Abnormal smooth muscle cell proliferation is a hallmark of vascular disease. Although growth factors are known to contribute to cell hyperplasia, the changes in metabolism associated with this response, particularly mitochondrial respiration, remain unclear. Given the increased energy requirements for proliferation, we hypothesized that PDGF (platelet-derived growth factor) would stimulate glycolysis and mitochondrial respiration and that this elevated bioenergetic capacity is required for smooth muscle cell hyperplasia. To test this hypothesis, cell proliferation, glycolytic flux and mitochondrial oxygen consumption were measured after treatment of primary rat aortic VSMCs (vascular smooth muscle cells) with PDGF. PDGF increased basal and maximal rates of glycolytic flux and mitochondrial oxygen consumption; enhancement of these bioenergetic pathways led to a substantial increase in the mitochondrial reserve capacity. Interventions with the PI3K (phosphoinositide 3-kinase) inhibitor LY-294002 or the glycolysis inhibitor 2-deoxy-d-glucose abrogated PDGF-stimulated proliferation and prevented augmentation of glycolysis and mitochondrial reserve capacity. Similarly, when L-glucose was substituted for D-glucose, PDGF-dependent proliferation was abolished, as were changes in glycolysis and mitochondrial respiration. Interestingly, LDH (lactate dehydrogenase) protein levels and activity were significantly increased after PDGF treatment. Moreover, substitution of L-lactate for D-glucose was sufficient to increase mitochondrial reserve capacity and cell proliferation after treatment with PDGF; these effects were inhibited by the LDH inhibitor oxamate. These results suggest that glycolysis, by providing substrates that enhance the mitochondrial reserve capacity, plays an essential role in PDGF-induced cell proliferation, underscoring the integrated metabolic response required for proliferation of VSMCs in the diseased vasculature.

Key words: metabolic flux, mitochondrion, phosphoinositide 3-kinase (PI3K), platelet-derived growth factor (PDGF), proliferation, vascular smooth muscle cell (VSMC).

INTRODUCTION

VSMC (vascular smooth muscle cell) proliferation stimulated by PDGF (platelet-derived growth factor) is instrumental in the progression of atherosclerosis, angioplasty-dependent restenosis, neo-intimal hyperplasia and other complications associated with vascular disease [1–4]. Previous studies suggest that PDGF-dependent VSMC proliferation involves stimulation of glycolysis [5–7]. Other studies have reported that a major metabolite produced by proliferating VSMCs is lactate [8], again supporting the concept that induction of glycolysis may be required for cell proliferation [5]. However, augmentation of glycolytic activity, supported by increased glycolytic protein expression and glucose utilization, has not been characterized further in the context of mitochondria-related metabolic changes. This is potentially important as the changes in metabolism associated with VSMC proliferation may contribute to the pathological changes associated with cell hyperplasia.

The increase in glycolytic flux due to growth factors has been shown to be critical in the bioenergetic shift that occurs during VSMC proliferation [9,10]. Several signalling pathways, including the PI3K (phosphoinositide 3-kinase)/Akt pathway, have been shown to regulate glucose metabolism, particularly through the GLUT4 (glucose transporter 4) channel and via activation of pathways that lead to increased glycolytic enzyme activity and expression [11–15]. Activation of the PI3K/Akt pathway in cells occurs rapidly upon PDGF exposure and has been shown to be a modulator of glycolytic activity. However, the role that mitochondria play in this proliferative response remains unclear. Establishing the role of cellular bioenergetics in proliferating cells is therefore critical for identifying new cellular targets that may help to prevent the uncontrolled proliferation of VSMCs, yet spare normal functioning vascular cells.

We hypothesized that glycolysis serves to increase the availability of metabolic substrates for oxidative phosphorylation and thereby supports the energy required for proliferation in growth factor-stimulated cells. Using extracellular flux technology, we examined mitochondrial oxygen consumption as well as glycolytic flux in intact VSMCs in response to PDGF. We show that PDGF increases both glycolysis and mitochondrial respiration in VSMCs. Interruption of glycolysis via direct inhibition of PI3K, glycolysis or LDH (lactate dehydrogenase) decreases the capacity of the cells to mount the bioenergetic response required for proliferation in response to PDGF. In addition, these studies suggest that the LDH pathway may play an important role in regulating the bioenergetic reserve of...
VSVMCs and therefore may be integral to the hyperplastic VSMC phenotype.

**EXPERIMENTAL**

**Reagents and antibodies**

Sodium pyruvate, D-glucose, L-glucose, sodium-L-lactate, NADH, 2-DG (2-deoxy-D-glucose), DTNB [5,5′-dithiobis-(2-nitrobenzoic) acid], dodecyl maltoside, potassium ferricyanide, ascorbate, oxaloacetate, acetyl-CoA, cytochrome c, sodium oxamate, ATP and ADP were purchased from Sigma–Aldrich. Chromatography grade TBAHS (tetrabutylammonium hydrogen sulfate), from Fluka Analytical, was also obtained through Sigma–Aldrich. Analytical grade perchloric acid (69 %), K2HPO4, KH2PO4, methanol, NaOH and orthophosphoric acid (85 %) were obtained from Fisher Scientific. LY-294002 was purchased from Calbiochem. Antibodies against Akt, phospho-Akt (Ser473), LDH, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), phospho-Rb (retinoblastoma) protein (Ser807,811), cyclin D1 and β-actin were purchased from Cell Signaling Technologies. Anti-phospho-PDH (pyruvate dehydrogenase)-E1-α (Ser293) antibody was purchased from Novus Biologicals. Anti-PDK-1 (pyruvate dehydrogenase kinase-1) antibody was purchased from Assay Designs, JC-1 dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide) was purchased from Invitrogen. Antibodies against complex I (39 kDa), tetraethylbenzimidazolyl-carbocyanine iodide) was purchased from Assay Designs. JC-1 dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide) was purchased from Invitrogen. Antibodies against complex I (39 kDa), complex II (70 kDa), complex III ceroid, cytochrome c oxidase (complex IV) subunit I, PDH-E1α and the VDAC (voltage-dependent anion channel) were purchased from MitoSciences. Antibodies against cytochrome c were purchased from BD Biosciences. The PDGF-BB homopeptide was purchased from R&D Biosciences.

**Cell culture and treatments**

Rat aortic smooth muscle cells were harvested from descending thoracic aortas and maintained at 37 °C under a 5 % CO2 atmosphere in DMEM (Dulbecco’s modified Eagle’s medium; Gibco) containing 1 g/l D-glucose, 4 mM GlutaMAXTM (Invitrogen), 1 mM pyruvate, 3.7 g/1 sodium bicarbonate and 10 % (v/v) FBS (fetal bovine serum) (Atlanta Biologicals) with 100 units/ml penicillin and 100 ng/ml streptomycin. Cells used in the present study were between passages four and 12. Experiments were performed in complete serum medium containing 10 % (v/v) FBS as described above unless specified. Glucose-free medium containing L-glutamate, in place of GlutaMAXTM, was used for treatments with 1 g/l L-glucose or 18 mM L-lactate. Sub-confluent cells grown in six-well, 12-well or Seahorse Bioscience V7 tissue culture plates were incubated with vehicle or inhibitor for 1 h prior to treatment with vehicle or PDGF for the indicated times.

**Measurement of cellular bioenergetic function using the XF-24 Extracellular Flux Analyzer**

The XF-24 Extracellular Flux Analyzer from Seahorse Biosciences was used to examine the effects of chronic PDGF treatment on glycolysis and mitochondrial function in rat aortic smooth muscle cells as described in [16–19]. A mitochondrial function assay based on inhibitors of the electron transport chain and uncoupling agents was used to identify changes in key aspects of respiratory function [20]. Briefly, three basal OCRs (oxygen consumption rates) are measured to obtain a baseline OCR, followed by the sequential injection and measurement of OCR with 1 μg/ml oligomycin, 1 μM FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) and 10 μM antimycin A after each intervention. In the present study, experiments were designed to determine ECAR (extracellular acidification rate) and OCR at the end of treatment without or with inhibitors and/or PDGF. The base medium was changed prior to the bioenergetic measurements to serum-free unbuffered (without sodium bicarbonate) DMEM medium base (Cellgro) supplemented with 4 mM L-glutamine, 5.5 mM D-glucose and 1 mM sodium pyruvate, at pH 7.4. In some experiments, L-glucose or L-lactate was substituted for D-glucose. Owing to the effects of PDGF on VSMC proliferation, total cellular protein was measured following each experiment using the Lowry method [21] and used to normalize mitochondrial function rates.

**Cell counting**

Cells treated in six-well plates were washed twice with PBS followed by a 3 min incubation with 300 μl of 0.25 % trypsin/EDTA at 37 °C. After the trypsin treatment, cells were resuspended in 1 ml of 10 % (v/v) FBS-containing medium and placed in Eppendorf tubes kept at 37 °C. Cells were immediately counted using a haemocytometer. At least five fields per replicate were counted to obtain the average cell number for each treatment group.

**LDH activity assay**

LDH activities in cell lysates and medium were used as indices of cell viability; activities from cell lysates alone were used to determine LDH specific activity. After the indicated treatments, the medium was collected and lysates were prepared by scraping cells in 100 μl of PBS containing 0.1 % Triton X-100. After centrifugation (20 800 g for 10 min at 4 °C) of the lysates and medium, LDH activity was measured spectrophotometrically at 340 nm for 4 min at 37 °C. Cytotoxicity was then estimated by dividing the change in absorbance of the medium by the values for combined LDH activity in the medium and the lysate. The values were then normalized relative to the control, which represented 100 % cell viability. Total protein in the cell lysates was measured using the Bradford method [22]. The absorption coefficient of NADH (6220 M−1·cm−1) was used to calculate the nmols NADH oxidized/min per mg of protein.

**Western blot analysis**

Cells were washed twice with PBS and lysed in protease-inhibitor- and phosphatase-inhibitor-containing cell lysis buffer [20 mM Heps, pH 7.0, 1 mM DTPA (diethylenetriaminepenta-acetic acid), 1 % (v/v) NP-40 (Nonidet P40) and 0.1 % SDS]. Cells lysates were collected and centrifuged at 14000 g for 10 min at 4 °C. Supernatants were collected and protein concentration was determined by the Lowry method [21] using BSA as a standard. Samples were mixed with Laemmli sample buffer and boiled for 10 min. Equal amounts of protein were resolved by SDS/PAGE (10 or 12 % gels) and transferred on to nitrocellulose membranes. Immunoblot analysis was performed using the antibodies listed above.

**Measurement of ΔψM (mitochondrial inner membrane potential)**

The inner membrane ΔψM was assessed using JC-1 dye. Briefly, VSMCs grown in 12-well culture plates were treated without or with PDGF for 24 h, after which 7.4 μM JC-1 dye was added directly to cells in culture medium and incubated for 30 min. Next, cells were washed with PBS and red/green fluorescence was measured using a fluorescence plate reader with
excitation/emission filters suitable for rhodamine (560/595 nm) and fluorescein (484/535 nm) respectively. Results are expressed as the ratio of red to green fluorescence.

**Citrate synthase activity assay**

Citrate synthase was assayed by monitoring the conversion of oxaloacetate and acetyl-CoA into citrate and CoA. The addition of DTNB promotes the chemical conversion of DTNB into TNB (2-nitro-5-thiobenzoic acid) by CoA. This reaction was monitored spectrophotometrically at 412 nm. VSMCs treated without or with PDGF for 24 h were lysed in PBS containing 0.2% dodecyl maltoside. At least 10 μg of whole cell lysate was added to a cuvette which contained buffer (100 mM Tris/HCl, pH 8.0, with 0.1% Triton X-100), 100 μM acetyl-CoA and 200 μM DTNB. The reaction was initiated by adding 200 μM oxaloacetate and the absorbance at 412 nm was monitored for 4 min to obtain the activity rate. The protein content was measured in cell lysates and used to normalize the activity rate.

**Complex IV activity assay**

Complex IV activity was assessed by monitoring the rate of cytochrome c oxidation spectrophotometrically [23]. VSMCs treated without or with PDGF for 24 h were lysed in PBS containing 0.2% lauryl maltoside. Purified reduced cytochrome c was used as the substrate in the assay and the concentration was determined using the absorption coefficient (19200 M\(^{-1}\)·cm\(^{-1}\)) at 550 nm. A reference cuvette containing 50 μM purified oxidized cytochrome c (by addition of 1 mM ferricyanide) in 10 mM phosphate buffer at pH 7.0 was used to subtract background absorbance. At least 5 μg of cell lysate was added to a cuvette containing 10 mM phosphate buffer, pH 7.0, and 50 μM cytochrome c. The rate of cytochrome c oxidation was then measured. Complex IV activity was calculated by plotting the natural log of the change in absorbance at 550 nm against time. The rate was then calculated and normalized to protein concentration.

**Nucleotide extraction**

Briefly, an aliquot of protein-precipitated lysate was obtained from VSMCs after the indicated treatments by scraping the cells after addition of 5% perchloric acid. This lysate was centrifuged (20800 g for 10 min at 4°C) and the precipitated protein pellet was stored and later resuspended in 1 ml of 0.5 M NaOH. Protein concentration was determined by the Bradford assay with BSA as a standard. Supernatant was transferred into an Eppendorf tube and neutralized by precipitating ClO\(_4\)- with K\(_2\)HPO\(_4\). The suspension was vortexed, kept on ice for 10 min and then centrifuged (as above) to remove salt. The supernatant was used immediately or stored at −80°C until analysis.

**HPLC separation and measurement of adenine nucleotides**

Nucleotide analysis was performed by modifying a method described previously [24,25]. The HPLC system consisted of a Gold HPLC model equipped with System Gold 168 Detector and System Gold Autosampler 507 from Beckman Coulter. The analytical column was a Supelcosil LC-18-T, (150 mm × 4.6 mm internal diameter, particle size 3 μm) from Sigma–Aldrich. Analytical runs were processed by 32 Karat Software (version 8.0) also from Beckman Coulter. The chromatographic separation was performed at ambient temperature with gradient elution. The mobile-phase flow rate was set at 0.9 ml/min and consisted of 65 mM potassium phosphate buffer and 3 mM TBAHS adjusted to pH 6.0 with orthophosphoric acid (buffer A) and 30% methanol in 65 mM potassium phosphate buffer with 4 mM TBAHS adjusted to pH 6.0 with orthophosphoric acid (buffer B). The buffers were delivered in a linear gradient as follows: 0–2 min, 30% buffer B; 2–16 min to 90% buffer B; 16–20 min to 90% buffer B; 20–21 min returned to 30% buffer B; and 21–24 min 30% buffer B using an 1 min equilibration between injections. The injection volume was 10 μl. Nucleotides were detected using a UV spectrum sweep between 200 and 400 nm, and detection peaks were compared with the UV spectra of ATP and ADP standards at different concentrations. Nucleotides were monitored at 254 and 262 nm. Standard ATP and ADP samples were prepared by dissolving in buffer A. Standards were prepared at a range of 2–100 μM to create a standard curve against which to compare the experimental samples. Standards were not filtered prior to injection. Experimental samples were prepared as follows: a volume of 150 μl of nucleotide extract suspension was mixed with 150 μl of buffer A and filtered prior to injection in HPLC.

**Statistical analysis**

Results are reported as means ± S.E.M. Comparisons between two groups were performed with unpaired Student’s t tests. Comparisons between multiple groups were performed by one-way ANOVA with Bonferroni post-hoc tests where applicable. A P value of less than 0.05 was considered statistically significant.

**RESULTS**

**PDGF increases glycolysis and smooth muscle cell proliferation**

In the first series of experiments, we examined acute changes in glycolysis caused by PDGF. PDGF was added to VSMCs and ECARs were recorded for a period of approx. 1 h. As shown in Figure 1(A), PDGF addition to VSMCs acutely increased ECAR, 2-fold over cells treated with vehicle control. Addition of oligomycin was then used to examine the glycolytic response to inhibition of mitochondrial ATP production. Interestingly, cells treated acutely with PDGF had an approx. 8-fold greater response to oligomycin compared with control cells, suggesting a greater ability to increase glycolytic flux under conditions of increased ATP demand.

Next, the chronic effects of PDGF on glycolytic flux were examined. VSMCs were seeded at five different cell densities (in the range of 2–6 × 10⁴ cells per well) and then treated with vehicle or PDGF for 48 h. The PDGF-containing medium was then removed and the glycolytic rates were examined by extracellular flux analysis. As PDGF is known to cause rapid proliferation of VSMCs, the ECAR values were normalized to total protein in cell lysates recovered after the assay. Consistent with increased cell proliferation, PDGF induced a significant increase in total protein over time compared with control cells (Figure 1B). Despite removal of the PDGF stimulus prior to the extracellular flux assay, cells treated with PDGF demonstrated elevated ECAR values, suggesting that metabolic reprogramming to a more glycolytic phenotype had occurred.

To examine cell proliferation induced by PDGF, cell proliferation was measured by cell counting after 48 h of PDGF treatment. Under these conditions, PDGF increased cell number approx. 1.3-fold (Figure 1C). An inhibitor of glycolysis, 2-DG, was used to determine whether glycolysis was required for the hyperplastic response. As shown in Figure 1(C), 2-DG inhibited both basal and PDGF-stimulated cell hyperplasia. To confirm that the changes in cell number were due to loss of a proliferative
response and not cytotoxicity, cell viability was assessed after 48 h and found to be unchanged (Supplementary Figure S1 available at http://www.BiochemJ.org/bj/428/bj4280255add.htm). The lower cell proliferation occurring in the cells treated with 2-DG was associated with a lower rate of glycolysis and an inability to mount a glycolytic response upon loss of mitochondrial ATP synthesis (Figure 1D).

**Effect of PI3K/Akt signalling on PDGF-dependent VSMC proliferation**

The PI3K/Akt signalling axis has been shown to be activated and required for PDGF-dependent VSMC proliferation [26]. As shown in Figure 2(A), PDGF increased PI3K-dependent Akt phosphorylation significantly compared with vehicle control; this effect was prevented by pre-treatment of VSMCs with 10 μM LY-294002 without changes in total Akt protein. We also examined the role of PI3K inhibition on VSMC proliferation and found that its activation is required for PDGF-dependent cell growth (Figure 2B). The lower cell counts in the LY-294002-treated cells were due to loss of a proliferative response and not cytotoxicity, as indicated by the LDH viability assay (Figure 2C).

**Effects of PI3K and glycolysis on VSMC cell cycle proteins**

PDGF-stimulated PI3K signalling and increased glycolytic activity have been shown to regulate cell cycle proteins that are responsible for growth [26]. We also examined the effect of PDGF on Rb protein phosphorylation and cyclin D1 expression in the absence or presence of LY-294002 and 2-DG. As shown in Supplementary Figure S2 (available at http://www.BiochemJ.org/bj/428/bj4280255add.htm), PDGF induced Rb phosphorylation at Ser807/811 and increased cyclin D1 expression significantly (approx. 2.5 fold compared with control). In response to PI3K inhibition by LY-294002, cyclin D1 expression, but not PDGF-dependent Rb phosphorylation, was inhibited. Similar results were shown previously [27]. As expected, 2-DG inhibited Rb phosphorylation, but increased cyclin D1 expression compared with controls. The 2-DG-dependent induction of cyclin D1 was not induced further by PDGF treatment. The reasons for increased cyclin D1 expression with 2-DG are still unclear. Nevertheless, these results are in agreement with reports published previously suggesting that both LY-294002 and 2-DG can affect cell cycle proteins and inhibit VSMC proliferation [10,28].

**PDGF increases LDH expression in a PI3K-dependent manner**

To determine whether the effects of PDGF on glycolysis were accompanied by reported changes in the levels of glycolytic enzymes, VSMCs were treated with PDGF for 24 h in the absence or presence of LY-294002 or 2-DG. LDH and GAPDH protein levels were then measured by Western blotting. As shown in Figure 3(A), PDGF had no effect on GAPDH protein expression; this finding is contrary to previous reports showing that PDGF increased GAPDH expression and activity in growth-arrested
As actively proliferating cells are expected to have higher energetic requirements than quiescent cells, we examined the levels of ATP and ADP in cells treated with and without PDGF. As shown in Supplementary Figure S3(A), ATP levels were similar between control and PDGF-treated cells. Interestingly, 2-DG caused a significant decrease in ATP, presumably due to the fact that 2-DG becomes phosphorylated by hexokinase upon entering the cells and hence cannot be metabolized and may inhibit other energy requiring processes [29]. In the presence of PDGF, 2-DG-treated cells had a similar level of ATP to that with 2-DG alone. Interestingly, LY-294002 treatment had no effect on ATP levels, whether in the presence or absence of PDGF treatment; Supplementary Figure S3(B) shows the ATP to ADP ratios for each treatment. PDGF increased the ATP to ADP ratio, and this was prevented by 2-DG and LY-294002 treatment.

**Inhibition of PI3K decreases PDGF-induced glycolytic flux**

We next examined how inhibition of PI3K affects glycolysis under basal and PDGF-stimulated conditions. VSMCs were pre-treated without and with LY-294002 and exposed to PDGF for 24 h. As shown in Figures 4(A)–4(C), LY-294002 alone mildly stimulated glycolysis; however, LY-294002 prevented the PDGF-dependent stimulation of glycolysis at 24 h, as well as the stimulation of glycolysis after oligomycin addition. When cells were treated acutely with LY-294002 for 1 h, the baseline levels of glycolysis were found to be no different from control cells (Figure 4D, left-hand panel). After baseline measurement of ECAR, 10 ng/ml PDGF was added to cells and glycolysis was assessed. As shown in Figure 4(D) (right-hand panel), PDGF treatment enhanced glycolysis significantly in VSMCs, within 5 min, and this effect was partially prevented by PI3K inhibition. Similarly, oligomycin-stimulated glycolysis, measured 90 min after injection with PDGF or vehicle, was enhanced in the presence of PDGF and inhibited by LY-294002 (Figure 4E).

**PDGF increases mitochondrial respiration**

Next, the effect of PDGF on mitochondrial oxygen consumption in the absence or presence of 2-DG was examined. VSMCs were treated with or without 2-DG for 1 h, followed by treatment with PDGF for 24 h. Mitochondrial function was assessed using the mitochondrial function assay described in Supplementary Figure S4 (available at http://www.BiochemJ.org/bj/428/bj4280255add.htm). As shown in Figures 5(A) and 5(B), treatment with PDGF significantly increased the basal rate of oxygen consumption. The presence of 2-DG inhibited the basal stimulation of OCR by PDGF; however, 2-DG alone did not affect the rate of oxygen consumption compared with control cells. Furthermore, the maximal respiratory capacity induced by FCCP was approx. 2-fold higher in PDGF-treated cells compared with control cells and 2-DG decreased the maximal respiratory capacity of cells treated with PDGF to levels comparable with control cells (Figures 5A and 5C). The increase in maximal respiratory capacity by PDGF resulted in a substantial increase in the mitochondrial reserve capacity and 2-DG abolished this mitochondrial bioenergetic reserve in PDGF-treated cells (Figure 5D).

As the PI3K/Akt pathway regulates glycolysis and therefore substrate supply to mitochondria, i.e. pyruvate [30], we examined the effect of LY-294002 on PDGF-induced changes in mitochondrial respiration (Figure 6A). Similar to direct inhibition of glycolysis with 2-DG, PI3K blockade with LY-294002 greatly decreased glycolysis and increased mitochondrial respiration compared with PDGF alone.
Figure 3  Effect of PDGF on GAPDH and LDH

VSMCs were treated with 10 ng/ml PDGF for 24 h in the absence or presence of 10 \( \mu \)M LY-294002 (LY) or 20 mM 2-DG. Lysates from the cells were used to examine GAPDH and LDH expression by immunoblotting. (A and B) Representative Western blots of GAPDH, LDH and \( \beta \)-actin expression. A densitometric analysis for each result set is shown below a representative immunoblot. (C) Intracellular LDH specific activity was measured in cell lysates 24 h after the indicated treatment. The rates of NADH oxidation were normalized to protein and expressed as nmols NADH oxidized/min per mg of protein. Results are presented as means ± S.E.M. (n = 3 per treatment group). N.S., non-significant differences between groups. *P < 0.05 compared with control; #P < 0.05 compared with PDGF.

abrogated the effect of PDGF on basal oxygen consumption (Figure 6B), maximal respiratory capacity (Figure 6C) and mitochondrial reserve capacity (Figure 6D). Collectively, these results suggest that PI3K-mediated stimulation of glycolysis is required for PDGF to increase mitochondrial respiration and VSMC proliferation.

Effect of glucose deprivation on glycolysis, mitochondrial respiration and VSMC proliferation

As 2-DG and PI3K inhibitors could have off-target effects, we further examined the role of glycolysis in this response by substituting L-glucose for D-glucose. Briefly, L-glucose, the non-metabolizable isomer of D-glucose, was added to the cell medium in place of D-glucose, and its effects on oxygen consumption, proliferation and glycolysis were examined. Interestingly, L-glucose increased basal oxygen consumption in VSMCs (Figures 7A and 7B); however, L-glucose did not support PDGF-induced increases in basal (Figure 7B) or maximal respiratory capacities (Figure 7C). The VSMCs treated with PDGF in L-glucose-containing medium showed a remarkably decreased mitochondrial reserve capacity (Figure 7D). Furthermore, as shown in Figure 7(E), cell proliferation induced by PDGF in D-glucose-containing medium was abolished completely in L-glucose-containing medium. In addition, PDGF was unable to increase ECAR in cells cultured in L-glucose-containing medium and these cells were not responsive to oligomycin (Figure 7F). It should be noted that in all experiments, pyruvate remained present in the medium (at 1 mM). Taken together, these results suggest that glycolysis is required for enhancing mitochondrial reserve capacity to support PDGF-stimulated VSMC growth.

PDGF does not affect mitochondrial membrane potential, mitochondrial mass or respiratory complex expression/activity

As the effects of PDGF could be explained by increases in mitochondrial mass or respiratory complex activity, we examined the direct effects of PDGF on mitochondria. JC-1 dye staining
Bioenergetic effects of PDGF

Figure 4  Inhibition of PI3K partially blocks PDGF-induced changes in glycolytic flux

ECAR values of VSMC treated with vehicle (CTRL) or 10 ng/ml PDGF in the absence or presence of 10 μM LY-294002 (LY). (A) Basal and oligomycin-stimulated ECAR values from cells treated for 24 h with vehicle or PDGF in the absence or presence of LY. The rates were normalized to total protein to give mU/h/min per μg of protein. (B) Baseline ECAR results for the treatment groups in (A). (C) Oligomycin-dependent ECAR values for treatment groups in (A). (D) Effects of acute PDGF exposure on glycolytic flux: VSMCs were exposed to vehicle or PDGF in the absence or presence of LY for 5 min and ECARs were measured. (E) Effects of acute PDGF exposure on glycolytic flux after oligomycin addition. After acute administration of PDGF, cells were exposed to 1 μg/ml oligomycin and ECARs were measured. Results are means ± S.E.M. (n = 3–5 per treatment group). *P < 0.05 compared with control; #P < 0.05 compared with PDGF.

was used to evaluate ΔΨM in VSMCs treated with PDGF for 24 h. As shown in Supplementary Figure S5 (available at http://www.BiochemJ.org/bj/428/bj4280255add.htm), PDGF did not affect the mitochondrial ΔΨM. Citrate synthase and cytochrome c oxidase activities were also measured after PDGF treatment. As shown in Supplementary Figures S5(B) and S5(C), PDGF increased citrate synthase activity by approx. 10%; however, there was no significant change in cytochrome c oxidase activity, suggesting that the activity of these key mitochondrial proteins was not increased to an appreciable extent due to PDGF treatment. We further examined several mitochondrial complex proteins (Supplementary Figure S5D) and observed no changes in their expression in response to PDGF.

To determine whether the effects of PDGF on mitochondrial oxygen consumption could derive from changes in PDH (pyruvate dehydrogenase) activation, protein phosphorylation of the E1-α subunit (Ser293) was examined after treatment with PDGF for 24 h in the absence or presence of LY-294002 or 2-DG. No changes in protein phosphorylation occurred (Supplementary Figure S5E), suggesting similar levels of PDH activation in all treatments. Similarly, total PDH expression and the levels of PDK-1 remained unchanged. These results are interesting in the light of published reports that suggest that PDH activity can be inhibited under certain conditions in proliferating cells [31].

Metabolism of L-lactate is sufficient to support PDGF-dependent increases in respiration and cell proliferation

We next examined the effect of L-lactate on PDGF-dependent mitochondrial function and VSMC proliferation. For this, 18 mM L-lactate was substituted for D-glucose in the culture medium. After a 1 h acclimation to substrate conditions, the cells were
Figure 5  Effects of PDGF on mitochondrial respiration

OCRs in VSMCs. (A) VSMCs were pre-treated without or with 20 mM 2-DG followed by exposure to vehicle (CTRL) or 10 ng/ml PDGF for 24 h. Baseline OCR values were first recorded followed by sequential injection of 1 μg/ml oligomycin, 1 μM FCCP and 10 μM antimycin A. The measurements were normalized to protein levels following the assay. (B) Basal OCR, (C) maximal respiratory capacity and (D) mitochondrial reserve capacity for each treatment group. Results are presented as means ± S.E.M. (n = 4–5 per treatment group). * P < 0.05 compared with control; # P < 0.05 compared with PDGF.

PDGF increased basal OCR in the presence of both glucose and lactate. There were no differences in the basal respiratory state between lactate and glucose medium alone; however, the maximal respiratory and reserve capacities induced by lactate were significantly higher than those observed with glucose (Figures 8B and 8C). Interestingly, basal and PDGF-stimulated cell proliferation was lower in cells cultured in lactate compared with those cultured in glucose (Figure 8D). Nevertheless, the stimulation of cell proliferation by PDGF was preserved in cells cultured in lactate.

The observation that PDGF increased LDH protein and activity (Figure 3) and that lactate was sufficient to support respiration and PDGF-dependent proliferation suggested that LDH itself may be one of the key factors regulating VSMC proliferation. To examine this, we pre-treated VSMCs with sodium oxamate (an inhibitor of LDH) in lactate-containing medium 1 h prior to PDGF treatment, and examined mitochondrial respiration after 24 h. It should be noted that oxamate, in other studies using the XF-24 Extracellular Flux Analyzer [16], inhibited extracellular acidification by approx. 80%, providing further confirmation that our acidification measurements were indeed associated with lactate production. Inhibition of LDH with oxamate resulted in loss of the mitochondrial reserve capacity in PDGF-treated cells (Figure 8E). LDH inhibition was also associated with loss of basal and PDGF-stimulated proliferation, without affecting cell viability (Figure 8F and results not shown).

DISCUSSION

PDGF stimulates cell migration and proliferation and has been studied extensively over the last 20 years [3,32–35]. Nevertheless, the mechanisms by which PDGF promotes proliferation in vascular disease have not been fully defined. In the present study, we examined the roles of glycolysis and mitochondrial respiration in PDGF-induced VSMC proliferation using extracellular flux analysis. We show for the first time that PDGF modulates LDH activity and mitochondrial oxygen consumption and that this regulation appears to be due, in part, to activation of the PI3K pathway. Consistent with published studies [10], glycolysis was found to be required for PDGF-induced proliferation. Importantly, the results shown in the present paper also reveal that mitochondrial respiration is enhanced in response to PDGF and that an enhanced mitochondrial reserve capacity is a feature common to the hyperplastic VSMC phenotype.

Glucose metabolism has been implicated as playing an important role in VSMC proliferation. Interestingly, in VSMCs, it was shown previously that 90% of the glucose transported into the cell is converted into lactate [36] and that glucose is the sole source for lactic acid production [8]. We exploited this characteristic of VSMCs in the present study by using...
extracellular flux technology to measure glycolytic flux. Under both acute and chronic treatment conditions, PDGF was found to increase extracellular acidification, suggesting that glycolytic flux was increased; this response was inhibited in the presence of 2-DG, a non-metabolizable glucose analogue (Figure 1). In addition, glycolytic flux was also inhibited in cells cultured in L-glucose instead of D-glucose (Figure 7F), adding validity to our glycolytic flux measurements and to the conclusion that glycolytic flux is increased by PDGF. Using oligomycin, an inhibitor of mitochondrial ATP synthase, we also examined the glycolytic response to inhibition of mitochondrial ATP synthesis. Acute treatment of VSMCs with PDGF resulted in a several-fold increase in the oligomycin-stimulated ECAR, indicating that the cells had a substantial glycolytic reserve that could be called upon to meet the energy demands of cell division. Chronic treatment of cells with PDGF resulted in higher basal levels of glycolytic flux, yet these cells did not demonstrate as large a glycolytic reserve as cells treated acutely with PDGF.

Previous studies have also shown that control of glycolysis occurs at different sites, demonstrating multi-site metabolic modulation [5]. We found that PDGF stimulation resulted in a significant up-regulation of LDH that led to a comparable increase in enzyme activity (Figure 3). This finding complements gene expression studies showing that the LDH enzyme is increased in rat aortic VSMCs with PDGF treatment [39]. Hence, our finding suggests that the LDH enzyme may be a critical point of modulation utilized by highly proliferating VSMCs. However, these results differ from those of Ranganna and Yatsu [6], where GAPDH was found to be up-regulated after PDGF treatment; we did not observe a comparable increase in GAPDH in the course of our study. The reasons for this disparity are unclear, but may relate to the culture conditions used during PDGF treatment. Diverging from the typical serum deprivation and growth arrest protocol, we incubated cells in PDGF under complete serum conditions. The reason for using this culture condition relates back to the original purpose of the present study, which was to measure the changes in metabolism caused by PDGF treatment and to determine their relationship with VSMC proliferation. Under low serum conditions (0.1 % FBS), basal mitochondrial function and glycolysis were below the level of detection. One interpretation of our findings is that the enhanced expression of LDH upon PDGF stimulation results in augmentation of the NAD+—dependent conversion of lactate into pyruvate, an activity generally associated with the H-type (also known as the B-type) isoform of LDH [41], which could increase substrate delivery to mitochondria.

A novel finding of the present study is that mitochondrial respiration is increased upon PDGF treatment. To the best of our knowledge, this is the first report demonstrating that PDGF modulates mitochondrial oxygen consumption. However, previous reports have shown that mitochondria could be directly affected by PDGF signalling. For example, PDGF signalling was shown to be responsible for the phosphorylation of the ATP synthase ∆ subunit, but the significance of that finding remains unclear [37,38]. Interestingly, in the present study, the treatments that inhibited PDGF-dependent changes in glycolysis (i.e. 2-DG, PI3K inhibitors, L-glucose and oxamate) were sufficient to prevent both proliferation and the augmentation of...
of mitochondrial oxygen consumption. In particular, treatment with PDGF resulted in a large increase in the mitochondrial reserve capacity. The possible explanations for this increase in mitochondrial spare respiratory capacity include: (i) that PDGF increased mitochondrial mass; (ii) that PDGF increased the activity of enzymes critical to energy transduction; or (iii) that PDGF increased respiratory substrates. To test the idea that PDGF could augment mitochondrial mass, MitoTracker Green and confocal microscopy were used to estimate mitochondrial mass (results not shown). No differences were apparent between the treatment groups. Moreover, key mitochondrial proteins showed no change in activity or expression after PDGF treatment (Supplementary Figure S5). We also examined PDH, an enzyme through which the bulk of substrates for ATP generation is produced via the oxidation of pyruvate in the tricarboxylic acid cycle. PDH demonstrated no significant changes in its expression or phosphorylation status in response to PDGF (Supplementary Figure 5E). Similarly, the expression of other PDH subunits (results not shown), as well as PDK-1 was found to remain constant upon PDGF treatment. Thus these findings partially dissociate our results from a ‘Warburg-like’ effect, where a combination of increased PDH phosphorylation and PDK protein expression inhibits mitochondrial respiratory function. Therefore our results suggest an alternative mechanism of action that may couple glycolytic and mitochondrial metabolism in the presence of PDGF.

It appears, then, that metabolic modulation of glycolysis through enhanced LDH may be key to delivering substrate to mitochondria, the hypothesis being that this increases the mitochondrial reserve capacity, which is critical for PDGF-dependent growth. This was examined further by omitting D-glucose from the culture medium, while culturing the cells in excess D-lactate during treatment. Under these conditions, the cells retained their ability to proliferate when stimulated with PDGF.
and demonstrated an enhanced mitochondrial reserve capacity (Figure 8). Interestingly, cells cultured in lactate had a higher maximal and spare respiratory capacity even in the absence of PDGF stimulation, yet maintained basal rates of oxygen consumption similar to that of cells cultured in D-glucose. This may be due to the fact that enzymatic conversion from lactate into pyruvate lacks the multifaceted allosteric control when compared with glycolysis, which would involve several points of control, including hexokinase, PFK (phosphofructokinase) and pyruvate kinase [5]. Control at LDH would then allow the cell to easily obtain pyruvate in one step rather than having to oxidize glucose through several steps. That oxamate inhibited both the proliferative response to PDGF and the mitochondrial metabolic response further supports the concept that LDH may be key to a hyperplastic VSMC phenotype. Therefore we propose a role for extracellular lactate as a ‘reservoir’ source for mitochondrial respiration in VSMCs in response to PDGF. We speculate that LDH may play an important role in lactate utilization to support oxidative phosphorylation through the conversion of lactate into pyruvate [41–43]. Indeed, evidence in the literature supports a role for lactate uptake and oxidation in muscle cells through the action of MCTs (monocarboxylate transport proteins) which can be coupled to LDH activity [42,44–46].

What remains to be examined in future studies is how this finely tuned and integrated metabolic response is controlled. It is well known that Akt signalling, stimulated upon PI3K activation, is required for proliferation in both cancer and VSMCs [12,47]. The present findings suggest that PI3K/Akt signalling is required...
for all steps leading to the proliferative VSMC phenotype, including the up-regulation of LDH (Figure 3), the increase in glycolytic flux (Figure 4) and the increase in mitochondrial oxygen consumption and reserve capacity (Figure 6), occurring upon PDGF stimulation. Moreover, we observed that inhibition of PI3K with LY-294002 prevented cyclin D1 up-regulation by PDGF (Supplementary Figure S2), which in itself could inhibit the cell cycle and suggests regulation beyond glycolysis. Hence, disentangling the relative contribution of PI3K-mediated changes in energy metabolism from other epi-phenoena regulating cell growth will be challenging. Nevertheless, these studies indicate that PDGF signalling co-ordinates glycolytic flux, cell cycle protein activity and mitochondrial metabolism to maximize cell proliferation. It appears that PI3K/Akt signalling and LDH are integral to PDGF-induced metabolic changes and the proliferative response. Targeting these bioenergetic responses could be a useful strategy for preventing or ameliorating the abnormal proliferation of VSMCs in vascular disease.

AUTHOR CONTRIBUTION

Jessica Perez, Bradford Hill, Brian Dranka and Gloria Benavides performed the experiments. Bradford Hill, Jessica Perez and Victor Darley-Usmar were the primary writers of the manuscript. Victor Darley-Usmar provided financial support for the study. All authors participated in the design of the study and preparation of the manuscript.

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Bioenergetic effects of PDGF


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SUPPLEMENTARY ONLINE DATA
Role of cellular bioenergetics in smooth muscle cell proliferation induced by platelet-derived growth factor

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Figure S1 Effect of 2-DG on cell viability
Cells were treated with PDGF or vehicle (CTRL) in the absence or presence of 20 mM 2-DG for 48 h. Cell viability was assessed by measuring LDH activity in the cell lysates and medium. Results are means ± S.E.M. (n = 3–5 per treatment group). N.S., non-significant differences between groups.

Figure S2 Changes in cell cycle proteins in VSMC treated with PDGF: effects of PI3K inhibitor and 2-DG
VSMCs were treated with or without 10 ng/ml PDGF for 24 h in the absence or presence of 10 μM LY-294002 (LY) or 2-DG (20 mM). (A) Levels of phospho-Rb (Ser807/811), cyclin D, β-actin (as a loading control) in cell lysates were measured by Western blotting. Grouped densitometric values of (B) phospho-Rb (Ser807/811) and (C) cyclin D Western blots. Results are means ± S.E.M. (n = 3 per treatment group). *P < 0.05 compared with control; #P < 0.05 compared with LY-294002.

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VSMCs pre-treated with 10 μM LY-294002 (LY) or 20 mM 2-DG for 1 h were exposed to 10 ng/ml PDGF for 24 h. Cellular nucleotides were isolated and analysed by HPLC. (A) Total levels of intracellular ATP. (B) ATP/ADP ratios. Results are means ± S.E.M. (n = 3 per treatment group) *P < 0.05 compared with control; #P < 0.05 compared with PDGF.

Figure S4 Mitochondrial function assay
Schematic showing parameters assessed by extracellular flux analysis. To probe individual components of respiration that contributed to the consumption of oxygen, 1 μg/ml oligomycin, 1 μM FCCP and 10 μM antimycin A were injected sequentially. This allowed for an estimation of the contribution of non-ATP-linked oxygen consumption (proton leak; Leak) and ATP-linked mitochondrial oxygen consumption (ATP). The maximal respiratory capacity was determined using the FCCP-stimulated rate. The reserve capacity is represented by the maximal respiratory capacity subtracted from the baseline OCR. The residual oxygen consumption that occurred after addition of antimycin A was ascribed to non-mitochondrial sources and was subtracted from all values in the analysis.
Figure S5  Effect of PDGF on mitochondrial membrane potential, protein expression and enzymatic activity

Mitochondrial membrane potential, citrate synthase activity, complex IV activity, mitochondrial protein expression, PDH E-1α subunit expression and phosphorylation, as well as PDK-1 expression levels were determined in VSMCs treated with vehicle (CTRL) with 10 ng/ml PDGF for 24 h. (A) Mitochondrial membrane potential was assessed using the ratio of red to green fluorescence for JC-1 dye. (B) Citrate synthase activity and (C) Cytochrome c oxidase activity determined after vehicle or PDGF treatment. Results for (A–C) are means ± S.E.M. (n = 3–6 per treatment group). *P < 0.05 compared with control. (D) Representative Western blots are shown for cytochrome c (Cyt-c), complex III core 2 protein (C III-core 2), complex II 70 kDa protein (C II-70kD), voltage-dependent anion channel (VDAC), complex IV subunit 1 (C IV-1), complex I 39 kDa protein (C I-39kD) and β-actin (as a loading control) after PDGF treatment for 24 h. Grouped densitometric values are shown to the right of each representative Western blot as the fold change (means ± S.E.M.) compared with control (n = 3 per treatment group). (E) Representative Western blots for PDH E-1α subunit phosphorylation at Ser293 and total protein expression, as well as PDK-1 expression, after pre-treatment of VSMCs with 10 μM LY-294002 (LY) or 20 mM 2-DG for 1 h followed by exposure to 10 ng/ml PDGF for 24 h. Results are fold change (means ± S.E.M.) compared with control (n = 3 per treatment group).

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