Biochemical and Biophysical Research Communications 471 (2016) 205–212

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Cobalt chloride-mediated protein kinase Cα (PKCα) phosphorylation induces hypoxia-inducible factor 1α (HIF1α) in the nucleus of gastric cancer cell

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ARTICLE INFO

*Article history:*
Received 10 January 2016
Accepted 22 January 2016
Available online 28 January 2016

*Keywords:*
Cobalt chloride
Gastric epithelial cell
HIF1α
Hypoxia
p300
PKC

ABSTRACT

Hypoxia promotes cancer progression, and metastasis. The major protein expressed in hypoxic solid cancer is hypoxia-inducible factor 1 (HIF1). We show that enhanced phosphorylation of a conventional protein kinase C isoform, PKCα, at threonine 638 ($T^{638}$) by hypoxia-mimetic cobalt chloride induces HIF1α in nuclei of gastric epithelial cells (GECs). Moreover, phospho-$T^{638}$-PKCα (P-PKCα) interacts with p300-HIF1α complex in the nuclei of hypoxic GECs and PKCα phosphorylation at $T^{638}$ enhances transcriptional activity of HIF1α. High P-PKCα expression in neoplastic gastric cancer biopsy samples as compared to nonneoplastic samples suggests that P-PKCα might act as an indicator of gastric cancer progression.



## 1. Introduction

Hypoxia induces a number of proteins which enable cells to adapt to low-oxygen environment. The master transcriptional regulator in hypoxic cells is a heterodimeric protein hypoxia-inducible factor 1 (HIF1). HIF1 binds to the hypoxia-response element (HRE) of the target gene to induce transcription. It comprises of α and β subunits. The β subunit is constitutively expressed but HIF1α is only detected in hypoxic cells or in cells that are under oxidative stress [1]. Prolyl hydroxylation of HIF1α in the normoxic cells catalyzed by prolyl hydroxylases (PHDs) leads to its binding with the pVHL-E3 ubiquitin ligase complex following degradation. Hypoxia inhibits prolyl hydroxylation of HIF1α and stabilizes it [2]. Cobalt chloride ($CoCl_2.6H_2O$) induces biochemical responses similar to hypoxia [3].

Blood circulation is inefficient in solid tumors and as a result, hypoxic regions are common at the core of solid tumors [4]. Prolonged hypoxia induces apoptosis but cells that can survive hypoxic assault, emerge as more drug-resistant and metastatic [5–7]. Besides HIF1, the PKC family of Ser/Thr kinases are also induced by hypoxia that can regulate various cellular functions including cell growth, differentiation and apoptosis [8]. Tissue ischemia or hypoxic stress leads to the activation of various protein kinase C (PKC) family members. PKCs protect against hypoxic injury and are implicated in ischemia-related diseases [9]. The PKC family has ten isoforms grouped in three subfamilies based on their differences in structure and catalytic domains and on their ability to respond to the cofactors $Ca^{++}$ and diacylglycerol (DAG). PKCα is a conventional or classical isoform and requires both $Ca^{++}$ and DAG for activation [10].

PKCα promotes invasiveness of various cancers including gastric

*Abbreviations:* $CoCl_2 \cdot 6H_2O$, Cobalt chloride hexahydrate; DAG, Diacylglycerol; DAPI, 4′,6-Diamidino-2-Phenylindole, Dilactate; DN, Dominant negative; GECs, Gastric epithelial cells; HDAC1, Histone-deacetylase 1; HIF1, Hypoxia-inducible factor 1; HRE, Hypoxia-response element; NLS, Nuclear localization signal; PHDs, Prolyl hydroxylases; PKC, Protein kinase C; PVDF, Polyvinylidene fluoride; SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; WT, Wild type.

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http://dx.doi.org/10.1016/j.bbrc.2016.01.140
0006-291X/

cancer [11,12]. But contradicting reports on PKCα downregulation in several cancers [11] and PKCα-mediated apoptosis induction in 12-O-tetradecanoyl phorbol-13-acetate-treated human gastric cancer cell lines [13] also exist. Cellular localization of PKCs determines their function and the effect is tissue or cell line-specific. For example, nuclear translocation of PKCδ in C5 cells induces apoptosis whereas its mitochondrial translocation is required for apoptosis in SP1 cells [14–16]. Phosphorylation and dephosphorylation influence PKC-mediated cellular signaling and impart specificities to PKC binding to distinct substrates as well [17]. Subcellular fractionation shows that phosphorylated active PKCα can translocate to the nucleus [18]. So, subcellular localization of PKCα might be the key factor determining its role in cancer progression. However, its expression status and role in hypoxic gastric cancer cells have not yet been studied.

This study identifies that PKCα phosphorylation at $T^{638}$ is induced in the nucleus of GECs after treatment with $CoCl_2$. Moreover, this study establishes that $T^{638}$-phosphorylated PKCα induces expression of HIF1α after $CoCl_2$ treatment in GECs. As human gastric neoplasia samples also showed highly-increased expression of P-PKCα as compared to noncancerous samples, P-PKCα might be useful as a molecular marker to study gastric cancer progression. Since fluctuations in blood flow during solid tumor development creates hypoxic state, our results implicate that P-PKCα might be a regulator of HIF1α activity in aggressively growing solid tumors.

## 2. Materials and methods

### 2.1. Cell culture and treatment

Cell lines and reagents used in this work are listed in supplementary materials.

### 2.2. Plasmids, site-directed mutagenesis and transfection

Wild type (WT) PKCα and its dominant negative (DN) mutant K368R (mutated in the ATP binding cassette and is kinase function-dead) were gifted by Bernard Weinstein (Addgene plasmids # 21232 and #21235, respectively) [19]. PKCα T638A mutant construct was generated from the WT PKCα construct using QuikChange site directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Mutagenesis primers and transfection method are described in supplementary materials.

### 2.3. Immunoprecipitation, western blotting and antibodies

Whole cell lysates were prepared by using standard protocol. Nuclear and cytoplasmic fractions were isolated using NE-PER kit (Thermo scientific, MI). Further details are provided in supplementary materials.

### 2.4. Immunofluorescence microscopy of gastric cancer biopsies

Immunofluorescence staining was performed to detect HIF1α, P-PKCα, PKCα proteins in human gastric cancer biopsy samples. Gastric neoplasia and metastatic biopsy samples from the antral gastric mucosa were collected from patients who were undergoing diagnostic esophagogastroduodenoscopy following a NISER Review Board-approved protocol. Biopsy samples from surrounding non-cancerous areas were used as control samples. Research was carried out in accordance with the Declaration of Helsinki (2013) of the World Medical Association. Written informed consent was obtained from all patients prior to the study. Primary antibodies used were HIF1α, P-PKCα and PKCα (1:500). Digital images were captured using a fluorescence microscope (Carl Zeiss, Jena, Germany).

### 2.5. Confocal microscopy

Detailed protocol for confocal microscopy is given in the supplementary materials section.

### 2.6. Dual luciferase assay

Dual luciferase assay was performed to study the effect of PKCα overexpression on the transcriptional activity of HIF1α according to manufacturer's instruction (Promega, CA) details of which can be found from the supplementary materials section.

### 2.7. Statistical analysis

All data were presented as mean ± SE from three or more independent experiments. Student's *t*-test was performed for statistical comparisons between two experimental groups. Statistical significance was determined at $P < 0.05$.

## 3. Results

### 3.1. Short treatment with $CoCl_2$ induces nuclear P-PKCα expression

Expression of PKCα and P-PKCα was assessed in normoxic (or control) and $CoCl_2$-treated AGS cells. Comparison of western blots ($n = 3$) of whole cell lysates prepared from control or 30 min, 1 h and 3 h $CoCl_2$-treated (200 μM) AGS cells did not show any change in expression of PKCα and P-PKCα (Fig. 1A). $CoCl_2$ at 200 μM was not cytotoxic to AGS cells (data not shown). Expression of HIF1α time-dependently increased with $CoCl_2$ treatment. Western blot results of the nuclear fractions identified significant ($n = 3$; $P < 0.05$) induction of P-PKCα in the nuclear fraction of $CoCl_2$-treated AGS cells as compared to control cells. Whereas, equal P-PKCα expression was observed in the cytosol of $CoCl_2$-treated and control cells at all time points. On the contrary, total PKCα was constitutively expressed in both compartments and was not changed after $CoCl_2$ treatment (Fig. 1B). GAPDH and HDAC1 immunoblots were used as cytosolic and nuclear loading controls, respectively. We next sought to determine whether 200 μM $CoCl_2$ was able to effectively induce P-PKCα expression in another gastric cancer cell line, NCI-N87. Western blot data confirmed that 200 μM $CoCl_2$ induced nuclear expression of P-PKCα in NCI-N87 cells as it did in AGS cells (Supplementary Fig. S1). Confocal microscopy data also showed the same trend of P-PKCα and PKCα expression in the nuclear and cytosolic compartments of control and $CoCl_2$-treated cells (Fig. 1C and Supplementary Fig. S2). Antral biopsy samples obtained from consenting patients were used to study expression of PKCα and P-PKCα proteins in gastric neoplasia. Comparison of fluorescence microscopy images obtained from neoplasia patients with images from non-cancer gastric “control” tissues showed very high P-PKCα expression in neoplasia samples but no difference was observed amongst these groups with respect to PKCα expression (Supplementary Fig. S3). However, we did not observe any difference between metastatic gastric cancer samples ($n = 4$) collected from antrum and adjacent non-cancer “control” samples ($n = 4$) with respect to PKCα expression but P-PKCα expression was substantially less in metastatic samples as compared to control samples (Supplementary Fig. S4). Next, we wanted to examine whether HIF1α expression differs in metastatic and neoplastic samples. Immunofluorescence microscopy data showed equivalent high expression of HIF1α in both neoplastic and metastatic gastric cancer tissues as compared to their non-cancer counterparts (Supplementary Fig. S5).