Role of protein kinase R in double-stranded RNA-induced expression of nitric oxide synthase in human astroglia

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Abstract Environmental factor(s), such as viral infection, has been implicated as one of the triggering events leading to neuroinflammation in multiple sclerosis. This study underlines the importance of double-stranded RNA (dsRNA), the active component of a viral infection, in inducing the expression of inducible nitric oxide synthase (iNOS) in human astroglia. DsRNA in the form of synthetic polyinosinic-polycytidylic acid (poly IC) induced expression of iNOS and iNOS promoter-driven luciferase activity through activation of nuclear factor (NF)-κB and CCAAT/enhancer-binding proteinβ (C/EBPβ). In addition, we show that inhibitors of protein kinase R attenuated iNOS by suppressing the activation of NF-κB but not C/EBPβ. In contrast, knock down of p38 mitogen-activated protein kinase (MAPK) attenuated iNOS by suppressing the activation of C/EBPβ but not NF-κB. This study delineates a novel role of dsRNA in inducing the expression of iNOS through dsRNA-activated protein kinase (PKR)-mediated activation of NF-κB and p38-mediated activation of C/EBPβ in human astroglia that may participate in virus-induced neurological abnormalities.

Key words: Double-stranded RNA; Human astroglia; Inducible nitric oxide synthase; Nuclear factor-κB; CCAAT/enhancer-binding proteinβ

1. Introduction

Multiple sclerosis (MS) is an autoimmune, inflammatory, demyelinating disease of the central nervous system (CNS) that generally afflicts persons between the ages of 20 and 50 years. The major pathological hallmark of MS is the presence of sclerotic plaques in the CNS which are characterized by demyelination associated with a broad-spectrum inflammatory reaction orchestrated by activated lymphocytes, macrophages, and endogenous glial cells (astroglia and microglia) [1]. Although the etiology of MS remains unknown, epidemiological studies suggest that both environmental and genetic factors play a role [1,2]. Environmental factor(s), particularly viral infection, has been implicated as one of the potential triggering events leading to demyelination in MS [2,3]. Demyelination in both humans and rodents can be initiated by infection with a diverse group of viruses. Consistently, both retroviral expression and cytotoxic factor production have been evidenced in MS monocyte/macrophage cultures and MS cerebrospinal fluid. Furthermore, human coronaviruses (HCoV), which cause common colds, can also infect neural cell cultures [4,5]. Coronaviruses induce an MS-like disease in rodents, and are neuroinvasive in humans [6,7]. Consistently, HCoV RNA was detected in MS patient brains [6,8] and in cerebrospinal fluid of MS and other neurological disease patients [7]. Coronavirus antigens were also detected in MS patient brains [8]. It is possible that a viral infection acquired during adolescence initiates MS after a long period of quiescence. However, the mechanism by which viral infection leads to neuroinflammation and demyelination in MS is poorly understood.

The active component of a viral infection that stimulates antiviral activities appears to be double-stranded RNA (dsRNA), which accumulates during the replication of many viruses [9]. Nitric oxide (NO) produced from the expression of inducible nitric oxide synthase (iNOS) participates in the pathogenesis of MS [10,11]. We herein report that dsRNA markedly induces the expression of iNOS in human astroglia.

2. Materials and methods

2.1. Reagents

Poly IC was obtained from Sigma. The dominant-negative mutants of CCAAT/enhancer-binding proteinβ (ΔC/EBPβ), p38 (Δp38) and dsRNA-activated protein kinase (PKR) (ΔPKR) were kindly provided by Dr. S. Smale of the University of California at Los Angeles, Dr. J. Alam of Alton Ochsner Medical Foundation at New Orleans, and Dr. R. Donis of the University of Nebraska at Lincoln, respectively. The pMT-K3L was obtained from Dr. B. L. Jacobs, Arizona State University at Tempe.

2.2. Astrocytes

Human primary astrocytes were prepared as described earlier [12,13]. Human U373MG astroglial cells were obtained from American Type Culture Collection (ATCC).

2.3. Assay for NO synthesis

Culture supernatants were assayed for nitrite, a stable reaction product of NO with molecular oxygen, using Griess reagent [12,13].

2.4. Immunoblot analysis for iNOS

Immunoblot analysis for iNOS was carried out as described earlier [12,13] using antibodies against mouse macrophage iNOS.

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2.5. RNA isolation and Northern blot analysis

Northern blot analysis for iNOS mRNA expression was carried out as described earlier [12,13] using ^{32}P-labeled cDNA probe.

2.6. Assay of iNOS promoter-driven reporter activity

Human 7.2-kb iNOS promoter-driven luciferase activity was monitored as described earlier [12,13] using phiNOS(7.2)Luc. Briefly, 24 h after transfection, cells were treated with stimuli for 12 h. Firefly and Renilla luciferase activities were obtained by analyzing total cell extract according to standard instructions provided in the Dual Luciferase Kit (Promega) in a TD-20/20 luminometer (Turner Designs).

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared and used for EMSA using ^{32}P end-labeled double-stranded nuclear factor (NF)-κB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ and C/EBP, 5′-TGC AGA TTG CGC AAT CTG CA-3′ oligonucleotides as described earlier [14]. Double-stranded mutated NF-κB, 5′-AGT TGA GGC GAC TTT CCC AGG C-3′, and C/EBP, 5′-TGC AGA GAC TAG TCT CTG CA-3′ (Santa Cruz Biotechnology, Inc.) oligonucleotides were used to verify the specificity of binding. For supershift analysis, 1 μg of antibodies against C/EBPβ (Santa Cruz Biotechnology, Inc.) were included in the binding reaction.

2.8. Assay of transcriptional activities of NF-κB and C/EBPβ

Transcriptional activities of NF-κB and C/EBPβ were assayed as described earlier [12–14] using pNF-κB-Luc (an NF-κB-dependent reporter construct) or pC/EBPβ-Luc (an C/EBPβ-dependent reporter construct).

3. Results

3.1. Poly IC induces the production of NO and the expression of iNOS in human U373MG astroglial cells and primary astroglia

Poly IC, a synthetic dsRNA copolymer of inosinic and cytidylic acids, has been often used as a tool to mimic the effects of dsRNA intermediates produced during viral infection of cells [9]. It is evident from Table 1 that poly IC alone markedly induced the production of NO in U373MG astroglial cells. Since poly IC is made up of two homopolymers of inosinic and cytidylic acids, we examined if each of these polymers could induce NO. In contrast to dsRNA, treatment of U373MG astroglial cells with single-stranded RNA (ssRNA) (either poly I or poly C) resulted in very little induction of NO production (Table 1) suggesting that the presence of RNA with double-stranded structures is required for the induction of NO. Furthermore, these results also suggested that the induction of NO production was not due to lipopolysaccharide (LPS) contamination in the RNA preparation, which is a potent inducer of NO production in different cells. The inhibition of poly IC-induced production of NO by arginase (an enzyme that degrades the substrate, L-arginine, of NOS) and L-NMMA (a competitive inhibitor of NOS) but not by D-NMMA (a negative control of L-NMMA) suggests that poly IC induces the production of NO through NOS-mediated arginine metabolism (Table 1). To understand the mechanism of induction of NO production, we examined the effect of poly IC on protein and mRNA levels of iNOS. Fig. 1 shows that poly IC dose dependently induced the production of NO (A) and the expression of iNOS protein (B) and mRNA (C). Similarly poly IC also induced iNOS promoter-driven luciferase activity in a dose-dependent fashion, and the maximum induction of about 3.7-fold was observed at 100 or 150 μg/ml of poly IC (Fig. 1D). Consistent to the induction of iNOS in U373MG astroglial cells, poly IC also dose dependently in-

Table 1
Poly IC induces the production of NO through NOS in human U373MG astroglial cells

Treatments	Nitrite (μg/mg protein/24 h)
Control	6.2 ± 0.8
Poly IC (100 μg/ml)	156.2 ± 19.7
Poly I (100 μg/ml)	9.5 ± 1.8
Poly C (100 μg/ml)	10.2 ± 1.5
Poly IC+arginase	7.4 ± 1.0
Poly IC+L-NMMA	17.4 ± 1.5
Poly IC+D-NMMA	154.9 ± 20.2

Cells were cultured for 24 h in serum-free Dulbecco's modified Eagle's medium (DMEM)/F-12 with the listed reagents; and nitrite concentration in the supernatants was measured. Arginase (100 units/ml), L-NMMA (0.1 mM) and D-NMMA (0.1 mM) were added to the cells together with poly IC. Data are means ± S.D. of three different experiments.

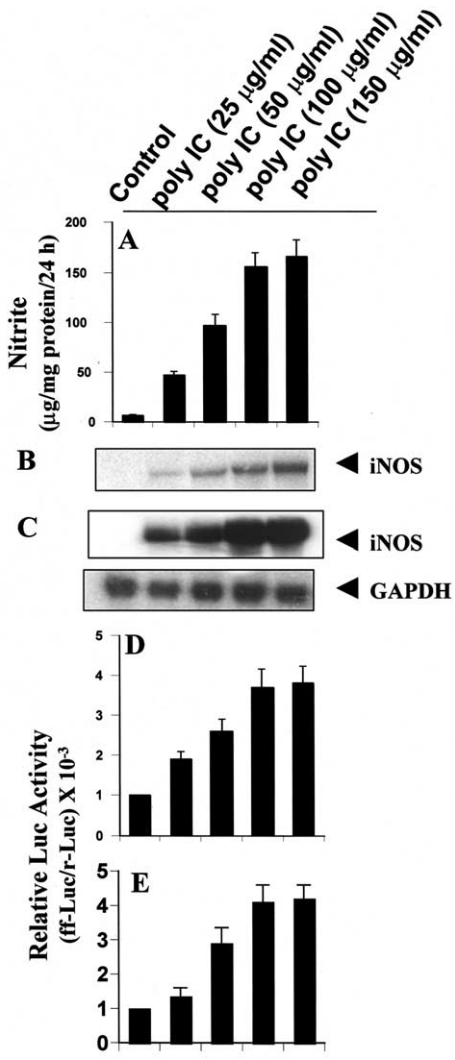

Fig. 1. Dose-dependent induction of iNOS by poly IC in human astrocytes. U373MG astroglial cells were stimulated with different concentrations of poly IC under serum-free condition. A: After 24 h, supernatants were used for nitrite assay. B: Cell homogenates were immunoblotted with antibodies against mouse macrophage iNOS. C: After 6 h of incubation, Northern blot analysis for iNOS mRNA was carried out. Human iNOS promoter-driven luciferase activity was assayed in U373MG astroglial cells (D) and primary astrocytes (E) as described in Section 2. Data are means ± S.D. of three different experiments.

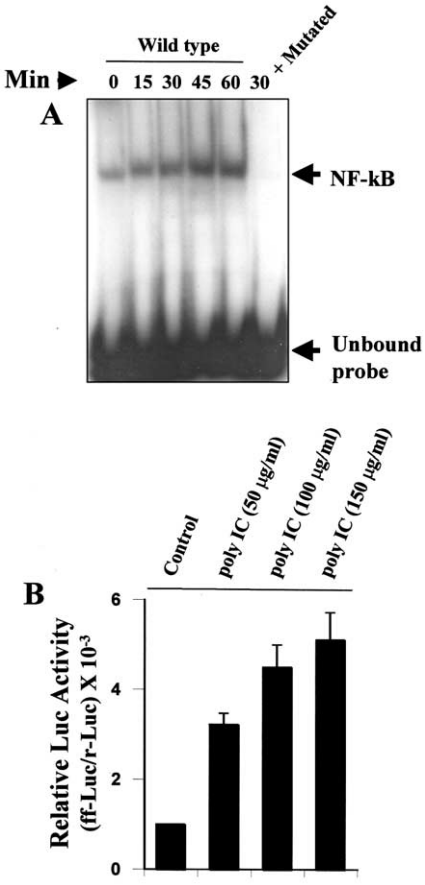

Fig. 2. Activation of NF-κB in poly IC-treated U373MG astroglial cells. A: After different minutes of stimulation with 100 μg/ml of poly IC under serum-free condition, nuclear proteins were examined for EMSA. The upper arrow indicates the induced NF-κB band, and the lower arrow indicates the unbound probe. B: Transcriptional activity of NF-κB was assayed as described in Section 2.

duced the production of NO (data not shown) and iNOS promoter-driven luciferase activity (Fig. 1E) in human primary astrocytes.

3.2. Involvement of NF-κB and C/EBPβ in poly IC-induced activation of iNOS promoter in human U373MG astroglial cells

Recent evidences that activation of both NF-κB and C/EBPβ plays a role in the expression of iNOS [12–15] prompted us to ask whether these two transcription factors may be responsible for poly IC-induced expression of iNOS. Stimulation of cells with poly IC led to the induction of DNA-binding activity of NF-κB at different minutes of stimulation (Fig. 2A). This gel shift assay detected a specific induced band (the upper band) in response to poly IC that was not found either in unstimulated control cells or in case of mutated double-stranded oligonucleotides. Consistent to the effect of poly IC on the DNA-binding activity of NF-κB, poly IC induced NF-κB-dependent transcription of luciferase (Fig. 2B).

Similar to the activation of NF-κB, poly IC also induced the DNA-binding activity of C/EBP at different minutes of stimulation (Fig. 3A). However, this gel shift assay detected two induced bands (the upper and the middle bands) in response to poly IC that were not found either in unstimulated control cells (Fig. 3A) or in case of mutated double-stranded oligonucleotides (Fig. 3B). Since C/EBPβ plays an important role in the activation of iNOS promoter [12–14], we examined if C/EBPβ is induced by poly IC. Supershift analysis using polyclonal antibodies against C/EBPβ (Santa Cruz Biotechnology, Inc.) showed that the intensity of the upper band but not of the middle one markedly reduced by anti-C/EBPβ antibodies (Fig. 3C). In contrast, non-specific antibodies had no effect on DNA-binding activities (Fig. 3C) suggesting that poly IC is able to induce the DNA-binding activity of C/EBPβ. Consistently, poly IC also induced the transcriptional activity of C/EBPβ (Fig. 3D).

Next we examined if activation of both NF-κB and C/EBPβ is important for poly IC-induced expression of iNOS. Overexpression of dominant-negative molecules provides an effective tool with which to investigate the in vivo functions of different transcription factors or signaling molecules. Therefore, NF-κB was inhibited by Δp65, a dominant-negative mutant of p65, and C/EBPβ was inhibited by ΔC/EBPβ [12,14]. Attenuation of iNOS promoter-driven luciferase activity by either Δp65 or ΔC/EBPβ but not by an empty vector in poly IC-stimulated cells (data not shown) suggests that the activation of both NF-κB and C/EBPβ is necessary for poly IC-induced expression of iNOS in astroglial cells.

3.3. Role of PKR and p38 mitogen-activated protein kinase (p38) in poly IC-induced activation of iNOS promoter in U373MG astroglial cells

Next we investigated mechanisms by which poly IC couples to NF-κB and C/EBPβ for the expression of iNOS. Several studies, e.g. [16], have shown that PKR plays an important role in biological activities of poly IC. To investigate if PKR is involved in poly IC-induced activation of human iNOS promoter, we inhibited the function of PKR by ΔPKR. It is evident from Fig. 4A that expression of the ΔPKR but not the empty vector significantly inhibited ($P < 0.01$) poly IC-induced activation of iNOS promoter. Interestingly, transcriptional activity of NF-κB (Fig. 4B) but not of C/EBPβ (Fig. 4C) was inhibited by ΔPKR. Vaccinia viral protein, K3L, inhibits PKR [17]. Similar to ΔPKR, pMT-K3L also attenuated the transcription of iNOS promoter (Fig. 5A) by suppressing the activation of NF-κB (Fig. 5B) but not of C/EBPβ (Fig. 5C). These studies suggest that ΔPKR inhibits the expression of iNOS by suppressing the activation of NF-κB but not of C/EBPβ.

Because p38 has been shown to be involved in cytokine-mediated expression of iNOS [18], we investigated the possibility if p38 plays a role in poly IC-mediated expression of iNOS. We used Δp38 to inhibit the function of p38. Similar to the effect of ΔPKR, Δp38 also inhibited human iNOS promoter-driven luciferase activity ($P < 0.01$) in poly IC-stimulated cells (Fig. 4A). However, in contrast to ΔPKR, Δp38 inhibited poly IC-induced activation of C/EBPβ (Fig. 4C) but not of NF-κB (Fig. 4B). Consistently, SB203580, a specific inhibitor of p38, also knocked down the activation of iNOS promoter by inhibiting the activation of C/EBPβ but not of NF-κB in poly IC-stimulated cells (data not shown). These results suggest that inhibition of p38 attenuates poly IC-induced expression of iNOS by impairing the activation of C/EBPβ but not of NF-κB. Since ΔPKR and Δp38 decreased poly IC-induced activation of iNOS promoter by acting on distinct transcription factors, we examined if these two dom-

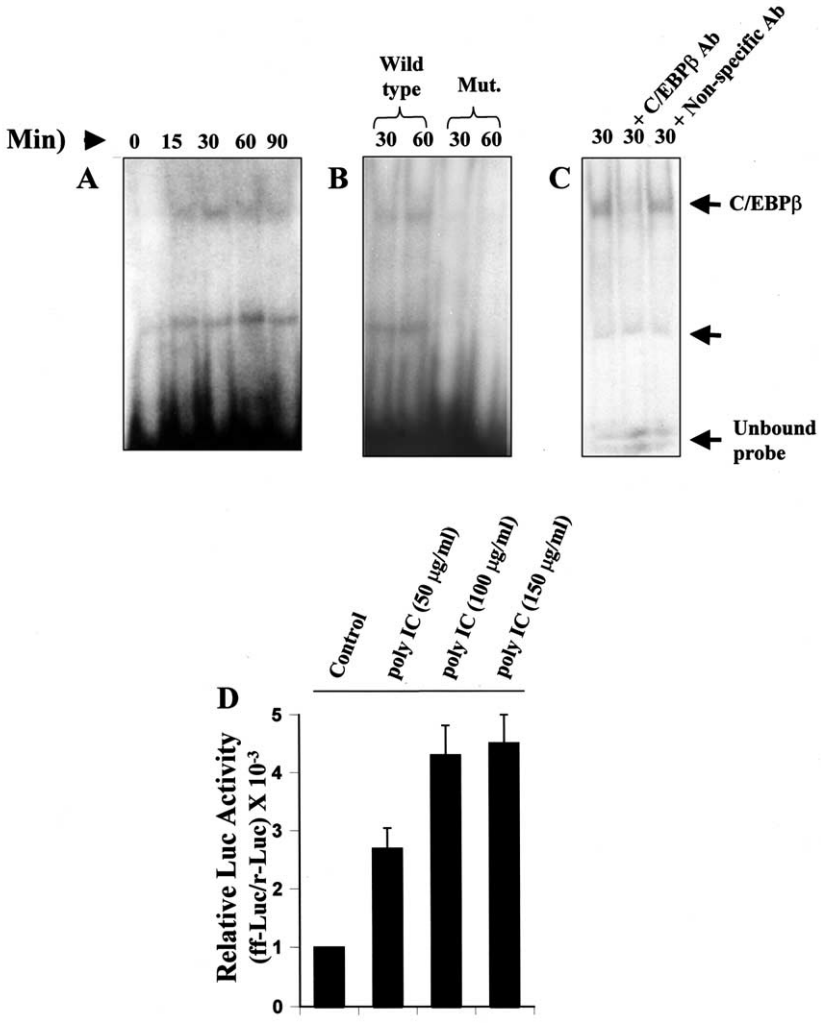

Fig. 3. Activation of C/EBPβ in poly IC-treated U373MG astroglial cells. A: At different minutes of stimulation with 100 μg/ml of poly IC, cells were taken out for EMSA. B: Nuclear proteins were examined for EMSA using wild-type and mutated C/EBP probes. C: 1 μg of antibodies against C/EBPβ or a non-specific protein were included in the binding reaction for supershift analysis. The upper, middle and lower arrows indicate the induced DNA-binding activity of C/EBPβ, the induced DNA-binding activity of another C/EBP isoform and the unbound probes respectively. D: Transcriptional activity of C/EBPβ was assayed as described above.

inant negatives exhibited any additive effect. However, after combining both forms of mutants during transient transfection, we were unable to achieve more than 40% inhibition (data not shown), and the reason behind this lies in the lack of achieving sufficient transfection efficiency. Earlier, we have shown that during successful transient transfection experiments by Lipofectamine Plus, transfection efficiency varies from 33 to 44% [19].

4. Discussion

Several human demyelinating disorders have a known viral etiology [20,21]. Although the association of a virus with MS has not been confirmed, based on detection of virions, viral nucleic acids, or viral proteins in CNS or the presence of antiviral antibodies in serum and/or cerebrospinal fluid (CSF), several viruses have been shown to be associated with MS [6–8]. A common viral structure that is almost universally recognized by eukaryotic cells is dsRNA (>100 bp). dsRNA is not present during the normal life cycle of eukaryotic cells; however, it is present in virally infected cells. However, the biological function of dsRNA within the CNS is poorly understood. Several lines of evidence presented here clearly support the conclusion that dsRNA is capable of inducing the expression of iNOS in human astroglia. Earlier studies have shown that dsRNA inhibits β-cell function and induces islet damage by stimulating β-cell production of NO [22]. Since the expression of iNOS within the CNS participates in the pathogenesis of MS, our findings could help explain why exacerbations of MS are often associated with a variety of different viral infections.

The signaling events for the induction of iNOS are not completely established so far. Proinflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1β, or interferon (IFN)-γ) and LPS bind to their respective receptors and induce the expression of iNOS via NF-κB activation [12–15]. Human iNOS promoter also contains 15 nucleotide sequences [23] that conform to the consensus C/EBP box TKNNGYAAK. Recently we have found that ΔC/EBPβ inhibits cytokine- or HIV-1 Tat-induced activation of human iNOS promoter in human astroglial cells [12,13] suggesting the involvement of C/EBPβ in cytokine- or Tat-induced ex-

pression of iNOS. Results presented in this manuscript clearly demonstrate that activation of both NF-κB and C/EBPβ is also essential for dsRNA-mediated expression of iNOS in human astroglial cells.

DsRNA-dependent protein kinase (PKR), a 68-kDa serine-threonine kinase, plays a primary role in mediating the antiviral activities of infected cells [17]. It has been shown that NF-κB activation in response to dsRNA appears to be dependent on functional PKR and that dsRNA-induced NF-κB activation is attenuated in mouse embryonic fibroblasts isolated from PKR-deficient mice [24]. Chu et al. [25] have shown that PKR may mediate dsRNA-induced NF-κB nuclear localization by activating IKK [24]. In addition, a Toll-like receptor (TLR) molecule (TLR3) has been reported to recognize dsRNA and activate NF-κB via PKR [26]. Consistently, we have found that inhibitors of PKR suppress dsRNA-induced activation of NF-κB, and thereby inhibit the activation of iNOS promoter in human astroglial cells. Earlier, Maggi et al. [27] have shown that PKR is required for dsRNA-induced expression of iNOS but not for activation of NF-κB in macrophages. In contrast, Blair et al. [28] have reported that PKR is not required for dsRNA-induced iNOS expression or NF-κB activation by islets. However, our studies have shown that PKR is required for dsRNA-induced expression of iNOS and activation of NF-κB in human astroglial cells. These discrepancies may be explained by the existence of different regulatory mechanisms in different cell types. In addition to the activation of NF-κB, dsRNA also induced the activation of C/EBPβ that has been also found to be necessary for dsRNA-induced expression of iNOS. However, similar to the inhibition of NF-κB activation, ΔPKR and

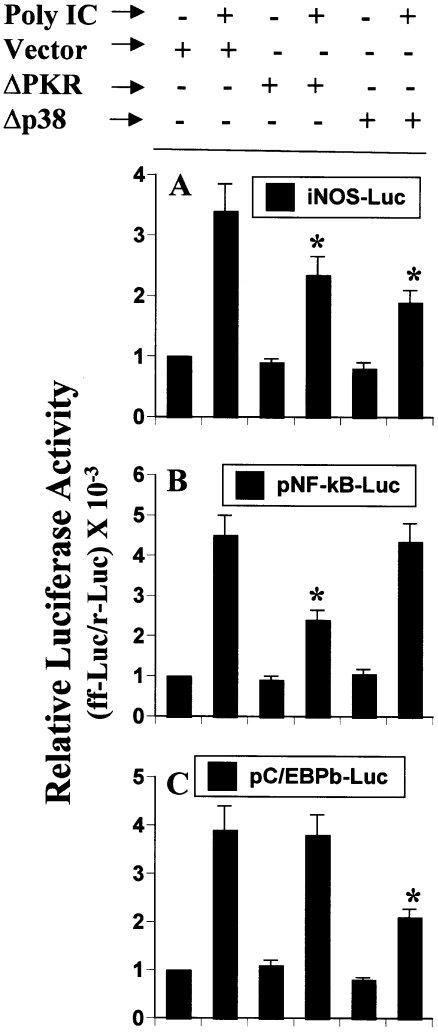

Fig. 4. Effect of ΔPKR and Δp38 on iNOS promoter-driven luciferase activity (A) and activation of NF-κB (B) and C/EBPβ (C) in poly IC-stimulated U373MG astroglial cells. A: Cells were cotransfected with 0.5 μg of phiNOS-Luc, 50 ng of pRL-TK and 0.5 μg of either ΔPKR, Δp38 or empty vector (pcDNA3). After 24 h of transfection, cells were stimulated with 100 μg/ml of poly IC for 12 h. Cells were cotransfected with 0.5 μg of either ΔPKR, Δp38 or empty vector, 50 ng of pRL-TK and 0.5 μg of either pNF-κB-Luc (B) or pC/EBPβ-Luc (C). After 24 h of transfection, cells were stimulated with 100 μg/ml of poly IC for 6 h. Firefly and Renilla luciferase activities were analyzed. $^*P < 0.01$ versus empty vector-transfected cells.

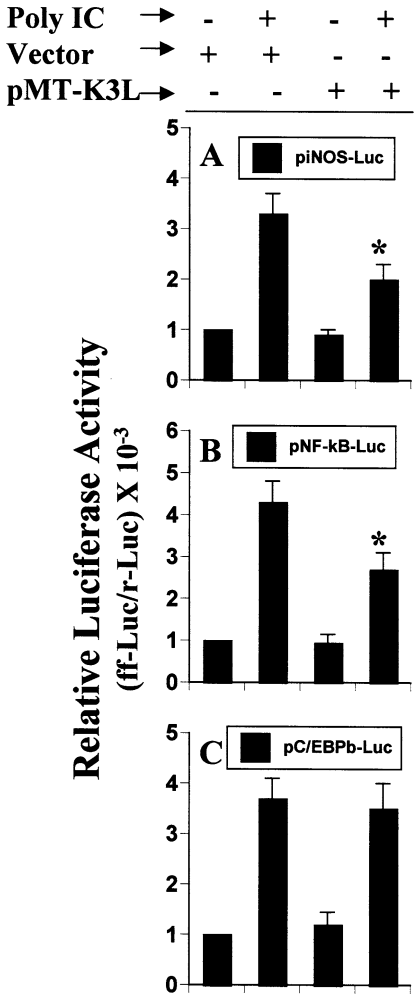

Fig. 5. Effect of pMT-K3L on iNOS promoter-driven luciferase activity (A) and the activation of NF-κB (B) and C/EBPβ (C) in poly IC-stimulated U373MG astroglial cells. A: Cells were cotransfected with 0.5 μg of either pMT-K3L or empty vector (pMT), 0.5 μg of phiNOS-Luc and 50 ng of pRL-TK. After 24 h of transfection, cells were stimulated with 100 μg/ml of poly IC for 12 h. Cells were cotransfected with 0.5 μg of either pMT-K3L or empty vector, 50 ng of pRL-TK and 0.5 μg of either pNF-κB-Luc (B) or pC/EBPβ-Luc (C). After 24 h of transfection, cells were stimulated with 100 μg/ml of poly IC for 6 h. Firefly and Renilla luciferase activities were analyzed. $^*P < 0.01$ versus empty vector-transfected cells.

pMT-K3L did not inhibit dsRNA-induced activation of C/EBPβ (Figs. 4 and 5). These studies suggest that PKR is coupled to the transcription of iNOS via activation of NF-κB but not that of C/EBPβ.

The role of mitogen-activated protein kinase (MAPK) and in particular p38 in modulating the expression of iNOS has been investigated [18]. We have found that Δp38 inhibited poly IC-induced activation of human iNOS promoter. However, interestingly, Δp38 inhibited dsRNA-induced activation of C/EBPβ but not of NF-κB. These studies suggest that p38 regulates dsRNA-induced expression of iNOS via C/EBPβ but not via NF-κB. Taken together, dsRNA-induced activation of NF-κB and C/EBPβ in human astroglial cells involves two distinct signaling pathways. PKR is coupled to the activation of NF-κB but not of C/EBPβ while p38 is coupled to the activation of C/EBPβ but not of NF-κB for dsRNA-induced expression of iNOS.

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