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Diclofenac Inhibits IFN-γ Plus Lipopolysaccharide-Induced iNOS Gene Expression via Suppression of NF-κB Activation in RAW 264.7 Macrophages

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Diclofenac, a phenylacetic acid derivative, is a widely used non-steroidal anti-inflammatory drug (NSAID) to provide effective relief of inflammation and pain. Nitric oxide (NO) synthesized by inducible nitric oxide synthase (iNOS) has been implicated as a mediator of inflammation. We examined the inhibitory effects of diclofenac on the induction of iNOS in RAW 264.7 macrophages which were activated with lipopolysaccharide (LPS) plus interferon-gamma (IFN-γ). Treatment of RAW 264.7 cells with diclofenac and other NSAIDs (aspirin and indomethacin) significantly inhibited NO production and iNOS protein expression induced by LPS plus IFN-γ. Also, diclofenac but not aspirin and indomethacin, inhibited iNOS mRNA expression and nuclear factor-kappa B (NF-κB) binding activity concentration-dependently. Furthermore, transfection of RAW 264.7 cells with iNOS promoter linked to a CAT reporter gene revealed that only diclofenac inhibited the iNOS promoter activity induced by LPS plus IFN-γ through the NF-κB sites of iNOS promoter. Taken together, these suggest that diclofenac may exert its anti-inflammatory effect by inhibiting iNOS gene expression at the transcriptional level through suppression of NF-κB activation.

Key Words: Diclofenac, NSAIDs, iNOS, NF-κB binding site, Macrophages

INTRODUCTION

Production of low levels of nitric oxide (NO) by constitutive isoform of NOS functions to regulate a number of homeostatic processes, whereas generation of larger quantities of NO from iNOS accounts for its proinflammatory and cytotoxic effects (Moncada et al, 1991; Nathan & Xie, 1994; Schmidt & Walter, 1994). In an inflammatory setting or in the presence of endotoxin and cytokines, however, the inducible nitric oxide synthase isoform (iNOS) is expressed in numerous cell types, including smooth muscle cells, hepatocyte and macrophages. Moreover, NO has recently been found to modulates cyclooxygenase (COX) activity, thereby increasing prostaglandin production that may enhance inflammation (Stoclet et al, 1999; Notoya et al, 2000). This effect of NO is probably due to its ability to activate the COX enzyme by unknown mechanism.

iNOS expression is very high in macrophages that are activated by a combination of LPS and IFN-γ (Lowenstein et al, 1993). The murine iNOS promoter contains various transcription factor binding sites, including nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1) sites, which are involved in cytokines or lipopolysaccharide (LPS) induction of other genes (Lowenstein et al, 1993). Analysis of the transcriptional activity of iNOS promoter using deletion mutants revealed the essential role of two NF-κB binding motifs in the control of iNOS expression (Lowenstein et al, 1993; Xie et al, 1993; Spink et al,

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1995). Bacterial LPS and cytokines are key mediators in the inflammatory response and have been shown to activate NF-κB that is critical for the inducible expression of multiple genes involved in inflammation (Baldwin, 1996; Baeuerle & Baichwal, 1997). NF-κB is, therefore, an obvious target for new types of anti-inflammatory treatments.

Some NSAIDs have been reported to inhibit the NF-κB binding and NF-κB mediated iNOS gene expression via COX-independent mechanism (Kopp & Ghosh, 1994; Farivar et al, 1996; Farivar et al, 1998; Sanchez et al, 1999). More recently, we also found that acetaminophen inhibits iNOS expression by suppressing of NF-κB binding activity, in mouse macrophages, whereas aspirin and sodium salicylate inhibit NO production without affecting NF-κB binding1(Ryu et al, 2000). Diclofenac, a dual inhibitor of COX-1 and COX-2, is a widely used anti-pyretic and anti-inflammatory drug (Insel, 1996; Berg et al, 1999). Although evidences suggest that anti-inflammatory effect of diclofenac is due to inhibition of COX as well as NO production, inhibitory effect of diclofenac on the iNOS expression and its underlying molecular mechanism have not yet been understood. In the present study, we examined whether diclofenac has an inhibitory effect on the iNOS gene transcription through the NF-κB site of the iNOS promoter.

METHODS

Cell culture and nitrite concentration assay

RAW 264.7, a mouse macrophage cell line (American Type Culture Collection, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies/BRL, Gaithersburg, MD, USA) supplemented with 2 mM L-glutamine, antibiotics (100 U/ml penicillin G and 100 g/ml streptomycin), and 10 heat-inactivated FBS. Accumulated nitrite (NO_2^-) in culture medium was measured using an automated colorimetric assay based on the Griess reaction by measuring absorbance at 540 nm in a microplate reader (Molecular Device, USA).

Western analysis

RAW 264.7 cells were incubated with LPS (1 μg/ml, Sigma, St. Louis, MO, USA) plus IFN-γ (10 U/ml, Genzyme, Cambridge, MA, USA) in the presence or absence of diclofenac or NSAIDs (aspirin, indomethacin) for 24 h. The cells were lysed in buffer containing 20 mM HEPES (pH 7.9), 0.1 M KCl, 0.3 M NaCl, 10 mM EDTA, 1 SDS, 1 2-mercaptoethanol, 1% Nonidet P-40, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml aprotinin. The supernatant after centrifugation at 15,000 g for 5 minutes was subjected to SDS-PAGE on 8% polyacrylamide gel and transferred to polyvinylidene difluride membrane (Amersham International) in 25 mM Tris, 20 methanol and 192 mM glycine. Blots were blocked at 4°C overnight by 5 nonfat milk dissolved in PBS containing 0.1 Tween-20 (PBST) and then incubated at room temperature with 0.5 μg/ml murine anti-iNOS antibody (Transduction Laboratories, Inc., Lexington, KY) for 1 h. The blots were washed and incubated with goat anti-mouse IgG conjugated to peroxidase in PBST containing 5% nonfat milk for 1 h. Following further washing in PBST, immunoreactive bands were visualized using ECL detection system (Amersham Co.).

Reverse transcription and polymerase chain reaction (RT-PCR)

RAW 264.7 cells were lysed with RNAzol B reagent (Tel-Test, Friendswood, TX), and the total cellular RNA was purified according to the manufacturer's recommended procedure. PCR primers for mouse iNOS and β-actin were as follows: iNOS sense primer, 5'-CAGAAGCAGAATGTGACCATC-3'; iNOS antisense primer, 5'-CTTC TGGTCGATG TCATGAGC-3'; β-actin sense primer, 5'-GTGGGGCGCCCCAGGCACCA-3'; β-actin antisense primer, 5'-CTCCTTAATGT CACGCACGATTTC-3'. PCR was performed in a 30 μl reaction volume containing 1×Taq polymerase buffer (10 mM Tris, pH 8.3), 200 μM each deoxynucleotide mixture, 1.5 mM $MgCl_2$, 0.5 U Taq polymerase, 0.5 μM each oligonucleotide primer, and 2 μl of RT products. After preincubation for 5 min at 94°C, 30 cycles of amplification (94°C for 2 min, 65°C for 2 min, and 72°C for 2 min) were performed and β-actin was used for an internal control. Each PCR reaction was analyzed by 1.2% agarose gel electrophoresis in Tris-acetate-EDTA buffer and stained with ethidium bromide.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts for EMSA were prepared from RAW 264.7 cells as previously described (Dignam et

al, 1983) with minor modification. For binding reaction, 5 μg of nuclear extract was incubated at room temperature for 20 min with reaction buffer containing 20 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 5% glycerol, 200 μg/ml BSA, and 2 μg of poly (dI-dC) · poly (dI-dC). Then, the ^{32}P-labeled double-stranded oligonucleotide (1 ng, ≥ 1×10^5 cpm) containing the NF-κB binding consensus sequence (5'-GGCAA CTGGGGACTCTCC-CTTT-3') was added to the reaction mixture for an additional 10 min at room temperature. The reaction products were fractionated on a nondenaturating 6% polyacrylamide gel, which was then dried and subjected to autoradiography. For competition assays, the excess oligonucleotide (100-fold molar excess) competitor was preincubated with nuclear extracts for 20 min at room temperature. A mutant NF-κB oligonucleotide used for the competition assay was as follows: 5'-GGCAACTGCTCA CTCTC CCTTT-3'; the mutated sequences are underlined.

Plasmid constructs

The piNOS 973 plasmid containing the regions from −973 to +82 of mouse iNOS promoter and piNOS m973 (mNF-κBu, mNF-κBd) plasmid containing the mutated upstream and downstream NF-kB sites in the same region of piNOS 973, which were linked to chloramphenicol acetyltransferase (CAT) reporter gene has been described earlier (Kim et al, 1997).

Transient transfection and CAT assay

RAW 264.7 cells were transfected by calcium phosphate-DNA co-precipitation method, as described previously (Gorman, 1986), with 20 μg of plasmid DNA containing the iNOS promoter construct. After 6 h, the cells were washed twice with 1×PBS followed by a 2 min shock with 15% glycerol and stimulated with LPS plus IFN-γ. At least 15 h later, the cells were lysed by freezing and thawing. Cell lysate was heated at 65°C for 10 min to inactivate CAT inhibitors. Protein content was determined by the Bradford assay (Bradford, 1976) and equal amounts of proteins were assayed for CAT enzyme activity by TLC method. As an internal control for transfection efficiency, all cells were cotransfected with 5 μg of pCH110 plasmid (Pharmacia, Piscataway, NJ) for β-galactosidase assay.

RESULTS

Diclofenac inhibits NO production, iNOS protein and iNOS mRNA expression induced by LPS plus IFN-γ

The effects of diclofenac and other NSAIDs (aspirin and indomethacin) on LPS plus IFN-γ-induced iNOS protein expression and NO production were examined (Fig. 1). Diclofenac inhibited iNOS protein expression and NO production in a dose-dependent manner. Aspirin was also found to inhibit iNOS pro-

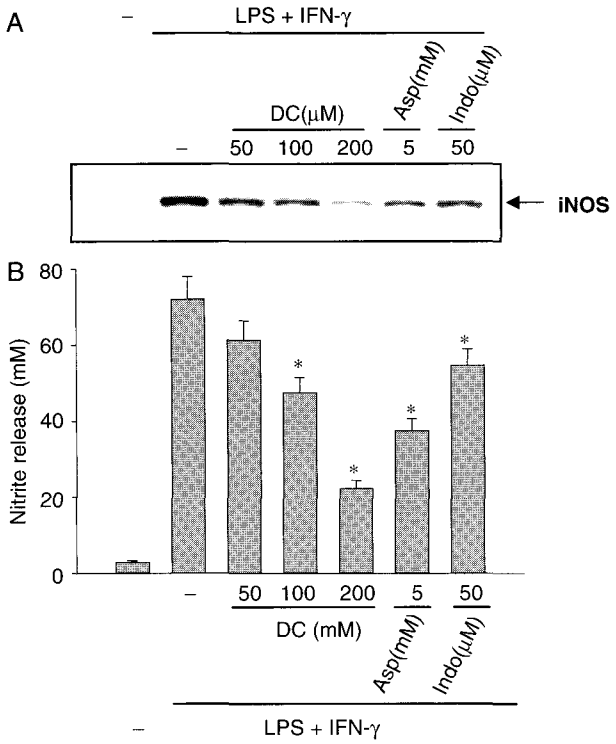

Fig. 1. Effects of diclofenac on LPS plus IFN-γ-induced iNOS protein expression and NO production in RAW 264.7 cells. RAW 264.7 cells were incubated with LPS (1 μg/ml) plus IFN-γ (10 U/ml) in the presence or absence of the indicated concentrations of diclofenac, aspirin or indomethacin. (A) Immunoblot analysis. After 24 h incubation, cell lysates (30 μg protein) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and blotted with a mouse monoclonal anti-iNOS antibody. This blot is a representative of three independent experiments. (B) Nitrite assay. The concentrations of nitrite released into media were measured by the Griess reaction. Each value is representative of mean SE from four independent experiments. Asterisks indicate significant difference ($P<0.05$), by Students t test, compared with that of LPS plus IFN-γ-treated cells.

tein expression and NO production markedly, whereas indomethacin was less active. Next, to examine whether the suppression of iNOS activity by diclofenac, aspirin and indomethacin was mediated by transcriptional regulation, their effects on iNOS mRNA expression were assessed by RT-PCR. As expected, treatment of RAW 264.7 cells with LPS plus IFN-γ for 5 h dramatically increased iNOS mRNA expression (Fig. 2), however, when the cells were incubated with various concentrations of diclofenac, there was a dose-dependent decrease of iNOS mRNA expression induced by LPS plus IFN-γ. In contrast, neither aspirin nor indomethacin treatment showed any inhibitory effect on iNOS mRNA expression.

Diclofenac inhibits LPS plus IFN-γ-induced iNOS promoter activity through suppression of NF-κB activation

It has been known that NF-κB activation is an essential process for the induction of iNOS gene transcription, and that iNOS promoter has two functional NF-κB binding sites (Lowenstein et al, 1993; Xie et al, 1993; Spink et al, 1995). Thus, we examined the effects of diclofenac on LPS plus IFN-γ-induced NF-κB binding activity by EMSA. As shown in Fig. 3, incubation of RAW 264.7 cells with LPS plus IFN-γ generated prominent NF-κB complex binding. This NF-κB binding was dose-dependently inhibited by diclofenac, but not by either aspirin or indomethacin. Addition of a 100-fold molar excess of unlabeled wild-type probe specifically inhibited the NF-kB binding, while a probe bearing the mutated NF-kB binding sequence did not, indicating the binding specificity of NF-κB complex.

To examine the effects of diclofenac on iNOS promoter activity, RAW 264.7 cells were transiently transfected with a plasmid containing the mouse iNOS promoter region (position from -973 to $+82$) linked to CAT reporter gene. Significantly enhanced CAT expression was observed in response to LPS plus IFN-γ (Fig. 4). As expected, diclofenac signi-

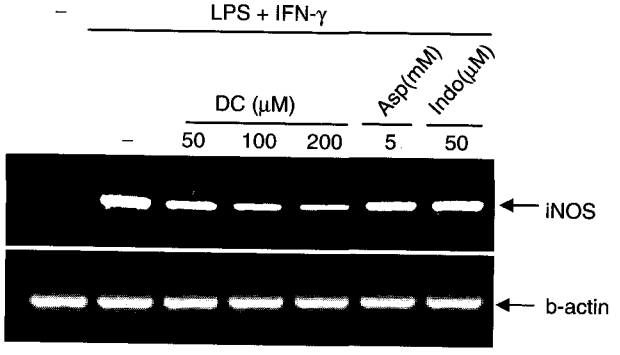

Fig. 2. Effects of diclofenac on LPS plus IFN-γ-induced iNOS mRNA expression. Total RNA (3 μg) from RAW 264.7 cells incubated with LPS (1 μg/ml) plus IFN-γ (10 U/ml) in the presence or absence of indicated concentrations of diclofenac, aspirin or indomethacin for 6 h was reverse transcribed and subjected to PCR to quantify the expression of iNOS and b-actin mRNA with specific primers. The PCR products were separated on a 1.2% agarose gel and stained with ethidium bromide. β-actin was used as an internal control.

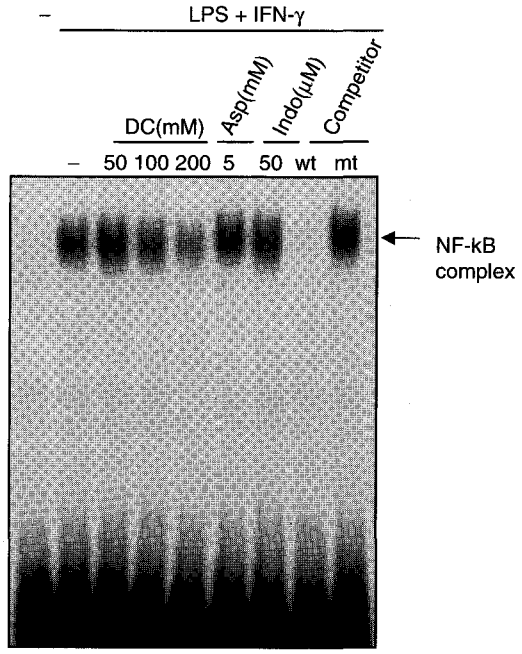

Fig. 3. Effects of diclofenac on LPS plus IFN-γ-induced NF-κB binding activity. RAW 264.7 cells were incubated with LPS (1 μg/ml) plus IFN-γg (10 U/ml) in the presence or absence of the indicated concentrations of diclofenac, aspirin or indomethacin. After 5 h incubation, nuclear proteins were prepared and subjected to EMSA for NF-kB binding as described under Materials and Methods. The specificity of bands depict the binding of NF-κB in nuclear extracts from cells treated with LPS plus IFN-γ for 5 h in the presence of 100-fold molar excess of unlabeled oligomers (wt) or oligomers containing the mutated NF-κB binding site (mt).

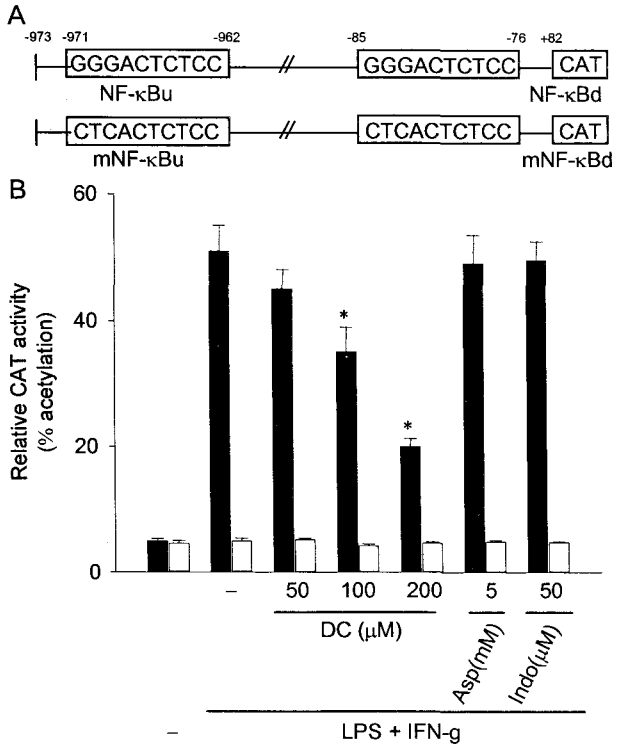

Fig. 4. Diclofenac inhibits LPS plus IFN-γ-induced iNOS promoter activity via NF-κB binding site. (A) CAT construct (piNOS m973) containing the mutated upstream and downstream NF-κB sites in the region from −973 to +82 of wild type piNOS 973 plasmid. The mutated nucleotides of the upstream and downstream NF-κB sites in CAT constructs are shown as bold characters. (B) The RAW 264.7 cells were transfected with piNOS 973 (solid bars) or a piNOS m973 (open bars), and treated with LPS (1 μg/ml) plus IFN-γ (10 U/ml) for 15 h in the presence or absence of indicated concentrations of diclofenac, aspirin or indomethacin. CAT activity was assayed by TLC. Results were indicated as percent acetylation and the means SE of four independent experiments. Asterisks indicate significant difference ($P<0.05$), by Students t test, compared with that of LPS plus IFN-γ stimulated cells.

ficantly decreased the CAT activity. The inhibitory effect of diclofenac on promoter activity was dose-dependent with a maximal effect at 200 μM. However, addition of neither aspirin (5 mM) nor indomethacin (50 μM) affected the iNOS promoter activity. In order to further determine the direct role of the two NF-κB sites on the control of iNOS promoter activity by diclofenac, we constructed mutant plasmid, piNOS m973 that contained three sites specific mutations within the upstream and downstream NF-κB sites of piNOS 973 (Fig. 4A). When RAW 264.7 cells were transfected with piNOS m973 and incubated with LPS plus IFN-γ, there was not only a marked reduction in CAT activities compared with those of cells transfected with the wild type piNOS 973, but also no difference in CAT activities by the addition of diclofenac, aspirin or indomethacin (Fig. 4B), suggesting an essential role of two NF-κB sites for the iNOS promoter activity.

DISCUSSION

The mechanism of action and side effects of aspirin or other NSAIDs are explained mainly on the basis of inhibition of COX and consequent diminished synthesis of prostaglandin (Insel, 1996). Although low doses of NSAIDs inhibit the biosynthesis of prostaglandin, high concentrations interfere with processes which are not dependent on prostaglandins. Previous studies including ours have shown that some NSAIDs such as aspirin or sodium salicylate inhibit the production of NO in macrophages, vascular smooth muscle cells and hepatocytes, and suggest that their anti-inflammatory effects are attributable to the inhibition of NO production (Amin et al, 1995; Sakitani, 1997; Katsuyama et al, 1999; 15, 22-24; Ryu et al, 2000). Aspirin and indomethacin are inhibitors of COX-1 and COX-2, but they show much more strong inhibitory effect on the COX-1 than on the COX-2, and COX-1 inhibition is thought to be responsible for the renal and gastrointestinal side effects of aspirin-like NSAIDs (Insel, 1996). Diclofenac, a derivative of phenylacetic acid, is known as an equivalent inhibitor of COX-1 and COX-2 as well as potent inhibitor of lipoxygenase, which is associated in the synthesis of leukotriene, a chemotactic agent (25). Therefore it shows more potent anti-inflammatory and less ulcergenic effects than aspirin or indomethacin (Battistini et al, 1994; Insel, 1996).

In this experiment, we observed that diclofenac and other NSAIDs (aspirin and indomethacin) significantly inhibited iNOS protein expression and NO production induced by LPS plus IFN-γ in RAW 264.7 macrophages (Fig. 1). Although these results are consistent with other studies in which diclofenac and aspirin-like NSAIDs inhibit NO production in diverse cultured cell types, nothing has yet been known about the precise inhibition mechanism of iNOS expression by diclofenac. Interestingly, diclo-

fenac inhibited LPS plus IFN-γ-induced iNOS mRNA expression, whereas aspirin and indomethacin did not affect on it. These results suggest that diclofenac and other NSAIDs (aspirin or indomethacin) may have a different inhibitory mechanism of iNOS expression in macrophages. Although the concentrations of diclofenac used in this study were supra-pharmacological compared with therapeutic plasma concentrations (10~20 μM) in patients with rheumatoid arthritis, osteoarthritis and ankylosing spondylitis (Insel, 1996), we did not observe any nonspecific or cytotoxic effect, as evaluated by MTT assay after 24 h incubation.

It has recently been reported that anti-inflammatory agents such as aspirin and salicylates inhibit cytokine-induced NF-κB activation in human Jurkat T, rat cardiac fibroblast cells and bovine vascular smooth muscle cells by preventing phosphorylation and degradation of inhibitory protein, IB (Kopp & Ghosh, 1994; Farivar et al, 1996; Farivar et al, 1998; Sanchez et al, 1999). However, we found that aspirin did not affect on iNOS mRNA expression and NF-κB activation induced by LPS plus IFN-γ in RAW 264.7 cells (Fig. 2, 3). Our results appear to be consistent with those of studies in which aspirin and salicylates inhibited cytokine-induced NO production without affecting iNOS mRNA expression in systems such as rat hepatocytes and vascular smooth muscle cells (Sakitani et al, 1997; Katsuyama et al, 1999). Apparent discrepancies among these results suggest that the inhibitory pathways of iNOS may be modulated differently by aspirin and salicylates in a cell- or tissue-specific manner. It had also been reported that the higher potency of aspirin to inhibit iNOS activity than other NSAIDs might be attributable to acetylation of functional components of the enzyme (Amin et al, 1995). Taken together, these data suggest that aspirin may suppress cytokine-induced NO production in RAW 264.7 cells at the translational or post-translational levels and directly the catalytic activity of iNOS.

Although diclofenac has been shown to inhibit the LPS plus IFN-γ-induced iNOS expression via suppression of NF-κB activation (Fig. 3), it remains unknown whether diclofenac might have other inhibitory mechanism to inhibit iNOS expression. In this regard, exogenous PGE_2 has been shown to down-regulate iNOS expression by inhibiting NF-κB activation in primary mesangial cell and J774 cells (Tetsuka et al, 1994; D'Acquisto et al, 1998). However, in our experimental condition, PGE_2 at any concentrations did not affect the LPS plus IFN-γ-induced NF-κB activation or iNOS mRNA expression (data not shown). Thus, the inhibitory effect of diclofenac on iNOS expression was most likely mediated by a mechanism independent of COX or PGE_2 production.

Two essential *cis*-acting element, a promoter proximal region (position -85 to -76, downstream NF-κB site) and a more distal region (position -971 to -962, upstream NF-κB site) are known to mediate transcriptional induction of iNOS (Lowenstein et al, 1993; Xie et al, 1993; Spink et al, 1995). Thus, the analysis of NF-κB activation is important to understand mechanisms by diclofenac controls iNOS gene transcription. In this experiment, diclofenac was found to significantly suppress NF-κB activation induced by LPS plus IFN-γ, whereas aspirin and indomethacin did not. In addition, by measuring iNOS promoter activity transfected in RAW 264.7 cells with piNOS 973 containing two NF-κB sites, we also found that diclofenac significantly inhibited iNOS promoter activity in a dose dependent manner, whereas no significant difference was observed in cells treated with aspirin and indomethacin (Fig. 4). The specific role of both NF-κB sites in the regulation of iNOS expression by diclofenac was in an agreement with the absence of diclofenac effect on the iNOS promoter with mutated NF-κB sites (piNOS m973). These observations suggest that diclofenac exerts its anti-inflammatory action by inhibiting iNOS gene transcription through the NF-κB sites in the iNOS promoter.

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