hypoxia for 24 h, and a ChIP assay was performed, as previously described (16). Briefly, the cells were fixed with 1% (v/v) formaldehyde (Sigma-Aldrich) for 10 min at room temperature. Crosslinking was blocked by the addition of 0.125 M glycine (Sigma-Aldrich) for 5 min at room temperature. The cells were then washed with ice-cold phosphate-buffered saline (PBS) and resuspended in cell lysis buffer provided in the EZ-Magna ChIP kit (cat. no. 17-409; EMD Millipore). The lysates were centrifuged at 10,000 x g at 4°C and the pellets were resuspended in nuclear lysis buffer provided in the EZ-Magna ChIP kit (EMD Millipore). The nuclear lysates were sonicated for chromatin fraction. The chromatin fraction was immunoprecipitated overnight at 4°C with 5 µg rabbit anti-human HIF-1α polyclonal antibody (cat. no. ab2185; Abcam) and 5 µg rabbit anti-human IgG polyclonal antibody (cat. no. ab2410; Abcam) respectively. The precipitated DNA was amplified by PCR with the following primers: P1, 5'-ACTCGGGCTTGGGACAGGGC-3'; P2, 5'-GCCAAGGCGGAAGGACGCGCGG-3'; P3, 5'-TGCATCAGCAGCTCACC-CC-3'; and P4, 5'-CTCGAGGGAAGTAAAGTGG-3'. PCR reactions were performed in a 20 µl system containing 1 µl of 10 µM primers (Sangon Biotech Co., Ltd.), 1 µl of 25 µM dNTP mixture (Beijing TransGen Biotech Co., Ltd.) and 1 µl DNA polymerase (Beijing TransGen Biotech Co., Ltd.) for 30 cycles consisting of denaturing for 10 sec at 94°C, annealing for 30 sec at 58°C and extension for 1 min at 72°C. As a positive control, the protein phosphatase 1, regulatory subunit 3C (PPP1R3C) promoter region, which contained a known HIF-1 binding site, was amplified using primers as previously described (17).

**Cell transfection with small interfering (si)RNA.** At 1 day prior to transfection, the cells were plated (5x10^3) into 6-well plates. The cells were grown to 50% confluence and then transfected with 25 nM (final concentration) of siGENOME non-targeting siRNA2, human HIF1A, siGENOME SMART pool, or human ADRP siGENOME SMART pool (Thermo Fisher Scientific, Inc.) using Dharma FECT1 transfection reagent, according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). Following 24 h incubation in normoxic conditions, the transfection medium was replaced with complete medium, and the cells were incubated in hypoxic conditions for another 24 h. The total RNA and cell lysates were collected for qPCR and western blotting, respectively.

Figure 1. ADRP is induced by hypoxia in a HIF-dependent manner. (A) mRNA levels of ADRP were analyzed using qPCR in MCF7 cells cultured in normoxia or hypoxia, or in medium containing 100 µM DFO for 24 h. The mRNA levels of ADRP were normalized to those of β-actin. The relative mRNA expression of ADRP is presented as the values in hypoxia, relative to normoxia. Data are expressed as the mean ± standard deviation.(B) Protein levels of HIF1α, ADRP and β-actin in the MCF7 cells were determined using western blotting under the different treatment conditions. Anti-β-actin antibody was used as a control for equal protein loading. (C) mRNA levels of ADRP and HIF1α were determined using qPCR in MCF7 cells transfected with siHIF1A and siCON. The relative mRNA expression levels are presented as the fold values of mRNA levels in cells transfected with siHIF1A cells transfected with siCON. Data are expressed as the mean ± standard deviation (D) Protein levels of HIF1α, ADRP and β-actin in the transfected MCF7 cells were determined using western blot assays, as above. *P<0.05. ADRP, Adipose differentiation-related protein; HIF1, hypoxia-inducible factor-1; N, normoxia; H, hypoxia; DFO, deferoxamine mesylate salt; si, small interfering RNA; CON, control.
Flow cytometric analysis. The cells were plated (5x10^5) into 6-well plate. Following incubation and treatment, the cells were digested with 0.25% trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.), collected and washed with PBS three times at room temperature. Subsequently, the cells were fixed with 3.7% formaldehyde (Sigma-Aldrich) for 30 min at room temperature. The cells were then rinsed immediately with PBS twice at room temperature, following which the cells were stained with 1 ml Nile Red working solution for 20 min at 37°C. The cells were then rinsed with PBS twice at room temperature, resuspended in PBS and analyzed immediately using an Accuri C6 flow cytometer system (Accuri cytometers, Ann Arbor, MI, USA). The Nile Red stock solution was purchased from Genmed Scientifics (Arlington, MA, USA), and was prepared by diluting 0.5 µl of the stock solution in 1 ml PBS, and mixing well.

Intracellular lipid qualification. The 1x10^5 MCF7 cells were plated into 24-well plate following incubation and treatment, and intracellular lipid quantification was performed using a Steatosis Colorimetric Assay kit (cat. no. 10012643-1; Cayman Chemical Co., Ann Arbor, MI, USA) according to the manu-
facture's protocol. Briefly, the cells were fixed with 1% (v/v) formaldehyde (Sigma-Aldrich) for 15 min at room temperature, washed twice with PBS (Thermo Fisher Scientific, Inc.) at room temperature for 5 min each, following which the well was dried completely and Oil Red O (Cayman Chemical Co.) working solution added to all cells, and incubated for 20 min at room temperature. The cells were washed with distilled water five times at room temperature for 5 min, and the cells were dried completely. Finally, dye extraction solution (Cayman Chemical Co.) was added to each well, gently mixed for 30 min at room temperature, and the absorbance was measured at 490 nm with a microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

Statistical analysis. The data were presented as the mean ± standard deviation, and subjected to one-way analysis of variance. Student's t test was used to compare the relative expression levels of target genes and the relative quantity of cellular lipid. The SPSS version 10.0 software package for Windows (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. P<0.05 was considered to indicate a statistically significant result.

Results

Hypoxia induces ADRP in an HIF1-dependent manner. To examine whether the gene expression of ADRP was oxygen regulated, the MCF7 cells were incubated under normoxic (21% O2) or hypoxic (1% O2) conditions, or in medium containing 100 µM deferoxamine mesylate salt (DFO) for 24 h. Subsequent qPCR assays (Fig. 1A) revealed an increase in the mRNA levels of ADRP in the cells under hypoxic conditions or following DFO treatment. The protein levels of ADRP in the MCF7 cells were determined using immunoblotting.
(Fig. 1B). Increased protein levels of ADRP were observed with increasing protein levels of HIF1A in the cells under hypoxic conditions or following DFO treatment. These results indicated ADRP as a hypoxia-inducible gene. In agreement, a significant decrease in the mRNA and protein levels of ADRP (Fig. 1C and D) were detected in the MCF7 cells transfected with siRNA targeting HIF1A, which suggested that inhibition of the HIF1A gene reduced the hypoxic induction of ADRP. Taken together, these results demonstrated that hypoxia induced the expression of ADRP in a HIF1-dependent manner.

Identification and validation of HRE in the human ADRP gene. To determine whether ADRP was a direct target of HIF1 under hypoxia, the present study examined the promoter region of the human ADRP gene for a consensus HRE sequence, as previously described (3). Several putative HREs were identified (Fig. 2A), however, only the HRE at ~−33 in position is conserved in human, mouse and rat (Fig. 2B). To determine whether this was a functional HRE, the promoter region of the ADRP gene was amplified and inserted it into the luciferase reporter plasmid, pGL3-promoter. The plasmid of the pGL3-promoter was used as a negative control. A construct of the pGL3-promoter with the insertion of the identified HRE of the EPO gene was used as a positive control. As shown in Fig. 2C, the region between -754 and +635 markedly increased luciferase activity in the MCF7 cells under hypoxia. Analysis of the deletion constructs suggested that the conserved HRE was functional (Fig. 2C). Mutation of the conserved HRE significantly impaired the induction of luciferase activity by hypoxia, however, mutation of the putative HRE in exon 1 did not impair the luciferase activity by hypoxia (Fig. 2D and E), which also suggested that the conserved HRE may be a functional HRE.

To determine whether HIF1 binds to the conserved HRE within living cells, the present study performed ChIP assays using antibodies against HIF1A (IgG as a negative control) in the normoxic- and hypoxic-cultured MCF7 cells. The sequence containing the potential functional HRE in the ADRP gene promoter was detected, and hypoxia significantly increased HIF1A binding to the HRE (Fig. 2F). The sequence containing the binding site of HIF1 in the known HIF1 target gene PPP1R3C, served as a positive control (17). The sequence between +745 and +894 in the ADRP promoter was detected, with primers P3 and P4 used as a negative control (Fig. 2F). Taken together, these results demonstrated that ADRP was a direct target of HIF1 under hypoxia.

ADRP is not important in hypoxia mediated LDL and VLDL uptake or lipid accumulation. Our previous study demonstrated that hypoxia significantly increases LDL and VLDL uptake, and enhances lipid accumulation in MCF7 cells (13), and another investigation demonstrated that ADRP also promotes lipid accumulation following overnight incubation with ox-LDL (9). Therefore, the present study aimed to determine whether ADRP induced by HIF1 also contributes to lipid accumulation under hypoxia. To investigate this, the MCF7 cells were transfected with ADRP siRNA and treated with LDL or VLDL, followed by exposure to normoxia or hypoxia for 24 h. Reductions in the mRNA and protein levels of ADRP were observed in the cells transfected with the specific siRNA targeting ADRP (Fig. 3A and B). Flow cytometry and a steatosis colorimetric assay kit were used to analyze intracellular lipid content. Consistent with our previous findings, the results showed that hypoxia induced lipid accumulation and LDL/VLDL uptake, in an HIF1-dependent manner (Fig. 4A and B). However, compared with the cells transfected with non-targeting siRNA (control), knockdown of ADRP did not reduce hypoxia-induced LDL and VLDL uptake or lipid accumulation (Fig. 4C-E). These results suggested that HIF1 promoted LDL and VLDL uptake, and lipid accumulation, but not through the induction of ADRP under hypoxia.

Discussion

ADRP is a structural protein of lipid droplets and it has been reported that ADRP is expressed at high levels and is associated with lipid accumulation in solid tumors, particularly in clear cell lesions (18,19). In step-wise carcinogenesis, the expression of ADRP is correlated with the proliferation rate and is upregulated during early tumorigenesis (18). The expression of ADRP is also associated with atherosclerosis (9). It has been reported that ADRP is induced under hypoxic conditions in several studies (10-12), however, whether ADRP is a direct HIF1 target gene remains to be elucidated. Thus, the present study examined whether ADRP is a direct target gene of HIF1. The present study demonstrated that ADRP was a HIF1 direct target gene, and identified a functional HRE in the ADRP gene promoter; results which are consistent with previous reports (10-12).

It has been reported that hypoxia significantly increases LDL and VLDL uptake, and enhances lipid accumulation in arterial SMCs (20-22), cardiomyocytes (23,24) and cancer cell lines (13). As ADRP promotes lipid accumulation and is upregulated under hypoxic conditions, the present study investigated whether ADRP also contributes to lipid accumulation under hypoxic conditions. The present study examined the impact of ADRP on LDL and VLDL uptake, and lipid accumulation under hypoxia. However, ADRP did not affect HIF1-mediated LDL and VLDL uptake or lipid accumulation under hypoxic conditions in the MCF7 cells. These data are not consistent with previous investigations, and the different experimental conditions may offer an explanation for the different results. The previous study was performed under normoxic conditions and used ox-LDL to incubate cells (9). In the present study, the cells were treated with LDL and VLDL under hypoxic conditions. In addition, lipid uptake and storage are two separate processes. Several proteins are involved in lipid storage. Hypoxia-inducible protein 2 (HIG2), a novel protein identified as being associated with lipid droplets, is upregulated by hypoxia and is a direct and specific target of HIF1. Normoxic overexpression of HIG2 is sufficient to increase lipid droplets in HeLa cells (25). When knocking down ADRP, other HIF1 target genes involving lipid storage, including HIG2, may compensate the function of ADRP. The combined knockdown of these genes may affect HIF1-mediated LDL and VLDL uptake, and lipid accumulation. Therefore, further investigations are required to identify the genes, which are involved in lipid storage and are regulated by HIF1 under hypoxic conditions.
Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant. nos. 31301076, 81201688 and 81401961 to Dr Guomin Shen, Dr Ning Ning and Dr Xiaoao Li, respectively), the Science and Technology Plan Foundation of Inner Mongolia Autonomous Region (grant. no. 20130404 to Dr Xingsheng Zhao), the Hospital Foundation of Inner Mongolia Autonomous Region People's Hospital (grant. no. 201301 to Dr Xi Liu) and the Wu-Lian-De Youth Science Foundation of Harbin Medical University (grant. no. WLD-QN1411 to Dr Xiaobo Li) and the Postdoctoral Scientific Research Development Fund of Heilongjiang Province (grant no. LBH-Q14104 to Dr Xiaobo Li).

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