Inhibition of Lipopolysaccharide-Induced Expression of Fractalkine by Methylprednisolone via NF-κB in Human Renal Tubular Epithelial Cells

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Abstract

Objective: to study the effect of the glucocorticoid, methylprednisolone (MP), in lipopolysaccharide (LPS)-induced fractalkine (FKN) expression in HK-2 cells and to determine the role of NF-κB in this signaling pathway.

Methods: HK-2 cells were stimulated by LPS to set up an in vitro inflammation model. The concentration of FKN in cell culture supernatant was measured by ELISA. FKN and p65 mRNA expression were detected by RT-PCR. FKN, p65 protein expression and the activity of the NF-κB were detected by immunofluorescence staining and western blotting. The effect of MP and SC-514 (a selective and reversible inhibitor of IKK beta) in FKN expression and NF-κB activation induced by LPS were evaluated.

Results: LPS induced FKN expression and secretion in HK-2 cells occurred in a time- and dose-dependent manner and correlated with the activation of NF-κB. MP was able to inhibit FKN expression and secretion as well as the NF-κB induced activation of LPS, whereas SC-514 abolished this effect.

Conclusions: MP inhibited FKN expression induced by LPS through the NF-κB pathway in human renal tubular epithelial cells in vitro. Use of HK-2 cells to study the renal inflammatory process will allow the further elucidation of the pathways involved in kidney disease.

Keywords: Human renal tubular epithelial cells; NF-κB; Fractalkine; Methylprednisolone; Inflammation

Introduction

Extensive studies have confirmed that inflammatory mechanisms play significant roles in the pathogenesis of kidney disease [1,2]. Fractalkine (FKN) is a unique member of the chemokines CX3C superfamily [3]. TNF-α, IL-1 and IFN-γ is known to induce the expression of FKN in endothelial cells [4]. FKN is involved in the acute inflammatory response [5] where it acts as an adhesion molecule mediating inflammatory lesions and participates in the occurrence and progression of a variety of renal diseases [6-8] and damage processes of renal cells [9-11], in particular, renal tubular epithelial cells [12,13]. The expression of FKN has been shown to be increased in renal diseases [14-16]. It is produced as a precursor protein in cells and transfers to the cell surface as a mature protein after glycosylation [16,17].

Lipopolysaccharide (LPS) is a proinflammatory factor which plays an important role in the regulation of endogenous expression of FKN in endotoxemia. LPS induces the expression of inflammation factors such as prostaglandin E2 and TNF-α in RAW264.7 cells. The expression of inflammatory factors and the activity of NF-κB are blocked by rosemary, when used as a medicinal herb [18]. LPS also stimulates the activation of NF-κB in human renal tubular epithelial cells [19]. The expression of MCP-1 increases and the expression of surface active protein D (SP-D) are reduced after incubation of human renal tubular epithelial cells with LPS. In addition, the LPS-induced MCP-1 expression is reduced by over-expression of SP-D in transfected cells [20]. LPS induces the expression of endogenous FKN protein in human umbilical vein endothelial cells whereas alpha-lipoic acid inhibits LPS-induced expression of endogenous FKN [21]. These results confirm that LPS induces the inflammatory reaction and FKN expression.

The activity of NF-κB, which includes Rel A (p65), p50, p52, Rel B and c-Rel, can be induced by pathogenic microorganisms, tumor growth factors and inflammatory factors. Activated NF-κB transfers from the cytoplasm to the nucleus, and then binds to specific DNA promoter regions and encodes proteins that regulates cell adhesion, proliferation and apoptosis [22]. Microarray analysis of renal tubular epithelial cells (HK-2) show that NF-κB plays a major role in the network structure of normal cells and that aristolochic acid has an inhibitory effect on the NF-κB activity [23]. Several studies have confirmed that the NF-κB signaling pathway is involved in the first step of inflammatory reaction induced by LPS [24]. TNF-α, stimulates the expression of FKN in an NF-κB-dependent and autocrine manner in arterial smooth muscle cells [25] and the NF-κB pathway has been shown to be associated with expression of FKN. The binding activity of NF-κB DNA has been correlated with the expression of FKN induced by LPS in rat endothelial cells and the NF-κB inhibitor, an adenovirus-mediated mutant of IkappaB inhibits the expression of FKN, suggesting that LPS induces expression of FKN in NF-κB dependent manner [26].

The glucocorticoid, methylprednisolone (MP), is a clinically effective drug that is not only used for the treatment of inflammatory diseases, but also the preferred drug against inflammation in kidney disease. But the mechanism for the beneficial effect of MP in renal inflammatory disease has still to be elucidated. Glucocorticoids have an inhibitory effect on FKN by reducing NF-κB gene promoter recruitment in respiratory epithelial A549 cells [27].

There is a need to investigate the protective effect of MP on the expression of FKN and the NF-κB pathway in the renal inflammatory process.

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process. The present study focuses on the effect of MP on LPS-induced expression of FKN and activation of NF-κB signaling pathway in HK-2 cells, and compares the effects seen in the presence of the NF-κB inhibitor, SC-514.

Materials and Methods

Cell culture and experimental protocol

This is an in vitro study using HK-2 cells purchased from China Center for Type Culture Collection (CCTCC, GDC0136). Cells were cultured in our laboratory in DMEM (Gibco, Life Technologies, USA) supplemented with 10% fetal calf serum, 100 mg/mL streptomycin in 75% in an atmosphere of 5% CO2 and 95% at 37°C. Cells from passages 1 to 5 were trypsinized with 0.25% trypsin containing 0.02% EDTA in phosphate buffered saline (PBS) and cultured in 6-well plates in 2 mL of DMEM. When cells were 85% to 95% confluent (days 3-4), old medium was removed and replaced with 1 mL of fresh medium supplemented with 1% fetal calf serum overnight. Cells were incubated with and without LPS (100 ng/ml; Sigma Chemical Co.), MP (10⁻⁸ M; Pfizer, Belgium) and SC-514 (30 μM; Sigma-Aldrich Chemical Co.) as required.

Measurement of FKN level in cell culture supernatant

The level of FKN in the supernatant was determined using enzyme-linked immunosorbent assay (ELISA) kits (RayBiotech, Inc., Norcross, GA 30092, USA) according to the manufacturer’s instructions.

RNA Isolation and Real-Time PCR analysis of FKN and NF-κB p65 mRNA

Total RNA of cells was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instructions. First-strand cDNA was then made from the total RNA using M-MLV reverse transcriptase (C28025, Invitrogen, Shanghai, China) with random primers. The expression levels of FKN and p65 were semiquantitatively measured by real-time PCR using SYBR Green I (204143, Qiagen, Germany) through forced denaturation at 95°C for 30 sec and 40 cycles of denaturation at 95°C for 10 sec, annealing and extension at 65°C for 30 sec. Primer sequences were designed using the Primer Premier 3.0 program. The optimal reference genes were B2M and HPRT1 in HK-2 cells, determined by geNorm 3.5 software as described previously [28]. Primer sequences (Invitrogen, Shanghai, China) were as follows: FKN forward 5’-CGGGAGAGACGGAGAGCACAG-3’; reverse 5’-ACCACAGACTCTGTCATTCC-3’; p65 forward 5’-CCGGGAGAGGCTATGCTAG-3’; reverse 5’-ACTGTCACCTGGGAAGCAGA-3’; B2M forward5’-CCGTGCAAATCGTGCTCTT-3’; reverse 5’-CTCCATGATGCTGCTTACA-3’; HPRT1 forward 5’-GGGGGACCCATTAGCTTGTG-3’; reverse 5’-AATCCACAGGCTAGCAGAAG-3’. The comparative gene expression was calculated by 2⁻ΔΔCt method as described previously [29].

Immunofluorescence staining for the detection of FKN and NF-κB p65 in HK-2 Cells

Immunofluorescence staining of cultured cells was performed as described previously [9]. The cells were incubated with the primary antibodies anti-FKN antibody (ab25088, Abcam Ltd, Hong Kong; 1:200 dilution) or anti-NF-κB p65 antibody (ab31481, Abcam Ltd, Hong Kong; 1:100 dilution) overnight at 4°C and then incubated with secondary antibody Alexa Fluor 488-conjugated goat anti-rabbit IgG (Beyotime Institute of Biotechnology, China, 1:200 dilution) for 60 min at 37°C. Cells stained with Alexa Fluor 488 conjugates of goat anti-mouse IgG antibody in order to detect HK-2 cells probed with human FKN or NF-κB p65 antibodies. Alexa fluor 488 appeared as green fluorescence and cells nuclei stained with PI appeared as red fluorescence. The image of immunofluorescence staining was scanned by Quantity One software and the original intensity was quantified with freeware image analysis software, NIH Image (National Institute of Health, Bethesda, Md., USA).

Western blot analysis of FKN, NF-κB p65 and phospho p65

Immunoblot analysis was performed to detect FKN protein, NF-κB p65 protein and phospho p65 in HK-2 cells. ELISA of FKN expression in culture supernatant of HK-2 cells at 0, 4, 12 and 24 h is shown in Figure 1. Compared with control HK-2 cells, the expression of FKN in culture supernatant of LPS-induced HK-2 cells was increased significantly at 4, 12 and 24 h (P<0.01), especially at 24 h. MP or SC-514 attenuated the increased expression of FKN induced by LPS at 4, 12 and 24 h (P<0.01), especially at 24 h. There was no significant change in the expression of FKN between control cells, MP-treated cells and SC-514-treated cells. The level of FKN in the supernatant was determined using enzyme-linked immunosorbent assay (ELISA) kits (RayBiotech, Inc., Norcross, GA 30092, USA) according to the manufacturer’s instructions. Data are reported as means ± SEM for normally distributed data and median (range) for nonparametric data. The comparisons of gene expression levels and demographic characteristics of the participants between groups were performed by using the one-way ANOVA for parametric data, F-test for equality of variances and Newman-Keuls test for heterogeneity of variance. All analyses were conducted with SPSS software, version 18.0. P value <0.05 was considered statistically significantly.

Results

Effect of MP or SC-514 on FKN expression in culture supernatant of LPS-Induced HK-2 cells

ELISA of FKN expression in culture supernatant of HK-2 cells at 0, 4, 12 and 24 h is shown in Figure 1. Compared with control HK-2 cells, the expression of FKN in culture supernatant of LPS-induced HK-2 cells was increased significantly at 4, 12 and 24 h (P<0.01), especially at 24 h. MP or SC-514 attenuated the increased expression of FKN induced by LPS at 4, 12 and 24 h (P<0.01), especially at 24 h. There was no significant change in the expression of FKN between control cells, MP-treated cells and SC-514-treated cells.

Effect of MP or SC-514 on FKN and p65 mRNA Expression in LPS-Induced HK-2 Cells

Real-time PCR analysis of FKN and p65 mRNA expression in HK-2 cells at 0, 4, 12 and 24 h is shown in Figures 2 and 3. Compared with control HK-2 cells, the expression of FKN mRNA in LPS-induced HK-2 cells was increased significantly at 4, 12 and 24 h (P<0.01), especially at 24 h. MP or SC-514 attenuated the increased expression of FKN mRNA induced by LPS at 4, 12 and 24 h (P<0.01). Compared with the normal HK-2 cells, the expression of p65 mRNA in LPS-induced HK-2 cells was increased significantly at 4, 12 and 24 h (P<0.01), especially at 24 h. There was no significant change in the expression of p65 between control cells, MP-treated cells and SC-514-treated cells.
33.6), the expression of FKN in LPS-induced HK-2 cells was increased significantly at 24 h (186.36 ± 56.25, P<0.01). MP or SC-514 attenuated the increasing expression of FKN induced by LPS at 24 h (88.7 ± 38.3, 81.3 ± 36.4; P<0.05). Compared with the normal HK-2 cells (74.5 ± 29.7), the expression of p65 in LPS-induced HK-2 cells was increased significantly at 24 h (156.9 ± 42.3, P<0.05). MP or SC-514 attenuated the increased expression of p65 induced by LPS at 24 h (78.6 ± 25.2, 77.6 ± 23.7, P<0.05).

Effect of MP or SC-514 on FKN, p65 protein and phospho p65 expression in LPS-Induced HK-2 cells

Western blot analysis of FKN in HK-2 cells at 24 h is shown in Figure 6. Compared with the normal HK-2 cells, the expression of FKN protein in LPS-induced HK-2 cells was increased significantly at 24 h (P<0.05). MP or SC-514 inhibited the increasing expression of FKN protein induced by LPS at 24 h (P<0.05).

Western blot analysis of p65 protein and phospho p65 in HK-2 cells is shown in Figure 7. Compared with the normal HK-2 cells, the ratio of phospho p65/p65 in LPS-induced HK-2 cells was increased significantly (P<0.05). MP or SC-514 down-regulated the increased ratio of phospho p65/p65 induced by LPS (P<0.05).

Discussion

Progressive renal interstitial fibrosis is the histological hallmark of chronic renal disease ultimately necessitating renal replacement therapy. Important biological roles of FKN in renal inflammation have been well documented in several tissues including the pancreas [31], joints and arteries [32] and more details are known for tubular and interstitial inflammation [33]. Because FKN has critical roles in renal inflammation, factors affecting its epithelial expression may be important in regulating inflammatory processes in renal interstitial fibrosis.

NF-κB is the predominant transcription factor that regulates a wide array of genes that encode pro-inflammatory cytokines, adhesion molecules and chemokines in kidney injury [34,35]. It is now well established that there are large numbers of genes activated via the NF-κB signaling pathway. Activation of NF-κB could play a central role in inflammatory cytokines-induced FKN expression at the transcriptional level [36]. LPS is a component of gram-negative bacteria that is known to cause pro-inflammatory cytokines production in renal tubular epithelial cells [37]. Previous work revealed that LPS stimulation increases FKN expression through the activation of the NF-κB signaling pathway in vitro [21].

In this study the mechanisms of LPS-induced expression of FKN and the effect of MP on LPS-induced expression of FKN and activation of NF-κB in HK-2 cells were investigated. HK-2 cells were incubated with LPS only or co-incubated with MP or the NF-κB inhibitor, SC-514. The NF-κB expression of FKN and activation of NF-κB were measured. The secretion levels of FKN in cells supernatant was increased significantly after induction by LPS in a dose- and time-dependent manner when compared with the control cells. MP and SC-514 inhibited LPS-induced FKN secretion. The expression of FKN and p65 mRNA was also detected in gene transcriptional level. Expression of FKN and p65 mRNA was decreased by MP and SC-514 in HK-2 cells pre-stimulated with LPS.

The expression of FKN and p65 protein in HK-2 cells was detected by immunofluorescence staining and western blotting. HK-2 cells can express FKN and p65 protein. The increased expression of FKN protein in HK-2 cells induced by LPS was accompanied with

immunofluorescence staining of FKN and p65 in HK-2 cells is shown in Figures 4 and 5. Positive staining for FKN and p65 proteins were detected in cytoplasmic and perinuclear regions of HK-2 cells. Compared with control HK-2 cells (78.2 ±

Effect of MP or SC-514 on FKN and p65 expression in LPS-Induced HK-2 cells

Immunofluorescence staining of FKN and p65 in HK-2 cells at 24 h is shown in Figures 4 and 5. Positive staining for FKN and p65 proteins were detected in cytoplasmic and perinuclear regions of HK-2 cells. Compared with control HK-2 cells (78.2 ±
increased expression of p65 protein. The results indicate that MP and SC-514 can inhibit LPS-induced expression of FKN, and p65 mRNA and protein in HK-2 cells.

The activation of NF-κB by induction with LPS was detected in HK-2 cells. Both MP and SC-514 was able to inhibit the activation of NF-κB induced by LPS. This suggests that the NF-κB signaling pathway was involved in the LPS induced FKN expression in HK-2 cells and that MP was able to prevent FKN-mediated renal inflammatory injury. The effect of MP was similar to NF-κB inhibitor, SC-514, suggesting that glucocorticoid MP may inhibit LPS-induced FKN expression via NF-κB pathway.

In summary, LPS induces the expression of FKN and activation of NF-κB in HK-2 cells. MP inhibits the expression of FKN and NF-κB activation induced by LPS in vitro. The inhibition of MP is consistent with NF-κB inhibitor, SC-514. MP inhibits LPS-induced FKN expression via the NF-κB pathway in vitro and this cell culture model may be used to further explore ways of inhibiting inflammation in kidney cells.

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