Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Indoxyl glucuronide, a protein-bound uremic toxin, inhibits hypoxia-inducible factor–dependent erythropoietin expression through activation of aryl hydrocarbon receptor

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ARTICLE INFO

Article history:
Received 3 September 2018
Accepted 4 September 2018
Available online xxx

Keywords:
Indoxyl glucuronide
Indoxyl sulfate
Aryl hydrocarbon receptor
Hypoxia-inducible factor
Erythropoietin

ABSTRACT

Renal anemia is common among chronic kidney disease (CKD) patients, and is mainly caused by inadequate erythropoietin (EPO) production from kidneys due to dysfunction of intracellular hypoxia-inducible factor (HIF) signaling in renal EPO-producing cells. We have previously shown that indoxyl sulfate (IS), a representative protein-bound uremic toxin accumulated in the blood of CKD patients, inhibits hypoxia-induced HIF activation and subsequent EPO production through activation of aryl hydrocarbon receptor (AHR). In this study, we further investigated the effects of other protein-bound uremic toxins on HIF-dependent EPO expression using EPO-producing HepG2 cells. We found that indoxyl glucuronide (IG) and IS, but not p-cresyl sulfate, phenyl sulfate, 3-indoleacetic acid or hippuric acid, inhibited hypoxia mimetic cobalt chloride-induced EPO mRNA expression. Furthermore, IG at concentrations similar to the blood levels in CKD patients inhibited the transcriptional activation of HIF induced by both cobalt chloride treatment and hypoxic culture. IG also induced CYP1A1 mRNA expression and nuclear translocation of AHR protein, indicating that IG activates AHR signaling. Blockade of AHR by a pharmacological antagonist CH-223191 abolished the IG-induced inhibition of HIF activation. Collectively, this study is the first to elucidate the biological effects of IG to inhibit HIF-dependent EPO production through activation of AHR. Our data suggests that not only IS but also IG contributes to the impairment of HIF signaling in renal anemia.

1. Introduction

Renal dysfunction leads to accumulation of uremic toxins in the blood of patients with chronic kidney disease (CKD). Approximately 90 compounds have been reported as uremic toxins. Based on the molecular weight and protein binding properties, these uremic toxins are classified into the following three groups: freely water-soluble low molecular weight molecules, protein-bound molecules and middle molecules [1,2]. In particular, protein-bound uremic toxins have attracted much attention since they are less efficiently removed by hemodialysis [3] and possibly contribute to CKD-associated complications [4,5]. Indoxyl sulfate (IS) and *p*-cresyl sulfate (PCS) are representative protein-bound uremic toxins and many studies have shown their contribution to the progression of CKD through inducing oxidative stress in renal tubular cells [6–8]. In addition, IS and PCS induce oxidative stress in vascular smooth muscle cells and endothelial cells [9–11], suggesting their involvement in cardiovascular diseases associated with CKD [12,13]. Numerous lines of evidence have shown that both IS and PCS play a role in the progression of CKD and CKD-associated complications; however, the biological effects of other protein-bound uremic toxins have been investigated to a much lesser extent.

Renal anemia occurs commonly in patients with CKD. Clinical studies indicate that renal anemia is an important risk factor for the progression of CKD [14] and mortality [15]. The main cause of renal anemia is insufficient erythropoiesis in the bone marrow due to the inadequate erythropoietin (EPO) production from interstitial fibroblast-like cells in the kidneys [16]. The transcription of EPO mRNA is mediated by hypoxia-inducible transcription factor (HIF), a heterodimeric complex composed of HIF-αs (including HIF-1α and HIF-2α) and aryl hydrocarbon receptor (AHR) nuclear translocator (ARNT). The expression of HIF-αs is strictly controlled by

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https://doi.org/10.1016/j.bbrc.2018.09.018
0006-291X/

cellular oxygen molecules. Under normoxia, oxygen-dependent prolyl-4 hydroxylase (PHD) hydroxylates the proline residues of HIF-αs triggering recruitment of the Von Hippel–Lindau (VHL) tumor suppressor protein, a component of E3 ubiquitin ligase complex, and facilitates proteasomal degradation of HIF-αs [17,18]. Under hypoxia, however, HIF-αs escape from the degradation pathway, consequently accelerating nuclear accumulation of HIF-αs and dimerization with ARNT, promoting the expression of target genes including EPO. In CKD, dysfunction of intracellular HIF signaling and transformation of renal interstitial EPO-producing cells to a pathologic fibrogenic state lead to loss of EPO-producing ability, and the inflammatory microenvironment is suggested as one of the possible causes [19,20]. However, the mechanisms of the impairment of HIF signaling and EPO production are not fully clarified.

Using an EPO-producing HepG2 cell line and rats with elevated EPO production by breeding, we have previously revealed that IS inhibits HIF-dependent EPO production through activation of AHR *in vitro* and *in vivo* [21]. These results prompted us to investigate the roles of other protein-bound uremic toxins in the impairment of HIF signaling. We evaluated protein-bound uremic toxins including indoxyl glucuronide (IG), PCS, phenyl sulfate, 3-indoleacetic acid and hippuric acid regarding their potential to inhibit the HIF-dependent EPO expression using HepG2 cells.

2. Materials and methods

2.1. Uremic toxins

IS potassium salt was purchased from Alfa Aesar (Ward Hill, MA), IG cyclohexylammonium salt from Glycosynth (Warrington, Cheshire, UK), and 3-indoleacetic acid potassium salt and hippuric acid sodium salt from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). PCS sodium salt and phenyl sulfate sodium salt (>98% purity) were synthesized at Eiweiss (Shizuoka, Japan). All these uremic toxins were dissolved in phosphate-buffered saline, filtered through Millex-GP (0.22 μm; Millipore, Burlington, MA) and then diluted with culture medium.

2.2. Cell culture, induction of hypoxic response and treatment with uremic toxins

The human hepatoma cell line HepG2 was purchased from DS Pharma Biomedical (Osaka, Japan). The cell line retains the ability to express EPO when cultured under hypoxic condition or treated with hypoxia-mimicking agents such as cobalt chloride [22]. The cells were cultured in MEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 1% non-essential amino acid solution (Sigma-Aldrich), 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Tokyo, Japan). Cells were cultured at 37 °C in a 5% CO_2 humidified incubator unless otherwise indicated. For induction of cellular hypoxic response, cells were treated with 50 μM cobalt chloride (Wako Pure Chemical Industries, Osaka, Japan). Alternatively, cells were cultured under hypoxic condition using an AnaeroPack System (Mitsubishi Gas Chemical, Tokyo, Japan) that depletes oxygen to < 1% within 1 h and provides a 5% CO_2 atmosphere without changing medium pH [23]. Uremic toxins were added 1 h prior to cobalt chloride treatment or immediately before hypoxic culture.

2.3. Cell viability assay

HepG2 cells (1×10^4/well) were seeded into 96-well plates and cultured overnight. After treatment with uremic toxins (0, 500, 1500 or 5000 μM) in the presence or absence of cobalt chloride for 24 h, 3 - (4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Dojindo Laboratories, Kumamoto, Japan) was added and cells were further cultured for 1 h. The formazan crystals were then dissolved in DMSO, and absorbance at 570 nm was determined.

2.4. RNA isolation and quantitative real-time PCR

HepG2 cells (2.5×10^5/well) were seeded into 12-well plates and cultured overnight. The culture medium was replaced with MEM containing 0.1% fetal bovine serum, and cells were further cultured overnight under serum starvation. Then, cells were treated with uremic toxins, and cultured with or without cobalt chloride or under hypoxic condition as described above. Total RNA was extracted using an Illustra RNA Spin Mini Kit (GE Healthcare, Tokyo, Japan) according to the manufacturer's instructions. Total RNA (250 ng in 10 μL reaction volume) was reverse transcribed with random hexamer primers using PrimeScript RT Master Mix (Takara Bio, Shiga, Japan). Real-time RT-PCR was then performed with fivefold-diluted cDNA using a KAPA SYBR fast qPCR kit (Nippon Genetics, Tokyo, Japan) and gene-specific primer pairs (35–40 cycles at 95 °C for 3 s, 60 °C for 30 s). Values for the mRNA of EPO, CYP1A1, HIF-1α and HIF-2α were normalized to HPRT mRNA expression. Analyses were conducted with a Thermal Cycler Dice Real Time System (Takara Bio). The sequences of primers used were described previously [21].

2.5. Transient transfection and hypoxia response element (HRE)-luciferase assay

The luciferase reporter plasmid for HRE (pGL4.42 [luc2P/HRE/Hygro] Vector) and control plasmid (pGL4.74 [hRluc/TK] Vector) were purchased from Promega (Madison, WI). HepG2 cells (1×10^4/well) were seeded into 96-well plate and cultured overnight. After co-transfection with both the HRE-luciferase and control plasmids using X-tremeGENE HP DNA Transfection Reagent (Roche diagnostics, Tokyo, Japan) and subsequent cultivation for 24 h, cells were treated with uremic toxins and cultured with or without cobalt chloride or under hypoxic condition as described above. Cells were then washed with ice-cold phosphate-buffered saline, and cellular luciferase activity was measured using a Dual Luciferase Reporter Assay System (Promega) with a Lumat LB9501 luminometer (Berthold Technologies, Bad Wildbad, Germany). The results are expressed as ratio of *Photinus pyralis* luciferase activity/*Renilla reniformis* luciferase activity. In some experiments, the AHR antagonist CH-223191 dissolved in DMSO was added at the same time of treatment with uremic toxins. The final concentration of DMSO in the medium was less than 0.1% in all experiments.

2.6. Nuclear protein extraction and immunoblot analysis

HepG2 cells (7.5×10^6/dish) were seeded into 10-cm dishes and cultured overnight. After treatment with uremic toxins and cobalt chloride for 4 h, cells were harvested and nuclear proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents and Halt Protease Inhibitor Cocktail with EDTA (both from Thermo Fisher Scientific, Kanagawa, Japan) according to the manufacturer's instructions. Protein concentrations of the nuclear protein extracts were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific) with BSA as standard. Nuclear protein extracts (5 μg) were mixed with reducing SDS sample buffer and heated for 3 min at 100 °C. The samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane

Please cite this article in press as: H. Asai, et al., Indoxyl glucuronide, a protein-bound uremic toxin, inhibits hypoxia-inducible factor–dependent erythropoietin expression through activation of aryl hydrocarbon receptor, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.09.018