Blockade of p38 Mitogen-activated Protein Kinase Pathway Inhibits Inducible Nitric-oxide Synthase Expression in Mouse Astrocytes*

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Nitric oxide (NO)1 is a short lived molecule that mediates a wide range of biologic effects. It acts as an intercellular messenger (for reviews, see Refs. 1–3) and plays a role in neurophysiological conditions such as infections (11), ischemia (12), and multiple sclerosis (13).

Little is known about the intracellular signaling pathways of iNOS induction. The murine iNOS promoter revealed the presence of 24 transcription factor binding sites, including NF-κB and AP-1 sites (14–16). Protein kinase C, and particularly protein kinase C-α, has been reported to induce iNOS (17). The role of the mitogen-activated protein kinase (MAPK) cascades, p42/p44 MAPK (ERK1 and ERK2), p38 MAPK, and p54 JNK in the control of iNOS expression has not yet been clearly defined (18, 19). p38 MAPK is activated by treatment of cells with lipopolysaccharide, cytokines, and stress (20, 21). MAPKAP kinase-2 was first identified as a p38 MAPK substrate, which in turn phosphorylated HSP-27 (22, 23). Subsequently, several transcription factors have been found to be activated by p38 MAPK. These transcription factors included ATF-2 (24); CHOP/GADD 153, a member of the C/EBP family expressed in stressed cells (25); Max, which is bound and phosphorylated by Mxi-2, a homologue to CSBP 1 and 2 (26), CREB, and ATF-1, reported to be under the control of MAPKAP kinase-2 and p38 MAPK (27, 28); and the myocyte enhancer factor 2C (MEF2C), belonging to the MADS family (29). However, p38 MAPK also has a major role in the regulation of gene expression at the post-transcriptional level (30), probably by a mechanism depending on the AUUUA sequence motifs in the 3′-untranslated region of their transcripts (31). Important roles of p38 MAPK have been shown in the control of TNF and IL-1 expression by THP-1 cells (30), of IL-8 expression in monocytes (32), and of IL-6 expression in TNF-treated astrocytes (33, 34). More recently, p38 MAPK has also been shown to play a role in the expression control of adhesion molecules in endothelial cells, including E-selectin (35) and VCAM-1 (36).

The present study investigates the role of p38 MAPK in the regulation of iNOS expression. The results show that a p38 MAPK-dependent pathway transduces iNOS expression signals elicited by combined TNF-α and IL-1α stimulation in mouse primary astrocytes. p38 MAPK activation is necessary but not sufficient, since stimulation by TNF-α or IL-1α alone fully activated p38 MAPK but was unable to induce iNOS transcription. The p38 MAPK-mediated signal acts indirectly, since iNOS gene transcription is blocked by protein synthesis inhibition.

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EXPERIMENTAL PROCEDURES

Mice—The TNF receptor-deficient mice (tnfr-1°, tnfr-2°, and tnfr-1°/tnfr-2°) have been previously reported (37–39). Wild type (C57BL/6 × 129/Sv), tnfr-1° (C57BL/6 × 129/Sv-tnfr-1°), tnfr-2° (C57BL/6 × 129/Sv-tnfr-2°), and tnfr-1°/tnfr-2° mice (C57BL/6 × 129/Sv-tnfr-1°/tnfr-2°) were sacrificed 4–5 days after birth.

Isolation and Culture of Mouse Neonate Astrocytes—Astrocyte precursors were isolated from 4–5-day-old mouse pups as described elsewhere (40). Briefly, cerebella were dissected and stripped of the meninges. The cerebellar cortex was isolated by a 3-day incubation in Dulbecco’s modified Eagle’s medium (DMEM) for astrocytes, the serum-free medium was exchanged with a medium containing 10% fetal calf serum. The astrocyte cultures were treated with TNF-α (100 ng/ml) and resuspended in serum-free medium (DMEM). The cultures were incubated for 24 h and harvested, and nitrite concentrations were determined by a colorimetric assay (41). The supernatants representing the nuclear extracts. The protein content was measured with the BCA protein assay kit (Pierce) with bovine serum albumin as a standard. Nuclear extracts were stored at −80 °C until use.

Preparation of Cell Lysates—Cells cultured in 10-cm Petri dishes were washed twice with phosphate-buffered saline and lysed in 300 μl of lysis buffer (50 mM Hepes, 100 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Nonidet P-40, 14 μg/ml leupeptin, 0.5 mM sodium orthovanadate, 10 mM sodium orthovanadate, 100 units/ml aprotinin, 100 mM NaF). After incubation for 30 min on ice, cell lysates were centrifuged (14,000 rpm, 10 min, 4 °C), and the supernatants were recovered. Protein concentrations were determined using the BCA colorimetric assay (Pierce), with bovine serum albumin as a standard.

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p38MAPK Mediates iNOS Expression

Fig. 1. p38MAPK activation is required for iNOS expression and NO production in mouse astrocytes. A and B, time course of iNOS expression and NO production. A, immunoblot detection of iNOS expression. Astrocyte cultures (3 × 10^5 cells/well) were treated with combined TNF-α/IL-1α (100 ng/ml each) for 0, 1, 3, 9, 24, and 48 h. Cellular extracts (20 μg of total protein) were separated by SDS-PAGE and transferred to PVDF membranes, and iNOS was detected by ECL. The filter was stripped and reprobed with anti-actin antibody to confirm equal protein loading. B, nitrite determination in cell culture supernatants reflecting NO release after 48 h of stimulation (results show one representative experiment of three, nitrite determination in duplicates, S.D. = 5% of the mean). C and D, effect of FHPI and PD98059 treatment on iNOS expression and NO production. Lane 1, unstimulated cells; lanes 2–4, cells stimulated with TNF-α/IL-1α without inhibitor (lane 2), with FHPI (lane 3), and with PD98059 (lane 4). C, immunoblot detection of iNOS expression. Cells were treated with FHPI (10 μM) or PD98059 (20 μM) for 45 min, and cell extracts were prepared after 24 h of TNF-α/IL-1α treatment. D, nitrite determination in cell culture supernatants reflecting NO release after 48 h of stimulation (results show one representative experiment of three, nitrite determination in duplicate, S.D. = 5% of the mean).

Fig. 2. Inhibition of iNOS expression and NO release by FHPI in TNF/IL-1-stimulated astrocytes. A, immunoblot detection of iNOS expression. Astrocytes were stimulated with TNF-α/IL-1α (100 ng/ml each) in the presence of the indicated amounts of FHPI for 24 h. Cytoplasmic extracts (20 μg of total protein) were separated by SDS-PAGE and transferred to PVDF membranes, and iNOS was detected by ECL. The filter was stripped and reprobed with anti-actin antibody to confirm equal protein loading. B, nitrite determination in cell culture supernatants reflecting NO release after 48 h of TNF-α/IL-1α treatment (results show one representative experiment of three, nitrite determination in duplicate, S.D. = 5% of the mean).

Colorimetric assay. An accumulation of nitrites in the supernatants consistent with the observed iNOS protein expression time profile was found (Fig. 1B).

To investigate whether a p38MAPK-dependent pathway mediates the TNF-α/IL-1α-stimulated iNOS induction, a specific inhibitor, FHPI, was used to block p38MAPK activity (30). As shown in Fig. 1C, preincubation of astrocytes with 10 μM FHPI reduced the TNF-α/IL-1α-induced iNOS protein expression to nondetectable levels at the 24-h time point. As a consequence, the stimulated NO release determined from the nitrite concentrations in the culture supernatants after 48 h was decreased by more than 80% to background levels (Fig. 1D). In contrast, iNOS expression 24 h after TNF-α/IL-1α stimulation was partially affected by the pretreatment with PD98059, a specific inhibitor of the MEK1-dependent pathway (Fig. 1C). As a consequence, the NO release 48 h after TNF-α/IL-1α stimulation was merely reduced by 15% in the presence of 20 μM of PD98059, a treatment that fully inactivates the MEK1 pathway (43). The FHPI dose dependence of the inhibition of iNOS expression was determined in astrocytes that had been preincubated for 45 min with various concentrations of p38MAPK inhibitor before TNF-α/IL-1α stimulation (Fig. 2A and B). iNOS expression was inhibited in a regular dose dependence, with a half-inhibitory concentration, IC_{50}, of about 0.5 μM, congruent with the IC_{50} of FHPI determined in assays using recombinant p38MAPK (data not shown). The NO production in astrocyte cultures in the presence of increasing concentrations of FHPI agreed well with the iNOS protein expression (Fig. 2B). The inhibition of NO release by PD98059 treatment saturated at about 40 μM at a level of about 10% inhibition.

p38MAPK Regulates iNOS Expression at the Transcriptional Level—The expression of iNOS had been previously shown to be regulated at the transcriptional level (16), but the dependence on p38MAPK activation and the presence of AUUUA motifs in the 3' untranslated region sequence of the iNOS mRNA suggested that a regulation at the translational level might also occur. To further investigate the molecular mechanisms of the regulation of iNOS gene expression by the p38MAPK pathway, iNOS mRNA levels were studied by Northern blot analysis in TNF-α/IL-1α-stimulated astrocytes in the presence or the absence of p38MAPK inhibitor. Combined TNF-α and IL-1α treatment resulted in a transient increase of the 4.4-kilobase pair iNOS transcript (Fig. 3A). The transcript was first detected at 3 h, reached a maximum at 6 h, and had substantially declined after 24 h. Pretreatment of the cells with 10 μM FHPI resulted in a 3–4-fold reduction in iNOS transcript level after 6 h, and only faint bands were detected at the 3-, 12-, and 24-h time points. The fact that residual iNOS mRNA levels are detected agrees well with incomplete inhibition of iNOS protein expression in the presence of 10 μM FHPI, the lowest inhibitor concentration causing a substantial reduction in iNOS protein expression (Fig. 2A). These findings support a dominant transcriptional control of iNOS by p38MAPK.

To test for a selective activity of FHPI on p38MAPK-mediated iNOS regulation, the Northern blots were reprobed for another inducible gene, the MCP-1 gene. The induction of TNF-α/IL-1α-stimulated MCP-1 transcription was found independent of FHPI treatment of the cells and therefore must be controlled by signal pathways independent of p38MAPK activity (Fig. 3A). Interestingly, the production of MCP-1 protein by the astrocytes was inhibited by FHPI treatment in a dose-dependent fashion, with an IC_{50} of 0.5 μM, despite the fact that the MCP-1 mRNA levels were unaffected, suggesting that p38MAPK exerts...
FIG. 3. p38MAPK controls iNOS expression in mouse astrocytes at the transcriptional level. A. Northern blot analysis of time-dependent iNOS transcription. Astrocytes pretreated with FHPI (10 μm) or excipient for 45 min were stimulated with combined TNF-α+IL-1α (100 ng/ml each) in the continued presence of the inhibitor for 0, 1, 3, 6, 12, and 24 h. 10 μg of total RNA were analyzed using a specific iNOS DNA probe. The filter was reprobed with a specific MCP-1 DNA probe and GAPDH DNA probe to confirm equal RNA loading. B, Northern blot analysis of the inhibition of iNOS transcription by FHPI. Astrocytes were pretreated with increasing concentrations of FHPI (0–20 μm) for 45 min as indicated followed by stimulation with TNF-α+IL-1α (100 ng/ml each) for 6 h. Total RNA was extracted and analyzed by Northern blotting for the transcription of iNOS, MCP-1, and GAPDH mRNA.

iNOS mRNA Induction Requires New Protein Synthesis—To investigate whether the induction of iNOS mRNA transcription by TNF-α+IL-1α treatment depended on new protein synthesis, the astrocytes were cultured in the presence of cycloheximide (1 or 10 μg/ml) added 1 h before the start of the cytokine treatment. The cells were harvested after 6 h of cytokine stimulation, at a time when in control cultures iNOS transcription was maximal. Cycloheximide at 1 and 10 μg/ml fully prevented induction of iNOS transcription as detected by Northern blot analysis (Fig. 4A), whereas transcription of MCP-1 and GAPDH was not affected by the cycloheximide treatment.

iNOS Induction Requires Combined TNF-α and IL-1α Stimulation—To dissect the role of the TNF-α and IL-1α in the iNOS expression, astrocyte cultures were stimulated with TNF-α, IL-1α, or combined TNF-α+IL-1α for 24 h. Cytoplasmic extracts were prepared and separated by 10% SDS-PAGE, and PVDF membrane filter blots were probed for iNOS protein by immunoblotting. iNOS expression was observed after combined TNF-α+IL-1α treatment but not after treatment with TNF-α or IL-1α or after osmotic shock by sorbitol used as control (Fig. 5A). Northern analysis showed that the astrocytes responded to TNF-α and IL-1α stimulation with induced transcription of the MCP-1 gene, but iNOS transcription required the combined activity of both cytokines (Fig. 5B). As a consequence, combined TNF-α+IL-1α stimulation is required for accumulating NO release after 48 h (Fig. 5C); preliminary studies had shown that the differences among TNF-α, IL-1α, combined TNF-α+IL-1α, and sorbitol stimulation are not due to different kinetic profiles of NO release from 12 to 72 h. Interestingly, sorbitol was not able to substitute for either TNF-α or IL-1α to induce the release of NO studied by nitrite determination.

TNF-α and IL-1α Alone Fully Activate p38MAPK—MAPKs are activated by upstream dual specificity kinases, which phosphorylate the threonine and tyrosine residues in the TEF, TGY, or TPY motifs of p42/44 MAPK, p38 MAPK, or p54JNK, respectively. Various cytokines, including TNF-α and IL-1α have been shown to activate these different pathways. To dissect the role of the different MAPK pathways in the TNF-α- and IL-1α-, and combined TNF-α+IL-1α-induced iNOS expression, immunoprecipitation kinase assays were performed. Astrocytes were stimulated with TNF-α, IL-1α, combined TNF-α+IL-1α, and sorbitol for various times. p38 MAPK, p42/44 MAPK, and p54JNK were immunoprecipitated sequentially from the cytoplasmic extracts with specific antibodies, and immune complex kinase assays were performed using [γ-32P]ATP and GST-ATF-2, MBP, or GST-c-Jun as specific substrates, respectively. The reactants were separated by SDS-PAGE, and the radioactivity incorporated in the respective bands was determined (Fig. 6). p38MAPK in TNF-α-stimulated astrocytes reached a maximal activity as early as 5 min and declined 1 h after the start of TNF-α treatment (Fig. 6). The p42/44 MAPK and p54JNK activity peaks were delayed when compared with p38 MAPK, reaching maxima about 15 min after TNF-α. IL-1α treatment also induced a transient activation of p38MAPK, p42/44 MAPK, and p54JNK with kinetics similar to those observed with TNF-α stimulation (Fig. 6). The induction of iNOS expression in the astrocytes required combined TNF-α and IL-1α stimulation (Fig. 1), yet both TNF-α and IL-1α independently were able to strongly activate p38MAPK. Furthermore, the treatment with both cytokines combined did not activate p38MAPK in a quantitatively or qualitatively significantly different manner from either TNF-α or IL-1α alone. Similarly, the level and time dependence of the activation of p42/44 MAPK and p54JNK were not affected by the presence of combined TNF-α+IL-1α (Fig. 6).

p38MAPK and p54JNK are also activated by stress factors such as osmotic shock. Sorbitol in hyperosmolar concentrations was therefore used to investigate the kinase responses to osmotic stress. Preliminary studies had shown a regular dose response of p38MAPK and p54JNK activation by sorbitol, first saturating at 300 mM. The further studies therefore were carried out at 300 mM sorbitol, which elicited a transient activation of p38MAPK and p54JNK (Fig. 6). Surprisingly, sorbitol was also able to activate the p42/44 MAPK pathway generally thought to be under the control of mitogenic or growth factor stimuli.

The above findings were in full agreement with the results of immunoblot analyses of p38MAPK and p42/44 MAPK activation. Cytoplasmic extracts of astrocytes were prepared; samples...
with calibrated amounts of total protein were separated by SDS-PAGE and transferred to PVDF membranes, and probed by two-layer antibody incubation and ECL detection. The filter was stripped and reprobed with anti-actin antibody to confirm equal protein loading.

**Fig. 5.** Combined TNF-α/IL-1α treatment is required for induced iNOS expression and nitrite release. Astrocyte cultures (3 x 10^5 cells/well) were left unstimulated (lane 1) or stimulated with TNF-α (100 ng/ml, lane 2), IL-1α (100 ng/ml, lane 3), combined TNF-α/IL-1α (100 ng/ml each, lane 4), or sorbitol (300 mM, lane 5). A, immunoblot analysis of iNOS expression. Cell lysates were separated by SDS-PAGE, transferred to PVDF membranes, and probed by two-layer antibody incubation and ECL detection. The filter was stripped and reprobed with anti-actin antibody to confirm equal protein loading. B, Northern blot analysis of iNOS transcription. 10 μg of total RNA were analyzed using sequentially iNOS, MCP-1, and GAPDH DNA probes. C, nitrite determination in cell supernatants reflecting NO release of the various stimulated cell cultures after 48 h of TNF-α/IL-1α treatment (results show one representative experiment of three, nitrite determination in duplicate, S.D. < 5% of the mean).

**Fig. 6.** p38MAPK, p42/44MAPK, and p54JNK are all activated by TNF-α, IL-1, combined TNF-α/IL-1α, and sorbitol in astrocyte cultures. Immunoprecipitation kinase assays of astrocytes left untreated or treated with TNF-α (100 ng/ml), IL-1α (100 ng/ml), combined TNF-α/IL-1α (100 ng/ml each), or sorbitol (300 mM) for the indicated times are shown. Specific polyclonal antibodies were used to immunoprecipitate p38 MAPK, p42/44MAPK, and p54JNK from cell lysates, and in vitro kinase assays were performed using 5 μCi of [γ-32P]-ATP and 1 μg of GST-ATF-2, MBP, or GST-c-Jun as substrate, respectively. The reagents were separated by SDS-PAGE. Normalized integrated band intensities determined by PhosphorImager counts are indicated below the respective bands.

The p38MAPK Pathway Does Not Regulate NF-κB Activity—Several transcription factors, such as NF-κB, have been implicated in mediating cytokine-induced iNOS expression (14, 16). NF-κB indeed participates in the control of genes of the cellular response to stress and inflammatory mediators. Therefore, the possibility that p38MAPK may couple the activation of NF-κB to the response to cytokines and stress in astrocyte cultures was explored. The effects of TNF-α, IL-1α, combined TNF-α/IL-1α, and sorbitol treatments in the presence or absence of FHPI on the activation of NF-κB were examined by electrophoretic mobility shift assays of astrocyte nuclear extracts using a double-stranded κB-specific 32P-labeled oligonucleotide probe. An NF-κB-specific band was detected with all TNF-α-, IL-1α-, and combined TNF-α/IL-1α-treated cells that was absent in untreated or sorbitol-treated nuclear extracts (Fig. 8). Interestingly, the inhibition of p38MAPK by 10 μM FHPI did not interfere with the intensities of these bands in any of the stimulated cells, suggesting that p38MAPK is not involved in the coupling of the activation and nuclear translocation of NF-κB to TNF-α and IL-1α receptor activation.

TNFR-1 but Not TNFR-2 Mediates iNOS Induction in Astrocytes—The activities of TNF-α are mediated through two distinct surface receptors, TNFR-1 and TNFR-2 (44). To dissect the role of the two TNF receptors in iNOS induction, primary astrocyte cultures from wild type, tnfr-1<sup>−/−</sup>, tnfr-2<sup>−/−</sup>, and tnfr-1<sup>−/−</sup>/tnfr-2<sup>−/−</sup> mice were stimulated with combined TNF-α/IL-1α. Fig. 9A shows an essential and exclusive role of TNFR-1 in the cooperative TNF-α/IL-1α stimulation to induce iNOS expression in the astrocytes, since stimulated iNOS expression is only detected in wild type and tnfr-2<sup>−/−</sup> astrocytes after TNF-α/IL-1α...
p38MAPK Mediates iNOS Expression

**FIG. 7. p38MAPK and p42/44MAPK phosphorylation by TNF-α, IL-1α, and combined TNF-α/IL-1α treatment.** Immunoblot analyses of cytoplasmic extracts of astrocytes left untreated or treated with TNF-α (100 ng/ml), IL-1α (100 ng/ml), or combined TNF-α/IL-1α (100 ng/ml each) for indicated times. Cell lysates were separated by SDS-PAGE and transferred to PVDF membranes. Phosphorylation of p38MAPK and p42/44MAPK was analyzed using anti-phospho-Tyr-182 p38MAPK (p38) or anti-phospho-Tyr-204 p42/44MAPK (ppERK1, -2) antibody. The same blots were reprobed for anti-p38MAPK (p38) or anti-p42/44MAPK (pERK1, -2) expression, indicating equal protein loading under all conditions.

**FIG. 8. p38MAPK does not regulate activation of NF-κB.** Electrophoretic mobility shift assays of NF-κB activation of astrocytes left unstimulated or stimulated for 15 min with TNF-α (100 ng/ml), IL-1α (100 ng/ml), combined TNF-α/IL-1α (100 ng/ml each), or sorbitol (300 mM) in the absence or presence of FHPI (10 μM) are shown. Nuclear extracts (10 μg of total protein) were incubated with a specific 32P-labeled oligonucleotide probe containing κB elements. The specificity of the band shift was shown in a parallel gel using a point-mutated κB probe.

Treatment. These results were further confirmed by nitrite measurements conducted after 48 h (Fig. 9B). Large amounts of accumulating nitrates were detected in supernatants of TNF-α/IL-1α-stimulated wild type and tnfr-2° astrocytes, whereas nitrite concentrations with tnfr-1° and tnfr-2° astrocytes remained at background levels. Interestingly, the TNF-α-stimulated activation of p38MAPK, p42/44MAPK, and p34NK in the astrocytes is also exclusively mediated by TNFR-1, in contrast to findings with primary fibroblasts isolated from these mice (39).

**DISCUSSION**

The necessity to stimulate cells with combined cytokines such as TNF-α plus IL-1β or IFN-γ or with cytokines combined with lipopolysaccharide to induce iNOS expression has been reported in several cell types such as astrocytes (45), fibroblasts (46), and endothelial cells (47), although a single cytokine can induce iNOS expression in some other cell systems including hepatocytes (48), islet cells (49), and vascular smooth muscle cells (50). In the present astrocyte cultures, combined stimulation with IL-1α and TNF-α was required to drive iNOS transcription; p38MAPK activation was necessary but not sufficient to transduce the signal, since either IL-1α or TNF-α alone fully activated p38MAPK. Osmotic stress also fully activated p38MAPK but could not substitute for either TNF-α or IL-1α in the TNF-α/IL-1α combination. Furthermore, combinations of either IL-1α or TNF-α with IFN-γ were ineffective in stimulating iNOS transcription, although IFN-γ activity enhanced the iNOS response to TNF-α/IL-1α. Combined TNF-α/IL-1α stimulation of the astrocytes was not a general requirement, since both TNF-α and IL-1α individually were fully competent to stimulate MCP-1 transcription in the astrocyte cultures.

The effect of FHPI treatment demonstrated that activation of the p38MAPK pathway is necessary for iNOS induction. It might have been argued that the requirement for two cytokines to induce iNOS transcription reflects a synergy leading to stronger and more extended p38MAPK activation. However, the extent and kinetics of p38MAPK as well as p42/44MAPK and p34NK kinase activation did not provide evidence for any such additive or enhancing effects by the TNF-α and IL-1α combination treatment. It must rather be proposed that in addition to the p38MAPK pathway a TNF-α- or IL-1α-activated second pathway is required to generate a sufficient signal for iNOS induction.

NF-κB has been reported to be involved in iNOS induction (17, 51). It was strongly activated by TNF-α as well as IL-1α in the present astrocytes. NF-κB and p38MAPK lie on two distinct pathways as shown by the present finding that p38MAPK inhibition did not interfere with activation of NF-κB, confirming previous reports (52, 53), and by the lack of evidence that NF-κB is upstream of p38MAPK. However, NF-κB may represent a second independent pathway for iNOS induction. NF-κB has been reported to be important for iNOS transcription, since the pretreatment of rat alveolar macrophages and glomerular mesangial cells with pyrrolidine dithiocarbamate, an inhibitor of NF-κB activation, completely blocked iNOS transcription (54, 55). Pyrrolidine dithiocarbamate treatment of the present astrocytes also suppressed iNOS transcription. However, given the time scale and dependence on new protein synthesis of iNOS induction, a more general or toxic effect of pyrrolidine dithiocarbamate could not be ruled out. The responses of the p38MAPK and NF-κB systems are fast and could both induce independently one or two separate genes, yet to be defined, whose products may lead to induced iNOS transcription. The present results do not allow us to definitely resolve the nature of the second pathway required for iNOS induction, but they provide clear evidence for a parallel signaling cascade in addition to p38MAPK to induce TNF-α/IL-1α-stimulated iNOS transcription.

A role of p34NK and p42/44MAPK must also be considered in the control of iNOS expression, since both kinases were shown to be activated by TNF-α and IL-1α in slightly delayed but otherwise similar kinetics as p38MAPK in the mouse astrocyte cultures. The lack of effect by PD98059 rendered the involvement of the p42/44MAPK pathway in iNOS control unlikely. This view was further supported by a recent report where the inhibition of IFN-γ-activation of ERK1 and ERK2 by PD98059 or by Ras dominant negative expression did not affect iNOS

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induction in C6 glioma cells (56). In contrast, inhibition of Ha-Ras farnesylation, which blocks Ha-Ras processing, correlated with inhibition of iNOS induction in vascular smooth muscle cells, suggesting a role of the Ras/ERK pathway in the control of iNOS induction by IL-1β (57). Furthermore, while the present data show that p38MAPK activity is required for the transcriptional induction of iNOS, it has been demonstrated in serum-starved mesangial cells that the inhibition of p38MAPK promoted IL-1β-induced iNOS expression and subsequent NO production (58). The most likely explanation for these seemingly inconsistent results is that the complex regulation of iNOS expression is tissue-specific. One further example of the intriguing cell type specificity of signal pathway connectivities is provided by the coupling of TNF receptors to the MAPK pathways. Studies of primary fibroblast cultures of the same mice from which the astrocyte cultures had been isolated showed that p38MAPK, p42/44MAPK, and p54NK are all fully activated by TNF-α treatment of wild type, tnfr-1−/−, and tnfr-2−/− fibroblasts (39), demonstrating that both TNFR-1 and TNFR-2 in these fibroblasts couple to all three MAPK pathways, whereas only TNFR-1-mediated signals accessed the three MAPK pathways in the astrocytes. Previous studies in tnfr-1−/− hepatic cells (59), peritoneal macrophages (60), and in vivo models (61) had reported TNFR-1 activity to be necessary to trigger NO release. With the present tnfr-2−/− astrocytes, TNF-α-activated pathways, when combined with IL-1α signals, were also sufficient to induce iNOS expression and produced measurable levels of iNOS protein and nitrates as wild type astrocytes, demonstrating the sufficient role of TNF-1. In contrast to the astrocytes, IL-1α stimulation alone suffices to induce iNOS expression in the fibroblasts of the same mouse lines, further demonstrating the complex and tissue-specific regulation of iNOS expression.
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p38MAPK Mediates iNOS Expression