Original Paper

High Glucose Induces VEGF-C Expression via the LPA_{1/3}-Akt-ROS-LEDGF Signaling Axis in Human Prostate Cancer PC-3 Cells

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Key Words

Vascular endothelial growth factor-C • Lysophosphatidic acid • Prostate cancer • Hyperglycemia • Lymphangiogenesis

Abstract

Background/Aims: Hyperglycemia has been shown to increase the incidence and metastasis in various types of cancers. However, the correlation between hyperglycemia and lymphatic metastasis in prostate cancer (PCa) remains unclear. Our previous study demonstrated that lysophosphatidic acid (LPA) enhances vascular endothelial growth factor-C (VEGF-C) expression, a lymphangiogenic factor, through activating its receptors LPA_{1/3} in prostate cancer (PCa) cells. Moreover, hyperglycemia up-regulates autotaxin (ATX) expression, a LPA-generating enzyme. Therefore, we propose that high glucose promotes VEGF-C expression through LPA signaling in PCa cells. **Methods:** Pharmacological inhibitors and siRNAs were utilized to investigate the molecular mechanism of high glucose-induced VEGF-C expression. Real-time PCR and Western blot were used to determine the mRNA and protein expressions, respectively. Cellular bioenergetics analysis was performed to determine the glycolysis levels. **Results:** We demonstrated that the expressions of VEGF-C, ATX, and calreticulin were increased upon high glucose treatments in PC-3 cells. Moreover, high glucose-induced VEGF-C expression was mediated through the LPA_{1/3}, PLC, Akt, ROS and LEDGF-dependent pathways. Additionally, high glucose enhanced the aerobic glycolysis via LPA_{1/3}. **Conclusion:** These results indicated that

hyperglycemia leads to LPA synthesis, and subsequent promoting pathological consequence of PCa. These novel findings could potentially provide new strategies for PCa treatments.

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Introduction

Prostate cancer (PCa) is one of the most common male cancers, and androgen-independent PCa cells are highly malignant and metastatic [1]. Since lymph nodes adjacent to the primary tumor, advanced PCa cells often invade into lymph nodes via lymphangiogenesis [2, 3] and then spread to other organs through the lymphatic system, which is known as lymphatic metastasis [4]. Therefore, how to inhibit PCa-induced lymphangiogenesis as well as lymphatic metastasis are critical to control PCa progression in patients.

Lysophosphatidic acid (LPA), a bioactive lysophospholipid, is implicated in several physiological functions, including cell proliferation, migration, angiogenesis and lymphangiogenesis [5-9]. To date, six G protein-coupled receptors, including LPA_{1~6}, were identified to mediate the cellular functions of LPA. Interestingly, PCa cells generate and secrete LPA [10]. Autotaxin (ATX) [11], the major LPA-synthesizing enzyme for extracellular LPA production [12] was highly expressed in PCa cells. These results indicated that PCa might utilize an autocrine mechanism to control LPA production and their-mediated cellular functions. In addition, our previous study showed that extracellular LPA up-regulates the expression of vascular endothelial growth factor (VEGF)-C, a lymphangiogenic factor, in PCa cells [5]. VEGF-C binds to VEGF receptor-3 (VEGFR-3) and activates the subsequent signaling events in lymphatic endothelial cells [13]. Moreover, we revealed that LPA-induced VEGF-C via generating reactive oxygen species (ROS) and lens epithelium-derived growth factor (LEDGF) [5, 9]. Since PCa-releasing VEGF-C has been shown to mediate lymphatic metastasis [14], blocking LPA-mediated signaling might be a potential strategy to confine metastasis of PCa.

Several studies have reported that elevated blood glucose (hyperglycemia) in type II diabetes mellitus (DM) is positively associated with cancer incidence and cancer progression [15, 16]. Hyperglycemia also increases the incidence and metastasis of various types of cancer [17-19]. However, the potential physiological links and the molecular mechanism between hyperglycemia and PCa remain unclear. It has been known that the proliferation of PCa cells is associated with elevated glucose consumption [20]. This might be due to malignant tumor cells typically have much higher glycolytic rates than normal tissues of origin [21]. Interestingly, hyperglycemia increases ATX expression in the adipocytes [22]. Furthermore, previous studies demonstrated that high glucose induces VEGF-A production in human synovial fibroblast [23] and HUVEC [24]. In addition, Ca²⁺ binding chaperone calreticulin (CRT), acts as a stress-related unfolding protein response (UPR) protein [25], plays a role in the pathophysiology of DM [26]. We recently revealed that CRT induces VEGF-A [27] in neuroblastoma cells. Since PCa cells express ATX and release VEGF-C in response to LPA, herein, we proposed that hyperglycemia induces ATX expression and therefore mediates LPA-VEGF-C signaling in PCa cells. In this study, we showed that high glucose treatments indeed increased ATX and VEGF-C in PC-3 cells. Moreover, high glucose-induced VEGF-C expression was mediated through the LPA_{1/3}, PLC, Akt, ROS and LEDGF-dependent pathways. Furthermore, high glucose also enhanced the aerobic glycolysis via LPA_{1/3}. Taken together, our results indicate that hyperglycemia promotes LPA synthesis via ATX and subsequently enhances VEGF-C expression. These novel findings proposed a correlation of DM to PCa-induced lymphatic metastasis and therefore provide new treatment strategies for PCa.

Materials and Methods

Reagents

LPA was purchased from Sigma-Aldrich (Santa Cruz, CA, USA). The antibodies against VEGF-C (Genetex, Irvine, CA, USA), ATX (Abcam), CRT (Upstate Biotechnology, Lake Placid, NY, USA), and GAPDH (Genetex) were used. The inhibitors for LPA_{1/3} (Ki16425), ROS (N-acetylcysteine; NAC), and ATX (S32826) were purchased from Cayman Chemical (Ann Arbor, MI, USA), Sigma-Aldrich (Santa Cruz, CA, USA) and Echelon (Salt Lake City, UT, USA), respectively. The shRNA expression vectors of LPA₁, LPA₃, and LEDGF were purchased from Origene Technology (Rockville, MD, USA) and CRT shRNA was acquired from the National RNAi Core Facility Platform, Academia Sinica (Taipei, Taiwan).

Cell culture

Human PCa cells, PC-3 and LNCaP, were obtained from American Type Culture Collection (Manassas, VA, USA), and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin in a humid atmosphere containing 5% CO₂ at 37°C. For the subcultures, cells were detached with 0.05% trypsin.

High glucose treatments

PCa cells (1×10^5) were seeded in 3.5 cm plates and cultured in RPMI 1640 medium without glucose nor FBS for 16~20 hrs. The control cells were treated with the serum-free medium with 10 mM D-glucose (Sigma-Aldrich) as normal concentration. Additional treatment of 10 and 20 mM of D-glucose were used to provide the high glucose culture environment. The D-mannitol (Sigma-Aldrich) was used as the osmotic control.

The short hairpin RNA (shRNA) transfection

The shRNA expression vectors (1 µg) were transfected into PC-3 cells using PolyJet *in vitro* DNA transfection reagent (Rockville, MD, USA) followed the manufacturer's instructions. After transfection, cells were selected with 0.5 µg/mL puromycin (Invivogen, San Diego, California, USA).

RNA isolation and reverse transcription (RT) PCR

Total RNA was extracted by TriPure isolation reagent (Indianapolis, IN, USA) followed the manufacturer's instructions. Complementary DNA (cDNA) was then synthesized from 1 µg of total RNA using the Toyobo RT-PCR kit (Osaka, Japan).

Real-time PCR

Real-time PCR was carried out using SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) with sequence-specific primers in the iCycler iQ Realtime detection system (Bio-Rad). The GAPDH mRNA expression was used as an internal control. Primer sequences for real-time PCR were: GAPDH (F-5'-AAGGTGAAGGTCGGAGTC and R-5'-TGT AGTTGAGGTCAATGAAGG); LPA₁ (F-5'-GTCTTCTGGGCCATTTTCAA and R-5'-TCATAGTCTCTGGCGAACA); LPA₃ (F-5'-GAAGCTAATGAAGAC GGTGATGA and R-5'-AGCAGGAACCACTTTTTCAC); ATX (F-5'-ACAACG AGGAGAGCTGCAAT and R-5'-ACAACGAGGAGAGCTGCAAT); VEGF-C (F-5'-AGTGTGAGGAGGAGCAACAAGA and R-5'-CTTCCTGAGCCAGGCATC TG); LEDGF (F-5'-GGGCCAAACAAAAAGCTAGA and R-5'-TTCATTGCT CTCCCCGTTAT); CRT (F-5'-CCTCCTCTTTGCGTTTCTTG and R-5'-CAG ACTCCAAGCCTGAGGAC).

Western blot analysis

PCa cells were lysed by RIPA buffer, and proteins concentrations were determined by Bradford assay as described previously [5]. Equal amounts of protein were separated by 10~12% SDS-PAGE. Then, proteins were transferred to polyvinylidene fluoride (PVDF) membranes and blotted by anti-VEGF-C (GeneTex, Irvine, CA, USA), anti-GAPDH (GeneTex), anti-ATX (GeneTex) and anti-CRT (Merck Millipore, Billerica, MA, USA) antibodies. The membranes were visualized by enhanced chemiluminescence (ECL) reagent (Advansta, Menlo Park, CA, USA). The fluorescent signals were detected by FluroChem M (ProteinSimple, San Jose, CA, USA) and quantified using the NIH ImageJ software.

Cellular bioenergetics
measurements

Cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were determined with the Seahorse XFe24 Analyzers (Agilent Technologies, Santa Clara, CA, USA) according to manufacturers' recommendations. Briefly, PC-3 cells were seeded in XF assay plate and incubated in a non-CO₂ incubator at 37°C for 120 min.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and Fisher's protected least significant difference (LSD) test (StatView; Abacus Concept, Berkeley, CA, USA). Each result was obtained after three to six independent experiments, and *P* value of < 0.05 was considered statistically significant.

Results

High glucose upregulates VEGF-C expression in PC-3 cells

Several studies have indicated that hyperglycemia increases the incidence and metastasis in many cancers [17-19]. Lymphangiogenesis and angiogenesis are two important characteristics in cancer progression [28]. Although some studies indicate that hyperglycemia induces VEGF production, which may leads to angiogenesis in human synovial fibroblasts and HUVECs [23, 24], little is known about the relationships between hyperglycemia

Fig. 1. High glucose up-regulates VEGF-C expression in PC-3 cells. (A) Serum-starved PC-3 cells were treated with or without an additional 20 mM of glucose or cultured in glucose-free RPMI medium for 4 hours, and the mRNA expressions of VEGF-A, VEGF-C, FGF-2, and PDGF-BB were then assessed. (B) PC-3 and (C) LNCaP cells were incubated with an additional 10 and 20 mM of glucose (B and C) or mannitol (D) for 2 or 4 hours, and the VEGF-C mRNA (B~D) and protein expressions (E) were then determined by real-time PCR and western blotting, respectively. Cells cultivation in 10 mM of glucose-containing RPMI media were used as the untreated control (set to 1.0), and data were shown as multiples of that. The results are expressed as mean $\pm$ SEM. **p*<0.05; ***p*<0.01 compared with untreated control (*n* $\geq$ 3). UT, untreated control.

and lymphangiogenesis. To investigate whether high glucose regulates lymphangiogenesis, several angiogenic factors, including VEGF-A, VEGF-C, FGF-2, and PDGF-BB, were monitored. Results showed that only the mRNA expression of VEGF-C was augmented upon high glucose stimulation (Fig. 1A). Moreover, the mRNA (Fig. 1B) and protein (Fig. 1E) expressions of VEGF-C were upregulated through high glucose (20 mM) stimulation for 4 and 8 hours, respectively, in PC-3 cells, but not in LNCaP cells (Fig. 1C). To exclude the side effects from the osmotic pressure, the same concentrations of *D*-mannitol were applied. Results indicated that *D*-mannitol had no effect on the VEGF-C mRNA levels in PC-3 and LNCaP cells (Fig. 1D). These results indicated that abnormally high glucose conditions upregulate the VEGF-C expression in malignant PCa cells.

High glucose upregulates the VEGF-C expression via induction of autotaxin in PC-3 cells

Previous studies revealed that hyperglycemia up-regulates ATX expression in diabetic mice [22]. Moreover, the ATX/LPA signaling axis is associated with tumor progression and metastasis in many cancers, including PCa [5]. Hence, we proposed that ATX might play an important role on high glucose-induced VEGF-C expression in PC-3 cells. To this end, ATX expression levels were firstly assessed following high glucose stimulation. The results showed that ATX mRNA (Fig. 2A) and protein (Fig. 2D) expression were upregulated following 20 mM of glucose stimulation for 4 and 8 hours, respectively, in the PC-3 cells, but not in the LNCaP cells (Fig. 2B). These results were not from the osmotic pressure (Fig. 2C). To further confirm that ATX is involved in high glucose-induced VEGF-C expression in PC-3 cells, an ATX inhibitor (S32826) and its specific siRNAs were applied. Results indicated that high glucose-

Fig. 2. High glucose up-regulates VEGF-C expression through inducing ATX expression in PC-3 cells. (A) Serum starved PC-3 and (B) LNCaP cells were incubated with an additional 10 and 20 mM of glucose (A and B) or mannitol (C) for 2 or 4 hours, and the ATX mRNA (A~C) and protein expressions (D) were then determined by real-time PCR and western blotting, respectively. Serum starved PC-3 cells were pretreated with 200 nM of S32826 for 1 hour, followed by incubation of an additional 20 mM of glucose for another 4 (E) or 8 hours (F), then assessing the VEGF-C mRNA (E) and protein (F) expressions by real-time PCR and western blotting, respectively. Cell cultivation in 10 mM of glucose-containing RPMI media was used as the untreated control (set to 1.0), and data were shown as multiples of that. The results are expressed as mean $\pm$ SEM. * p <0.05; ** p <0.01 compared with untreated control ($n \geq 3$). # p <0.05 compared with glucose-treated group. UT, untreated control.

Fig. 3. High glucose up-regulates VEGF-C expression though inducing CRT expression in PC-3 cells. (A) Serum starved PC-3 and (B) LNCaP cells were incubated with an additional 10 and 20 mM of glucose (A and B) or mannitol (C) for 2 or 4 hours, and the CRT mRNA (A~C) and protein expressions (D) were then determined by real-time PCR and western blotting, respectively. (E) PC-3 cells were transfected with CRT (shCRT) shRNA for 24 hours, and the mRNA expressions of CRT were then determined to show the knockdown efficiency. PC-3 cells which are CRT knockdown, were treated with an additional 20 mM of glucose for 4 (F) or 8 (G) hours, followed by assessing the expressions of VEGF-C mRNA (F) as well as ATX, CRT, and VEGF-C protein (G) through real-time PCR and western blotting, respectively. PC-3 cells were transfected with either mock control vector, CRT shRNA (G and H), or ATX siRNA (I and J), followed by treatment with an additional 20 mM of glucose for another 4 hours (H and I) or 8 hours (G and I), and the mRNA expressions of ATX (H) and CRT (J), as well as the protein expressions of ATX, CRT, or VEGF-C (H) were then determined. Cells with or without transfection with the mock vector cultivation in 10 mM of glucose-containing RPMI media were used as the untreated control (set to 1.0), and data were shown as multiples of that. The results are expressed as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with untreated control ($n \geq 3$). # $p < 0.05$; ## $p < 0.01$ compared with glucose-treated group. UT, untreated control.

induced VEGF-C mRNA and protein expression were both suppressed upon either S32826 co-treatment (Fig. 2E and 2F) or ATX siRNA transfection in the PC-3 cells. Taken together, these results indicated that ATX is responsible for high glucose-induced VEGF-C expression in PC-3 cells.

High glucose upregulates VEGF-C expression via induction of calreticulin in PC-3 cells

Previous studies have shown that CRT expression levels are significantly upregulated in PCa [29]. Moreover, we recently demonstrated that CRT induced VEGF-A [27] and VEGF-C (unpublished results) expression in neuroblastoma cells. Furthermore, CRT also played a role in LPA-induced VEGF-A expression in PC-3 cells (unpublished results). These results indicated that CRT might contribute significantly to PCa progression. Thus, we hypothesized that CRT may also participate in high glucose-induced VEGF-C expression in PC-3 cells. Results showed that 10 and 20 mM glucose induced CRT mRNA expression within 2 hours (Fig. 3A), and also enhanced its protein expression after 8 hours (Fig. 3D) in PC-3 cells, but not in LNCaP cells (Fig. 3B). To further confirm the role of CRT in high glucose-induced VEGF-C expression in PC-3 cells, CRT shRNA was applied. Results showed that the high glucose-induced VEGF-C mRNA (Fig. 3F) and protein (Fig. 3G) expressions were abolished upon seeing the CRT knockdown (Fig. 3E), indicating that CRT is involved in high glucose-induced VEGF-C expression in PC-3 cells.

To investigate whether CRT-induced VEGF-C expression resulted from the regulation of ATX under the high glucose condition, the ATX protein expression was monitored during the CRT shRNA application. Results showed that ATX protein expression was suppressed following the knockdown of CRT, leading to a decrease of VEGF-C expression in PC-3 cells (Fig. 3H). On the other hand, knockdown of ATX attenuates the VEGF-C expression, but not the CRT expression (Fig. 3I and 3J). Taken together, these results indicated that CRT is an upstream regulator for ATX and is responsible for the high glucose-induced VEGF-C expression in PC-3 cells.

High glucose-induced VEGF-C expression is mediated through the LPA_{1/3} receptors signaling in PC-3 cells

It has been well-known that ATX is a critical molecule for LPA production, which is considerably correlated to cancer progression [30]. Moreover, our previous studies have revealed that the LPA upregulated VEGF-C expression in human endothelial cells and PC-3 cells [5, 6, 9]. To determine whether the high glucose-induced VEGF-C is LPA-dependent, Ki16425, a potent LPA_{1/3} antagonist, and their specific shRNAs were applied. The results showed that the high glucose-induced VEGF-C mRNA expression was obviously abolished by Ki16425 co-treatment in PC-3 cells (Fig. 4A), indicating that LPA₁ or LPA₃ signaling is responsible for the high glucose-induced VEGF-C expression. To further confirm whether LPA₁ and/or LPA₃ are involved in the high glucose's effects on VEGF-C induction, the LPA₁ and LPA₃ stably knockdown of PC-3 cells were generated (Fig. 4B and 4C). Results showed that both the LPA₁ and LPA₃ knockdown cells attenuated the high glucose-induced VEGF-C expression (Fig. 4D). To further investigate which G protein is involved in LPA_{1/3}-dependent signaling in high glucose-induced VEGF-C expression, various G protein inhibitors were applied. Results showed that the GP antagonist-2 and GP antagonist-2A, but not Y-27632, were suppressed through the high glucose-induced VEGF-C mRNA expression (Fig. 4E), indicating that high glucose-induced VEGF-C expression is mediated through G_i and G_q, but not G_{12/13} protein in PC-3 cells. To further decipher the molecular mechanism of LPA on the high glucose-induced VEGF-C expression, PLC and Akt, two intracellular signaling pathways, were assessed. Results showed that the high glucose-induced VEGF-C expression was abolished once the U73122 and LY294002 treatments in the PC-3 cells were applied. (Fig. 4F). These results indicated that the high glucose-induced VEGF-C expression is mediated through the LPA_{1/3}-PLC-Akt signaling pathway in PC-3 cells.

Fig. 4. High glucose up-regulates VEGF-C expression though the $LPA_{1/3}$ -dependent signaling in PC-3 cells. (A) Serum starved PC-3 cells were pretreated with 5 μ M of Ki16425 for 1 hour prior to co-treatment with additional 10 or 20 mM of glucose for another 4 hours, the VEGF-C mRNA expression was then assessed by real-time PCR analysis. PC-3 cells were transfected with either mock vector, LPA_1 shRNA (sh LPA_1) or LPA_3 shRNA (sh LPA_3) for 24 hours, and the mRNA expressions of LPA_1 (B) or LPA_3 (C) were then determined to show the knockdown efficiency. (D) PC-3 cells were transfected with either the mock control vector, LPA_1 shRNA, or LPA_3 shRNA, followed by treatment with an additional 20 mM of glucose for 4 hours. Then the VEGF-C mRNA expressions

were assessed. PC-3 cells were pre-incubated with (E) 10 μ M of GP antagonist-2A, GP antagonist-2, Y-27632, or (F) 2 μ M of U73122, 10 μ M of LY294002 for 1 hour prior to treatment with an additional 20 mM of glucose for another 4 hours. The VEGF-C mRNA expressions were then determined by real-time PCR analysis. Cells with or without transfection with the mock vector cultivation in 10 mM of glucose-containing RPMI media were used as the untreated control (set to 1.0), and data were shown as multiples of that. The results are expressed as mean $\pm$ SEM. *p<0.05; **p<0.01; ***p<0.001 compared with untreated control (n $\geq$ 3). #p<0.05; ###p<0.001 compared with glucose-treated group. UT, untreated control.

High glucose-induced VEGF-C expression is mediated through the $LPA_{1/3}$ -PLC-Akt-ROS-LEDGF-dependent signaling axis in PC-3 cells

The reactive oxygen species (ROS) and lens epithelium-derived growth factor (LEDGF) were downstream of the LPA signals that are involved in the LPA-induced VEGF-C expression in PC-3 cells [5]. To decipher the intracellular signaling mechanism of high glucose-induced VEGF-C expression, ROS and LEDGF signaling were determined. We showed that the ROS was augmented through 10 and 20 mM glucose stimulation in PC-3 cells (Fig. 5A). To confirm whether high glucose-enhanced ROS is responsible for the VEGF-C induction in PC-3 cells, the ROS inhibitor, N-acetylcysteine (NAC), was applied. Compared to the control group,

Fig. 5. High glucose up-regulates VEGF-C expression through the ROS-LEDGF signaling axis in PC-3 cells. (A) Serum starved PC-3 cells were pre-incubated with 10 μ M of 2',7'-dichlorofluorescein diacetate (DCFDA) for 10 min, followed by treatment with an additional 10 and 20 mM of glucose for another 10 min, and the ROS generation was then monitored by flow cytometry. (B) Serum starved PC-3 cells were pre-incubated with 10 mM of N-acetylcysteine (NAC) for 1 hour, followed by treatment with an additional 10 and 20 mM of glucose for another 4 hours. MTT analysis was used to assess the cell viability (B), and the VEGF-C mRNA expressions were then determined (C). (D) Serum starved PC-3 cells were treated with an additional 10 and 20 mM of glucose, and the LEDGF mRNA expressions were then monitored. (E) PC-3 cells were transfected with either the mock control vector or LEDGF shRNA (shLEDGF) for 24 hours, and the LEDGF mRNA expression was then monitored to show the knockdown efficiency. PC-3 cells were transfected with LEDGF shRNA (F) for 24 hours or pre-incubated with 5 μ M of Ki16425 (G), followed by treatment with an additional 10 and 20 mM of glucose for another 4 hours. The mRNA expressions of VEGF-C (F) or LEDGF (G) were then monitored. PC-3 cells were pre-incubated with 10 μ M of LEDGF for 10 min, followed by treatment with 5 μ M of Ki16425 (H) or 10 mM of NAC (K) for another 1 hour prior to co-treatment with an additional 20 mM of glucose for another 10 min. The ROS generation was then assessed by flow cytometry. PC-3 cells were transfected with either the mock control vector, LPA₁ shRNA, or LPA₃ shRNA, and the ROS generation was then monitored through pre-incubation with 10 μ M of LEDGF for 10 min prior to treatment with an additional 20 mM glucose for 10 min (J) or the LEDGF mRNA expression was then determined with an additional 20 mM of glucose stimulation for 4 hours (I). Cells with or without transfection with the mock vector cultivation in 10 mM of glucose-containing RPMI media were used as the untreated control (set to 1.0), and data were shown as mean $\pm$ SEM. * p <0.05; ** p <0.01; *** p <0.001 compared with untreated control ($n \geq 3$). # p <0.05; ## p <0.01; ### p <0.001 compared with glucose-treated group. UT, untreated control.

pretreatment of NAC suppressed the high glucose-induced VEGF-C mRNA expression (Fig. 5C), but not affecting the cell viability (Fig. 5B). Next, we monitored the LEDGF expression following the high glucose stimulation in PC-3 cells. Our results indicated that the LEDGF mRNA significantly increased following the 10 and 20 mM glucose treatment in the PC-3 cells (Fig. 5D). Moreover, knockdown of the LEDGF by its specific shRNA (Fig. 5E) abolished the effects of the high glucose on the VEGF-C induction (Fig. 5F). To determine whether the effects of ROS and LEDGF on the high glucose-induced VEGF-C expression were LPA-dependent, the LPA receptor signaling was blocked. The results showed that high glucose-induced LEDGF and ROS were suppressed through either Ki16425 stimulation or LPA_{1/3} shRNA transfection (Fig. 5G~J). To further study the signaling axis of ROS and LEDGF on high glucose-induced VEGF-C expression, the LEDGF mRNA expression was monitored via NAC treatment. The results showed that the high glucose-induced LEDGF mRNA expression was obviously attenuated through NAC pretreatment (Fig. 5K). Taken together, these results indicated that high glucose-induced VEGF-C expression is mediated through the LPA_{1/3}-ROS-LEDGF signaling axis in the PC-3 cells.

LPA_{1/3} is involved in the high glucose-upregulated aerobic glycolysis in PC-3 cells

Alteration of metabolism is one of the hallmarks of cancer cells, referred to as aerobic glycolysis or the Warburg effect [21]. Additionally, hyperglycemia is believed to be an independent risk factor for the development of many cancers [31]. Moreover, it has been shown that LPA up-regulates hexokinase activity and glycolysis in ovarian cancer cells [32]. To investigate the roles of hyperglycemia and LPA on metabolism in PCa cells, cellular bioenergetics were determined. The results showed that 20 mM glucose up-regulates the extracellular acidification rate (ECAR), an indicator of glycolysis (Fig. 6A), and the cellular oxygen consumption rate (OCR), an indicator of mitochondrial respiration (Fig. 6B). Meanwhile, LPA also showed a similar effect on cellular bioenergetics (Fig. 6A and 6B), indicating that both high glucose and LPA up-regulates glycolysis in PC-3 cells. To further confirm whether high glucose-upregulated glycolysis is LPA-dependent, Ki16425 was applied. The results revealed that both high glucose-augmented ECAR and OCR were abolished through Ki16425 co-treatment in PC-3 cells (Fig. 6C and 6D). Interestingly, not only the basal levels of ECAR but also basal OCR were both reduced when LPA_{1/3} signaling is blocked by Ki16425 treatment (Fig. 6E and 6F). Cells were then treated oligomycin, carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP), and antimycin & rotenone in order to obtain the levels of ATP production and spare respiratory capacity. Both ATP production and spare respiratory capacity were also decreased upon Ki16425 treatment (Fig. 6G). Reduced oxygen consumption may result in the reduced mitochondrial ROS production. Moreover, our result is consistent with our previous study indicated that LPA_{1/3} signals are necessary for the ROS production. Meanwhile, we performed Glycolysis Stress Test in Ki16425-pretreated cells. After Ki16425 treatment, cells were incubated in serum-free medium for additional 1h, and then sequentially treated with glucose, oligomycin, and 2-deoxyglucose (2-DG). However, Ki16425 did not alter the glycolysis in PC-3 cells (Fig. 6H). Taken together, our results imply that LPA_{1/3} is critical for the mitochondrial respiration rather than glycolysis in PC-3 cells.

Fig. 6. High glucose up-regulates glycolysis though the LPA_{1/3}-dependent signaling in PC-3 cells. PC-3 cells were seeded in XF^e 24-well cell culture plates 24 hours prior to the assays. Assays were initiated by removing the growth medium and replacing it with assay medium which contains 5 μ M of Ki16425 prior to treatment with an additional 20 mM of glucose (C and D) or containing an additional 20 mM of glucose or 5 μ M of LPA alone (A and B). The extracellular acidification rate (ECAR) (A and C) and cellular oxygen consumption rate (OCR) (B and D) were then determined within 1 hour. PC-3 cells were treated with Ki16425 (5 μ M) or DMSO for 1 hour, and subjected into Mito Stress Test and Glycolysis Stress test. The real-time OCR (E) and ECAR (F) were detected every 8 min within 100 min by Seahorse XF^e 24 Analyzer. Oligomycin (1 μ M), FCCP (0.5 μ M), and rotenone/antimycin A (0.5 μ M) were injected at indicated times. Basal respiration, basal ECAR, ATP production, and spare respiration capacity were analyzed by Wave software. (H) The real-time glycolysis rate were analyzed by Glycolysis Stress Test. Ki16425- and DMSO-treated cells were cultured in glucose-free medium for 1 hour prior to Glycolysis Stress Test. Subsequently, glucose, oligomycin, and 2-DG were injected in order to the cells. The results are expressed as mean $\pm$ SEM. * p <0.05; ** p <0.01; *** p <0.001 compared with untreated control ($n \geq 3$). # p <0.05; ## p <0.01; ### p <0.001 compared with glucose-treated group. UT, untreated control.

Discussion

Several clinical studies and meta-analyses have revealed that hyperglycemia showed the positive correlation with many types of cancers, including liver, pancreas, colorectal, kidney, bladder, endometrial, and breast cancers [15, 16]. Additionally, hyperglycemia increases the incidence and mortality of cancers [33]. Moreover, high glucose promotes the growth and metastasis of cancer cells *in vivo* [20, 34]. However, the effect of high glucose on PCa is still controversial. Although some studies indicate that type II DM increases the incidence of PCa because of the lower levels of androgen in diabetic patients [34, 35], others showed the opposite results [14]. Furthermore, high glucose promotes the growth of androgen-independent

PCa DU145 cells, but not on androgen-dependent PCa LNCaP cells [28], suggesting that high glucose facilitates the malignant PCa growth. These results were similar with our observations showing that high glucose induces VEGF-C expression in PC-3 cells, but not in LNCaP cells (Fig. 1).

LPA has been reported to induce VEGF-A and VEGF-C expressions [5, 7-9], as well as enhance tumor cell proliferation and migration [36], indicating that LPA is critical for tumor progression and metastasis. On the other hand, previous studies indicated that hyperglycemia increases LPA production by up-regulating ATX expression in diabetic mice [22]. Moreover, elevated serum levels of VEGF-C were found in obese patients and its level contributes to the development of insulin resistance [37]. These results were similar with our observations indicating that high glucose induces ATX and VEGF-C expression which is mediated through LPA_{1/3} (Fig. 4). These results suggested that the blockage of VEGF-C or LPA/ATX may be a suitable approach to alleviate the development of type II diabetes.

VEGFs have been implicated in angiogenesis and lymphangiogenesis [4, 24, 38, 39] that play critical roles during the progression of cancer. Thus, the anti-angiogenic or anti-lymphangiogenic molecules exert their beneficial effects on cancer therapy [40]. Our previous studies have revealed that LPA-induced VEGF-C expression is mediated through LPA_{1/3}, PLC, protein kinase-C (PKC), ROS and LEDGF signaling pathway [5, 7-9]. Additionally, high glucose increases ROS production via PKC-dependent pathways in smooth muscle and endothelial cells [41]. Herein, we showed for the first time that high glucose induces VEGF-C expression via the LPA_{1/3}-ROS-LEDGF-dependent signaling in PC-3 cells (Fig. 4 and 5). On the other hand, *in silico* analysis indicated that the LEDGF binding sites were identified in the promoter region of the human VEGF-C [42]. Furthermore, LEDGF enhances VEGF-C expression *in vitro* and enhances angiogenesis and lymphangiogenesis *in vivo* [42]. These results suggested that LEDGF may serve as a potential target for anticancer therapy.

CRT, a multifunctional chaperone protein, regulates various biological functions, including cell adhesion and post-transcription regulation [43, 44]. The expression levels of CRT correlated with cancer progression and lymphatic metastasis [45]. On the other hand, CRT destabilizes the glucose transporter-1 mRNA expression in vascular endothelial and smooth muscle cells under high-glucose conditions [46]. Moreover, our unpublished data suggested that CRT expression is regulated by LPA activation. Herein, we showed that high glucose enhances CRT and ATX expressions in PC-3 cells (Fig. 2 and 3). In addition, ATX interacts with tumor cells by binding to integrin $\alpha_v\beta_3$ to mediate LPA production in breast cancer cells [47]. Through analyzing the AU-rich element (ARE) database, we found that ATX has 4 AU-rich domains (data not shown). Meanwhile, CRT plays as an upstream regulator for high glucose-induced ATX expression in PC-3 cells (Fig. 3I). Hence, we hypothesized that CRT might regulate ATX expression by enhancing its mRNA stability or regulating its integrin interactions, which leads to LPA production and subsequent induction of VEGF-C in PC-3 cells.

Glucose-lowering therapy is a method for diabetes treatment by using anti-hyperglycemic medications to control blood sugar levels. Recent studies have suggested that these hypoglycemic drugs, such as metformin, decrease the prostate specific antigen (PSA) level, epithelial-mesenchymal transition (EMT) and proliferation of PCa [48-50]. These studies confirm our present study showing that hyperglycemia drives PCa progressions. Therefore, the combination with anti-hyperglycemic drugs and anti-lymphangiogenic therapy could potentially provide a new strategy for preventing lymphangiogenesis in PCa.

Conclusion

In conclusion, these results suggested that abnormal glucose metabolism might lead to LPA synthesis and therefore up-regulates aerobic glycolysis and VEGF-C production in PCa. These novel findings could potentially provide new evidence for glucose-lowering therapies in PCa.

Disclosure Statement

No conflict of interests exists.

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