Journal of Molecular and Cellular Cardiology 62 (2013) 1–7

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Original article

ADAM17 silencing by adenovirus encoding miRNA-embedded siRNA revealed essential signal transduction by angiotensin II in vascular smooth muscle cells

Katherine J. Elliott [1], Allison M. Bourne [1], Takehiko Takayanagi, Akira Takaguri, Tomonori Kobayashi, Kunie Eguchi, Satoru Eguchi *

Cardiovascular Research Center and Department of Physiology, Temple University School of Medicine, Philadelphia, PA 19140, USA

ARTICLE INFO

Article history:
Received 24 February 2013
Received in revised form 24 April 2013
Accepted 6 May 2013
Available online 17 May 2013

Keywords:
ADAM17
Epidermal growth factor receptor
Angiotensin II type 1 receptor
Signal transduction
Vascular biology

ABSTRACT

Small interfering RNA (siRNA) mediated gene silencing has been utilized as a powerful molecular tool to study the functional significance of a specific protein. However, due to transient gene silencing and insufficient transfection efficiency, this approach can be problematic in primary cell culture such as vascular smooth muscle cells. To overcome this weakness, we utilized an adenoviral-encoded microRNA (miRNA)-embedded siRNA "mi/siRNA"-based RNA interference. Here, we report the results of silencing a disintegrin and metalloprotease 17 (ADAM17) in cultured rat vascular smooth muscle cells and its functional mechanism in angiotensin II signal transduction. 3 distinct mi/siRNA sequences targeting rat ADAM17 were inserted into pAd/CMV/V5-DEST and adenoviral solutions were obtained. Nearly 90% silencing of ADAM17 was achieved when vascular smooth muscle cells were infected with 100 multiplicity of infection of each ADAM17 mi/siRNA encoding adenovirus for 3 days. mi/siRNA-ADAM17 but not mi/siRNA-control inhibited angiotensin II-induced epidermal growth factor receptor trans-activation and subsequent extracellular signal-regulated kinase activation and hypertrophic response in the cells. mi/siRNA-ADAM17 also inhibited angiotensin II-induced heparin-binding epidermal growth factor-like factor shedding. This inhibition was rescued with co-infection of adenovirus encoding mouse ADAM17 but not by its cytosolic domain deletion mutant or cytosolic Y702F mutant. As expected, angiotensin II induced tyrosine phosphorylation of ADAM17 in the cells. In conclusion, ADAM17 activation via its tyrosine phosphorylation contributes to heparin-binding epidermal growth factor-like factor shedding and subsequent growth promoting signals induced by angiotensin II in vascular smooth muscle cells. An artificial mi/siRNA-based adenoviral approach appears to be a reliable gene-silencing strategy for signal transduction research in primary cultured vascular cells.

1. Introduction

Angiotensin II (Ang II), the major bioactive hormone of the renin-angiotensin system, has long been implicated in contributing to various cardiovascular diseases such as hypertension, atherosclerosis and heart failure. Within the vasculature, vascular smooth muscle cells (VSMCs) primarily express the Ang II type 1 (AT_1) receptor, which mediates most of the pathological functions of Ang II [1,2]. In VSMC, AT_1 receptor signal transduction is known to involve many signaling proteins and pathways to mediate Ang II-induced VSMC hypertrophy, proliferation, and/or migration [2,3]. A disintegrin and metalloproteases (ADAMs) are a family of membrane-anchored, zinc-dependent metalloproteases [4]. Among many ADAMs, ADAM17 is primarily responsible for the ectodomain shedding of several critical substrates including precursors of epidermal growth factor (EGF) receptor ligands [4,5]. Our data from VSMC permanently over-expressing a catalytically inactive dominant-negative mutant of ADAM17 suggest the importance of this metalloprotease in mediating heparin-binding EGF-like growth factor (HB-EGF) generation and subsequent "trans"-activation of EGF receptor induced by Ang II [6].

Recent studies suggest potential roles of ADAM17 in mediating cardiovascular diseases. ADAM17 expression is enhanced in atherosclerotic lesions in apoE−/− mice and humans [7,8]. We have shown that over-expression of dominant-negative mutant ADAM17 by adenovirus reduced VSMC hyperplasia in response to arterial injury [9]. ADAM17 is also implicated in hypertensive cardiac hypertrophy in rodents [10]. Moreover, an ADAM17 polymorphism was associated with cardiovascular mortality [11].

* Corresponding author at: Room 1051 MERB, Temple University School of Medicine, 3500 N. Broad Street, Philadelphia, PA 19140, USA. Tel.: +1 215 707 0169; fax: +1 215 707 5737.
E-mail address: seguchi@temple.edu (S. Eguchi).
[1] The first 2 authors contributed equally to this work.

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http://dx.doi.org/10.1016/j.yjmcc.2013.05.005

Despite these studies, the molecular mechanism by which ADAM17 activity is enhanced in the cardiovascular system such as by Ang II remains largely unclear. However, post-transcriptional modification including Thr735 phosphorylation has been proposed as one potential mechanism by which extracellular stimuli increase ADAM17 activity [12]. Given more than 30 ADAM family members, many of them ubiquitously expressed, the over-expression approach such as with an ADAM17 dominant-negative mutant likely involves adverse effects influencing other ADAMs in the system. The approach is also unsuitable for mechanistic studies to look for the ADAM17 catalytic regulation by Ang II. While small interfering RNA (siRNA) technology offers a way to specifically silence target protein expression, the limitations of siRNA include poor transfection efficiency in primary cultured cells and a transient nature of gene silencing [13]. Viral vector-based siRNA technology in particular, an engineered microRNA (miRNA)-embedded siRNA "mi/siRNA" approach, should offer the flexibility to select various potent promoter drivers and a high transduction efficiency to specifically silence proteins [14–16] with a long half life such as ADAM17 [17] in primary cultured cells.

In this study, we made adenoviral vectors encoding engineered mi/siRNAs to target rat ADAM17 and significantly suppressed ADAM17 expression in rat VSMC. We showed that silencing ADAM17 expression using this adenovirus inhibited Ang II-mediated HB-EGF shedding and subsequent growth promoting signal transduction. We then tested our hypothesis that Ang II activates ADAM17 via tyrosine phosphorylation by silencing endogenous rat ADAM17 with ADAM17 mi/siRNA adenovirus in VSMC and replacing it with a mouse Y702F ADAM17 mutant.

2. Materials and methods

2.1. Reagents

HEK293A cell line and all cloning materials were purchased from Invitrogen. Ang II was purchased from Sigma-Aldrich. FuGene 6 was purchased from Roche. Antibodies against Tyr204-phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), ERK2, and ADAM17 (sc-7383, sc-154, and sc-13973) were purchased from Santa Cruz Biotechnology. Antibody for Tyr1068-phosphorylated EGF receptor (44788G) was purchased from Invitrogen. Antibody for GAPDH (MRB374) was purchased from Millipore. Antibody for ADAM9 (M61420) was purchased from BD Biosciences. Antibody for phospho-tyrosine (05-321) was purchased from Millpore. Antibody for phospho-serine/threonine (61-8100) was purchased from Zymed.

2.2. Generation of recombinant adenoviruses

Replication-incompetent recombinant adenoviruses for RNA interference expressing engineered pre-miRNA encoding murine miR-155 stem loop and embedded siRNAs targeting rat ADAM17 mRNA were constructed using the BLOCK-iT™ Adenoviral RNAi Expression System (Invitrogen) according to the manufacturer's instructions. In this system, the virally encoded construct contains, in order: 21 nucleotide antisense target sequence, 19 nucleotide miR-155 derived loop sequence and sense target sequence nucleotides 1–8 and 11–21 (Supplementary Fig. A1). This sequence forms the basis of the synthetic "siRNA-embedded pre-miRNA" and contains the correct stem loop structure. This cassette is flanked by pri-miRNA sequence based on native miR-155. The entire expression cassette will form a stem-loop precursor/pri-miRNA and be processed by Drosha and Dicer to form pre-miRNA and the mature mi/siRNA, respectively. The mi/siRNA would then be incorporated into RNA-induced silencing complex (RISC) for cleavage of target mRNA [18]. Three distinct 21mer oligonucleotides encoding for the mi/siRNAs perfectly complementary to coding regions of rat ADAM17 mRNA (Accession: NM_020306 GI: 9945329) were designed using the Invitrogen BLOCK-iT™ RNAi online designer program and were subsequently cloned into the pcDNA™ 6.2-GW/EmGFP-miR vector. The regions of rat ADAM17 coding sequences matching the mi/siRNAs, their homologies to mouse and rat ADAM17, and a Blast search results covering rat gene coding regions as well as 3′UTRs are shown in Supplemental Fig. A2. Note that the silencing system of mi/siRNAs is mechanistically distinct from endogenous miRNAs, which reduce expression of multiple targets via interaction to target 3′UTRs with imperfect complementarity [19]. For convenience, we abbreviated the miRNA-embedded ADAM17 mi/siRNA as miA17. These sequences included: miA17-94 to target residue 94–114 of ADAM7 mRNA: 5′-CTT GAG AAG CTT GAT TCT TTG-3′, miA17-450 to target residue 450–470 of ADAM7 mRNA: 5′-GCC ACT TTG GAG GTT TGT TAA-3′, and miA17-724 to target residue 724–744 of ADAM7 mRNA: 5′-GGA GAA GAG AGC ACT ACT ACA-3′. The pcDNA™6.2-GW/EmGFP-miR control plasmid (Invitrogen) with a 21mer sense target sequence: 5′-GTC TCC ACG CGC AGT ACA TTT-3′, which is predicted not to target any known mammalian gene, was used as a scramble control (referred to as miCon). Adenoviruses encoding these mi/siRNAs were generated using the ViraPower™ Adenoviral Expression System (Invitrogen) according to the manufacturer's instructions to produce crude adenoviral stocks. Adenoviral vectors encoding wild-type mouse ADAM17 (wtADAM17), its cytoplasmic domain deletion mutant (1-699) and Y702F mutant were created using the mammalian expression vectors as the template [9,20]. Viral titers were calculated as previously described [21] and are expressed in units of multiplicity of infection (MOI).

2.3. VSMC culture and adenoviral infection

VSMC were prepared from the thoracic aorta of male Sprague–Dawley rats (~350 g) by the explant method and cultured as previously described [22]. Prior to infection, VSMCs from passages 3 to 10 at 80–90% confluence in culture wells were made quiescent by incubation with serum-free medium for 2–3 days. VSMCs were infected with adenovirus as described [23] with modification to include 3% FuGENE6 to enhance infection efficiency as reported in other cell lines [24]. To avoid any potential phenotypic alteration, VSMCs have been renewed every 2–3 months and VSMCs from frozen stock were never used.

2.4. Plasmid transfection in HEK293 A cells

Rat ADAM17 cDNA was cloned by a PCR reaction from rat VSMC mRNAs and subcloned into pcDNA3 IRES DsRed vector. C-terminus HA tag was included by PCR reactions. The pcDNA3 rat ADAM17-HA DsRed vector (1.25 μg) and pcDNA™ 6.2-GW/EmGFP-miR vector encoding ADAM17 mi/siRNAs (3.75 μg) or control mi/siRNA (3.75 μg) were co-transfected to 5×10^5 HEK293A cells seeded on a 6 well plate using 1% lipofectamin in 1 mL optiMEM medium for 3 h under manufacturer's instruction. After the transfection, 1 mL DMEM with 10% fetal bovine serum was added and the cells were incubated for 2 days.

2.5. Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as previously described [22]. Cell lysates or immunoprecipitation lysates were subjected to SDS PAGE gel electrophoresis and transferred to nitrocellulose membranes by electrophoresis. Membranes were incubated with primary antibodies as indicated at 4 °C overnight, exposed to horseradish peroxidase conjugated secondary antibodies for 1–2 h and proteins of interest were detected using chemiluminescent substrate (Thermo Scientific) according to manufacturer's instructions.

2.6. Hypertrophy assay

To assess Ang II-induced VSMC hypertrophy directly, we measured cell protein accumulation [21], but did not use a radiolabeled leucine incorporation assay in order to avoid unnecessary use of a radioactive