ARTICLE

Glucose lowers the threshold for human aortic vascular smooth muscle cell migration: inhibition by protein phosphatase-2A

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Abstract

Aims/hypothesis Atherosclerosis, which occurs prematurely in individuals with diabetes, incorporates vascular smooth muscle cell (VSMC) chemotaxis. Glucose, through protein kinase C-β_{II} signalling, increases chemotaxis to low concentrations of platelet-derived growth factor (PDGF)-BB. In VSMC, a biphasic response in PDGF-beta receptor (PDGF-βR) level occurs as PDGF-BB concentrations increase. The purpose of this study was to determine whether increased concentrations of PDGF-BB and raised glucose level had a modulatory effect on the mitogen-activated protein kinase/extracellular-regulated protein kinase pathway, control of PDGF-βR level and chemotaxis.
Methods Cultured aortic VSMC, exposed to normal glucose (NG) (5 mmol/l) or high glucose (HG) (25 mmol/l) in the presence of PDGF-BB, were assessed for migration (chemotaxis chamber) or else extracted and immunoblotted.
Results At concentrations of PDGF-BB <540 pmol/l, HG caused an increase in the level of PDGF-βR in VSMC (immunoblotting) versus NG, an effect that was abrogated by inhibition of aldose reductase or protein kinase C-β_{II}. At higher concentrations of PDGF-BB (>540 pmol/l) in HG, receptor level was reduced but in the presence of aldose reductase or protein kinase C-β_{II} inhibitors the receptor levels increased. It is known that phosphatases may be activated at high concentrations of growth factors. At high concentrations of PDGF-BB, the protein phosphatase (PP)2A inhibitor, endothall, caused an increase in PDGF-βR levels and a loss of biphasicity in receptor levels in HG. At higher concentrations of PDGF-BB in HG, the chemoattractant effect of PDGF-BB was lost (chemotaxis chamber). Under these conditions inhibition of PP2A was associated with a restoration of chemotaxis to high concentrations of PDGF-BB.
Conclusion/interpretation The biphasic response in PDGF-βR level and in chemotaxis to PDGF-BB in HG is due to PP2A activation.

Abbreviations

AR	aldose reductase
DAG	diacylglycerol
ERK	extracellular-regulated protein kinase
HG	high glucose
MAPK	mitogen-activated protein kinase
NG	normal glucose
PDGF	platelet-derived growth factor
PDGF-βR	PDGF beta receptor
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PP	protein phosphatase
PTP	protein tyrosine phosphatase
siRNA	small interfering RNA
VSMC	vascular smooth muscle cells

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Introduction

Atherosclerosis is a major cause of morbidity and mortality in diabetes [1]. During atheroma formation the levels of platelet-derived growth factor (PDGF)-BB, a chemoattractant for vascular smooth muscle cells (VSMC), are raised locally within the subendothelial space [2, 3]. Since PDGF-BB concentrations are also supraphysiologically raised in diabetes and PDGF-beta receptor (PDGF-βR) production is increased by glucose [4–6], the importance of PDGF-BB signalling may be increased in VSMC migration and atheroma formation in diabetes.

VSMC chemotaxis to PDGF-BB (~360 pmol/l) is dependent on activation of phosphatidylinositol 3-kinase (PI3K; p110β isoform) and cross-talk between the PI3K and mitogen-activated protein kinase (MAPK) pathways [7]. We have shown that glucose increases Akt and extracellular-regulated protein kinase (ERK) activation levels in the PI3K and MAPK pathways, respectively, and enhances that caused by PDGF-BB; in these conditions MAPK/ERK is downstream of PI3K [7, 8]. Glucose causes increased polyol pathway flux leading to de novo synthesis of diacylglycerol (DAG), which is both a cofactor for protein kinase C (PKC) and a direct activator of MAPK/Ras [8, 9]. In aortic VSMC, DAG-dependent signalling directly augments MAPK/ERK activity through Ras and indirectly by PKCβ$_{II}$ promoting crosstalk between PI3K and MAPK [8].

PDGF-BB-associated ERK activation also stimulates activator protein-1 resulting in *PDGF-βR* (also known as *PDGFRB*) upregulation [10]. Inhibition of either PI3K or MAPK interrupts receptor upregulation and chemotaxis to 360 pmol/l PDGF-BB [10, 11]. Interestingly, therapeutic statin concentrations also interrupt these responses by inhibiting MAPK/Ras farnesylation [7, 8].

The elevated PDGF-BB concentrations found in atheromas, mainly released from the cell types involved [12], are associated with a fivefold increase in PDGF-BB transcription in the plaque compared with normal arteries [13]. It has been suggested that the chemoattractant effect on VSMC (saphenous vein-derived) is lost at high PDGF-BB concentrations (glucose concentration not stated) [14]. The activation state of signalling pathways depends on the balance between phosphorylation and dephosphorylation of key molecules. If chemotaxis is lost at higher PDGF-BB concentrations then phosphatases may have inactivated the regulating pathways (PI3K and MAPK). Serine/threonine phosphatases, including protein phosphatase (PP)2A, can dephosphorylate MAPK and PI3K pathway proteins [15, 16].

It is not known whether PP2A activity alters in response to glucose and/or PDGF-BB in VSMC. If PP2A activation is glucose-dependent, then diabetic therapies targeting glucose-dependent signalling may leave ERK activity unopposed in elevated PDGF-BB conditions and so potentiate migration (a key event in atheroma formation).

Therefore the aim of the present study was to determine the effects of glucose on PDGF-BB signalling and on the deactivation of signalling pathways involved in human aortic VSMC chemotaxis.

Methods

For an expanded Methods section, please see Electronic supplementary material (ESM).

Materials Chemicals and antibodies were commercially available except LY379196 (gift from Eli Lilly, Indianapolis, IN, USA) and sorbinil (gift from Pfizer, New York, USA).

Cell culture Explanted human aortic VSMC, obtained with consent from transplant donors, were cultured, in normal glucose (NG; 5 mmol/l D-glucose) and 10% FCS conditions as described [11, 17].

Treatments Unless otherwise stated, cells (passage 3) were washed (serum-free medium, three times) and set up for 24 h in NG or high glucose (HG) (NG+20 mmol/l D-glucose) in basal conditions (0.5% BSA) or in the presence of 36 to 3,600 pmol/l PDGF-BB±inhibitors. Inhibitors were used at concentrations effective in VSMC or were titrated for effect using kinase assays [8, 11, 18–22]. Cells were not serum-starved during these experiments, since this intervention upregulates receptors and potentiates migration to reduced growth factor levels [23, 24]. Treatment did not affect cell viability or proliferation during these experiments but glucose decreased marginally (see ESM, Methods, Treatment effects on cell viability, glucose utilisation and proliferation).

Microinjection Cells (20,000) were seeded in Petri dishes containing etched coverslips as described [8]. All cells within the etched area (2×2 mm) were microinjected (InjectMan; Eppendorf, Hamburg, Germany) 24 h later with control (non-specific IgG) antibody (0.1 mg/ml) without/with affinity-purified specific antibodies (0.1 mg/ml) or small interfering (si)RNA (100 nmol/l) and rested (2 h) before treatments commenced.

Immunoblotting After treatment, lysate extraction (phospho-protective buffer), protein concentration determination, immunoprecipitation, western blot and chemiluminescence measurements (densitometry) were done as described [11]. ERK and/or Coomassie Blue-staining of membranes were used as loading controls.

Chemotaxis Cells on coverslips were observed (during the treatment period) in stable PDGF-BB gradients in the Dunn

chamber (Weber Scientific International, Teddington, UK) in various glucose conditions without/with exposure to pharmacological agents or after microinjection. Pools of cells derived from individual donors produced a consistent migratory response at early passage; therefore, individual experiments were defined by donor source. Cell movement was tracked using image capture software (Metamorph 6.1; Universal Imaging Corporation, West Chester, PA, USA) connected to an IX51 Olympus microscope (×10 objective; Olympus, Tokyo, Japan). Cell movement was assessed as described [11, 22, 25]. In the absence of a chemoattractant gradient, cells move randomly (net percentage of cell migration is approximately 0%). The Rayleigh test is employed to separate out unidirectional movement (i.e. directly towards the growth factor source) from general cellular migration.

Immunofluorescence After treatment, cells on coverslips were fixed, permeabilised and actin localised as described [11]. For localisation of other proteins, the antibody (1:200) was co-incubated with phalloidin then FITC-conjugated secondary antibody added (1 h). Confocal laser-scanning microscopy (Micro Radiance; Bio-Rad, Hercules, CA, USA) was used for imaging and acquiring semi-quantitative measurements (in-built software) of masked slides. Results are presented as mean target protein signal for $n>30$ cells from each of more than three independent experiments (with local background subtraction) relative to the equivalent actin signal.

Kinase assays MAPK, Akt (both Cell Signaling Technology, Beverly, MA, USA) and PP2A (Upstate, Charlottesville, VA, USA) assay kits were used according to the manufacturers' instructions. The PKCβ_{II} activity assay (PepTag; Promega, Madison, WI, USA) was carried out with those modifications described [8].

Statistics When relevant ANOVA and Fisher's LSD was employed. Data are presented as mean ± SEM with $p<0.05$ considered statistically significant.

Note that results for migrating cell percentages refer to the net percentage of cells moving in the direction of the PDGF-BB: a result of 0% indicates no net migration (as many cells move towards PDGF-BB as away from it). To detect significant chemotaxis (unidirectional movement) the Rayleigh test for unimodal clustering of directions was used: $p<0.01$ was chosen as the criterion for rejecting the null hypothesis of random directionality [7, 11].

Results

PDGF-BB and glucose effects on PDGF-βR protein level We previously demonstrated that raising glucose from

normal fasting level (5 mmol/l) to 25 mmol/l increases PDGF-βR levels in aortic VSMC exposed to 360 pmol/l PDGF-BB concentrations [7, 11].

To determine whether human aortic VSMC demonstrate a biphasic receptor response and identify how glucose modifies this, cells were exposed to basal conditions (0.5% BSA) or increasing PDGF-BB concentrations in normal or raised glucose conditions. Total receptor protein level was determined (Fig. 1a,b). At NG (5 mmol/l D-glucose), PDGF-BB caused a dose-dependent increase in PDGF-βR level until 1,080 pmol/l PDGF-BB; above this concentration the receptor level fell. By contrast at HG (25 mmol/l D-glucose, 24 h), the maximum receptor level occurred at a PDGF-BB concentration of 540 pmol/l, above which the receptor level fell sharply. At concentrations ≤720 pmol/l PDGF-BB, the receptor level was greater in HG than in NG. The HG effect was not due to an osmotic effect of glucose since the non-metabolisable analogue L-glucose (NG plus 20 mmol/l L-glucose, 24 h) was similar to NG. An intermediate D-glucose concentration (10 mmol/l) also had maximum receptor levels at 540 pmol/l (mean 2.6±0.15-fold above basal; $n=4$, not shown).

The results clearly show a biphasic PDGF-βR response to PDGF-BB for aortic VSMC. Glucose caused a left-shift in the biphasic response to PDGF-BB and increased the maximum receptor level. Similar responses to glucose and PDGF-BB were found for PDGF-βR level at the cell surface, where PDGF-BB binding occurs (assessed by cell membrane immunoblotting and immunofluorescence of non-permeabilised cells) and in mRNA level (RT-PCR; see ESM Figs 1 and 2a,b).

PDGF-BB and glucose effects on ERK and Akt phosphorylation PDGF-βR regulation in HG at 360 pmol/l PDGF-BB concentrations depends on MAPK/ERK pathway signalling with crosstalk from the PI3K/Akt pathway [7]. As was found for the receptor, phospho-ERK1/2 (Fig. 1c,d) and to a lesser degree phospho-Akt (Fig. 1e,f) showed biphasic responses to PDGF-BB, whereas the total protein levels remained constant. Glucose increased the maximum phosphorylation level and left-shifted the curves for phospho-ERK and phospho-Akt. ERK signals to the transcription factor, activator protein-1 (c-fos and c-Jun), which has a *PDGF-βR* gene promoter binding site [10, 26]. In assays employing nuclear extracts c-fos and c-Jun binding to an immobilised oligonucleotide reflected the changes in PDGF-βR level under similar conditions (ESM Fig. 2c–e).

PDGF-BB and glucose effects on chemotaxis The major functional VSMC response in atherosclerosis is migration into the plaque. Figure 1g shows a biphasic response for PDGF-BB-induced chemotaxis. Glucose caused a left-shift in the response with chemotaxis being detected in NG (or

Fig. 1 Effect of PDGF-BB and glucose (24 h) on PDGF-βR production (**a**, **b**), ERK phosphorylation (**c**, **d**), Akt phosphorylation (**e**, **f**) and chemotaxis (**g**). **a**–**f** Relative densitometric results for PDGF-βR protein (**a**), phospho-ERK (**c**) and phospho-Akt (**e**), respectively (mean±SEM), following immunoprecipitation and western blotting (**b**, **d**, **f**) in VSMC (passage 3) lysates. VSMC had been previously incubated in NG (5 mmol/l D-glucose), HG (NG plus 20 mmol/l D-glucose, 25 mmol/l D-glucose) or NG plus 20 mmol/l L-glucose (LG; osmotic control) under basal conditions (B; 0.5% BSA) or 36–3600 pmol/l (1–100 ng/ml) PDGF-BB for 24 h (*n*=30 independent experiments). Total ERK and Akt protein (**d**, **f**) (representative blots) did not alter and were employed as loading controls.
g Corresponding Dunn chemotaxis results. Percentages represent net percentage of observed cells (>98% viability for each condition) that moved >30 μm up the PDGF-BB concentration gradient (*n*>30 cells tracked from each of five independent experiments). Grey squares/line, NG; black triangles/continuous line, HG; black circles/dotted line, LG. Analysis: ANOVA. *$p<0.05$ and **$p<0.01$ vs basal; †$p<0.05$ and ††$p<0.01$ vs NG (and LG); ‡‡$p<0.01$ in Rayleigh test for unidirectional movement=significant chemotaxis
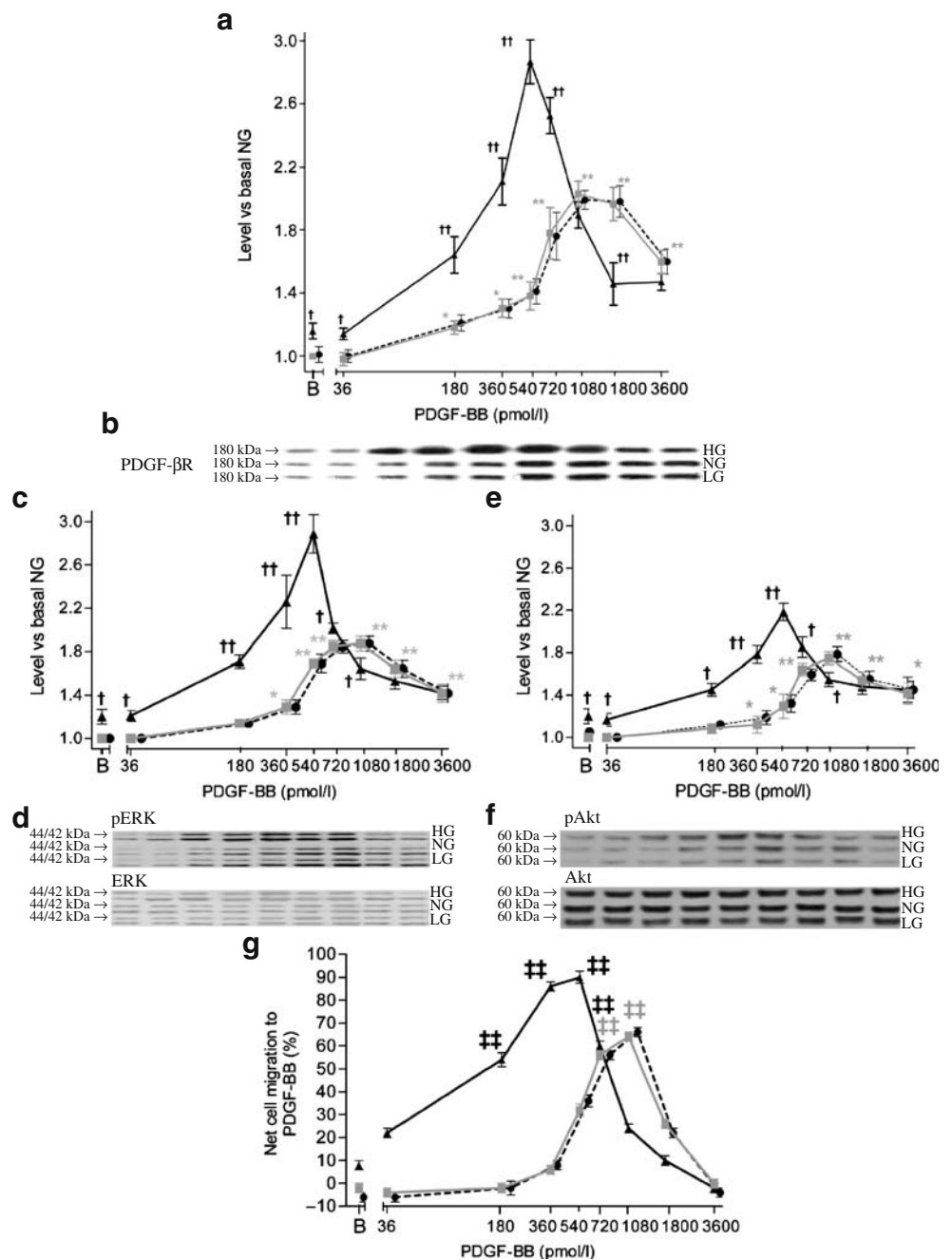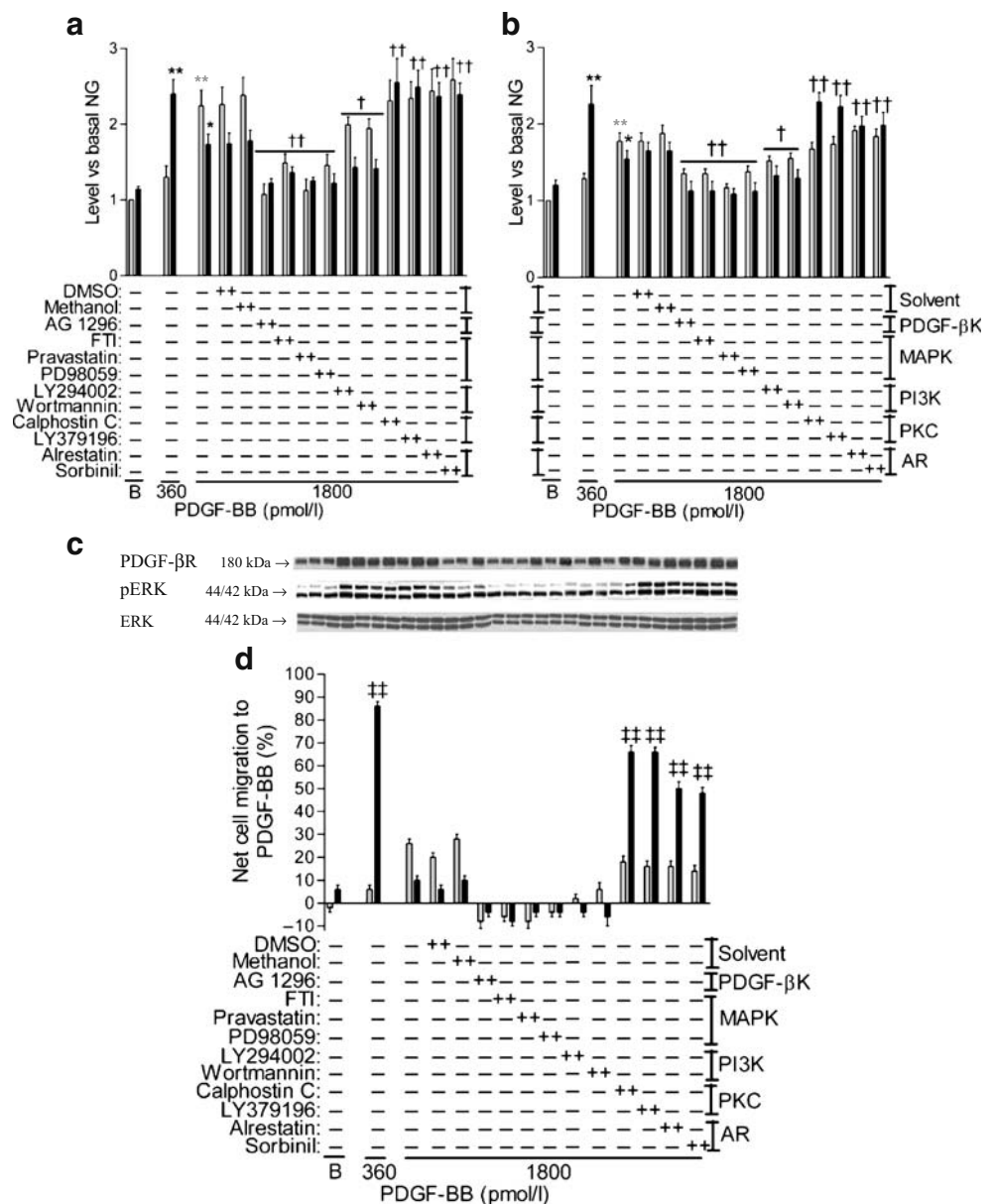

NG+20 mmol/l L-glucose) between 720–1,080 pmol/l PDGF-BB, and in HG between 180–720 pmol/l. At 1,800–3,600 pmol/l PDGF-BB, no significant net migration occurred at either glucose level. In atheromas, PDGF-BB levels may not match those found to induce migration in Fig. 1g; other factors may modify migration in vivo [2, 3, 27].

Chemotaxis experiments were run for 18 h, and the above relate to the condition at the end of that period. However, cell movement recording allows the results to be interrogated at various time intervals. At 6 h the PDGF-BB concentrations causing chemotaxis in NG and HG were the same as at 18 h (not shown).

These results indicate that phospho-ERK, phospho-Akt and their targets PDGF-βR protein levels and chemotaxis follow similar biphasic response patterns to PDGF-BB concentrations as glucose changes.

PDGF-βR level control in the presence of PDGF-BB and glucose Since we have previously shown that MAPK/Ras, PI3K, PKCβ$_{II}$ and aldose reductase (AR; the rate-limiting polyol pathway enzyme) control receptor level, phospho-ERK and chemotaxis at 360 pmol/l PDGF-BB [7, 8], these pathways were investigated here at higher PDGF-BB concentrations (Fig. 2).

Experiments carried out to identify how total receptor level was reduced in the presence of elevated PDGF-BB concentrations in NG and HG employed chemical inhibitors (Fig. 2a,c). Solvent controls did not alter receptor level. In the presence of PDGF-BB 1,800 pmol/l, PDGF-βR kinase inhibition (AG1296) reduced the receptor to basal level in both NG and HG, showing that PDGF-βR activation is a controlling factor of receptor protein level. MAPK inhibition at the level of Ras (farnesyltransferase inhibitor and pravastatin: non-specific inhibitors of Ras-dependent signalling in aortic VSMC) [7, 8] or MAPK kinase 1/2 (PD98059) as well as PI3K inhibition (LY294002 and wortmannin; inhibitors with different profiles) reduced receptor level in 1,800 pmol/l PDGF-BB at both glucose levels.

Glucose has a major effect on PDGF-βR level in response to PDGF-BB. Since HG increases polyol pathway flux and activates PKC$_{\beta II}$ [8, 28], the influence of this pathway and PKC$_{\beta II}$ on receptor level were investigated. A major finding was that at the higher PDGF-BB concentration (1,800 pmol/l) in HG, PKC inhibitors (calphostin C: general PKC inhibitor; or LY379196: PKC$_{\beta II}$-specific inhibitor at 200 nmol/l [8]) or AR inhibitors (alrestatin and sorbinil) increased receptor levels, eliminating the difference between NG and HG. (It should be noted that at 360 pmol/l PDGF-BB in HG, AR/PKC inhibition decreases PDGF-βR receptor level [8].) As expected AR/PKC inhibition had no effect on receptor level in NG.

The results so far show that the biphasic PDGF-βR response can be abrogated by AR/PKC$_{\beta II}$ inhibition at high

PDGF-BB concentrations (1,800 pmol/l) in HG. To see whether AR/PKCβ_{II} inhibition could also restore phospho-ERK under these conditions the following experiments were undertaken.

ERK phosphorylation control in the presence of PDGF-BB and glucose To identify whether the biphasic response in PDGF-βR expression occurs above the ERK signalling level, phospho-ERK was assessed under 1,800 pmol/l PDGF-BB conditions (Fig. 2b). Inhibition of PDGF-βR kinase or MAPK significantly decreased phospho-ERK in NG and HG. PI3K inhibitors also decreased phospho-ERK level in 1,800 pmol/l PDGF-BB, but to a lesser degree. MAPK inhibitors did not alter phospho-Akt level under these conditions, indicating that PI3K to MAPK crosstalk, present at 360 pmol/l PDGF-BB concentrations [7], also occurs at 1,800 pmol/l PDGF-BB (data not shown).

By contrast, PKC inhibition significantly increased phospho-ERK in HG. AR inhibition also significantly increased phospho-ERK in HG, but to a lesser degree. As expected, AR/PKC inhibition did not alter phospho-ERK in NG. The contrasting pharmacological inhibitor results for AR/PKC were confirmed both by microinjection techniques (ESM Fig. 3a,b; phospho-PKC$\beta_{II}$$^{(Ser660)}$ antibodies or AR siRNA microinjected) and by ERK activity assay (ESM Fig. 3c). These results indicate that ERK activity is reduced by AR/PKCβ_{II} signalling in HG at 1,800 pmol/l PDGF-BB.

Chemotaxis control in the presence of PDGF-BB and glucose Chemotaxis results (Fig. 2d) were obtained for those conditions investigated in Fig. 2a–c. In HG 1,800 pmol/l PDGF-BB, PDGF-βR kinase, MAPK or PI3K inhibitors prevented chemotaxis, but AR/PKCβ_{II} inhibition restored it and its related morphology (including filopodia formation; ESM Fig. 4a). Chemotaxis results from the microinjection experiments noted above confirmed the inhibitor results (ESM Fig. 4b).

These results (Fig. 2) are consistent with the effect of glucose on the biphasic response in PDGF-βR and chemotaxis occurring above the level of ERK signalling, with AR/PKCβ_{II} signalling promoting ERK activation at low PDGF-BB levels [8], but contributing to inhibition at high levels.

Activation of PKCβ$_{II}$ by PDGF-BB and glucose Since the negative regulation of PDGF-βR level, ERK activity and chemotaxis in high PDGF-BB concentrations in HG was PKCβ_{II}-dependent (Fig. 2), the control of phospho-PKCβ_{II} was investigated next (Fig. 3a,b).

In NG, a small significant reduction in phospho-PKC$\beta_{II}$$^{(Ser660)}$ occurred at high PDGF-BB levels. In HG, in the absence of PDGF-BB, phospho-PKCβ_{II} was significantly elevated compared with NG and rose further (monophasic response) as PDGF-BB concentrations increased.

Pathway inhibitor effects on phospho-PKCβ_{II} were next investigated. MAPK and PI3K inhibition had no effect on phospho-PKCβ_{II} (Fig. 3c,d), confirming that PKCβ_{II} activation occurs upstream of MAPK and PI3K.

Diacylglycerol (a cofactor for PKCβ_{II} activation) can be generated from two distinct intracellular pools: (1) PDGF-βR signalling causes phospholipase C (PLC) γ activation, which hydrolyses phosphatidylinositol bisphosphate to form DAG (glucose-independent DAG) and inositol 1,4,5-triphosphate; and (2) in elevated glucose conditions de novo synthesis of DAG (glucose-dependent DAG) also occurs through an AR-dependent pathway. Inhibition of PDGF-βR kinase resulted in a small (significant) phospho-PKCβ_{II} decrease in both glucose conditions: since this effect was glucose-independent, inhibition of PLCγ-signalling was considered to be the probable cause. Indeed PLCγ inhibition (U-73122) had a similar effect to PDGF-βR kinase inhibition. AR inhibition had no effect in NG but abolished the HG effect confirming a glucose-dependent response in phospho-PKCβ_{II}. PKCβ_{II} phosphorylation therefore is receptor/PLCγ-dependent in NG and HG; this is augmented by AR-dependence in HG.

The AR effect was confirmed with a PKCβ_{II} activity assay (Fig. 3e,f). PKCβ_{II} requires membrane binding for full activity [8]. In PDGF-BB 1,800 pmol/l PKCβ_{II} activity in isolated membranes was 2.5-fold greater in HG than in NG. This effect was blocked by AR inhibition. Cytoplasmic PKCβ_{II} activity remained unchanged.

The above results indicated that the glucose-potentiated left-shift in the biphasic response to PDGF-BB was due to negative effects associated with PKCβ_{II} signalling in high PDGF-BB concentrations, but the mechanism was unknown. Phosphatases activated in HG, diabetes or atherosclerosis, as well as those known to affect the MAPK and PI3K pathways were investigated to identify putative effectors of the biphasic response.

Phosphatase inhibitor effects on PDGF-βR level Total PDGF-βR production was assessed (cell lysate immunoblotting) after VSMC were exposed to PP inhibitors (PP1, PPI2; PP2A, endothall and okadaic acid; PP2B, fenvalerate) or protein tyrosine phosphatase (PTP) inhibitors (PTP1B, PTPI; general PTPs, vanadate). In NG, only PTP1B inhibition significantly increased the receptor level at 1,800 pmol/l PDGF-BB (Fig. 4a,b); in HG PTP1B elicited a similar response, indicating a glucose-independent effect. However in HG (1,800 pmol/l PDGF-BB), PP2A inhibition significantly augmented PDGF-βR level, whereas in NG the effect was minimal (non-significant), indicating a glucose-dependent effect.

Phosphatase inhibitor effects on chemotaxis to PDGF-BB Matched cells to those investigated (Fig. 4a,b) were

Fig. 3 Effect of glucose and
PDGF-BB on PKCβ$_{II}$
phosphorylation. **a** Relative
densitometric results for phos-
pho-PKCβ$_{II}^{Ser660}$ (mean±SEM)
for immunoblotted VSMC
lysates from cells treated as in
Fig. 1 (n=30 independent
experiments) or with added
inhibitors (**c**) (n=8 independent
experiments). **b, d** Respective
immunoblots. Loading control:
total ERK protein. **e, f** PKCβ$_{II}$
activity results for alrestatin
(cf. **a–d**) for membranous (**e**)
and cytoplasmic (**f**) fractions
(n=4 independent experiments).
Inhibitors: PLCγ (U-73122:
5 μmol/l) and its negative
control (U-73343: 5 μmol/l).
For other inhibitors and treat-
ment conditions, see Fig. 2.
Grey squares/grey line, NG;
black triangles/continuous line,
HG; black circles/dotted line,
NG+ 20 mmol/l L-glucose
(LG); grey bars, NG; black bars,
HG. Analysis: ANOVA. *p<
0.05 and **p<0.01 vs basal (B);
$^{††}p$<0.01 vs NG; $^{‡}p$<0.05 and
$^{‡‡}p$<0.01 vs DMSO at same
glucose level and PDGF-BB
concentration
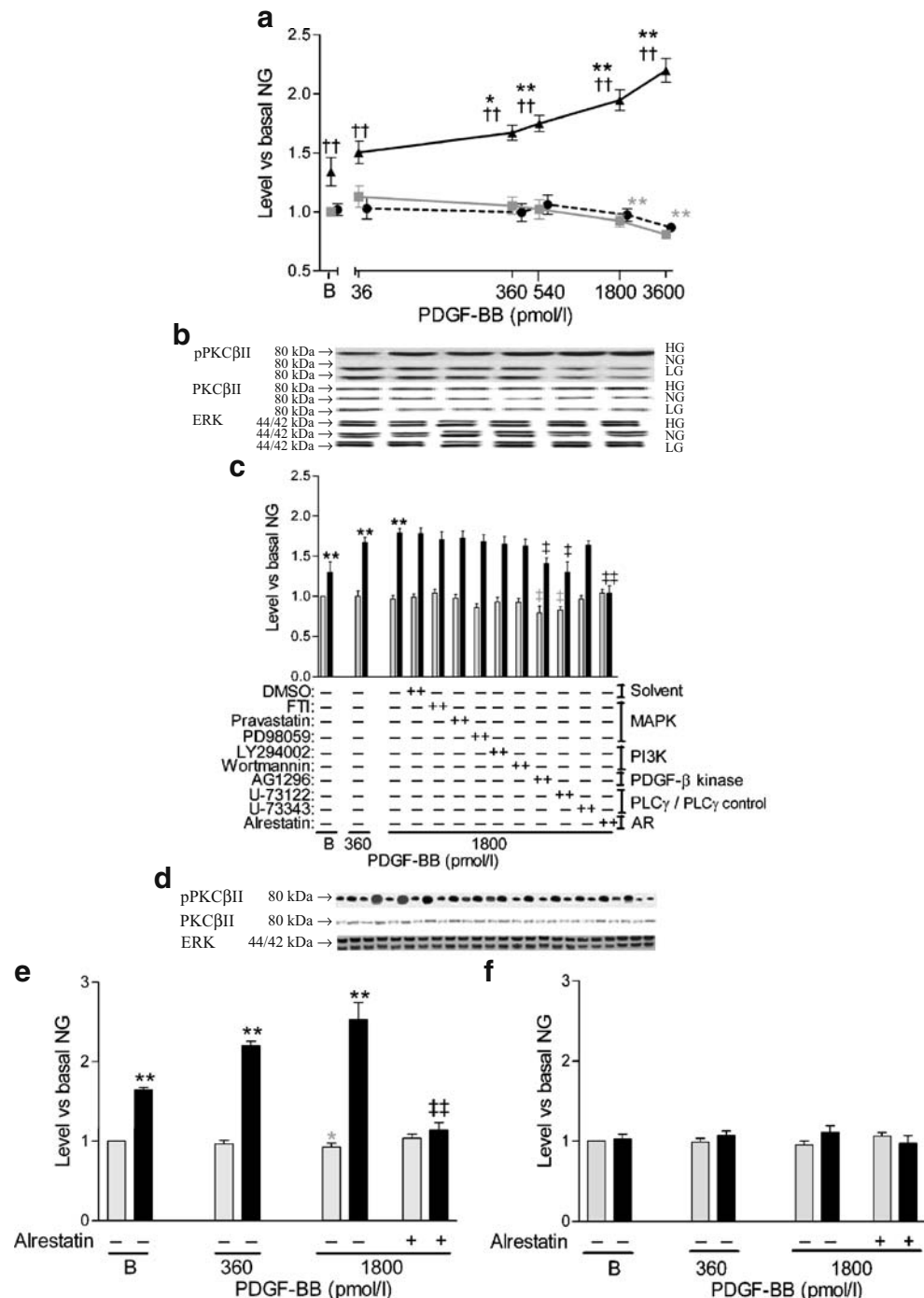
assessed for chemotaxis (Fig. 4c). Although PTP1B inhibition had a small effect in increasing PDGF-βR level (Fig. 4a,b), significant chemotaxis was not detected (Fig. 4c; Rayleigh test, p=0.21). However, the significant increase in receptor numbers in HG due to PP2A inhibition, a known MAPK modulator, was associated with restored chemotaxis; PP2A had little effect in NG.

To confirm the inhibitor results and to exclude a role for MAPK phosphatases (known to be glucose-independent [29]), monoclonal antibody microinjection studies were undertaken

(Fig. 4d). In 360 pmol/l PDGF-BB, PP2A or PTP1B antibody microinjection had no effect on chemotaxis. At 1,800 pmol/l PDGF-BB, anti-PTP1B did not restore chemotaxis at either glucose level. Anti-PP2A restored chemotaxis in HG; the effect was specific since antibody preabsorption could not mimic the effect. The MAPK phosphatase antibodies had no significant effect.

Similar results to those shown for antibody microinjection (Fig. 4d) were found with *PP2A* or *PTP1B* siRNA microinjection (not shown). Microinjection of active PTP1B

peptide (10 nmol/l) to cells in HG at 360 pmol/l or 1,800 pmol/l PDGF-BB did not significantly alter migration (net cell migration 75±4 and 5±2% respectively).

The biphasic PDGF-βR and chemotaxis responses appear to be due to negative regulation in HG and high PDGF-BB conditions mainly by PP2A activity. The biphasic responses in NG were less dependent on any one phosphatase, with most inhibitors or antibodies having a marginal ability to increase receptor level (significant for PTP1B) or chemotaxis.

Phosphatase inhibitor effects on ERK, Akt and PKCβ$_{II}$ activation If PP2A critically regulates the biphasic responses noted in this study, then PP2A blockade in HG should have a similar effect on ERK signalling, as with receptor level and chemotaxis. In PDGF-BB 360 pmol/l, neither PP2A nor PTP1B inhibition altered phospho-ERK level in NG or HG (Fig. 5a,b). In PDGF-BB 1,800 pmol/l and NG, PTP1B inhibition increased phospho-ERK by a small, significant amount; changes due to PP2A inhibition were not significant. In PDGF-BB 1,800 pmol/l and HG, PTP1B inhibition resulted in a small increase in phospho-ERK, while PP2A inhibition resulted in a marked phosphorylation increase; the phospho-ERK response to PP2A inhibition was matched by an increase in ERK activity (Fig. 5c). ERK activity also increased with PP2A inhibition at PDGF-BB 1,800 pmol/l in NG, but to a lesser degree than in HG.

Akt also has a biphasic phosphorylation (Fig. 1e,f) and activity (Fig. 5d) profile, but is less affected (fold changes) by PDGF-BB and glucose than ERK. Akt is a PKCβ$_{II}$ signalling target involved in crosstalk to MAPK [8] and is a known PP2A target. PP2A inhibition increased Akt signalling in PDGF-BB 1,800 pmol/l and HG (Fig. 5d), but to a lesser degree than that seen in ERK activity (Fig. 5c).

In similar conditions we investigated the PP2A and PTP1B inhibition effects on PKCβ$_{II}$ phosphorylation (Fig. 5e,f) and activity (Fig. 5g,h). PKCβ$_{II}$ activation did not depend on PP2A or PTP1B.

Phospho-PP2A: modification by PDGF-BB and glucose Decreased phospho-PP2A^{Tyr307} level is associated with increased PP2A activity [30]. In NG, phospho-PP2A was
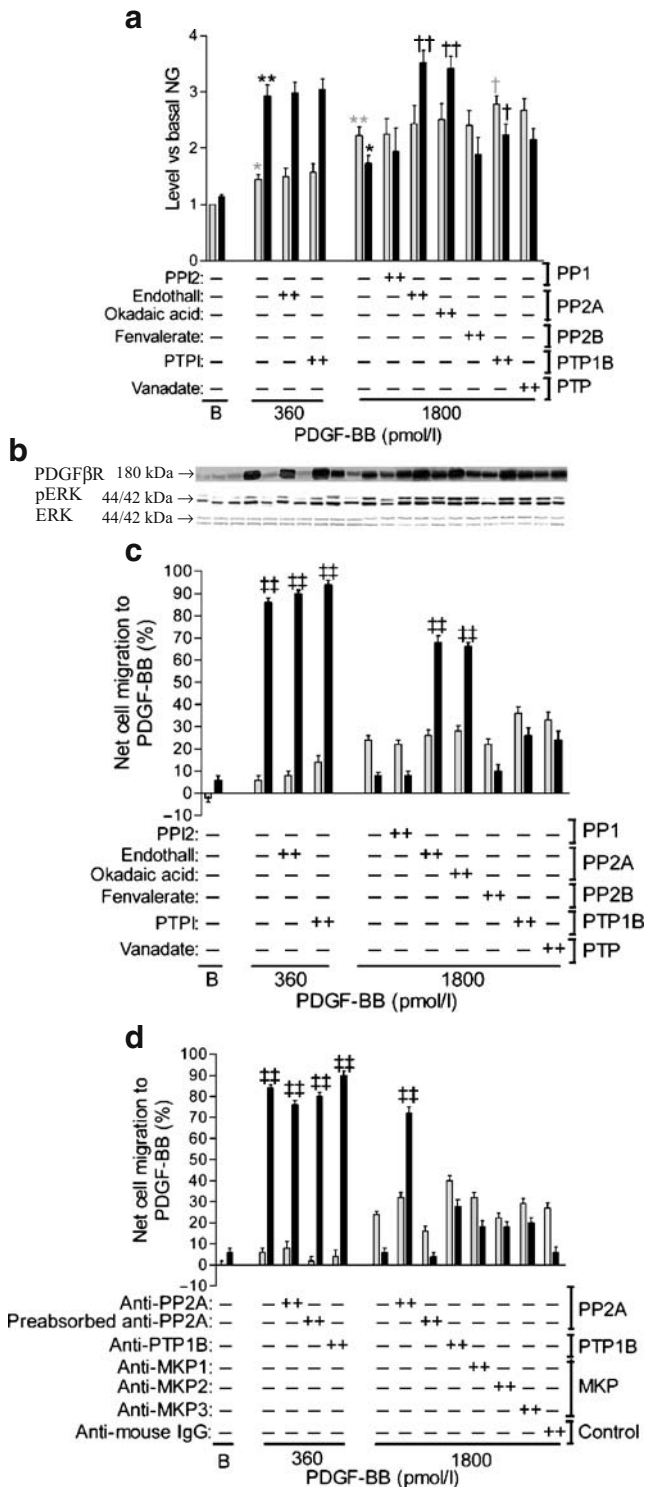

◀ **Fig. 4** Effect of phosphatase inhibitors on PDGF-βR protein and chemotaxis. **a** Relative densitometric results (mean±SEM) and (**b**) immunoblot respectively for PDGF-βR protein in immunoblotted lysates of cells treated as in Fig. 1 plus phosphatase inhibitors (*n*=8 independent experiments). **c** Corresponding Dunn chamber chemotaxis results (*n*>30 cells tracked from each of five independent experiments). **d** Confirmatory chemotaxis results (for **c**) using micro-injected phosphatase antibodies, preabsorbed PP2A antibody (pre-exposed to molar excess of cognate peptide before injection), non-specific antibodies and sham injection. Micro-injected/sham-injected VSMC were rested (2 h) then placed in the Dunn chemotaxis chamber (18 h) and tracked (*n*>30 cells tracked from each of four independent experiments). Inhibitors: PP1 (PPI2: 2 nmol/l), PP2A (endothall: 90 nmol/l; okadaic acid: 1 nmol/l), PP2B (fenvalerate: 2 nmol/l), PTP1B (PTPI: 40 μmol/l) and non-specific PTP (vanadate: 10 mmol/l). MKP, MAPK phosphatase. Grey bars, NG; black bars, HG. Analysis: ANOVA. **p*<0.05 and ***p*<0.01 vs basal (B); †*p*<0.05 and ††*p*<0.01 vs DMSO at same glucose level and PDGF-BB concentration; ‡‡*p*<0.01 in Rayleigh test for unidirectional movement= significant chemotaxis

Fig. 5 Effect of phosphatase inhibitors on ERK (**a**–**c**), Akt (**d**) and PKCβ_{II} (**e**–**h**) activity. **a** Relative densitometric results (mean±SEM) and respective blot (**b**) for phospho-ERK in immunoblotted lysates; cells were treated as in Fig. 1 without/with inhibitors (*n*=8 independent experiments). **c** Confirmatory protein activity results for endothall under similar conditions to those for phospho-ERK (**a**) (*n*=4 independent experiments). **d** Akt activity under conditions of panel **c** (*n*=4 independent experiments). **e** Relative densitometric result and respective blot (**f**) for phospho-PKCβ_{II} matching conditions for ERK (**a**). **g**, **h** Confirmatory PKCβ_{II} activity results for membranous (**g**) and cytoplasmic (**h**) fractions, respectively under similar conditions to those for phospho-PKCβ_{II} (**e**) (*n*=4 independent experiments). Inhibitors: see Fig. 4 legend. Grey bars, NG; black bars, HG. Analysis: ANOVA. *$p<0.05$ and **$p<0.01$ vs basal (B); †$p<0.05$ and ††$p<0.01$ vs DMSO at same glucose level and PDGF-BB concentration
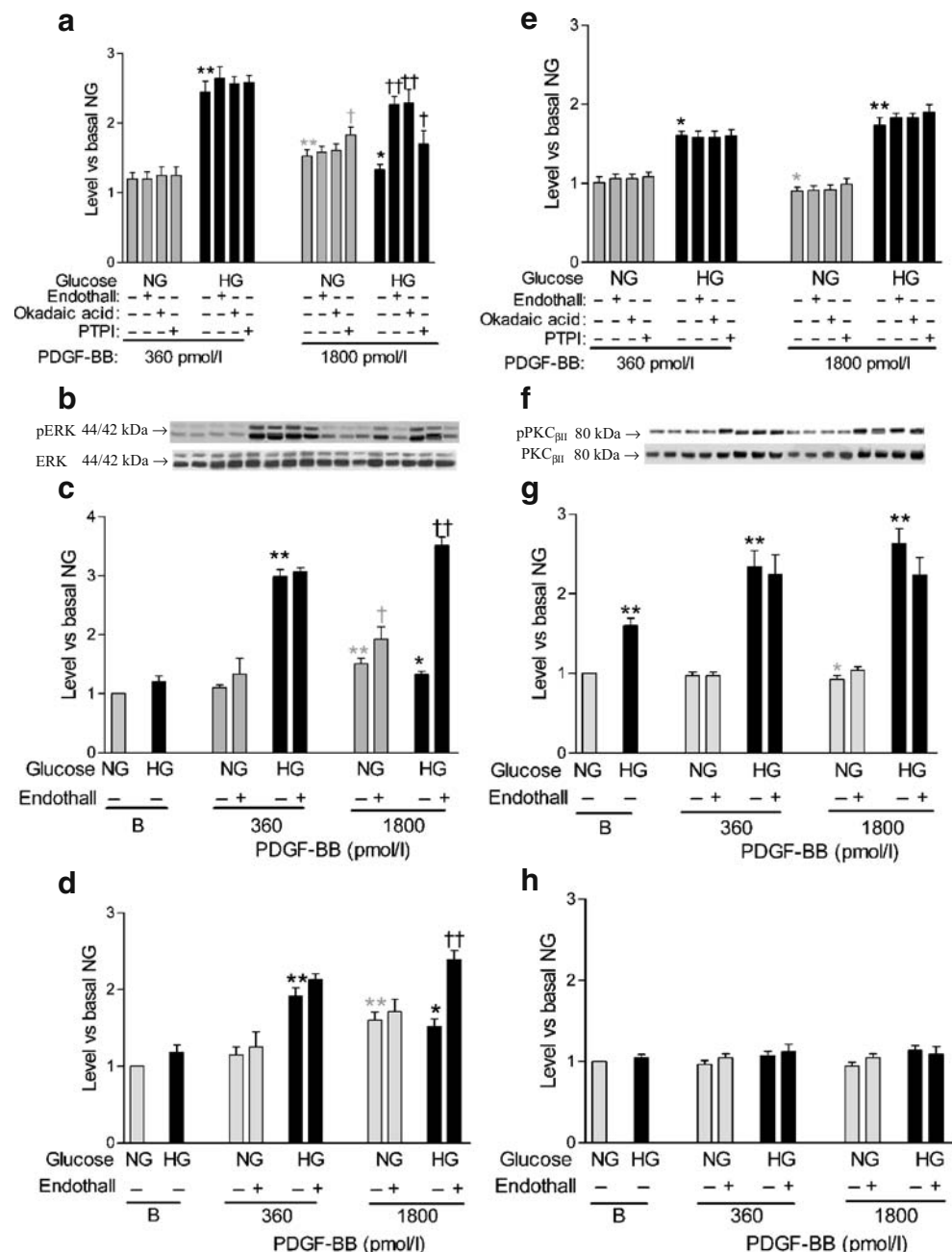
relatively unaltered by PDGF-BB (Fig. 6a,b). By contrast, in HG phospho-PP2A was significantly decreased at PDGF-BB concentrations above 540 pmol/l; therefore active PP2A levels increase where HG and high PDGF-BB concentrations coexist. Total PP2A protein levels remained unchanged throughout.

Phospho-PP2A control in the presence of PDGF-BB and glucose Neither MAPK inhibition by PD98059 (Fig. 6c,d) nor pravastatin (not shown) nor PI3K inhibition altered phospho-PP2A. PKCβ_{II} or AR inhibition (Fig. 6e,f) or microinjection of PKCβ_{II} antibody or AR siRNA (not

shown) increased phospho-PP2A level (i.e. inactivated it) in HG at 1,800 pmol/l PDGF-BB. PLCγ and PTP1B inhibition had only a marginal effect on phospho-PP2A under these conditions.

To confirm that the increased PKCβ_{II} activity brought about by HG regulates PP2A activity, an activity assay was undertaken (Fig. 6g). PKCβ_{II} or AR inhibition significantly decreased PP2A activity in 1,800 pmol/l PDGF-BB in HG conditions while having no significant effect in 360 pmol/l PDGF-BB.

The present results indicate that as PDGF-BB level increases in HG, PKCβ_{II} activates PP2A, thereby reducing

Fig. 6 Effect of inhibitors on PP2A phosphorylation (inactivation). **a** Relative densitometric results for phospho-PP2A$^{(Tyr307)}$ (mean±SEM) and respective blot (**b**), in immunoblotted lysates of cells previously treated as in Fig. 1 ($n=30$ independent experiments). Loading control: total ERK protein. **c–f** Results (phospho-PP2A$^{(Tyr307)}$) for lysates from cells treated as **a, b** plus with inhibitors ($n=8$ independent experiments). **g** Confirmatory PP2A activity results for inhibitors that significantly increased phospho-PP2A$^{(Tyr307)}$ (**e, f**) ($n=6$ independent experiments). Inhibitors: see legends Figs 2 and 4. Grey squares/grey line, NG; black triangles/continuous line, HG; black circles/dotted line, NG+20 mmol/l L-glucose (LG). Grey bars, NG; black bars, HG. Analysis: ANOVA. *$p<0.05$ and **$p<0.01$ vs basal (B); $^{†}p<0.05$ and $^{††}p<0.01$ vs NG; $^{‡}p<0.05$ and $^{‡‡}p<0.01$ vs solvent at same glucose level and PDGF-BB concentration
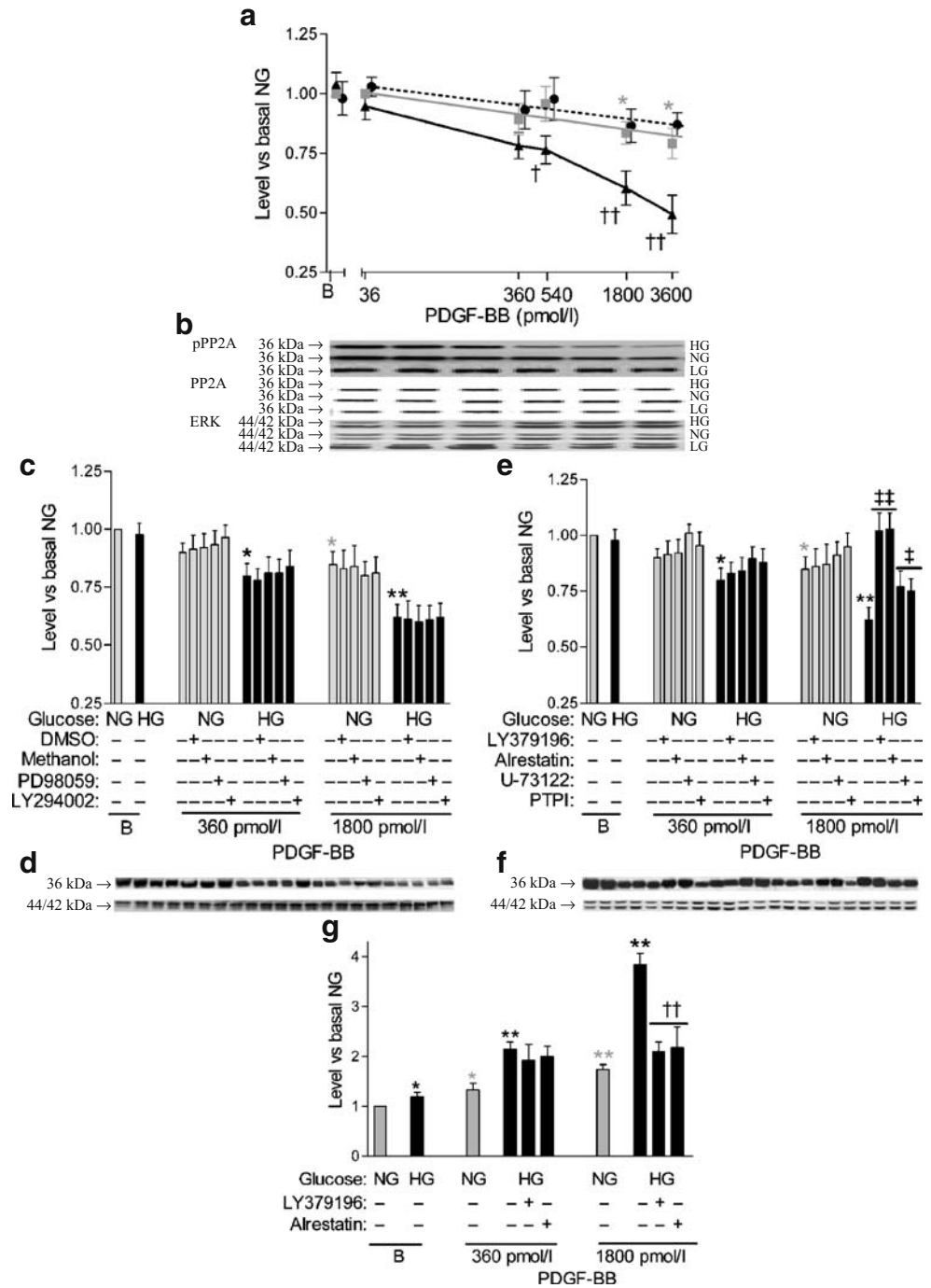
Akt and ERK signalling (under these conditions phospho-PKCβ$_{II}$ and PP2A physically associate, as does PP2A, with ERK and Akt; ESM Fig. 5).

Discussion

The results clearly show that a biphasic response to PDGF-BB exists for both PDGF-βR expression and chemotaxis in human aortic VSMC. We demonstrated previously that PDGF-βR level and chemotaxis depend on signalling through PI3K/Akt to the MAPK/ERK pathway in response to low PDGF-BB concentrations [7, 8, 11]. In the present work Akt and ERK activation followed similar biphasic changes to those that occurred with receptor expression/production and chemotaxis.

Atherosclerosis, which critically involves VSMC migration into the growth factor-rich plaque, occurs prematurely in individuals with diabetes. Ambient glucose concentration has a major influence on responses to PDGF-BB. VSMC
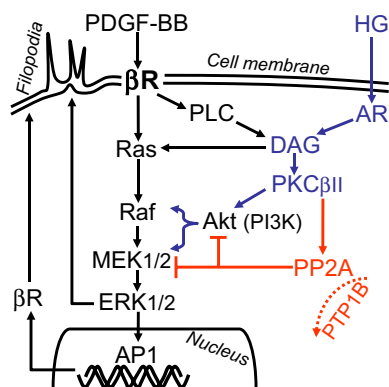
Fig. 7 DAG-dependent pathways activated in aortic VSMC by PDGF-BB and HG. In NG, PDGF-BB signals through the PDGF-βR kinase to activate the MAPK/ERK pathway encouraging formation of direction-sensing filopodia and upregulating PDGF-βR. As PDGF-BB concentration increases, VSMC migration, a key factor in atheroma formation, occurs. However, as PDGF-BB increases further, phosphatases, including PTP1B, become activated inhibiting these responses (not shown). In raised glucose conditions, a consistent finding in diabetes, the DAG/PKCβ$_{II}$/PI3K pathway and DAG/MAPK pathway both lead to ERK activation and migration. We have previously shown that Akt activates the MAPK pathway under these conditions [7], but the point between Ras and ERK1/2 where this occurs has not been demonstrated. Glucose lowers the threshold for migration to PDGF-BB to occur, indicating that even minor vessel wall injuries that release PDGF-BB may more readily trigger atheroma formation. Atherosclerosis is already known to have a premature onset in individuals with diabetes. If, in addition to elevations in glucose level, the PDGF-BB level is increased, PP2A becomes activated in a PKCβ$_{II}$-dependent pathway inhibiting ERK and Akt activation. Decreases in ERK activation are associated with lack of filopodia formation, reduced cellular migration and lower transcriptional regulation of the PDGF-βR. PTP1B has a minor role in activating PP2A in conditions of raised glucose and PDGF-BB. Therapies that indirectly target PP2A under conditions of elevated glucose and elevated PDGF-BB may remove a critical inhibitory effect on ERK-dependent events and risk potentiating VSMC migration AP-1, activator protein 1

showed no net migration to low PDGF-BB concentrations in NG, but in raised glucose (>10 mmol/l) migration surpassed that in NG for all PDGF-BB concentrations ≤720 pmol/l. Thus, raised glucose levels, a consistent finding in diabetes, may potentiate VSMC migration into growth factor rich plaques.

Arterial tissue alterations in diabetes include increased bone-related molecule production, e.g. osteoprotegerin [31]. Elevated osteoprotegerin levels occur early in atheroma formation and are PDGF-inducible [32, 33]. By potentiating PDGF-induced signalling, hyperglycaemia may encourage arterial alterations associated with atheroma formation. It is likely that even minor vessel wall insults producing small changes in chemoattractant levels can potentiate VSMC migration and significant atheroma formation in individuals with diabetes.

HG caused a left-shift in the biphasic dose response for PDGF-βR levels, phospho-Akt and phospho-ERK in re-

sponse to PDGF-BB. HG activates PKCβ$_{II}$ in VSMC; in these conditions, ERK is stimulated indirectly through the effect of PKCβ$_{II}$ on PI3K/Akt and directly through DAG acting on Ras [8]. The peak phospho-Akt level in HG (~25% above that for NG) was only half the difference found for phospho-ERK (peak increase >50%). The greater effect of glucose on MAPK/ERK activity reflects the dual glucose/AR-dependent input to this pathway (Fig. 7). In a previous report on saphenous vein-derived VSMC, PDGF-BB was reported to have a biphasic effect on PDGF-βR level and chemotaxis, but the pathways involved were not identified and the ambient glucose level not given [14].

We identified phosphatases that could potentially decrease phospho-ERK in VSMC at high PDGF-BB concentrations. At high PDGF-BB concentrations, both PP2A (a known negative regulator of ERK [34]) inhibition and PTP1B inhibition increased ERK activity and phosphorylation respectively in NG and HG. PTP1B demonstrated a small glucose-independent but PDGF-BB concentration-dependent response. In contrast, combining HG and high PDGF-BB concentrations greatly increased PP2A activation; this was less marked in NG.

The present results show that PKCβ$_{II}$ activation is independent of the activation status of PP2A, PI3K/Akt and MAPK/ERK; however, PKCβ$_{II}$ activates PP2A [35, 36]. In HG, when PP2A activity is increased (high PDGF-BB concentrations), PKCβ$_{II}$ inhibition resulted in PP2A inactivation and so increased Akt and ERK activation. Thus when both glucose and PDGF-BB are high, PKCβ$_{II}$ activates PP2A, which in turn leads to dephosphorylation of Akt and ERK as well as loss of chemotaxis. It is clear that PDGF-BB cannot cause VSMC chemotaxis when ERK phosphorylation/activation is low from whatever cause.

Increased polyol pathway flux in hyperglycaemia alters intracellular signalling and VSMC migration. The clinical use of AR inhibitors to reduce the vascular complications of diabetes has given disappointing results, perhaps for several reasons [37]. In the present investigation, AR/PKCβ$_{II}$ inhibition in HG and high PDGF-BB levels actually caused, rather than preventing chemotaxis. Since HG levels and supraphysiological PDGF-BB levels co-exist, in the area of the atheromatous plaque in diabetes [38], these results point to one mechanism that may help explain the lack of success of AR inhibitors in preventing the vascular complications of diabetes.

In contrast to the outcome with AR inhibitors, statin therapy is effective for cardiovascular disease prevention in diabetes [39]. Their major effect is to reduce cholesterol synthesis. However, we have shown here and previously [7] that statins directly inhibit the MAPK pathway (prevent Ras farnesylation) and VSMC migration at clinically relevant concentrations.

In signalling pathways, the critical balance between phosphorylation and dephosphorylation is altered by glu-

cose, with implications for VSMC chemotaxis and atheroma formation. If the same chemotactic forces identified in this study are exerted in vivo, VSMC may be attracted into the atheromatous plaque at lower concentrations of PDGF-BB in the presence of raised glucose concentrations; this points to another mechanism by which atheroma formation may be accelerated in diabetes.

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