Biochimica et Biophysica Acta 1793 (2009) 1174–1181

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Epidermal growth factor activates Na^{+}/H^{+} exchanger in podocytes through a mechanism that involves Janus kinase and calmodulin

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ARTICLE INFO

Article history:
Received 24 September 2008
Received in revised form 28 February 2009
Accepted 19 March 2009
Available online 31 March 2009

Keywords:
Cytosensor microphysiometer
ECAR
Phosphorylation
AG1478

ABSTRACT

Sodium-proton exchanger type 1 (NHE-1) is ubiquitously expressed, is activated by numerous growth factors, and plays significant roles in regulating intracellular pH and cellular volume, proliferation and cytoskeleton. Despite its importance, little is known about its regulation in renal glomerular podocytes. In the current work, we studied the regulation of NHE-1 activity by the epidermal growth factor receptor (EGFR) in cultured podocytes. RT-PCR demonstrated mRNAs for NHE-1 and NHE-2 in differentiated podocytes, as well as for EGFR subunits EGFR/ErbB1, Erb3, and ErbB4. EGF induced concentration-dependent increases in proton efflux in renal podocytes as assessed using a Cytosensor microphysiometer, were diminished in the presence of 5-(N-methyl-N-isobutyl) amiloride or in a sodium-free solution. Furthermore, pharmacological inhibitors of Janus kinase (Jak2) and calmodulin (CaM) attenuated EGF-induced NHE-1 activity. Co-immunoprecipitation studies determined that EGF induced formation of complexes between Jak2 and CaM, as well as between CaM and NHE-1. In addition, EGF increased levels of tyrosine phosphorylation of Jak2 and CaM. The EGFR kinase inhibitor, AG1478, blocked activation of NHE-1, but did not block EGF-induced phosphorylation of Jak2 or CaM. These results suggest that EGF induces NHE-1 activity in podocytes through two pathways: (1) EGF → EGFR → Jak2 activation (independent of EGFR tyrosine kinase activity) → tyrosine phosphorylation of CaM → CaM binding to NHE-1 → conformational change of NHE-1 → activation of NHE-1; and (2) EGF → EGFR → EGFR kinase activation → association of CaM with NHE-1 (independent of Jak2) → conformational change of NHE-1 → activation of NHE-1.

1. Introduction

Epidermal growth factor (EGF) plays a number of key roles in the kidney, contributing to cellular proliferative and survival pathways, renal metabolism [1], regenerative hyperplasia [2], tubulointerstitial injury [3], tubulogenesis [4], transport [5], renal cyst formation [6] and renal development [7]. EGF also has been implicated in the genesis and development of polycystic kidney disease [8,9]. Despite the importance of EGF in many renal functions, particularly in renal tubules and mesangial cells, little is known about its effects in glomerular podocytes. Podocytes are critical for the maintenance of normal glomerular structure [10,11], and aberrant podocyte function has been implicated in the pathogenesis of chronic renal diseases [12–17]. These highly specialized cells are characterized by the formation of foot processes that are interconnected by the slit diaphragm, which is a critical component of the glomerular filtration barrier [18,19]. Podocytes are emerging as the focus of intense investigation because they have been implicated as both targets and instigators of renal injury in various progressive renal diseases [18,20–23]. Therefore, it would be desirable to elucidate signaling pathways in podocytes, which may be important in delineating the mechanisms through which podocytes contribute to the progression of glomerular injury.

Because of the emerging role of the sodium-proton exchanger type 1 (NHE-1), also known as product of SLC9A1, solute carrier family 9A, type 1 in the regulation of the cytoskeleton [24], apoptosis and cellular proliferation [25], cell cycle control [26], and development and maintenance of the transformed cellular phenotype [27], we thought that it might be of interest to develop a better understanding of its regulation in podocytes. Previous studies have demonstrated that EGF stimulates NHE-1 in non-renal cells [28–30], but the signaling pathways involved in the regulation have not been fully elucidated. Moreover, currently there are no data on the regulation of NHE-1 in podocytes. In that regard, recent studies from our laboratory have shown that NHE-1 can be activated by G protein-coupled receptors or hypertonic medium, through Janus kinase 2 (Jak2)-dependent phosphorylation of CaM, and subsequent interaction between CaM and NHE-1 [31–33]. Therefore, we wanted to determine whether EGF is important for regulating NHE-1 activity

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doi:10.1016/j.bbamcr.2009.03.006

in podocytes, and to identify key components of the signal transduction pathway linking EGF and NHE-1. In the current study, we demonstrate that EGF stimulates NHE-1 activity in podocytes. In addition, we have shown that Jak2 and CaM play critical roles in the activation of NHE-1 by EGF in podocytes.

2. Materials and methods

2.1. Cell culture

The podocyte cell line was kindly provided by Dr. Peter Mundel of Mt. Sinai School of Medicine. Podocytes were cultured as previously described [34]. Undifferentiated podocytes were maintained in RPMI-1640 medium containing 10 U/ml of mouse recombinant γ-interferon, 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin at 33 °C in 95% air and 5% CO_2. To induce differentiation, podocytes were maintained in the same medium as undifferentiated podocytes without γ-interferon at 37 °C in 95% air and 5% CO_2 for 14 days. All experiments were conducted using differentiated podocytes, unless stated otherwise.

2.2. Immunofluorescence microscopy

Immunolabeling was performed as previously described [34]. Cells were seeded in 35-mm collagen-coated glass-bottom culture dishes (MatTek Corporation) and fixed with 2% paraformaldehyde, 4% sucrose in phosphate-buffered saline (PBS) for 10 min at room temperature. Subsequently, cells were permeabilized with 0.3% Triton X-100 (Sigma) in PBS for 5 min, following which nonspecific binding sites were blocked with 2% fetal calf serum, 2% BSA and 0.2% gelatin in PBS for 1 h. Incubations with the appropriate dilutions of primary and secondary antibodies (as directed by the manufacturer) were performed in blocking solution. The primary and secondary antibodies used were: anti-WT1 (1:50) (Santa Cruz Biotechnology); anti-synaptopodin (Research Diagnostics, Inc.) and Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes). Confocal microscopy was performed using a Zeiss LSM 510 META laser-scanning microscope (Carl Zeiss, Inc.).

2.3. Microphysiometry

NHE-1 activity studies were conducted on a Cytosensor microphysiometer as previously described for other cell types [32,35,36]. Cells were plated on transwell membranes (3 μm pore size, 12 mm size) at a density of 300,000 cells per insert, and serum starved overnight on the day prior to experimentation. On the day of the experiment, the cells were washed with serum-free, bicarbonate-free F-12 medium, prior to being placed into microphysiometer chambers. The chambers were perfused at 37 °C with serum-free media or balanced salt solutions. After establishment of a stable baseline for at least five cycles, cells were exposed to the drugs for 4 cycles (360 s). Podocytes had low basal proton efflux levels (−10 to −40 μV/s), which roughly corresponds to millipH units/minute according to the Nernst equation). The extracellular acidification rate (ECAR, rate of accumulation of protons during a stop-flow period) was measured at peak stimulation after initiation of drug treatment, as is standard for microphysiometry studies. This typically occurred after two or three cycles (180 or 270 s) of exposure to EGF. Rate data were expressed as percentages of baseline values.

2.4. RT-PCR and PCR

RNA was prepared from differentiated and undifferentiated podocytes using Trizol reagent following the manufacturer's protocol (Invitrogen). Five micrograms of total RNA were used for first strand cDNA synthesis (SuperScript III RT-PCR kit, Invitrogen). EGFR/ErbB transcripts were identified using SuperArray's Multigen-12 RT-PCR profiling kit. To analyze NHE-1 message an already published primer pair was used: 5′-TCTGCCGTCTCAACTGTCTCTA-3′ sense, 5′-CCCTTCAACTCCTCATTCACCA-3′ antisense [37], which generated a 422 base pair (bp) product. For analyzing other NHE messages, new PCR primer pairs were designed: NHE-2 (5′-ACACACAACATCCGGGT-CATT-3′ sense, 5′-CGCTTGTGTCTGCCGTCA-3′ antisense) resulted in a 982 bp product; NHE-3 (5′-CGCCTGGAGTCCTTTAAGTC-3′ sense, 5′-GGAGAACACGGGATTATCAAT-3′ antisense) generated a 294 bp product, and NHE-4 (5′-GAGGACATAGAAGCGGTGGAC-3′ sense, 5′-AGGAGAAAGCCGCTTGATTC-3′ antisense) resulted in a 993 bp PCR product. Target specificities of the PCR primers were confirmed by sequencing the PCR products using the MUSC sequencing core facility.

2.5. Immunoprecipitation

For immunoprecipitation of Jak2 and NHE-1, quiescent differentiated podocytes grown on 100 mm collagen-coated tissue culture dishes were pretreated with 50 μM of AG490 or 20 μM of AG1478 for 30 min prior to treatment with 10 ng/ml of EGF or vehicle for 5 min, and then lysed in 1 ml/dish of RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA) supplemented with protease inhibitors (1 mM AEBSF, 0.8 μM aprotinin, 50 μM bestatin, 15 μM E-64, 20 μM leupeptin, 10 μM pepstatin A, 1 mM sodium orthovanadate, 1 mM sodium fluoride and 1 mM PMSF). Equal amounts of proteins (1.5 mg) were precleared by incubation with protein A/G sepharose beads for 30 min at 4 °C. After a brief centrifugation, the supernatants were removed and incubated with either agarose conjugated anti-JAK2 antibody (Upstate) or anti-NHE-1 antibody (Chemicon) overnight at 4 °C. Immunoprecipitates were captured with 50 μl of protein A/G beads at 4 °C for 1 h. Then, the samples were centrifuged and washed

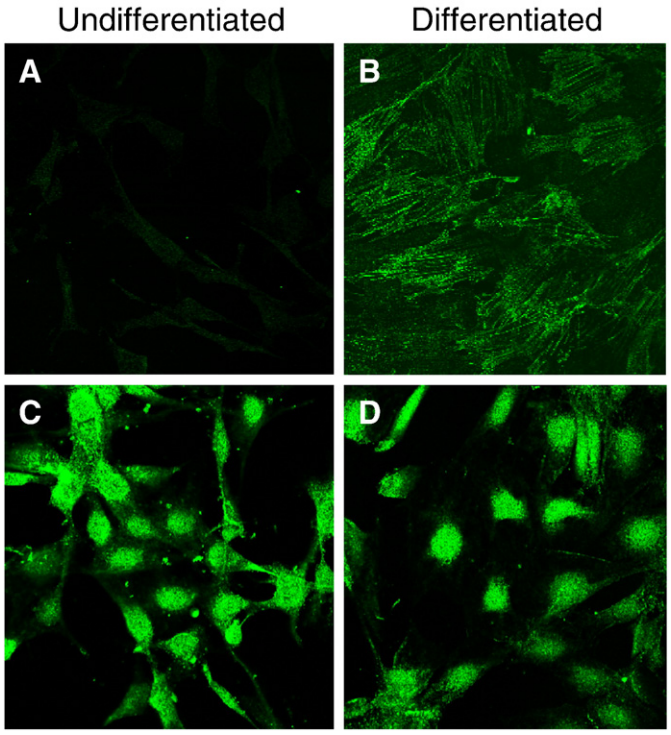

Fig. 1. Immunofluorescence analysis of podocyte markers. Cells were fixed and then stained for synaptopodin or WT-1 to confirm differentiated from undifferentiated podocytes. Undifferentiated podocytes did not stain for synaptopodin (A). In contrast, differentiated podocytes stained for synaptopodin (B). Undifferentiated and differentiated podocytes stained for WT-1 (C and D).