High glucose inhibits insulin-stimulated nitric oxide production without reducing eNOS Ser1177 phosphorylation in human aortic endothelial cells

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SUMMARY:

Recent studies have indicated that insulin activates eNOS by PKB-mediated phosphorylation at Ser1177 in endothelial cells. As hyperglycaemia contributes to endothelial dysfunction and decreased NO availability in types 1 and 2 diabetes mellitus, we have studied the effects of high glucose (25 mM, 48 h) on insulin signalling pathways that regulate NO production in human aortic endothelial cells. High glucose inhibited insulin-stimulated NO synthesis but was without effect on NO synthesis stimulated by increasing intracellular Ca^{2+} concentration. This was accompanied by reduced expression of IRS-2 and attenuated insulin-stimulated recruitment of PI3K to IRS-1 and IRS-2 yet insulin-stimulated PKB activity and phosphorylation of eNOS at Ser1177 were unaffected. Inhibition of insulin-stimulated NO synthesis by high glucose was unaffected by an inhibitor of PKC. Furthermore, high glucose downregulated the expression of CAP and Cbl, and insulin-stimulated Cbl phosphorylation, components of an insulin signalling cascade previously characterised in adipocytes. These data suggest high glucose specifically inhibits insulin-stimulated NO synthesis and downregulates some aspects of insulin signalling, including the CAP-Cbl signalling pathway, yet this is not a result of reduced PKB-mediated eNOS phosphorylation at Ser1177. Therefore, we propose that phosphorylation of eNOS at Ser1177 is not sufficient to stimulate NO production in cells cultured at 25 mM glucose.
INTRODUCTION:

Endothelium-derived nitric oxide (NO), produced by endothelial nitric oxide synthase (eNOS), promotes vasodilation and inhibits platelet aggregation, leukocyte adherence and vascular smooth muscle proliferation (1). Decreased NO bioavailability is well-described in patients with type 1 diabetes (2,3), type 2 diabetes (4,5) and also in cardiovascular disorders such as hypertension and atherosclerosis associated with insulin resistance (6,7).

Insulin is a direct-acting vasodilator in intact vessels (8,9) and has been demonstrated to stimulate wortmannin-sensitive NO production in cultured endothelial cells (10). Recently, several laboratories have demonstrated activation of eNOS by phosphorylation at Ser1177 (human sequence) in response to shear stress, VEGF, IGF-I and insulin (11-15). Phosphorylation at this site is accompanied by a decrease in the dependence of eNOS for Ca\(^{2+}\)/CaM (12,16). Incubation of bovine aortic endothelial cells (BAECs) with insulin has been demonstrated to result in the rapid stimulation of insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation, IRS-1-associated Phosphatidylinositol-3’-kinase (PI3K) activity, phosphorylation and activation of protein kinase B (PKB), eNOS phosphorylation at the equivalent bovine site to Ser1177 and NO production (15). It is proposed that insulin stimulates PKB-mediated phosphorylation and activation of eNOS via PI3K recruitment to IRS-1 (15,16).

Hyperglycaemia contributes to endothelial dysfunction and decreased NO availability in types 1 and 2 diabetes mellitus, but the mechanisms remain uncharacterised. It has been proposed that high glucose inhibits basal eNOS expression, activity and NO production in cultured endothelial cells (17,18). However other studies have demonstrated the opposite effects of experimental hyperglycaemia on NO production in human aortic endothelial cells (HAECs) (19). Additionally, a recent report has suggested that acute (15-30 min) stimulation
with high glucose inhibits insulin-stimulated NO production as a result of reduced eNOS serine phosphorylation in human umbilical vein endothelial cells (HUVECs) (20).

The proposed pathway of insulin-stimulated NO synthesis exhibits striking similarities with the characterised mechanism of insulin-stimulated glucose transport in adipocytes. PI3K stimulation is necessary, but not sufficient for insulin-stimulated glucose transport in adipocytes (21). Similarly, it has been proposed that PKB stimulation is necessary, but not sufficient for insulin-stimulated NO synthesis in endothelial cells (16). Recent studies in adipocytes have demonstrated a novel insulin-signalling pathway required in parallel with PI3K activation to stimulate glucose transport (22,23). In this pathway, Cbl is recruited to the insulin receptor (IR) by interaction with the adapter protein CAP. Upon insulin stimulation Cbl becomes tyrosine phosphorylated and the CAP-Cbl complex dissociates from the IR and moves to a caveolin- and flotillin-rich lipid raft subdomain of the plasma membrane (22). Phosphorylated Cbl recruits the CrkII-C3G complex to lipid rafts where C3G specifically activates the small G-protein TC10 (23).

In the present study, we investigated the effects of prolonged (48 h) experimental hyperglycaemia on insulin-stimulated NO production in HAECs. Our results suggest that insulin-stimulated NO production is specifically abolished by culture in high glucose. Importantly, we found that there was no reduction in PKB activity, total phosphorylation of eNOS or specific phosphorylation of eNOS at Ser1177 in response to insulin that could account for this inhibition. Therefore, insulin-stimulated phosphorylation of eNOS at Ser1177 is not sufficient to stimulate NO production in endothelial cells cultured in 25 mM glucose. We also demonstrate that HAECs express CAP, Cbl, C3G and flotillin. Culture of HAECs in high glucose concentrations resulted in reduced expression of CAP and Cbl, and inhibited insulin-stimulated Cbl phosphorylation. We propose that the CAP-Cbl pathway
may represent a candidate parallel pathway required in addition to PKB activation for insulin-stimulated NO synthesis.
EXPERIMENTAL PROCEDURES:

Materials

Cryopreserved HAECs and cell culture media were obtained from TCS Cellworks (Bucks, UK). Rabbit anti-eNOS antibody were supplied by Sigma (Poole, Dorset, UK). L-NMMA was from Alexis (San Diego, CA, USA). Insulin (Actrapid) was from Novo-Nordisk (Copenhagen, Denmark). A23187, bisindolylmaleimide I and mouse anti-eNOS antibodies were obtained from Calbiochem (San Diego, CA, USA). Sheep isoform-specific anti-PKB antibodies, rabbit anti-IRS-1, anti-IRS-2, anti-PI3K (p85 subunit), anti-PDK-1, anti-PTEN, anti-phospho-Thr495eNOS and anti-CAP antibodies were from Upstate Biotechnology (Lake Placid, NY, USA). Anti-caveolin-1 antibodies were from Becton Dickinson (Oxford, UK). Anti-flotillin antibodies were from Transduction Laboratories (San Diego, CA). Rabbit isoform-specific anti-PI3K (p110 subunit), anti-C3G and anti-Cbl antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). PKB substrate peptide (RPRAATF) was kindly supplied by Dr. R. Plevin, University of Strathclyde, UK. Sheep anti-phospho-Ser1177eNOS antibody was a generous gift from Prof. D. G. Hardie, University of Dundee, UK. All other reagents were from sources described previously (24).

HAEC cell culture

HAECs were grown in large vessel endothelial cell medium at 37°C in 5% CO₂ and passaged when at 80% confluence. Prior to use cells were maintained in medium containing 5 mM or 25 mM glucose for 48 hours. During this period, culture media containing 5 mM glucose was supplemented with 20 mM mannitol to maintain osmolarity. Cells were used for experiments between passages 3 and 6.

Nitric Oxide Release
Cells cultured in 6-well plates in media containing either glucose concentration were preincubated for 1 h at 37°C in 2 ml/well Krebs Ringer HEPES (KRH) buffer (119 mM NaCl, 20 mM Na HEPES, pH 7.4, 5 mM NaHCO₃, 4.7 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 1 mM KH₂PO₄, 100 µM L-arginine, 5 or 25 mM glucose) at 37°C. The media was removed and replaced with 1 ml fresh KRH buffer in the presence or absence of insulin or A23187. Aliquots (100 µl) of the medium were removed after various incubation times and refluxed in glacial acetic acid containing NaI. Under these conditions, NO₂⁻, a stable breakdown product of NO, is quantitatively reduced to NO. NO-specific chemiluminescence was then analysed using a Sievers 280A NO meter. The appropriate control experiments performed in the presence of the eNOS inhibitor, L-NMMA. NO production (adjusted for L-NMMA-insensitive production) is expressed per mg of protein.

Preparation of HAEC lysates

Cells cultured in 10 cm diameter dishes in media containing either glucose concentration were preincubated for 1 h at 37°C in 5 ml KRH buffer containing 5 or 25 mM glucose. The medium was replaced with 5 ml KRH buffer containing test substances and incubated for various durations at 37°C. The medium was removed and 0.5 ml buffer A (50 mM Tris-HCl, pH 7.4 at 4°C, 150 mM NaCl, 50 mM NaF, 5 mM Na pyrophosphate, 2 mM Na orthovanadate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM benzamidine, 0.1 mM PMSF, 5 µg/ml SBTI, 1% (v/v) Triton X-100, 1% (v/v) glycerol) added. The cell extract was scraped off and transferred to a microcentrifuge tube. Extracts were vortex mixed and centrifuged (14,000 x g, 3 min, 4°C). Supernatants were snap-frozen in liquid N₂ and stored at -80°C prior to use.

³²P labelling of HAECs
Cells cultured in 10 cm diameter dishes in media containing either glucose concentration were preincubated for 1 h at 37°C in 5 ml phosphate-free KRH buffer containing 0.2 mCi/ml $^{32}$PO$_4^-$ and 5 or 25 mM glucose. After incubation in the presence or absence of insulin (100 nM) for 5 minutes, the medium was removed and cell lysates prepared.

**Immunoprecipitation of IRS-1 and IRS-2**

HAEC lysate (50 µg) was added to 20 µl 25% (v/v) Protein A-Sepharose pre-bound to 2.5 µg rabbit anti-IRS-1 or anti-IRS-2 antibodies, the volume made up to 300 µl with buffer A and mixed for 2 h at 4°C on a rotating mixer. The mixture was then centrifuged (14,000 x g, 30 s, 4°C), and the pellet washed three times with 1 ml buffer A at 4°C. Immunoprecipitated protein was separated by SDS-PAGE and transferred to nitrocellulose.

**Immunoprecipitation and assay of PKB**

HAEC lysate (50 µg) was added to 20 µl 25% (v/v) Protein G-Sepharose pre-bound to 5 µg sheep anti-PKB antibody, the volume made up to 300 µl with buffer A and mixed for 2 h at 4°C on a rotating mixer. The mixture was then centrifuged (14,000 x g, 30 s, 4°C), and the pellet washed and assayed as described previously by incorporation of $^{32}$P into the RPRAATF substrate peptide (24).

**Immunoprecipitation of $^{32}$P-labelled Cbl**

HAEC lysate (300 µg) from $^{32}$P-labelled cells was added to 20 µl 25% (v/v) Protein A-Sepharose pre-bound to 2µg rabbit anti-Cbl, the volume made up to 300 µl with buffer A and mixed for 2 h at 4°C on a rotating mixer. The mixture was then centrifuged (14,000 x g, 30 s, 4°C), and the pellet washed three times with 1 ml buffer A supplemented with 0.5 M NaCl, and three times with 1 ml buffer A at 4°C. Immunoprecipitated protein was separated
by SDS-PAGE, the resultant gel dried and $^{32}$P-labelled proteins visualised by autoradiography.

*Immunoprecipitation of $^{32}$P-labelled eNOS*

HAEC lysate (200 µg) from $^{32}$P-labelled cells was added to 20 µl 25% (v/v) Protein G-Sepharose pre-bound to 5µg mouse anti-eNOS, the volume made up to 300 µl with buffer A and mixed for 2 h at 4°C on a rotating mixer. The mixture was then centrifuged (14,000 x g, 30 s, 4°C), and the pellet washed three times with 1 ml buffer A supplemented with 0.5 M NaCl, and three times with 1 ml buffer A at 4°C. Immunoprecipitated protein was separated by SDS-PAGE, the resultant gel dried and $^{32}$P-labelled proteins visualised by phosphorimage analysis using a Fuji FLA-5000 analyser.

*Statistics*

Unless stated otherwise, results are expressed as means ± SD. Statistically significant differences were determined using a 2-tailed independent-samples Student's t-test, using $p<0.05$ as significant using Statview software.
RESULTS:

To study the effects of experimental hyperglycaemia on insulin-stimulated NO production in HAECs, we examined the ability of insulin to stimulate NO synthesis in HAECs cultured for 48 h in 5 mM or 25 mM glucose using a Sievers 280A NO meter (Fig. 1A). As shown, insulin (100 nM) rapidly stimulated NO synthesis in HAECs cultured in 5 mM glucose. Insulin-stimulated NO production could be demonstrated at concentrations as low as 5 nM and was maximal at 100 nM (not shown), a concentration at which all further experiments were performed. In contrast, insulin had no significant effect on NO synthesis in HAECs cultured in 25 mM glucose. There was no significant difference in basal NO synthesis at either glucose concentration. In order to investigate whether this inhibitory effect of hyperglycaemia was restricted to insulin-stimulated NO synthesis, we next examined the effect of culture glucose concentration on NO synthesis stimulated by the Ca$^{2+}$ ionophore, A23187 (Fig. 1B). As shown, A23187 rapidly stimulated NO synthesis in HAECs cultured in either 5 mM or 25 mM glucose. There was no significant difference between A23187-stimulated NO production at either glucose culture concentration.

In an attempt to identify differences between HAECs cultured in either glucose concentration that could account for the observed inhibition of insulin-stimulated NO production, we characterised the expression of proteins involved in insulin signalling in HAECs cultured in either glucose concentration. Quantitative analysis of western blots illustrated that there was no significant difference in the expression of IRS-1 (Fig. 2), PI3K (p85 and p110β subunits), PTEN, PDK-1, PKBα or eNOS in HAECs cultured in either 5 mM or 25 mM glucose (data not shown). However, expression of IRS-2 in cells cultured in 25 mM glucose was reduced to 29 ± 19% (n=6, p<0.01) of that in HAECs cultured in 5 mM glucose (Fig. 2). No expression of the p110α/γ subunit isoforms of PI3K or PKBβ protein could be demonstrated in HAECs.
To determine whether there were any differences in the activity of the previously characterised insulin signalling pathway in HAECs cultured in 5 or 25 mM glucose, we characterised the recruitment of PI3K to IRSs in HAECs cultured in either glucose concentration under basal and insulin-stimulated conditions. Insulin-stimulated recruitment of PI3K to IRS-1 is reduced by 24 ± 7 % (n=6, p<0.01) in HAECs cultured in 25 mM glucose, compared to those cultured in 5 mM glucose (Fig. 3). Insulin also stimulated the recruitment of PI3K to IRS-2 in cells cultured in 5 mM glucose, but could not be demonstrated in cells cultured in 25 mM glucose (Fig. 3A). We next characterised the effects of insulin on PKB activity in HAECs cultured in either glucose concentration. Stimulation of HAECs with insulin resulted in the time-dependent activation of PKBα, maximal by 10 min (not shown). Insulin-stimulated PKB activity was maximal at 100 nM, and could be demonstrated at concentrations as low as 1 nM (data not shown). PKBβ activity could not be detected in HAECs by immunoprecipitation kinase assays with a PKBβ-specific antibody. Culture of HAECs at 25 mM glucose had no significant effect on total insulin-stimulated PKBα activity, compared to HAECs cultured in 5 mM glucose, although basal PKB activity was significantly increased (2.4-fold, p<0.01) (Fig. 4).

To determine whether phosphorylation of eNOS was altered by culture glucose concentration we determined the total phosphorylation of immunoprecipititates from 32P-labelled HAECs. Insulin stimulated eNOS phosphorylation in cells cultured in either 5 mM or 25 mM glucose (1.6-fold, p<0.05 and 1.4-fold, p<0.05 respectively, n=3), (Fig. 5). There was no significant difference in basal or insulin-stimulated eNOS phosphorylation at either glucose concentration. To determine whether insulin stimulated tyrosine phosphorylation of eNOS, we used a phosphotyrosine antibody on western blots of eNOS immunoprecipitates from HAEC lysates. No anti-phosphotyrosine-reactive species of the correct molecular mass was detected in lysates prepared from cells cultured in either glucose concentration in the
presence or absence of insulin (not shown). To determine whether phosphorylation of eNOS at Ser1177 was altered by culture glucose concentration we used a phospho-Ser1177-specific antibody. Insulin stimulated phosphorylation of eNOS at Ser1177 in HAECs cultured in either 5 mM or 25 mM glucose (1.6-fold, p<0.05 and 2.4-fold, p<0.01 respectively, n=6), (Fig. 6). There was no significant difference in basal or insulin-stimulated Ser1177 phosphorylation at either glucose concentration.

Activation of protein kinase C (PKC) has been demonstrated in vascular cells exposed to elevated glucose (25). Furthermore, it has been proposed that activation of PKBβ1 inhibits insulin-stimulated eNOS expression in BAECs (26) and that phosphorylation of eNOS at Thr495 by PKC inhibits activity (27). We therefore characterised the expression of PKC isoforms and phosphorylation of eNOS at Thr495 in HAECs cultured in either glucose concentration. Phosphorylation of eNOS at Thr495 was unaltered by medium glucose concentration in HAECs as judged by quantitative analysis of western blots using a phospho-Thr495-specific anti-eNOS antibody (not shown). We next determined whether inhibition of PKC with bisindolylmaleimide I would reverse the inhibition of insulin-stimulated NO production in HAECs cultured in 25 mM glucose. Preincubation of cells for 1 hour with bisindolylmaleimide I had no significant effect on basal or insulin-stimulated NO production in cells cultured in 5 mM glucose (Fig. 7). Additionally, bisindolylmaleimide I had no effect on the inhibition of insulin-stimulated NO production by culture in 25 mM glucose (Fig. 7).

Recent studies in adipocytes have illustrated a novel insulin-signalling pathway that localises to caveolin and flotillin-rich lipid raft domains and is required for insulin-stimulated glucose transport, in parallel with PI3K activation (22,23). We therefore determined whether proteins involved in this pathway were expressed in HAECs cultured at 5 or 25 mM glucose. There was no significant difference in the expression of C3G, caveolin or flotillin in HAECs cultured in either 5 mM or 25 mM glucose (Fig. 8). However, expression of CAP and Cbl in
cells cultured in 25 mM glucose was reduced to 54 ± 12% (n=9, p<0.05) and 60 ± 9% (n=9, p<0.01) respectively of that in HAECs cultured in 5 mM glucose (Fig. 8). Next we determined the phosphorylation of Cbl in response to insulin in HAECs cultured in either glucose concentration. Insulin stimulated Cbl phosphorylation in cells cultured in 5 mM glucose (1.5-fold, n=3, p<0.01) but was ineffective in cells cultured in 25 mM glucose (Fig. 9).
DISCUSSION:

Abnormal vascular endothelial function, as defined by measures of NO bioavailability, is well described in patients with types 1 and 2 diabetes and in cardiovascular disorders that are associated with insulin resistance including hypertension and atherosclerosis (6,7,28-30). Although endothelial dysfunction may occur in association with insulin resistance even in normoglycaemic subjects (31), hyperglycaemia is an obvious candidate contributor. This prompted us to determine the effects of experimental hyperglycaemia on insulin-stimulated NO production in HAECs.

Culture of HAECs in 22.2 mM glucose for 5 days was previously reported to stimulate basal eNOS expression and NO production (19). In contrast, other groups have demonstrated reduced basal NO production in response to high glucose, with a concomitant reduction in eNOS expression in human coronary artery endothelial cells and BAECs (17,18). In the present study we could not demonstrate any effect of culture glucose concentration on eNOS expression in HAECs. These differences may be due to the different endothelial cell types used or the varied duration of experimental hyperglycaemia applied.

In this study, we have demonstrated that insulin-stimulated NO production was abolished in HAECs cultured in 25 mmol/l glucose, yet stimulation of eNOS by the Ca^{2+} ionophore, A23187 was unaffected by culture glucose concentration, which suggests the inhibitory effect of experimental hyperglycaemia is specific to insulin stimulation and that eNOS is still capable of being activated by increased intracellular Ca^{2+}. We therefore hypothesized that this inhibition of insulin-stimulated NO production was a consequence of reduced expression or activity of insulin signalling proteins. It is proposed that insulin stimulates PKB-mediated phosphorylation and activation of eNOS via PI3K recruitment to IRS-1 (15,16). In this study, we find that in cells cultured in 5 mM glucose, the time course and dose-dependence of insulin-stimulated NO production in HAECs is comparable to that
previously reported in HUVECs and BAECs (10,15). We have also shown that insulin stimulates PI3K recruitment to IRS-1, PKB activity and NO phosphorylation on Ser1177 as previously reported in BAECs (15). Our data also represents, to our knowledge, the first demonstration of insulin-stimulated PI3K recruitment to IRS-2 in endothelial cells. The importance of IRS-2 in endothelial cell insulin signalling has yet to be determined, although studies in knockout mice have demonstrated that IRS-2 appears to mediate whole body insulin action, whereas IRS-1 is of importance primarily in muscle (32).

The reduction in insulin-stimulated PI3K recruitment to IRS-1 mediated by experimental hyperglycaemia is consistent with recent data in HUVECs (21), and not a result of decreased IRS-1 or PI3K expression. The total inhibition of PI3K recruitment to IRS-2 observed in this study is likely to be a result of the markedly reduced expression of IRS-2. This reduction of PI3K recruitment did not, however, result in reduced insulin-stimulated PKB activity in HAECs subjected to high glucose. Although this may seem surprising, it has recently been demonstrated that PKB activation is normal in insulin-resistant mice with impaired insulin-stimulated IRS-1 phosphorylation and PI3K activity (33). There are several potential explanations for the seemingly divergent effects of elevated culture glucose concentration on insulin stimulated PI3K recruitment to IRSs and PKB activity. Firstly, only partial activation of PI3K may be necessary for full stimulation of PKB. Secondly, changes in total IRS-associated PI3K activity and PKB activity may not reflect changes within specific membrane compartments. Thirdly, a PI3K-independent mechanism may be able to offset the decrease in recruitment of PI3K to IRSs.

In contrast to our study, a recent report has demonstrated that basal eNOS activity and phosphorylation at Ser1177 were reduced in BAECs cultured in 30 mM glucose for 48 h (18). Du and co-workers interpreted their findings as demonstrating increased O-linked N-acetylglucosamine modification of eNOS (18). Additionally, inhibition of insulin-stimulated
eNOS serine phosphorylation by acute exposure of HUVECs to high glucose concentration has recently been reported (20). In the present study, chronic exposure of HAECs to 25 mM glucose had no effect on either insulin-stimulated total eNOS phosphorylation or specific Ser1177 phosphorylation. These contrasting effects are likely to reflect the very different durations of experimental hyperglycaemia applied, or the different sources of endothelial cells used. It should also be noted that Schnyder and co-workers used an anti-phosphoserine antibody, rather than a phospho-Ser1177-specific antibody in their study (20); thus, the hyperglycaemia-induced reduction in insulin-stimulated eNOS serine phosphorylation that they reported may represent phosphorylation at sites other than Ser1177.

These data suggest that there is an as yet uncharacterised pathway that regulates eNOS, at least in high glucose concentrations. This may be an alternative inhibitory phosphorylation site on eNOS that is activated under hyperglycaemic conditions and specifically interferes with activation mediated by Ser1177 phosphorylation, yet does not inhibit eNOS activation by Ca\(^{2+}/\)CaM. In the current study, culture glucose concentration had no effect on total eNOS phosphorylation, arguing against the mediation of inhibition of insulin-stimulated NO production by an alternative phosphorylation site. However, these data do not rule out changes in the extent of eNOS phosphorylation at sites other than Ser1177 that result in no alteration in the total extent of eNOS phosphorylation. It has been demonstrated that hyperglycaemia stimulates PKC activity (25) and proposed that phosphorylation of eNOS at Thr495 by PKC inhibits activity (27). However, the observed inhibition of insulin-stimulated NO synthesis was not affected by the PKC inhibitor, bisindolylmaleimide I, and was not due to increased phosphorylation of Thr495, which is unaltered by medium glucose concentration in this study. These data indicate that neither PKC activation nor inhibitory phosphorylation of eNOS at Thr495 mediate inhibition of insulin-stimulated NO production by high culture glucose concentration.
Alternatively, an alternative insulin signalling pathway, which is impaired in hyperglycaemic conditions, may be required for eNOS activation in parallel with PKB activation. The proposed pathway of insulin-stimulated NO synthesis exhibits striking similarities with the mechanism of insulin-stimulated glucose transport in adipocytes. PI3K stimulation is necessary, but not sufficient for insulin-stimulated glucose transport in adipocytes (21). Similarly, it has been proposed that PKB stimulation is necessary, but not sufficient for insulin-stimulated NO synthesis in endothelial cells (16). Studies in adipocytes have demonstrated a novel insulin-signalling pathway that localises to flotillin-rich lipid raft domains in the plasma membrane, required in parallel with PI3K stimulation for insulin-stimulated glucose transport (22,23). We therefore reasoned that this novel insulin-signalling pathway characterised in adipocytes might be activated by insulin in parallel with PKB activation in endothelial cells, and impinge on insulin-stimulated eNOS activity. We demonstrate that flotillin, CAP, Cbl and C3G, components of this pathway are present in endothelial cells. Intriguingly, culture in elevated glucose concentrations markedly reduced the expression of CAP and Cbl and insulin-stimulated Cbl phosphorylation, required for the recruitment of the CrkII-C3G complex to lipid rafts in adipocytes (23). The regulation of CAP and Cbl expression is largely uncharacterised. Both CAP gene expression and Cbl tyrosine phosphorylation have been demonstrated to be stimulated by the thiazolidinedione insulin-sensitizing drugs in adipocytes (34). The regulation of these genes by culture glucose concentration is a novel finding which requires further study in other tissues such as adipocytes.

In summary, we have shown that culture in high glucose quantitatively inhibits insulin-stimulated NO production in HAECs. This is not due to a general reduced responsiveness of eNOS, as A23187-stimulated NO production is unaffected. Importantly, however, there is no effect of high glucose on insulin-stimulated PKB activation or eNOS
phosphorylation at Ser1177. We therefore propose that PKB-stimulated phosphorylation of eNOS at Ser1177 is not sufficient to stimulate NO synthesis in cells exposed to high glucose for 48 hr. Moreover, the CAP-Cbl insulin signalling pathway is downregulated by high glucose in these cells. The role of the CAP-Cbl pathway in insulin signalling in endothelial cells is unclear, but provides a candidate pathway required for insulin-stimulated NO production in parallel with PKB activation, as is the case in insulin-stimulated glucose transport in adipocytes. Future experiments are required to characterise the role of the CAP-Cbl pathway in insulin signalling and insulin-stimulated NO production in HAECs, to help understand the mechanism of hyperglycaemia-induced endothelial dysfunction.
REFERENCES:


Footnotes

The abbreviations used are: NO, nitric oxide; eNOS, endothelial nitric oxide synthase; PKB, protein kinase B; BAECs, bovine aortic endothelial cells; CaM, calmodulin; IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3′-kinase; HAECs, human aortic endothelial cells; HUVECs, human umbilical vein endothelial cells; IR, insulin receptor; DTT, dithiothreitol; L-NMMA, \( \text{N}^{\text{G}} \)-monomethyl-L-arginine; PMSF, phenylmethylsulphonyl fluoride; SBTI, soybean trypsin inhibitor.

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FIGURE LEGENDS:

Fig 1. Effect of culture glucose concentration on insulin and A23187-stimulated NO synthesis in HAECs

Cells were cultured in medium containing 5 or 25 mM glucose for 48 h. Prior to NO measurement, the medium was removed and replaced with KRH buffer containing 5 (square symbols) or 25 mM glucose (circular symbols) in the presence or absence of 0.1 mM L-NMMA. A) Aliquots of media were removed and assayed for NO$_2$ content after incubation in the presence (filled symbols) or absence (open symbols) of insulin (100 nM) for various durations as described in methods. B) Aliquots of media were removed and assayed for NO$_2$ content after incubation in the presence (filled symbols) or absence (open symbols) of A23187 (3 µM) for various durations as described in methods. The data shown represents the mean ± SD L-NMMA-sensitive NO synthesis from five independent experiments.

Fig 2. Effects of culture glucose concentration on expression of IRS isoforms.

HAEC lysates were prepared from cells cultured for 48 h in medium containing 5 or 25 mM glucose. A) Lysate proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with the antibodies indicated. A western blot is shown of three independent samples. B) Densitometric analysis of western blots reveals no significant difference of IRS-1 expression, but IRS-2 expression (*) was reduced to 29 ± 19%, n=6, p<0.01 of that in HAEC lysates cultured in 5 mM glucose.

Fig 3. Effects of culture glucose concentration on insulin-stimulated recruitment of PI3K to IRSs.
HAEC lysates were prepared from cells cultured for 48h in medium containing 5 or 25 mM glucose. A) IRS-1 or IRS-2 were immunoprecipitated from HAEC lysates prepared after incubation with 100 nM insulin for 5 minutes. The immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose and probed with rabbit anti-PI3K (p85 subunit) antibodies. A representative western blot is shown of three independent samples. B) Quantification of PI3K recruitment to IRS-1 revealed that insulin stimulated PI3K recruitment to IRS-1 in cells cultured in 5 or 25 mM glucose (2.4-fold, n=6, p<0.01 and 1.4-fold, n=6, p<0.01 respectively). Insulin-stimulated PI3K recruitment to IRS-1 was reduced in cells cultured in 25 mM glucose to 71 ± 19% (n=6, p<0.01 compared to 5 mM glucose + insulin).

Fig 4. Effects of culture glucose concentration on insulin-stimulated PKBα activity

HAEC lysates were prepared from cells cultured for 48h in medium containing 5 or 25 mM glucose. PKBα was immunoprecipitated from HAEC lysates prepared from cells incubated in the presence (hatched bars) or absence (filled bars) of 100 nM insulin for 5 minutes. PKBα activity was assayed as described in methods. The results shown are the mean ± SD from three independent experiments. Insulin-stimulated PKB activity was not significantly different after culture in either glucose concentration. Basal PKB activity was increased in cells cultured in 25 mM glucose by 2.4-fold (p<0.05 compared to 5 mM glucose).

Fig 9. Effects of culture glucose concentration on insulin-stimulated eNOS phosphorylation.

HAEC lysates were prepared from cells cultured for 48h in medium containing 5 or 25 mM glucose prior to incubation in media containing 0.2 mCi/ml $^{32}$PO$_4^{3-}$ and subsequent
incubation with insulin (100 nM) for 5 min. eNOS was immunoprecipitated from HAEC lysates, the immunoprecipitated proteins were resolved by SDS PAGE and subjected to western blotting with anti-eNOS antibodies or phosphorimage analysis. A) A representative western blot and autoradiograph is shown. B) Quantification of eNOS phosphorylation. Insulin stimulated eNOS phosphorylation in cells cultured in 5 or 25 mM glucose (1.6-fold, p<0.05 and 1.4-fold, p<0.05 respectively, n=3). There were no significant differences between basal eNOS phosphorylation at either glucose concentration. Similarly there were no significant differences between insulin-stimulated eNOS phosphorylation at either glucose concentration.

Fig 6. Effects of culture glucose concentration on insulin-stimulated eNOS phosphorylation at Ser1177.

HAEC lysates were prepared from cells cultured for 48h in medium containing 5 or 25 mM glucose and incubated with insulin (100 nM) for 5 min. 10 µg of protein were resolved by SDS PAGE, transferred to nitrocellulose and probed with sheep anti-phospho-Ser1177 antibodies. A) A representative western blot is shown of three independent samples. B) Quantification of Ser1177 phosphorylation. Insulin stimulated Ser1177 phosphorylation in cells cultured in 5 or 25 mM glucose (1.6-fold, p<0.05 and 2.4-fold, p<0.01 respectively, n=6). There were no significant differences between basal Ser1177 phosphorylation at either glucose concentration. Similarly there were no significant differences between insulin-stimulated Ser1177 phosphorylation at either glucose concentration.

Fig 7. Effects of PKC inhibition on insulin-stimulated NO production

Cells were cultured in medium containing 5 or 25 mM glucose for 48 h. Prior to NO measurement, the medium was removed and cells preincubated in KRH buffer containing 5
(open bars) or 25 mM glucose (filled bars) in the presence or absence of 0.1 mM L-NMMA and 0.1 µM bisindolylmaleimide I for 1 h. Media was removed and replaced with fresh KRH, aliquots of media were removed and assayed for NO₂⁻ content after incubation in the presence or absence of insulin (100 nM) or bisindolylmaleimide I (100 nM) for 5 minutes. The data shown represents the mean ± SEM L-NMMA-sensitive NO synthesis from six independent experiments.

**Fig 8. Expression of CAP/Cbl signalling proteins in HAECs cultured in 5 or 25 mM glucose**

HAEC lysates were prepared from cells cultured for 48hr in medium containing 5 or 25 mM glucose. Lysate proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with the antibodies indicated. A western blot is shown of three independent samples. There was no significant difference in the expression of the proteins examined, except for CAP expression (*), reduced to 54 ± 12%, n=6, p<0.05 of that in HAEC lysates cultured in 5 mM glucose and Cbl expression (†), reduced to 60 ± 9%, n=6, p<0.01 of that in HAEC lysates cultured in 5 mmol/l glucose.

**Fig 9. Effects of culture glucose concentration on insulin-stimulated Cbl phosphorylation.**

HAEC lysates were prepared from cells cultured for 48h in medium containing 5 or 25 mM glucose prior to incubation in media containing 0.2 mCi/ml ^32^PO_4^- and subsequent incubation with insulin (100 nM) for 5 min. Cbl was immunoprecipitated from HAEC lysates, the immunoprecipitated proteins were resolved by SDS PAGE, the gel dried and subjected to autoradiography. A) A representative autoradiograph is shown. B) Quantification of Cbl phosphorylation. Insulin stimulated Cbl phosphorylation in cells...
cultured in 5 mM glucose (1.5-fold, n=3, p<0.01). There was no significant alteration in Cbl phosphorylation in response to insulin in those cells cultured in 25 mM glucose.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5

Panel A: Western blot analysis showing Anti-eNOS and 32P-eNOS with 5mM and 25mM insulin treatment.

Panel B: Bar graph illustrating the arbitrary units of 5mM and 25mM with and without insulin treatment.
Fig. 6
Fig. 7

Insulin
- + - +

Bisindolylmaleimide I
- - + +

pmol NO$_2$ produced/mg protein
<table>
<thead>
<tr>
<th></th>
<th>5mM</th>
<th>25mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>*</td>
<td>anti-CAP</td>
<td></td>
</tr>
<tr>
<td>†</td>
<td>anti-Cbl</td>
<td>anti-C3G</td>
</tr>
<tr>
<td></td>
<td>anti-caveolin</td>
<td>anti-flotillin</td>
</tr>
</tbody>
</table>
**Fig. 9**

**A**

<table>
<thead>
<tr>
<th>Insulin</th>
<th>5mM</th>
<th>25mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
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<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**B**

![Graph showing arbitrary units for different conditions of 5mM and 25mM with and without insulin](image)

- **5 mM**
- **5 mM + insulin**
- **25 mM**
- **25 mM + insulin**
High glucose inhibits insulin-stimulated nitric oxide production without reducing eNOS Ser1177 phosphorylation in human aortic endothelial cells
Ian P. Salt, Valerie A. Morrow, Fiona M. Brandie, John M. C. Connell and John R. Petrie

J. Biol. Chem. published online March 18, 2003

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