Biochimica et Biophysica Acta 1863 (2016) 2624–2636

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Low dose ouabain stimulates Na—K ATPase α1 subunit association with angiotensin II type 1 receptor in renal proximal tubule cells

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ARTICLE INFO

Article history:
Received 29 April 2016
Received in revised form 15 July 2016
Accepted 29 July 2016
Available online 03 August 2016

Keywords:
Na—K ATPase
Ouabain
AT1R
Proteomics
FRET-TIRF microscopy
Human kidney cells

ABSTRACT

Our laboratory has recently demonstrated that low concentrations of ouabain increase blood pressure in rats associated with stimulation of Na—K ATPase activity and activation of the Src signaling cascade in NHE1-dependent manner. Proteomic analysis of human kidney proximal tubule cells (HKC11) suggested that the Angiotensin II type 1 receptor (AT1R) as an ouabain-associating protein. We hypothesize that ouabain-induced stimulation of Na—K ATPase activity is mediated through AT1R. To test this hypothesis, we examined the effect of ouabain on renal cell angiotensin II production, the effect of AT1R inhibition on ouabain-stimulated NKA activity, and the effect of ouabain on NKA-AT1R association. Ouabain increased plasma angiotensin II levels in rats treated with ouabain (1 μg/kg body wt./day) for 9 days and increased angiotensin II levels in cell culture media after 24 h treatment with ouabain in human (HKC11), mouse (MRPT), and human adrenal cells. Ouabain 10 pM stimulated NKA-mediated ^{86}Rb uptake and phosphorylation of EGFR, Src, and ERK1/2. These effects were prevented by the AT1R receptor blocker candesartan. FRET and TIRF microscopy using Bodipy-labeled ouabain and mCherry-NKA or mCherry-AT1R demonstrated association of ouabain with AT1R and NKA. Further our FRET and TIRF studies demonstrated increased association between AT1R and NKA upon treatment with low dose ouabain. We conclude that ouabain stimulates NKA in renal proximal tubule cells through an angiotensin/AT1R-dependent mechanism and that this pathway contributes to cardiac glycoside associated hypertension.

1. Introduction

Sodium potassium ATPase (NKA) is a heterotrimeric enzyme consisting of an α, a β, and a γ subunit [1]. NKA is localized to the basolateral membrane in epithelial cells of renal tubules where it is responsible for the active transport of sodium and potassium. NKA maintains intracellular levels of sodium and energizes the secondary active transport of sodium into the cells from the apical membrane. NKA activity is tightly regulated in the renal proximal tubules. We and others have demonstrated that cardioglycosides like ouabain exert a biphasic effect on NKA activity, low concentrations stimulating and higher concentrations inhibiting the activity of NKA [2–5]. Our laboratory has demonstrated that ouabain-mediated stimulation of NKA involves an NHE1 dependent activation of a signaling complex that includes EGFR, Src kinase, ERK, and Akt [3,6]. We have also demonstrated that low concentrations of ouabain increase blood pressure in Sprague Dawley rats in an NHE1 dependent manner [3].

Cardiac glycosides have been identified predominantly as inhibitors of NKA, and their use in the treatment of cardiac disease has exploited this property. Ouabain and other cardioglycosides are endogenously synthesized in humans and other mammals and expressed at very low plasma (~100 pM) concentrations [7]. The physiologic relevance of endogenous ouabain production remains poorly understood. Recent studies have demonstrated that plasma levels of ouabain increase in individuals on a high salt diet [8]. Endogenous ouabain levels are also significantly increased in patients with several forms of hypertension [9], chronic heart disease [10], chronic kidney disease [11], and preeclampsia [12] to approximately 900 pM [13], a concentration that is well above normal but not sufficient to inhibit NKA activity. The synthesis of ouabain in the adrenal glands is regulated by adenocorticotropic hormone (ACTH), α adrenergic and dopaminergic stimulation, angiotensin II (Ang II) through stimulation of angiotensin receptor type 2 (AT2R),

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http://dx.doi.org/10.1016/j.bbamcr.2016.07.008
0167-4889/

hypoxia, and physical exercise [14,15], suggesting a role in regulation of blood pressure. Ouabain increases circulating levels of angiotensin II through pathways involving the sympathetic nervous system [16,17]. Blaustein et al. suggested that endogenous ouabain regulates blood pressure through a complex pathway involving aldosterone, the epithelial Na^+ channel, NKA α2 subunit, and Ang II. This pathway modulates the activity of brain cardiovascular control centers that regulate the BP set point and induce sustained changes in sympathetic nerve activity [18].

A recent report suggests that ouabain can bind proteins other than NKA. Fujita-Sato et al. demonstrated that cardioglycosides can bind to retinoic acid-related orphan nuclear receptor γt to suppress the differentiation of Th17 cells [19]. Based on these findings as well as our previous studies, we hypothesized that ouabain regulated blood pressure by increasing proximal tubule sodium reabsorption through mechanisms involving the renin angiotensin system. In the present study, using proteomic analysis of human kidney proximal tubule cell proteins immunoprecipitated treated with low dose ouabain, we identified the angiotensin receptor type I (AT1R) and type 2 (AT2R) receptors as potential binding partners (Tables 1–3). Using pharmacologic and genetic approaches, our results demonstrate that ouabain-stimulated signaling and NKA-mediated ion transport is AT1R dependent. Our results further demonstrate that NKA α1 subunit and AT1R can directly associate with each other and this association is enhanced by treatment with low dose ouabain.

2. Materials and experimental methods

2.1. Materials

Ouabain and candesartan were purchased from Tocris (St. Louis, MO). Digibind, an FDA approved antibody against cardioglycosides was purchased from GlaxoSmithKline (Parma, Italy). The monoclonal antibody against NKA (α6F) developed by Dr. D.M. Fambrough was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of NIHCD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Phospho- and total EGFR, Src, and ERK1/2 antibodies were purchased from Cell Signaling. Antibodies against angiotensin converting enzyme (catalog number GTX100923, ACE) were purchased from GeneTex (Irvine, CA) and against angiotensinogen (catalog number ab198180, AGT) were purchased from Abcam (Cambridge, MA). HRP-linked secondary antibodies were purchased from Vector laboratories. Streptavidin agarose resin was purchased from Pierce Biotechnology (Rockford, IL). Phosphatase inhibitor cocktail-1 and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). All other chemicals were purchased from Sigma, unless otherwise specified.

Table 1
Identification of ouabain-binding proteins in cells treated with 10 pM ouabain.

Proteins pulled down from cells treated with 10 pM ouabain
ADAM metallopeptidase domain 10
Adenosine kinase
Adenosine A2a receptor
Adenosine A2b receptor
Angiotensin II receptor, type 1
Angiotensin II receptor, type 2
Alanine-glyoxylate aminotransferase
Aryl hydrocarbon receptor
Aryl-hydrocarbon receptor repressor
Ankyrin 2, neuronal
Ankyrin 3, node of Ranvier (ankyrin G)
Apelin receptor
Androgen receptor
ERBB4 Isoform JM-A of Receptor tyrosine-protein kinase erbB-4
Rho GTPase activating protein 4
Fas ligand (TNF superfamily, member 6)
Ras homolog gene family, member A
Ras homolog gene family, member C
RYR1 Isoform 2 of Ryanodine receptor 1
Shroom family member 2
Solute carrier family 16, member 2 (monocarboxylic acid transporter 8)

Table 2
Identification of ouabain-binding proteins in cells treated with 100 nM ouabain.

Proteins pulled down from cells treated with 100 nM ouabain
Adenylate cyclase 3
Adducin 3 (gamma)
AE binding protein 1
Advanced glycosylation end product-specific receptor
Anaplastic lymphoma receptor tyrosine kinase
Arachidonate 12-lipoxygenase, 12R type
Arachidonate 15-lipoxygenase, type B
Arachidonate 5-lipoxygenase-activating protein
ALX homeobox 3
Alpha-1-microglobulin/bikunin precursor
Annexin A8-like 2
ERBB2 Isoform 1 of Receptor tyrosine-protein kinase erbB-2
Rho GTPase activating protein 5
FRMPD3 FERM and PDZ domain-containing protein 3
H^+—K^+ ATPase non colonic
Ras homolog gene family, member B
SLC4A4 Isoform 2 of Electrogenic sodium bicarbonate cotransporter 1

2.2. Animal model

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Louisville. Sprague Dawley rats weighing 200–250 g were stabilized on the standard rat chow and water ad libitum for a week prior to experiments. Animals were divided into groups (6 animals in each group). The vehicle group received PBS intraperitoneally and the ouabain group received 1 μg/kg body weight/day ouabain for 9 days. Blood was collected from carotid artery and serum was separated after measuring blood pressure (blood pressure data was reported previously) [3,6].

2.3. Cell culture

Human kidney proximal tubule cells HKC11 (a gift from Dr. Lorraine Racusen, Johns Hopkins University Baltimore, MD), mouse renal proximal tubule cells (MRPT, a gift from Dr. Jeffrey R. Schelling, Case

Table 3
Identification of ouabain-binding proteins common in cells treated with 10 pM and 100 nM ouabain.

Proteins pulled common in cells treated with 10 pM and 100 nM ouabain
Actinin, alpha 2
Activin A receptor, type I
Activin A receptor type II-like 1
Adenylate cyclase 9
Adenylate cyclase activating polypeptide 1 (pituitary)
Adenylate cyclase activating polypeptide 1 (pituitary) receptor type I
Adducin 3 (gamma)
Adrenergic, alpha-1D-, receptor
Adrenergic, alpha-2A-, receptor
Adrenergic, beta-1-, receptor
Adrenergic, beta-3-, receptor
Adrenergic, beta, receptor kinase 1
Adaptor-related protein complex 2, alpha 1 subunit
Adaptor-related protein complex 2, alpha 2 subunit
Solute carrier family 39 (zinc transporter), member 4
Cofilin
Na—K ATPase α1 subunit
NHE1
Src
EGFR