Communication

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Aspirin, a potential GLUT1 inhibitor in a vascular endothelial cell line

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Abstract: Recent epidemiological and preclinical studies have revealed that aspirin possesses antitumor properties; one of the mechanisms results from inhibition of angiogenesis. However, the underlying mechanisms of such action remain to be elucidated, in particular, the effect of aspirin on glucose metabolism of vascular endothelial cells (ECs) has not yet been reported. Herein, we demonstrate that glucose transporter 1 (GLUT1), a main glucose transporter in ECs, can be down-regulated by aspirin. Exposure to 4-mM aspirin significantly decreased GLUT1 at the mRNA and protein level, resulting in impaired glucose uptake capacity in vascular ECs. In addition, we also showed that exposure to 4-mM aspirin led to an inhibition of intracellular ATP and lactate synthesis in vascular ECs, and a down-regulation of the phosphorylation level of NF-κB p65 was observed. Taken together, these findings indicate 4-mM aspirin inhibits glucose uptake and glucose metabolism of vascular ECs through down-regulating GLUT1 expression and suggest that GLUT1 has potential to be a target for aspirin in vascular ECs.

Keywords: Aspirin (ASA); Endothelial cells (ECs); Glucose transporter 1 (GLUT1); Glucose metabolism

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1 Introduction

Aspirin (acetyl salicylic acid, ASA) is a drug that was originally extracted from willow bark; it exhibits various pharmacological effects such as anti-inflammatory, antipyretic, analgesic, and antithrombotic properties. Aspirin has been used in clinical practice to relieve rheumatic diseases and to reduce the risk of heart attack and stroke [1-2]. A series of epidemiological studies also showed that aspirin prevents cancer metastasis and reduces the incidence rate and mortality of cancer, especially in colorectal cancer patients [3-5]. Currently, aspirin is the only non-steroidal anti-inflammatory drug that has been recommended for primary prevention of colorectal cancer by a clinical guideline of the 2016 U.S. Preventive Services Task Force [6].

Earlier studies have demonstrated that prostaglandins are the primary target of aspirin [7] and that aspirin acts as an inhibitor of cyclooxygenase (COX) iso-enzymes 1 and 2 [8]. In addition, aspirin blocks biosynthesis of prostaglandins through inhibiting the activity of prostaglandin G/H-synthase [9]. Recent studies have also reported that aspirin shows anti-angiogenetic effects, such as preventing angiogenesis in tumor-bearing mice [10,11], inhibiting tube formation of ECs [11,12], reducing risk of tumor metastasis [4], and modulating angiogenic protein expression in breast cancer patients [13]. Although these studies have revealed that anti-angiogenic properties of aspirin are responsible for its inhibitory effects on tumor growth and metastasis, the underlying mechanisms whereby aspirin inhibits angiogenesis remain to be elucidated.

It is now well established that angiogenesis plays a central process in tumor growth and metastasis [14-15]. In 2004, bevacizumab (avastin) was approved by the U.S. Food and Drug Administration as the first antiangiogenic drug for treatment of metastatic colon cancer, starting an era of anti-angiogenic therapy. However, after years of efforts, researchers discovered that current anti- VEGF therapeutic strategy has its drawbacks and limitations [16-17]. A new concept has been proposed, namely that

the altered glycolysis of vascular ECs promotes the formation of the new vasculature [18-20]. Researchers have found that glycolysis in parallel with VEGF signaling in driving angiogenesis may lead to the inadequate efficacy of VEGF-pathway inhibitors [21]. Several glycolytic inhibitors also showed anti-angiogenic properties, including 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), 2-deoxy-D-glucose (2-DG) and 3-bromopyruvate (3BP) [22-24]. These findings indicate that targeting glucose metabolism of vascular ECs may be one approach that might overcome the challenges of current anti-VEGF therapy. Considering that these drugs have not yet been approved for clinical application, it is necessary to assess whether clinically available drugs, such as aspirin, can be used to interfere glucose metabolism of vascular ECs.

As far back as the 1960s, aspirin was found to affect glucose metabolism and transport in platelets [25]. Recent studies have also demonstrated that aspirin targets glycolytic key enzymes, including 6-phosphofructo-1-kinase (PFK), 6-phosphofructo-2-kinase/ fructose-2,6-biphosphatase 3 (PFKFB3), and glucose-6-phosphate dehydrogenase (G6PD) [26-28]. However, the effect of aspirin on glucose metabolism of vascular ECs has not yet been reported. Herein, we begin by investigating whether aspirin affects glucose metabolism of vascular ECs and suggesting a potential mechanism by which aspirin works.

2 Materials and methods

2.1 Reagents and cell line

Acetyl salicylic acid (Sigma-Aldrich, St. Louis, Missouri, USA), SEND cell line (a murine vascular endothelium cell line) was provided by the Medical Research Center, The First Affiliated Hospital of Zhengzhou University. SEND cells were checked to be mycoplasma-free and grown in a 37°C incubator with a 5% CO_2 atmosphere in High Glucose Dulbecco's modified Eagle's medium (Hyclone, #SH30022.01) containing supplemented with 10% fetal bovine serum (FBS, PAN, #P30-3302) and 1% antibiotics (penicillin plus streptomycin, Hyclone, # SV30010). SEND cells were passaged only for up to three times prior to use and were cultured for at least 12 hours prior to addition of aspirin.

2.2 Quantification of mRNA with real-time PCR

Intact RNA was isolated from SEND cells by TRIzol reagent (Invitrogen, #15596018) according to manufacturer's protocol. 1 μg of intact RNA was used for the real-time PCR experiments, which were performed following the protocol of the real-time PCR (RT-PCR) kit (Takara, Dalian, China). mRNA expressions were measured in real time using the primers listed in Table 1 (Sangon Biotech, China). The expression levels of each gene were adjusted with β-actin as an internal control. All RT-PCR assays were performed with Sybr-Green-based technology (Taraka, Dalian, China). Primers were tested for equal efficiency using serial dilutions of purified targets.

2.3 Western blotting

Intact proteins were extracted from SEND cells using RIPA buffer (Solarbio, China) contained 1% phenylmethanesulfonylfluoride fluoride (PMSF) and a 2% protease inhibitor cocktail (Sigma-Aldrich, USA). Concentrations of the proteins were measured using a BCA method. An equal amount of proteins was separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Invitrogen, #HATF00010). Next, the membranes were blocked with 3% to 5% (w/v) nonfat milk powder in TBST (Tris-buffered saline with 0.1% Tween-20), followed by overnight incubation at 4℃ with the following antibodies: anti-GLUT1 (Millipore; #2956779; 1:1000), anti-PFBFK2 (OriGene; #TA314335; 1:1000), anti-HK2 (CST; #2867S; 1:1000), anti-

Table 1: Primers used for RT-PCR

Name of primers	Primer sequence (5'to3')
GLUT1(F)	CAGTTCGGCTATAACACTGGTG
GLUT1(R)	GCCCCCGACAGAAAGATG
PFKFB2(F)	CGCCTCAAATCTGCGAAGG
PFKFB2(R)	ACAATGAGTGTTGGGGAGTTG
PFKFB3(F)	CCCAGAGCCGGGTACAGAA
PFKFB3(R)	GGGGAGTTGGTCAGCTTCG
LDHA(F)	TGTCTCCAGCAAAGACTACTGT
LDHA(R)	GACTGTACTTGACAATGTTGGGA
PKM2(F)	GCCGCCTGGACATTGACTC
PKM2(R)	AGCCGAGCCACATTCATTCC
HK2(F)	GGAACCGCCTAGAAATCTCC
HK2(R)	GGAGCTCAACCAAAACCAAG
β-actin(F)	GAAGTGTGACGTTGACATCCGTA
β-actin(R)	CTCAGGAGGAGCAATGATCTTGA

HIF-1α (Proteintech; #20960-1-AP; 1:1000), anti-NF-κB p65 (CST; #8242S; 1:1000), anti-phospho-NF-κB p65 (CST; #3033S; 1:1000), anti-Erk1/2 (CST; #4695S; 1:1000), anti-phospho-Erk1/2 (Cell Signaling Technology; #4370S; 1:1000), anti-phospho-AKT (CST; #13038S; 1:1000), anti-AKT (CST; #9272S; 1:1000), anti-β-actin (Abclonal; #AC004(48509); 1:1000). Membranes were washed and then incubated with horseradish peroxidase-conjugated secondary antibodies (Proteintech; USA and OriGene; USA) for 1 h at room temperature. The membranes were treated with the eECL Western Blot Kit (CwBio, China), and the intensities of signals were determined using a Quantity One Image Analyzer (Bio-Rad, Hercules, California, USA).

2.4 Measurement of 2-NBDG uptake

SEND cells were plated at $5x10^4$ per well in 24-well plates and then incubated in a 37°C incubator with a 5% CO_2 atmosphere. Next, cells were treated with different concentrations of aspirin for 24 h. Cells were subsequently washed with PBS and then were incubated in fresh glucose-free DMEM containing 100 μM 2-NBDG (2-[N-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl) amino]-2-deoxyglucose) (Life Technologies, USA, #N13195) for 45 mins. Cells in each well were subsequently resuspended in 200 μl precold PBS and then were dyed with concentration of 1 μg/ml Propidium Iodide (PI, BD Pharmingen, #51-66211E) before flow cytometry analysis. Total operation time was within 30 min. For each measurement, data from over 35,000 single cell events were collected using a FACSCanto (BD, USA) flow cytometer. The proportion of PI^-2NBDG^{hi} cells reflects the glucose analogues 2-NBDG uptake capacity by living cells.

2.5 ATP and lactate assays

The productions of intracellular ATP and lactate were measured using the ATP Colorimetric/Fluorometric Assay Kit (Mountain View, CA, USA) and Lactate Colorimetric/Fluorometric Assay Kit (Mountain View, CA, USA) following the manufacturer's instructions. Briefly, SEND cells were treated with 4 mM aspirin for 24 h. Next, cells were lysed and centrifuged at 12,000 rpm for 5 min. The supernatant (50 μL) was transferred to a 96-well plate and then mixed with colorimetric assay work solution (50 μL). Luminescence signals were measured by a microplate reader (BMG Labtech), and ATP concentrations were calculated using an ATP standard curve. The protein concentrations of each group were also measured using a BCA method. The relative ATP levels were then calibrated by protein concentrations to eliminate cell quantity variance among tests. The measurements and calculations of relative lactate levels were done using the same procedure.

2.6 Statistical analysis

In this experiment, data were exhibited as mean ±SEM for at least three independent experimental tests; statistical analysis for comparison between two means was performed using the Student's *t*-test. All data were statistically analyzed using a GraphPad Prism software version 6.01 (GraphPad Software, La Jolla, CA). Differences were considered statistically significant at P values less than 0.05 (*), 0.01 (**), or 0.001 (***).

3 Result

3.1 The effect of aspirin on glycolysis-related mRNA expressions

To investigate whether aspirin affects the glucose metabolism of vascular ECs, we reviewed published literature and screened out several relevant genes, including GLUT1, Hexokinase 2 (HK2), PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (PFKFB2), PFK1 and lactate dehydrogenase A (LDHA) [19,29]. Three concentrations of aspirin (0.25 mM, 1 mM, 4 mM) were chosen for the following measurements.

After a 12-hour exposure to aspirin, mRNA levels were detected using RT-PCR (Fig. 1A-F). Compared with the control group, exposure to aspirin (0.25 mM, 1 mM, 4 mM) has no effect on other glycolysis-related mRNA expressions except for a significant down-regulation of GLUT1 mRNA expression in the 4 mM aspirin treatment group (Fig. 1B, $p<0.001$). This result indicates aspirin may inhibit GLUT1 mRNA expression in a concentration-dependent manner.

3.2 Aspirin inhibits GLUT1 expression of SEND cells

Since 4 mM aspirin significantly down-regulated GLUT1 mRNA expression, we next checked if this change was reflected at protein level. To find the optimal treatment time, we chose 4 mM aspirin to treat SEND cells for differ-

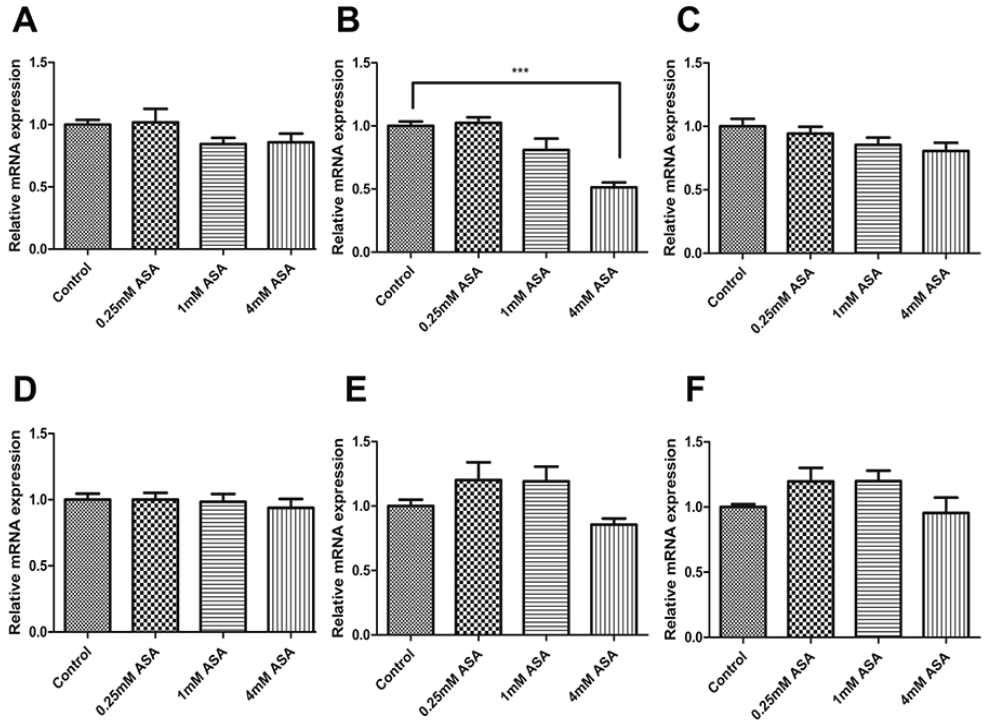

Figure 1: Effect of aspirin on glycolysis-related mRNA expressions in SEND cells. A-F shows the relative mRNA expressions of (A) HK2; (B) GLUT1; (C)LDHA; (D) PFK1; (E) PFKFB2, and (F) PFKFB3. After a 12-hour exposure to three concentrations (0.25 mM, 1 mM, 4 mM) of aspirin, these mRNA expressions were measured by RT-PCR. Data are shown as means±SEM of three independent experiments. *** represents $P < 0.001$.

ent length of time. As shown in Fig. 2A, GLUT1 expression levels were measured at six time points (3 h, 6 h, 12 h, 24 h, 48 h, 72 h), and the lowest expression level was detected after exposure to 4 mM aspirin for 24 h. This result shows a single-dose administration of 4 mM aspirin induced repression of GLUT1 protein expression in a time-dependent manner, which may be related to depletion and decomposition of aspirin during long-time (over 24 h) incubations. Together, these results suggest that 4 mM aspirin significantly reduces GLUT1 at mRNA and protein level.

Next, we treated SEND cells with different concentrations (0.25 mM, 1 mM and 4 mM) of aspirin for 24 h, then measured the expression levels of two glycolysis-related enzymes as well as the primary glucose transporter GLUT1. Of all measured proteins, only GLUT1 showed a visible down-regulation (Fig. 2B). In agreement with RT-PCR results, exposure to 4 mM aspirin decreases GLUT1 protein expression in SEND cells.

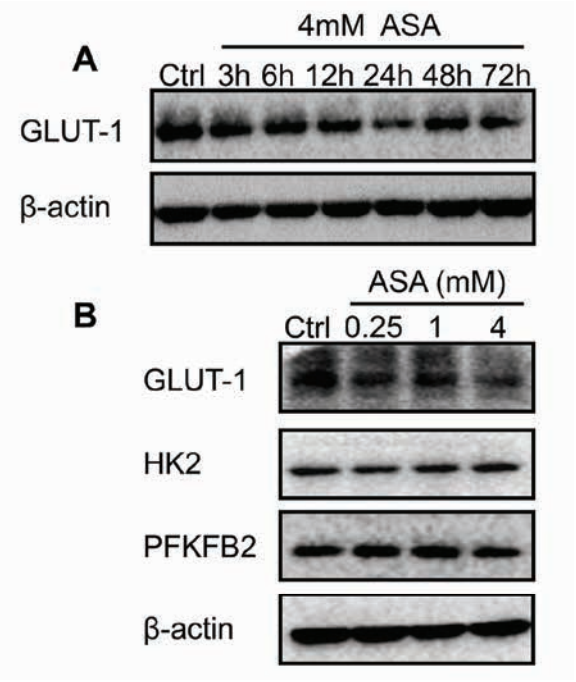

Figure 2: Aspirin-induced repression of GLUT1 expression in SEND cells. (A) Western-blotting analyzed the inhibitory effect of 4 mM aspirin on GLUT1 expression in SEND cells at six time points. (B) Exposure to different concentrations (0.25 mM, 1 mM and 4 Mm) of aspirin for 24 h affected expression of several key glycolytic proteins (GLUT1, HK2, and PFKFB2) in SEND cells.

Table 2: 2-NBDG uptake assay

Group	Subgroup of PI- 2-NBDGhi	P value
Control	83.07±2.13%	—
0.25mM ASA	74.87±3.22%	0.1008
1mM ASA	66.20±2.21%	0.0054**
4mM ASA	50.73±3.63%	0.0015**

* represents P value <0.05 ; ** represents P value <0.01 ; *** represents P value <0.001

3.3 The inhibitory effect of aspirin on glucose uptake of SEND cells

GLUT1 has been confirmed to be the main route of glucose uptake in ECs [29]. As a fluorescent glucose analog, 2-NBDG has been used to monitor glucose uptake by living cells [30]; this can be used to reflect the glucose transport function of GLUT1 in SEND cells.

As shown in Table 2 and Fig. 3A-D, we found the proportion of PI-2-NBDG^{hi} subgroup in the 4 mM aspirin treatment group was significantly lower than that in control group (50.73±3.63% vs 83.07±2.13%, p<0.01). This result showed that exposure to 4 mM aspirin significantly reduces 2-NBDG uptake in SEND cells; this suggests that decreased GLUT1 mRNA and protein expression levels lead to an impaired glucose-uptake capacity in SEND cells.

3.4 Aspirin reduces intracellular ATP and lactate synthesis of SEND cells

The growth factor-induced switch of vascular ECs from a quiescent phenotype to an angiogenic phenotype is known to be driven by changes of glucose metabolism [18,20,29]. During glycolysis, glucose is converted to lactate with production of adenosine triphosphate (ATP) [29]. Therefore, ATP and lactate can be measured to reflect intracellular glycolysis level.

To evaluate whether the inhibitory effect of aspirin on glucose uptake affects glucose metabolism in SEND cells, we measured intracellular ATP and lactate levels. Exposure to 4 mM aspirin for 24 h significantly reduced intracellular ATP (Fig. 3E, p<0.05) and lactate (Fig. 3F, p<0.05) in SEND cells. These results suggest that aspirin-induced

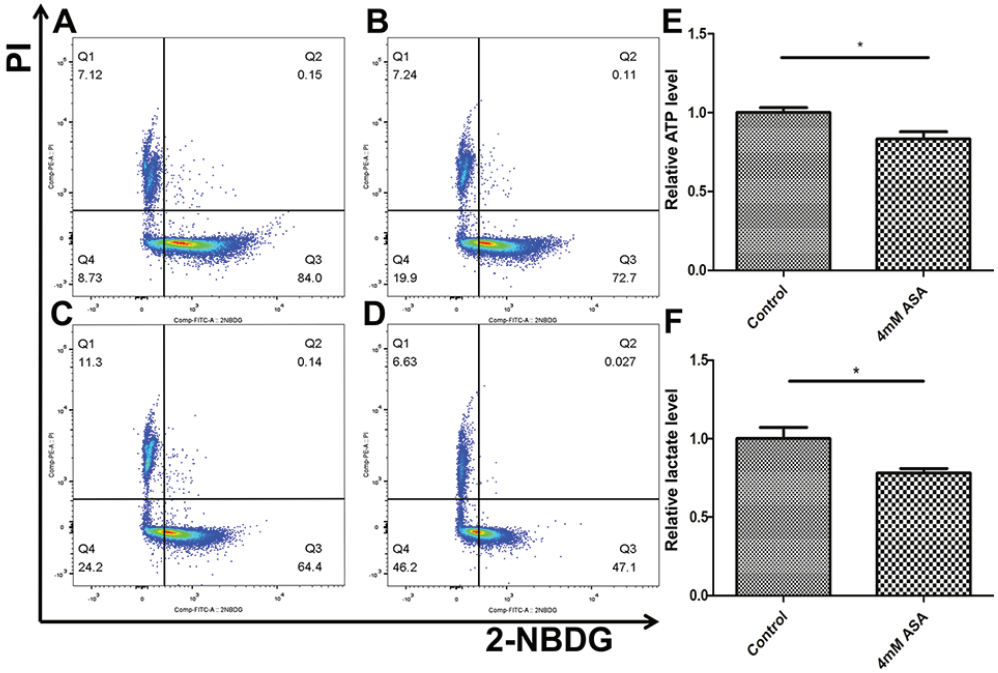

Figure 3: Aspirin inhibits glucose uptake and glucose metabolism in SEND cells. A-D show the 2-NBDG uptake detected by flow cytometry. After exposure to different concentrations (0.25 mM, 1 mM and 4 mM) of aspirin for 24 h, the proportion of PI-2NBDGhi cells was detected to reflect the glucose uptake by living cells. (A) The proportion of PI-2NBDGhi cells was 84.0% in the control group; (B) 72.7% in the 0.25 mM aspirin group, (C) 64.4% in the 1 mM aspirin group; (D) 47.1% in the 4 mM aspirin group. E-F showed inhibition of 4 mM aspirin on (E) ATP and (F) lactate synthesis. The relative ATP and lactate levels were calculated based on standard curves and were calibrated by protein concentrations with a BCA method.(* represents P < 0.05).

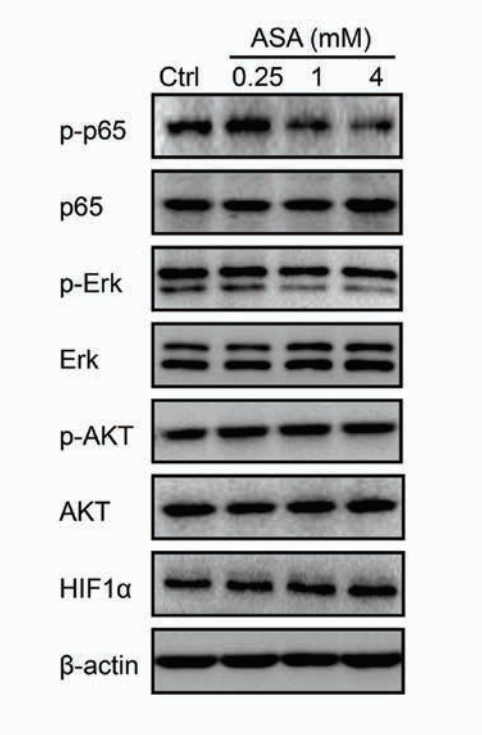

Figure 4: Inhibition of phosphorylation of NF-κB p65 (p-p65) in SEND cells by aspirin. SEND cells were treated with three concentrations (0.25 mM, 1 mM, and 4 mM) of aspirin for 24 h. Equal amounts of proteins of SEND cells were evaluated by western-blotting analysis for HIF-1α, NF-κB p65, Erk, AKT, as well as for phosphorylation level of NF-κB p65, Erk, and AKT.

decrease of glucose uptake leads to an inhibition of intracellular ATP and lactate synthesis.

3.5 The effect of aspirin on glycolysis-related protein expressions

To further investigate the inhibitory effect of aspirin on glycolysis in SEND cells, we assessed the expressions of a panel of glycolysis-related protein expressions [16-17]. Equal amounts of proteins were evaluated for HIF-1α, NF-κB p65 (p65), Akt, Erk expressions, as well as for the phosphorylation level of NF-κB p65, Akt, and Erk1/2 (p-NF-κB p65 (p-p65), p-Akt and p-Erk).

SEND cells were treated with different concentrations (0.25 mM, 1 mM and 4 mM) of aspirin for 24 h. Compared to control group, p-p65 expression was markedly down-regulated in the 4 mM aspirin group (Fig.4). Besides, we also examined HIF-1α, a key transcription factor of glycolytic genes [18]. However, compared to control group, no visible differences were observed regardless of concentrations of aspirin.

4 Discussion

Tumor angiogenesis is a necessary event for tumor growth and metastasis. Without nutrition support of neovascularization, it would be difficult for a tumor to expand beyond 2 to 3 mm in size [14]. Over 80% of ATP in ECs is produced by glycolysis [29] and glycolysis provides essential energy during the switch from quiescent to an angiogenic phenotype and promotes vascular sprouting during the process of tumor-induced angiogenesis [21]. Although ECs express both glucose transporters (GLUTs) and sodium-glucose linked transporters (SGLTs), only the high affinity GLUT1 transporter has been identified as the primary glucose transporter [29]. In ECs, hypoxia promotes GLUT1 expression but down-regulates SGLT1 and SGLT2 expressions [31], and elevated expression of GLUT1 is related to tumor angiogenesis [32]. It was also reported that elevated expression of SGLT2 is induced by high glucose [33], indicating that SGLT2 is mainly useful as a target for the treatment of diabetes mellitus. Additionally, there is no convincing evidence that other members of GLUT families, such as GLUT 2, 3, 4, and 5, are involved in tumor-induced angiogenesis and glycolytic disorder in ECs. Instead, VEGF signaling promotes glycolytic flux mainly through GLUT1 and PFKFB3 [29]. That evidence, taken together, indicates that the GLUT1 transporter plays a key role in tumor-induced glycolytic disorder in ECs.

In this study, we observed that a high-concentration (4 mM) aspirin down-regulates GLUT1 expression and inhibits glucose metabolism in vascular ECs. We reviewed previous publications and found that aspirin-induced inhibition of GLUT1 expression has been reported in tumor tissue and hepatocellular carcinoma cell lines [26,34]. It has also been reported that 4 mM aspirin down-regulates GLUT1 expression and glucose consumption in hepatoma cells possibly through targeting NF-κB or NF-κB/HIF1α signaling [35], which coincides with our findings. A clinical report also showed that long-term use of aspirin inhibits F-18 fluorodeoxyglucose (FDG) uptake in colorectal cancer lesions [36]. Finally, we found a visible down-regulation of p-p65, indicating that 4 mM aspirin may inhibit activation of p65 subunit of NF-κB. Previous studies showed that NF-κB signaling pathway affects glucose metabolism through regulating GLUT1 [37,38]. Thus, the decreased GLUT1 expression and impaired glucose uptake capacity in SEND cells may be involved with the down-regulation of p-p65. However, to show the direct link between NF-κB signaling pathway and glucose metabolism of SEND cells requires more investigation.

In clinical practice, low-dose (lower than 0.1 g/d) aspirin is the most commonly recommended regimen

to reduce the risk of heart attack, stroke, and colorectal cancer [6], whereas high-dose (between 3.6 to 5 g/d) aspirin is usually used to relieve rheumatic diseases [1]. In the treatment of Kawasaki diseases, aspirin regimen is recommended at a high-dose of 80 to 100 mg/kg per day by the American Heart Association [39]. The gastrointestinal safety of high-dose aspirin administration with a median dose of 3 g/day has also been established [40]. Of course, this evidence may not completely dispel our concerns about the safety of high-dose aspirin application, which needs to be explored further.

Although preventing angiogenesis by targeting glucose metabolism of vascular ECs has already been proposed [20,29], the clinical application of glucose metabolism inhibitors is still a long way off. WZB117, an inhibitor of GLUT1, shows a remarkable anti-tumor efficiency in mice model, but also causes metabolic disorders such as hyperglycemia and lipodystrophy [41]. It was found that affinity of WZB117 to the insulin-sensitive glucose transport (GLUT4) is higher than its affinity for the major glucose transporter (GLUT1) in tumor cells and vascular ECs. Another obstacle is that WZB117 inhibits glucose uptake in human erythrocytes, which may limit its therapeutic concentration [42]. However, exposure to aspirin at high doses, even reaching 90 mM, does not disturb glucose uptake and glycolysis in human erythrocytes [43]. Different from the possibility that WZB117 can cause metabolic disorders, it was reported that high-dose aspirin (approximately 7 g/d) improves glucose metabolism in type 2 diabetes [44]. Although the mechanisms of these phenomena remain unclear, there is no doubt that high-dose aspirin is more advantageous than current GLUT1 inhibitors, indicating the necessity for further investigation.

In summary, this study has shown that aspirin-induced repression of GLUT1 expression led to impaired glucose uptake capacity, as well as to an inhibition of intracellular ATP and lactate synthesis in SEND cells. These findings revealed GLUT1 has potential to be a target of aspirin and may provide a new perspective to understand the pharmacological efficacy of aspirin on vascular ECs; this suggests a need for further investigation of the phenomenon.

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