Cellular Signalling xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Cellular Signalling

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

Regulation of contractile signaling and matrix remodeling by T-cadherin in vascular smooth muscle cells: Constitutive and insulin-dependent effects

Agne Frismantiene [a,b,1], Dennis Pfaff [a,b,1], Audrey Frachet [a,b], Matteo Coen [c], Manjunath B. Joshi [a,b,2], Kseniya Maslova [a,b], Marie-Luce Bochaton-Piallat [c], Paul Erne [d], Therese J. Resink [a,b,*], Maria Philippova [a,b]

[a] Department of Biomedicine, Laboratory for Signal Transduction, University Hospital of Basel, Basel, Switzerland
[b] Department of Biomedicine, Laboratory for Signal Transduction, University of Basel, Basel, Switzerland
[c] Department of Pathology and Immunology, University of Geneva, Faculty of Medicine, Switzerland
[d] Hirslanden Klinik St Anna, Luzern, Switzerland

ARTICLE INFO

Article history:
Received 9 April 2014
Received in revised form 29 April 2014
Accepted 2 May 2014
Available online xxxx

Keywords:
T-cadherin
Vascular smooth muscle cells
Insulin resistance
Signal transduction
Remodeling

ABSTRACT

Expression of GPI-anchored T-cadherin (T-cad) on vascular smooth muscle cells (VSMCs) is elevated in vascular disorders such as atherosclerosis and restenosis which are associated with insulin resistance. Functions for T-cad and signal transduction pathway utilization by T-cad in VSMC are unknown. The present study examines the consequences of altered T-cad expression on VSMC for constitutive and insulin-induced Akt/mTOR axis signaling and contractile competence. Using viral vectors rat (WKY and SHR) and human aortic VSMCs were variously transduced with respect to T-cad-overexpression (Tcad+-VSMC) or T-cad-deficiency (shT-VSMC) and compared with their respective control transductants (E-VSMC or shC-VSMC). Tcad+-VSMC exhibited elevated constitutive levels of phosphorylated Akt^{ser473}, $GSK3\beta^{ser9}$, $S6RP^{ser235/236}$ and $IRS\text{-}1^{ser636/639}$. Total IRS-1 levels were reduced. Contractile machinery was constitutively altered in a manner indicative of reduced intrinsic contractile competence, namely decreased phosphorylation of $MYPT1^{thr696\ or\ thr853}$ and $MLC_{20}{}^{thr18/ser19}$, reduced RhoA activity and increased iNOS expression. Tcad+-VSMC-populated collagen lattices exhibited greater compaction which was due to increased collagen fibril packing/reorganization. T-cad+-VSMC exhibited a state of insulin insensitivity as evidenced by attenuation of the ability of insulin to stimulate Akt/mTOR axis signaling, phosphorylation of MLC_{20} and MYPT1, compaction of free-floating lattices and collagen fibril reorganization in unreleased lattices. The effects of T-cad-deficiency on constitutive characteristics and insulin responsiveness of VSMC were opposite to those of T-cad-overexpression. The study reveals novel cadherin-based modalities to modulate VSMC sensitivity to insulin through Akt/mTOR axis signaling as well as vascular function and tissue architecture through the effects on contractile competence and organization of extracellular matrix.

1. Introduction

Insulin resistance is classically defined as the inability of insulin to exert its metabolic actions. In vascular tissue insulin has non-metabolic functions crucial to vascular and hemodynamic homeostasis, including preservation of endothelial-dependent vasorelaxation, reduction of endothelial cell (EC) apoptosis [1], and maintenance of the differentiated, contractile vascular smooth muscle cell (VSMC) phenotype [2]. Pathophysiological consequences of vascular insulin resistance encompass endothelial dysfunction (ED), hypertension, microvessel disease, vascular inflammation and atherogenesis [1,3]. Thus it is important to understand the cell-specific actions of insulin in vascular cells as well as the mechanisms leading to impaired insulin responsiveness in vascular cells and functional consequences thereof. Acquisition of vascular insulin resistance with its concomitant disturbance of vascular homeostasis is linked to ED, characterized by an impaired ability of

Abbreviations: E, empty vector control transductant; EC, endothelial cell; ECM, extracellular matrix; ED, endothelial dysfunction; Ins, insulin; IRS-1, insulin receptor substrate-1; MLC_{20}, myosin light chain 20; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; MYPT1, myosin phosphatase targeting subunit 1; shC, non-coding shRNA control transductant; shT, T-cadherin shRNA silenced transductant; T-cad, T-cadherin; Tcad+, T-cadherin overexpressing transductant; VSMC, vascular smooth muscle cell; VSMC-PCL, vascular smooth muscle cell-populated collagen lattice.

* Corresponding author at: University Hospital of Basel, Department of Biomedicine, Laboratory for Signal Transduction, Hebelstrasse 20, CH 4031 Basel, Switzerland. Tel.: +41 612652422; fax: +41 612652350.
E-mail address: Therese-J.Resink@unibas.ch (T.J. Resink).

[1] These authors contributed equally to this study.
[2] Current address: Department of Biotechnology, Manipal Life Sciences Center, Manipal University, Manipal, India.

http://dx.doi.org/10.1016/j.cellsig.2014.05.001
0898-6568/

insulin to induce eNOS activation [1,3]. However, controversy remains regarding whether ED arises because of the whole-body milieu typical of insulin resistant states (*e.g.* inflammation, oxidative stress, hyperinsulinemia, hyperglycemia) or whether insulin resistance in EC *per se* disturbs endothelial function [4]. VSMC-specific effects contributing to, or arising because of, vascular insulin resistance are difficult to infer from clinical studies and *in vivo* experimental models because of confounding influences on VSMC arising secondarily to ED [4].

We previously described a novel cadherin-based mechanism for regulation of insulin sensitivity in EC [5]. GPI-anchored T-cadherin (T-cad) is constitutively expressed on EC and functions to promote survival, migration, proliferation and angiogenesis [6–8]. Central to these protective functions of T-cad in EC is its ability to stimulate signal transduction *via* the PI3K/Akt pathway [7,9]. However, T-cad upregulation on EC induces constitutive PI3K/Akt/mTOR pathway hyperactivation [9] which has deleterious consequences of persistent activation of the negative feedback loop of the insulin cascade and enhanced IRS-1 degradation [5]. Accordingly chronic T-cad upregulation engenders a state of insulin resistance in EC, manifest as blunted insulin-stimulated eNOS activation, migration and angiogenesis [5].

It has long been recognized that VSMC also constitutively expresses T-cad [10] but remarkably little is known regarding its functions in this cell type. Histological studies demonstrated the upregulation of T-cad in intimal VSMC during atherosclerosis, most prominently within lesions of high disease severity [11], and also during the period of active tissue reparation/restenosis following arterial injury [12]. Consistent with these immunohistological findings are *in vitro* observations that T-cad expression on VSMC is higher during proliferation than at quiescence [13] and that ectopic T-cad overexpression increases proliferation [6]. VSMC proliferation induced by some tyrosine kinase-linked receptor agonists (*e.g.* PDGF) and G-protein-coupled receptor agonists (*e.g.* Ang II, ET-1) has been associated with attenuated insulin responsiveness through agonist-promoted PI3K/Akt/mTOR axis signaling and subsequent feedback serine phosphorylation and degradation of IRS-1/IRS-2 [14–16]. Since T-cad upregulation on VSMC occurs in vascular disorders that are linked to insulin resistance and in association with increased proliferation [11,12] we questioned whether the upregulation of T-cad might also participate in the development of insulin resistance in VSMC.

Signal transduction pathway utilization by T-cad in VSMC has never been studied. The PI3K/Akt/mTOR pathway is a major intracellular target of T-cad in EC, and its constitutive hyperactivation in T-cad overexpressing EC was identified as the mechanism underlying attenuation of insulin signaling in EC [5,7,9]. Further, PI3K/Akt/mTOR signaling axis plays a central role in VSMC proliferation and contractile competence [17] and in the development of insulin resistance [18]. Therefore, we investigated the influence of altered T-cad expression on constitutive and insulin-induced Akt/mTOR pathway activity and on contractile competence in VSMC.

2. Materials and methods

2.1. Cell culture and transduction

VSMC was normally maintained in DMEM (containing 5.5 mM glucose) supplemented with 10% FCS. The isolation and characterization of VSMC from the descending thoracic aorta of 20-week old male Wistar Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats have been detailed before [19]. T-cad was stably overexpressed (Tcad+) in rat VSMC using pLVX-puro vector carrying full length human T-cad cDNA (empty pLVX-puro vector served as control (E)) [5]. For each transduction of WKY-VSMC and SHR-VSMC, 4–5 different isolates at passages 2–3 were randomly selected from our cryostocks of VSMC isolates from 8 WKY to 8 SHR animals and pooled. Human aortic VSMC (Hu-VSMC) cultures were obtained from Lonza (Basel, Switzerland) and transduced following expansion to passage 3. Hu-VSMC was transduced to either transiently or stably overexpress T-cad using Adeno-X [6] or pLVX-puro [5] vectors; respective empty vectors served as the controls (E). Additionally, stable transductants of T-cad-deficient Hu-VSMC (shT) were generated using pLKO.1-puro vector carrying human T-cad shRNA or non-target shRNA as control (shC) [5]. We used both transient adenovirus-mediated (adeno) and stable lentivirus-mediated (lenti) transduction because (1) it enabled us to exclude any artifacts related to transduction protocols and number of cell passages, and (2) lentiviral approach allows generation of stable T-cad-silenced VSMC transductants. Transient and/or stable transduction protocols were performed on at least three separate occasions for any given VSMC species or strain. Transiently transduced Hu-VSMC was used at up to 2–3 passages after transfection. Stably transduced rat-VSMC and Hu-VSMC were used at up to passages 8–10 after puromycin (3.6 μg/ml) selection.

2.2. Immunoblotting

VSMC was seeded (2×10^5 cells/well in 6-well plates), allowed overnight adherence and further cultured for 24 h in DMEM/0.1% BSA, followed by DMEM/0.1% BSA refreshment and a 1 h resting incubation period before exposure to insulin (Actrapid, Novo Nordisk, Mainz, Germany). Whole cell lysates were prepared and analyzed by immunoblotting as described [9]. Primary antibodies against the following proteins/epitopes were used: T-cad (R&D Systems Europe Ltd., Abingdon, UK), phospho (p)-Akt^{ser473}, Akt, p-GSK3β^{ser9}, GSK3β, S6RP, p-S6RP$^{ser235/236}$, IRS-1, p-IRS-1$^{ser636/639}$, and p-MLC$_{20}^{thr18/ser19}$ (Cell Signalling, New England Biolabs GMBH, Frankfurt, Germany), p-MYPT1^{thr696}, p-MYPT1^{thr853}, and β-actin (Santa Cruz Biotechnology Inc., Heidelberg, Germany), iNOS/NOS Type II (BD Biosciences, Allschwil, Switzerland) and GAPDH (Abcam, Cambridge, UK). β-Actin, GAPDH or total Akt was variously used as loading controls. Representative blots are shown.

2.3. VSMC-populated collagen lattice compaction assay

To prepare VSMC-populated collagen lattices (VSMC-PCLs) VSMC suspensions in DMEM/0.1% BSA (4×10^5 cells/ml) were mixed 1:1 with neutralized and diluted (3 mg/ml) collagen solution (BD Biosciences), aliquoted into 48-well plates (250 μl/well, triplicate wells per condition) and allowed to polymerize. The gel–dish interface was released using a curved spatula followed by the addition of DMEM/0.1% BSA (0.5 ml/well) without or with inclusion of insulin to wells. After incubation (20 h) VSMC-PCL areas were measured morphometrically using Cell^P software (Soft Imaging System GmbH, Munich, Germany).

2.4. In-gel proliferation and viability assays

To assay proliferation freshly prepared VSMC-PCLs were overlaid with DMEM/0.1% BSA containing a 1:25 dilution of Alamar Blue solution (AbD Serotec, Düsseldorf, Germany) and absorbance (560/590 nm) was measured after 3 h (baseline) and 20 h of incubation. Live–dead assay for viability was performed at the end of the 20 h incubation period. VSMC-PCLs were washed twice with PBS, and after the addition of PBS containing calcein-AM (10 μM) (Life Technologies, Basel, Switzerland) and propidium iodide (500 nM) (Sigma-Aldrich, Buchs, Switzerland) the lattices were incubated for 10 min at 37 °C. Images of 3 randomly selected fields/gel were captured under an inverted fluorescence Olympus IX50 microscope. Proportions of calcein-AM-positive (live) and propidium iodide-positive (dead) cells were calculated after enumeration using Cell^P software.

2.5. Picrosirius Red staining and image analysis

VSMC-PCLs were incubated (20 h) either as free-floating lattices or as unreleased lattices (*i.e.* without disturbance of the gel–dish interface), washed with PBS, fixed in-plate (4% paraformaldehyde, 10 min), rinsed twice with PBS and embedded in paraffin. Sections (10 μm) were stained with Picrosirius Red to label collagen and analyzed by

Please cite this article as: A. Frismantiene, et al., Cellular Signalling (2013), http://dx.doi.org/10.1016/j.cellsig.2014.05.001