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NOX4 supports glycolysis and promotes glutamine metabolism in non-small cell lung cancer cells

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ABSTRACT

Our previous studies have confirmed that NADPH oxidase 4 (NOX4) is abundantly expressed in non-small cell lung cancer (NSCLC) and contributes to cancer progression. Nevertheless, the comprehensive mechanisms for NOX4-mediated malignant progression and oxidative resistance of cancer cells remain largely unknown. This study found that NOX4 directed glucose metabolism not only to the glycolysis but also to pentose phosphate pathway (PPP) pathway for production of NADPH in non-small cell lung cancer (NSCLC) cell lines. Besides, we also found that NOX4 promoted glutaminolysis into total GSH synthesis. Specifically, the data showed that ectopic NOX4 expression did not induce apoptosis of NSCLC cells; however, inhibition of GSH production resulted in obvious apoptotic death of NOX4-overexpressed NSCLC cells. Furthermore, we demonstrated that NOX4-induced glycolysis probably via ROS/PI3K/Akt signaling-dependent c-Myc upregulation. The selective NOX4 inhibitor, GKT137831, significantly inhibited glucose and glutamine metabolic phenotypes both in vitro and in vivo, and itself or combination with 2-DG, a synthetic glycolytic inhibitor, suppressed cancer cell growth both in vivo and in vitro. Elimination of NOX4-derived H_2O_2 effectively reversed NOX4 overexpression-mediated metabolic effects in NSCLC cells. NOX4 levels were significantly correlated with increased glucose and glutamine metabolism-related genes, as well as Akt phosphorylation and c-Myc expression in primary NSCLC specimens. In conclusion, these results reveal that NOX4 promotes glycolysis, contributing to NSCLC growth, and supports glutaminolysis for oxidative resistance. Therefore, NOX4 may be a promising target to reverse malignant progression of NSCLC.

1. Introduction

Lung cancer is the most common cause of cancer mortality. Non-small cell lung cancer (NSCLC) accounts for up to 80% of all lung cancer cases with poor prognosis [1]. There are increasing evidences suggesting that cancer cells exhibit special metabolic phenotypes, such as enhanced glycolysis and glutaminolysis, that are essential for them to sustain high proliferative rates and resist cell death signals [2,3].

NADPH oxidases (nicotinamide adenine dinucleotide phosphate oxidase, NOXs) are a family of enzymes with the primary function to generate superoxide ($O_2^{\cdot-}$) or hydrogen peroxide (H_2O_2). They consist of seven members, represented by different catalytic subunits: NADPH oxidase 1 (NOX1), NOX2 ($gp91^{phox}$), NOX3, NOX4, NOX5, Duox1, and Duox2 [4]. NADPH oxidases (NOXs) have been confirmed to be correlated with progression of many diseases, especially for cancer [5]. Specially for NOX4, it is the most frequent NOX isoform in several cancer cell lines [4]. In NSCLC, we found that NOX4 is abundantly expressed and contributes to NSCLC progression either by itself or cooperation with IL-6 [6,7]. Nevertheless, the comprehensive mechanisms for NOXs-mediated malignant progression of cancer remain largely unknown.

Recently, increasinging evidences indicate a critical role of NOXs in regulation of cancer metabolism. Lu et al. reported that induction of mitochondrial respiratory defect in pancreatic cancer cells caused

Abbreviations: NOX4, NADPH oxidase 4; PPP, pentose phosphate pathway; NSCLC, non-small cell lung cancer; ROS, reactive oxygen species; OXPHOX, oxidative phosphorylation; Glut1, glucose transporter 1; LDHA, lactate dehydrogenase A; PKM2, pyruvate kinase M2; Gln, glutamine; GLS, glutaminase; GS, glutathione synthetase; GSH, glutathione

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increased NOX-mediated ROS generation and subsequent a metabolic shift from oxidative phosphorylation to glycolysis [8]. In glioblastoma cells, inhibition of NOX4 suppressed the glycolytic phenotype and synergized with 2-deoxy-D-glucose to inhibit cancer cell growth [9]. However, the precise functions of NOXs in cancer cell metabolism and what the role of NOX4 in metabolism of NSCLC remains still unidentified.

On the other hand, NOX4, in contrast to that other NOXs mostly produce $O_2^{\cdot-}$, uniquely produces large amount of H_2O_2 constitutively [10]. It has been confirmed that an increase in the intracellular $O_2^{\cdot-}$ concentration in the absence of cytotoxic production of H_2O_2 does not kill cells while inhibits activation of the apoptotic pathway. On the contrary, higher concentration of H_2O_2 is directly responsible for cell apoptosis under oxidative stress [11]. We previously found that ectopic NOX4 expression in NSCLC cells did not induce cell apoptosis but promoted cell growth [7]. How does NSCLC cells adapt to NOX4-mediated oxidative stress and display apoptosis resistance?

In this study, we illustrated that NOX4 promoted glucose metabolism to glycolysis and the pentose phosphate pathway (PPP) in NSCLC cells. Specifically, we discovered an unexpected function of NOX4 that supported the glutamine metabolism for GSH production, contributing to oxidative adaption of NSCLC cells. Inhibition of NOX4 reversed metabolic reprogramming and suppressed cancer cell growth in vivo. Therefore, NOX4 may be a promising target to reverse metabolic reprogramming and thus malignant progression of NSCLC.

2. Materials and methods

2.1. Materials

LY294002 (PI3K inhibitor) was obtained from Merck. GKT137831 (NOX4 inhibitor) was purchased from Selleckchem. DPI (NADPH oxidase inhibitor), BSO (a specific inhibitor of GSH synthesis), 2-Deoxy-D-glucose (2-DG) and PEG-catalase (polyethylene glycol-catalase) were purchased from Sigma. Cell culture reagents were obtained from Invitrogen. All other reagents were from Sigma unless stated otherwise.

2.2. Cell lines, plasmids, and transfection

A549 and H460 cell lines (originally purchased from ATCC) were used. Cells were incubated at 37 °C in an atmosphere of 5% CO_2 in DMEM supplemented with 10% FBS, penicillin-streptomycin and glutamine (2 mM). Cells were transfected with 100 nM of a control siRNA, two individual siRNA against NOX4 (OriGene, SR309388) or a pCMV-NOX4 cDNA plasmid to overexpress the human NOX4 protein according to our previous study [6], together with Lipofectamine 2000 (Invitrogen, 11668-019) overnight according to manufacturer's instructions. The NOX4 siRNA sequences used in present study as follows: siRNA1: AGAGTATCACTACCTCCACCAGATGTTGG, and siRNA2: AACCTCTTCTTTGTCTTCTACATGCTGCT. Additionally, cells were transfected with 50 nM of c-Myc siRNA (Santa, sc-29226) using Lipofectamine 2000.

2.3. Real-time RT-PCR

Total RNA was extracted from cells using Trizol Reagent (Invitrogen), and then complementary DNA (cDNA) was synthesized using ReverTra Ace reverse transcriptase (TOYOBO, Japan, FSQ-301) according to the manufacturer's instructions. Real-time RT-PCR was performed with the SYBR Green Realtime PCR Master Mix (TOYOBO, Japan, QPK-201) on an iCycler (Bio-Rad) following the manufacturer's instructions. The primers used in real-time quantitative PCR are shown in Supplementary Table S1.

2.4. Western blotting

Western blotting protocol was according to our previous report [6]. The membrane was first probed primary antibodies as follows: NOX4 (ab133303), Glut1 (ab40084), PKM2 (ab150377), LDHA (ab47010), G6PD (ab87230), GS (ab176562), GLS (ab156876), c-Myc (ab32072) and β-Tubulin (ab6046) purchased from Abcam. The secondary antibody were Goat anti-mouse or Goat anti-rabbit IgG (Proteintech, USA, SA00001-1 and SA00001-2), respectively. The bands in the membrane were visualized and analyzed by UVP BioImaging systems.

2.5. Measurement of ATP production, glucose consumption, lactate production

Cellular ATP, normalized by the cell number, was determined with the use of fluorometric-based assay (Sigma, MAK190). Glucose and lactate concentrations in the cultured medium were measured with the cell-based assay kit (Cayman Chemical Co., Ann Arbor, MI, USA, 600470) and lactate assay kit (Sigma, MAK064), respectively. Glucose uptake and lactate production were corrected by amounts of cellular protein.

2.6. Measurement of GSH, GSSG, NADPH, and $NADP^+$ production

Intracellular levels of GSH and ratio of GSH to GSSG were determined by using GSH-Glo Glutathione Assay Kit (Promega, V6912 and V6911). Intracellular nucleotides $NADP^+$ and NADPH were measured using the $NADP^+$ and NADPH assay kit (Abcam, ab65349).

2.7. NADPH oxidase activity assay

Cells were subjected to the indicated treatments and subsequently lysed by sonication in a cold protease inhibitor buffer, centrifuged at 1000*g* for 10 min at 4 °C to remove cell debris. The pellet was suspended in a protease inhibitor buffer and the protein concentration was measured. The NADPH oxidase activity was measured by lucigenin-enhanced chemiluminescence after addition of NADPH. Duplicate samples were also incubated with catalase and the catalase-inhibitable chemiluminescence was measured in a THERMO Multiskan GO Spectrofluorimeter.

2.8. Measurement of H_2O_2 production

Amplex Red® hydrogen peroxide assay kit (Invitrogen) was used to determine extracellular steady-state generation of H_2O_2. After treatment, cellular H_2O_2 production was determined using 10-acetyl-3,7-dihydroxyphenoxazine which is a substrate for horseradish peroxidase (HRP) that enables selective detection of H_2O_2. In the presence of peroxidase, this reagent reacts with H_2O_2 to produce resorufin. Resorufin fluorescence was measured in the plate reader with excitation at 530 nm and emission at 590 nm at 37 °C.

2.9. Flow cytometry (FCM) analysis of apoptosis and caspase3/7 activity assay

Apoptotic cell death was determined by flow cytometric analysis of cells double stained with Annexin V-FITC and propidium iodide (PI) using an assay kit (BD, PharMingen, San Diego, CA). Briefly, after BSO incubation, cells were collected, washed with cold PBS, suspended in 5 μL of Annexin V binding buffer and stained with 5 μL of PI. The cells were mixed gently and incubated in the dark for 20 min, washed. The samples were analyzed with a FACS (Beckman Coulter, CA). For detection of Caspase activity, after transfection with NOX4 plasmid, 5×10^4 A549 or H460 cells were seeded in 96-well plates and incubated overnight. Cells were switch to fresh culture medium in the presence of control solvent (0.1% DMSO) or BSO. After 48 h incubation, the