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A role for heterotrimeric GTP-binding proteins and ERK1/2 in insulin-mediated, nitric-oxide-dependent, cyclic GMP production in human umbilical vein endothelial cells

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Abstract *Aims/hypothesis:* Insulin is known to stimulate endothelial nitric oxide synthesis, although much remains unknown about the intracellular mechanisms involved. This study aims to examine, in human endothelial cells, the specific contribution of heterotrimeric G_i proteins and extracellular signal-regulated protein kinases 1/2 (ERK1/2) in insulin signalling upstream of nitric-oxide-dependent cyclic GMP production. *Methods:* Human umbilical vein endothelial cells were treated with 1 nmol/l insulin in the presence or absence of inhibitors of tyrosine kinases (erbstatin), G_i proteins (pertussis toxin) or ERK1/2 (PD098059 or U0126), and nitric oxide production was examined by quantification of intracellular cyclic GMP. Activation/phosphorylation of ERK1/2 by insulin was examined by immunoblotting with specific antibodies, and direct association of the insulin receptor with G_i proteins was examined by immunoprecipitation. *Results:* Treatment of cells with a physiological concentration of insulin (1 nmol/l) for 5 min increased nitric-oxide-dependent cyclic GMP accumulation by 3.3-fold, and this was significantly inhibited by erbstatin. Insulin-stimulated cyclic GMP production was significantly reduced by pertussis toxin and by the inhibitors of ERK1/2, PD098059 and U0126. Immunoblotting indicated that insulin stimulated the phosphorylation of ERK1/2 after 5 min and 1 h, and that this was completely abolished by pertussis toxin, but insensitive to the nitric oxide synthase inhibitor L-NAME. No direct interaction of the insulin

receptor β with G_{i α 2} could be demonstrated by immunoprecipitation. *Conclusions/interpretation:* This study demonstrates, for the first time, that nitric oxide production induced by physiologically relevant concentrations of insulin, is mediated by the post-receptor activation of a pertussis-sensitive GTP-binding protein and subsequent downstream activation of the ERK1/2 cascade.

Keywords Cyclic GMP · ERK1/2 · Insulin · Nitric oxide · Pertussis toxin

Abbreviations ECGS: endothelial growth factor supplement · eNOS: endothelial nitric oxide synthase · ERK1/2: extracellular signal-regulated protein kinases 1/2 · G protein: GTP-binding protein · IR β : insulin receptor β subunit · MAPK: mitogen-activated protein kinase · MTT: 3-[4,5]Dimethylthiazol-2,5diphenyltetrazolium bromide · NOS: nitric oxide synthase · PI3K: phosphatidylinositol 3-kinase · PMSF: phenylmethylsulphonylfluoride · PTX: pertussis toxin

Introduction

Insulin is an essential hormone that regulates metabolism, growth and differentiation. The biological actions of insulin are initiated when insulin binds to the extracellular domain of its cognate cell surface receptor. The subsequent activation of the intracellular tyrosine protein kinase activity of the receptor triggers autophosphorylation of the β -subunit of the receptor as well as phosphorylation of a number of intracellular insulin receptor substrates, including IRSs and Shc. This gives rise to the activation of Ras and phosphatidylinositol 3-kinase (PI3K), and hence to the activation of a number of serine/threonine protein kinases. Many of these kinases are arranged in cascades, including a cascade that results in activation of mitogen-activated protein kinase (MAPK) and another that may result in activation of protein kinase B (Akt) [1].

As well as being present on traditional insulin-responsive cells, i.e. adipocytes and skeletal muscle, insulin re-

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ceptors have been demonstrated on endothelial cells of both large and small blood vessels [2, 3]. Insulin has been shown to strongly influence the function of the vascular endothelium and in vitro studies indicate that its functions include increases in amino acid transport, gene expression and enhancement of nitric oxide release [4, 5]. A number of studies have also provided convincing evidence that, in addition to its metabolic effects, insulin has nitric-oxide-dependent vasodilator actions in vivo [6–8]. This is supported by the observation that endothelial nitric oxide synthase (eNOS) knockout mice are insulin-resistant compared to the wild type [9], and that the vascular action of insulin is altered in insulin-resistant states, such as diabetes and obesity [10].

Despite extensive investigation, much remains unknown about how insulin exerts its effect on nitric oxide synthesis. In vitro studies in cultured human endothelial cells have demonstrated that the signalling pathways activated during insulin-stimulated nitric oxide production include the insulin receptor tyrosine kinase, phosphatidylinositol 3-kinase (PI3K) and Akt [5, 11], molecules which these pathways share with the metabolic actions of insulin, such as translocation of the insulin-sensitive glucose transporter GLUT4. In addition, insulin has also been reported to regulate the Ras/MAPK cascade, specifically the extracellular signal-regulated protein kinases 1/2 (ERK1/2) isoforms that are ubiquitously expressed and are activated by dual-specificity MAPK kinases (MEK1/MEK2) [12]. MAPK activation in response to insulin is classically considered to be associated with regulation of gene expression and cell proliferation, but the possibility of a MAPK contribution to insulin-induced nitric oxide synthesis has not been investigated. This possibility is supported by the observation that the ERK1/2 signalling pathway is involved in nitric oxide production in human endothelial cells stimulated by other agonists including adenosine [13].

Endothelium-derived nitric oxide is synthesised by the endothelial calcium/calmodulin-dependent form of nitric oxide synthase (eNOS) that is one of three isoenzymes that convert L-arginine to L-citrulline, forming nitric oxide in the process. Activation of nitric oxide synthase (NOS) and release of nitric oxide results in stimulation of a soluble guanylyl cyclase leading to a profound increase of cyclic GMP levels within target cells and thus vasodilatation [14]. The generation of nitric oxide represents a common component of the signal transduction pathways of a number of agonists (e.g. acetylcholine, serotonin, ATP, bradykinin, histamine) that act via binding to receptors coupled to heterotrimeric GTP-binding proteins (G proteins). Agonist binding to the receptor leads to exchange of GDP to GTP, resulting in dissociation of α -GTP and $\beta\gamma$ -subunits [15]. Both the α - and $\beta\gamma$ -subunits can transduce several events in cells. Previously it has been reported that pertussis toxin (PTX), which ADP-ribosylates the α -subunit of members of the G_i/G_o family and uncouples them from receptors, inhibited a number of insulin-stimulated events, such as glucose transport and metabolism in the insulin target cells [16, 17]. In addition, G proteins sensitive to pertussis toxin

have been reported to be involved in nitric oxide synthesis triggered by other agonists including endothelin [18] and acetylcholine [19]. However, the involvement of pertussis-sensitive G-proteins in insulin-mediated nitric oxide responses of human endothelial cells remains unknown.

This study was designed to examine the specific contribution of PTX-sensitive G-proteins and ERK1/2 in the intracellular signalling pathways activated during insulin stimulation of nitric oxide synthesis in human endothelial cells. We provide, for the first time, evidence that nitric oxide production induced by physiologically relevant concentrations of insulin is mediated by the post-receptor activation of G-proteins and determined by a downstream initiation of the MAPK cascade.

Materials and methods

Materials Unless stated otherwise, materials were obtained from Sigma Chemicals (Poole, UK).

Endothelial cell culture Human umbilical cords were obtained within 12 h of birth from normal pregnancies. The anonymous collection of umbilical cords for this project was approved by the North and East Devon Medical Research Ethics Committee (study 1301). Human umbilical vein endothelial cells were isolated from the vein by collagenase (0.3 mg/ml) digestion [20] and cultured in medium 199 (M199) containing 5 mmol/l glutamine, 20% (v/v) fetal bovine serum, 0.05 mg/ml gentamicin, 20 μ g/ml endothelial growth factor supplement (ECGS) and 90 μ g/ml heparin at 37°C in 5% CO₂/air. Confluent monolayers were trypsinised (trypsin–EDTA, 0.1–0.02% w/v), and the cells resuspended in M199 and cultured for the cyclic GMP assay on gelatine (1%, w/v)-coated 24-well plates. For immunoblotting, cells were grown on 21-cm² plastic Petri dishes (Greiner, Gloucester, UK). The identity of endothelial cells in culture was confirmed by the presence of the typical “cobblestone” morphology and by the expression of von Willebrand factor, PECAM and ACE as determined by immunocytochemical staining and western blotting. All cells were used at passage one at 85–90% confluency. Cell viability during passage procedure was routinely assessed by trypan blue exclusion.

Nitric oxide production Nitric oxide production was assessed by quantification of intracellular cyclic GMP levels, which allowed a functional cellular response to be studied. Cells were grown on 24-well plates in supplemented M199 in the presence or absence of inhibitors as indicated. Cells were then washed and pre-incubated for 15 min in Krebs buffer (37°C), containing 100 μ mol/l L-arginine and 0.5 mmol/l of the phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine. Pre-incubation media were removed and in some experiments cells were additionally challenged with 1 nmol/l human recombinant insulin (Calbiochem, Nottingham, UK) in Krebs solution for 5 min. Stimulation was terminated

by treatment of cells with 0.1N HCl on ice for 60 min (300 μ l per well). The HCl cell extract was assayed for cyclic GMP accumulation by radioimmunoassay following acetylation (Amersham, Little Chalfont, UK). The sensitivity of the assay is less than 2 fmol/l per 50 μ l; the intra-assay coefficient of variation is 9.8% ($n=6$). Cell protein was assayed with Bradford reagent [21] after cell solubilisation in 1 mol/l NaOH. The intracellular cyclic GMP concentration was initially expressed in fmol/ μ g protein. Due to the variation in increases in cyclic GMP levels between cell populations, intracellular cyclic GMP concentration is presented in the text as percentage of insulin-stimulated cyclic GMP content.

Immunoblotting For the examination of total and phosphorylated levels of ERK1/2, HUVECs were starved of fetal bovine serum and ECGS for 16 h and then treated with insulin (1 nmol/l) and/or drugs for times indicated. This period of serum starvation is similar to that used by other investigators [13, 22], and was necessary to reduce baseline activation of ERK1/2 by components in the serum. Similar results were obtained with shorter periods of serum starvation (i.e. 4 h), however, the baseline levels of phosphorylated ERK1/2 were higher. For total and phosphorylated eNOS cells were serum-starved for 4 h. For the examination of total $G_{\alpha 12}$ cells were not serum-starved. HUVECs were then washed twice with ice-cold PBS and lysed for 1 min in lysis buffer: 50 mmol/l Tris (pH 8.0), 150 mmol/l NaCl, 1 mmol/l EGTA (pH 8.0), 100 mmol/l NaF, 100 mmol/l $MgCl_2$, 10% w/v glycerol, 1% w/v Triton X₁₀₀, 1 mmol/l phenylmethylsulfonylfluoride (PMSF), 1 mmol/l sodium orthovanadate, and 50 μ l/ml of protease inhibitor cocktail consisting of 20 μ g/ml leupeptin, 20 μ g/ml pepstatin A, 200 μ g/ml benzoyl-L-arginine methyl ester, 200 μ g/ml *p*-tosyl-L-arginine methyl ester, 200 μ g/ml trypsin inhibitor, 200 μ g/ml L-1-tosylamide-2-phenylethylchloromethyl ketone and 200 μ g/ml aprotinin. The lysates were subjected to one cycle of freezing/thawing, mild ultrasonication for 5 min in a sonicleaner and protein content measurement. Protein samples were solubilised in Laemmli buffer [23], boiled for 3–4 min and centrifuged for 2 min at 20,000 $\times$ g to remove insoluble materials. 5 μ g protein per lane was separated by SDS-PAGE (10%) and transferred to 0.2- μ m nitrocellulose membrane. The blocked membranes were blotted for 2 h at room temperature with antibodies against the phosphorylated form of ERK1/2 (1:1,000)/total ERK1/2 (1:1,000) (Santa Cruz, Calne, UK), or for 24 h at 4°C against phosphorylated eNOS (1/1,000)/total eNOS (1/1,000) (Cell Signaling Technology, New England Biolabs, Hitchin, Herts, UK). The membranes were incubated with secondary antibody conjugated with HRP (1:15,000), and immunoreactive bands were visualised using enhanced chemiluminescence detection reagent. The immunoreactive bands corresponding to total eNOS, phosphorylated eNOS or the combined total for the phosphorylated ERK1/2 signal on X-ray film were quantified in densitometric analysis and expressed as an integrated density value.

Immunoprecipitation Following insulin treatment HUVECs were washed with ice-cold PBS and incubated on ice at 4°C for 10 min in RIPA buffer, containing: 50 mmol/l Tris (pH 8.0), 1 mmol/l EDTA, 150 mmol/l NaCl, 0.1% w/v SDS, 1% w/v NP-40, 0.5 w/v sodium deoxycholate, 1 mmol/l PMSF, 1 mmol/l sodium orthovanadate, 50 μ l/ml protease inhibitor cocktail (as described). The cells were disrupted by repeated aspiration through a 21-gauge needle and cellular debris was pelleted by centrifugation at 10,000 $\times$ g for 10 min at 4°C. The supernatant was pre-cleared with normal rabbit IgG together with protein A-agarose, and the mixture was incubated at 4°C for 30 min. Centrifugation at 1,000 $\times$ g for 5 min at 4°C allowed a full separation of agarose beads. The supernatant (cell lysate) was transferred to two parallel fresh tubes and insulin receptor β subunit (IR β) rabbit polyclonal antibody (Santa Cruz, Calne, UK) was added to one with normal non-immune rabbit serum added to another as immunoprecipitation control. The immune complexes were incubated overnight whilst mixing at 4°C. Next day protein A-agarose was added, followed by additional incubation for 3 h at 4°C. Immunoprecipitates were collected by centrifugation at 1,000 $\times$ g for 5 min at 4°C. The supernatant was carefully aspirated and pellets were washed four times with RIPA buffer, each time repeating the centrifugation step. After the final wash, pellets were resuspended in Laemmli sample buffer, boiled for 2–3 min and centrifuged at 20,000 $\times$ g for 2 min. The resulting supernatant was separated by SDS-PAGE electrophoresis (5 μ g protein/well) and analysed by western blotting with anti-IR β (1:1,000) or with anti- $G_{\alpha 12}$ (1:1,000) antibody (Upstate Biotechnology, Botolph Claydon, UK).

Colorimetric 3-[4,5]dimethylthiazol-2,5-diphenyltetrazolium bromide assay Since the concentration of PD098059 used (50 μ mol/l) has been previously reported to be cytotoxic for some cell types [24], we tested whether the viability of HUVECs was influenced by PD098059 in the present experimental conditions. Cells were seeded in 96-well plates at a density of 5 $\times$ 10⁴ cells/cm². The following day, the culture medium was changed to medium supplemented with 50 μ mol/l PD098059 (treated cells) or to regular medium with DMSO as a vehicle, at 1:1,000 dilution (control cells). Following 30-min incubation, cell viability was measured using the quantitative colorimetric 3-[4,5]dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay [25]. The tetrazolium ring of MTT is cleaved only in active mitochondria, and so the reaction occurs only in living cells. Plates were analysed in an ELISA plate reader at 540 nm.

Statistical analysis Statistical analyses were carried out on raw data using the Mann–Whitney test and a *p* value of less than 0.05 was considered statistically significant. Values are expressed as means $\pm$ SD. For all data, *n* represents the number of wells or dishes tested under each condition and also the results from at least two cell populations.

Results

Nitric-oxide-dependent cyclic GMP production in HUVECs is tyrosine-kinase mediated and dependent on a pertussis-toxin-sensitive G-protein Previous data from our laboratory indicate that insulin activates nitric-oxide-dependent cyclic GMP production in a manner that is dependent on time and concentration [26]. A maximal response occurs at 1 nmol/l insulin with a highly significant response observed at 5 min and increased production sustained for at least 1 h [26]. Thus, for all of the experiments reported in this study insulin was used at a final concentration of 1 nmol/l. Incubation of HUVECs with insulin (1 nmol/l) for 5 min resulted in a significant increase of cyclic GMP levels above the unstimulated control level (100% vs 35.96 ± 14.77 , $p \leq 0.001$) (Fig. 1). Previously, we proved that the observed changes in cyclic GMP reflected the increased production of nitric oxide, since the specific competitive NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) (100 μ mol/l, 15 min) [27] completely abrogated the insulin-stimulated cyclic GMP level [26].

Since the insulin receptor is reported to be a tyrosine kinase receptor [28], initial experiments were carried out to confirm that nitric-oxide-dependent cyclic GMP production is dependent on tyrosine kinase in HUVECs, using the specific tyrosine kinase inhibitor erbstatin [29]. Insulin-stimulated cyclic GMP accumulation was significantly inhibited by pretreatment with 3 μ mol/l erbstatin for 15 min (Fig. 1). Insulin-stimulated levels were reduced from 100 to $43.9 \pm 16.89\%$ ($p = 0.001$), which is not sig-

Fig. 1 The effect of pertussis toxin (PTX) and erbstatin on insulin-stimulated cyclic GMP production in HUVECs. Cells were preincubated for 2 h in the presence or absence of 500 ng/ml PTX, then washed and incubated for a further 15 min in Krebs solution in the presence of PTX and/or 3 μ mol/l erbstatin, or DMSO as a vehicle and additionally for 5 min in buffer of the same composition with or without 1 nmol/l insulin. Cells were lysed and analysed for cyclic GMP content. Data are mean cyclic GMP levels expressed as percentage of the insulin-stimulated cyclic GMP level $\pm$ SD ($n \geq 6$). * $p < 0.001$ vs control; ** $p = 0.01$ vs control; # $p < 0.001$ vs insulin-stimulated level; ## $p = 0.05$ vs insulin+PTX

nificantly different to the unstimulated control value of $35.96 \pm 14.77\%$.

In order to examine the involvement of G-proteins in insulin-stimulated nitric oxide production, HUVECs were exposed to 500 ng/ml PTX, which selectively inhibits the transmission of G_i -protein-mediated agonist effects [30]. Incubation with PTX for 2 h prior to insulin treatment reduced insulin-stimulated cyclic GMP levels by approximately 50% (Fig. 1). Interestingly, basal cyclic GMP production in these experiments was also inhibited by PTX. When cultured cells were challenged with a combination of PTX and erbstatin, the inhibitory effect of the combined inhibitors on insulin-stimulated cyclic GMP levels was not significantly different to that of erbstatin alone, but was significantly lower than the levels observed with PTX alone (Fig. 1). This confirms that tyrosine phosphorylation of the insulin receptor is the upstream event in insulin signalling leading to nitric-oxide-dependent cyclic GMP generation.

Insulin-stimulated nitric-oxide-dependent cyclic GMP production is not dependent on PI3K Several studies have implicated PI3K in insulin-stimulated nitric oxide production [5, 11, 22]. In order to examine the role of PI3K in insulin-stimulated cyclic GMP production in our experimental model, cells were pre-incubated with the PI3K inhibitors wortmannin (100 nmol/l, 15 min) or LY294002 (10 μ mol/l, 15 min) prior to insulin stimulation. Interestingly, wortmannin only reduced insulin-stimulated cyclic GMP production by $17.5 \pm 26\%$ ($p < 0.05$, $n = 9$) and the more specific inhibitor LY294002 had no statistically significant effect ($98 \pm 36.23\%$ vs insulin-stimulated level of 100%, $n = 9$) (data not shown). Other reports have suggested that insulin-stimulated nitric oxide production is associated with phosphorylation of eNOS on ser¹¹⁷⁷ [31, 32] downstream of PI3K. To investigate this relationship in the experimental system described here, serine phosphorylation of eNOS was examined in HUVECs in response to 5-min stimulation with 1 nmol/l insulin. Figure 2 indicates that insulin does not stimulate eNOS phosphorylation on ser¹¹⁷⁷. The ratio of total eNOS/phosphorylated eNOS was 1:0.82 for control cells and 1:0.88 for insulin-treated cells.

Insulin receptor β and $G_{\alpha i2}$ are not directly associated The observation that PTX significantly inhibits insulin-stimulated nitric oxide production raised the possibility that IR β can transduce insulin signalling via direct dynamic regulation of a PTX-sensitive $G_{\alpha i}$ protein. Therefore, the possibility of a direct physical interaction between the IR β and the $G_{\alpha i}$ protein in HUVECs was explored by immunoprecipitation of the IR β and western blotting of the immunoprecipitate for $G_{\alpha i}$. It has previously been reported that HUVECs express type 2 $G_{\alpha i}$ [33]. In order to confirm this, an immunoblotting procedure of the whole cell lysate with an anti- $G_{\alpha i2}$ antibody was performed and a specific immunoreactive band, corresponding to the molecular weight (40 Mr) of $G_{\alpha i2}$ was found (Fig. 3a). Initial control

Fig. 2 Insulin (1 nmol/l) does not induce eNOS phosphorylation on ser¹¹⁷⁷ in HUVECs. HUVECs were deprived of serum and ECGS for 4 h and then incubated for 5 min in Krebs solution with 1 nmol/l insulin (*Ins*) or in medium alone as control (*C*). Cells were then lysed, and total eNOS protein and then eNOS phosphorylated on ser¹¹⁷⁷ were assessed by western blot analysis using specific antibodies. The intensity of relevant protein bands was measured by densitometry and quantified as integrated density values (*IDV*), which are represented as the ratio of *IDV* value for total eNOS to the *IDV* value for eNOS phosphorylated on ser¹¹⁷⁷. Lysates of bovine aortic endothelial cells (*BAEC*) treated with or without vascular endothelial growth factor (*VEGF*) were included as a positive control for phosphorylated eNOS. Images shown are representative of two experiments carried out on two separate cell populations with identical results obtained on each occasion

Fig. 3 G_{αi2} is not co-immunoprecipitated with insulin receptor β (*IRβ*). **a** Untreated HUVECs were lysed and the cell lysate western blotted (WB) with anti-G_{αi2}. The results from two separate cell lysates are shown. **b** Untreated HUVECs were lysed and immunoprecipitated (IP) with either non-immune (NI) serum or with anti-IRβ antiserum (AS) and immunoprecipitates were western blotted with anti-IRβ. **c** HUVECs were incubated in the presence or absence of 1 nmol/l insulin for 5 min as indicated. Cells were then lysed and immunoprecipitated (IP) with anti-IRβ antibody or non-immune (NI) control serum. Immunoprecipitates were western blotted (WB) with G_{αi2} antibody. Images shown are representative of two to three experiments carried out on three separate cell populations with identical results obtained on each occasion

experiments were then carried out to confirm that IRβ could be successfully immunoprecipitated using our experimental protocol. Figure 3b demonstrates that a specific immunoreactive band was detected after immunoprecipitation with the IRβ antiserum, but not with the pre-immune control serum. However, G_{αi2} could not be detected in the immunoprecipitate after immunoprecipitation with IRβ in

Fig. 4 The effect of PD098059 and U0126 on insulin-stimulated cyclic GMP production in HUVECs. HUVECs were preincubated for 15 min in the presence of 50 μmol/l PD098059 or vehicle (DMSO) (a), or for 1 h with 10 μmol/l U0126 or vehicle (DMSO) (b). All cells were then washed and incubated for 15 min in Krebs solution in the presence of PD098059/DMSO (a) or U0126/DMSO (b) and additionally for 5 min in buffer of the same composition ±1 nmol/l insulin. Cells were lysed and analysed for cyclic GMP content. Results are presented as mean cyclic GMP levels expressed as percentage of the insulin-stimulated cyclic GMP level ±SD (n=9 or 12). *p<0.001 vs control; **p<0.05 vs control; #p=0.01 vs insulin-stimulated level

control, untreated cells or after insulin treatment (Fig. 3c), indicating that no direct association of IR β and G α_{i2} could be demonstrated in HUVECs.

Insulin stimulates MAPK-dependent cyclic GMP production and ERK1/2 phosphorylation in HUVECs We then examined the possibility that MAPK activation is required for insulin-induced nitric-oxide-dependent cyclic GMP production. Pre-incubation with cell-permeable inhibitors of the ERK signalling cascade, PD098059 (50 μ mol/l, 30 min) [34] and U0126 (10 μ mol/l, 1 h) [35], reduced the increase in insulin-stimulated cyclic GMP generation from 100% (insulin-stimulated level) to $72.89 \pm 22.51\%$, $p < 0.005$ for PD098059 and from 100% (insulin-stimulated level) to $73.94 \pm 23.3\%$ ($p = 0.005$) for U0126 (Fig. 4). Interestingly pretreatment with PD098059 significantly reduced basal cyclic GMP levels. However, the inhibitory effect of PD098059 on insulin-stimulated cyclic GMP remained significant even when this reduction in baseline was taken into account (data not shown). To eliminate the possibility of a potentially detrimental effect of PD098059 on cell viability, we did an MTT colorimetric cytotoxicity assay. The results demonstrated that PD098059 at 50 μ mol/l for 30 min did not cause any statistically significant cytotoxic effect on living HUVECs ($n = 3$, $p = 1$, Mann-Whitney test, treated vs untreated cells; data not shown).

These experiments indicated a possible role for ERK1/2 in insulin-stimulated nitric oxide generation. Therefore, further studies were carried out to investigate the functional activity of ERK following insulin stimulation. Since the activation of ERK1/2 involves phosphorylation of tyrosine and threonine residues [36], activation was monitored by western blotting using an antibody specific for the phosphorylated form of the protein. Immunoblotting revealed that although levels of total ERK1/2 were unaltered by insulin treatment, insulin did induce a dramatic increase in the intensity of phosphorylated ERK1/2 (Fig. 5). This effect was evident from short (5 min) and prolonged (1 h) insulin exposure, although 1 h of treatment seemed to result in a larger amplitude of stimulation (Fig. 5a). The specificity of ERK1/2 activation was demonstrated by pre-incubation of endothelial cells with PD098059, which resulted in the almost complete elimination of basal and insulin-stimulated levels of phosphorylated ERK1/2 (Fig. 5b).

To examine whether nitric oxide was involved in insulin-induced ERK1/2 activation, cells were incubated in the

Fig. 5 ERK1/2 phosphorylation is induced by insulin and is nitric oxide-independent. HUVECs were deprived of serum and ECGS for 16 h and then preincubated with: **a**, control medium, 50 μ mol/l PD098059 or vehicle (DMSO) for 30 min; **b** control medium or 100 μ mol/l L-NAME for 1 h, followed by incubation $\pm$ 1 nmol/l insulin as indicated. Cells were then lysed and total ERK1/2 protein was assessed by western blot analysis using anti-non-phosphorylated total ERK1/2 antibody (**a**) or phosphorylation/activation of ERK1/2 was detected by western blot analysis with phospho-specific anti-phosphoERK antibody (**a-c**). Images shown are representative of three experiments carried out on three separate cell populations with identical results obtained on each occasion. The integrated density values (IDV) presented are the mean of the three experiments

presence or absence of the NOS inhibitor L-NAME prior to insulin treatment. As demonstrated in Fig. 5c, the phosphorylated levels of ERK1/2 following insulin treatment were not altered in cells pretreated with L-NAME compared with cells stimulated with insulin following pretreatment with carrier alone (media).

ERK1/2 phosphorylation induced by insulin is sensitive to pertussis toxin In order to further dissect the elements of the signal transduction pathway upstream of insulin-induced ERK activation, the role of a G_i protein sensitive to PTX was considered. Figure 6a illustrates that PTX completely abrogated the stimulatory effect of insulin (1 nmol/l, 5 min) on ERK1/2 phosphorylation, and also inhibited the basal

phosphorylation of ERK1/2. Similar results were obtained after prolonged 1-h incubation with insulin (Fig. 6b), although interestingly at this time point PTX alone reduced the baseline below those levels observed with PTX plus insulin.

Discussion

This study extends previous knowledge of the mechanism of insulin-stimulated nitric oxide production in human endothelial cells treated with physiologically relevant levels of insulin, and demonstrates for the first time that G_i -proteins and the activation of the ERK1/2 MAPK cascade mediate insulin-induced nitric-oxide-dependent cyclic GMP production.

Previously, work from this laboratory has reported that rapid (i.e. within 5 min) nitric-oxide-dependent cyclic GMP production occurred at insulin levels closely corresponding to the range of binding and activation of insulin receptors in endothelial cells and at the concentration in normal human plasma [26]. The current study demonstrates that this insulin-stimulated nitric-oxide-dependent production of cyclic GMP is completely blocked by erbstatin, a specific inhibitor of tyrosine kinases, extending previous data using the less specific tyrosine kinase inhibitor genistein [11]. These results indicate that synthesis of nitric oxide by endothelial NOS is regulated downstream of a tyrosine kinase, presumably the insulin receptor.

The G_i -protein inhibitor, PTX, also significantly reduced insulin-stimulated endothelial cell nitric oxide production, clearly implicating, for the first time, a role for a pertussis-sensitive heterotrimeric G_i protein downstream of the insulin receptor tyrosine kinase in this signalling pathway. There is increasing evidence that insulin signalling via several intracellular pathways may be mediated by a heterotrimeric G protein downstream of the insulin receptor, although the G protein subunit involved appears to differ depending on the cell type and the cellular response to insulin studied [17, 37–40]. Pertussis toxin sensitivity of insulin-dependent functions has previously been demonstrated for the metabolic effects of the hormone in its target tissues including the stimulation of diacylglycerol production and glucose uptake in murine myocytes [17, 39], and inhibition of adenylate cyclase activity in hepatocytes [40]. However, whether G-proteins that are sensitive to PTX transduce insulin-stimulated vasoactive effects in human vascular endothelial cells was previously unknown. Nevertheless, a PTX-sensitive mechanism has been reported to be involved in the insulin-induced enhancement of α_2 -adrenergic vasorelaxation in rat aorta and in nitric oxide production in rat aorta endothelial cells although no direct nitric oxide response to insulin stimulation could be demonstrated [41].

In order to further examine the relationship between insulin and G_i -proteins in the experimental model used here, we examined the possibility that, similar to classic G-protein-coupled receptors, the insulin receptor may interact directly with the G-protein. Indeed, it has been documented

Fig. 6 The effect of pertussis toxin (PTX) on insulin-induced ERK phosphorylation. HUVECs were cultured for 16 h in the absence of serum and ECGS, challenged for the last 2 h with 500 ng/ml PTX, and subsequently treated with or without 1 nmol/l insulin for 5 min (a) or 1 h (b) in the presence or absence of PTX. Cells were then lysed and phosphorylation/activation of ERK1/2 was detected by western blot analysis with phospho-specific anti-pERK. Images shown are representative of two to three experiments with identical results obtained on each occasion. The integrated density values (IDV) presented are the mean of experiments performed using two to three different cultures of HUVECs

that the insulin receptor is associated with α -subunits of non-pertussis-sensitive heterotrimeric G-proteins ($G_{\alpha_{q/11}}$) [37] in 3T3-L1 adipocytes, with an unusual, 65-M_r, putative GTP-binding protein [42, 43] in human placenta, and with $G_{\alpha_{i2}}$ in human adipocytes [44]. In the present study no association of $G_{\alpha_{i2}}$ with the insulin receptor in HUVECs could be demonstrated by co-immunoprecipitation, even though immunoblotting confirmed the presence of $G_{\alpha_{i2}}$ in these cells. Since PTX inhibits the action of all G_i -proteins, this implies that other G_{α_i} isoforms expressed in endothelial cells may be involved in insulin receptor interactions. It is also possible that the insulin receptor, acting as a classic receptor tyrosine kinase, initiates a cascade of phosphorylation-mediated signalling events, where G_i -proteins act as an integrated element of the signalling chain and the activation of $G_{i\alpha}$ could represent a downstream response to (a) putative adapter protein(s), and not a direct physical coupling to the insulin receptor. Finally, it is possible that the interaction between $G_{\alpha_{i2}}$ and the insulin receptor was too transient to be detected by the experimental methods utilised in the present study.

In insulin-triggered signalling events upstream of cell differentiation and gene expression, insulin regulates activation of the Ras/MAPK pathway. This study therefore examined whether insulin-stimulated nitric oxide production is also mediated by MAPKs, specifically ERK1/2. Insulin-mediated nitric oxide production was found to be significantly inhibited by the MEK inhibitors PD098059 and U0126, demonstrating, for the first time, that the MAPK pathway does mediate insulin-stimulated NOS activation. The stimulatory and specific effect of insulin on ERK1/2 activation was further confirmed by the finding that insulin induced a dramatic and sustained increase in expression levels of the phosphorylated forms of the enzymes. Although the prolonged (1 h), effect of insulin on MAPK activation in human vascular endothelial cells has recently been described [22], acute, i.e. within minutes, insulin stimulation has not been reported previously.

The reduction in intracellular cyclic GMP accumulation with the MEK inhibitors suggests that nitric oxide synthesis is a consequence of MAPK cascade activation. In order to confirm this, the effect of insulin on ERK1/2 protein phosphorylation during NOS inhibition was investigated. The results indicate that, as assessed by western blotting, insulin-stimulated ERK1/2 activation was unaffected by pretreatment of the cells with the NOS inhibitor L-NAME, suggesting that nitric oxide production is downstream of MAPK activation. Therefore, it appears that the functional relationship between nitric oxide and MAPK is not due to the stimulatory effect of nitric oxide on MAPK phosphorylation/activation. This is in contrast to previous reports in the literature indicating that nitric oxide promotes rapid cyclic-GMP-mediated MAPK activation in rat mesangial cells [45] and triggers MAPK activation induced by VEGF in postcapillary endothelium [46].

The relationship between insulin-triggered mechanisms sensitive to PTX and ERK1/2 was then investigated. Insulin-stimulated ERK1/2 activation was completely abolished by PTX, indicating that MAPK activation is downstream

of heterotrimeric G-proteins in insulin signalling. The literature suggests that activation of the insulin receptor may initiate two parallel signalling pathways leading to MAPK activation: one is well characterised for insulin action and is Ras-mediated, modulating cell growth and gene expression; the other is G-protein-mediated, also involving MAPK. The second pathway, reported to be both Ras-dependent and -independent [47–49], appears to be prevalent in vascular endothelial cells as indicated by the complete inhibition of insulin-stimulated MAPK activation with PTX in the present experimental model.

The observation that PTX did not fully inhibit insulin-stimulated nitric oxide production, coupled to the fact that MAPK inhibitors did not fully prevent nitric oxide production at concentrations which fully inhibited MAPK phosphorylation, suggests that other pathways are additionally activated by insulin downstream of the insulin receptor. Several studies have implicated PI3K in insulin-stimulated nitric oxide production [5, 11, 22]. However, in our experimental model the PI3K inhibitors wortmannin and LY294002 had little or no effect on insulin-stimulated, nitric-oxide-dependent cyclic-GMP production. The reasons why these data do not support other reports in the literature are unknown. However, the concentration of insulin (1 nmol/l) used here to induce a robust level of nitric oxide production is at least an order of magnitude lower than those in other studies using native human endothelial cells. This raises the possibility that at low concentrations of insulin, nitric oxide production is PI3K-independent or that at low levels of insulin wortmannin- and LY 294002-insensitive PI3K isoforms are involved. Since class II PI3K is known to be relatively resistant to the action of both inhibitors [50], it cannot be excluded that this class of PI3K is involved in insulin-stimulated nitric oxide production in the assay described here. It is interesting to note that other reports have suggested that insulin-stimulated nitric oxide production is associated with phosphorylation of eNOS on ser¹¹⁷⁷ downstream of PI3K. However, in the experimental conditions described here, no phosphorylation of eNOS on ser¹¹⁷⁷ was observed. These results suggest that insulin activates multiple signalling pathways in endothelial cells, some of which are yet to be fully identified and defined.

The signalling events of eNOS activation remained largely beyond the scope of this study, but it is conceivable that MAPKs and G-proteins may be regulatory elements of insulin-induced eNOS activation, as has been shown for sphingosine 1-phosphate [51]; this requires further investigation.

In summary, these data are the first demonstration that insulin at physiologically relevant concentrations activates nitric oxide synthesis in vascular endothelial cells via G-proteins that are sensitive to PTX and are activated downstream of the insulin receptor tyrosine kinase and upstream of MAPK. In association with the described alterations in activity and expression of insulin receptors [52], MAPK [53] and heterotrimeric G-proteins [54, 55] in diabetes, our findings raise the possibility that disruption of insulin transduction via these signalling molecules may contribute to impairment of vasodilatory responses in diabetes.

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