Expression of a constitutively active form of phosphatidylinositol 3-kinase inhibits the induction of nitric oxide synthase in human astrocytes

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Abstract The present study underlines the importance of phosphatidylinositol 3-kinase (PI 3-kinase) in attenuating the induction of nitric oxide synthase (iNOS) in human astrocytes. Proinflammatory cytokines induced the production of nitric oxide (NO) and the expression of iNOS in human U373MG astrocytoma cells and primary astrocytes. Expression of a catalytically active p110 subunit (p110*) of PI 3-kinase but not that of a kinase-deficient mutant of p110 (p110-kd) induced an increase in PI 3-kinase activity and inhibited cytokine-induced production of NO and expression of iNOS. However, expression of p110* had no effect on the activation of NF-kB, suggesting that p110* inhibits the expression of iNOS without inhibiting the activation of NF-kB.

Key words: Astrocyte; Cytokine; Phosphatidylinositol 3-kinase; Inducible nitric oxide synthase; NF-kB

1. Introduction

A series of proinflammatory cytokines including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) activate glial cells (astrocytes and microglia) in the central nervous system (CNS) to induce the expression of inducible nitric oxide synthase (iNOS) [1–4]. Nitric oxide (NO) produced by iNOS is of particular importance in pathophysiologies of inflammatory neurological diseases including demyelinating disorders (e.g. multiple sclerosis, experimental allergic encephalomyelitis, X-adrenoleukodystrophy) and in ischemia and traumatic injuries associated with the production of proinflammatory cytokines [5–7]. Astrocytes in the healthy brain do not express iNOS but following ischemic, traumatic, neurotoxic or inflammatory damage, the reactive astrocytes express iNOS in the mouse, rat and human [1–4].

Characterization of intracellular pathways required to transduce the signal from the cell surface to the nucleus for the induction of iNOS is an active area of investigation. Phosphatidylinositol 3-kinase (PI 3-kinase), a dual protein and lipid kinase, is a key signaling molecule implicated in the regulation of a broad array of biological responses including receptor-stimulated mitogenesis, oxidative burst and cell survival [8,9]. PI 3-kinase is composed of a 110 kDa catalytic subunit (p110) associated with an 85 kDa regulatory subunit (p85). The p85 regulatory subunit acts as an interface by interacting with the IRS-1 through its SH2 domain and thus recruits the p110 catalytic subunit to the cell membrane through its SH2 domain [8,9]. The p110 then catalyzes the reaction to release phosphatidylinositol (3,4,5)-triphosphate as the second messenger using phosphatidylinositol (4,5)-bisphosphate as the substrate and activates downstream signaling molecules like Akt/protein kinase B and p70 ribosomal S6 kinase [8,9]. Recently, we have found that inhibition of PI 3-kinase by either chemical inhibitors such as wortmannin and LY294002 or a dominant-negative mutant of the regulatory subunit p85α induces/stimulates the expression of iNOS in LPS- or cytokine-stimulated C_6 glial cells and rat primary astrocytes [10], suggesting that activation of PI 3-kinase may transduce an inhibitory signal for the expression of iNOS.

In this manuscript, we report that activation of PI 3-kinase by expression of the catalytically active p110 subunit inhibits the production of NO and the expression of iNOS in human U373MG astrocytoma cells and primary astrocytes. These results suggest that PI 3-kinase signal transduction pathway is a negative regulator of the expression of iNOS in human astrocytes.

2. Materials and methods

2.1. Reagents

Fetal bovine serum and DMEM/F-12 were from Gibco, USA. Human recombinant IL-1β, IFN-γ and TNF-α were purchased from R&D, USA. Phosphatidylinositol and phosphatidylserine were purchased from Matreya Inc., USA. Antibodies against regulatory subunit of PI 3-kinase (p85α) and mouse macrophage iNOS were obtained from Calbiochem, USA. [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham, USA.

2.2. Preparation of human astrocytes

Fetal CNS tissue was obtained from the Human Embryology Laboratory, University of Washington, Seattle, WA, USA. The CNS tissue from each fetal specimen was processed separately and independently, as were subsequent cell cultures; there was no pooling of CNS tissue from distinct fetal specimens. These cells were grown in a serum-free, defined medium (B16) enriched with 5 ng of basic fibroblast growth factor per ml for optimal growth of astrocytes and for the suppression of fibroblast growth [11]. By immunofluorescence assay, these cultures homogeneously expressed GFAP. Cells were trypsinized, subcultured and stimulated with cytokines in serum-free DMEM/F-12. The human U373MG astrocytoma cell line was purchased from the American Type Culture Collection.

2.3. Transfection of U373MG glial cells and human primary astrocytes

The cell monolayer (50–60% confluent) was transfected by Lipofectamine Plus (Gibco, USA) following the manufacturer's protocol. To monitor the transfection efficiency, cells were cotransfected with a

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pCAT 3-control plasmid (Promega, Madison, WI, USA). A radioisotopic method was used to assay CAT activity using a kit (Promega) as described by the manufacturer's protocol [10,12]. As revealed by the CAT assay, transfection efficiency ranged within 33–40% in U373MG glial cells and within 38–44% in human primary astrocytes.

2.4. Assay of PI 3-kinase

Cells were lysed with ice-cold lysis buffer containing 1% v/v NP-40, 100 mM NaCl, 20 mM Tris (pH 7.4), 10 mM iodoacetamide, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethyl sulfonyl chloride and 1 μg/ml of each of leupeptin, antipain, aprotinin and pepstatin A. Lysates were incubated at 4°C for 15 min, followed by centrifugation at 13 000×*g* for 15 min. The supernatant was precleared with protein G-Sepharose beads (Pharmacia Biotech Inc.) for 1 h at 4°C followed by the addition of 1 μg/ml of p85α monoclonal antibody. After 2 h of incubation at 4°C, protein G-Sepharose beads were added and the resulting mixture was further incubated for 1 h at 4°C. The immunoprecipitates were washed twice with lysis buffer, once with phosphate-buffered saline, once with 0.5 M LiCl, 100 mM Tris, pH 7.6, once in water, and once in kinase buffer (5 mM $MgCl_2$, 0.25 mM EDTA, 20 mM HEPES, pH 7.4). PI 3-kinase activity was determined as described earlier [10,13]. Briefly, a lipid mixture of 100 μl of 0.1 mg/ml phosphatidylinositol and 0.1 mg/ml phosphatidylserine was dispersed by sonication in 20 mM HEPES, pH 7.0, and 1 mM EDTA. The reaction was initiated by the addition of 20 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) and 100 μM ATP and terminated after 15 min by the addition of 80 μl of 1 N HCl and 200 μl of chloroform:methanol (1:1). Phospholipids were separated by thin layer chromatography and visualized by exposure to iodine vapor and autoradiography [10,13].

2.5. Assay for NO synthesis

Synthesis of NO was determined by assay of culture supernatant for nitrite, a stable reaction product of NO with molecular oxygen, using Griess reagent as described earlier [2–4].

2.6. Immunoblot analysis for iNOS

Cells were homogenized in 50 mM Tris–HCl (pH 7.4) containing protease inhibitors (1 mM PMSF and 5 μg/ml of each of aprotinin, antipain, pepstatin A and leupeptin). After electrophoresis, the proteins were transferred onto a nitrocellulose membrane, and the iNOS band was visualized by immunoblotting with antibodies against mouse macrophage iNOS and ^{125}I-labeled protein A [3,4].

2.7. RNA isolation and Northern blot analysis

Total RNA was isolated using Ultraspec II RNA reagent (Biotecx Laboratories Inc.) according to the manufacturer's protocol. For Northern blot analyses, 20 μg of total RNA was electrophoresed on 1.2% denaturing formaldehyde-agarose gels, electrotransferred to Hybond-Nylon Membrane (Amersham) and hybridized at 68°C with ^{32}P-labeled cDNA probe using Express Hyb hybridization solution (Clontech) as described by the manufacturer. The cDNA probes have been described earlier [3,4]. After hybridization, filters were washed two or three times in solution I (2×SSC, 0.05% sodium dodecyl sulfate (SDS)) for 1 h at room temperature followed by solution II (0.1×SSC, 0.1% SDS) at 50°C for another hour. The membranes were then dried and exposed to X-ray films (Kodak). The same amount of RNA was hybridized with probe for actin.

2.8. Assay of transcriptional activity of NF-kB

To assay the transcriptional activity of NF-kB, cells were transfected with pNF-kB-Luc, an NF-kB-dependent reporter construct (obtained from Stratagene), using the Lipofectamine Plus method [10,12]. After 24 h of transfection, cells were treated with different stimuli for 12 h. Total cell extracts were used to measure luciferase activity in a TD-20/20 Luminometer (Turner Designs, USA) using an assay kit from Stratagene.

3. Results

3.1. Expression of a catalytically active p110 subunit of PI 3-kinase (p110) inhibits the induction of NO production in cytokine-stimulated human U373MG astrocytoma cells*

PI 3-kinase consists of a catalytic subunit (p110) of 110 kDa and a regulatory subunit (p85) of 85 kDa. In the constitutively active mutant of PI 3-kinase (p110*), the inter-SH2 domain of p85 is ligated to the NH_2-terminus of p110 whereas in the kinase-deficient mutant of p110 (p110-kd), the ATP binding site is mutated [14]. It has been reported that expression of p110* but not that of p110-kd is sufficient to promote Glut 4 translocation in adipocytes [14]. Therefore, to stimulate PI 3-kinase activity in human astrocytes, we transfected astrocytes with p110*. Fig. 1 shows that expression of the p110* but not that of the p110-kd stimulated the lipid kinase activity of PI 3-kinase. About a 2-fold increase in PI 3-kinase activity was observed in cells transfected with 1 μg of p110*. Then we examined the regulation of iNOS expression by activation of PI 3-kinase. Different combinations of proinflammatory cytokines induced the production of NO in U373MG glial cells (Fig. 2A). Consistent to our earlier observation that inhibition of PI 3-kinase by the expression of a dominant-negative mutant of p85α induces/stimulates the expression of iNOS in rat C_6 glial cells [10], here we have observed that activation of PI 3-kinase by the expression of p110* inhibited the induction of NO production in cytokine-stimulated U373MG glial cells (Fig. 2A). However, the expression of p110-kd had no effect on the induction of NO production in cytokine-stimulated cells. About 37–42% inhibition of NO production was found in cells transfected with p110* compared to cells transfected with p110-kd (Fig. 2A). To understand the mechanism of

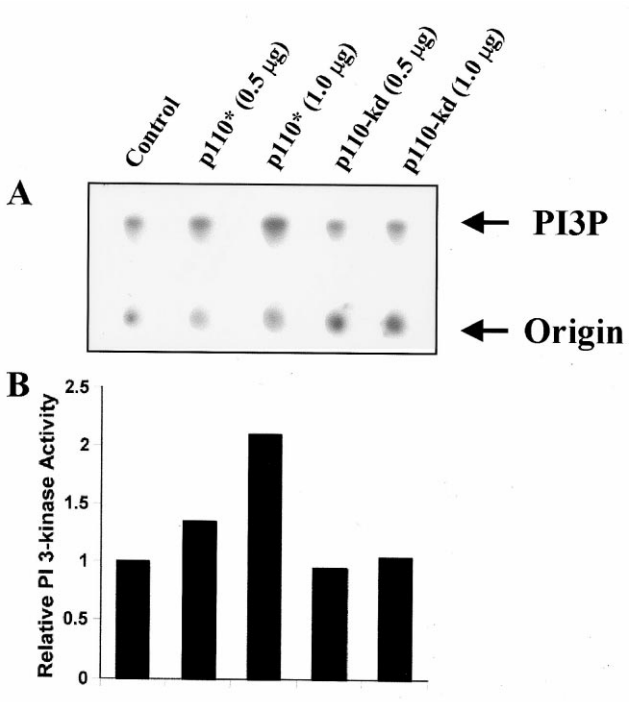

Fig. 1. Expression of a catalytically active p110 subunit of PI 3-kinase stimulates PI 3-kinase activity in human U373MG astrocytoma cells. Cells plated at 50–60% confluence in six-well plates were transfected with 1 μg of either p110* or p110-kd using Lipofectamine Plus as described in Section 2. Control cells did not receive any DNA but received only Lipofectamine and Plus reagents. 24 h after transfection, cells were lysed, immunoprecipitated with monoclonal antibodies against p85α, and the lipid kinase activity of immunoprecipitated PI 3-kinase was assayed as described under Section 2. Lipids were detected by exposure to film at −70°C (A) and lipid bands corresponding to PI 3-P were quantitated by densitometry (B). Data are from a single experiment representative of at least three others.

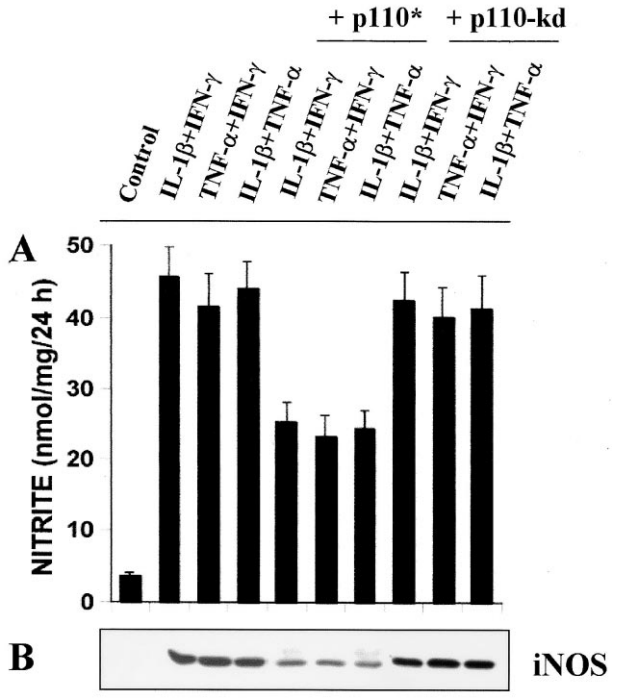

Fig. 2. Expression of p110* inhibits the induction of NO production in cytokine-stimulated U373MG glial cells. Cells plated at 50–60% confluence in six-well plates were transfected with 1 μg of either p110* or p110-kd using Lipofectamine Plus. Control cells did not receive any DNA but received only Lipofectamine and Plus reagents. 24 h after transfection, cells were stimulated with different cytokines under serum-free conditions. (A) After 24 h of stimulation, nitrite concentrations were measured in the supernatants. Data are mean ± S.D. of three different experiments. (B) Cell homogenates were electrophoresed, transferred on nitrocellulose membrane and immunoblotted with antibodies against mouse macrophage iNOS as described under Section 2. Concentrations of different stimuli were: TNF-α, 10 ng/ml; IL-1β, 10 ng/ml; IFN-γ, 10 U/ml.

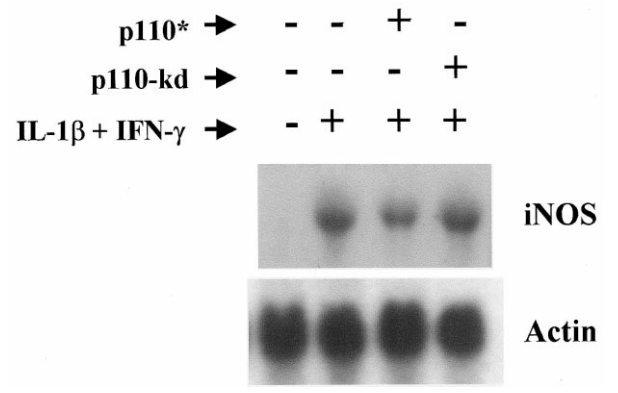

Fig. 3. Effect of p110* on the expression of iNOS mRNA in cytokine-stimulated U373MG glial cells. Cells plated at 50–60% confluence in a 100 mm dish were transfected with 4 μg of either p110* or p110-kd using Lipofectamine Plus. Control cells did not receive any DNA but received only Lipofectamine and Plus reagents. 24 h after transfection, cells were stimulated with the combination of IL-1β (10 ng/ml) and IFN-γ (10 U/ml). After 6 h of stimulation, cells were taken out directly by adding Ultraspec II RNA reagent (Biotecx Laboratories Inc.) to the plates for isolation of total RNA, and Northern blot analysis for iNOS mRNA was carried out as described in Section 2.

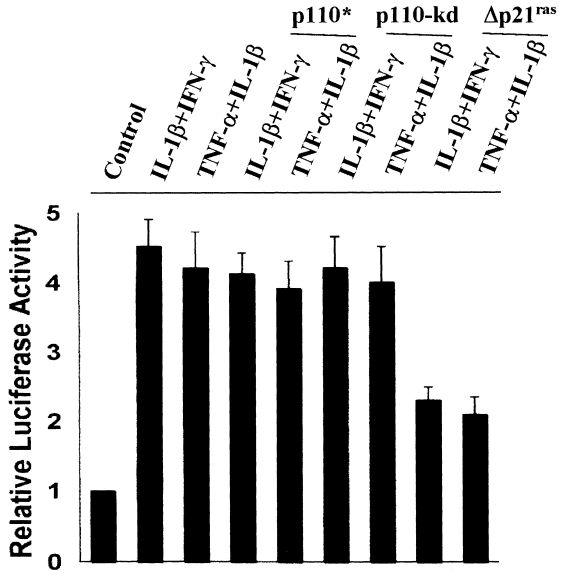

Fig. 4. Effect of p110* on the activation of NF-kB in cytokine-stimulated U373MG glial cells. Cells plated at 50–60% confluence in six-well plates were cotransfected with 1 μg of pNF-kB-Luc (an NF-kB-dependent reporter construct) and 1 μg of p110* or p110-kd or $\Delta p21^{ras}$ using Lipofectamine Plus. Control cells did not receive any DNA but received only Lipofectamine and Plus reagents. After 24 h of transfection, cells were stimulated with different proinflammatory cytokines for 12 h. The expression of luciferase reporter was quantitated as described in Section 2. Data are mean ± S.D. of three different experiments. Concentrations of different stimuli were: TNF-α, 10 ng/ml; IL-1β, 10 ng/ml; IFN-γ, 10 U/ml.

p110*-mediated inhibition of NO production, we examined the effect of p110* on the protein level of iNOS by Western blot analysis. Consistent to the inhibition of NO production, p110* but not p110-kd inhibited the expression of iNOS protein (Fig. 2B) in cytokine-stimulated glial cells. Similarly, p110* but not p110-kd also attenuated the expression of iNOS mRNA in cells stimulated with the combination of IL-1β and IFN-γ (Fig. 3). These results indicate that activation of PI 3-kinase provides an inhibitory signal for cytokine-induced production of NO and expression of iNOS in human astrocytes.

3.2. Expression of p110 does not inhibit the activation of NF-kB in cytokine-stimulated U373MG cells*

Proinflammatory cytokine-induced expression of iNOS in glial cells depends on the activation of NF-kB [3,4,10,12]. Therefore, to gain further insight into the mechanism of action of activated PI 3-kinase, we investigated the effect of activation of PI 3-kinase on the activation of NF-kB. Activation of NF-kB was monitored by assaying the transcriptional activity of NF-kB [10,12]. Cells were cotransfected with pNF-kB-Luc, an NF-kB-dependent reporter construct, and either p110* or p110-kd followed by the stimulation with proinflammatory cytokines. Proinflammatory cytokines induced the activation of NF-kB by about 4-fold as revealed from the luciferase activity (Fig. 4). As expected, the expression of p110-kd had no effect on NF-kB-dependent reporter activity. However, in contrast to the inhibition of iNOS expression, p110* did not inhibit NF-kB-dependent luciferase activity in cytokine-stimulated cells (Fig. 4). Consistent to our earlier observation [15], $\Delta p21^{ras}$, a dominant-negative mutant of $p21^{ras}$,

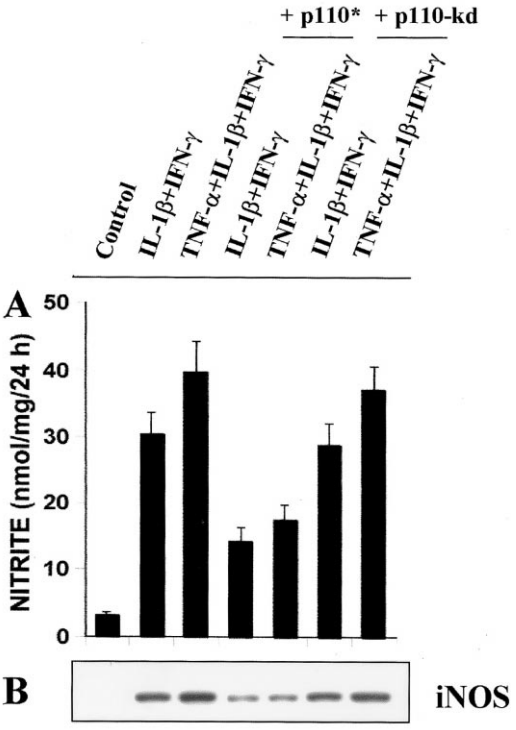

Fig. 5. Expression of p110* inhibits the expression of iNOS in cytokine-stimulated human primary astrocytes. Cells plated at 50–60% confluence in six-well plates were transfected with 1 μg of either p110* or p110-kd using Lipofectamine Plus. Control cells did not receive any DNA but received only Lipofectamine and Plus reagents. 24 h after transfection, cells were stimulated with proinflammatory cytokines under serum-free conditions. (A) After 24 h of stimulation, nitrite concentrations were measured in supernatants. Data are mean ± S.D. of three different experiments. (B) Cell homogenates were immunoblotted with antibodies against mouse macrophage iNOS. Concentrations of different stimuli were: TNF-α, 10 ng/ml; IL-1β, 10 ng/ml; IFN-γ, 10 U/ml.

inhibited NF-kB-dependent luciferase activity as a positive experimental control (Fig. 4). These experiments suggest that activation of PI 3-kinase inhibits the expression of iNOS without inhibiting the activation of NF-kB.

3.3. Expression of p110 inhibits the expression of iNOS in cytokine-stimulated human primary astrocytes*

Human primary astrocytes have been shown to induce the expression of iNOS in the presence of different proinflammatory cytokines [15]. Since the activation of PI 3-kinase inhibited the expression of iNOS in human U373MG astroglial cells, we examined the effect of p110* on cytokine-induced expression of iNOS in human primary astrocytes. Different combinations of TNF-α, IL-1β and IFN-γ induced the production of NO (Fig. 5A) and the expression of iNOS protein (Fig. 5B). However, expression of p110* but not that of p110-kd attenuated cytokine-induced expression of production of NO and expression of iNOS protein, suggesting that similar to U373MG glial cells, activation of PI 3-kinase inhibits the induction of iNOS in human primary astrocytes.

4. Discussion

The signaling events transduced by proinflammatory cytokines for the induction of iNOS are poorly understood. A complete understanding of the cellular signaling mechanisms involved in the induction of iNOS should identify novel targets for the therapeutic intervention in NO-mediated neuroinflammatory and neurodegenerative diseases. In this study, we overexpressed the catalytically active p110 subunit to investigate the role of PI 3-kinase in the induction of iNOS and production of NO. Consistent to our earlier observation that inhibition of PI 3-kinase stimulates or induces the expression production of NO and the expression of iNOS in C_6 glial cells and rat primary astrocytes [10], we have found that activation of PI 3-kinase inhibits the induction of NO production and the expression of iNOS in U373MG glial cells and human primary astrocytes.

Proinflammatory cytokines (TNF-α, IL-1β or IFN-γ) bind to their respective receptors and induce iNOS expression via activation of NF-kB [10,12,16]. The presence of consensus sequence in the promoter region of iNOS for the binding of NF-kB [16] and the inhibition of iNOS expression with the inhibition of NF-kB activation establish an essential role of NF-kB activation in the induction of iNOS [3,4,10,12]. Activation of NF-kB by various cellular stimuli involves the proteolytic degradation of IkBα and the concomitant nuclear translocation of the liberated NF-kB heterodimer [17]. Although the biochemical mechanism underlying the degradation of IkBα remains unclear, it appears that various mitogens and cytokines activate IKKs, kinases which phosphorylate IkBα on serines 32 and 36 [18]. Upon phosphorylation, IkBα which is still bound to NF-kB apparently becomes a high affinity substrate for an ubiquitin-conjugating enzyme [19]. Following phosphorylation-controlled ubiquitination, the IkBα is rapidly and completely degraded by the 20 S or 26 S proteosome and NF-kB heterodimer is targeted to the nucleus [19]. Earlier, we have also observed that cAMP derivatives that activate protein kinase A, mevalonate inhibitors that inhibit the $p21^{ras}$ or antioxidants like *N*-acetyl cysteine inhibit the expression of iNOS by inhibiting the activation of NF-kB [3,4,20]. On the other hand, cell-permeable ceramide analogs and inhibitors of protein phosphate 1/2A stimulate the expression of iNOS in rat primary astrocytes by stimulating the activation of NF-kB [12,21]. However, recently, we have found that inhibition of PI 3-kinase by either chemical inhibitors or dominant-negative mutant of p85α stimulates/induces the expression of iNOS in cytokine-stimulated rat astrocytes without modulating cytokine-induced activation of NF-kB, suggesting that PI 3-kinase-mediated regulation of iNOS expression is independent of NF-kB activation. Consistently, here we have demonstrated that activation of PI 3-kinase by the expression of a catalytically active p110 subunit inhibits the expression of iNOS in human astrocytes without inhibiting the activation of NF-kB.

Recently, PI 3-kinase-associated signaling events have been shown to prevent apoptosis in a number of cell types including cerebellar granule neurons and hematopoietic cells [8,9]. Consistent with the apoptotic activity of NO [22] and the anti-apoptotic activity of activated PI 3-kinase [8,9], the observed attenuation of cytokine-induced expression of iNOS and production of NO by activated PI 3-kinase in human astrocytes indicates that the negative regulation of iNOS expression by PI 3-kinase may contribute to its anti-apoptotic activity.

There are considerable evidences for the transcriptional induction of iNOS (the high-output isoform of NOS) in the CNS that is associated with autoimmune reactions, acute in-

fection and traumatic brain injury [5,7,22]. Once NO is formed, it spontaneously reacts with O_2^- to form peroxynitrite ($ONOO^-$), the most reactive derivative of NO known so far [23]. Both NO and peroxynitrite are potentially toxic molecules to neurons and oligodendrocytes that may mediate toxicity through the formation of iron–NO complexes of iron-containing enzyme systems [24], oxidation of protein sulfhydryl groups [23], nitration of proteins, and nitrosylation of nucleic acids and DNA strand breaks [25]. In the CNS, iNOS is expressed mainly by activated astrocytes and microglia, the two glial cell types involved in intracerebral immune regulation. Astrocytes are the major glial cell population in the CNS; therefore, induction of iNOS in astrocytes may be an important source of NO in CNS inflammatory disorders associated with neuronal and oligodendrocytes death. Therefore, activation of PI 3-kinase may represent an avenue for therapeutic intervention in neuroinflammatory and neurodegenerative disorders.

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