Biochimica et Biophysica Acta 1813 (2011) 1475–1482

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Regulation of heparanase by albumin and advanced glycation end products in proximal tubular cells

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ARTICLE INFO

Article history:
Received 18 November 2010
Received in revised form 3 May 2011
Accepted 4 May 2011
Available online 12 May 2011

Keywords:
Heparanase
Albuminuria
AGE
PI3K/AKT
Syndecan-1
Diabetic nephropathy

ABSTRACT

Diabetic nephropathy is one of the main causes of end-stage renal disease, in which the development of tubular damage depends on factors such as high glucose levels, albuminuria and advanced glycation end-product. In this study, we analyzed the involvement of heparanase, a heparan sulfate glycosidase, in the homeostasis of proximal tubular epithelial cells in the diabetic milieu. In vitro studies were performed on a wild-type and stably heparanase-silenced adult tubular line (HK2) and HEK293. Gene and protein expression analyses were performed in the presence and absence of diabetic mediators. Albumin and advanced glycation end-product, but not high glucose levels, increased heparanase expression in adult tubular cells via the AKT/PI3K signaling pathway. This over-expression of heparanase is then responsible for heparan sulfate reduction via its endoglycosidase activity and its capacity to regulate the heparan sulfate-proteoglycans core protein. In fact, heparanase regulates the gene expression of syndecan-1, the most abundant heparan sulfate-proteoglycans in tubular cells. We showed that heparanase is a target gene of the diabetic nephropathy mediators albumin and advanced glycation end-product, so it may be relevant to the progression of diabetic nephropathy. It could take part in several processes, e.g. extracellular-matrix remodeling and cell–cell crosstalk, via its heparan sulfate endoglycosidase activity and capacity to regulate the expression of the heparan sulfate-proteoglycan syndecan-1.

1. Introduction

End-stage renal disease (ESRD) is increasing in western countries and diabetic nephropathy (DN) is one of the most frequent causes [1]. The pathophysiological changes in DN include glomerular hyperfiltration, abnormal permeability of the glomerular sieve to albumin, and cellular and extracellular changes in the glomerulus and tubulo-interstititium, which lead to a worsening renal function and ultimately to ESRD [2]. As in most chronic renal diseases, even if the primary damage is restricted to the glomerulus, the renal tubule and interstitium become more and more involved as the disease progresses. It is now recognized that the stage of disease and its prognosis correlate better with the severity of tubulo-interstitial damage than with glomerular sclerosis [3,4].

In diabetes, tubular injury in the kidney is due to several factors, particularly high glucose levels, albuminuria, and the presence of advanced glycation end-product (AGE)-modified proteins. Exposure of the proximal tubular epithelial cells (PTECs) to high albumin concentrations and AGE-modified proteins leads to the activation of multiple signaling cascades, with an ensuing abnormal regulation of several growth factors and cytokines, and an abnormal synthesis and degradation of extracellular matrix (ECM) proteins, giving rise to a pro-fibrotic environment distinctive of the failing kidney [5–7].

Recent findings suggest an involvement of glomerular heparanase-1 (HPSE) in the pathogenesis of proteinuric disorders, including DN [8]. HPSE is a heparan sulfate (HS)-specific endo-β-D-glucuronidase that catalyzes the hydrolytic cleavage of the β(1,4)-glycosidic bond between glucuronic acid and glucosamine residue. HPSE participates in ECM remodeling and degradation, regulating the release of many HS-bonded molecules, such as growth factors, chemokines, cytokines, and enzymes involved in inflammation, wound healing and tumor invasion. Heparanase is expressed in numerous cell types and tissues, and it is abundantly expressed by several different tumor cells. Its expression

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doi:10.1016/j.bbamcr.2011.05.004

and regulation is cell-type specific [9,10]. It is also emerging that HPSE may have non-enzymatic activities, playing a part in different signaling cascades, selected protein kinase activation, and gene transcription [11].

While HPSE has been investigated mainly in the glomeruli, when it comes to the renal tubule we only know that its tubular expression is found increased in biopsies from patients with DN and this correlates with a decline in HS tubular staining [12]. Given the crucial role of tubular cells in the progression of DN, we thoroughly investigated the effect of the diabetic milieu on HPSE regulation in the tubular cell, and its consequences downstream. In particular, we analyzed: the effect of high glucose, albumin and glycated albumin levels on the expression, synthesis and activity of HPSE; the signaling pathways involved; the effect of an altered HPSE expression on HS and the above mechanisms; and the interaction between HPSE, HS and syndecan-1 (SDC1), which is the most abundant HSPG in the proximal tubule.

2. Methods

2.1. Cell cultures

HK2 (human kidney 2) is an immortalized proximal tubule epithelial cell line whereas HEK293 is a human embryonic tubular cell line. HK2 cells were grown in DMEM-F15 (EuroClone, Pavia, Italy) (17.5 mM glucose) and HEK293 cells in MEM (EuroClone), supplemented with 10% fetal bovine serum (Biochrom AG, Berlin, Germany), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml), and maintained at 37 °C in a 5% CO_2 water-saturated atmosphere.

2.2. Reagents

Bovine serum albumin (BSA, Sigma, Milano Italy), D-(+)-glucose and D-(+)-mannitol (Sigma), NF-kB inhibitor ammonium pyrrolidine dithiocarbamate (PDTC, Sigma), PI3 kinase inhibitor LY294002, p38 kinase inhibitor SB203580, Jak2 inhibitor AG490, MEK1 and MEK2 inhibitors PD98059 and U0126 (Calbiochem, San Diego, USA) were all purchased commercially. Sulodexide was a kind gift from Alfa Wassermann, Bologna, Italy.

2.3. Preparation of albumin-derived AGE

BSA-derived AGE was prepared by incubating 0.5 M glucose with 20 mg/ml BSA and 0.05 mg/ml NaN_3 in 0.4 M phosphate buffer (pH 7.5) for 15 days at 56 °C under sterile conditions. After incubation, unbound glucose was removed by dialysis against phosphate buffer. AGE protein concentration was evaluated using the Bio-Rad Protein Assay (Bio-Rad, Milan, Italy). The mixture turned into a stable viscous brown product that yielded fluorescence of 440 nm following excitation at 370 nm. The control BSA sample (non-glycated BSA) was incubated under identical conditions, but without any glucose. AGE and control (non-glycated BSA) were filter sterilized, aliquoted and stored at −20 °C until they were used.

2.4. Transfection of HPSE shRNA plasmid

To obtain stably *HPSE* silenced cell lines, we used four different shRNAs targeting human heparanase (NM_006665), purchased from OriGene (Rockville, MD, USA). We performed a transfection reaction for each plasmid, plus a reaction in which all four plasmids have been transfected together. As a negative control, we used an shRNA pRS non-effective GFP plasmid (TR30003) and an empty vector, shRNA pRS plasmid (TR20003). The shRNAs were reconstituted with 50 μl of RNAse-free water (Gibco, Invitrogen, Milano, Italy) to prepare a 100 ng/μl stock solution. HK2 cells were seeded in 6-well plates at a density of 1.5×10^5 cells per well. After 24 h, the cells had reached 70–80% of confluence. The medium was removed and replaced with two milliliters per well of fresh complete growth medium. A mixture prepared with 188 μl of serum free medium and 12 μl of TransIT-LT1 transfection reagent (Mirus, Madison, WI) was vortexed and incubated at room temperature for 20 min. Three micrograms of plasmids DNA were added to the mixture, which was then incubated at room temperature for another 20 min before adding it dropwise to the cells. Control cells were treated with the same amount of TransIT-LT1 transfection reagent (Mock). Forty-eight hours after transfection, the cells underwent several weeks of selection with 0.75 μg/ml of puromycin (Sigma).

2.5. Transfection of AKT1/2 siRNA

To silence AKT1 and AKT2 we use AKT1/2 siRNA (sc43609, Santa Cruz Biotechnology Heidelberg, Germany) as well as a control si-RNA. The siRNAs were reconstituted with 330 μl of RNAse-free water to prepare a 100 μM solution. HK2 wt cells were plated in 6-well plates at a density of 1.5×10^5 cells per well in 2 ml complete growth medium. After 24 h, we prepared a mixture with 188 μl of serum-free medium and 12 μl of TransIT-LT1 transfection reagent (Mirus). The solution was vortexed and incubated at room temperature for 20 min, then we added 6.6 μl of siRNAs and the mixture was then incubated at room temperature for another 20 min before adding it dropwise to the cells. After 24 h the medium was replaced with fresh complete growth medium. Cells were incubated for an additional 24 h and then starved, treated with BSA and AGE and assayed for *HPSE* gene expression and AKT protein expression described as follows.

2.6. mRNA expression analysis

The cells were grown to sub-confluence, starved for 24 h in serum-free medium, and then cultured in serum-free medium with the different treatments for another 6 h.

The cells were exposed to normal glucose (17.5 mM), a high mannitol as an osmolarity control (25 mM), high glucose levels (25 mM), and BSA or AGE (30 or 60 μg/ml), with or without the inhibitors: LY294002 (25 μM), SB203580 (20 μM), PD98059 (25 μM), U0126 (10 μM), AG490 (25 μM) and PDTC (10 μM). All these inhibitors were added 30 min before the BSA and AGE treatments, except for LY294002 (which was added 2 h beforehand).

Total RNA was extracted from cell monolayers using the "GenElute Mammalian Total RNA Miniprep" Kit (Sigma), including DNAse treatment (DNASE70, Sigma), according to the manufacturer's instructions. Yield and purity were checked using Nanodrop (EuroClone) and 1 μg of total RNA from each sample was reverse transcribed into cDNA using 500 ng random primers and 200 U SuperScript II Reverse Transcriptase (Invitrogen).

Then we performed a quantitative real-time PCR to measure *HPSE*, *SDC1* or *GAPDH* expression. The assays were performed on 96 multi-well PCR plates in the Applied Biosystems 7500 real-time PCR system, in a final volume of 12.5 μl containing 10 ng of cDNA, 6.25 μl of Power SYBR Green Master Mix 2× (Applied Biosystems, Carlsbad, USA), 5 μmol of forward or reverse primers (HPSE: d—ATTTGAATGGACGGACTGC r—GTTTCTCCTAACCAGACCTTC; SDC1: d—GAAGATCAAGATGGCTCTGGG r—GTTCTGGAGACGTGGGAATAG; GAPDH: d—ACACCCACTCCTCCACCTTT r—TCCACCACCCTGTTGCTGTA), and water q.s. The reaction underwent denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing and elongation at 60 °C for 1 min.

mRNA expression levels were evaluated by relative quantitative real-time PCR and normalized to *GAPDH*. The comparative Ct method (ΔΔCt) was used to quantify gene expression and the relative quantification (RQ) was calculated as $2^{-\Delta\Delta Ct}$. We performed melting curves analysis to avoid any interference of aspecific amplification products.

2.7. Western blot

To analyze HPSE and GAPDH protein expression, cells were grown to subconfluence for 24 h in serum-free medium, then washed with PBS, lysed and scraped in lysis buffer (50 mM Tris–HCl [pH 5.0], 150 mM