Journal of Korean Association
of Cancer Prevention
2001; 6(4): 240-247

NF-κB and Cyclooxygenase-2 are Negatively Regulated by Glucocorticoid in C6 Glioma Cells

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Down-regulation of NF- B-dependent gene expression by glucocorticoids may be a key underlying mechanism for its anti-inflammatory and immunosuppressive effects, since the presence of NF-κB-responsive elements are required for expression of proinflammatory cytokines. It has been shown that glucocorticoid modulates gene expression of cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) by inhibiting NF-κB. It is also known that a synthetic glucocorticoid, dexamethasone (Dexa) inhibits NF-κB activation and increases the level of the inhibitor, IκB-α. In the present study, we attempted to delineate the molecular action of Dexa on the NF-κB-DNA binding activity and the modulation of NF-κB gene expression in C6 glioma cells. We also assessed the protein level of IκB-α to elucidate its action mechanism. Results showed that the NF-κB binding activity was inhibited in a dose-dependent manner by Dexa. Lipopolysaccharide-induced luciferase activity in transfected cells with luciferase reporter plasmid containing NF-κB site of immunoglobulin κB promoer was also inhibited. Similarly, the NF-κB-dependent gene, COX-2 expression was effectively down-regulated by Dexa. A salient finding of our study was that the NF-κB inhibition by Dexa does not require a newly synthesized IκB-α, implying the involvement of some other regulatory process. Thus, our data provide a new insight on the molecular effect of glucocorticoid on NF-κB and COX-2 in C6 glioma cell, which can lead to a better understanding of its possible therapeutic usage.

Key Words: NF-κB, COX-2, Glucocorticoid, C6 glioma cells, Dexamethasone

INTRODUCTION

Ubiquitously expressed NF-κB which is one of better-studied, oxidative stress-responsive transcription factors[1)] regulates the expressions of many genes related to the immune and inflammatory processes.[2)] Furthermore, a variety of stimuli are known to activate NF-κB for the expression of various genes.[3)] Structually, NF-κB exists as a complex form of a homodimer (e.g., p50-p50), or heterodimer (e.g., p50-p65), which are bound to a member of the inhibitor proteins, IκB family.[2,4)]

In the brain, several targeted genes of NF-κB encode proteins that are involved in immune-related activities. Evidence has been accumulated that NF-κB has roles unique to the brain in such processes as neuronal plasticity, neurodegeneration and neuronal development.[3)] For instance, NF-κB activation was

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increased in the ischemic cortex due to ischemia-reperfusion injury.[5] The potential role of NF-κB in neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease has been suspected, as shown by the neurotoxic peptide Aβ, that is deposited in plaques, can activate NF-κB in neuroblastoma cells[6] and cerebellar granule cells.[7]

Glucocorticoid with well-known action against inflammation, suppresses NF-κB-dependent gene activation, in which glucocorticoid acts on NF-κB-responsive elements that are existing in many cytokine promoters for its anti-inflammatory and immunosuppressive effects.[2] For example, NF-κB-responsive elements in IL-6 and IL-8 promoters have been implicated in glucocorticoid-mediated suppression.[8,9] More relevant to its anti-inflammatory action of glucocorticoid, has shown to modulate gene expression of COX-2 and iNOS through the suppression of NF-κB.[10-12] It has further been shown that Dexa suppresses NF-κB activity by increasing inhibitory IκB-α protein in some immune cells,[13] indicating a possible regulatory site of the NF-κB inhibition.

In the present study, we investigated, using C6 glioma cells, the modulatory effects of Dexa on the modulation of NF-κB activity and COX-2 gene expression, and the underlying mechanism by assessing the status of IκB-α.

MATERIALS AND METHODS

1) Materials

Antibodies for Western blotting were obtained from Santa Cruz Biotechnology (Santa Cruz, USA), and luciferase assay kit was from Promega (Madison, USA). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore Corporation (Bedford, MA, USA). Other chemicals were purchased from Sigma (St. Louis, MO, USA).

2) Cell culture

C6 glioma cell (rat glial cell tumor; ATCC No. CCL-107) was purchased from American Type Culture Collection (ATCC, Maryland, USA). Cells were cultured in DMEM medium (Nissui Co., Tokyo, Japan) supplemented with 15% heat-inactivated (56°C for 30 min) horse serum (Gibco, Grand Island, NY, USA), 2.5% heat-inactivated (56°C for 30 min) fetal bovine serum, 5.84 mg/mL glutamine, 0.25μg/mL amphotericin and 100 unit/mL penicillin-streptomycin solution (Gibco, Grand Island, NY, USA) and adjusted to pH 7.4～7.6 with $NaHCO_3$. Cells were grown at 37°C in a 5% CO_2 atmosphere and maintained by subculturing every other day.

3) Preparation of nuclear extract and electrophoretic mobility shift assay

Nuclear extracts and cytosolic fractions were prepared by modified method of Dignam et al.[14] after C6-glioma cells were treated with lipopolysaccharide (1μg/mL) for 2 hr in the presence or absence of Dexa (0.1, 1, 10μM) or pyrrolidine dithiocarbamate (0.1 μM). The nuclear extract was frozen at -70°C in aliquots until electrophoretic mobility shift assay (EMSA) was done. The concentration of total protein in samples was measured using bicinchoninic acid containing the protein assay reagent kit of Sigma.

The oligonucleotide (5'-AGCTTCAGA*GGGGATTTCC*GAGAGG-3) containing NF-κB binding site (specified in italic letters) from immunoglobulin κ gene promoter was used to detect the DNA binding activities of NF-κB.[15] Complementary oligonucleotides synthesized separately were annealed in 20 mM Tris-HCl (pH 7.6), 50 mM NaCl, and 10 mM $MgCl_2$ after end-labeling with T4 polynucleotide kinase (Promega) and the radionucleotide [γ-^{32}P]-ATP (Amersham). Nuclear extract (10μg) was reacted in 20μl reaction mixture containing 5% glycerol, 10 mM Tris/HCl, pH 7.5, 50 mM NaCl 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 1.0% (v/v) Nonidet P40 and 1μg poly (dI-dC) on ice for 15 min and then incubated with ～1.6 pmol (～50,000 cpm) of ^{32}P-end-labeled oligonucleotide at room temperature for 20 min. In other experiments, nuclear extracts were incubated with a 200-fold excess unlabeled NF-

κB oligonucleotides or the antibodies raised against components of NF-κB such as p65 and p50 (Santa Cruz) for 15 min before the addition of labeled probe. DNA-protein complexes were separated on a 6%-nondenaturing polyacrylamide gel in a running buffer of 0.5×TBE (50 mM Tris, pH 8.0, 45 mM borate, 0.5 mM EDTA). After suitable separation was achieved, the gel was vacuum dried for autoradiography and exposed to a Fuji X-ray film at -80°C for 1~2 days.

4) Transient transfection

Transient transfection was performed according to the manufacturer's suggest protocol (Boehringer Mannheim, Mannheim, Germany). Briefly, cells (1×10^5) were plated on 6-well plates 18 to 20 hr prior to transfection. Five hundred nanogram of luciferase reporter plasmid containning NF-κB site of immunoglobulin κB promoter (pBIIX-Luc) was mixed with FuGENE™ 6 transfection reagent diluted in Opti-MEM medium (Gibco) and added to the cell. After 24 hr of incubation, the medium was replaced with fresh medium. The cells were further cultured in the presence of LPS (1μg/mL) for 24 hrs 15 hr after preincubation with Dexa (0.1, 1, 10μM). bgalactocidase reporter plasmid was used as the control cDNA.

5) Luciferase assay

Cells were washed twice with PBS and solubilized with 100μl of reporter lysis buffer. Cell lysates were removed by centrifugation at 900 g for 10 min. Luciferase assay was performed on 50μl of cell lysate as suggested by the manufacturer's protocol of luciferase assay system (Promega, Madison, USA). The light intensity was measured for 30 sec using a luminometer. The luciferase activity was normalized by total protein amount in lysates by Lowry's method [15] using bovine serum albumin as a standard.

6) Assay of PGE_2 production

Glioma cells were plated in six-well plates and incubated with Iμg/mL of LPS for 6 hr in the presence of Dexa (0.1μM) or pyrrolidine dithiocarbamate (0.1μM), and then the supernatant culture medium was collected to determine the amount of PGE_2 with EIA.[17]

7) Western blotting procedure

Western blotting was carried out by the method of Habib *et al.*[18] Total protein-equivalents (60μg) for each sample were separated by sodium dodecyl sulphate (SDS)-polyacrylamide mini-gel electrophoresis and transferred to PVDF membrane at 15 V for 1.5 hr in a semi-dry transfer system. Nonspecific binding to the membrane was blocked by 1% non-fat milk-blocking buffer of 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% at room temperature for 1 hr. The membrane was washed and then probed with the polyclonal goat antibody against COX-1 (1 : 500) and COX-2 (1 : 200), and the polyclonal rabbit antibody against IκB-α (1 : 200) overnight at room temperature. Bands were visualized with horseradish peroxidase-conjugated donkey anti-goat antibody for COX-1 and COX-2 (Santacruz, 1 : 2000) and horseradish peroxidase-conjugated donkey anti-rabbit antibody (Amersham, 1 : 2000) for IκB-α and enhanced Chemiluminescence assay (Amersham) according to manufacturer's protocol. Pre-stained protein markers were used for molecular weight determinations.

RESULTS

1) Effect of dexamethasone (Dexa) on NF-κB binding activity

To investigate the effect of Dexa on NF-κB binding activity, electrophoretic mobility shift assay (EMSA) was performed with nuclear extracts of LPS-induced C6 glioma cells after preincubation with or without Dexa. LPS-induced NF-κB binding activity was inhibited in a dose-dependent manner by

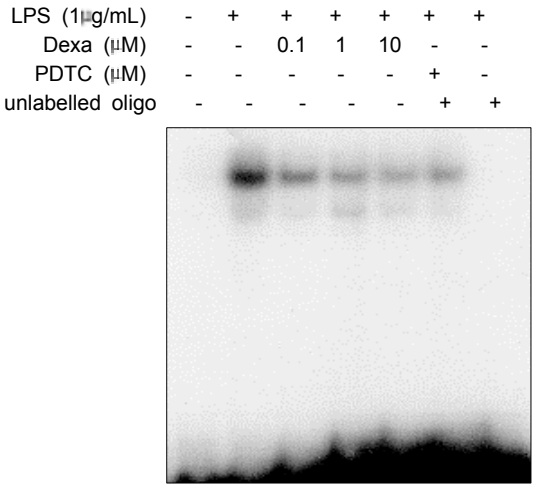

Fig. 1. Effect of Dexa on NF-κB binding activity induced by LPS in C6 glioma cell. Cells were induced by LPS after pretreatment of Dexa (0.1, 1, and 10μM) for 12 hr or PDTC (0.1μM), and then, the nuclear extract from cells was isolated to determine the binding activity of NF-κB with EMSA. Dexa, Dexamethasone; PDTC, pyrolidine dithiocarbamate.

pretreatment with Dexa. The specificity of the complex was verified by competition assay with excess unlabeled oligonucleotide that blocked non-specific binding of unlabeled oligonucleotide (Fig. 1). Pyrrolidine dithiocarbamate (PDTC), a well-known inhibitor of NF-κB, was used as a positive control. Specificity of NF-κB binding was confirmed by supershift analysis as treatment with p50 antibody resulted in faint bands, and the upper band disappeared by treatment with p65 antibody (Fig. 2). These data were taken as a clear evidence showing both the induction of heterodimer (p50-p65) of NF-κB complex by LPS and at the same time down-regulation of NF-κB by the glucocorticoid.

2) Effect of dexamethasone (Dexa) on NF-κB-dependent gene expression

To determine the effect of Dexa on NF-κB-dependent gene expression, C6 glioma cells were transfected with NF-κB-luciferase reporter plasmid, and the luciferase activity of the cells was measured

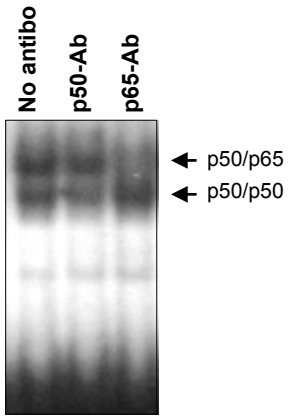

Fig. 2. Supershift analysis to determine specificity of NF-κB binding. Nuclear extracts were incubated with the antibodies raised against p65 (0.1μg) and p50 (0.1 μg) for 20 min before the addition of labeled probe, and then, EMSA was performed.

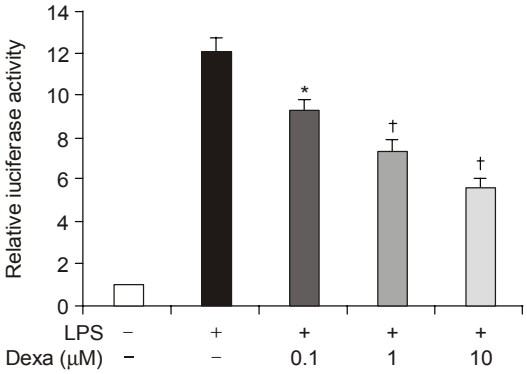

Fig. 3. Effect of Dexa on the luciferase activity of transient transfected C6 glioma cells with luciferase reporter plasmid containning NF-κB site of immunoglobulin κB promoter (pBIIX-Luc). Cells were transfected with luciferase reporter plasmid containning NF-κB site of immunoglobulin κB promoter (pBIIX-Luc), and then the cells were treated with LPS (1μg/mL) for 24 hrs 15 hr after preincubation with Dexa (0.1, 1, 10μM). bgalactocidase reporter plasmid was used as the control. The luciferase activity of the cells was measured by luminometer. Statistical significance: *$p < 0.05$ and †$p < 0.01$ vs. only LPS-treated group, respectively. Dexa, Dexamethasone; LPS, lipopolysaccharide.

by a luminometer. As shown in Fig. 3, LPS-induced luciferase activity was about 12 fold higher than control. And the level of this LPS-stimulated lu-

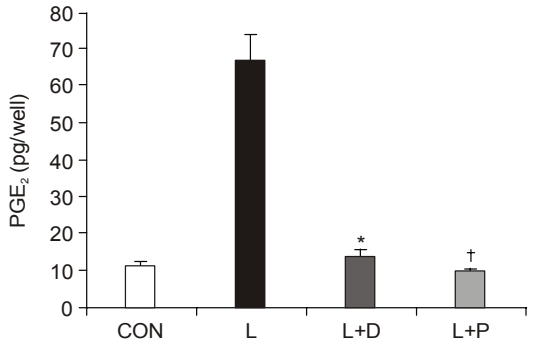

Fig. 4. Effects of Dexa on PGE_2 production in C6 glioma cells. Cells were plated in six-well plates and induced by LPS (1μg/mL) after pretreatment with Dexa (0.1μM) and PDTC (0.1μM) or without. The supernatant of culture medium was then collected to determine the amount of PGE_2 with EIA. Each value is the mean±S.E. of three samples. Statistical significance: *$p<0.01$ and †$p<0.001$ vs. only LPS-treated group, respectively. CON, control; L, lipopolysaccharide; D, dexamethasone; P, pyrrolidine dithiocarbamate.

ciferase activity was attenuated in a dose-dependent manner by Dexa pretreatment as shown in the Fig. 3.

3) Effect of Dexa on LPS-induced COX-2 gene expression and the activity

We determined the effect of Dexa on the protein amount of COX-2 and the PGE_2 level whose production is NF-κB-dependent. In LPS-stimulated cells, the PGE_2 production and protein level of COX-2 increased significantly as compared to control (Fig. 4, 5). The preincubation of the cell with 1μM Dexa effectively suppressed both COX-2 expression and production of PGE_2. However, in contrast, constitutive COX-1 was neither affected by LPS nor suppressed by Dexa pretreatment (Fig. 5). Our data also showed pyrrolidine dithiocarbamate (PDTC), a well-known inhibitor of NF-κB, suppressed COX-2 induction and PGE_2 production (Fig. 4, 5). The inhibitory effects of both Dexa and PDTC on COX-2 induction and PGE_2 production strongly suggest the requirement of activated NF-κB in the both prostanoid biosynthesis.

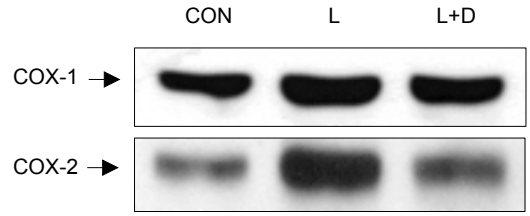

Fig. 5. Effects of Dexa on the protein levels of COX-1 and COX-2 in C6 glioma cells. Cells were plated in six-well plates and induced by LPS (1μg/mL) for 6 hr 15 hr after pretreatment with Dexa or without. Cells were then harvested. Cell extracts were resolved in a 7.5% SDS-polyacrylamide gel electrophoresis and Western blot was performed by using antibodies of COX-1, and COX-2. Bands were visualized using ECL procedure. CON, control; L, lipopolysaccharide; D, Dexamethasone, COX, cyclooxygenase

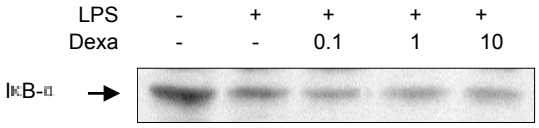

Fig. 6. Effect of Dexa on the protein level of cytoplasmic IκB-α in C6 glioma cells. Cells were stimulated by LPS for 2 hr 15 hr after pretreatment of Dexa (0.1, 1, and 10μM), and then, the cytoplasmic fraction was isolated from cells. To determine the protein of IκB-α, Western blotting was performed. Dexa, dexamethasone; LPS, lipopolysaccharide.

4) Effect of Dexa on the protein level of IκB-α

To further define the possible inhibitory mechanisms of Dexa on NF-κB binding activity and NF-κB-dependent gene expression in C6 glioma cell, its modulatory protein, IκB-α was assessed. As shown in Fig. 6, Dexa had no effect on LPS-induced decrease of IκB-α protein level. These results were taken as an evidence that the inhibitory effects of Dexa on NF-κB are likely caused through direct interaction between NF-κB and glucocorticoid receptor in C6 glioma cell without influencing IκB-α status.

DISCUSSION

Glucocorticoids are among the most potent anti-inflammatory and immunosuppressive agents that modulate pathophysiologic and pro-inflammatory activities. As most cases of the steroid-receptor mediated interaction, the activated receptor modulates transcriptional activity by eliciting the activation and repression of selected genes.[19,20] It has been shown, but yet to be proven, that gene repression of activated glucocorticoid receptor can be either initiated by binding to negative glucocorticoid-response element sites, nGRE,[21] and/or by interfering the binding with other stimulating transcription factors like NF-κB. Recently, indeed, it bas been shown that inhibitory interactions exists between the activated glucocorticoid receptor and NF-κb,[8,9] and AP-1.[19] In the present study, we demonstrated that a potent anti-inflammatory Dexa inhibits LPS-induced NF-κB binding activity and NF-κB-dependent gene expressions in C6 glioma cells, providing further supporting evidence of the glucocorticoid-mediated repression of NF-κB.

Consensus sequences of NF-κB exist in the promoter regions.[10,22,23] The gene of COX-2 which is a key enzyme in the pro-inflammatory prostanoids synthesis shares that NF-κB region has been known to be involved in the induction of COX-2 by LPS.[10] For this reasons, we looked into the effect of Dexa on the protein level of COX-2 and the production of PGE_2. Our data show that in LPS-stimulated cells, the protein level of COX-2 and the PGE_2 production increased compared to control. The Dexa pretreatment inhibited these increases, and furthermore a potent inhibitor of NF-κB, PDTC also resulted in inhibition of COX-2 induction and PGE_2 production. These results clearly indicate that the suppressed COX-2 induction and the PGE_2 production may be caused by blocking of a common step, namely NF-κB activation.

Putting together, two possible pathways can be envisioned for the molecular mechanisms of the repression of NF-κB by the activated glucocorticoid receptor.[24] One possibility is the inhibition of NF-κB by modulation of inhibitory action of IκB by glucocorticoid.[8,13,25] However, our current data do not support this, similar to those found in other cell types including mouse fibroblast L929, human fibroblast 293, monkey COS, human T-cell CEM-C7, mouse endothelial TC10, and BAEC cells where IκB-α protein levels do not increase following Dexa treatment.[26-29] The second possibility is the interaction model, in which the repressive glucocorticoid action on NF-κB is initiated by direct interaction between glucocorticoid receptor and the subunit of NF-κB, p65.[30-32] Since in our study, Dexa had no effect on LPS-induced IκB-α degradation and IκB-α synthesis, this second pathway would be more likely. This view is in line with the work of Brostjan *et al.* who showed that in endothelial cells, the induction of IκB-α is not involved in glucocorticoid-mediated repression of NF-κB activity.[33]

In summary, our results demonstrate that in C6 glioma cell Dexa inhibits COX-2 expression as well as NF-κB activity and this inhibitory effect is not involved in the modulation of IκB-α synthesis. Our data further suggests that glucocorticoid in the brain may play an important role in suppression of inflammatory process by modulating NF-κB.

ACKNOWLEDGEMENTS

This work was supported by a grant (PF002201-08) from the Plant Diversity Research Center of the 21st Frontier Research Program funded by the Ministry of Science and Technology of the Korean government.

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