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INSULIN INDUCED EPIDERMAL GROWTH FACTOR ACTIVATION IN VASCULAR SMOOTH MUSCLE CELLS IS ADAM-DEPENDENT

Elisa Roztocil, Suzanne M. Nicholl, and Mark G. Davies

Vascular Biology and Therapeutics Program, Methodist DeBakey Heart Center, Department of Cardiovascular Surgery, The Methodist Hospital, Houston, Texas

Abstract

Background—With the rise in metabolic syndrome, understanding the role of insulin signaling within the cells of vasculature has become more important but yet remains poorly defined. The study examines the role of insulin actions on a pivotal cross-talk receptor, Epidermal Growth Factor Receptor (EGFR). EGFR is transactivated by both G-protein-coupled receptors and receptor linked tyrosine kinases and is key to many of their responses.

Objective—To determine the pathway of EGFR transactivation by insulin in human coronary smooth muscle cells (VSMC)

Methods—VSMC were cultured *in vitro*. Assays of EGFR phosphorylation were examined in response to insulin in the presence and absence of the plasmin inhibitors (ε-aminocaproic acid and aprotinin) matrix metalloprotease (MMP) inhibitor GM6001, the ADAM (A Disintegrin And Metalloproteinase Domain) inhibitors TAPI-0 and TAPI-1, Heparin binding epidermal growth factor (HB-EGF) inhibitor, CRM197, HB-EGF inhibitory antibodies, EGF inhibitory antibodies and the EGFR inhibitor AG1478.

Results—Insulin induced time-dependent EGFR phosphorylation, which was inhibited by AG1478 in a concentration dependent manner. Application of the plasmin inhibitors did not block the response. EGFR phosphorylation by insulin was blocked by inhibition of MMP activity and the ligand HB-EGF. The presence of the ADAM inhibitors, TAPI-0 and TAPI-1 significantly decreased EGFR activation. EGFR phosphorylation by EGF was not interrupted by inhibition of plasmin, MMPs TAPIs, or HB-EGF. Direct blockade of the EGFR prevented activation by both insulin and EGF.

Conclusion—Insulin can induce transactivation of EGFR by an ADAM-mediated, HB-EGF dependent process. This is the first description of crosstalk via ADAM between insulin and EGFR in vascular SMC. Targeting a pivotal cross-talk receptor such as EGFR, which can be transactivated by both G-protein-coupled receptors and receptor tyrosine kinases is an attractive molecular target.

Keywords

Insulin; epidermal growth factor receptor; transactivation; vascular smooth muscle cell

Correspondence: Mark G. Davies, MD, PhD, Methodist DeBakey Heart Center, Department of Cardiovascular Surgery, The Methodist Hospital, 6560 Fannin, - Scurlock Tower - Suite 1006, Houston, Texas 77030.

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INTRODUCTION

With the rise in metabolic syndrome, understanding the role of insulin signaling within the cells of vasculature is important but yet remains poorly defined (1,2). Insulin has been shown to regulate vascular smooth muscle cell (VSMC) quiescence and inhibit VSMC migration. This activity is mediated in part by phosphatidylinositol 3' kinase (PI3K) and mitogen activated protein kinase (MAPK) pathways (3). Insulin can also modulate the responses of VSMC to both G-protein-coupled and receptor-linked tyrosine kinase receptor agonists. Epidermal Growth Factor Receptor (EGFR) is transactivated by both G-protein-coupled receptors and receptor-linked tyrosine kinases and is the key to many of their responses (4). Insulin resistance, a feature of metabolic syndrome, results in a loss of the regulation of VSMC quiescence and promotion of VSMC migration. VSMC migration is a pivotal process in the development of atherosclerosis and wound healing. Insulin has been shown to modulate epidermal wound healing through Epidermal Growth Factor Receptor (EGFR) signaling (5). The role of EGFR during insulin signaling in VSMC is not defined. The aim of this study is to determine the pathway of EGFR transactivation by insulin in human coronary smooth muscle cells (VSMC).

MATERIALS AND METHODS

Experimental Design

Human coronary VSMC (passages 3–6, Clontech) were cultured *in vitro*. Cell migration in response to insulin (0.1–100nM) alone and in combination with PDGF (10μM) and S-1-P (100nM) were examined. Assays of EGFR phosphorylation were examined in response to insulin in the presence and absence of the plasmin inhibitors (ε-aminocaproic acid -EACA- and aprotinin) the matrix metalloprotease (MMP) inhibitor GM6001, the ADAM (A Disintegrin And Metalloproteinase Domain) inhibitors TAPI (Tumour necrosis factor-α protease inhibitor)-0 and TAPI-1, Heparin binding epidermal growth factor (HB-EGF) inhibitor, CRM197, HB-EGF inhibitory antibodies, EGF inhibitory antibodies the insulin growth factor receptor inhibitor (IGFR) AG1024 (50nM) and the epidermal growth factor receptor inhibitor (EGFR) AG1478 (10nM).

Materials

Insulin, EGF, EACA, and aprotinin were purchased from Sigma Chemical Co (St. Louis, MO). AG1024, AG1478 and CRM197 were purchased from Calbiochem (La Jolla, CA). GM6001, was purchased from Chemicon International, Inc (Temecula, CA). CRM197 TAPI-0 and TAPI-1 were purchased from Biomol. The Anti-HB-EGF antibody was purchased from R&D Systems, Inc (Minneapolis, MN). The Anti-EGFR antibody (151-IgG) developed by Dr Ann Hubbard was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa (Department of Biological Sciences, Iowa City, IA). Peroxidase-conjugated antirabbit IgG antibody (raised in goat) and peroxidase-conjugated antimouse IgG antibody (raised in goat) were purchased from Jackson Immuno Research Laboratories, Inc (West Grove, Pa). Phospho-ERK1/2 antibody was purchased from Promega, Inc (Madison, Wis). Total ERK1/2 antibody was purchased from BD Transduction Laboratories (Lexington, Ky). Phospho- EGFR (Y1068), Phospho-akt (ser472) and total EGFR and akt antibodies were obtained from Cell Signaling Technology, Inc (Beveley, MA). Dulbecco modified Eagle minimal essential medium (DMEM) and Dulbecco phosphate-buffered saline were purchased from Mediatech (Herndon, VA).

Wound Assay

The wound assay was performed with VSMC as previously described (6,7). Trials with insulin were performed in six separate dishes, and the results were averaged. Cells were then allowed to migrate over 24 hours at 37°C in DMEM with or without Insulin.

Boyden Chamber

Chemotaxis was measured using a 48-well Boyden chamber (Neuroprobe Inc.) and polycarbonate filters (Neuroprobe, Inc., 10 µm pore size, 25 × 80 mm, PVP free) with VSMC as previously described (6,7). Insulin was added to the lower wells. Trials included eight or twelve wells per reagent per trial, and were repeated no fewer than three times.

Western Blotting

Cells were allowed to grow to 80% confluence and starved for 48 hours. Cells were then stimulated with insulin or EGF alone and in the presence of pharmacological and peptide inhibitors; cells were harvested at time points from 0 to 30 minutes. Western blotting was performed as previously described (8).

Data and Statistical Analysis

All data are presented as the mean ± standard error of the mean (s.e.m.) and statistical differences between groups were tested with a Kruskal-Wallis nonparametric test with post hoc Dunn's multiple comparison correction, where appropriate. A p-value less than 0.05 was regarded as significant. Non-significant p-values were expressed as p=ns.

RESULTS

Consistent with other reports, Insulin did not induce significant smooth muscle cell migration in the linear scratch or boyden chamber assays (Fig 1A and 1B). To determine if Insulin would inhibit agonist mediated migration, we incubated VSMC with insulin (10nM) for 30 minutes prior to exposure to PDGF and S-1-P. Insulin inhibited both PDGF- and S-1-P-mediated VSMC migration in both assays (Fig 1C). Insulin did activate both PI3K and ERK1/2 in VSMC in a time dependent manner (Fig 2A). Application of EGF produced a similar series of results. When the activation of EGFR as measured by phosphorylation of Y1068, Insulin induced a 6-fold increase in EGFR phosphorylation; this response was inhibited in a concentration-dependent manner by increasing concentrations of the EGFR inhibitor AG1478 (0.1–1000nM) (Fig 2B). Incubation with the IGFR inhibitor, AG1024, had no effect on EGF or insulin activation of EGFR (Fig 2C). EGFR phosphorylation by insulin was blocked by the inhibitor of the cell surface tethered ligand HB-EGF with the HBEGF inhibitor CRM197 (Fig 3). This would suggest that EGFR activation is through release of a tethered ligand. To confirm this we incubated the cell with anti HBEGF and Anti EGFR antibodies and uses an isotype IgG as a control. Incubation with the HB-EGF antibody blocked the insulin but not the EGF mediated activation of EGFR (Fig 3). Direct blockade of the EGFR prevented activation by both insulin and EGF (Fig 3). IgG had no effect. As PAI-1, the endogenous inhibitor of plasmin activation is elevated in metabolic syndrome, we tested whether plasmin had a role in insulin signaling to EGFR. The EGFR activation in response to insulin was not dependent on the proteolytic activity of plasmin since both plasmin inhibitors (aprotinin and EACA) had no effect (Fig 4). MMP have been implicated in tethered ligand release. Blockade of metalloproteinases with GM6001 did inhibit EGFR phosphorylation in response to insulin (Fig 4). EGFR phosphorylation by EGF was not interrupted by the presence of inhibitors of plasmin or MMP (Fig 4). Insulin signals through insulin receptor complexes. ADAM are transmembrane molecules that can be activated by intracellular pathways to induce release of a tethered extracellular ligand. To test whether ADAMs are involved, VSMC were incubated with the

ADAM inhibitors TAPI-0 (20 μ M) and TAPI-1 (10 μ M) and both blocked EGFR activation by insulin but had no effect on EGFR activation by EGF (Fig 4). Reports in the literature have suggested that Insulin can mediate some of its actions through intracellular oxygen free radical generation and oxygen free radicals have been implicated in EGFR transactivation. Inhibition of oxygen free radical generation with N-acetylcysteine and NAD(P)H oxidase with DPI inhibited EGFR activation in response to insulin (Fig 5). Finally to determine if inhibition of ADAM could block the inhibitory effect insulin on agonist mediated migration in response to S-1-P or PDGF, we incubated VSMC with insulin (10nM) and either ADAM inhibitors TAPI-0 (20 μ M) or TAPI-1 (10 μ M) for 30 minutes prior to exposure to PDGF and S-1-P. The presence of the ADAM inhibitors removed the insulin inhibition of PDGF-mediated VSMC migration in the Boyden chamber (Fig 6). A similar response was not seen during S-1-P-mediated VSMC migration in the presence of insulin as both the control and insulin treated cells in the presence of TAPI-0 and TAPI-1 were equivalent suggesting that S-1-P may also use ADAM to signal to EGFR during VSMC migration.

DISCUSSION

There is divergence on the role of insulin in VSMC migration under normal conditions. Insulin has been shown to inhibit, to have no effect, and to stimulate cultured VSMC migration depending on the experimental conditions (9–13). The present study has demonstrated that insulin can induce a low level of migration in human coronary smooth muscle cells in two distinct assays. However, under normal glucose conditions and in keeping with insulin's role as a modulator of quiescence, the presence of insulin inhibited stronger chemotactic agents such as receptor linked tyrosine kinase agonist PDGF-BB and G-protein coupled receptor Sphingosine-1-Phosphate. Insulin is considered to maintain VSMC quiescence and differentiation via a PI3-K dependent pathway and induce migration via the MAPK pathway (3). We have demonstrated that insulin can activate both PI3-K and ERK1/2 pathways but in these cells under these conditions, there was no significant migration. Signal transduction in both pathways is initiated by insulin binding to the insulin receptor. Propagation of this signal through the pathway that involves IRS proteins, PI3-K, and Akt is responsible for most of the metabolic aspects of insulin action (14,15). Signaling through Ras, Raf, to ERK1/2 is responsible for the migratory aspects of insulin (16,17). The inhibitory effect on migration may be the result of altering the PI3K or MAPK systems prior to the action of the stronger agonist. Several investigators have also observed in experimental in vitro systems, in animal models of insulin resistance, and in insulin-resistant humans that in the presence of metabolic insulin resistance, that many insulin target tissues, including vascular cells, display a significant defect of PI3-K signaling but retain normal sensitivity to insulin via the MAPK signaling pathway (18–20).

We have also shown that insulin will induce EGFR phosphorylation. The EGFR/ErbB family is composed of four receptors tyrosine kinases: ERB1/EGFR, ErbB2/neu, ErbB3 and ErbB4, each of which consists of a glycosylated ligand binding domain, a single transmembrane domain and a cytoplasmic tyrosine kinase domain (21) ErbB1 has been studied in vascular smooth muscle cells but there is no data available to implicate the other ErbB receptors in vascular remodeling. EGFR is expressed in the injured vessel wall (22). In the rat carotid injury model, blockade of EGFR with an antibody will reduce intimal hyperplasia development (23). Both PDGF-BB and S-1-P have been shown to utilize EGFR: PDGF by dimerization with ERGR and S-1-P by transactivation. Insulin activation of EGFR may disrupt these interactions and through the premature activation of EGFR decrease the number of receptors available for interaction and activation by RTK and GPCR agonists. The data presented in this report demonstrated that EGFR activation appears to occur through the release of the tethered ligand HB-EGF and that this mechanism is MMP dependent. All ErbB ligands are synthesized as transmembrane precursors that are proteolytically cleaved to release biologically active

soluble growth factors, which act in an autocrine and/or paracrine manner. HB-EGF can stimulate growth and migration of smooth muscle cells (24). It also requires cell surface heparan sulphate proteoglycans as co-receptors and HBEGF binding to EGFR can be antagonized in a dose dependent manner by heparin (25). HB-EGF is expressed in atherosclerotic lesions (26,27) and in restenotic lesions following angioplasty (24,28). The data in this report suggests that MMP not plasmin activity is required for HB-EGF release and that disruption of MMPs, HB-EGF or the extracellular part of the EGFR will disrupt insulin mediated EGFR phosphorylation. These features are suggestive of receptor transactivation.

Receptor transactivation is the process, whereby activation of a given receptor activates a heterologous receptor (29). This situation is commonly associated with GPCR and receptor tyrosine kinases (RTK). ET-1, LPA and thrombin induce rapid phosphorylation of the EGFR, and suppression of this EGFR activation leads to reduced MAPK activation (29,30). At present, the triple membrane passing signaling (TMPS) mechanism of GPCR induced EGFR activation is a widely accepted model of RTK transactivation (29). In this model, there is a sequence of 3 transmembrane signaling events: GPCR activation followed by MMP activation, and subsequent activation of the EGFR by heparin binding EGF, or other latent ligands of the EGFR. Several different MMPs have been identified with HB-EGF release: ADAM-10 (A Disintegrin And Metalloproteinase Domain), ADAM-12, ADAM-17 (TNF- α converting enzyme) and MMP3 (31). ADAM are approximately 70 to 90 kDa (mature proteins; the unprocessed precursors are about 20 kDa larger due to their prodomain). They feature a common modular ectodomain structure, encompassing a variable stalk region; a cysteine-rich domain, a disintegrin domain binding to integrin-class cell adhesion molecules and a Zn-binding metalloprotease domain. ADAM-12, ADAM-9, ADAM-10, ADAM-17 have been reported to be involved in the ectodomain shedding of proHB-EGF (32,33). Activation of these proteases pathway has been shown to involve one or more of the following signaling molecules: src, intracellular calcium, and protein kinase C (29). An alternative mechanism may be inactivation of protein tyrosine phosphatases due to the generation of oxygen free radicals by the NAD(P)H oxidase complex. We have shown that insulin will induces EGFR phosphorylation through an ADAM-mediated HB-EGF transactivation process. Activation of this process appears to be dependent on oxygen free radicals likely from NAD(P)H oxidase. Insulin has been shown to modestly stimulate NAD(P)H oxidase activity in cultured rat VSMCs (34) and it is known that reactive oxygen species contribute to VSMC migration (35–37). It appears that in the case of Angiotensin II induced migration insulin can sensitize the cells through a reactive oxygen species mediated process. The insulin-stimulated component of migration of Angiotensin II-treated cells can be blocked by inhibitors of NAD(P)H oxidase. This would indicate that insulin's stimulation of NAD(P)H oxidase activity in Angiotensin II-treated VSMC is required for increased migration. Insulin plus Angiotensin II-stimulated NAD (P)H oxidase activity has been shown to cause NO scavenging and inhibition of NO-stimulated cGMP production and thus a loss of modulation (10). This finding of enhancement of Angiotensin II cell migration by insulin is in contradistinction to the effects we witnessed with PDGF and S-1-P and suggests that insulin's effects are receptor family specific.

Conclusion

Insulin can induce transactivation of EGFR by an ADAM-mediated, HB-EGF dependent process. This is the first description of crosstalk via ADAM between insulin and EGFR in vascular SMC. Targeting a pivotal cross-talk receptor such as EGFR, which can be transactivated by both G-protein-coupled receptors and receptor tyrosine kinases is an attractive molecular target.

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1A

1B

1C

Figure 1.

Insulin (0.1–1000nM) did not induce significant smooth muscle cell migration in either the wound assay (A) and the Boyden chamber assays (B) of migration compared to S-1-P (100nM) and PDGF (10nM). Pre-incubation of Insulin (10nM) prior to S-1-P (100nM) and PDGF (10nM) did inhibit both S-1-P- and PDGF-mediated VSMC migration in both assays. Values are the mean±SEM (n=6, **p<0.01 compared to control).

2A

2B

Figure 2.

Insulin induced both PI3K and ERK1/2 phosphorylation in the VSMC in a time dependent manner (A). Insulin induced a 6-fold increase in EGFR phosphorylation (A) and this response was inhibited in a concentration-dependent manner by increasing concentrations of AG1478 (0.1–1000nM) (B). Incubation of insulin with the EGFR inhibitor AG1478 (EGFR inhibitor but not AG1024 (IGFR inhibitor) inhibited EGFR phosphorylation (C). Values are the mean $\pm$ SEM of the ratio of the phosphorylation of the respective protein relative to the total unphosphorylated protein (n=6, **p<0.01 compared to control). Representative Western blots are shown above the graphs.

Figure 3.

EGFR phosphorylation by insulin (10nM) was inhibited by blockade of the ligand HB-EGF with the HBEGF inhibitor CRM197 and an Anti-HB-EGF antibody. IgG had no effect. EGF-mediated activation of EGFR was not blocked by these inhibitors. Direct blockade of the EGFR prevented activation by both insulin and EGF. Values are the mean $\pm$ SEM of the ratio of the phosphorylation of the respective protein relative to the total unphosphorylated protein (n=6, *p<0.05 compared to control). Representative Western blots are shown above the graphs.

Figure 4.

Insulin (10nM) induced EGFR phosphorylation was not inhibited by the plasmin inhibitors (ϵ -aminocaproic acid -EACA- and aprotinin). Pre-incubation with the matrix metalloprotease inhibitor GM6001 and the ADAM inhibitors TAPI-0 and TAPI-1 blocked Insulin induced EGFR phosphorylation. Values are the mean $\pm$ SEM of the ratio of the phosphorylation of the respective protein relative to the total unphosphorylated protein (n=6, *p<0.05 compared to control). Representative Western blots are shown above the graphs.

Figure 5.

Insulin (10nM) induced EGFR phosphorylation was inhibited by the oxygen free radical generation inhibitor N-acetylcysteine and the NAD(P)H oxidase inhibitor DPI. Values are the mean $\pm$ SEM of the ratio of the phosphorylation of the respective protein relative to the total unphosphorylated protein (n=6, **p<0.01 compared to control).

Figure 6.

Pre-incubation of with insulin (10nM) and either ADAM inhibitors TAPI-0 (20 μ M) or TAPI-1 (10 μ M) for 30 minutes prior to exposure to prior to S-1-P (100nM) and PDGF (10nM). The presence of the ADAM inhibitors removed the insulin inhibition of PDGF-mediated VSMC migration in the Boyden chamber. S-1-P-mediated VSMC migration in the presence of insulin was unchanged but both the response in the control and insulin treated cells in the presence of TAPI-0 and TAPI-1 was equivalent. Values are the mean $\pm$ SEM (n=6, *p<0.05, **p<0.01 compared to control).