Glucose-Potentiated Chemotaxis in Human Vascular Smooth Muscle Is Dependent on Cross-Talk Between the PI3K and MAPK Signaling Pathways

Malcolm Campbell, William E. Allen, Carol Sawyer, Bart Vanhaesebroeck, Elisabeth R. Trimble

Abstract—Atheroma formation involves the movement of vascular smooth muscle cells (VSMC) into the subendothelial space. The aim of this study was to determine the involvement of PI3K and MAPK pathways and the importance of cross-talk between these pathways, in glucose-potentiated VSMC chemotaxis to serum factors. VSMC chemotaxis occurred in a serum gradient in 25 mmol/L glucose (but not in 5 mmol/L glucose) in association with increased phosphorylation (activation) of Akt and ERK1/2 in PI3K and MAPK pathways, respectively. Inhibitors of these pathways blocked chemotaxis, as did an mTOR inhibitor. VSMC expressed all class IA PI3K isoforms, but microinjection experiments demonstrated that only the p110β isoform was involved in chemotaxis. ERK1/2 phosphorylation was reduced not only by MAPK pathway inhibitors but also by PI3K and mTOR inhibitors; when PI3K was inhibited, ERK phosphorylation could be induced by microinjected activated Akt, indicating important cross-talk between the PI3K and ERK1/2 pathways. Glucose-potentiated phosphorylation of molecules in the p38 and JNK MAPK pathways inhibited these pathways but did not affect chemotaxis. The statin, mevinolin, blocked chemotaxis through its effects on the MAPK pathway. Mevinolin-inhibited chemotaxis was restored by farnesylpyrophosphate but not by geranylgeranylpyrophosphate; in the absence of mevinolin, inhibition of farnesyltransferase reduced ERK phosphorylation and blocked chemotaxis, indicating a role for the Ras family of GTPases (MAPK pathway) under these conditions. In conclusion, glucose sensitizes VSMC to serum, inducing chemotaxis via pathways involving p110β-PI3K, Akt, mTOR, and ERK1/2 MAPK. Cross-talk between the PI3K and MAPK pathways is necessary for VSMC chemotaxis under these conditions.

Key Words: diabetes ■ atherosclerosis ■ Akt ■ ERK ■ statin

Cardiovascular disease is responsible for most of the complications associated with diabetes. Impairment of endothelium and muscle contributes to abnormalities of vasodilation. Expansion of the subendothelial space occurs with the development of atheromatous plaques, which contain cellular components that have migrated from the endothelium and the tunica media, in addition to invading inflammatory cells and macrophages. Both PI3K and MAPK have been implicated in chemotaxis.

PI3K enzymes regulate many cellular responses, including proliferation, protection from apoptosis, intracellular vesicular transport, cytoskeletal rearrangements, and cell migration. PI3Ks signal via the production of 3-phosphoinositides, lipid second messengers that bind to lipid-binding domains in a wide variety of proteins, including protein kinases (such as Akt/PKB) and regulators of small GTPases. The pleckstrin homology lipid-binding domain of Akt binds to the lipid products of PI3K; Akt is then recruited to the plasma membrane and phosphorylated at T308 and S473 to yield a fully activated kinase. PI3Ks are heterodimers consisting of a 110-kDa catalytic subunit (p110α, p110β, or p110δ) in complex with a regulatory adapter molecule (p85α and its splice variants, p85β and p55γ), which contains Src-homology-2 (SH2) domains. Whereas p110α and p110β are ubiquitously expressed, p110δ was previously thought to be restricted to leukocytes but has recently been found in other cell types. Many studies implicating PI3K activity have not distinguished between the 3 p110 isoforms, and little is known about their distinct signaling roles. All isoforms have the same lipid substrate, but the protein kinase substrates are isoform-specific. Functional inactivation of individual PI3K isoforms (microinjection of isoform-specific antibodies) revealed isoform-specific roles in mediating cellular responses to specific extracellular signals. In this study, we aimed to identify the isoform(s) of PI3K involved in glucose-potentiated vascular smooth muscle cell (VSMC) chemotaxis.

Many molecules with chemotactic properties activate the MAPK pathways, in addition to the PI3K pathway. The 3...
main human MAPK pathway cascades terminate in ERK1/2, p38, and JNK, respectively. The ERK1/2 MAPK pathway, which involves sequential activation of Ras, Raf, MEK1/2, and ERK1/2, is involved in platelet-derived growth factor (PDGF)–induced12-13 and tumor necrosis factor-α-induced14 VSMC chemotaxis. By contrast, p38 has been implicated in sphingosine-1-phosphate-stimulated chemotaxis of rat VSMC.15 Glucose activates the ERK1/2 pathway in VSMC16,17 and the p38 pathway in mesothelial cells, Schwann cells,21 and endothelial cells.17 In addition, glucose is also associated with activation of ERK1/2 and JNK.20

Cross-talk occurs among the different MAPK pathways, the degree of which depends on both the conditions and the tissues involved;21 however, in many publications only 1 of the pathways has been investigated. Cross-talk between the PI3K and MAPK pathways has been implicated in phagocytic cell chemotaxis.22,23 Furthermore, glucose can alter the cross-talk between signaling systems, as has been shown for the insulin and β-adrenergic signaling systems in VSMC.24

The aims of the present study were to investigate the involvement of the PI3K and MAPK in glucose-potentiated chemotaxis of VSMC to serum factors and to determine whether cross-talk between these pathways played an important role in chemotaxis under these conditions.

### Materials and Methods

#### Chemicals and Antibodies

The following chemicals were purchased: D-glucose and L-glucose (BDH, Poole, England); Wortmannin and LY294002 (PI3K inhibitor), rapamycin (mTOR inhibitor), mevinolin (lovastatin), geranylgeranylpyrophosphate, and farnesylpyrophosphate (Sigma); PD98059 (MEK1/2 inhibitor), SP600125 (JNK inhibitor), and nylgeranylpyrophosphate, and farnesylpyrophosphate (Sigma); Wortmannin (10 nmol/L) or LY294002 (10 μmol/L); rapamycin (mTOR inhibitor), mevinolin (lovastatin), geranylgeranylpyrophosphate, and farnesylpyrophosphate (Sigma); PD98059 (MEK1/2 inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor) (Calbiochem, La Jolla, Calif); the farnesyltransferase inhibitor H-D-Trp-D-Met-p-chloro-D-Phe-Gla-NH2 (BDH, Poole, England); antibodies to p110α, p110β, and phosphorylated p38 MAPK (Calbiochem); HRP-conjugated antimouse or antirabbit IgG (DAKO, Ely, UK); and rabbit or mouse IgG (Sigma). C-terminal antibodies were pre-absorbed with 30 molar excess of its cognate peptide before injection. Chemotaxis was blocked only in cells injected with antibody to p110α, and p110β, and preabsorption of this antibody with excess p110α abrogated its effect on chemotaxis (Table 1).

For an expanded Materials and Methods section, see the online data supplement available at http://circres.ahajournals.org.

### Results

#### Glucose-Potentiated Chemotaxis Is Dependent on the p110β Isoform of PI3K

We have previously shown that VSMC will show chemotaxis in fetal calf serum (FCS) gradient without previous starvation in the presence of 9 to 25 mmol/L glucose16 (see online Figure A). PI3K inhibitors, wortmannin (10 μmol/L) or LY294002 (10 μmol/L), blocked chemotaxis of VSMC in a gradient of FCS in 25 mmol/L glucose, with the Rayleigh test demonstrating no significant chemotaxis; the solvent, DMSO 0.1% (v/v), did not affect chemotaxis (Table 1). The present results show that VSMC express all class IA PI3K isoforms (see online Figure B). To determine the roles of these isoforms in VSMC chemotaxis, isofrom-specific neutralizing antibodies were microinjected. After recovery (2 hours), cells were placed in a chemotaxis chamber with 25 mmol/L glucose and FCS gradient; for controls, nonspecific IgG was injected. As additional controls, each antibody was pre-absorbed with excess p110α, p110β, and p110δ; used for microinjection and Western blotting, have been described elsewhere.1-3

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### Table 1. Glucose-Potentiated Chemotaxis is PI3K-Dependent (p110β), Akt-Dependent, mTOR-Dependent, and ERK1/2-Dependent

<table>
<thead>
<tr>
<th>Glucose Level + Treatment</th>
<th>Chemotaxis</th>
<th>Rayleigh Test P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mmol/L glucose (no treatment)</td>
<td>No</td>
<td>0.102</td>
</tr>
<tr>
<td>25 mmol/L glucose (no treatment)</td>
<td>Yes</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>25 mmol/L glucose + 0.1 % DMSO</td>
<td>Yes</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**PI3K pathway (all 25 mmol/L glucose)**

| + 10 nmol/L wortmannin | No | 0.136 |
| +10 μmol/L LY294002 | No | 0.142 |
| Anti-p110α | Yes | <0.001 |
| Anti-p110β | Yes | <0.001 |
| Anti-p110δ | Yes | <0.001 |
| Anti-Akt (rabbit) | No | 0.107 |
| + Nonspecific IgG (rabbit) | Yes | <0.001 |
| + 1 nmol/L Rapamycin | No | 0.127 |

**MAPK pathways (all 25 mmol/L glucose)**

| +600 mmol/L SB203580 (p38) | Yes | <0.001 |
| +90 nmol/L SP600125 (JNK) | Yes | <0.001 |
| +10 μmol/L PD98059 (MEK1/2) | No | 0.140 |
| Anti-ERK (mouse) | No | 0.237 |
| + Nonspecific IgG (mouse) | Yes | <0.001 |

**Glucose-Potentiated Chemotaxis Is Dependent on Akt and mTOR**

To test the effect of 25 mmol/L glucose on pathways mediated by PI3K, the activity of Akt a downstream target for PI3K, was investigated. No specific Akt inhibitors are available; therefore, we microinjected an antibody directed against the N terminus of Akt, which was previously used to demonstrate Akt involvement in insulin-stimulated glucose transport in adipocytes.25 Cells injected with the anti-Akt antibodies were microinjected. After recovery (2 hours), cells were placed in a chemotaxis chamber with 25 mmol/L glucose and FCS gradient; for controls, nonspecific IgG was injected. As additional controls, each antibody was pre-absorbed with 30 molar excess of its cognate peptide before injection. Chemotaxis was blocked only in cells injected with antibody to p110β, and preabsorption of this antibody with excess p110β abrogated its effect on chemotaxis (Table 1).
antibody showed no chemotaxis in 25 mmol/L glucose and FCS gradient, whereas those injected with a nonspecific IgG control antibody did show chemotaxis (Table 1).

A pathway closely linked to the PI3K pathway is that of mTOR, leading to activation of p70-S6 kinase. The mTOR inhibitor, rapamycin, also blocked chemotaxis (Table 1).

**Glucose Increases the Expression and Phosphorylation Levels of MAPK Proteins**

We tested the impact of 24-hour incubation in 25 mmol/L d-glucose on the expression levels and phosphorylation states of p38, JNK, and ERK1/2 members of the MAPK family by immunoprecipitation and Western blotting of lysates using total and phospho-specific antibodies and densitometric evaluation (Table 2). Incubation in 25 mmol/L glucose caused a significant increase in total protein level of all 3 proteins (P<0.05 in all cases). Both p38 and JNK showed significant increases (21% and 28%, respectively) in their phosphorylation level (P<0.05), but these did not differ significantly from the increases seen in total protein level (27% and 22%, respectively). A significant increase in ERK1/2 phosphorylation was seen in 25 mmol/L glucose (P<0.01 versus 5 mmol/L glucose), which was greater than 3-fold the increase seen in ERK1/2 total protein level (95% versus 30%, respectively).

**Which MAPK Pathway Is Required for Glucose-Potentiated Chemotaxis?**

To determine the role of the 3 main MAPK signaling pathways in d-glucose–mediated chemotaxis, VSMC were placed in a gradient of FCS in 25 mmol/L glucose in the presence of 600 mmol/L SB203580 (a p38 inhibitor), 90 mmol/L SP600125 (a JNK inhibitor), or 10 μmol/L PD98059 (a MEK1/2 inhibitor). VSMC treated with SB203580 or SP600125 still showed chemotaxis, but PD98059 blocked chemotaxis (Table 1), suggesting that only the MEK/ERK is involved in glucose-potentiated chemotaxis. To investigate this further, an anti-ERK antibody (directed to the C-terminus) was microinjected into VSMC. Anti-ERK antibody blocked chemotaxis whereas nonspecific IgG did not (Table 1).

**Glucose-Potentiated Akt Activation Is PI3K-Dependent but not MAPK-Dependent**

We next investigated how the PI3K and MAPK pathways interact to control chemotaxis in VSMC. Total and Ser473-phosphorylated Akt were immunoprecipitated from lysates of cells treated with 25 mmol/L d-glucose with or without the PI3K inhibitor LY29402, the mTOR inhibitor rapamycin, the MEK1/2 inhibitor PD98059, or the Ras farnesylation inhibitor, mevinolin. None of these inhibitors and/or l-glucose controls (latter not shown) caused a significant change in total Akt protein (Figure 1A and 1D). The level of Ser473-phosphorylated Akt (Figure 1B and 1E) increased significantly in 25 mmol/L glucose (versus 5 mmol/L glucose, \(P<0.05\)). Akt Ser473 phosphorylation was reduced by the PI3K inhibitors LY294002 (\(P<0.05\)) and wortmannin (not shown, \(P<0.05\)) but not by rapamycin, PD98059, or mevinolin (Figure 1B and 1E), indicating that the effects of glucose on the PI3K to Akt pathway are independent of mTOR and MAPK. We next tested the activity of immunoprecipitated Ser473-phosphorylated Akt by assessing its ability to phosphorylate GSK-3, a downstream target of Akt. The results were similar to those found for phosphorylation of Akt. Akt activity increased (80%) in cells cultured in 25 mmol/L glucose for 24 hours (Figure 1C and 1F); this was inhibited by co-incubation with the PI3K inhibitor, LY294002, further confirming that glucose-stimulated Akt activation is PI3K-dependent.

**Glucose-Potentiated ERK1/2 Activation Is Affected by MEK/MAPK Pathway Inhibitors and by Inhibitors of the PI3K and mTOR Pathways**

To determine whether ERK activation in VSMC depends on PI3K activation, total and phosphorylated ERK1/2 were immunoprecipitated from lysates of cells treated with 25 mmol/L d-glucose with or without wortmannin and LY294002. Additionally, rapamycin, PD98059, or mevinolin were also used. The small but significant increase in total ERK1/2 protein level in 25 mmol/L glucose (\(P<0.05\) versus 5 mmol/L glucose alone) was not altered by rapamycin or mevinolin (Figure 2A and 2D) but was reduced by LY294002 and PD98059 (\(P<0.05\) versus 25 mmol/L glucose alone). The increased level of ERK phosphorylation (Figure 2B and
2E) in 25 mmol/L glucose (compared with 5 mmol/L glucose, $P<0.05$) was blocked by LY294002, wortmannin (data not shown), rapamycin, PD98059, and mevinolin ($P<0.05$ for all), indicating that the effect of glucose on the MAPK pathway leading to ERK1/2 is dependent on PI3K, mTOR, and, as expected, on Ras and MEK1/2 (Figure 2E). For dose-response to mevinolin, see online Figure C. Furthermore, an inhibitor of farnesyltransferase (FTI; 2 nmol/L), H-D-Trp-D-Met-p-chloro-D-Phe-Gla-NH$_2$, also reduced glucose-induced ERK phosphorylation (online Figure D). To investigate the relationship between ERK phosphorylation under these experimental conditions, a MAPK activity assay was undertaken; phosphorylated ERK1/2 was isolated from cell lysates and assessed for its ability to phosphorylate its target, ELK1. The results were similar to those found for phosphorylation of ERK1/2 (Figure 2C and 2F) with $\approx 300\%$ increase in ERK1/2 activity after culture in 25 mmol/L glucose for 24 hours compared with 5 mmol/L glucose conditions. The PI3K inhibitor (LY294002), rapamycin, PD98059, and mevinolin all significantly reduced the activity of ERK1/2 in 25 mmol/L glucose conditions ($P<0.05$ for all).

To investigate further the effects of glucose activation of the PI3K pathway on the MAPK pathway, VSMC were treated with the PI3K inhibitor and then microinjected with activated Akt to see whether phosphorylated Akt could induce phosphorylation of ERK. At the inhibitor concentration used, the glucose-induced increase in PI3K Akt was totally inhibited (online Figure E). Microinjection of activated Akt into VSMC pretreated with LY294002 resulted in a 4-fold increase in phosphorylated ERK in cells incubated in either 5 mmol/L or 25 mmol/L glucose (Figure 3).
Glucose-Potentiated Chemotaxis Is Ras-Dependent

Statins, in addition to inhibition of cholesterol synthesis, also cause impaired farnesylation of Ras and impaired geranylation of Rho family proteins.27 Mevinolin acts as a MAPK inhibitor because of its ability to prevent Ras farnesylation and therefore subsequent recruitment to the membrane and activation. To investigate the role of Ras and Rho family members in glucose-mediated chemotaxis, VSMC were placed in a gradient of FCS in 25 mmol/L glucose in the presence of various concentrations of mevinolin (0.1 nmol/L to 10 μmol/L); concentrations as low as 1 nmol/L inhibited chemotaxis (not shown). To investigate whether mevinolin was acting through the Ras or Rho family of proteins, geranylgeranylpoyrophosphate (10 μmol/L), the activated substrate for Rho geranylation, or farnesylpyrophosphate (10 μmol/L), required for Ras farnesylation, was placed in the chemotaxis chamber with mevinolin-treated cells; farnesylpyrophosphate, but not geranylgeranylpoyrophosphate, restored chemotaxis (Table 3). This suggests that under the conditions of these experiments, it is the interference of mevinolin with farnesylation of Ras that blocked activation of the ERK1/2 MAPK pathway and prevented chemotaxis. Furthermore, the inhibitor of FTI, in the absence of mevinolin, prevented chemotaxis to FCS in 25 mmol/L glucose (Table 3).

**Figure 2.** The effect of PI3K and MAPK pathway inhibitors on total protein level and activation of the downstream target of the MAPK pathway, ERK1/2. This figure shows a representative immunoblot of (A) total ERK1/2, (B) Tyr204-phosphorylated ERK1/2, and (C) phosphorylated ELK-1 (a measure of ERK1/2 activation) under conditions of 5 or 25 mmol/L glucose, with or without the addition of inhibitors. Graphs (D), (E), and (F) give results for the same measurements for 4 to 7 (n=4 to 7) independent experiments. The ability of isolated ERK1/2 to phosphorylate its substrate ELK1 under the indicated conditions was assessed using a MAPK assay (F). Inhibitors: LY (LY294002, 10 μmol/L), Rap (rapamycin, 1 nmol/L), PD9 (PD98059, 10 μmol/L), and Mev (mevinolin, 10 μmol/L).
Morphological Changes Associated With Glucose-Potentiated Chemotaxis Are Ras-Dependent

Confocal microscopy was used to assess the morphological alterations associated with Ras inhibition in 25 mmol/L glucose conditions. In the sample micrographs shown in Figure 4, a novel technique was used where the pre-etched coverslip from the chemotaxis experiment was gently removed after identifying the direction of the FCS gradient. Cells were stained with TRITC-phalloidin. The images have been rotated so that the FCS source (chemoattractant) is located at the top.

Figure 4A shows a cell in 5 mmol/L glucose in an FCS gradient. Lamellipodia are present without an obvious leading edge or filopodia (insert). In Figure 4B, a cell in 25 mmol/L glucose in FCS gradient shows that direction-sensing filopodia are present on the lamellipodia at the leading edge of the cell. In Figure 4C, a cell in 25 mmol/L glucose treated with mevinolin in a FCS gradient shows actin cables but lacks filopodia (insert) along the extensive lamellipodium, which is not restricted to the leading edge. In Figure 4D, a cell exposed to 25 mmol/L glucose in FCS gradient and treated with mevinolin and geranylgeranylpyrophosphate is shown; filopodia are absent along the lamellipodium. In Figure 4E, a cell exposed to 25 mmol/L glucose and treated with mevinolin and farnesylpyrophosphate in a FCS gradient is shown. The cell has a distinct leading edge in the direction of the chemoattractant (FCS), with filopodia (insert) along the lamellipodium. It would therefore appear that Ras farnesylation is a prerequisite for formation of filopodia in VSMC under these experimental conditions.

Discussion

The results clearly show that both the p110β-PI3K and MEK1/2 MAPK pathways are involved in glucose-potentiated VSMC chemotaxis to serum and that there is

### Table 3. Ras Farnesylation Is Necessary for Glucose-Potentiated Chemotaxis to FCS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Addition</th>
<th>Chemotaxis</th>
<th>Rayleigh Test P Value</th>
</tr>
</thead>
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<tr>
<td>10 μmol/L</td>
<td>None</td>
<td>No</td>
<td>0.205</td>
</tr>
<tr>
<td>10 μmol/L</td>
<td>Geranylgeranylpyrophosphate 10 μmol/L</td>
<td>No</td>
<td>0.190</td>
</tr>
<tr>
<td>10 μmol/L</td>
<td>Farnesylpyrophosphate 10 μmol/L</td>
<td>Yes</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Farnesyltransferase inhibitor 2 nmol/L</td>
<td>No</td>
<td>0.260</td>
<td></td>
</tr>
</tbody>
</table>

Chemotaxis experiments were performed in the presence of the Ras inhibitor mevinolin. Activated products of the cholesterol synthesis pathway farnesyl-pyrophosphate and geranylgeranylpyrophosphate were co-incubated with mevinolin to determine which small GTPases are involved in the control of chemotaxis in these experiments.

In the absence of mevinolin, the farnesyltransferase inhibitor (FTI; 2 nmol/L), prevented chemotaxis to FCS in 25 mmol/L glucose.
The expression of p110 required for glucose-potentiated VSMC chemotaxis to serum. To a few cell types, 5,6,8 has been found here for the first time. The p110 isoform of PI3K (but not p110α or p110β) was required for glucose-potentiated VSMC chemotaxis to serum. The expression of p110β, previously thought to be confined to a few cell types, 5,6,8 has been found here for the first time to our knowledge in human aortic VSMC. Our observation that p110β, but not p110α or p110δ, mediates glucose-potentiated VSMC chemotaxis to serum indicates that these isoforms are not functionally redundant, and it is confirmed by previous studies of the roles of the p110 isoforms. 6,8-10 However, although p110 isoforms play distinct roles, these vary with cell type and stimuli. For example, in porcine aortic endothelial cells, PDGF-stimulated actin reorganization is p110α-dependent, whereas insulin-induced actin reorganization is p110β-dependent.10

PI3K inhibitors also reduced glucose-induced phosphorylation of ERK1/2 and MAPK activity, showing that there was significant cross-talk between these 2 pathways. An important site of the cross-talk centered on Akt. This was shown in 2 sets of experiments: microinjection of Akt antibodies blocked chemotaxis, and microinjection of activated Akt into PI3K-inhibited cells resulted in phosphorylation of ERK. Conversely, inhibition of MEK1/2 did not affect Akt phosphorylation or activity.

Microinjection of antibodies to ERK1/2 prevented chemotaxis. Additionally, inhibitors of both MEK1/2 and PI3K resulted in reduced phosphorylation of ERK and reduced MAPK activity. Mevinolin, a member of the statin group of drugs that inhibits Ras, also inhibited chemotaxis and ERK1/2 phosphorylation, but not Akt phosphorylation. Similar observations were made with rapamycin, an mTOR inhibitor. Although mTOR is known to be PI3K-dependent, 28 it is not clear how mTOR relates to the PI3K-Akt pathway with respect to VSMC chemotaxis. The effect of rapamycin on VSMC migration has been shown, in part, to be p27Kip1-dependent. 29 However, the complete signaling pathway that links mTOR to VSMC migration is not yet fully delineated. 30 PI3K inhibitors and rapamycin inhibited ERK activation to a similar degree.

There have been conflicting reports in the literature of the importance of PI3K and MAPK in VSMC chemotaxis. Cospedal et al 31 found that PDGF-mediated chemotaxis of VSMC was dependent on MAPK but not PI3K. This was unexpected because PI3K has been implicated in cell migration in numerous cell types and with various stimuli, whereas MAPK has often been associated with proliferation but not chemotaxis. 31 A few studies have investigated both effects under the same experimental conditions. Nelson et al 32 found that when VSMC were stimulated with PDGF, MAPK-dependent migration occurred much more rapidly than did MAPK-dependent proliferation, suggesting distinct phases of enzyme activation for these different cellular phenomena, a point of view supported by other work showing a long lag phase for glucose effects on VSMC, such as thymidine uptake 33,34 or cell multiplication. 35 We also have found that VSMC replication does not significantly change until after 8 to 10 days in 25 mmol/L glucose, 35 and thymidine uptake in 5 or 25 mmol/L glucose conditions are similar over the course of 72 hours (unpublished results). Chemotaxis induced by thrombospondin-1 has also been found to be both PI3K-dependent 36 and MAPK-dependent. 37 It is of interest that in the other important cell of the vascular wall, the endothelial cell, glucose activates JNK but not ERK1/2 or p38, and it is associated with a reduction in endothelial cell proliferation. 38 As further evidence of the diversity of glucose-related chemotactic responses, it has been shown that neural crest cell migration in rat embryos is inhibited by elevated glucose concentrations. 39

The inhibitory effect of mevinolin on chemotaxis was of particular interest. Statin drugs are in widespread use for the
reduction of cholesterol levels; their primary action is to inhibit the rate-limiting enzyme in cholesterol synthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase). It has become clear that this group of drugs has additional effects that can be ascribed to the reduction of intermediates in the cholesterol synthesis pathway downstream of HMG CoA reductase,\textsuperscript{40–42} which are required for prenylation of small GTPase proteins. A significant proportion of subjects with diabetes have dyslipidemias that is treated with a drug of the statin group. It seems probable that in those patients, statins may help to prevent the development of atherosclerosis by a lipid-lowering action and by interruption of the signaling pathway involved in glucose-enhanced chemotaxis of VSMC from the tunica media to the subendothelial space of the arterial wall. In this study, the statin influence on chemotaxis could be detected at concentrations as low as 1 nmol/L and was mediated via Ras rather than via the Rho family of GTPases. This was deduced from the fact that addition of farnesylphosphate, required for the prenylation of Ras, and not geranylgeranylphosphate, required for prenylation of Rho A, restored chemotaxis in VSMC treated with mevinolin. Furthermore, the use of a farnesyltransferase inhibitor in the absence of a statin reduced ERK phosphorylation and prevented chemotaxis. By contrast, in neonatal rat cardiomyocytes, statins have been shown to downregulate Rac1 and Rho A.\textsuperscript{43} In addition to these mechanisms, a previous study\textsuperscript{40} suggested that statins may also inhibit chemotaxis through an associated inhibition of oxidative stress. Another area of variability in statin action relevant to the present study is the effects on the PI3K pathway. It is clear that mevinolin neither activated nor suppressed PI3K activity under the conditions of the present investigations. Different statins and conditions are associated with different activation profiles. Atorvastatin treatment during oxidative stress associated with reperfusion injury in mouse myocardium is associated with increased phosphorylation of Akt.\textsuperscript{44} Simvastatin treatment reduces PDGF-induced Akt phosphorylation in the aortas of low-density lipoprotein receptor-deficient mice subjected to coronary angioplasty.\textsuperscript{45} Pravastatin (100 nmol/L) has been shown to inhibit PI3K and phosphorylation of Akt in the A10 aortic VSMC cell line.\textsuperscript{46} We have observed a significant reduction in glucose-induced Akt phosphorylation in primary VSMC with concentrations of pravastatin as low as 10 nmol/L (ERK phosphorylation is reduced at 1 nmol/L), and pravastatin blocks glucose-potentiated chemotaxis to serum (unpublished). Thus, there are differences in the inhibitory action of statins on the PI3K pathway, even under the same experimental conditions of glucose-potentiated chemotaxis to serum. In conclusion, this study has shown that glucose-potentiated VSMC chemotaxis to serum involves the p110\(eta\) catalytic subunit of PI3K and the ERK1/2 MAPK pathway, and that mTOR and Ras are involved. The results reveal significant cross-talk between the PI3K and MAPK pathways, with PI3K/Akt having a major influence on the extent of activation within the ERK1/2 pathway in VSMC. Statins, a widely used group of drugs in diabetic subjects with dyslipidemias, block glucose-dependent chemotaxis of VSMC.

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References
15. Campbell M, Allen WE, Silversides JA, Trimble ER. Glucose-induced phosphorylation of Akt is associated with increased phosphorylation of Akt.\textsuperscript{44} Simvastatin treatment reduces PDGF-induced Akt phosphorylation in the aortas of low-density lipoprotein receptor-deficient mice subjected to coronary angioplasty.\textsuperscript{45} Pravastatin (100 nmol/L) has been shown to inhibit PI3K and phosphorylation of Akt in the A10 aortic VSMC cell line.\textsuperscript{46} We have observed a significant reduction in glucose-induced Akt phosphorylation in primary VSMC with concentrations of pravastatin as low as 10 nmol/L (ERK phosphorylation is decreased at 1 nmol/L), and pravastatin blocks glucose-potentiated chemotaxis to serum (unpublished). Thus, there are differences in the inhibitory action of statins on the PI3K pathway, even under the same experimental conditions of glucose-potentiated chemotaxis to serum.

In conclusion, this study has shown that glucose-potentiated VSMC chemotaxis to serum involves the p110\beta catalytic subunit of PI3K and the ERK1/2 MAPK pathway, and that mTOR and Ras are involved. The results reveal significant cross-talk between the PI3K and MAPK pathways, with PI3K/Akt having a major influence on the extent of activation within the ERK1/2 pathway in VSMC. Statins, a widely used group of drugs in diabetic subjects with dyslipidemias, block glucose-dependent chemotaxis of VSMC.
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Materials and Methods

Chemicals and Antibodies

The following chemicals were purchased: D-glucose and L-glucose (BDH, Poole, England); Wortmannin and LY294002 (PI3K inhibitors), rapamycin (mTOR inhibitor), mevinolin (lovastatin), geranylgeranylpyrophosphate and farnesylpyrophosphate (Sigma); PD98059 (MEK1/2 inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor) (Calbiochem, La Jolla, CA); the farnesyltransferase inhibitor H-D-Trp-D-Met-p-chloro-D-Phe-Gla-NH₂ (Bachem, St. Helens, UK); PDGF-β receptor antibody (R&D System, Minneapolis, MN); antibodies to p110β (sc-602), p85α, total and phosphorylated ERK, total and phosphorylated JNK (Santa Cruz Biotechnologies, CA); antibodies to total and Ser473-phosphorylated Akt/PKB (Cell Signaling Technology); antibodies to total and phosphorylated p38 MAPK (Calbiochem); HRP-conjugated anti-mouse or anti-rabbit IgG (DAKO, Ely, UK); rabbit or mouse IgG (Sigma). C-terminal-directed, affinity-purified antibodies to p110α, p110β and p110δ, used for microinjection and Western blotting, have been described elsewhere.\textsuperscript{1;2;3}

Cell Culture

Human VSMC were obtained from the infrarenal portion of the abdominal aorta of transplant donors with ethical approval as previously described.\textsuperscript{4} Cells were cultured in Dulbecco’s modified Eagle’s medium (GibcoBRL, Life Technologies, Paisley, UK) containing 5 mmol/L D-glucose (unless stated otherwise) and 10% fetal calf serum (FCS) and used in passages 4-7.

Microinjection

For microinjection, 2 x 10⁴ cells were seeded in 25-mm Petri dishes containing 18-mm square No. 3 glass coverslips (Chance-Propper, Smethwick, UK), which had been pre-etched with a
glass pen on the reverse side. All the cells within the marked area were microinjected 24 h after seeding. Anti-p110 isoform antibodies were injected at 1 mg/mL, and anti-Akt and anti-ERK1/2 antibodies at 0.1 mg/mL. Where indicated, antibodies were pre-incubated for 1 h at room temperature with an x30 molar excess of cognate peptide before microinjection.

**Activated Akt Microinjection Assay**

For microinjection, 2x10⁴ cells were seeded in 25-mm Petri dishes containing 13-mm diameter No. 1 glass coverslips (Chance-Propper, Smethwick, UK). Cells were incubated in either 5 mmol/L glucose or 25 mmol/L glucose with or without LY294002 (10 µmol/L) for 2 hours before being injected with 1 mg/mL non-specific rabbit IgG and 0.4 µg/mL activated Akt (Upstate Biotechnology, Ca) or rabbit IgG alone (control). The cells were then incubated for 8 hours before being fixed and permeabilised as previously described.⁵ Injected rabbit IgG was localised with anti-rabbit FITC-conjugated secondary antibody (1:400, Sigma). Phosphorylated ERK was localised by use of a phosphorylated ERK specific antibody (1:200, Santa Cruz) and an anti-mouse TRITC-conjugated secondary antibody (1:400, Sigma). Micrographs were obtained using a confocal laser-scanning microscope (Model Micro Radiance; Bio-Rad). Phosphorylated ERK expression was measured by image analysis of micrographs using the confocal software.

**Chemotaxis Assays**

Chemotaxis was assessed by direct observation and recordings in stable concentration gradients of 10% FCS using the Dunn chemotaxis chamber (Weber Scientific, Teddington, UK). The direction of migration of individual cells was measured in relation to the direction of the gradient, and the time course of the response followed, as previously described.²³ Cells were exposed to 25 mmol/L glucose and inhibitors (dissolved in DMSO) within the chamber in a darkened room: Several inhibitors were light sensitive.
**Immunofluorescence**

Cells were paraformaldehyde fixed and permeabilised as previously described. Actin filament localisation: cells were incubated with 0.1 mg/mL TRITC-labelled phalloidin (Sigma) for 60 minutes. Injected IgG was localised with species-specific FITC-conjugated secondary antibodies (1:400, Sigma). Micrographs were obtained using a confocal laser-scanning microscope (Model Micro Radiance; Bio-Rad).

**Immunoprecipitation and Western Blotting**

Immunoprecipitation and Western blotting of protein from cell lysate was carried out as previously described with the following modifications. For phosphorylated Akt detection, 1 µmol/L okadaic acid, 5 µmol/L fenvalerate and 5 µmol/L potassium bisperoxo (1,10-phenanthroline) oxovanadate (V) (all from Calbiochem) were added to the lysis buffer. Primary antibodies (1:500 dilution) were detected with HRP-conjugated secondary antibodies diluted 1:1,000, followed by enhanced chemiluminescence (Supersignal Ultra; Pierce & Warriner, Chester, UK). Analysis was by densitometry (model GS-670, Bio-Rad).

**Kinase Assays**

The MAP kinase and Akt kinase assay kits (Cell Signaling Technology) were used as described in the manufacturer’s instructions. Isolation of the activated (phosphorylated) protein kinase (ERK1/2 or Akt) from cell lysates, was followed by the addition of substrates for those proteins [ELK1 and Glycogen synthase kinase-3 (GSK3), respectively] in a kinase assay and Western blotting for phosphorylated product using phosphospecific antibodies. The PI3-Kinase activity assay Elisa kit (Echelon Bioscience Incorporation, Utah) was used as described in the manufacturer’s instructions.
Statistics

Chemotaxis was assessed using the Rayleigh test for unimodal clustering of directions; 
$p<0.01$ was chosen as the criterion for rejecting the null hypothesis of random directionality. 
When significant unimodal clustering occurred, the mean direction and its 95% confidence 
interval was calculated. Experimental data were analyzed by ANOVA and Student’s $t$ test 
for multiple comparisons between groups and expressed as mean±SEM. $^{2;3;5}$ A $p$ value of less 
than 0.05 was considered statistically significant.
### Results on line

**Online Fig. A**

<table>
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<th>Glucose</th>
<th>Chemotaxis</th>
<th>Rayleigh test of unimodal clustering p value</th>
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<tbody>
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#### Effect of glucose on chemotaxis in 10% FCS gradient

Chemotaxis assay result for VSMC grown in 5 mmol/L glucose and set up in 5 or 25 mmol/L glucose conditions in FCS gradient within the chamber without prior incubation or serum starvation. The circular histograms show the proportion of VSMC migrating in each direction, with the FCS source at the top. The direction of chemotaxis, if present, is indicated by an arrow, with the 95% confidence interval shown as a wedge. The Rayleigh test for unimodal clustering demonstrated that cells exposed to 5 mmol/L glucose did not show chemotaxis, while those exposed to 25 mmol/L glucose within the chamber showed chemotaxis. Cells incubated in 5 mmol/L D-glucose together with 20 mmol/L of the non-metabolizable analogue, L-glucose, showed no chemotaxis (results not shown); indicating that metabolism of glucose is required for glucose-potentiated chemotaxis. Number of cells, 30-42, from 3-5 independent experiments. The p value equals the Rayleigh test probability that chemotaxis has occurred.
Online Fig. B

(a) Class IA PI3Ks are heterodimers made up of a regulatory subunit, usually 85 kDa (p85), and a 110 kDa catalytic subunit (p110).

(b) Western blot showing the expression of p110α, -β and -δ in VSMC. Lanes marked VSMC are cell lysates, while those marked Rec. p110 are recombinant p110 protein controls.

p110α, p110β and p110δ PI3K isoforms are expressed in human VSMC
The dose response in ERK phosphorylation when VSMC are exposed to mevinolin

The graph shows the results from n=3 independent experiments when VSMC were exposed for 24 h to concentrations of mevinolin ranging from 0.1–10,000 nmol/L. The level of ERK phosphorylation was assessed by immunoprecipitation and measured by densitometry. The results are shown as percentage change relative to the uninhibited control. There was a significant decrease in ERK phosphorylation for all concentrations above 0.1 nmol/L as measured by Student’s t test. p<0.05 at 1 nmol/L vs. uninhibited.
The effect of Farnesyltransferase inhibition on ERK phosphorylation
The graph shows the densitometry results from n=4-6 independent immunoprecipitation experiments, where the effect of glucose and the FTI inhibitor H-D-Trp-D-Met-p-chloro-D-Phe-Gla-NH₂ (2 nmol/L) for 24 h on the phosphorylation of ERK was assessed.
The effect of PI3K inhibitors on PI3K activity in 25 mmol/L glucose conditions
The graph shows results from n=5-6 independent experiments using a PI3K activity kinase (Elisa) assay for whole cell lysates in 5 mmol/L glucose or exposed to 25 mmol/L glucose conditions in the presence or absence of PI3K inhibitors. Inhibitors: LY2 (LY294002 10 µmol/L) and Wort (wortmannin 10 nmol/L).

Online References


