Original Paper

PDGF Promotes the Warburg Effect in Pulmonary Arterial Smooth Muscle Cells via Activation of the PI3K/AKT/mTOR/HIF-1 α Signaling Pathway

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Key Words

Warburg effect • Pulmonary arterial smooth muscle cells • Platelet-derived growth factor

Abstract

Background/Aims: The enhanced proliferation of pulmonary arterial smooth muscle cells (PASMCs) is a central pathological component in pulmonary arterial hypertension (PAH). Both the Warburg effect and platelet-derived growth factor (PDGF) are involved in the proliferation of PASMCs. However, the mechanism underlying the crosstalk between the Warburg effect and PDGF during PASMC proliferation is still unknown. We hypothesized that PDGF promotes the Warburg effect via activating the phosphatidylinositol 3-kinase (PI3K) signaling pathway and hypoxia-inducible factor 1- α (HIF-1 α) in proliferative PASMCs. **Methods:** PASMCs were derived from pulmonary arteries of SD rats; cell viability, the presence of metabolites, and metabolic enzyme activities assay were determined by MTT assays, kit assays and western blot analysis, respectively. **Results:** PDGF promoted PASMC proliferation in a dose- and time-dependent manner, accompanied by an enhanced Warburg effect. Treatment with PDGFR antagonists, Warburg effect inhibitor and PDK1 inhibitor significantly inhibited PI3K signaling activation, HIF-1 α expression and PASMC proliferation induced by PDGF, respectively. Furthermore, treatment with PI3K signaling pathway inhibitors remarkably suppressed PDGF-induced PASMC proliferation and the Warburg effect. **Conclusion:** The Warburg effect plays a critical role in PDGF-induced PASMC proliferation and is mediated by activation of the PI3K signaling pathway and HIF-1 α .

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Introduction

Pulmonary arterial hypertension (PAH) is a pulmonary vascular remodeling disease with a continuous and notable elevation of pulmonary arterial pressure (PAP) and pulmonary vascular resistance (PVR) resulting in right heart failure and death [1]. Pulmonary arterial vascular smooth muscle cell (PASM C) proliferation is a pivotal part in the pathological vascular remodeling of pulmonary arterial hypertension (PAH) [1], whose mechanism is still a source of controversy. The Warburg effect, also named aerobic glycolysis, is a metabolic shift toward glycolysis and has been identified as central to malignant transformation in a number of tumor types. The effect is characterized by production of lactate to form an acid environment that creates a protective effect for cancer cells [2, 3]. In recent decades, many researchers have found that cancer and PAH share not only a similarly poor prognosis but also a major pathophysiologic mechanism, including cell proliferation and the abnormal metabolic pathway that constitutes the Warburg effect [4-6], which indicates that the Warburg effect may play an essential role in cell proliferation related to the vascular remodeling process of PAH. However, it remains unclear whether the Warburg effect is involved in PASM C proliferation.

Increased expression of platelet-derived growth factor (PDGF) and platelet-derived growth factor receptor (PDGFR) were found in lung tissue of patients and animals with PAH. PDGFR is a receptor tyrosine kinase [7], which exerts its actions via binding PDGF and then activating a downstream signaling pathway to promote PASM C proliferation and migration [8]. The phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway is downstream of PDGFR [9] and plays an essential part in provoking the Warburg effect in tumor cells [10, 11] by upregulating hypoxia-inducible factor 1 α (HIF-1 α) and then increasing pyruvate dehydrogenase kinase 1 (PDK1) expression, which suppresses pyruvate dehydrogenase (PDH) activity and shifts metabolism to aerobic glycolysis [5, 12, 13]. Studies have shown that PI3K inhibitors and Warburg effect inhibitors attenuate PDGF-induced aorta vascular smooth muscle cell (AVSM C) proliferation and aerobic glycolysis [14, 15]; moreover, AKT and mTOR inhibitors suppress PDGF-induced PASM C proliferation [16]. Thus, we hypothesized that PDGF upregulates the Warburg effect via binding PDGFR and then activating the PI3K/AKT/mTOR/HIF-1 α signaling pathway to promote PASM C proliferation.

In this study, we investigated the effect of exogenous PDGF in the Warburg effect and PASM C proliferation *in vitro*. Our data indicated that PDGF caused an increase of the Warburg effect in the proliferation state of PASM Cs; the mechanism involved activation of downstream PI3K/Akt/mTOR/HIF-1 α signaling pathways.

Materials and Methods

Materials

Primary antibodies against PCNA, Glut1, Glut4, PDK1, PDH, LDH, PI3K, p-PI3K, and HIF-1 α were purchased from Abcam. Primary antibodies against AKT, p-AKT, mTOR, and p-mTOR were purchased from Cell Signaling Technology and MCT4 and β -actin antibodies were obtained from Santa Cruz Biotechnology Inc. Imatinib, LY294002, RAPI, and PDGF were purchased from Sigma Alorich, and Sorafenib, 2-deoxy-D-glucose (2-DG), dichloroacetate (DCA) were purchased from Kang Baotai (Wuhan, China). Lactate and glucose assay kits were bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). ATP assay kit was purchased from Beyotime Institute of Biotechnology (Haimen, China). MTT was obtained from Solarbio Life Science (Beijing, China).

Animals

Male Sprague-Dawley (SD) rats (weighing 110–150 g) were obtained from the Laboratory Animal Center at the University of South China (Hengyang, China). All live animals were handled in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the approval of the University of South China Animal Care and Use Committee.

Cell culture

PASMCs were isolated from the pulmonary arteries of 10-week-old male SD rats using our laboratory's previously described method [14]. The cells were cultured at 37°C under 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS). PASMCs were identified by immunohistochemical staining using an antibody against smooth muscle α -actin [14]. PASMCs between passages 3 and 8 were used for the experiments.

Cell viability assays

Cell viability was analyzed using MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl- tetrazolium bromide) assays. PASMCs were cultured in 96-well culture plates and exposed to different treatment factors according to the PSMC experiments. Then, MTT dye was added and incubated for 4 hr. The optical density (OD) values were measured at 570 nm using BioTek's Gen5™ microplate reader (Biotek, Winooski, VT) after removing the supernatant and dissolving the formazan in DMSO.

Detection of extracellular glucose consumption, lactic acid (LD) level and cellular ATP

Levels of extracellular glucose consumption, lactic acid (LD) and cellular ATP were determined by a commercially available colorimetric glucose assay kit, and LD assay kit, or an ATP assay kit according to the manufacturer's instructions. ATP levels were normalized to protein levels.

Western blot analysis

Protein was extracted from PASMCs with lysis buffer (RIPA buffer: PMSF=6:1), and equal amounts of protein from each sample (20 μ g or 40 μ g) were separated by 12% or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. The membranes were then incubated with primary antibodies overnight at 4°C and horseradish peroxidase-coupled goat anti-rat or anti-rabbit secondary antibody. The chemiluminescence signals were detected with a chemiluminescent HPR substrate (Millipore Corporation, Billerica, USA). The densitometric analysis was conducted with Alpha Imager 2000 (Alpha Innotech Corporation, San Leandro, USA).

Statistical analysis

All data were expressed as arithmetic mean $\pm$ SEM. Statistical analysis was performed with an unpaired Student's t-test or one-way ANOVA followed by a Student-Newman-Keuls test where appropriate. Statistical significance was defined as $P < 0.05$. The number of experiments per group is indicated in the figure legends.

Results

PDGF promotes PSMC proliferation in a dose- and time- dependent manner

PASMC proliferation induced by PDGF has been described using cell viability and the expression of PCNA as evaluated using MTT assays and western blotting, respectively. The results showed that PDGF increased PSMC viability and expression of PCNA protein in a concentration-dependent and time-dependent manner (Fig. 1A-1D).

PASMC proliferation accompanied by enhanced Warburg effect

To explore the relationship between PDGF and the Warburg effect in proliferative PASMCs, the PASMCs were treated with PDGF in the present experiments, and glucose consumption and lactate production in the culture medium were analyzed using a kit. The results showed a pronounced increase in glucose consumption and lactate production and a marked decrease in ATP production in the proliferative cells (Fig. 2A-2C), which indicated that the metabolic activity of PASMCs exhibited the Warburg effect in cell proliferation. The expression of lactate dehydrogenase (LDH), one of key proteins of the Warburg effect, was increased after treatment with PDGF (Fig. 2D), whereas expression of pyruvate dehydrogenase (PDH), another key protein of the Warburg effect, was attenuated after treatment with PDGF (Fig. 2E), which further suggested that the Warburg effect in PASMCs was enhanced by PDGF. Consistent with this, glucose transporter 1 (Glut1), glucose transporter 4 (Glut4)

Fig. 1. The relationship of PDGF dose and time to the promotion of PASM C proliferation. Incubation of cells with PDGF for 12 hr increased cell viability (A) and expression of PCNA protein (C) in a concentration-dependent manner, with a peak at 15 ng/mL; # and ## indicate $P < 0.05$ and $P < 0.01$, respectively, compared with 0 ng/mL. Treatment with 15 ng/mL PDGF increased cell viability (B) and expression of PCNA (D) in a time-dependent manner, with a peak at 24 hr; # and ## indicate $P < 0.05$ and $P < 0.01$, respectively, compared with 0 hr. Bars represent means $\pm$ SEM of $n = 3$.

Fig. 2. PDGF promotes the Warburg effect in proliferative PASM Cs. (A-C) Glucose consumption, lactate and ATP production were determined by kit analysis. Representative immunoblots and relative densitometric analysis of (D) LDH, (E) PDH, (F) Glut1, (G) Glut4 and (H) MCT4 with β -actin as loading control. Bars represent means $\pm$ SEM of $n = 3$. ## and ### indicate $P < 0.01$ and $P < 0.001$, respectively, compared with the control.

and monocarboxylate transporter 4 (MCT4) were markedly increased in the PDGF-treated PASM Cs (Fig. 2F-2H).

To further determine whether PDGF promotes the Warburg effect in these cells, PDGFR antagonists (sorafenib, imatinib) were used in pretreatment of the PDGF-treated PASM Cs. In addition, the Warburg effect inhibitor (2-DG) and PDK1 inhibitor (DCA) were used as positive controls. The results showed that sorafenib and imatinib markedly decreased glucose consumption and lactate production, with markedly increased ATP production (Fig. 3A-3C). Expression of LDH was significantly decreased, yet

Fig. 3. PDGFR inhibitors attenuated the Warburg effect in proliferative PASM Cs. The glucose consumption (A) and the production of lactate (B) and ATP (C) were determined by kit analysis. Representative immunoblots and relative densitometric analysis of (D) LDH, (E) PDH, (F) Glut1, (G) Glut4 and (H) MCT4 with β -actin as loading control. Bars represent means $\pm$ SEM of $n = 3$. #, ## and ### indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, compared with the control; \$, \$\$ and \$\$\$ indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, compared with PDGF.

PDH protein expression was remarkably increased (Fig. 3D-3E). Furthermore, the Warburg effect was dramatically reversed in PASM Cs simultaneously treated with imatinib and 2-DG (Fig. 3A-3H). Consistently, PDGFR antagonists markedly decreased the expression of Glut1, Glut4 and MCT4 in PASM Cs (Fig. 3F-3H). Interestingly, we also found that PDGFR antagonists and Warburg effect inhibitors markedly attenuated PDGF-induced PASM C proliferation (Fig. 4A-4B), which suggests that PDGF promotes the Warburg effect in proliferative PASM Cs and that the Warburg effect plays a key role in PASM C proliferation.

PI3K/Akt/mTOR/HIF-1 α signaling pathway mediates the Warburg effect in proliferative PASM Cs

The PI3K/Akt/mTOR signaling pathway is an essential mechanism by which tumor cells regulate the Warburg effect and is downstream of the PDGFR signaling pathway [10,

Fig. 4. Inhibiting PDGFR and the Warburg effect suppresses PDGF-induced PASM C proliferation. (A) Cell viability of PASM Cs was measured by MTT assay (B) Representative immunoblots and relative densitometric analysis of PCNA with β -actin as loading control. Bars represent means $\pm$ SEM of $n = 3$. # indicates $P < 0.05$ compared with the untreated control; \$, \$\$ and \$\$\$ indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, compared with PDGF.

Fig. 5. PDGF causes activation of the PI3K/AKT/mTOR/HIF-1 α signaling pathway in proliferative PASM Cs. Representative immunoblots and relative densitometric analysis of PI3K, AKT, mTOR phosphorylation levels, HIF-1 α and PDK1 in PASM Cs treated with PDGFR antagonists, a Warburg effect inhibitor or a PDK1 inhibitor after treatment with PDGF. (A) PI3K phosphorylation level, (B) AKT phosphorylation level, (C) mTOR phosphorylation level, (D) HIF-1 α expression, and (E) PDK1 expression were measured by western blotting. Bars represent means $\pm$ SEM of $n = 3$. # and ## indicate $P < 0.05$ and $P < 0.01$, respectively, compared with the untreated control; \$, \$\$ and \$\$\$ indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, compared with PDGF; NS indicates no significance.

11]. Here, we documented a trend toward an elevation in the phosphorylation levels of PI3K, AKT and mTOR in PDGF-induced PASM C proliferation (Fig. 5A-5C) but a marked reduction with PDGFR antagonists (Fig. 5A-5C), whereas the Warburg effect inhibitor did not significantly affect PI3K, AKT or mTOR phosphorylation level. Moreover, increased expression of HIF-1 α and its target protein, PDK1, were found in PDGF-induced PASM C proliferation (Fig. 5D-5E),

Fig. 6. PI3K and mTOR inhibitors attenuated the Warburg effect in PDGF-induced PASM C proliferation. PASM Cs were isolated from rats and treated with LY294002 or RAPI for 24 hr after treatment with PDGF for 6 hr. (C-E) Glucose consumption, lactate and ATP production were measured by kit assays. Representative immunoblots and relative densitometric analysis of (A) PI3K, (B) mTOR, (F) LDH, (G) PDH, (H) Glut1, (I) Glut4 and (J) MCT4 with β -actin as loading control. Bars represent means $\pm$ SEM of $n = 3$. #, ## and ### indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, compared with the untreated control; \$, \$\$ and \$\$\$ indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, compared with PDGF; NS indicates no significance.

which was reversed by PDGFR antagonists and Warburg effect inhibitors (Fig. 5D-5E).

As shown in Fig. 6A-6J, a PI3K inhibitor (LY294002) and an mTOR inhibitor (RAPI) made a marked reduction in the Warburg effect in PDGF-induced PASM C proliferation. Meanwhile, we also found significant decreases in HIF-1 α and PDK1 expression in PASM Cs treated with PI3K and mTOR inhibitors (Fig. 7A-7B). Furthermore, PASM Cs treated with PI3K and mTOR inhibitors showed attenuated PDGF-induced proliferation (Fig. 7C-7D). These results indicate that the PI3K/AKT/mTOR signaling pathway activated by PDGF is involved in promoting the Warburg effect in proliferative PASM Cs and further suggest that the Warburg effect plays a key role in PDGF-induced PASM C proliferation.

Fig. 7. PI3K and mTOR inhibitors suppress HIF-1 α and PDK1 expression and inhibit PDGF-induced PASM C proliferation. PASM Cs were isolated from rats and treated with LY294002 or RAPI for 24 hr after treatment with PDGF for 6 hr. Representative immunoblots and relative densitometric analysis of (A) HIF-1 α , (B) PDK1 and (D) PCNA with β -actin as loading control. (C) Cell viability of PASM Cs was measured by MTT assay. Bars represent means $\pm$ SEM of $n = 3$. # and ## indicate $P < 0.05$ and $P < 0.01$, respectively, compared with the untreated control; \$ and \$\$ indicate $P < 0.05$ and $P < 0.01$, respectively, compared with PDGF.

Fig. 8. Working model: A link between PDGF and the Warburg effect in PASM C proliferation. PDGF promotes PASM C proliferation by activating PI3K/AKT/mTOR/ HIF-1 α signaling pathway and increasing PDK1 expression that leading to metabolic way shift toward the Warburg effect.

Discussion

Our study established a connection between PDGF and the Warburg effect in proliferative PASM Cs *in vitro*, specifically, PDGF causes an increase in the Warburg effect and proliferation state of PASM Cs. In addition, the mechanisms whereby PDGF promotes PASM C proliferation involve activation of downstream PI3K/Akt/ mTOR /HIF-1 α signaling pathways followed by enhancement of the Warburg effect (Fig. 8).

Abnormal PASM C proliferation is a pivotal component of pulmonary vascular remodeling and leads to medial hyperplasia in PAH [17, 18]. Meanwhile, activation of PDGFR is linked to the pathogenesis of PAH related to abnormal proliferation of PASM Cs [19, 20]. Indeed, research has reported that continuous PDGFR activation results in pulmonary vascular remodeling and pulmonary arterial hypertension in chronic hypoxia-induced PAH mice [21]. Consistently, our results showed that exogenous PDGF promotes PASM C proliferation in a dose- and time-dependent manner, which further demonstrates that PDGF and PDGFR play an important role in the pathogenesis of PAH associated with abnormal PASM C proliferation.

In addition, a key finding of our study was that PDGF promotes PASM C proliferation accompanied with increase of cellular lactate production and LDH, Glut1, Glut4 and MCT4 expression, but decrease of cellular ATP production and PDH expression, which suggested that the metabolic way of PASM Cs shift toward the Warburg effect. To study whether the Warburg effect have a cross talk with PDGFR in proliferative PASM Cs, we employed PDGFR antagonists (sorafenib, imatinib), our results showed that imatinib and sorafenib inhibited PDGFR to inactivate downstream signaling pathways, hence to downregulate the Warburg effect and inhibit PASM C proliferation. In addition, we used Warburg effect inhibitors (2-DG) and found that 2-DG blocked the Warburg effect and inhibited PASM C proliferation, whereas, inhibiting the Warburg effect by 2-DG did not affect PDGFR and its downstream signaling pathway in PASM Cs, which indicated that the Warburg effect plays a role of bridge between PDGFR signaling pathway and PASM C proliferation. Indeed, research has reported that PDGF promotes ASM C proliferation accompanied by enhanced aerobic glycolysis [14, 15], and a Warburg effect inhibitor attenuated PDGF-induced vascular smooth muscle cell (VSMC) proliferation [14]. These results strongly suggest that the Warburg effect is involved in PDGF-induced PASM C proliferation.

The Warburg effect, also known as aerobic glycolysis, is a metabolic phenomenon by which cancer cells produce lactate from glucose even under non-hypoxic conditions [3]. Studies reported that altered mitochondrial metabolism associated with the Warburg effect occurred in animal models of PAH and PAH patients [13]; moreover, animal models of PAH and extravascular tissue of PAH patients also exhibit the Warburg effect [5, 6]. PDK1 plays an important role in the Warburg effect by inactivating PDH and then converting pyruvate to lactate, thereby further shifting the metabolic pathway toward aerobic glycolysis [5, 22]. Moreover, research has reported that PDK1 inhibitors reverse the Warburg effect and ameliorate PAH [5]. Meanwhile, activation of PDGFR increases the activity of PDK1 in PASM Cs [23, 24]. Our study also found that PDGF increases PDK1 expression and that the effect was attenuated by PDGFR antagonists. In addition, our results showed that PDK1 inhibitor (DCA) attenuated the Warburg effect by inhibiting PDK1 expression to increase PDH expression, which results in the metabolic way of PASM C shift toward oxidative phosphorylation. Moreover, blocking PDK1 by DCA did not affect PDGFR and its downstream signaling pathway, but attenuated PASM C proliferation induced by PDGF. These results showed that PDGF promotes the Warburg effect by regulating key enzymes of glucose metabolism in proliferative PASM Cs. However, the signaling between PDGFR and the Warburg effect pathway needs to be elucidated.

Data accumulated thus far have shown that the PI3K/Akt/mTOR signaling pathway is a classic pro-survival pathway in various types of cells [9-11]; furthermore, a critical role of the PI3K/AKT/mTOR signaling pathway in regulating the Warburg effect has also been reported, mainly in tumor cells, and this signaling pathway is also downstream of the PDGFR signaling pathway [10, 11]. Our results showed that PDGF promotes the Warburg effect by activating PI3K, AKT, and mTOR in PASM Cs, which suggested that the PI3K/AKT/mTOR signaling pathway is involved in PDGF-induced PASM C proliferation, at least in part by promoting the Warburg effect. Moreover, it has been reported that HIF-1 α plays a pivotal role in regulating the Warburg effect in tumor cells [25]; HIF-1 α suppresses the tricarboxylic acid cycle (TCA) by directly activating the gene encoding PDK1 [26]. Increased PDK1 then inhibits PDH expression and enhances the Warburg effect [5, 12, 13]. Furthermore, there is cross-talk between the PI3K/AKT/mTOR pathway and HIF-1 α ; thus, we sought to determine whether HIF-1 α serves as the downstream molecule in PDGF-induced PASM C proliferation. We observed that blocking PI3K and mTOR with a specific inhibitor suppressed HIF-1 α expression, whereas suppression of HIF-1 α expression improved the Warburg effect and PASM C proliferation, which indicates that HIF-1 α serves as a downstream regulator of the PI3K/AKT/mTOR signaling pathway in PDGF-induced PASM C proliferation. Thus, we speculated that increased expression of HIF-1 α via the PI3K/AKT/mTOR signaling pathway activated by PDGF stimulation, which leads to upregulation of PDK1 expression, would ultimately enhance the Warburg effect.

Our results indicated that PDGF promotes PASM C proliferation accompanied by an enhanced Warburg effect. In addition, we found the PI3K/AKT/mTOR/HIF-1 α signaling pathway to be involved in promoting the Warburg effect. Therefore, we conducted experiments in order to confirm whether the PI3K/AKT/mTOR/HIF-1 α signaling pathway mediates the Warburg effect in PDGF-induced PASM C proliferation. We showed that blocking PI3K and mTOR attenuated the Warburg effect stimulated by PDGF, which demonstrated that PDGF promotes the Warburg effect by activating the PI3K/AKT/mTOR/HIF-1 α signaling pathway in PASM Cs. Interestingly, we also found feedback regulation between the Warburg effect and HIF-1 α : our results showed that blocking the PI3K/AKT/mTOR/HIF-1 α signaling pathway decreased the Warburg effect, whereas blocking the Warburg effect with 2-DG in turn suppressed HIF-1 α levels, furthermore, recent research has also reported that blocking the Warburg effect with 2-DG in turn represses the Akt/mTOR/HIF-1 α signaling pathway in antiestrogen-resistant breast cancer cells [27]. These results demonstrated the existence of feedback regulation between the Warburg effect and HIF-1 α .

Although we demonstrated that PDGF promotes the Warburg effect by activating the PI3K/AKT/mTOR/HIF-1 α signaling pathway in PASM C proliferation, whether other parallel signaling pathways are also regulated by PDGF to promote the Warburg effect in PASM C proliferation needs to be further explored. Additionally, relevant *in vivo* experiments should be performed in future studies to confirm the important role played by PDGF in PAH associated with abnormal PASM C proliferation.

In conclusion, our study reveals that PDGF promotes PASM C proliferation accompanied by an enhanced Warburg effect, and the regulation mechanism is involved in activation of the PI3K/AKT/mTOR/HIF-1 α signaling pathway. Hence, the molecular mechanism connecting the Warburg effect to PASM C proliferation is clarified in our study, which will enable novel avenues for therapeutic intervention for PAH.

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Disclosure Statement

The authors declare no conflict of interest.

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