MG132 reduces the pro-inflammatory effect of cyclooxygenase 2 (COX-2) on glomerular mesangial cells

Hai-ying Liu¹, Tong Wu², Fang-fang Huang¹ and Tao Wu¹*

¹The Second Hospital of Shandong University, 247 Beiyuan Road, Jinan 250033, Shandong, P. R. China.
²Shandong Polytechnic University, Daxue Road, Western University Science Park, Jinan 250353, Shandong, P. R. China.

Accepted 21 September, 2012

Cyclooxygenase 2 (COX-2) overexpression is involved in the proliferation of mesangial cells in the pathogenesis of glomerulonephritis, and nuclear factor kappa B (NF-κB) signaling participates in the induction of COX-2 gene expression in these cells. This study explores the effect of MG132, a NF-κB proteasome inhibitor, on the pro-inflammatory effect of glomerular COX-2 gene overexpression. A COX-2-overexpressing (COX-2+) mesangial cell line was established in this study. The expression of chemokine (C-C motif) ligand 2 (CCL2) and NF-κB were determined by quantitative real-time polymerase chain reaction (qRT-PCR). The activation of NF-κB was detected by electrophoretic mobility shift assay (EMSA), and the transcriptional activity of NF-κB was determined by luciferase-based reporter gene assay. CCL2 expression in NF-κB-activated cells was suppressed by MG132 (P < 0.05), especially in NF-κB-activated COX-2+ cells. The binding of NF-κB to DNA increased in MG132-treated cells and was accompanied by an increase in NF-κB p50 and p65 subunits. The transcriptional activity of NF-κB in MG132-treated cells significantly decreased, especially in COX-2+ cells (P < 0.05). Therefore, MG132 could reduce pro-inflammatory effects in glomerular mesangial cells with COX-2 gene overexpression and suppress the CCL2 expression by interfering with the dismantling of nuclear NF-κB p65.

Key words: Nuclear factor kappa B (NF-κB) inhibitor, MG132, proteasome inhibitor, cyclooxygenase 2, glomerulonephritis.

INTRODUCTION

Glomerulonephritis (GN) is a serious renal disease characterized by inflammation of the glomeruli or small blood vessels in the kidneys (Hricik et al., 1998), leading to a significant reduction in life expectancy, provoking a wide range of incidental and consequential diseases, and seriously impairing the quality of life. Glomerulonephritis remains a common cause of end-stage kidney failure worldwide (Chadban and Atkins, 2005). Therefore, clarification of the pathogenesis of glomerulonephritis is both urgent and important.

Various types of GN, such as immunoglobulin A (IgA) nephropathy, are associated with the strong proliferation of the mesangium (Hricik et al., 1998). Several researchers have found that cyclooxygenase 2 (COX-2) expression is transiently up-regulated in the human GN (Komers and Epstein, 2002). COX pathways are capable of producing the characteristic signs of inflammation, vasodilatation, hyperemia, pain, edema and cellular filtration which include in the pathogenesis of glomerulonephritis (Rahman et al., 2012). COX-2

*Corresponding author. E-mail: wutaocn@yeah.net.
expression in rat mesangial cells is regulated *in vitro* by inflammatory mediators, including interleukin 1 (IL-1), lipopolysaccharide (LPS), and serotonin (Schneider and Stahl, 1998). However, the COX-2 gene seems not to only have pro-inflammatory effects, but also glomerular inflammation-promoting effects. A possible mechanism by which COX-2 exerts its effects is the anti-inflammatory function of COX-2 metabolite-prostanoids (Gilroy et al., 1999; Nasri et al., 2012). Since the COX-2 gene has been shown to be a pathogenetic enzyme for the development of GN (Komers and Epstein, 2002), the anti-inflammatory effects of COX-2 inhibitors were tested in the present study *in vitro*. Nuclear factor kappa B (NF-kB), a protein complex that controls this transcription of DNA, is found in almost all animal cell types and is involved in cellular responses to various stimuli, such as stress, cytokines, free radicals, ultraviolet irradiation, and oxidized low density lipoproteins, among others. Incorrect regulation of NF-kB has been associated with the development of cancer, inflammatory, and autoimmune diseases, and septic shock, to name a few (Gilmore, 2006; Perkins, 2007). This study explored the anti-inflammatory effect of MG132, a NF-kB inhibitor, on glomerular COX-2 gene overexpression. However, it was necessary to investigate how the hyper-inflammatory effect, which was observed in COX-2+ mesangial cells after treatment with LPS, was limited. The anti-inflammatory potency of MG132 (MG) against COX-2+ mesangial cells was also evaluated with emphasis on the expression of chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemotactic protein-1 (MCP-1).

**MATERIALS AND METHODS**

**Cells**

COX-2-overexpressing mesangial cell lines (COX-2+) and the vector control cell lines (VCs) were used. A pre-polymerase chain reaction (PCR) construct of rat COX-2 was cloned into the mammalian expression vector pcDNA3.1/Zeo (+) (Invitrogen, Carlsbad, CA, USA). Primary rat mesangial cells (MCs) were established from glomeruli isolated from the kidneys of male Wistar rats (provided by Shandong University, China) as previously described (Stahl et al., 1990). VCs were established by the expression vector pcDNA3.1/Neo (+), which bears a neomycin-resistant gene instead of a zeocin-resistant gene (Zahnner et al., 2002), without transfection of the COX-2 PCR construct. For the selection of successfully transfected cells, the medium was supplemented with 200 g/ml zeocin (Invitrogen) for COX-2+ and with 200 mg/ml genetecin (Invitrogen) for the VCs.

**Quantitative real-time PCR**

Purification of the total RNA from transfected cells was performed using the NucleoSpin RNA Kit I (Macherey-Nagel, Germany) according to the manufacturer’s recommendations. The cDNA corresponding to 50 ng of RNA was added to the SYBR-Green JumpStart Taq Ready Mix (Sigma-Aldrich, St. Louis, MO, USA). Afterwards, the cDNA for the 18S rRNA gene was diluted due to the quantitative superiority of the ribosomal RNA. A duplicate was made for each sample. Quantitative real-time PCR (Mx4000, Stratagene, Netherlands) was performed using a three-step protocol followed by melting curve analysis to verify the homogeneity of the amplified PCR products. Calculation of CCL2 expression was carried out after the ∆∆Ct method with 18S rRNA as the reference gene to determine variations in the samples. The sequence of oligonucleotides used for real-time PCR is listed as follows: CCL2 sense: 5' CTCAGCCAGATGCA GTTAATG 3' and antisense: 5' TTCTCCACGGCAGCTATTGG 3'; 18S rRNA sense: 5' CACGGCCGGTACAGTGAAC 3' and antisense: 5' AGAGGAGCCGAGCGACC AAA 3'.

**Electrophoretic mobility shift assay (EMSA)**

[γ32P] ATP end-labeled double-stranded siRNA (luciferase) was used as a probe for EMSA. Protein complexess/recombinant proteins were added to the reaction containing 15 nM siRNA in the same buffer used for the pre-miRNA processing and were incubated for 20 min at 30°C. The protein complexes were resolved by 4% native polyacrylamide gel electrophoresis (PAGE) at 4°C.

**Transfection**

About 200,000 cells were seeded and cultured overnight in an incubator. Cotransfection was performed with 1.25 μg of pNF-kB-hrGFP plasmid (Stratagene). In addition to the internal control, cells were co-transfected with 1.25 μg of the luciferase reporter pGL3- Basic/ad,prom and hrGFP plasmid. About 500 μl of Opti-MEM (Invitrogen, Carlsbad CA, USA) with 2.5 mg of plasmid DNA and 6.25 μl of Lipofectamine™ LTX reagent (Invitrogen, Carlsbad CA, USA) were used for the cell transfection. The pre-complexation protein was incubated for 30 min at room temperature. Meanwhile, about 60% of the confluent cells were washed with transfection medium. Transfection was then performed according to the manufacturer's manual.

**Luciferase assay**

Approximately, 55 μl of luciferase lysate was added to cells washed with PBS. The lysates were incubated overnight at 4°C and then were stored and transferred for several days at -20°C. About 90 μl of an adenosine 5'-triphosphate (ATP) solution was added to 25 ml of the lysate. Determination of luciferase activity was carried out in white 96-well plates. About 25 ml of luciferin (Promega, USA) was added into the samples, after which the luminescence of the solutions was measured over 10 s. The fluorescence of the samples was also determined using FITC excitation and emission filters.

**Immunocytochemistry**

The cells were seeded on two-chamber slides for immunocytochemistry. Cells were washed with phosphate buffer saline (PBS) after treatment with LPS and MG and then fixed in humid chambers for 10 min at 20°C. Non-specific binding sites of fixed cells were subsequently masked with 600 μl of blocking buffer (1% bovine serum albumin (BSA) in PBS). Unmasking of antigen-binding sites was performed by overnight incubation with 200 μl of diluted primary antibody at 4°C. The following day, slides were first washed three times with PBS and then incubated for 35 min with 200 μl of the diluted fluorescent-labeled secondary antibody.
Coverslips were also washed extensively before mounting onto glass microscope slides with Ultra-Cruz mounting medium (Santa Cruz Biotechnology). Cells were viewed using a fluorescence microscope (Axiophot, Zeiss) at 200× and 400× magnification.

**Statistical analysis**

The values shown represent mean ± standard error (SE). For the normality test, Kolmogorov-Smirnov method was used, then data were analyzed using a one-way analysis of variance (ANOVA) followed by a Bonferroni post-test. P value < 0.05 was considered significant.

**RESULTS**

**CCL2 expression in the treatment of LPS and MG132**

VC and COX-2+ cells were incubated for 1, 3, 6, 16, and 24 h with LPS or MG + LPS. CCL2 RNA expression was then determined by quantitative real-time polymerase chain reaction (qRT-PCR). CCL2 expression increased in LPS-treated COX-2+ cells, reaching a maximum after 3 h with an increase of 637.2 ± 360.0-fold (Figure 1A); in contrast, the expression of CCL2 in VCs only increased 12.9 ± 5.9-fold at 3 h. CCL2 maximum expression of CCL2 in VCs was achieved after 6 h with an increase of 33.7 ± 18.3-fold. CCL2 expression in the MG + LPS-treated COX-2+ group decreased to 0.46 ± 0.11-fold after 3 h of treatment (Figure 1B) and then to 0.53 ± 0.25-fold after 6 h of treatment. Interestingly, MG did not inhibit CCL2 expression in MG + LPS-treated VC cells.

A concentration-time curve analysis of CCL2 expression under MG + LPS was performed. A significant reduction in CCL2 expression was observed in the COX-2+ group when compared with the VCs group (Figure 2). The expression of CCL2 decreased most at 10 µM for the MG-treated group. However, the combination of MG and LPS treatment did not significantly reduce CCL2 expression in the VC group.
Figure 3. The result of binding of NF-κB to DNA. VC and COX-2+ cells incubated with LPS in 6 h, EMSA method test the binding of NF-κB to DNA sequence.

Figure 4. Supershift of the p50 and p65 subunits of NF-κB. COX-2+ cells were incubated with LPS, or MG alone, or MG + LPS for 3 and 6 h. Supershift method test the expression of NF-κB p50 and p65.

Binding ability of NF-κB to DNA

The EMSA result of NF-κB is as shown in Figure 4. After 6 h of incubation with LPS, both VCs and COX-2+ cells showed a significant increase in NF-κB to DNA sequence binding. The LPS-induced increase in nuclear localized NF-κB to DNA binding was more significant in the COX-2+ cell group than in the VC group. Interestingly, sole treatment with MG significantly increased the binding of NF-κB to DNA in COX-2+ cells when compared with basal levels. However, the combination treatment of MG and LPS had no effect on the binding of NF-κB to DNA in COX-2+ cells (Figure 3).

Supershift of NF-κB p50 and p65

Only COX-2+ cells were examined to investigate the influence of NF-κB inhibitor on shifts between NF-κB p50 and p65 subunits, because MG + LPS treatment was effective in inhibiting the expression of CCL2 in the COX-2+ group.

LPS stimulation caused a massive increase in NF-κB to DNA binding in the COX-2+ group (Figure 3). The increased binding of NF-κB to DNA binding was accompanied by increases in p50 and p65 supershifts of NF-κB (Figure 4). LPS-treated COX-2+ showed only a partial shift of NF-κB p65. As shown in Figure 4, the binding of NF-κB to DNA increased under the sole treatment of MG. Increased binding of NF-κB to DNA was accompanied by an increase in NF-κB p50 subunits (Figure 4). The combined treatment of MG and LPS also led to significant increases in NF-κB to DNA binding (Figure 4). Interestingly, the ratio of NF-κB p50 and p65 subunits did not significantly shift under the combined treatment of MG and LPS. The DNA binding of NF-κB p50 and NF-κB p65 was similarly induced by LPS (Figure 4), and sole treatment with LPS led to a largely complete
Figure 5. Intracellular distribution of NF-κB subunit p65 in COX-2+ treated with LPS and MG132. COX-2+ cells were incubated with LPS, or MG alone, or MG + LPS for 3 and 6 h. The expression and location of NF-κB p65 were determined under fluorescence microscopy.

supershift of NF-κB p65.

Effect of MG132 on NF-κB p65

COX-2+ cells were incubated with either LPS or MG alone or MG + LPS for 3 and 6 h and then were fixed and incubated overnight. There were then incubated with a NF-κB p65 antibody for intracellular localization. Detection of NF-κB p65 was carried out with a CY-2-coupled secondary antibody by fluorescence microscopy. NF-κB p65 was cytoplasmically predominant in COX-2+ under control conditions (Figure 5). Stimulation with LPS induced a massive translocation of NF-κB p65 to the nucleus. The nuclear accumulation of NF-κB was the highest at 3 h. Sole treatment with MG provided minimal effects on the intracellular distribution of NF-κB; thus, cytoplasmic predominance of the p65 subunit was observed. MG and LPS combination therapy led to a significant nuclear translocation of NF-κB p65, similar to the LPS solo treatment, but with lower nuclear accumulation.

Transcriptional activity of NF-κB

The LPS treatment of both VCs and COX-2+ cells increased the transcriptional activity of NF-κB (Figure 6A) showing a 5.6 ± 2.7-fold increase in COX-2+ and 1.4 ± 0.3-fold increase in VCs. MG treatment led to a strong inhibition of transcriptional activity of NF-κB (Figure 6B) which decreased to 0.12 ± 0.07-fold in the COX-2+ group and 0.16 ± 0.13-fold in the VC group. MG and LPS combination therapy resulted in a significant decrease in transcriptional activity of NF-κB in both VCs and COX-2+ cells (Figure 6C), decreasing to 0.02 ± 0.028-fold in the COX-2+ group and 0.12 ± 0.08-fold in the VC group.

DISCUSSION

This study explored the influence of MG132, a NF-κB inhibitor, on the proinflammatory effect of glomerular COX-2 gene overexpression. CCL2 expression significantly increased in LPS-treated COX-2+ cells when compared with LPS-treated VC cells. CCL2 expression in the MG + LPS-treated COX-2+ group decreased significantly, but MG did not inhibit CCL2 expression in MG + LPS-treated VCs. VCs and COX-2+ cells showed a significant increase in NF-κB to DNA sequence binding after LPS treatment, while MG treatment had no negative effect. Immunocytochemical studies on COX-2+ cells show that MG treatment significantly reduces the massive LPS-induced translocation of NF-κB p65 into the nucleus (Figure 5). LPS treatment significantly increased the transcriptional activity of NF-κB in COX-2+ cells when compared with VC cells, whereas MG treatment significantly decreased the transcriptional activity of NF-κB in both COX-2+ and VC cells. The combination treatment of MG and LPS led to strong inhibition of the transcriptional activity of NF-κB in both VC and COX-2+ cells (Figure 6), and the effect of NF-κB inhibitor was more significant in COX-2+ cells than in VC mesangial cells.

In this study, the NF-κB inhibitor could significantly decrease the activity of NF-κB without impairing the

**Figure 6.** Transcriptional activity of NF-κB. VC and COX-2+ cells were incubated with LPS, or MG alone, or MG + LPS. (A) The treatment of LPS, both in VC and in COX-2+ cells group. (B) The treatment of MG both in VC and in COX-2+ cells group. (C) The treatment of LPS plus MG both in VC and in COX-2+ cells group. Values are means ± SE. *P < 0.05.

The chemokine CCL2 was selected as the target gene, because it is upregulated in the early part of glomerulonephritis (Rovin, 1999) and correlates with the extent of renal damage (Viedt and Orth, 2002). Treatment with NF-κB inhibitors caused a partial, but not significant inhibition of the CCL2 expression in LPS-stimulated COX-2+ cells after 3 and 6 h, whereas CCL2 expression in LPS-stimulated VCs slightly increased (Figure 1). Surprisingly, treatment with only the NF-κB inhibitor led to non-locking, but increased CCL2 expression (Figure 2). Aside its anti-inflammatory effect, NF-κB inhibitor also exerts pro-inflammatory effects, as demonstrated by Nakayama et al. (2001). Similar results also show that NF-κB inhibitor enhances the glomerular expression of CCL2 in rats (Zahner et al., 2007). The results of the luciferase assay are limited since only a single transcription factor was estimated while the regulation of genes such as CCL2 or NF-κB was subjected to various transcription factors (Roebuck et al., 1999).

CCL2 expression is involved in all the interactions of transcription factors and adapter protein, the transactivation of a NF-κB-regulated gene, such as CCL2, after the binding of NF-κB to DNA of various coactivators (p300/CREB and PCAF) is required (Quivy and Lint, 2004). Before p300/CREB can accumulate, NF-κB must be initially phosphorylated by different kinases (Hoberg et al., 2004); thus, it is important to activate the transcription of a target gene after assembly of the activator-coactivator complex acetylation of the NF-κB p65 subunit (Hoberg et al., 2004). The coactivators p300/CREB and PCAF are characterized by intrinsic histone acetyltransferase activity, which involves local chromatin restructuring, ultimately causing target gene transactivation (Kiernan et al., 2003). Thus, it may be reasonably conceived that stabilization of NF-κB p65 in the nucleus with altered tertiary structure of the assembly prevents the activator from activating.

The ubiquitination of cellular proteins is a highly complex process that remains incompletely understood. Recent findings indicate that protein ubiquitination is required not only by a variety of ubiquitylating enzymes, but also by deubiquitylating enzymes (DUBs). Among the DUBs, for example, A20 protein, which functions as an E3 ligase during ubiquitination, as well as a domain with DUB activity, interferes with NF-κB signaling pathways (Sutcliffe et al., 2009). Since the processes of ubiquitination and deubiquitylation are very similar and are sometimes even mediated by the same enzyme (Nkomo et al., 2010), it is conceivable that the MG-induced accumulation of polyubiquitinated proteins, such as IκB-α and nuclear NF-κB p65, leads to a balance shift by ubiquitination, and ultimately, activation of DUBs. Some researchers have shown that A20 as a DUB is able...
to deubiquitylate both K48 and K63 polyubiquitinated target proteins in vitro (Sutcliffe et al., 2009). Therefore, the NF-κB inhibitor MG132 could lead to the activation of DUBs by a preponderance of ubiquitinated proteins, which could then have a different effect on the deubiquitylation of K63 ubiquitin chains between protein-protein interactions.

Conclusively, our findings suggest that MG132 could reduce the pro-inflammatory effect of COX-2 gene overexpression in glomerular mesangial cells and suppress CCL2 expression by interfering with the dismantling of nuclear NF-κB p65.

ACKNOWLEDGEMENTS

This work was supported by National Science Foundation Grant (No. 260102051311103), Doctoral Foundation of Ministry of Education Grant (No. 26010205471001) and Shandong Province Young Scientist Award Grant (No. 26010204021123).

REFERENCES


