Hypoxia-inducible factor 1-alpha up-regulates the expression of phospholipase D2 in colon cancer cells under hypoxic conditions

Maoxi Liu · Kunli Du · Zhongxue Fu · Shouru Zhang · Xingye Wu

Received: 18 November 2014/Accepted: 20 November 2014/Published online: 29 November 2014
© Springer Science+Business Media New York 2014

Abstract Hypoxia is a common characteristic of solid tumors. Recent studies confirmed that phospholipase D2 (PLD2) plays significant roles in cancer progression. In this study, correlation between the expression of PLD2 and the change in the protein level of hypoxia-inducible factor 1-alpha (HIF1-α) was studied. Thirty human colon cancer tissues were examined for the expression of HIF1-α and PLD2 protein, and mRNA levels. SW480 and SW620 cells were exposed to normoxia (20 %) or hypoxia (<1 %). HIF1-α and PLD2 protein, and mRNA levels were analyzed by Western blot and qRT-PCR, respectively. Growth studies were conducted on cells with HIF1-α inhibition through xenograft tumor model. Finally, PLD2 protein was detected by Western blot analysis in vivo. There was a positive correlation between HIF1-α and PLD2 in colon cancer tissues. Hypoxic stress induced PLD2 mRNA and protein expression in SW480 and SW620 cells. Cells transfected with HIF1-α siRNA showed attenuation of hypoxia stress-induced PLD2 expression. In vivo growth decreased in response to HIF1-α and PLD2 inhibition. These results suggest that PLD2 expression in colon cancer cells is up-regulated via HIF1-α in response to hypoxic stress and underscores the crucial role of HIF1-α-induced PLD2 in tumor growth.

Keywords HIF1-α · Up-regulation · PLD2 · Colon cancer cells · Hypoxia

Introduction Colon cancer is one of the most lethal solid tumors of the gastrointestinal tract. Among all cancer types, it ranks second in females and third in males [1]. In 2008, over 1.2 million new cases and 608,700 deaths due to colon cancer have been estimated worldwide [2]. A great progress has been made in the treatment for colon cancer, but the whole survival rate is still very low. So understanding the mechanism of colon cancer is essential for an effective strategy to improve the present situation.

Although molecular mechanisms linking genetic changes to the nature of colon cancer are poorly understood, many growth factors are over-expressed in this disease. PLD are a family of signal-transmitting enzymes that are best known for their role in signal transduction by catalytic hydrolysis of phosphatidylcholine to generate phosphatidic acid (PA) and choline. Mounting evidence suggests that PLD plays important roles in many physiological processes [3], including membrane trafficking, cell senescence [4], proliferation [5], differentiation and apoptosis [6], under various growth factor stimuli. To date, two types of human PLD, with different mechanisms of activation and subcellular localization, have been identified [7, 8]. Our understanding of the role of PLD2 in cancer is still limited, although many studies have suggested that PLD2 may have a universal role in cancer. There is a positive association between PLD2 expression and hypoxia disease, and its expression is up-regulated in various tissues of patients with ischemia–reperfusion injury, including brain [9] and heart [10]. PLD2 expression is also increased during hypoxia-induced death of PC12 cells [11]. Moreover, up-regulated expression of PLD2 has been detected in many tumor cell lines or tumors, such as gastric carcinoma, lung cancer, glioma, kidney cancer and breast cancer [12–16].
Henkels et al. [17] found that over-expression of PLD2 drives tumor growth. Conversely, inhibition of PLD2 signaling delays tumor growth and enhances radiosensitivity of breast cancer [18].

Hypoxia is a typical feature of solid tumors. HIF1-α is a major survival factor for tumor cells growing in a low-oxygen environment. Moreover, it plays a critical role in inducing hypoxia-related gene expression and cellular responses [19]. HIF1 is a heterodimer composed of an inducible HIF1-α subunit and HIF1-β subunit. While HIF1-β is constitutively expressed, HIF1-α is tightly regulated by oxygen. Therefore, the whole activity of HIF1 is determined by the intracellular level of HIF1-α. HIF1-α protein degradation is regulated by O2-dependent prolyl hydroxylation [20]. Hydroxylated HIF1-α is bound specifically by the von Hippel–Lindau (VHL) tumor suppressor protein, which is induced by E3 ubiquitin-protein ligases through ubiquitylation. Ubiquitylated HIF1-α is quickly degraded by the ubiquitin-proteasomal pathway under normoxia condition [21]. But HIF1-α becomes stable and translocates into nucleus and then, after combining with HIF1-β, takes part in many pathological and physiological processes as a transcription factor under hypoxia conditions [22]. HIF1-α can transcript one hundred genes. The functions of these genes include cell growth promotion, cell transformation, angiogenesis, apoptosis and energy metabolism [23–25]. DNA microarray analysis suggests that more than 2 % of all human genes in arterial endothelial cells are regulated by HIF1-α [26]. Indeed, recent studies have confirmed that HIF1-α over-expression is associated with poor prognosis in various cancer patients [27, 28]. PLD2 induces HIF1-α expression in chronic autoimmune arthritis, which suggests that cross-talk between HIF1-α and PLD2 may exist [29].

PLD2 has a significant correlation with colon cancer [30–33] according to previous reports. We hypothesized that PLD2 expression may be up-regulated by HIF1-α to facilitate colon tumor growth. In this case, regulation of HIF1-α-induced PLD2 expression might represent a novel therapeutic target in colon cancer.

Methods and materials

Patients and samples

Tissue samples were obtained from 30 patients with colon cancer (16 men and 14 women, age 30–72 years) who underwent surgery at the Department of Gastrointestinal Surgery, The First Affiliated Hospital of Chongqing Medical University (Chongqing, China), from March 2014 to May 2014. All patients had moderately differentiated cancer according to the results of pathology diagnosis. None of the patients underwent radiotherapy and chemotherapy before operation. Fresh tissue samples were obtained from all of the resected specimens and were rapidly frozen at −80°C for storage until analysis. Tumor stage was defined according to the CRC staging standard by the International Union Against Cancer (UICC). The Principal Committee of The First Affiliated Hospital of Chongqing Medical University authorized this study.

Immunohistochemical analysis

Tissues were fixed in 4 % paraformaldehyde and embedded with paraffin. Every tissue sample was sectioned at 5 μm and mounted on polylysine-coated slides. Experiments were performed using immunohistochemical SP9000 kit (Zhongshan Golden Bridge, Beijing, China) according to manufacturer’s instructions. Briefly, slides were heated for antigen retrieval at 95°C for 15 min in 10 Mm citric acid buffer (pH 6.0). After quenching endogenous peroxidase activity by treatment with 3 % H2O2 for 10 min, slides were blocked using goat serum and then incubated with primary antibodies against HIF1-α (1:100; Abcam Biotechnology, Cambridge, UK) and PLD2 (1:80; Abcam Biotechnology, Cambridge, UK), respectively, under 4°C for not <12 h. Following incubation with biotinylated secondary goat anti-rabbit antibody (Zhongshan Golden Bridge, Beijing, China) and an avidin–biotin–peroxidase complex (Zhongshan Golden Bridge, Beijing, China) for 30 min at 37°C, respectively, slides were colored using diaminobenzidine (DAB) (Zhongshan Golden Bridge, Beijing, China) and nuclei were counterstained with Mayer’s modified hematoxylin and mounted with PVP. Phosphate-buffered saline (PBS) rather than the primary antibody was used as a negative control. Images of at least ten fields of view were acquired from the stained sections, and positive cells were counted using ImageJ software. Evaluation of immunohistochemistry results was performed by two observers independently to determine the percentage of positive cells after inspection of all fields in the sections, as described previously [34].

Cell lines and cell culture

SW480 and SW620 cell lines were purchased from the Shanghai Cell Bank at the Chinese Academy of Sciences (Shanghai, China). All the cells were cultured in RPMI-1640 medium (Gibco, GrandIsland, NY) supplemented with 10 % fetal bovine serum (Hyclone, Shanghai, China) and 1 % penicillin/streptomycin (Beyotime, Jiangsu, China) at 37°C in a humidified incubator containing 5 % CO2. Hypoxia culture was performed in a three-chamber air incubator flushed with a gas mixture of 5 % CO2 and 94 % N2 at 37°C. The final O2 pressure of the medium was measured at a range of 0.5–1 %. The above method is widely used to induce hypoxic stress.
Transfection of colon cancer cells with interference RNA

Human-specific HIF1-α interference RNA (siRNA) was designed, constructed and purified by Hanheng Biotech Co (Shanghai, China). HIF1-α siRNA composed of sense 5’-GGAAATGAGAGAAATGCTTAC-3’. After determining the best effect of interference and the best transfer MOI (60) through fluorescence camera, RT-PCR and Western blot analysis, SW480 and SW620 cells were seeded in six-well plates at a concentration of 0.5 x 10⁵ per well (20–30% confluence) on the day before siRNA transfection. The ad-HIF1-α-siRNA was transfected into cells (siRNA group). Simultaneously, the flank vector of adenovirus was transfected into cells as negative control to control the impact of adenovirus vector. After incubation for 2 h, medium was replaced with fresh RI-1640. According to the indicated time, the cells were harvested for subsequent studies.

Western blot analysis

Total proteins were extracted from harvested cells or tissue samples by protein extract kit (Beyontime, Jiangsu, China). All the proteins were degenerated by boiling, separated by SDS-PAGE and transferred onto PVDF membranes. Then, membranes were blocked with 5% milk. Next, the membranes were incubated by first antibodies overnight at 4°C. The next day, after washing by TBS containing 0.05% Tween-20, the membranes were incubated by second antibody (goat anti-rabbit) for 2 h under 37°C incubator. Finally, all the membranes were visualized by a chemiluminescence kit (Beyontime, Jiangsu, China) on a Bio-Rad imaging system. Primary antibodies used in this study are as follows: HIF1-α (1:1,000 dilution, Abcam Biotechnology, Cambridge, UK), PLD2 (1:1,000 dilution, Abcam Biotechnology, Cambridge, UK) and β-actin (1:500 dilution, Boster, Biotechnology, Wuhan, China).

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA from cells or tissues was isolated using RNA isolation reagent (TaKaRa, Dalian, China) according to manufacturer’s instructions. After evaluating the concentration and purity of total RNA by using an UV spectrophotometer (Ultrospec 2100 pro, Amersham, USA), total RNA was reverse-transcribed using the Prime Script RT Reagent Kit (TaKaRa, Dalian, China). Quantitative real-time PCR was performed using SYBR Premix Ex Taq™ II (TaKaRa, Dalian, China) with CFX96™ Real-Time System (BIO-RAD, USA), with each sample analyzed in triplicate. Relative levels of mRNA expression were normalized for β-actin mRNA expression and calculated according to the formula 2^(-ΔΔCt). Primer sequence for the genes analyzed are as follows: HIF1-α: forward 5’-CCACAGAAACTACCTTCAACTCC-3’ and reverse 5’-GTGGGCGATGAGATTGTGGAC-3’; PLD2: forward 5’-GTGGGCGATGAGATTGTGGAC-3’ and reverse 5’-CAGGATTGAATCCCCGCAAG-3’; and β-actin: forward CACGAAAATTACCTCAACTCC and reverse GTGATCCTTTCTGCATCCTGT.

Animal study

Five-week-old female BALB/C nude mice were purchased from the Laboratory Animal Center of Chongqing Medical University and were maintained in specific pathogen-free units under isothermal conditions. All experimental procedures were carried out in accordance with the National Institute of Health Guide for the care and use of laboratory animals. SW620 and SW480 cells (5 x 10⁶) were subcutaneously injected and length (L) and width (S) of the tumor were measured every 3 days. After the volume of tumor reached 50–70 mm³, which was calculated according to the formula V = 0.5 x L x S², the animals were divided into interference group and flank control group according to the injection of siRNA adenovirus or flank adenovirus. The first injection was followed by one injection every week, four times altogether. Then, animals were killed, and the tumor was dissected for further study.

Statistical analysis

All the experiments were repeated in triplicate. Analyses were carried out using SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) and data are displayed as mean ± standard deviation (SD). Student’s t test or one-way ANOVA was used to analyze quantitative data. Associations between expression levels of HIF1-α and PLD2 were analyzed by the Pearson’s correlation coefficient. Relationships between HIF1-α and PLD2 immunohistochemical staining and clinicopathological features were analyzed using χ² test or Fisher’s exact probability test when appropriate. Differences were considered significant at p < 0.05.

Results

Immunohistochemical staining of HIF1-α and PLD2 and correlation with clinicopathological features in human colon cancer

To investigate the association between HIF1-α and PLD2 expression in colon cancer, we performed immunohistochemical staining for these proteins in tissue specimens from 30 colon cancer patients. Staining for HIF1-α and
Fig. 1 Immunohistochemical (IHC) staining of HIF1-α (a, b) and PLD2 (c, d) in tumor colon cancer tissue and corresponding normal colon tissue, respectively. In the normal colon cancer tissue (b, d), HIF1-α and PLD2 were not or faintly expressed. In tumor tissue (a, c), HIF1-α and PLD2 were strongly expressed. HIF1-α was mainly expressed in cytoplasm and nucleus (a). PLD2 was mainly expressed in cytomembrane and cytoplasm (c). Positive expression is visualized by brown staining. Original magnification, ×200

Table 1 Correlation between HIF1-α and PLD2 expression and clinicopathological features of colon cancer

<table>
<thead>
<tr>
<th>Clinicopathological Features</th>
<th>Total</th>
<th>HIF1-α expression</th>
<th>PLD2 expression</th>
<th>p</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive (n = 23)</td>
<td>Negative (n = 7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤65</td>
<td>18</td>
<td>13</td>
<td>5</td>
<td>0.403</td>
<td>12</td>
</tr>
<tr>
<td>&gt;65</td>
<td>12</td>
<td>10</td>
<td>2</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16</td>
<td>14</td>
<td>2</td>
<td>0.143</td>
<td>13</td>
</tr>
<tr>
<td>Female</td>
<td>14</td>
<td>9</td>
<td>5</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤3</td>
<td>11</td>
<td>5</td>
<td>6</td>
<td><strong>0.004</strong></td>
<td>4</td>
</tr>
<tr>
<td>&gt;3</td>
<td>19</td>
<td>18</td>
<td>1</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>10</td>
<td>4</td>
<td>6</td>
<td><strong>0.002</strong></td>
<td>8</td>
</tr>
<tr>
<td>Present</td>
<td>20</td>
<td>19</td>
<td>1</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I, II</td>
<td>13</td>
<td>6</td>
<td>7</td>
<td><strong>0.001</strong></td>
<td>9</td>
</tr>
<tr>
<td>III, IV</td>
<td>17</td>
<td>17</td>
<td>0</td>
<td></td>
<td>13</td>
</tr>
</tbody>
</table>

Bold-faced items indicate p < 0.05
PLD2 in representative clinical samples is shown in Fig. 1. Among the 30 colon cancer specimens, 76.67 % (23/30) and 73.33 % (22/30) were positive for HIF1-α and PLD2 expression, respectively. HIF1-α was predominantly expressed in the cytoplasm and nucleus of the tumor cell (Fig. 1a), while PLD2 was largely expressed in the cytomembrane and cytoplasm (Fig. 1c). But there was no expression or weak expression of them in the corresponding normal tissue (Fig. 1b, d). The expression level of HIF1-α and PLD2 was significantly higher in colon cancer tissues compared with corresponding normal colon tissues \((p < 0.01)\). Co-expression of HIF1-α and PLD2 was detected in 19 (63.33 %) patients. Indeed, we observed a positive correlation between HIF1-α and PLD2 expression in 30 colon cancer specimens \((r = 0.56, p < 0.05)\). The association between HIF1-α and PLD2 expression, and clinicopathological features of patients is shown in Table 1. We observed that the size of the tumor was significantly higher in HIF1-α and PLD2 expression positive group than in the negative group \((p < 0.05)\). No significant difference between HIF1-α and PLD2 expression and other clinicopathological features, including
patient age, gender, tumor stage and lymph node metastasis.

HIF1-α and PLD2 mRNA and protein expression in colon cancer tissues

We next examined the expression of HIF1-α and PLD2 mRNA and protein level in 30 colon cancer patients using qRT-PCR and Western blot analysis. qRT-PCR analysis revealed that HIF1-α and PLD2 mRNA levels were significantly higher in tumor tissues compared with corresponding normal colon tissues (p < 0.01, Fig. 2a). Consistent with mRNA levels, HIF1-α and PLD2 protein levels were also significantly higher in tumor tissues compared with corresponding normal colon tissues (p < 0.01, Fig. 2b).

Hypoxic stress-induced PLD2 expression in colon cancer cells

The messenger RNA (mRNA) levels of PLD2 were determined by qRT-PCR when cells were exposed to hypoxia for 0, 12, 24 and 48 h. Hypoxia enhanced the levels of PLD2 mRNA in a time-dependent manner (Fig. 3a). Protein levels of PD2 were markedly increased after exposure to hypoxia for 24 h (p < 0.01, Fig. 3b).
HIF1-α is involved in the hypoxia-induced expression of PLD2 in vitro

To characterize the hypoxia-induced PLD2 expression, we tested the role of HIF1-α using HIF1-α siRNA. The siRNA targeting HIF1-α reduced the level of HIF1-α mRNA (p < 0.01, Fig. 4a) and the expression of HIF1-α protein (p < 0.01, Fig 4b). Up-regulation of PLD2 mRNA, induced by hypoxia, was significantly decreased when HIF1-α expression was knocked down by siRNA (p < 0.01, Fig. 5a). Consistent with this observation, HIF1-α siRNA also decreased PLD2 protein expression in response to hypoxia (p < 0.01, Fig. 5b).

Down-regulation of HIF1-α significantly reduces tumor volume and the expression of PLD2 in vivo

In vivo growth study was performed by SW620 and SW480 cells. We set up xenograft tumor model according to the method introduced in Materials and Methods. Growth of the xenograft tumor was studied by tumor growth curve. Expression of PLD2 in xenograft tumor was detected by Western blot analysis. From the tumor growth curve, we found that there was a statistical difference in tumor growth between HIF1-α interference group and flank control group after 21 days (p < 0.01, Fig. 6a). Moreover, it is interesting that the growth of xenograft tumor had no
difference between HIF1-a interference group and flank control group in the early stage such as at 15 days \((p > 0.05)\). From Western blot analysis, we found that there was a significant difference between interference group and flank control group in the expression of PLD2 in colon cancer xenograft tumor \((p < 0.01, \text{Fig. 6b})\).

**Discussion**

Previous evidence suggests that PLD plays important roles in progression of cancer as a signal-transmitting enzyme. PLD2 is an important member of PLD family and is examined high expression in tumor, such as breast cancer [12] and colorectal cancer [31, 32]. Expression of PLD2 in tumor cells plays a role in apoptosis, facilitating tumor growth and chemoresistance [17]. However, the expression of PLD2 is not static but dynamic in response to environment such as hypoxic stress. It is not yet clear whether hypoxic stress induces PLD2 in colon cancer cells.

In this study, we have demonstrated that expression of PLD2 was up-regulated in SW480 cells and SW620 cells that were exposed to hypoxic stress. Up-regulation of
PLD2 mRNA and protein in SW480 and SW620 cells was observed after exposure to 12 h and 24 h of hypoxic stress. Yamakawa et al. [11] showed that protein expression of PLD2 was induced by a relatively short-term exposure to hypoxic environment for 12 h in human PC12 cells. However, PLD2 mRNA level was decreased in the hippocampus after ischemia [35]. Although difference in the duration of hypoxia and the types of cells may account for differential cellular responses in PLD2 expression, these results suggest that expression of PLD2 can be dynamically regulated by hypoxic stress. PLD2 initiates signals through catalytic hydrolysis of phosphatidylcholine to generate PA and choline, which could take part in signal transduction as a second messenger and then activate downstream mediators, such as NF-κB, to activate genes that encode apoptosis-correlated proteins. NF-κB is a multifunctional transcription factor that effects tumor growth and apoptosis. Kang DW proved that NF-κB-mediated PLD promote the growth of breast cancer cells [36]. Utilizing in vitro systems, researchers have reported that there is a cross-talk between HIF1-α and NF-κB [37, 38]. Park et al. [39] demonstrated that PLD2 signaling influenced invasion and metastasis and was a critical upstream activator of NF-κB. Kang et al. [29] reported that PLD2 induces HIF1-α expression in chronic autoimmune arthritis. These studies are consistent with a role for the PLD2 signaling pathway in regulating HIF1-α transcription. Here, we found that the hypoxia-induced PLD2 expression was HIF1-α dependent, as demonstrated by the ability of HIF1-α siRNA to inhibit hypoxia-induced PLD2 up-regulation in SW480 and SW620 cells. These results suggest that HIF1-α promotes PLD2 expression under hypoxic conditions. Together, these results suggest that PLD2 induction by hypoxia was determined, at least in part, by HIF1-α.

HIF1-α siRNA down-regulation indicates that the protein is required for sustained colon cancer growth. We also indicate that the reduction in HIF1-α had an obvious effect on reducing the expression of PLD2 of colon cancer cells in vivo. These results further support a role for HIF1-α in mediating PLD2 expression in colon cancer and imply that PLD2 and HIF1-α together may play a role in the growth of colon tumor. Dong Woo Kang reported that positive feedback regulation between PLD and Wnt signaling promotes the growth of colorectal cancer cell [40]. Furthermore, one of the targets of PLD signaling is mammalian target of rapamycin (mTOR), which is a critical regulator of cell cycle progression and cell growth [41]. Those reports indicates HIF1-α up-regulate PLD2 inhibit the growth of colon cancer may through those signaling pathways.

The present study revealed a new mechanism through which HIF1-α up-regulates PLD2 expression in colon cancer and provided evidence that hypoxia increased the expression of PLD2 likely by modulating HIF1-α gene expression. These results underscore the important role of HIF1-α-induced PLD2 in tumor growth. However, further studies are needed to elucidate the mechanism by which HIF1-α drives PLD2 up-regulation and the effects of them on colon cancer cell growth.

Acknowledgments This work was supported by Grants from the National Natural Science Foundation of China (No. 81201916). The authors gratefully thank Laboratory Research Center of The First Affiliated Hospital of Chongqing Medical University for providing equipment support.

Conflict of interest All the authors indicate no potential conflicts of interests.

References


学霸图书馆

www.xuebalib.com

本文献由“学霸图书馆-文献云下载”收集自网络，仅供学习交流使用。

学霸图书馆（www.xuebalib.com）是一个“整合众多图书馆数据库资源，提供一站式文献检索和下载服务”的24小时在线不限IP图书馆。

图书馆致力于便利、促进学习与科研，提供最强文献下载服务。

图书馆导航：

图书馆首页 文献云下载 图书馆入口 外文数据库大全 疑难文献辅助工具