Life Sciences 92 (2013) 143–148

Contents lists available at SciVerse ScienceDirect

Life Sciences

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

Indoxyl sulfate upregulates renal expression of ICAM-1 via production of ROS and activation of NF-κB and p53 in proximal tubular cells

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ARTICLE INFO

Article history:
Received 1 August 2012
Accepted 13 November 2012

Keywords:
ROS
NF-κB
p53
Tubulointerstitial injury
Chronic kidney disease

ABSTRACT

Aims: Intercellular adhesion molecule 1 (ICAM-1) plays an important role in adhesion of monocytes/macrophages to injured tubulointerstitial tissue. The present study aimed to determine if indoxyl sulfate, a uremic toxin, regulates renal expression of ICAM-1.
Main methods: The effect of indoxyl sulfate on expression of ICAM-1 was determined using human proximal tubular cells (HK-2 cells) and the following animals: (1) Dahl salt-resistant normotensive rats (DN), (2) Dahl salt-resistant normotensive indoxyl sulfate-administered rats (DN + IS), (3) Dahl salt-sensitive hypertensive rats (DH), and (4) Dahl salt-sensitive hypertensive indoxyl sulfate-administered rats (DH + IS).
Key findings: DN + IS, DH, and DH + IS rats showed significantly increased mRNA expression of ICAM-1 in the kidneys compared with DN rats. DH + IS rats showed significantly increased mRNA expression of ICAM-1 in the kidneys compared with DH rats. Immunohistochemistry revealed that ICAM-1 was localized in the cytoplasm of renal tubular cells, and was most prominently expressed in DH + IS rats. Indoxyl sulfate upregulated mRNA and protein expression of ICAM-1 in HK-2 cells. Inhibitors of NADPH oxidase (diphenylene iodonium chloride), NF-κB (isohelenin) and p53 (pifithrin-α,*p*-nitro) suppressed indoxyl sulfate-induced expression of ICAM-1 mRNA and protein in HK-2 cells.
Significance: Indoxyl sulfate upregulated renal expression of ICAM-1 through production of reactive oxygen species (ROS) such as superoxide, and activation of NF-κB and p53 in proximal tubular cells. Further, administration of indoxyl sulfate promoted ICAM-1 expression in rat kidneys. Thus, accumulation of indoxyl sulfate in chronic kidney disease might be involved in the pathogenesis of tubulointerstitial injury through induction of ICAM-1 in the kidney.

Introduction

Indoxyl sulfate, a uremic toxin, is synthesized by the liver from indole, which is generated from tryptophan in dietary proteins by intestinal flora. Progressive deterioration of renal function in chronic kidney disease (CKD) leads to accumulation of indoxyl sulfate in serum as a uremic toxin (Niwa and Ise, 1994; Niwa et al., 1994; Miyazaki et al., 1997). Administration of indoxyl sulfate to 5/6-nephrectomized rats promoted glomerular sclerosis and tubulointerstitial fibrosis in the remnant kidneys with a decline in renal function (Niwa and Ise, 1994; Niwa et al., 1994). Furthermore, it upregulated expression of fibrotic genes such as transforming growth factor-β1 (TGF-β1), tissue inhibitor of metalloproteinases-1 (TIMP-1) and type-I collagen in the remnant kidneys (Miyazaki et al., 1997). Indoxyl sulfate induced reactive oxygen species (ROS) in proximal tubular cells in the kidney, and reduced superoxide scavenging activity in the kidneys of normal and CKD rats (Owada et al., 2008). Indoxyl sulfate accumulated in serum is incorporated into renal proximal tubular cells through the basolateral membrane via organic anion transporters (OATs) 1 and 3 (Enomoto et al., 2002). After incorporation into proximal tubular cells, indoxyl sulfate induces ROS, and thereby activates nuclear factor-κB (NF-κB) and p53 (Shimizu et al., 2010, 2011b). Indoxyl sulfate-induced activation of NF-κB promotes expression of fibrotic genes, TGF-β1 and α-smooth muscle actin (α-SMA), through upregulation of p53, whereas expression of klotho is downregulated (Shimizu et al., 2011a). Further, indoxyl sulfate-induced activation of NF-κB leads to cellular senescence through upregulation of p53. Thus, indoxyl sulfate accumulates in the proximal tubular cells, and thereby induces nephrotoxicity.

Intercellular adhesion molecule 1 (ICAM-1) also known as CD54 contributes to the regulation of interactions with immune cells such as lymphocytes and monocytes/macrophages. This interaction with target cells is mediated through β2 integrins: lymphocyte function associated antigen-1 (LFA-1) expressed on T-cells, B-cells, macrophages

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http://dx.doi.org/10.1016/j.lfs.2012.11.012

and neutrophils, and macrophage-1 antigen (Mac-1) expressed on macrophages (Rothlein and Wegner, 1992; Mao et al., 2011). ICAM-1 is produced in many cell types including tubular and interstitial cells, and is upregulated at sites of inflammation (Aoyama et al., 2002; Arrizabalaga et al., 2003). ICAM-1 is the most important adhesion molecule in infiltration of macrophages which plays a key role in the pathogenesis of tubulointerstitial injury in CKD.

In the present study, we investigated the effects of indoxyl sulfate on ICAM-1 expression in rat kidneys and human proximal tubular cells (HK-2 cells). Further, we determined whether ROS, NF-κB and p53 are involved in indoxyl sulfate-induced ICAM-1 expression in the proximal tubular cells.

Materials and methods

Reagents

Indoxyl sulfate was from Alfa Aesar (Lancashire, England, UK). Diphenylene iodonium chloride (DPI), an inhibitor of NADPH oxidase, isohelenin (ISO), an NF-κB inhibitor, and pifithrin-α,*p*-nitro (PFTα), a p53 inhibitor, were from Calbiochem (La Jolla, CA, USA). Dulbecco's modified Eagle's medium (DMEM)/F12 was purchased from Wako (Osaka, Japan). Trypsin-EDTA, fetal bovine serum (FBS) and insulin–transferrin–selenium (ITS) were from Gibco (Grand Island, NY, USA). Penicillin and streptomycin were purchased from Nacalai Tesque (Kyoto, Japan). Antibodies were obtained from the following suppliers: anti-ICAM-1 (CD54) antibodies were from Cell Signaling Technology (Beverly, MA, USA) for immunoblotting and from Abcam (Cambridge, UK) for immunohistochemistry; anti-α-tubulin was from Calbiochem (La Jolla, CA, USA).

Design of experimental rats

Experimental rats were produced by Kureha Corporation as reported previously (Adijiang et al., 2010, 2011). Briefly, the rat groups consisted of (1) Dahl salt-resistant normotensive rats (DN), (2) Dahl salt-resistant normotensive indoxyl sulfate-administered rats (DN + IS) (200 mg/kg of indoxyl sulfate in drinking water), (3) Dahl salt-sensitive hypertensive rats (DH), and (4) Dahl salt-sensitive hypertensive indoxyl sulfate-administered rats (DH + IS) (200 mg/kg of indoxyl sulfate in drinking water). There were no differences in water intake between DN and DN + IS, or between DH and DH + IS. We have chosen the dose of indoxyl sulfate to increase its serum levels similar to those found in hemodialysis patients (5.3 mg/dl) (Niwa and Ise, 1994).

After 32 weeks, the rats were anesthetized intraperitoneally with pentobarbital (35 mg/kg body weight) (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan), and then renal cortices were isolated visually with a razor. The Animal Care Committee of Kureha Biomedical Research Laboratories approved the study, which proceeded according to the Guiding Principles for the Care and Use of Laboratory Animals of the Japanese Pharmacological Society.

Immunohistochemistry

Paraffin-embedded sections of kidney tissues were deparaffinized and hydrated as described previously (Adijiang et al., 2008, 2010). For immunohistochemisty, sections were stained according to the streptavidin–biotin complex method. Sections were incubated overnight with anti-ICAM-1 antibody (diluted 1:10) to determine their localization in kidney tissues. Antigen was retrieved by microwave heating twice in 0.01 M citrate buffer (pH 6.0) for 5 min at 600 W. All sections were examined by light microscopy (DN100, E600, Nikon, Tokyo, Japan).

Cell culture

We used proximal tubular cells (HK-2) to examine the effect of indoxyl sulfate on ICAM-1 expression, because indoxyl sulfate mainly accumulates in the proximal tubular cells in the kidney (Enomoto et al., 2002). HK-2 cells purchased from ATCC (Manassas, VA, USA) were maintained in DMEM/F12 supplemented with 10% FBS, ITS, 100 U/ml penicillin and 100 μg/ml streptomycin. Serum-starved HK-2 cells were incubated at 37 °C under 5% CO_2 humidified atmosphere with indoxyl sulfate (250 μM) for 1 to 48 h (mRNA), or 72 h (protein) to determine the expression of ICAM-1 mRNA or protein. Then, we determined whether DPI, ISO and PFTα suppress indoxyl sulfate-induced upregulation of ICAM-1 mRNA and protein expression. Serum-starved HK-2 cells were incubated at 37 °C under 5% CO_2 humidified atmosphere with or without DPI (10 μM), ISO (10 μM) or PFTα (10 μM) for 30 min followed by indoxyl sulfate (250 μM) for 48 h (mRNA) or 72 h (protein).

Quantitative real time PCR

Quantitative real time PCR was performed as described previously (Shimizu et al., 2011a; Tumur et al., 2010). In brief, total RNA was isolated from HK-2 cells using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan). Total RNA was extracted from renal cortex trimmed from fresh frozen renal tissues, by the acid guanidiniumthiocyanate–phenol–chloroform method using an ISOGEN RNA extraction kit (Nippon Gene, Tokyo, Japan). In detail, renal cortex (30 mg) was homogenized with 1 ml ISOGEN, and RNA-containing aqueous phase was obtained by centrifugation. Following the precipitation of RNA by centrifugation with 0.8 volume isopropanol and purification with 70% ethanol, total RNA solution was obtained by dissolution in deionized distilled water.

First-strand cDNAs were synthesized from template RNA (2 μg) using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real time PCR was performed using Syber Premix Ex Taq™ II Green (Takara Bio, Shiga, Japan) and the LightCycler PCR system (Roche, Mannheim, Germany), according to the manufacturer's protocol with the following oligonucleotide primers: human ICAM-1 5′-CTGTCACTCG AGATCTTGAGG-3′ (forward) and 5′-CCTGCAGTGCCCATTATGA-3′ (reverse); human GAPDH, 5′-ATGGGGAAGGTGAAGGTCG-3′ (forward) and 5′-GGGGTCATTGATGGCAACAATA-3′ (reverse); rat ICAM-1 5′-TC GGTGCTCAGGTATCCATCCA-3′ (forward) and 5′-CACAGTTCTCAAAG CACAGTG-3′ (reverse); rat GAPDH, 5′-AGGTTGTCTCCTGTGACTTC-3′ (forward) and 5′-CTGTTGCTGTAGCCATATTC-3′ (reverse). The expression of mRNA levels was measured as the ratio of each mRNA to GAPDH mRNA.

Immunoblotting

Immunoblotting was performed as described previously (Shimizu et al., 2009, 2011a). In brief, cell lysates were fractionated by SDS-PAGE on polyacrylamide gels, and proteins were transferred to PVDF membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA). ICAM-1 was detected using a specific antibody and normalized to α-tubulin. The protein bands were visualized using the enhanced Chemi-Lumi One system (Nacalai Tesque, Kyoto, Japan). Intensity of ICAM-1 bands normalized to the amount of α-tubulin is expressed as ratios (fold increase) of control value.

Statistical analysis

Results are expressed as mean ± SE. Values between groups were compared using the analysis of variance (ANOVA) and Fisher's protected least significance difference test. Results were considered statistically significant at $p<0.05$.