Biochemical Pharmacology 84 (2012) 1643–1650

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

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

The (pro)renin receptor ((P)RR) can act as a repressor of Wnt signalling

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ARTICLE INFO

Article history:
Received 11 July 2012
Accepted 18 September 2012
Available online 26 September 2012

Keywords:
(Pro)renin receptor
Wnt
Dvl
Beta-catenin
Furin
Aliskiren

ABSTRACT

The (pro)renin receptor ((P)RR) and Wnt signalling are both involved in different diseases ranging from cardiac and renal end-organ damage to cancer. (P)RR function involves signalling via the transcription factor promyelocytic leukemia zinc finger protein (PLZF) as well as the furin-mediated generation of vacuolar proton-translocating ATPase (V-ATPase)-associated and soluble (P)RR isoforms. Recently, the (P)RR was described as adaptor protein of Wnt (co)receptors. The aim of this study was to analyse the contribution of these distinct (P)RR functions to Wnt signalling.

Using Tcf/Lef reporter gene systems in HEK293T and HepG2 cells and quantification of endogenous axin2 mRNA and protein levels in HEK293T cells we were able to demonstrate that full-length (P)RR acts as a repressor of Wnt signalling in a system preactivated either by Wnt3a stimulation or by constitutively active β-catenin. These repressive effects are mediated by Dvl but are independent of the mutation status of β-catenin. Furthermore, the V-ATPase complex, but not PLZF translocation or renin enzymatic activity, is necessary for the induction of Tcf/Lef-responsive genes by Wnt3a.

Our data indicate interference of (P)RR and Wnt cascades, a fact that has to be considered concerning pathophysiology of cardio-renal and oncological entities as well as in drug development programs targeting (P)RR or Wnt pathways.

1. Introduction

The gene encoding the (pro)renin receptor ((P)RR also termed RER) was initially discovered in a (pro)renin independent context as vacuolar proton-translocating ATPase (V-ATPase) membrane sector-associated protein M8-9 (ATP6M8-9, also known as ATP6AP2) [1]. Four years later, the ATP6AP2 gene obtained its actual name (pro)renin receptor based on the discovery that binding of prorenin and renin to this receptor initiate an angiotensin-independent signal transduction cascade involving mitogen-activated protein kinases (MAPKs) [2]. Our group revealed one (pro)renin-dependent (P)RR signal transduction pathway mediated by physical interaction between the receptor and the transcription factor promyelocytic leukemia zinc finger protein (PLZF) [3,4].

Furthermore, a soluble isoform of the (P)RR (s(P)RR) corresponding to the extracellular (intravesicular) part of this receptor, which is generated by furin and/or ADAM19, was described [5–7]. The remaining transmembrane and cytoplasmic portion of the (P)RR most likely corresponds to the M8-9 protein [1,7–9].

Besides the ligand-mediated effects, there is more and more evidence that indicates an additional constitutive (i.e., (pro)renin-independent) function of the (P)RR. In invertebrates such as *C. elegans*, which in contrast to mammalian and non-mammalian vertebrates do not express the ligand renin, the (P)RR gene product is essential for survival [10,11] suggesting a ligand-independent function of the (P)RR in early phylogenesis. Regarding ontogenesis, cardiomyocyte-specific [12] as well as podocyte-specific [13,14] (P)RR knockout mice are characterised by cell death phenotypically similar to V-ATPase dysfunction [15]. The importance of the constitutive activity is further supported by the fact that plasma (pro)renin levels [16] as well as tissue (pro)renin concentrations [15,17] are too low to be of biological relevance with respect to (P)RR activation. In 2010, the (P)RR was described as adaptor protein linking Wnt (co)receptors with the V-ATPase complex [18] and as being essential for canonical as well as non-canonical Wnt signalling [18–20]. The Wnt pathway itself was named after the *Drosophila* gene wingless (Wg) and vertebrate protooncogene integration site-1 (int-1) [21,22]. There is experimental evidence that the (P)RR function within the Wnt pathway is (pro)renin-independent [18]. In consequence, it was even questioned whether the (pro)renin receptor deserves its name [15].

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http://dx.doi.org/10.1016/j.bcp.2012.09.020

The (P)RR seems to be a crucial determinant for the development of renal and cardiac end-organ damage in diabetes mellitus and arterial hypertension because an inhibition of its ligand activation prevents or attenuates these entities and related parameters such as proteinuria, glomerulosclerosis, left ventricular mass index and cardiac fibrosis [7,23,24].

Moreover, (P)RR expression is upregulated on the mRNA and protein levels in cardiomyopathy [25] and diabetic nephropathy [26,27]. Besides its important role in oncology, the Wnt pathway is also linked to cardiovascular diseases. For example, Dvl and β-catenin knockouts are characterised by a reduced cardiac hypertrophy and an improved post infarction ventricular function, respectively, [28] and podocyte-specific knockout of β-catenin protects against development of albuminuria [29].

The impact of the different (P)RR facets on Wnt function is currently unknown. Therefore, the aim of this study was to analyse the contribution of the distinct (P)RR functions to Wnt signalling using siRNA approaches as well as the specific V-ATPase inhibitor bafilomycin A1 [30], the PLZF translocation blocker genistein [31] and the renin inhibitor aliskiren [32].

2. Materials and methods

2.1. Cell culture

HepG2 cells (DSMZ, Braunschweig, Germany) were grown in RPMI 1640 medium (Life Technologies, Darmstadt, Germany). HEK293T cells (DSMZ) were cultured in High Glucose DMEM (Life Technologies). All media contained 10% foetal bovine serum (Life Technologies), 100 U/ml penicillin and 100 μg/ml streptomycin (A2212 Penicillin/Streptomycin; Biochrom, Berlin, Germany). All cell lines were cultured in a humidified incubator at 5% CO_2 and 37 °C.

2.2. Preparation of conditioned media

Mouse L cells (CRL-2648; ATCC, Manassas, USA) and mouse L cells stably transfected with a mouse Wnt3a expression vector (CRL-2647; ATCC) were cultured in High Glucose DMEM containing 10% foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin and in case of the Wnt3a-transfected cells additional 500 μg/ml G-418 sulphate (PAA, Pasching, Austria) for selection. Conditioned media were obtained as proposed by the supplier in absence of G-418 and stored at 4 °C. All experiments were carried out with the same batch used as a 1:1 mixture with fresh High Glucose DMEM medium.

2.3. Western blotting

Immunoblotting of the conditioned media was performed as described recently [33]. Recombinant human Wnt3a (5036-WNP/CF; R&D Systems, Minneapolis, USA) served as positive control. Anti-Wnt3a (#2391; Cell Signalling, Frankfurt a. M., Germany) was used as primary antibody and detection of the horseradish peroxidase (HRP)-labelled secondary antibody (swine anti-rabbit-IgG HRP; P0217; Dako Diagnostica GmbH, Hamburg, Germany) was performed with an enhanced chemoluminescence (ECL) reagent containing a 1:1 mixture of solution A (100 mM Tris base, pH 8.5; 2.5 mM luminol (Sigma–Aldrich, Taufkirchen, Germany); 0.4 mM p-coumaric acid (Sigma–Aldrich)) and solution B (100 mM Tris base, pH 8.5; 0.02% H_2O_2).

For analysis of (P)RR and axin2, cell lysis and Western blotting were performend as described recently [33] using anti-ATP6AP2 (rabbit; HPA003156; Sigma–Aldrich) and anti-axin2 (rabbit; ab32197; Abcam, Cambridge, UK) antibodies, respectively. An anti-β-actin anitbody (mouse; sc-81178; Santa Cruz, Heidelberg, Germany) served for standardisation. Swine anti-rabbit-IgG HRP (P0217; Dako Diagnostica GmbH) and rabbit anti-mouse-IgG HRP (P0161; Dako Diagnostica GmbH) were used as secondary antibodies.

2.4. Subcloning and transient transfection experiments

A sequence of 7 Tcf/Lef motifs and the corresponding 7 mutated motifs were subcloned into the pGL4.14 firefly luciferase vector (Promega, Mannheim, Germany) using a synthesized double-stranded oligonucleotide (144 bp including restriction sites, 138 bp after restriction digest; Eurofins, Hamburg, Germany) containing the mutated Tcf/Lef sequence derived from Super 8x FOPFlash vector [34] (5′-GAGCTCTTACGCGCCTTTGGCCTGGG-TACCTTTGGCCTGGGTACCTTTGGCCTGGCGCGCCTTTGGCCTGGG-TACCTTTGGCCTGGGTACCTTTGGCCTGGG-TACCTTTGGCCTGGCGCGCCCGCGTGCTAGCCCGGGCTCGAG-3′ (sense strand)) and the restricted non-mutated Tcf/Lef motifs (138 bp) of Super 8x TOPFlash vector [34] (5′-GAGCTCTTACGCGA-GATCAAAGGGGGTAAGATCAAAGGGGGTAAGATCAAAGGGGCGCGA-GATCAAAGGGGGTAAGATCAAAGGGGGTAAGATCAAAGGGGGTAA-GATCAAAGGGGCGCGCCCTCGTGCGGGCCCGGGCTCGAG-3′ (sense strand)) (plasmid 12456; Addgene, Cambridge, USA), respectively.

The full-length human furin coding sequence was cloned into pCEP4 vector (Life Technologies) using the primers 5′-AGGAAGCT-TATGGAGCTGAGGCCCTGGTT-3′ (forward) and 5′-CCTGC-GGCCGCTCAGAGGGCGCTCTGGTCTTTGATAAAGG-3′ (reverse). Transient transfection experiments were perfomed using Lipofectamin 2000 (Life Technologies) transfection reagents according to manufacturer's protocols with a total amount of 25 ng plasmid/cm^2 (20 ng/cm^2 firefly-encoding plasmid plus 5 ng/cm^2 renilla-encoding plasmid phRL-null (Promega)); for cotransfections additional 25 ng/cm^2 of the expression vector pCEP4 or the respective insertless control were used.

SiRNA experiments were performed with siRNA against the (P)RR [3] [5′-GCUCCGUAAUCGCCUGUUU-3′ (sense strand); 20 nM final] or scrambled control siRNA [5′-UUUACCGUCGCCUUGAGCU-3′ (sense strand)] (Eurogentec, Köln, Germany) and against dishevelled (Dvl) [35] [Dvl-1/-3: 5′-AACAAGAUCACCUUCUCC-GAGtt-3′ (sense strand); 10 nM and Dvl-2: 5′-AACUUUGAGAA-CAUGAGCAACtt-3′ (sense strand); 10 nM] (Eurogentec) or control siRNA (sc-37007, 20 nM; Santa Cruz, Heidelberg, Germany) using Lipofectamin 2000 (Life Technologies).

For all experiments 1.3×10^4 cells per well were seeded in 48-well plates (corresponding to a confluence of about 10%) in High Glucose DMEM medium. First siRNA transfection was performed the next day followed by a second siRNA transfection 24 h later with cotransfection of Tcf/Lef reporter plasmids and expression vectors when indicated. 48 h after the first transfection stimulation with conditioned media and compounds (bafilomycin A1 (Enzo Life Science, Lörrach, Germany), genistein (Carl Roth, Karlsruhe, Germany), aliskiren (Novartis, Nürnberg, Germany)) was carried out. Luciferase activity was assessed another 48 h later (i.e., 96 h after the first siRNA transfection).

2.5. RNA isolation

Isolation of total RNA of cultured cells was performed using the Nucleo-Spin RNA II Kit (Macherey-Nagel, Düren, Germany) following the instructions of the supplier. RNA concentration and integrity were measured by Nanodrop ND-1000 Spectrophotometer (PeqLab, Erlangen, Germany).

2.6. Real-time PCR

Reverse transcription was performed using M-MLV reverse transcriptase (RNase H minus, Promega, Mannheim, Germany) and