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Expression of a Dominant-Negative Mutant of p21^{ras} Inhibits Induction of Nitric Oxide Synthase and Activation of Nuclear Factor-κB in Primary Astrocytes

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Abstract: The present study underlines the importance of p21^{ras} in regulating the inducible nitric oxide synthase (iNOS) in primary astrocytes. Bacterial lipopolysaccharides induced the GTP loading of p21^{ras}, and the expression of a dominant-negative mutant of p21^{ras} (Δp21^{ras}) inhibited lipopolysaccharide-induced GTP loading in rat primary astrocytes. To delineate the role of p21^{ras} in the induction of iNOS, we examined the effect of Δp21^{ras} on the expression of iNOS and the production of nitric oxide. It is interesting that expression of Δp21^{ras} markedly inhibited the production of nitric oxide and the expression of iNOS in lipopolysaccharide- and proinflammatory cytokine (tumor necrosis factor-α, interleukin-1β; interferon-γ)-stimulated rat and human primary astrocytes. Inhibition of iNOS promoter-derived chloramphenicol acetyltransferase activity by Δp21^{ras} suggests that p21^{ras} is involved in the transcription of iNOS. As activation of nuclear factor-κB (NF-κB) is necessary for the transcription of iNOS, we examined the effect of Δp21^{ras} on the activation of NF-κB. Expression of Δp21^{ras} inhibited the DNA binding as well as the transcriptional activity of NF-κB in activated astrocytes, suggesting that Δp21^{ras} inhibits the expression of iNOS by inhibiting the activation of NF-κB. These studies also suggest that inhibitors of p21^{ras} may be used as therapeutics in nitric oxide- and cytokine-mediated neuroinflammatory diseases. **Key Words:** Astrocytes—Cytokines—Lipopolysaccharides—p21^{ras}—Inducible nitric oxide synthase—Nuclear factor-κB.
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Nitric oxide (NO), a vascular and neuronal messenger and a cytotoxic and cytostatic agent, is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS) (Nathan, 1992). Basically, NOS is classified into two groups. One type, constitutively expressed (cNOS) in several cell types (e.g., neurons, endothelial cells), is regulated predominantly at the posttranscriptional level by calmodulin in a calcium-dependent manner (Nathan, 1992; Jaffrey and Snyder, 1995). In contrast, the activity of the inducible isoform (iNOS), expressed in response to different stimuli in various cell types including macrophages, hepatocytes, and neutrophils, is independent of calcium (Xie et al., 1992; Vos et al., 1999). Glial cells in the CNS like astrocytes and microglia have also been shown to induce iNOS in response to a series of proinflammatory cytokines including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and bacterial lipopolysaccharide (LPS) (Simmons and Murphy, 1992; Zielasek et al., 1992; Lee et al., 1993; Pahan et al., 1997*b*). NO derived from the activation of iNOS in astrocytes and microglia under the influence of proinflamatory cytokines is assumed to contribute to oligodendrocyte degeneration in demyelinating diseases and neuronal death during ischemic and traumatic brain injury (Koprowski et al., 1993; Merrill et al., 1993; Cross et al., 1994; Endoh et al., 1994).

Therefore, characterization of intracellular pathways evoked to transduce the signal from the cell surface to the nucleus for the induction of iNOS in activated glial cells is an active area of investigation. Identification of the DNA-binding site for nuclear factor-κB (NF-κB), a proinflammatory transcription factor, in the promoter region of iNOS (Xie et al., 1994), and inhibition of iNOS

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Abbreviations used: CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; FTase, farnesyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBSS, Hanks' balanced salt solution; IFN-γ, interferon-γ; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TNF-α, tumor necrosis factor-α.

expression by inhibitors of NF-κB activation (Nishiya et al., 1995; Pahan et al., 1997*a*,*b*, 1998*a*) have established an essential role of NF-κB activation in the induction of iNOS. Suppression of NF-κB activation and inhibition of iNOS expression by inhibitors of tyrosine kinase in glial cells suggest the possible involvement of tyrosine phosphorylation in the activation of NF-κB and the induction of iNOS (Nishiya et al., 1995). Recently, we have found that inhibitors of the mevalonate pathway (lovastatin and sodium phenylacetate) inhibit the expression of iNOS and the activation of NF-κB in activated glial cells (Pahan et al., 1997*b*). Inhibition of LPS-induced expression of iNOS and activation of NF-κB in rat primary astrocytes by sodium phenylacetate and its reversal by farnesyl pyrophosphate, but not by mevalonate (Pahan et al., 1997*b*), indicate that the farnesylation reaction is involved in the activation of NF-κB and the induction of iNOS. As farnesylation is a necessary step for the activation of $p21^{ras}$, the inhibition of expression of iNOS and activation of NF-κB by mevalonate inhibitors suggested a possible role of $p21^{ras}$ in these proinflammatory processes.

The present study was undertaken to investigate the cellular regulation of the induction of iNOS and the activation of NF-κB by $p21^{ras}$ in rat and human primary astrocytes. We herein report that the activation of NF-κB and the induction of iNOS in rat and human primary astrocytes are uniquely sensitive to the knockdown of $p21^{ras}$ by the expression of a dominant-negative mutant of $p21^{ras}$, suggesting that the activation of $p21^{ras}$ may be an important target for therapeutic intervention in neuroinflammatory diseases.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM)/F-12, Hanks' balanced salt solution (HBSS), and NF-κB DNA binding protein detection kit were from Life Technologies, Inc. (U.S.A.). LPS (*Escherichia coli,* serotype (111:B4) was from Sigma. Antibodies against $p21^{ras}$ and antibodies against mouse macrophage iNOS were obtained from Transduction Laboratories (U.S.A.). Rat and human recombinant TNF-α, IL-1β, and IFN-γ were obtained from R&D (U.S.A.). [γ-^{32}P]ATP (3,000 Ci/mmol) and trisodium [^{32}P]orthophosphate (200 mCi/mmol) were purchased from ICN (U.S.A.).

Preparation of rat astrocytes

Rat primary astrocytes were prepared from rat cerebral tissue as described by McCarthy and de Vellis (1980). Cells were maintained in DMEM/F-12 medium containing 10% FBS. After 10 days of culture, astrocytes were separated from microglia and oligodendrocytes by shaking for 24 h in an orbital shaker at 240 rpm. To ensure the removal of oligodendrocytes and microglia, the shaking was repeated twice after a gap of 1 or 2 days. By immunofluorescence assay, these cultures homogeneously expressed glial fibrillary acidic protein, the definitive marker for astrocytes. Cells were trypsinized, subcultured, and stimulated with LPS or different cytokines in serum-free DMEM/F-12 medium.

Preparation of human astrocytes

Fetal CNS tissue was obtained from the Human Embryology Laboratory (University of Washington, Seattle, WA, U.S.A.). 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/ml basic fibroblast growth factor for optimal growth of astrocytes and for the suppression of fibroblast growth (McCarthy et al., 1995). By immunofluorescence assay, these cultures homogeneously expressed glial fibrillary acidic protein. Cells were trypsinized, subcultured, and stimulated with LPS or different cytokines in serum-free DMEM/F-12 medium.

Expression of $\Delta p21^{ras}$ in astrocytes

In the dominant-negative form of $p21^{ras}$ ($\Delta p21^{ras}$), the Ser residue at position 17 was changed to Asn (S17N mutant). This mutant binds preferentially to GDP and acts as the dominant inhibitor of $p21^{ras}$ function presumably by blocking access to exchange factors (Qiu et al., 1995; Garnovskaya et al., 1996). The engineering of the construct and description of the vector driving the expression of the proteins have been published previously (Garnovskaya et al., 1996). Rat and human primary astrocytes were transiently transfected with either $\Delta p21^{ras}$ or an empty vector by Lipotaxi (Stratagene) following the manufacturer's protocol. In brief, for transfection of each well of a six-well plate, 2 μg of DNA was allowed to form a complex with 20 μl of Lipotaxi and serum-free DMEM/F-12 in a total volume of 300 μl by incubating at room temperature in a sterile Eppendorf for 30 min. In the meantime, the cell monolayer (50–60% confluent) was washed twice with HBSS and replenished with 450 μl of serum-free DMEM/F-12. Then the entire mixture of Lipotaxi, DMEM/F-12, and DNA was added dropwise to the cells while swirling. After an incubation of 6 h, 750 μl of DMEM/F-12 containing 20% FBS was added to a total volume of 1.5 ml and cells were incubated further for 24 h. For transfection of cells in 60-mm dishes, 4 μg of DNA was complexed with 50 μl of Lipotaxi, whereas for cells in 100-mm dishes, 8 μg of DNA was complexed with 150 μl of Lipotaxi. During cotransfection in six-well plates, 2 μg of either $\Delta p21^{ras}$ or the empty vector and 1 μg of the other DNA were complexed together with 30 μl of Lipotaxi. After transfection, cells were stimulated with different stimuli for the indicated time periods under serum-free conditions. To monitor the transfection efficiency, cells were cotransfected with a pCAT 3-control plasmid (Promega, Madison, WI, U.S.A.). A radioisotopic method was used to assay chloramphenicol acetyltransferase (CAT) activity using a kit (Promega) as described by the manufacturer's protocol (Pahan et al., 1998*b*, 1999). As revealed by the CAT assay, transfection efficiency ranged between 38 and 54%.

Assay of $p21^{ras}$ activation

Activation of $p21^{ras}$ was assayed by GTP loading as described by Downward et al. (1990) with slight modifications. In brief, cells were labeled with [^{32}P]orthophosphate in phosphate-free medium for 16 h and incubated with different stimuli under serum-free conditions. At various points of stimulation, cells were lysed with ice-cold lysis buffer containing 1% (vol/vol) Nonidet P-40, 100 m*M* NaCl, 20 m*M* Tris, pH 7.4, 10 m*M* iodoacetamide, 10 m*M* NaF, 1 m*M* sodium orthovanadate, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A. Lysates were precleared with protein G-Sepharose beads (Phar-

macia Biotech Inc.) for 1 h at 4°C, followed by the addition of 2 μg/ml $p21^{ras}$ 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 three times with 20 m*M* Tris, pH 7.4, 500 m*M* NaCl, 5 m*M* $MgCl_2$, 0.1% Triton X-100, and 0.005% sodium dodecyl sulfate (SDS). Nucleotides (GTP and GDP) associated with ras were eluted with 2 m*M* EDTA, 2 m*M* dithiothreitol (DTT), 0.2% SDS, 0.5 m*M* GTP, 0.5 m*M* GDP at 68°C for 20 min (Downward et al., 1990). The eluted nucleotides were separated on polyethylenimine TLC plates using 0.75 *M* KH_2PO_4, pH 3.4, as a solvent and exposed to x-ray film.

Assay for NO synthesis

Synthesis of NO was determined by assay of culture supernatants for nitrite, a stable reaction product of NO with molecular oxygen. In brief, 400 μl of culture supernatant was allowed to react with 200 μl of Griess reagent (Green et al., 1982; Pahan et al., 1997*a*) and incubated at room temperature for 15 min. The optical density of the assay samples was measured spectrophotometrically at 570 nm. Fresh culture medium served as the blank in all experiments. Nitrite concentrations were calculated from a standard curve derived from the reaction of $NaNO_2$ in the assay. Protein was measured by the procedure of Bradford (1976).

Immunoblot analysis for iNOS

Following 24 h of incubation in the presence or absence of different stimuli, cells were scraped off, washed with HBSS, and homogenized in 50 m*M* Tris-HCl, pH 7.4, containing protease inhibitors (1 m*M* PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 5 μg/ml 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 (Pahan et al., 1997*a*, 1998*a*).

RNA isolation and northern blot analysis

Cells were taken out from culture dishes directly by adding Ultraspec-II RNA reagent (Biotecx Laboratories Inc.), and total RNA was isolated 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 fragment for iNOS was amplified by PCR using two primers (forward primer: 5′-CTC CTT CAA AGA GGC AAA AAT A-3′; reverse primer: 5′-CAC TTC CTC CAG GAT GTT GT-3′) and cloned in pGEM-T vector (Geller et al., 1993; Pahan et al., 1997*a*, 1998*a*). The clone was confirmed by DNA sequencing, and the insert was used as probe. After hybridization, filters were washed two or three times in solution I (2× saline–sodium citrate, 0.05% SDS) for 1 h at room temperature, followed by solution II (0.1× saline–sodium citrate, 0.1% SDS) at 50°C for another hour. The membranes were then dried and exposed to x-ray films (Kodak). The same filters were stripped and rehybridized with probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or the same amount of RNA was electrophoresed and hybridized for GAPDH.

Construction of reporter plasmid, transfection, and assay of CAT activity

The CAT under the control of iNOS promoter was created by subcloning the 1.5-kb promoter from pGEM-NOS at *Sph*I and *Sal*I restriction sites of pCAT-basic vector (Promega) (Pahan et al., 1998*b*, 1999). Full-length promoter (Eberhardt et al., 1996) was amplified by using two primers (forward: 5′-GAG AGT GTG CAA GTA TTT GTA GGA G-3′; reverse: 5′-AAG GTG GCT GAG AAG TTT CA-3′) from rat genomic DNA and cloned in pGEM-T vector (Promega) to produce pGEM-NOS. The clone was confirmed by restriction mapping and sequencing. The cells were transfected with reporter plasmid by using the Lipotaxi (Stratagene) method, as described in the manufacturer's protocol. Twenty-four hours after transfection, cells were treated with different stimuli for 14 h and harvested. A radioisotopic method was used to assay CAT activity using a kit (Promega) as described by the manufacturer's protocol.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts from stimulated or unstimulated astrocytes (1×10^7 cells) were prepared using the method of Dignam et al. (1983) with slight modification. Cells were harvested, washed twice with ice-cold phosphate-buffered saline, lysed in 400 μl of buffer A (10 m*M* HEPES, pH 7.9, 10 m*M* KCl, 2 m*M* $MgCl_2$, 0.5 m*M* DTT, 1 m*M* PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin) containing 0.1% Nonidet P-40 for 15 min on ice, vortex-mixed vigorously for 15 s, and centrifuged at 14,000 rpm for 30 s. The pelleted nuclei were resuspended in 40 μl of buffer B [20 m*M* HEPES, pH 7.9, 25% (vol/vol) glycerol, 0.42 *M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* DTT, 1 m*M* PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin]. After 30 min on ice, lysates were centrifuged at 14,000 rpm for 10 min. Supernatants containing the nuclear proteins were diluted with 20 μl of modified buffer C [20 m*M* HEPES, pH 7.9, 20% (vol/vol) glycerol, 0.05 *M* KCl, 0.2 m*M* EDTA, 0.5 m*M* DTT, and 0.5 m*M* PMSF] and stored at −70°C until use. Nuclear extracts were used for the EMSA in 6% nondenaturing polyacrylamide gel (Pahan et al., 1997*a*,*b*) using the NF-κB DNA binding protein detection system kit (GibcoBRL), according to the manufacturer's protocol.

Assay of transcriptional activity of NF-κB

To assay the transcriptional activity of NF-κB, cells were transfected with pNF-κB-Luc, an NF-κB-dependent reporter construct (obtained from Stratagene), using the Lipotaxi method. Twenty-four hours after transfection, cells were treated with different stimuli for 5 h. Total cell extracts were used to measure luciferase activity in a Turner Design luminometer (TD-20/20) using an assay kit from Stratagene (Pahan et al., 1998*b*, 1999).

RESULTS

Knockdown of $p21^{ras}$ function by the expression of $\Delta p21^{ras}$ inhibits the expression of iNOS in rat primary astrocytes

To investigate the involvement of $p21^{ras}$ in the expression of iNOS in glial cells, we inhibited the function of this small G protein in rat primary astrocytes by a dominant-negative mutant of $p21^{ras}$ ($\Delta p21^{ras}$) in which the Ser residue at position 17 was changed to Asn (S17N mutant) (Qiu et al., 1995; Garnovskaya et al., 1996). First, we examined the effect of expression of $\Delta p21^{ras}$ on the function of $p21^{ras}$ as measured by GTP loading. LPS induced GTP loading of $p21^{ras}$ within minutes in rat primary astrocytes, whereas expression of $\Delta p21^{ras}$ mark-

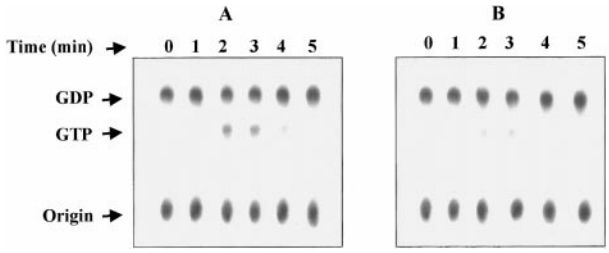

FIG. 1. Expression of $\Delta p21^{ras}$ inhibits LPS-induced GTP loading in rat primary astrocytes. Cells plated at 50–60% confluence in 60-mm dishes were transfected with 4 μg of either an empty vector (**A**) or $\Delta p21^{ras}$ (**B**) using Lipotaxi as described in Materials and Methods. At 24 h after transfection, cells were labeled with [^{32}P]orthophosphate for 16 h in phosphate-free medium and then stimulated with 1 μg/ml LPS. At different points of stimulation, cells were lysed with the lysis buffer, lysates were immunoprecipitated with antibodies against $p21^{ras}$, and nucleotides (GTP and GDP) released from immunoprecipitates were analyzed by TLC as described in Materials and Methods.

edly inhibited LPS-induced GTP loading (Fig. 1), suggesting that, as in other cell types (Qiu et al., 1995; Garnovskaya et al., 1996), $\Delta p21^{ras}$ is able to inhibit the function of $p21^{ras}$ in astrocytes. Then we assessed the effect of expression of $\Delta p21^{ras}$ on the induction of NO production in rat primary astrocytes. Figure 2A shows that $\Delta p21^{ras}$ markedly inhibited the production of NO in LPS-stimulated primary astrocytes, whereas expression of the empty vector "dummy" had no effect. To understand the mechanism of this inhibitory effect of $\Delta p21^{ras}$ on LPS-mediated nitrite production, we examined the effect of $\Delta p21^{ras}$ on protein and mRNA levels of iNOS. Western blot analysis with antibodies against murine macrophage iNOS and northern blot analysis for iNOS mRNA of LPS-stimulated astrocytes clearly show that $\Delta p21^{ras}$ significantly inhibited the LPS-mediated induction of iNOS protein (Fig. 2B) and mRNA (Fig. 2C). Farnesyltransferase (FTase) inhibitor I is known to prevent farnesylation of $p21^{ras}$, thus resulting in its inability to associate with other signaling components in the cell (Garcia et al., 1993). To confirm the involvement of $p21^{ras}$ in the induction of iNOS from a different angle, we examined the effect of FTase inhibitor I (Calbiochem) on LPS-induced production of NO. About 55% inhibition of NO production was observed with 50 μM FTase inhibitor I (data not shown), suggesting again the involvement of $p21^{ras}$ in the induction of NO production. Similar to LPS, proinflammatory cytokines (TNF-α, IL-1β, and IFN-γ) are also known to induce the expression of iNOS in rat primary astrocytes (Nishiya et al., 1995; Pahan et al., 1997*b*, 1998*a*). To examine whether cytokine-induced NO production is also inhibited by the expression of $\Delta p21^{ras}$, rat primary astrocytes were transiently transfected with $\Delta p21^{ras}$, and 24 h after transfection cells were stimulated with different combinations of TNF-α, IL-1β, and IFN-γ. All the combinations of cytokines induced the production of NO significantly; however, the expression of $\Delta p21^{ras}$ potently inhibited the NO production (Fig. 3A) and the induction of iNOS protein (Fig. 3B), suggesting that, similar to LPS, cytokine-mediated expression of iNOS also requires the involvement of $p21^{ras}$.

$\Delta p21^{ras}$ inhibits iNOS promoter-derived CAT activity in cytokine-stimulated rat primary astrocytes

Inhibition of the induction of iNOS protein and mRNA in astrocytes by the expression of $\Delta p21^{ras}$ suggests that the knockdown of $p21^{ras}$ may inhibit the transcription of iNOS gene. Therefore, to understand the effect of $\Delta p21^{ras}$ on the transcription of iNOS gene, astrocytes were cotransfected with either $\Delta p21^{ras}$ or an empty vector and piNOS-CAT, a construct containing the iNOS promoter fused to the CAT gene, and activation of this promoter was measured after the cells were stimulated with different cytokines. Consistent with the effect of $\Delta p21^{ras}$ on the production of NO and the expression of endogenous iNOS protein and mRNA,

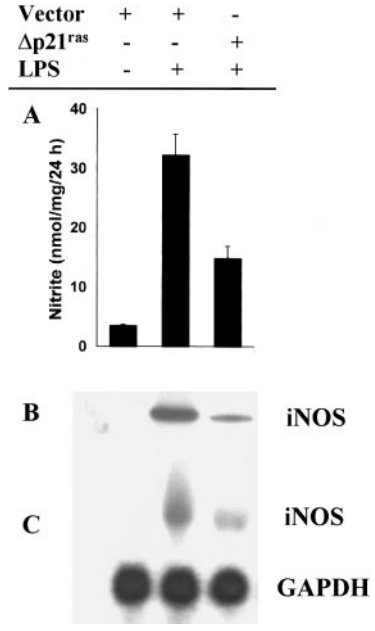

FIG. 2. Expression of $\Delta p21^{ras}$ inhibits the expression of iNOS and production of NO in LPS-stimulated rat primary astrocytes. Cells plated at 50–60% confluence in six-well plates were transfected with 2 μg of either $\Delta p21^{ras}$ or an empty vector using Lipotaxi as described in Materials and Methods. At 24 h after transfection, cells were stimulated with 1 μg/ml LPS. **A:** After 24 h of stimulation, the production of nitrite was measured in supernatants using Griess reagent as mentioned in Materials and Methods. Data are means ± SD of three different experiments. **B:** Cell homogenates were electrophoresed, transferred onto nitrocellulose membrane, and immunoblotted with antibodies against mouse macrophage iNOS as described in Materials and Methods. **C:** Cells plated at 50–60% confluence in 100-mm dishes were transfected with 8 μg of either $\Delta p21^{ras}$ or an empty vector using Lipotaxi. At 24 h after transfection, cells were stimulated with 1 μg/ml LPS for 5 h. 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 Materials and Methods.

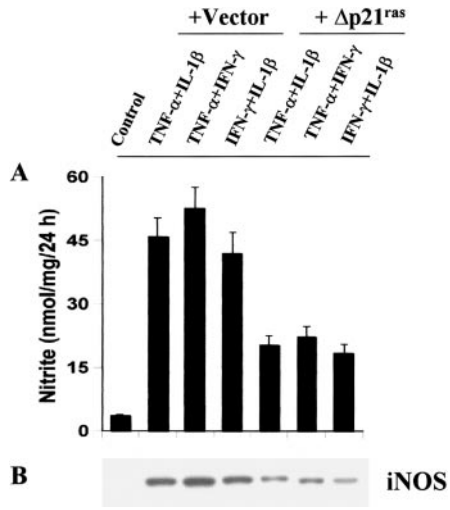

FIG. 3. Effect of $\Delta p21^{ras}$-mediated knockdown of $p21^{ras}$ on the expression of iNOS in cytokine-stimulated rat primary astrocytes. Cells plated at 50–60% confluence in six-well plates were transfected with 2 μg of either $\Delta p21^{ras}$ or an empty vector using Lipotaxi. At 24 h after transfection, cells were stimulated with different combinations of proinflammatory cytokines for 24 h. The production of nitrite was measured in supernatants (**A**). Data are means ± SD of three different experiments. Cell homogenates were analyzed for iNOS protein by an immunoblotting technique (**B**). Concentrations of different stimuli were as follows: TNF-α, 10 ng/ml; IL-1β, 10 ng/ml; IFN-γ, 10 U/ml.

$\Delta p21^{ras}$ markedly inhibited the induction of iNOS promoter-derived CAT activity in cytokine-stimulated astrocytes (Fig. 4), suggesting the conclusion that $\Delta p21^{ras}$ inhibits the transcription of iNOS gene in astrocytes.

Expression of $\Delta p21^{ras}$ inhibits the activation of NF-κB in rat primary astrocytes

As the activation of NF-κB is necessary for the transcription of iNOS gene (Xie et al., 1994; Nishiya et al., 1995; Pahan et al., 1998*a*,*b*), to understand the basis of $\Delta p21^{ras}$-mediated inhibition of iNOS expression, we examined the effect of $\Delta p21^{ras}$ on the activation of NF-κB. Treatment of astrocytes with 1 μg/ml LPS resulted in the activation of DNA-binding activity of NF-κB (Fig. 5). This gel shift assay detected a specific band in response to LPS that was competed off by an unlabeled NF-κB probe, but not by an unlabeled AP-1 probe (Fig. 5A). Expression of $\Delta p21^{ras}$ or the empty vector alone failed to induce the DNA-binding activity of NF-κB. However, $\Delta p21^{ras}$ markedly inhibited LPS-induced DNA-binding activity of NF-κB (Fig. 5B). We then tested the effect of $\Delta p21^{ras}$ on NF-κB-dependent transcription of luciferase in astrocytes in the presence or absence of different cytokines, using the expression of luciferase from a reporter construct, pNF-κB Luc (Stratagene), as an assay (Pahan et al., 1998*b*, 1999). Consistent with the effect of $\Delta p21^{ras}$ on the DNA-binding activity of NF-κB, $\Delta p21^{ras}$ also inhibited NF-κB-dependent transcription of luciferase in astrocytes, whereas expression of the empty vector had no effect on the transcriptional activity of NF-κB (Fig. 5C), suggesting that the inhibition of iNOS expression by $\Delta p21^{ras}$ is due to the inhibition of NF-κB activation.

Involvement of $p21^{ras}$ in the expression of iNOS in human primary astrocytes

As in rat primary astrocytes, proinflammatory cytokines are also able to induce the production of nitrite, as well as the expression of iNOS, in human primary astrocytes (Lee et al., 1993). To investigate whether $p21^{ras}$ is also involved in the induction of iNOS in human primary astrocytes, cells were transfected with $\Delta p21^{ras}$. Different combinations of proinflammatory cytokines induced the production of NO (Fig. 6A) and the expression of iNOS protein (Fig. 6B), whereas the expression of $\Delta p21^{ras}$ alone had no effect on NO production. However, $\Delta p21^{ras}$ significantly inhibited cytokine-stimulated production of NO (Fig. 6A) and expression of iNOS protein (Fig. 6B), suggesting that in both rat and human primary astrocytes, the induction of iNOS requires the involvement of $p21^{ras}$.

DISCUSSION

$p21^{ras}$, a membrane-associated small guanine nucleotide-binding protein, plays a central role in transmitting extracellular signals within the cell and in controlling cellular proliferation and differentiation (Gomez et al., 1998). Here we present evidence that activation of $p21^{ras}$ is involved in the activation of NF-κB and the induction of iNOS in activated primary astrocytes. Our conclusion is based on the following observations. First, LPS and

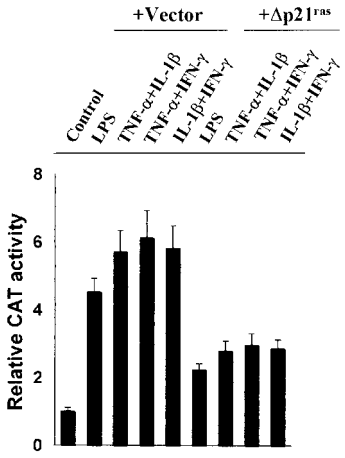

FIG. 4. Effect of $\Delta p21^{ras}$-mediated knockdown of $p21^{ras}$ on iNOS promoter-derived CAT activity in cytokine-stimulated rat primary astrocytes. Cells plated at 50–60% confluence in six-well plates were cotransfected with 1 μg of piNOS-CAT (the construct containing the rat iNOS promoter fused to the CAT gene) and 2 μg of either $\Delta p21^{ras}$ or an empty vector using Lipotaxi. At 24 h after transfection, cells were stimulated with LPS and proinflammatory cytokines for 14 h under serum-free conditions. The activity of CAT was measured using the radioisotopic assay kit (Promega) following the manufacturer's protocol. The concentrations of different stimuli were as follows: LPS, 1.0 μg/ml; TNF-α, 10 ng/ml; IL-1β, 10 ng/ml; IFN-γ, 10 U/ml.

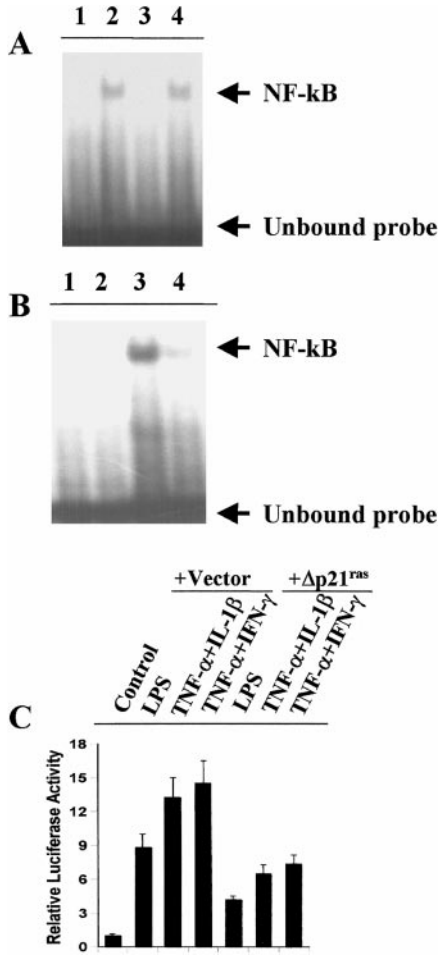

FIG. 5. Expression of $\Delta p21^{ras}$ inhibits the activation of NF-κB in LPS- and cytokine-stimulated rat primary astrocytes. **A:** Cells incubated in serum-free medium were treated with 1 μg/ml LPS. After a 30-min incubation, cells were taken out to prepare nuclear extracts, and nuclear proteins were used for the EMSA as described in Materials and Methods. Lane 1, nuclear extract of control cells; lane 2, nuclear extract of LPS-treated cells; lane 3, nuclear extract of LPS-treated cells incubated with a 100-fold excess of unlabeled NF-κB probe; and lane 4, nuclear extract of LPS-treated cells incubated with a 100-fold excess of unlabeled AP-1 probe. The upper arrow indicates the induced NF-κB, whereas the lower arrow indicates the unbound probe. **B:** Cells plated at 50–60% confluence in six-well plates were transfected with 2 μg of either $\Delta p21^{ras}$ or an empty vector using Lipotaxi. At 24 h after transfection, cells received 1 μg/ml LPS. After 30 min of stimulation, EMSA for NF-κB was carried out in nuclear extracts as described. Lane 1, nuclear extract of vector-transfected cells; lane 2, nuclear extract of $\Delta p21^{ras}$-transfected cells; lane 3, nuclear extract of vector-transfected cells treated with LPS; and lane 4, nuclear extract of $\Delta p21^{ras}$-transfected cells treated with LPS. The upper arrow indicates the induced NF-κB, whereas the lower arrow indicates the unbound probe. **C:** Cells plated at 50–60% confluence in six-well plates were cotransfected with 1 μg of pNF-κB-Luc (an NF-κB-dependent reporter construct) and 2 μg of either $\Delta p21^{ras}$ or empty vector using Lipotaxi. At 24 h after transfection, cells were stimulated with LPS and cytokines for 4 h, and the expression of luciferase reporter was quantified as described in Materials and Methods. Data are means ± SD of three different experiments. The concentrations of different stimuli were as follows: LPS, 1.0 μg/ml; TNF-α, 10 ng/ml; IL-1β, 10 ng/ml; IFN-γ, 10 U/ml.

proinflammatory cytokines induced the GTP loading of $p21^{ras}$, the activation of NF-κB, and the expression of iNOS. Second, expression of a dominant-inhibitory mutant of $p21^{ras}$ ($\Delta p21^{ras}$) knocked down LPS- and cytokine-induced GTP loading of $p21^{ras}$ and inhibited LPS- and cytokine-induced activation of NF-κB and the expression of iNOS. These studies demonstrate that $p21^{ras}$ is involved in the activation of NF-κB and the induction of iNOS in primary astrocytes. As NO synthesized from iNOS has been implicated in the pathogenesis of demyelinating and neurodegenerative diseases (Koprowski et al., 1993; Merrill et al., 1993; Cross et al., 1994; Endoh et al., 1994; Schubert et al., 1998), our results indicate that activation of $p21^{ras}$ may be a critical signaling step in the regulation of neuroinflammatory and neurodegenerative diseases, and pharmacological compounds capable of counteracting the activation of $p21^{ras}$ may ameliorate neural injury.

Signaling mechanisms for the induction of iNOS are poorly understood. The presence of a consensus DNA-binding site for NF-κB in the promoter of iNOS and inhibition of the LPS-induced expression of iNOS by inhibitors of NF-κB activation suggest that LPS induces the expression of these inflammatory molecules via activation of NF-κB (Xie et al., 1994; Nishiya et al., 1995; Pahan et al., 1998*a*,*b*). Earlier studies of Pahan et al. (1997*b*) demonstrating the inhibition of NF-κB activation and expression of iNOS by lovastatin and sodium phenylacetate suggested a role of protein farnesylation in the activation of NF-κB and induction of inflammatory molecules. Consistent with this observation, we demonstrate here the involvement of $p21^{ras}$ in the activation of

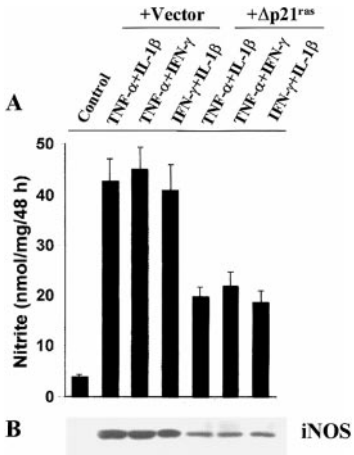

FIG. 6. $\Delta p21^{ras}$-mediated knockdown of $p21^{ras}$ inhibits the expression of iNOS and production of NO in human primary astrocytes. Cells plated at 50–60% confluence in six-well plates were transfected with 2 μg of either $\Delta p21^{ras}$ or an empty vector using Lipotaxi. At 24 h after transfection, cells were stimulated with different combinations of proinflammatory cytokines for 48 h. The production of nitrite was measured in supernatants (**A**). Data are means ± SD of three different experiments. Cell homogenates were analyzed for iNOS protein by an immunoblotting technique (**B**). The concentrations of different stimuli were as follows: TNF-α, 10 ng/ml; IL-1β, 10 ng/ml; IFN-γ, 10 U/ml.

NF-κB and the induction of iNOS in LPS- and cytokine-stimulated primary astrocytes. The Ras protooncogene proteins, a family of GTP-binding proteins, function by binding to the cytoplasmic surface of the plasma membrane. This membrane localization of $p21^{ras}$ involves prenylation of cysteine in CAAX motif present at the C-terminus, proteolytic removal of AAX tripeptide, and then carboxymethylation of C-terminal cysteine (Hancock et al., 1991). The activation of $p21^{ras}$ by receptor tyrosine kinase occurs through conversion of the GDP-bound inactive form to the GTP-bound active form by Sos and Grb2 and then transduction of signal to downstream effector molecules (Kikuchi and Williams, 1994). The GTP-bound form is converted to the inactive form by the intrinsic GTPase activity, which is accelerated by GTPase-activating proteins (Boguski and McCormick, 1993). The mutated $p21^{ras}$ protein translated from the expression of $\Delta p21^{ras}$ binds preferentially to GDP and acts as the dominant inhibitor of $p21^{ras}$ function presumably by blocking access to exchange factors and, in turn, inhibiting the signal transmission to the downstream signaling molecules (Qiu et al., 1995; Garnovskaya et al., 1996). One such downstream candidate is Raf-1 (serine-threonine kinase). $p21^{ras}$ interacts directly with Raf-1 and is believed to function by positioning Raf-1 at the plasma membrane in the vicinity of its activator, and tyrosine phosphorylation of Raf-1 seems to be essential for $p21^{ras}$-induced activation of Raf-1 (Jelinek et al., 1996). Raf-1, in turn, phosphorylates and activates MEKs and ERKs (members of mitogen-activated protein kinase cascade). Therefore, the observed inhibition of NF-κB activation and induction of iNOS by $\Delta p21^{ras}$ may be due to decrease or lack of signal transmission from receptor tyrosine kinase to Raf/mitogen-activated protein kinase cascade via $p21^{ras}$.

NO, a diffusible free radical, plays many roles as a signaling and as an effector molecule in diverse biological systems, including neuronal messenger, vasodilation, and antimicrobial and antitumor activities (Nathan, 1992; Jaffrey and Snyder, 1995). In the nervous system, the NO appears to have both neurotoxic and neuroprotective effects and may have a role in the pathogenesis of stroke and other neurodegenerative diseases (e.g., Alzheimer's disease) and in demyelinating conditions (e.g., multiple sclerosis, experimental allergic encephalopathy, X-adrenoleukodystrophy) associated with the activation of glial cells and the production of proinflammatory cytokines (Koprowski et al., 1993; Merrill et al., 1993; Cross et al., 1994; Endoh et al., 1994; Schubert et al., 1998). NO and peroxynitrite (reaction product of NO and O_2^-) are potentially toxic to neurons and oligodendrocytes that may mediate toxicity through the formation of iron–NO complexes of iron-containing enzyme systems (Drapier and Hibbs, 1988), oxidation of protein sulfhydryl groups (Radi et al., 1991), nitration of proteins, nitrosylation of nucleic acids, and DNA strand breaks (Wink et al., 1991). Although monocytes/macrophages are the primary source of iNOS in inflammation, LPS and other cytokines induce a similar response in astrocytes and microglia (Simmons and Murphy, 1992; Zielasek et al., 1992; Pahan et al., 1997*b*). NO derived from activated microglia and astrocytes has been implicated in the damage of myelin-producing oligodendrocytes in demyelinating disorders like multiple sclerosis and neuronal death during neurodegenerating conditions, including brain trauma and Alzheimer's disease (Koprowski et al., 1993; Merrill et al., 1993; Cross et al., 1994; Endoh et al., 1994; Schubert et al., 1998). The studies described in this article suggest that inhibition of $p21^{ras}$ activation may represent a possible avenue of research for therapeutics directed against cytokine- and NO-mediated neuroinflammatory and neurodegenerative disorders.

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