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Downregulation of connective tissue growth factor by LPS/IFN-γ-induced nitric oxide is reversed by aristolochic acid treatment in glomerular mesangial cells via STAT-1α and NF-κB signaling

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ABSTRACT

Aristolochic acid (AA) is a common cause of Chinese herb nephropathy. The mechanisms involved in the pathogenesis of AA nephropathy (AAN) are intricate. One well-documented effect of AA in the kidney is its pro-fibrotic activity. Nitric oxide (NO), a messenger gas generated from L-arginine, is the product of nitric oxide synthase (NOS). NO is involved in renal hemodynamics and exerts cytoprotective effects against renal injury. In the present study, the role of NO in AAN was investigated in MES-13 cells, a glomerular mesangial cell line. NO endogenously generated by the induction of inducible nitric oxide synthase (iNOS) with lipopolysaccharide (LPS)/interferon-γ (IFN-γ) significantly downregulated connective tissue growth factor (CTGF) protein expression in MES-13 cells. AA significantly suppressed LPS/IFN-γ-induced NO production and reversed CTGF expression that was downregulated by LPS/IFN-γ. AA decreased iNOS gene and protein expressions in a concentration-dependent manner. AA caused declines in LPS/IFN-γ-induced signal transducer and activator of transcription-1α (STAT-1α) phosphorylation and interferon response factor-1 (IRF-1) mRNA expression. Furthermore, AA attenuated IκB phosphorylation and reduced NF-κB translocation to the nuclear fraction. Taken together, our data indicate that AA reversed the CTGF expression inhibited by LPS/IFN-γ treatment via suppression of NO and iNOS expressions in MES-13 cells through inhibition of the JAK/STAT-1α and NF-κB signaling pathways. NO potentially exerts antifibrotic activity by down regulation of CTGF in MES-13 cells and inhibition of the *iNOS* gene by AA might partially account for the fibrotic effects of AA in nephropathy.

1. Introduction

Nitric oxide (NO) is a labile radical gas that exhibits a broad range of functions in biology [1–3]. NO is formed from L-arginine by three NO synthases (NOSs) including inducible (i)NOS, neuronal (n)NOS, and endothelial (e)NOS [1–3]. NO signaling is primarily mediated by cGMP, which is synthesized by NO-activated guanylyl cyclases and broken down by cyclic nucleotide phosphodiesterases (PDEs) [1–3]. In cGMP-independent mechanisms, NO either reacts with oxygen and superoxide radicals to form peroxynitrite or modifies the target protein through S-nitrosylation of cysteine thiol residues, which appear to be emerging mechanisms of NO signaling in diverse functions [1–3]. NO is involved in a variety of physiological processes in the kidney, including mediating tubular ion transport, regulating extracellular fluid volume, renin release and exerting cytoprotective effects against renal injury [4]. Under

Abbreviations: AA, aristolochic acid; AAN, AA nephropathy; CTGF, connective tissue growth factor; CHN, chinese herb nephropathy; CKD, chronic kidney disease; CM, cytokine mixtures; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; GAS, gamma-activated sequence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1β; IFN-γ, interferon-γ; γ-IRE, interferon-γ response element; iNOS, inducible nitric oxide synthase; IκB, inhibitory factor-κB; IKK, IκB kinase; IRF-1, interferon response factor; JAK, janus kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MES-13 cells, mouse glomerular mesangial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NF-κB, nuclear factor κB; NO, nitric oxide; PDEs, nucleotide phosphodiesterases; PLA_2, phospholipase A_2; PVDF membrane, polyvinylidene difluoride membrane; Q-RT-PCR, quantitative real-time reverse transcription polymerase chain reaction; STAT-1α, signal transducer and activator of transcription-1α; TLR4, toll-like receptor; TGF β, transformation growth factor β; TNF α, tumor necrosis factor α; TBS, tris-buffered saline; TBST, tris-buffered saline containing Tween 20.

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pathophysiologic conditions, NO is involved in renal hypertension, immune-mediated glomerulonephritis, glomerulosclerosis, interstitial fibrosis, diabetic nephropathy and transplant rejection [4].

Aristolochic acid (AA) produced from *Artistolochia fangchi* is a common cause of Chinese herb nephropathy (CHN) and Balkan endemic nephropathy [5]. AA-induced nephropathies (AAN) are associated with urothelial cancer development through AA-specific DNA adducts [6]. Patients with AAN show a rapidly progressive decline in renal function, resulting in end-stage renal failure requiring hemodialysis [7]. Pathologically, AAN is associated with tubular atrophy, severe anemia, inflammation and cortical interstitial fibrosis with interstitial nephritis [8]. The mechanisms involved in the pathogenesis of AAN are complicated.

Connective tissue growth factor (CTGF) is a secreted protein comprised of four conserved cysteine residue-rich domains [9]. Each functional domain has the capacity to interact with multiple biological ligands, such as growth factors, cell surface receptors and extracellular matrix proteins [9]. CTGF mediates the stimulatory actions of transformation growth factor β (TGF β) on extracellular matrix (ECM) synthesis and is strongly upregulated in fibrotic tissue [10]. CTGF is a key factor in the onset and progression of kidney damage and is involved in several forms of nephropathy [11,12]. CTGF might be a potential therapeutic target for AAN.

The overall production of NO is reduced in chronic kidney disease (CKD) [13]. Pharmacologic intervention by replenishing NO production has been used to lessen the complications of CKD [13]. The protective role of NO in renal injury has been reported in several animal models of renal disease [14]. Due to the meager information regarding the role of NO in AAN, whether NO exhibits antifibrotic effects in AAN and the underlying mechanisms were investigated in MES-13 cells in this study.

In the present study, we demonstrated that NO endogenously generated by iNOS downregulated CTGF expression in MES-13 cells. AA significantly suppressed LPS/IFN-γ-induced NO production and reversed the CTGF expression that was downregulated by iNOS. We further demonstrated that AA inhibited LPS/IFN-γ-stimulated NO production in MES-13 cells via JAK/STAT-1α and NF-κB pathways. Our data indicate that the downregulation of *iNOS* gene by AA might partially account for the pro-fibrogenic activity of some plants from the genus *Aristolochia*.

2. Materials and methods

2.1. Materials

Fetal bovine serum was from Highclone (Logan, UT). DMEM medium and Ham's F12 medium and medium supplements were obtained from Gibco BRL (Gaithersburg, MD). LPS and AA were obtained from Sigma Chemical Company (St. Louis, MO). IFN-γ was purchased from PeproTech EC Ltd. (London, UK). The specific antibodies for iNOS, CTGF, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), inhibitor κBα (IκB-α) and IRF-1 were products from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for p65 (NF-κB) was acquired from BD Biosciences (Franklin Lakes, NJ). Phosphorylated STAT-1α, STAT-1α, phosphorylated IκB-α antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Oligonucleotide primer sequences of iNOS, IRF-1 and GAPDH for reverse-transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time reverse transcription polymerase chain reaction (Q-RT-PCR) were selected by using primer select (MD Bio, Inc). The nylon membranes (Hybond N^+) for electrophoretic mobility shift assay (EMSA) were purchased from Amersham Pharmacia Biotech Inc., (Piscataway, NJ). PGE_2 EIA kit was purchased from Cayman Chemical Company (Ann Arbor, Michigan).

2.2. Cell culture

The MES-13 cell line (glomerular mesangial cells from an SV40 transgenic mouse) was obtained from the American Type Culture Collection (CRL-1927; Manassas, VA, USA) and maintained in culture medium with a 3:1 mixture of DMEM medium and Ham's medium, supplemented with 14 mM HEPES, 2 mM glutamine, antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin) and 5% fetal bovine serum at 37 °C. The NRK-52E cell line (a normal rat kidney proximal tubular epithelial cell line) was obtained from the American Type Culture Collection (CRL-1571; Manassas, VA, USA) and maintained in DMEM medium with 4 mM glutamine, antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin) and 5% fetal bovine serum at 37 °C. The incubation chamber was equilibrated with 5% CO_2–95% air.

2.3. Nitrite assay

The nitrite assay was performed to measure NO production in MES-13 cells after different treatments. NO is rapidly converted into nitrite as the end product. Thus, the nitrite accumulation in culture supernatant was used as an indirect measure of the amount of NO produced. The Griess assay [15] was used and the nitrite level was measured in triplicate. Briefly, an aliquot of 100 μl of the culture supernatant of MES-13 cells was mixed with 100 μl of Griess reagent (one part 0.1% *N*-(1-naphtyl) ethylenediamine dihydrochloride in water and one part 1% sulfanilamide in 5% H_3PO_4; both purchased from Sigma Chemicals). The mixture was incubated for 10 min at room temperature in the dark. The absorbance at 540 nm was measured and the nitrite concentration was calculated by comparison to standard curves of sodium nitrite in culture medium.

2.4. Preparation of whole cell extract and Western blot analysis

To detect the protein levels of iNOS and CTGF after exposure to different stimuli, MES-13 cells were washed with 1× PBS, scraped out, and incubated with lysis buffer. The lysis buffer contained 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail tablets (Roche Applied Science, Mannheim, Germany). Cells suspended in lysis buffer were sonicated. The homogenate was centrifuged at 13,000 rpm for 40 min at 4 °C, and the cell supernatant was collected. The protein concentration was measured using a Bio-Rad protein assay kit. Cell lysate was combined with 5× sample buffer containing 100 mM Tris–HCl (pH 6.8), 20% glycerol, 7% SDS, 5% β-mercaptoethanol, and 0.1% bromophenol blue. The sample was boiled for 5 min and centrifuged to remove the debris. Equal amounts of protein samples (60 μg) were subjected to SDS–PAGE using 10% polyacrylamide gels. Following electrophoresis, the gel was transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with 5% skim-milk in Tris-buffered saline (TBS) containing 10 mM Tris (pH 8.0) and 150 mM NaCl, then incubated with primary antibody at 4 °C overnight. TBS containing 0.02% Tween 20 (TBST) was used to wash out the nonspecific binding material on the PVDF membrane. Finally, the membrane was incubated with secondary antibody for 1 h at room temperature. After washing with TBST, the immunoreactive bands were visualized with a light-emitting kit (ECL, Amersham, UK). The protein amount was quantified by measuring the area of the iNOS and CTGF bands using densitometric analysis with AlphaEaseFC.

2.5. Preparation of cytosolic and nuclear extracts

To determine cytoplasmic IκB phosphorylation and the nuclear NF-κB protein level, cytoplasmic and nuclear proteins of MES-13