Molecular and Cellular Endocrinology xxx (2018) 1–11

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

Epidermal growth factor receptor/extracellular signal-regulated kinase pathway enhances mineralocorticoid receptor transcriptional activity through protein stabilization

Yuko Mitsuishi [a], Hirotaka Shibata [a, b, *], Isao Kurihara [a], Sakiko Kobayashi [a], Kenichi Yokota [a], Ayano Murai-Takeda [a], Takeshi Hayashi [a, c], Rie Jo [a], Toshifumi Nakamura [a], Mitsuha Morisaki [a], Hiroshi Itoh [a]

[a] Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
[b] Department of Endocrinology, Metabolism, Rheumatology and Nephrology, Faculty of Medicine, Oita University, 1-1 Idaigaoka, Hasamamachi, Yufu 879-5593, Oita, Japan
[c] Division of Diabetes Metabolism and Endocrinology, Department of Internal Medicine, The Jikei University School of Medicine, 3-19-18 Nishishimbashi, Minato-ku, Tokyo 105-8471, Japan

ARTICLE INFO

Article history:
Received 9 August 2017
Received in revised form
12 January 2018
Accepted 16 January 2018
Available online xxx

Keywords:
Mineralocorticoid receptor
Epidermal growth factor receptor
Extracellular signal-regulated kinase pathway

ABSTRACT

Activation of mineralocorticoid receptor (MR) is evoked by aldosterone, and it induces hypertension and cardiovascular disease when it's concomitant with excessive salt loading. We have proposed the notion of "MR-associated hypertension", in which add-on therapy of MR blockers is effective even though serum aldosterone level is within normal range. To elucidate its underlying molecular mechanism, we focused on the effect of epidermal growth factor receptor (EGFR)/extracellular signal-regulated kinase (ERK) activation on MR activity. Epidermal growth factor (EGF) administration increased MR transcriptional activity through EGFR/ERK pathway and increased protein level by counteracting MR ubiquitylation *in vitro*. EGF administration *in vivo* also increased MR protein level and target gene expression in kidney, which were decreased by EGFR inhibitor. In addition, the administration of EGFR inhibitor lowered systolic blood pressure and MR activity in DOCA/salt-treated mice. In conclusion, EGFR/ERK pathway activation is considered as one of the underlying mechanisms of aberrant MR activation and EGFR/ERK pathway blockade could be an alternative approach for the prevention of MR-related cardiovascular events.

1. Introduction

According to the large clinical trials such as randomized aldactone evaluation study (RALES), eplerenone post-acute myocardial infarction heart failure efficacy and survival study (EPHESUS), eplerenone in mild patients hospitalization and survival study in heart failure (EMPHASIS-HF) and 4E-Left ventricular hypertrophy study (4E-LVH), MR blockers improved the prognosis of heart failure and hypertension-related organ damages (Pitt et al., 1999, 2003a,b, Zannad et al., 2011). We have recently proposed the notion of "MR-associated hypertension", in which add-on therapy of MR blockers is effective in the treatment of hypertension (Shibata and Itoh, 2012). The MR-associated hypertension is classified into two subtypes, that with elevated plasma aldosterone level and that with normal plasma aldosterone level. The former subtype includes primary aldosteronism (PA), which is the most common cause of secondary hypertension. The activation of MR accelerates cardiovascular inflammation and aggravates its related disorders such as cerebrovascular disease, heart failure and chronic kidney disease, which are more prevalently observed in patients with PA than those with essential hypertension (Catena et al., 2008,Sechi et al., 2006). On the other hand, even though the plasma aldosterone concentration is normal, MR blockers are effective in some clinical contexts, such as obesity, diabetes mellitus and chronic kidney diseases (Pimenta and Calhoun, 2007,Sowers et al., 2009,Tirosh et al., 2010), which are classified as the latter

* Corresponding author. Department of Endocrinology, Metabolism, Rheumatology and Nephrology, Faculty of Medicine, Oita University, 1-1 Idaigaoka, Hasamamachi, Yufu 879-5593, Oita, Japan.
E-mail address: hiro-405@cb3.so-net.ne.jp (H. Shibata).

https://doi.org/10.1016/j.mce.2018.01.007
0303-7207/

subtype of MR-associated hypertension. The underlying mechanism of MR activation in MR-associated hypertension with normal plasma aldosterone level is considered to be mediated by increased MR activity. To elucidate the molecular mechanism of this aberrant MR activation, we had focused on the factors which directly interact with MR and alter its transcriptional activity, and then we newly identified Ubc9 as a coactivator and NF-YC as a corepressor of MR through yeast-two hybrid screening (Murai-Takeda et al., 2010,Yokota et al., 2007). However, all of the mechanisms underlying MR-associated hypertension might not be explained by such cofactors. A previous paper has reported the role of Rho/Rho-kinase pathway on MR activity in obesity-related renal injury (Tokuyama et al., 2012). Likewise several studies have shown that the interaction between membrane receptors and nuclear receptors is important in the regulation of nuclear receptor activities (Lange, 2004,Weigel and Zhang, 1998).

EGFR/ERK pathway, one of the representative membrane signals, is known to be clinically important in the pathogenesis of several lines of cancers and also has the merging evidence of its clinical importance in the development of hypertension and cardiovascular comorbidities (Forrester et al., 2016). The blockade of EGFR reduced blood pressure, prevented left ventricular hypertrophy, renal vascular and glomerular fibrosis and inhibited AII-induced cardiac hypertrophy and hypertension of *in vivo* model (Francois et al., 2004,Jamroz-Wisniewska et al., 2008,Kagiyama et al., 2002, 2003). EGFR could be activated by not only EGF, but also by several factors including high glucose (Konishi and Berk, 2003), osmotic stress (Lezama et al., 2005), inflammation factors such as TGF-α (Singh and Coffey, 2014) and TGF-β (Samarakoon et al., 2013), oxidative stress, obesity-related factors such as leptin (Jamroz-Wisniewska et al., 2008). In addition to that, the factors which are well known as inducers of hypertension, such as catecholamine, angiotensinⅡand endothelin-1 also activate EGFR through the transactivation of G-protein-coupled receptor (GPCR) (Daub et al., 1996,Shah and Catt, 2003). Activation of EGFR causes fibrosis and inflammation which result in vascular damages in *in vivo* model (Dreux et al., 2006,Florian et al., 2001,Forrester et al., 2016; J. and Lowicka, 2009).

The beneficial effects by EGFR inhibitor and MR antagonist on cardiovascular complications have the similar spectrum therefore we could assume that the interaction between MR and EGFR is involved in the development of hypertension-related comorbidities, cardiac hypertrophy or heart failure. Actually, there were some reports about the association between MR and EGFR. It has been reported that MR binds to the EGFR promoter, and upregulates EGFR *in vitro* (Grossmann et al., 2007, 2010). As for *in vivo* reports, the administration of MR blocker attenuated EGFR activation and organ damage in stroke-prone rats (Dorrance et al., 2001) and MR blocker prevented hypertensive cardiovascular injury in salt-sensitive hypertensive rats (Nakamura et al., 2011) in which activated EGFR are observed (Ying and Sanders, 2005). However, there was no report addressing whether EGFR influences MR activity.

Thus, we focused on the effect of EGFR/ERK signaling on MR activity to explain one of the possible mechanisms which account for increased MR activity under normal aldosterone level. We examined in this study whether EGFR/ERK pathway enhances MR transcriptional activity and whether the crosstalk between EGFR and MR is involved in MR-associated hypertension.

2. Materials and methods

2.1. Plasmid constructs

MR (1-984) was subcloned into pcDNA3.1/His vector as described previously (Yokota et al., 2007) and 3 × mineralocorticoid-responsive element (MRE)-Elb-Luc was a generous gift from Dr. Bert W. O'Malley (Baylor College of Medicine, Houston).

2.2. Cell culture, transient transfections, and luciferase assays

COS 7 cells and HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). For luciferase reporter assay, COS 7 cells were transiently transfected using Lipofectamine LTX (Invitrogen) with pcDNA3.1/His-MR (1-984), pRL-null, and 3 × MRE-E1b-Luc reporter. Just before transfection, we changed the medium with FBS to the medium with 2% charcoal-stripped serum. Twenty-four hours after transfection, cells were treated with serial concentrations of aldosterone (10^{-11} M to 10^{-7} M) and EGF (1 ng/ml to 500 ng/ml), and cells were harvested 24 h after the treatment with aldosterone. Cell extract was assayed for both Firefly and Renilla luciferase activities with a dual-luciferase reporter assay system (Promega). We used Cytation5 (Bio Tek) to measure Firefly and Renilla luciferase activities, and Relative luciferase activities were determined as ratio of Firefly/Renilla luciferase activities. Detailed information described previously (Luciferase Assay System Technical Bulletin, Promega Corporation).

2.3. Generation of human MR expressing cell line

Because MR protein expression is very low in native cells, we established HEK 293-MR cells which express human MR stably (Yokota et al., 2007). HEK 293-MR cells were routinely maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone). We changed the medium with FBS to the medium with 2% charcoal-stripped serum 24 h before aldosterone and/or EGF treatment.

2.4. Western blot and co-immunoprecipitation assay

Whole cell lysates were prepared by homogenization in buffer composed of 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride. Western blot analyses were performed before immunoprecipitation (IP) steps to confirm the initial expression level of protein by several corresponding antibodies (mouse anti-MR antibody (Perseus Proteomics), mouse anti-α-tubulin (Calbiochem), mouse anti-ubiquitin antibody (Invitrogen) and rabbit anti-EGFR (Cell Signaling) for *in vitro* experiments, mouse anti-MR monoclonal antibody (a gift from Gomez-Sanchez (Gomez-Sanchez et al., 2011)) and rabbit anti-MR antibody (Santa Cruz Biotechnology) (results for MR expression were confirmed by both anti-MR antibodies), rabbit anti-serum- and glucocorticoid-regulated kinase 1 (Sgk1) (Millipore) and rabbit anti-β-actin antibody (Cell Signaling) for *in vivo* experiments). For co-IP, the same samples for the Western blot analysis were diluted into 1.5 ml in IP buffer (20 mM Tris-HCL (pH 7.5), 150 mM NaCl, 10 mM dithiothreitol, 5 ng/μl aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% Tween 20) and pre-cleared with protein G plus-agarose beads (Santa Cruz Biotechnology), and the primary antibody (anti-MR) were added and incubated overnight. Immune complexes were absorbed to protein G plus-agarose beads and washed three times in IP buffer. Proteins were then separated on 7.5% polyacrylamide gels and transferred onto Hybond ECL nitrocellulose membrane (Amersham Biosciences).

2.5. RNA extraction and quantitative real-time reverse transcription-PCR

Total RNA was extracted from HEK 293-MR cells or the kidney

Please cite this article in press as: Mitsuishi, Y., et al., Epidermal growth factor receptor/extracellular signal-regulated kinase pathway enhances mineralocorticoid receptor transcriptional activity through protein stabilization, Molecular and Cellular Endocrinology (2018), https://doi.org/10.1016/j.mce.2018.01.007