Microvascular Research 79 (2010) 121–127

Contents lists available at ScienceDirect

Microvascular Research

journal homepage: www.elsevier.com/locate/ymvre

Regular Article

High glucose and elevated fatty acids suppress signaling by the endothelium protective ligand angiopoietin-1

Harprit Singh [a], Nicholas P.J. Brindle [a,*], Victor A. Zammit [b]

[a] University of Leicester, Department of Cardiovascular Sciences, RKCSB, PO Box 65, Leicester LE2 7LX, UK
[b] University of Warwick, Warwick Medical School, Gibbet Hill Campus, Coventry CV4 7AL, UK

ARTICLE INFO

Article history:
Received 21 December 2009
Accepted 7 January 2010
Available online 15 January 2010

Keywords:
Glucose
Vascular endothelium
Angiopoietin-1
Receptor (s)

ABSTRACT

Pre-diabetes is characterized by hyperglycemia and dyslipidemia; it is associated with increased cardiovascular disease and endothelial dysfunction. Angiopoietin-1 (Ang1), a ligand for endothelial receptor, is a potent vascular protective factor important in maintaining normal endothelial function. The aim of the study was to examine the influence of elevated glucose and fatty acid concentrations on angiopoietin signaling in human cardiac microvascular endothelial cells. Incubation with 30 mM glucose caused 50% suppression in the ability of Ang1 to activate Tie2-receptor phosphorylation without any decrease in Tie2 expression or increased internalization in microvascular endothelial cells. Examination of downstream signaling revealed inhibition of Ang1-dependent Akt phosphorylation. By contrast, Ang1 activation of Erk1/2 signaling was not affected by hyperglycemia. Similar suppression of Ang1-dependent activation of Akt by hyperglycemia was observed in large vessel human endothelial cells. Incubation of microvascular endothelial cells with 200 μM palmitic acid significantly inhibited Ang1-dependent Akt phosphorylation without affecting phosphorylation of the Tie-2 receptor or of ERK1/2. Therefore, contrary to hyperglycemia, palmitate acted exclusively downstream of the receptor. The present findings suggest a mechanism by which increased glucose or fatty acids may suppress vascular protection by Ang1 and predispose to endothelial dysfunction and vascular disease.

Introduction

Diabetes is associated with a range of vascular diseases including atherosclerosis, myocardial infarction and heart failure resulting from endothelial dysfunction (Deedwania, 2003; Fornoni and Raij, 2005). Glucose intolerance and obesity are the main components of the metabolic syndrome related to increased cardiovascular risk (Eckel et al., 2005). There are a number of mechanisms by which hyperglycemia and lipid disturbances can contribute to defects in endothelial function. In particular, increased endothelial permeability, elevated leukocyte adhesion and impaired nitric oxide action are associated with hyperglycemia (Algenstaedt et al., 2003; Du et al., 2001; Morigi et al., 1998).

The angiopoietins are a family of four secreted glycoprotein ligands, angiopoietin-1 to -4, originally identified as important in blood vessel formation (Davis et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999). The best characterized of these ligands are angiopoietin-1 (Ang1) and angiopoitein-2 (Ang2). Both Ang1 and Ang2 are certainly essential for correct vascular development, with Ang1 acting primarily to enhance vascular maturation following the initial stages of neovessel formation (Suri et al., 1996). Ang2 functions to destabilize microvessels ahead of vascular growth and remodeling (Gale et al., 2002). The angiopoietins bind to the receptor tyrosine kinase Tie2 which is expressed predominantly in vascular endothelial cells (Dumont et al., 1992). Ang1 activates Tie2 receptor and its downstream signaling pathways, including the phosphatidylinositol-3-kinase and Erk1/2 pathways (Harfouche et al., 2003; Kim et al., 2000a,b). By contrast, Ang2 can act as a context-dependent antagonist, preventing Ang1 binding and suppressing Ang1 signaling (Maisonpierre et al., 1997). Overexpression of Ang2 in transgenic mice results in a similar phenotype as that seen in mice lacking Ang1, consistent with Ang2 acting as an Ang1 antagonist in vivo (Maisonpierre et al., 1997). Tie2 physically interacts with Tie1 on the endothelial cell surface in hetero-oligomeric complexes (Marron et al., 2000). Interaction between Tie2 and Tie1 allows Ang1 to stimulate Tie1 signaling via transactivation by Tie2 (Saharinen et al., 2005). In addition, Tie1 regulates the ability of Ang1 to activate Tie2, and allows Tie2 signaling to be coordinated with that of VEGF, and with inflammatory and physical effectors in the endothelial microenvironment (Marron et al., 2007; Singh et al., 2009).

In recent years, it has become evident that in addition to their involvement in vascular development the angiopoietins have crucial roles in regulating vascular function and phenotype post-development (Kim et al., 2001; Thurston et al., 2000). In the established

* Corresponding author. Fax: +44 0 116 252 3179.
E-mail address: npjb1@le.ac.uk (N.P.J. Brindle).

0026-2862/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.mvr.2010.01.005

vasculature Ang1 is a potent vascular protective agonist, acting to suppress vascular leakage, maintain endothelial survival and inhibit vascular inflammation (Brindle et al., 2006; Kim et al., 2000a,b; Thurston et al., 2000). Indeed, Ang1 is produced by perivascular cells to maintain a tonic vascular protective action on the endothelium in healthy vessels (Davis et al., 1996; Thurston et al., 2000). In agreement with the molecular observations, the vascular protective effects of Ang1 can be antagonized by Ang2 which increases vessel leakage, turnover and inflammation (Fiedler et al., 2006; Roviezzo et al., 2005). Ang2 is released from endothelial cells in response to stimuli such as thrombin (Fiedler et al., 2004) and it is the molecular ratio of Ang1:Ang2, as well as that of Tie1:Tie2 that determine the level of vascular protective signaling due to Tie2 in the endothelium (Kim et al., 2006; Scharpfenecker et al., 2005). A decrease in Ang1: Ang2 ratio, primarily due to increased Ang2, is seen in a range of diseases affecting the vasculature, including sepsis and adult respiratory distress syndrome (Parikh et al., 2006).

Given the importance of angiopoietin signaling in vascular protection, it is possible that defects in its signal transduction mechanism could contribute to endothelial dysfunction associated with conditions such as diabetes. Indeed, increased production of Ang2 has been implicated in the pericyte dropout associated with diabetic retinopathy (Hammes et al., 2004). Furthermore, Chen and Stinnett (2008) recently reported a reduction of Tie2 levels and a corresponding decrease in Tie2 signaling in db/db mice subjected to myocardial ischemia. Therefore, in the present study we have investigated the direct effects of elevated glucose and free fatty acid concentrations on angiopoietin signaling in human microvascular endothelial cells.

Materials and methods

Reagents

Recombinant angiopoietin1 and antibodies against Tie2 and Tie1 ectodomain were purchased from R&D systems. PhosphoDetect™ Tie2 antibody (pTyr $^{1102/1108}$) was purchased from Merck. Phosphotyrosine-specific, PY99 antibody was from Santa Cruz Biotechnology. Antibodies against Phospho-Tie2 (pTyr^{992}), AKT, Phospho-Ser-473-AKT, Erk1/2 and Phospho-Erk1/2 (Thr 202/Tyr 204) were from Cell Signaling Technology. Sodium Palmitate was purchased from Sigma Aldrich.

Fatty acid

Palmitate mixtures were prepared by dissolving 150 mM sodium palmitate in a 1:1 ratio of ethanol–water (1:1) at 50 °C and then incubated with 10% fatty-acid-free BSA for 1 h at 37 °C. The BSA–Palmitate complex was filter-sterilized and stored at −20 °C.

Cell culture and incubation

Human microvascular cardiac endothelial cells (HCMEC) and human umbilical vein endothelial cells (HUVEC) were purchased from PromoCell and cultured according to the manufactures' instructions. Cells were either treated with 5 or 30 mM glucose for 72 h. Cells treated with palmitate-albumin were incubated with the equivalent of 200 μM palmitate for 16 h. Before stimulation, cells were serum-starved for 1 h followed by addition of Ang1 at 100 ng/ml.

Biotinylation of cell surface proteins

Cells were cultured with either 5 or 30 mM glucose for 72 h after which cell surface proteins were biotinylated by washing cells in PBS and incubating for 30 min on ice with 250 ng/ml sulfo-NHS-SS-biotin in PBS. Cells were then quenched in 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, rinsed in PBS and lysed. Biotinylated proteins were recovered with streptavidin–agarose before performing SDS–PAGE and immunoblotting.

Immunoprecipitation and Western blotting

Cells were washed with PBS and lysed using ice-cold lysis buffer (50 mM Tris, pH 7.4; 50 mM NaCl, 1 mM sodium fluoride, 1 mM EGTA, 1 mM sodium orthovanadate, 1% Triton X-100, complete protease inhibitor mixture) followed by centrifugation at 13,000×*g* for 10 min. For immunoprecipitation, lysates containing equal amounts of protein, determined by Bradford assay, were incubated with 2 μg of antibody for 3 h in the presence of protein G agarose. Immunoprecipitates were recovered by centrifugation at 10,000×*g* for 1 min and washed 3 times with wash buffer (50 mM Tris, pH 7.4; 50 mM NaCl; 1 mM sodium fluoride, 1 mM EGTA, 1 mM sodium orthovanadate, 0.1% Triton X-100, complete protease inhibitor mixture). Proteins were eluted by the addition of Laemmli sample buffer containing 100 mM dithiothreitol and boiled for 5 min before resolved by SDS–PAGE. For the analysis of whole cell protein extracts, lysates were mixed with Laemmli buffer as above. Proteins were transferred to nitrocellulose membranes, probed with the relevant antibodies and visualized using chemiluminescent detection.

Results

Effects of hyperglycemia on Tie2 signaling in cardiac endothelial cells

Tie2 expression has been shown significantly attenuated in db/db mouse hearts subjected to myocardial ischemia and mouse heart endothelial cells subjected to hyperglycemia (Chen and Stinnett, 2008). We were interested therefore to examine the effects of elevated glucose concentration on Tie2 expression in human microvascular cardiac endothelial cells (HCMEC). To do this, HCMEC were cultured in the presence of 5 mM glucose or 30 mM glucose for 24 or 72 h before cell lysis. After 24 h hyperglycemia, the expression levels of both Tie1 and Tie2 were similar to those in control cells, with only a 10% decrease in Tie2 levels after incubation with 30 mM glucose for 72 h, which failed to reach statistical significance (Figs. 1A and B).

To test whether changes in Tie signaling occurred in the absence of changes in protein expression, we cultured HCMEC for 72 h in the presence of 5 or 30 mM glucose before stimulating them with Ang1 and analyzing receptor activation by monitoring tyrosine phosphorylation of Tie2. Immunoprecipitation of Tie2 was performed on cell lysates followed by immunoblotting with phosphotyrosine-specific antibody. Ang1-mediated Tie2 phosphorylation was significantly impaired in endothelial cells cultured in the presence of elevated glucose (Fig. 1C). Indeed the ability of Ang1 to activate Tie2 receptors was suppressed by approximately 50% under these conditions (Fig. 1D). Immunoblots of whole cell lysates visualized for specific phospho-Tie2 tyrosine residues demonstrated impaired Ang1-mediated tyrosine phosphorylation of residues 1102 and 992 (Fig. 1E). As a control, an equivalent amount of mannitol to that of high glucose was used; it showed no effect on Ang1-induced Tie2 phosphorylation (data not shown).

Suppression of the ability of Ang1 to activate Tie2 would be anticipated to attenuate its downstream signaling. This was examined by analysis of the effects of elevated glucose on Ang1 activation of Akt and Erk1/2, well-characterized signaling intermediates in the Ang1/Tie2 signal transduction cascade (DeBusk et al., 2004; Harfouche et al., 2003; Kim et al., 2000a,b). HCMEC were incubated for 72 h with 5 or 30 mM glucose before stimulation with Ang1. To monitor stimulation of Akt, whole cell lysates were prepared and levels of Akt phosphorylated on serine 473 determined by