OGFOD1, a member of the 2-oxoglutarate and iron dependent dioxygenase family, functions in ischemic signaling

Ken Saito a, Noritaka Adachi b, Hideki Koyama b, Masayuki Matsushita a,c,*

a Mitsubishi Kagaku Institute of Life Sciences (MITILS), 11 Minamiooya, Machida, Tokyo 194-8511, Japan
b Graduate School of Nanobioscience, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan
c Molecular and Cellular Physiology, University of the Ryukyus, Graduate School of Medicine, 207 Uehara, Nishihara, Okinawa 903-0215, Japan

Abstract

The 2-oxoglutarate and iron dependent dioxygenase family are crucial for cellular adaptation to changes in oxygen concentration. We found that cells with OGFOD1 gene silencing in this family showed resistance to cell death under ischemia, and cDNA microarray analysis of OGFOD1 knockout human cells revealed downregulation of ATPAF1. Although reintroduction of the OGFOD1 wild-type gene to OGFOD1 KO cells restored ATPAF1 mRNA levels, the catalytically inactive OGFOD1 mutants did not. Furthermore, introduction of ATPAF1 gene to OGFOD1 KO cells induced ischemic cell death. Thus, OGFOD1 plays an important role in ischemic cell survival and an OGFOD1 iron binding residue is required for ATPAF1 gene expression.

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1. Introduction

Metazoans can sense the oxygen concentration and respond to hypoxia with adaptive changes in gene expression relating to angiogenesis and glycolysis. This gene expression is induced by hypoxic inducible factor (HIF) [1,2]. Under normoxic conditions, HIF (HIF-1α and HIF-2α) are hydroxylated by prolyl hydroxylase (PHDs) of the 2-oxoglutarate and iron dependent dioxygenase (2-OG-Fe(II) dioxygenase) family, and then hydroxylated HIF is degraded by a proteasome through interaction with the pVHL E3 ubiquitin ligase complex [3]. Another 2-OG-Fe(II) dioxygenase, factor inhibiting HIF (FIH) hydroxylates an asparagine residue of HIF [4,5]. FIH modulates the transcriptional activation rather than stabilization of HIF, preventing its interaction with the transcriptional co-activator p300 that potentially reduces HIF transcriptional activity [5,6]. However, oxygen-dependent hydroxylation of HIF is reduced in hypoxia, resulting in the stabilization, activation [3,5,7], and induction of gene expression such as glucose transporters and vascular endothelial growth factor (VEGF). In the hypoxic and ischemic cardiomyocyte, the expression of glucose transporters was increased and cardiomyocytes serve to enhance glucose uptake and metabolism for protection from hypoxic/ischemic injury [8,9]. VEGF also protects motor neurons from growth factor deprivation and hydrogen peroxide treatment in vitro and from ischemia in vivo [10,11]. Drug studies have reported that inhibitor, dimethylxalylglycine (DMOG), for PHDs and FIH delay cell death caused by NGF deprivation in a stroke model [12,13]. DMOG is 2-oxoglutarate analogs and inhibit the activity of 2-oxoglutarate-dependent dioxygenases through the competitive binding with 2-oxoglutarate to active site [4]. Thus, it is thought that regulation of PHDs and FIH might also be one of the survival responses under hypoxia/ischemia.

To discover the novel 2-OG-Fe(II) dioxygenases candidate in ischemic survival, we screened this family using an siRNA library. In this study, OGFOD1 (FLJ10826) gene-silenced cells showed significant resistance to cell death in ischemia, indicating a role of OGFOD1 in ischemic cell survival.

2. Materials and methods

See Supplementary data.

3. Results

3.1. siRNA library screening

Ischemia is complex physiological processes, in which multiple changes contribute to cellular adaptation. To study cell survival
under these component stimuli, HeLa cells were cultured under oxygen and glucose deprivation (OGD) conditions to induce ischemia. Cell death under OGD was prevented by the 2-OG-Fe(II) dioxygenase inhibitor DMOG in a dose-dependent manner (Fig. 1A). Therefore, we used the siRNA library of the 2-OG-Fe(II) dioxygenase family (Table S1) to discover significant genes responsible for cell survival under OGD. HeLa cells were added to the well containing mixture of siRNA and transfection reagent (Fig. 1B). After 48 h of culture, cells were exposed to OGD conditions and cell survival was measured. We established a cut-off point (absorbance of 2.3) in the siRNA library screening based on the value of cell viability using 2 mM DMOG, and obtained knockdown of ALKBH, DEPC-1, PHF8, ASPH, FLJ10826, and JMJD2B genes in HeLa cells with the same or more viability as 2 mM DMOG-treated cells under OGD (Fig. 1A and C). While knockdown of HIF1AN and EGLN2 (also known as FIH and PHD1) showed the absorbance of 1.7 and 1.9, respectively. They were slightly increased survival under OGD conditions, but knockdown of EGLN1 and EGLN3 (also known as PHD2 and PHD3) induced cell death (Fig. 1C). Among these genes, function of OGFOD1 (FLJ10826) is unclear and it was selected as the most viable for further validation and characterization. Cells with a silenced OGFOD1 gene survived under both normoxic and OGD conditions (Fig. 2A and B). We also confirmed that OGFOD1 protein levels were decreased by siRNA under our experimental conditions (Fig. 2C).

3.2. Cellular localization of OGFOD1

OGFOD1 has >80% identity between human (542 amino acids) and mouse (545 amino acids). From the NCBI database, the N-terminal region in OGFOD1 was predicted to contain the prolyl 4-hydroxylase (P4H) domain (Fig. 3A). A BLAST search and alignment analysis indicated that the P4H domain of OGFOD1 shares 35–40% identity with the Schizosaccharomyces pombe Ofd1 or Saccharomyces cerevisiae PRO1 domain.

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**Fig. 1.** Screening of genes for cell survival under ischemia. (A) Cells were treated with the DMSO or dimethyloxalylglycine (DMOG). Cell viability under normoxic (black bars) and ischemic (white bars) conditions was measured by the absorbance of formazan products at 490 nm. Results represent mean ± S.D. (n = 3). (B) Outline of the transfection and screening condition. (C) Ischemic cell survival in the knockdown of 2-OG-Fe(II) dioxygenase family genes was measured. Cut-off point (absorbance of 2.3) was indicated by dotted line. Results represent mean ± S.D. (n = 3).
*cerevisiae* TPA1 genes (Fig. 3B); regions other than the P4H domain have little homology (<20% identity). The N-terminal region of OGFOD1 also contains a positively charged amino acid sequence (Fig. 3A, underlined), most likely correlating to a nuclear localization signal. To determine the cellular localization of OGFOD1, EGFP-OGFOD1 (wild-type) and nuclear localization signal-deleted EGFP-OGFOD1 (ΔNLS) expression plasmids were transfected into HeLa cells. Wild-type expression was localized to the nucleus, while the mutant OGFOD1 (ΔNLS) expression was not (Fig. 3C). OGFOD1 translocation in response to changes in oxygen concentration resulted in similar nuclear localization of endogenous OGFOD1 under both normoxic and ischemic conditions, indicating that OGFOD1 functions in the nucleus (Fig. 3D).

3.3. Downregulated genes in OGFOD1 KO cells

To investigate OGFOD1 function, we generated an OGFOD1 knockout cell line using human Nalm6 cells. The Nalm6 cell line provides convenient and high-efficiency gene targeting [14]. We disrupted exons 5–8 of the P4H domain (Fig. 4A) by substitution with both hygromycin and puromycin resistance genes. In Southern blot analysis, OGFOD1hyg/+ and OGFOD1hyg/puro cells revealed bands of 7.0 kb and 8.6 kb, respectively (Fig. 4B, left). Wild-type cells, but not OGFOD1 KO (OGFOD1hyg/puro) cells expressed OGFOD1 (Fig 4B, right). This OGFOD1 KO cells had more viability than wild-type cells under ischemia (Fig. 3D).

Next, to understand how OGFOD1 KO results in ischemic cell survival, we analyzed gene expression by cDNA microarray and qRT-PCR. Our cDNA microarray analysis showed genes that were up- and downregulated by at least threefold in OGFOD1 KO cells (OGFOD1hyg/puro) compared to wild-type cells (Table S2, GEO accession number: GSE21557). Quantification of mRNA levels using qRT-PCR showed a 1.5-fold change that corresponded to the microarray data, except for the NAALADL2 and COX1 genes (Table S3). Taken together, the expression levels of many genes were downregulated in OGFOD1 KO cells.

3.4. Regulation of ATPAF1 expression by OGFOD1

To confirm whether OGFOD1 modulates the downregulation of genes, we reintroduced a OGFOD1 wild-type gene into the OGFOD1 KO cells (revWT) and found that revWT expression recovered only ATPAF1 mRNA levels among the downregulated genes (Table S4). This result indicates that the intact OGFOD1 appears to dominantly regulate ATPAF1 mRNA levels over the other downregulated genes. Therefore, we focused on the ATPAF1 gene for further studies of OGFOD1 function and examined whether the structure and regulation of OGFOD1 are important for expression of the ATPAF1 gene.

We generated an OGFOD1 H155A mutant that substitutes the conserved His with Ala, a residue that along with Asp is located within the P4H domain and is important for iron coordination in the active site (Fig. 5A) [15,16]. We also generated an OGFOD1 C and OGFOD1 N mutants (Fig. 5A). Although these mutants were stably introduced into OGFOD1 KO cells, re-expression of ATPAF1 was not restored by revH155A, revN, or revC (Fig. 5B); the N-terminal domain of OGFOD1 (revN) is an unstable protein fragment (Fig. 5C). Furthermore, we examined the cell survival of revartant cell lines under OGD conditions. The cells of revC or revH155A significantly reduced the cell death under OGD compared with wild-type cells (Fig. 5D). The cell survival of revC was the same as that of
OGFOD1 KO cells. These results confirm that the iron-coordinating residue is required for ATPAF1 expression and cell death under OGD conditions.

Under OGD conditions, endogenous ATPAF1 and OGFOD1 mRNA levels decreased in wild-type cells (Fig. S1A). To examine whether decreased ATPAF1 mRNA levels under OGD conditions correlate with OGFOD1 protein expression levels, FLAG-OGFOD1 was overexpressed in HeLa cells (Fig. S1B). We found that the expression levels of FLAG-OGFOD1 did not increase ATPAF1 mRNA levels in normal and OGD condition (Fig. S1C). In the cell survival assay under OGD, increased expression of FLAG-OGFOD1 had no effect on cell survival (Fig. S1D). Thus, we concluded that ATPAF1 mRNA expression required the activity of OGFOD1 P4H domain rather than OGFOD1 protein expression levels.

The yeast homolog of ATPAF1, ATP11p, has been reported to be a mitochondrial F1-ATPase assembly factor [17,18]. We confirmed that ATPAF1 was co-localized with the mitochondrial maker, DsRed-Mito, in HeLa cells (Fig. 6A, left). Moreover, introduction of the ATPAF1 gene into OGFOD1 KO cells (Fig. 6A, right) significantly increased cell death under OGD compared with OGFOD1 KO cells (Fig. 6B).

4. Discussion

We identified the OGFOD1 gene, a member of the 2-OG-Fe(II) dioxygenase family, that plays an important role in ischemic survival. It is well known that loss of PHDs and FIH (EGLN1, 2, 3 and HIF1AN) stabilize the HIF for hypoxic adaptation. However, knockdown of EGLN1, 2, 3 and HIF1AN genes in our siRNA library screening had no significant effect on ischemic survival compared to knockdown of Ogfod1 gene (Fig. 1). We found that HIF-1α expression in OGFOD1 KO cells was slightly lower compared to wild-type cells and PHDs expression were not different between OGFOD1 wild-type and KO cells (Fig. S2A), suggesting that PHDs, FIH, and OGFOD1 might recognize different substrates and play different roles in cell survival.

The S. pombe ortholog of OGFOD1, Ofd1 degrades the Sre1 mature form (Sre1N) and an Ofd1-deficient strain accumulates Sre1N [19], which is an ortholog of the human sterol regulatory element binding protein (SREBP). Ofd1 has a molecular mechanism whereby the C-terminal region has catalytic activity under normoxia and the activity was suppressed by the binding of Nro1 protein under hypoxia [20]. In our study, OGFOD1 KO cells had little influence on the accumulation of SREBP (Fig. S2B) and loss of OGFOD1
decreased the ATPAF1 expression as well as the revH155A and revC cell lines (Fig. 5). Thus, we found that the iron-coordinating residue is required for ATPAF1 gene expression. If OGFOD1 has the same intramolecular or intermolecular mechanism with Ofd1, it is thought that OGFOD1 accumulate a negative regulator of ATPAF1 expression under ischemia. Further investigation is needed to determine the molecular mechanism underlying the decrease in ATPAF1 in OGFOD1 KO cells.

Although the substrate of OGFOD1 is currently unclear, several reports provide speculative evidence that OGFOD1 functions in nucleus (Fig. 3). Many 2-OG-Fe(II) dioxygenases in the nucleus function as demethylases for the methylated DNA, RNA and histones [21]. Studies of TPA1, an OGFOD1 ortholog in S. cerevisiae, have suggested control of translation termination, mRNA poly(A) tail length, and mRNA stability [22]. TPA1 has also been co-purified with the NuA3 histone H3 HAT complex [23]. Thus, OGFOD1 may cooperates with the translation termination or histone modification complex. As shown in Fig. S2C, we found that Lys 9 methylation on histone H3 levels in OGFOD1 KO cells was lower compared to wild-type cells. Therefore, OGFOD1 may regulates transcription through the

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**Fig. 4.** OGFOD1 KO cell line. (A) The human OGFOD1 gene is located on chromosome 16q13 and is composed of 13 exons (black boxes). Two targeting vectors are shown as pOGFOD1-Hyg and pOGFOD1-Puro. The diphteria toxin A (DT-A), puromycin (Puro), and hygromycin (Hygr) resistance genes are marked by white boxes and arrows. Triangles represent floP sequences. (B) The left panel shows southern blot of wild-type (+/+), heterozygous (+/hyg) and homozygous (puro/hyg) mutants. The right panel shows OGFOD1 expressions in wild-type and KO cells. (C) OGFOD1 wild-type and KO cells were stably transfected with EGFP expression plasmid. The cells were cultured under ischemia for five days. Cell death was indicated by PI staining (left panels). Bar = 50 μm. The right panel shows the number of PI stained cells. Results represent means ± S.D. (n = 9, ***P < 0.0001).
chromatin structure. It will be interesting to investigate whether histone H3 modifications can regulate ATPAF1 transcription, and whether mitochondrial ATPAF1 expression contributes to the reduction of ADP/ATP ratio (Fig. S3) and cell death (Fig. 6). We also could not rule out the possibility that loss of OGFOD1 reduces cell death by mechanism other than controlling ATPAF1 expression, since ATPAF1 levels in revN cell lines were not coincident with cell death (Fig. 5).

Finally, we propose that intact OGFOD1 iron binding residues are required for ATPAF1 gene expression and that changes in the activity/structure of OGFOD1 induce cell survival under ischemic conditions. These results and further studies of OGFOD1 will improve the understanding of cell survival under ischemic conditions including brain stroke, as OGFOD1 was found to be more abundant in the brain (Fig. S4).

Fig. 5. Regulation of ATPAF1 mRNA level by OGFOD1. (A) The construction of 3 × FLAG-tagged OGFOD1 variants. (B) ATPAF1 mRNA levels in OGFOD1 revertant cells (revWT, revH155A, revN, and revC) were detected by qRT-PCR. Results represent means ± S.D. (n = 6, ***P < 0.0001). The same results were also obtained in other revertant clones. (C) Expression levels of OGFOD1 variants in OGFOD1 revertant cells were detected by western blotting using anti-FLAG antibody. (D) Cell survival of OGFOD1 revertant cell lines under ischemia for five days were shown. Cell death was indicated by PI staining (left panels). Bar = 100 μm. The right panel shows the number of PI stained cells. Results represent means ± S.D. (n = 3, *P < 0.05).
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.06.015.

References


Fig. 6. Cell death in the expression of ATPAF1. (A) Localization of ATPAF1 was shown (left panel). Scale bar = 50 µm. The right panel shows ATPAF1 mRNA levels in ATPAF1-overexpressing OGFOD1 KO cells (ATPAF1). Results represent means ± S.D. (n = 6, ***P < 0.0001). The same results were also obtained in other clones. (B) PI staining of OGFOD1 KO (KO) and ATPAF1-overexpressing OGFOD1 KO cells cultured under ischemic conditions for four days. Cell death is indicated by PI staining (left) and the number of PI stained cells are shown (right). Bar = 50 µm. Results represent means ± S.D. (n = 6, **P < 0.01).

