Inhibitory and Stimulatory Effects of Lactalacystin on Expression of Nitric Oxide Synthase Type 2 in Brain Glial Cells

THE ROLE OF IxB-β*

Expression of inflammatory nitric oxide synthase (NOS2) is mediated by transcription factor NFκB. By using the specific proteasome inhibitor lactacystin to examine IxB degradation, we observed a paradoxical increase in lipopolysaccharide- and cytokine-dependent NOS2 expression at low concentrations or when lactacystin was added subsequent to cytokines. Lactacystin reduced the initial accumulation of NOS2 mRNA but reduced its subsequent decrease. Lactacystin increased NOS2 promoter activation after 24 h, but not after 4 h, and similarly prevented initial NFκB activation and at later times caused NFκB reactivation. Lactacystin reduced initial degradation of IxB-α and IxB-β, however, at later times selectively increased IxB-β, which was predominantly non-phosphorylated. Expression of full-length rat IxB-β, but not a carboxyl-terminal truncated form, inhibited NOS2 induction and potentiation by lactacystin. Lactacystin increased IxB-β expression in the absence of NOS2 inducers, as well as expression of heat shock protein 70, and the heat shock response due to hyperthermia increased IxB-β expression. These results suggest that IxB-β contributes to persistent NFκB activation and NOS2 expression in glial cells, that IxB-β is a stress protein inducible by hyperthermia or proteasome inhibitors, and that delayed addition of proteasome inhibitors can have stimulatory rather than inhibitory actions.

In brain, inflammatory responses of astroglial cells occur during disease, infection, trauma, and ischemia. These responses include release of pro-inflammatory cytokines as well as synthesis and release of nitric oxide (NO).1 In astrocytes, NO is primarily biosynthesized by the calcium-independent isoform of NO synthase (NOS2) which is normally not present but whose expression is induced by a variety of inflammatory stimuli. Increasing evidence points to a role for NOS2-derived NO in the pathogenesis of a variety of human neurological diseases and brain trauma (1). In human brain, astrocytes express NOS2 during demyelinating diseases (2–4), cerebral ischemia (5, 6), viral infection (7), traumatic brain injury (8), Alzheimer’s disease (9), and possibly during AIDS dementia (10). Beneficial aspects of preventing or reducing NOS2 expression have been demonstrated in animal studies of experimental autoimmune encephalomyelitis (EAE) (11) and cerebral ischemia (12) demonstrating that suppression of astroglial NOS2 can be of therapeutic value in the prevention of neurological damage.

Primary cultures of astrocytes from rat (13, 14), mouse (15), and human (16) have been used extensively to characterize the inductive process of astroglial NOS2 expression. In vitro studies have demonstrated that stimulation of cells with bacterial endotoxin lipopolysaccharide (LPS) or with a combination of cytokines which nominally includes IL1-β leads to de novo expression of NOS2. Extensive characterization of glial NOS2 expression and regulation has also been carried out with the rat C6 glioma cell line (17, 18), which shares many properties with primary astrocyte cultures, including expression and regulation of the same NOS2 gene, mRNA, and protein (17).

As found for many other cell types, the induction of astroglial NOS2 requires activation of transcription factor NFκB (19), as determined by use of pharmacological inhibitors of NFκB and by analysis of the NOS2 promoter region (20–26). The activation pathway of NFκB has been well characterized (19, 27–30). NFκB is a dimeric complex consisting of two members of the Rel protein family including p50, p52, Rel A (p65), Rel B, and the oncogene c-rel. NFκB is maintained in the cytoplasm by association with members of the IxB protein family (36) whose ankyrin repeat domains bind to, and mask, nuclear localization sites present in NFκB subunits. Inflammatory stimulation by cytokines or lipopolysaccharides (LPS) results in activation of IK kinases, leading to serine phosphorylation of IxBs at their amino termini (28). Phosphorylated IxBs are ubiquitinated at amino-terminal lysine residues (31) which targets them for degradation by the 26 S proteasome (28, 30). This allows the rapid translocation of NFκB to the nucleus, where it binds to κB DNA motifs present in a variety of promoter regions including that of NOS2 (26) as well as that of IxB-α (32). The de novo transcription of IxB-β results in a rapid increase of IxB-α

* This worked was supported in part by grants from the National Institutes of Health and from the National Multiple Sclerosis Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Anesthesiology, University of Illinois, 900 South Ashland Ave., MC513, Chicago, IL 60607. Tel.: 312-355-1665; Fax: 815-333-0449; E-mail: dfeins@uic.edu.

‡ Both authors contributed equally to this work.

§ The abbreviations used are: NO, nitric oxide; NOS2, the calcium-independent isoform of nitric oxide synthase, also referred to as iNOS; CIS, competitive internal standard; HSP, heat shock protein; HSR, heat shock response; LPS, lipopolysaccharide; IFN, interferon-γ; TPCK, N-tosyl-l-phenylalanine chloromethyl ketone; ZIE, benzoyloxycarbonyl-leucinal; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; RT-PCR, reverse transcriptase-polymerase chain reaction; kb, kilobase pair; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; EMSA, electrophoretic mobility shift assay; ANOVA, analysis of variance; IL, interleukin; bp, base pair.
protein expression, which can reassociate with active NF-κB (both in the cytoplasm as well as in the nucleus) thus reducing ongoing NF-κB activity. The expression patterns and phosphorylation kinetics of IkB proteins are therefore key to the overall level of NF-κB activation. However, the characterization of IkB proteins in astrocytes is limited (33–35).

Recently, the cloning of the second major member of the IkB family, IkB-β, has allowed molecular characterization of the interaction of this isoform with NF-κB (29). In contrast to IkB-α, the IkB-β gene is not induced upon NF-κB activation, presumably due to lack of b1-binding sites in its promoter region (29). Instead, alternative signals, not yet well characterized, lead to increased IkB-β transcription and de novo expression of hypophosphorylated IkB-β. Non-phosphorylated IkB-β can bind to NFκB but does not mask its nuclear localization site nor transcription activation domain. NFκB-IkB-β complexes can enter the nucleus and exhibit transcriptional activity, and it has been proposed that this mechanism is responsible for persistent NFκB activation (37–40). Phosphorylation of IkB-β at its carboxyl-terminal PEST domain (41) converts IkB-β to an inhibitor molecule similar in properties to IkB-α. Thus, the phosphorylation state of IkB-β dictates its role as an inhibitory versus a stimulatory molecule. It is not yet clear what maintains the hypophosphorylated state of newly made IkB-β during persistent activation, but it has been suggested that LPS and IL-1β induce an IkB-β-specific phosphatase (38).

We have previously used a nonspecific serine protease inhibitor (TPCK) of the 26 S proteasome, as well as the more selective peptide aldehyde inhibitor benzylxoxy carbonyl-Ile-Glu (O-t-butyI)-Ala-leucinal (ZIE), to investigate NFκB activation in astrocytes and C6 cells (22). ZIE inhibited NOS2 induction only when present during the initial times of incubation with NOS2 inducers, whereas TPCK inhibited NOS2 expression regardless of the presence of NOS2 inducers and lactacystin, relative to the activity of control cells (incubated in media alone).

The preparation of astrocyte cultures—C6 glioma and human U172 astrocyoma cells were grown in DMEM containing 10% FCS and antibiotics (penicillin and streptomycin). Cells were passaged once a week and used for 3–4 days at which point they were 90–95% confluent. Primary astrocytes were from cerebral cortices of postnatal day 1 Harlan Sprague Dawley rats prepared in 96-well plates as described previously (13). Media were changed every 3 days. After 2 weeks growth in complete media (DMEM with 10% FCS) the cultures consisted of 95–98% astrocytes and 2–3% microglia.

NOS2 Induction and Activity Assay—NOS2 expression was induced in C6 cells by overnight incubation in DMEM containing 1% FCS and NO2 inducers as indicated (final concentrations of inducers were as follows: IL-1β, 100 unit/ml; IFN-γ, 200 unit/ml; TNF-α, 25 unit/ml; LPS, 10 μg/ml). NOS2 activity was assayed in primary astrocyte cultures in DMEM containing 1% FCS and LPS. After 24 h, NOS2 induction was assessed indirectly by accumulation of nitrite in the culture media. An aliquot of media (100 μl) was mixed with 50 μl of Griess reagent (48), incubated at room temperature for 10 min, and then the absorbance at 546 nm determined. Solutions of NaNO2 served as standards. Background nitrite accumulation in unstimulated cells (cells cultured overnight in the absence of cytokines or LPS) was not significantly different from nitrite accumulation measured in media alone.

Preparation of Stable Transfected C6 Cell Lines—A 2,168-bp fragment of the rat NOS2 promoter was amplified from Harlan Sprague Dawley liver genomic DNA by PCR using primers derived from the published rat NOS2 promoter sequence (49) (forward, 5′-GAG ATG TCC ACA CAA CAA-3′, corresponding to bases 1108–1127; reverse, 5′-GTA CCA GTC CCC TCA CCA A-3′, corresponding to bases 3259–3277), and its identity was confirmed by DNA sequence analysis. Briefly, the 2.2-kb fragment was ligated into the pGL3 basic luciferase vector (Promega, Madison, WI) and co-transfected into C6 cells with plasmid pCMV-Neo (containing a neomycin resistance gene) using Lipofectamine (Life Technologies, Inc.). After 2 days, stable transfectants were selected by growth in 0.4 mg/ml G418 (Sigma). The resulting cell line, C6-2.2, has a low level of basal luciferase activity, which can be induced between 4- and 10-fold upon incubation with LPS plus IFN-γ.

 Luciferase Assays—Cells were incubated with the indicated NOS2 inducers in DMEM containing 1% FCS and the indicated concentrations of lactacystin. After varying the incubation times, the media were removed, and the cells were washed once with cold phosphate-buffered saline. To prepare lysates, 50 μl of CHAPS buffer (10 mM CHAPS, 10 mM Tris, pH 7.4) were added to each well, the plate frozen at −80 °C, thawed, and shaken on a rotary shaker for 10–15 min at room temperature. Aliquots of cell lysates (10–20 μl) containing equal amounts of protein (∼20 μg) were placed into wells of an opaque white 96-well microplate. An equal volume of luciferase substrate (Steady Glo reagent, Promega) was added to all samples, and the luminescence was measured in a microplate luminometer (Roys-Anthos, Austria). The data are presented as the percentage of luciferase activity measured in the presence of NOS2 inducers and lactacystin, relative to the activity of control cells (incubated in media alone).

Preparation of Nuclear Extracts—Nuclear extracts were prepared from C6 cells as described previously (33). Following stimulation with NO2 inducers, cells were washed in cold phosphate-buffered saline, pelleted, and resuspended in hypotonic buffer (10 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM KCl, 1 mM PMSF) and protease inhibitors (10 μg/ml aprotinin and leupeptin, 100 μM TPCK, 1 mM PMSF). After 15 min on ice, Nonidet P-40 was added to a final concentration of 0.6%, and the lysates were incubated for 5 min and then subjected for 15 min at 12,000 × g to pellet the nuclei. The nuclei were washed once in the same buffer by gentle resuspension and centrifugation. Nuclear extracts were prepared by extraction of the nuclear pellet for 15 min at 4°C in 50–100 μl of 400 mM NaCl, 10 mM HEPES, pH 7.9, 1 mM EDTA, 2% Y. Gavrilivuk and D. L. Feinstein, unpublished results.
were resuspended directly into 5 volumes of 8 M urea. Aliquots were frozen at −80 °C, and 5% CO2. Following incubation, the cells were returned to humidity, and 5% CO2. Incubation was initiated by a hot start method, and conditions were 35 cycles of denaturation at 94 °C, annealing at 56 °C, and extension at 72 °C. The PCR products were electrophoresed for 2.5 h at 200 V in a cold box, after which the gels were dried and exposed to x-ray film at room temperature for up to 2 days.

For competition assays, the binding reaction was carried out in binding buffer (50 mM NaCl, 200 ng of poly(dI-dC), 1 mM DTT, 5% glycerol, 10 mM HEPES, pH 7.9, 1 mM FMSF, 50 μg/ml aprotinin) containing 20–50,000 cpm of 32P probe. After 20 min incubation at room temperature, the samples were applied to non-denaturing polyacrylamide gels that have been pre-run for 1 h at 100 V at 4 °C. The running buffer and gel buffer were both 0.5 M Tris-Cl, pH 6.8, 0.2% SDS, 10% β-mercaptoethanol, 10 mM EDTA, 50% glycerol and boiled for 5 min. Protein samples (approximately 20 μg) were separated through SDS-10% polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes by semi-dry electrophoretic transfer. The membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 (TBST), and 5% dry milk (1 h), rinsed, and incubated with primary antibodies in TBST containing 0.5% bovine serum albumin overnight with gentle shaking at 4 °C. The primary antibody was removed, membranes washed 4 times in TBST, and 0.1 μg/ml peroxidase-labeled goat secondary antibodies added for 1 h. Following 4 washes in TBST, bands were visualized by incubation in enhanced chemiluminescence reagents and exposure to x-ray film. Quantitative assessment of band intensities was obtained by imaging of autoradiographs with an Alpha Innotech (Temecula, CA) Imaging 2000 system. Band intensities were determined from autoradiographs with an Alpha Innotech (Temecula, CA) Imaging 2000 system. Band intensities were determined from autoradiographs obtained from at least two different exposure times, and background intensities (determined from an equal-sized area of the film immediately below the band of interest) were subtracted.

For in vitro phosphorylation using either 1B-β or a aliquot of whole cell lysate was diluted 5-fold to reduce urea concentration, calf intestine alkaline phosphatase (CIP) buffer added, and samples incubated for 30 min at 37 °C with 10 units of CIP. Samples were separated by SDS-10% polyacrylamide electrophoresis gels that also contained 4 μM urea, which we found increases band resolution. Gels were run at 50 V for approximately 1 h with 16 changes of the running buffer occurring. Bands were visualized by autoradiography.

RT-PCR Analysis of NOS2 mRNA—Total cytoplasmic RNA was prepared from cells by the Nonidet P-40 lysis procedure, and levels of NOS2 mRNA were estimated by competitive RT-PCR assay (50). The primers used for NOS2 detection were 1704F (5'-CAAGT TACG GCCAT CACCA AAG) and 1065R (5'-TCCAG GGGATT TGTGGCAA T) which yield a 230-bp product. PCR was done in the presence of 2 μg of a 180-bp NOS2 C5 (50) which is amplified with the same efficiency as the cDNA template. The mRNA levels of glyceraldehyde-3-phosphate dehydrogenase were determined in parallel aliquots of cDNA, with primers 796F (5'-GGCTACGATTGAGATGAAAG) and 1058R (5'-TCCAGGGATT TGTGGCAA T) which yield a 264-bp product. PCR was initiated by a hot start method, and cycles were 35 cycles of denaturation at 93 °C for 35 s; annealing at 63 °C for 45 s; and extension at 72 °C for 45 s; followed by 10 min at 72 °C done in a Hybaid Thermoblotter (Franklin, MA) controlled by tube temperature. PCR products were separated by electrophoresis through 2% agarose gels containing 0.1 μg/ml ethidium bromide. Band intensities were determined using the Alpha Innotech 2000 software.

RESULTS

Biphasic Effects of Proteasome Inhibitors on Nitrite Production—As previously reported (13), incubation of primary cultures of rat astrocytes with LPS induced NOS2 expression as indicated by the accumulation of nitrites, a stable metabolite of NO, in the cell culture media (Fig. 1A). Simultaneous incubation with the selective proteasome inhibitor ZIE (23) reduced the LPS-dependent nitrite production when present at concentrations of 30 nM and above, with complete inhibition observed at concentrations of 200 nM and above (not shown). This is similar to the reported inhibition of NOS2 expression by ZIE in C6 cells (22) and in smooth muscle cells (23) where ZIE was used at micromolar concentrations. We reproducibly noticed that at lower concentrations of ZIE, there was no inhibition observed, and in some cases there was an increase in the nitrites produced. Although the increase in astrocyte nitrite production did not reach statistical significance, there was a tendency toward increases at ZIE concentrations near 7.5 nM. Since ZIE can inhibit the activity of other proteases (43, 44), we tested the highly specific proteasome inhibitor lactacytin that does not cross-react with any other protease tested (44). As the case with ZIE, inhibition of astroglial nitrite production by lactacytin followed a biphasic pattern. Decreased nitrite levels were observed at concentrations of 1.85 μM, with complete inhibition at concentrations of 15.3 μM and above. At lower doses of lactacytin, the reaction was again vector dependent to increase in nitrite levels, with a statistically significant increase (220 ± 25% of control values, n = 4) observed at roughly 800 nM (p < 0.05 versus control cells). These results confirm that NOS2-dependent nitrite production in astrocytes requires proteasome activation, as previously concluded using less specific proteasome inhibitors, and suggest that low doses of proteasome inhibitors...
inhibitors potentiate, rather than inhibit, NOS2 expression.

Incubation of rat C6 glioma cells with LPS plus IFN results in expression of the same NOS2 as astrocytes (17, 18) (Fig. 1B). As observed in primary astrocytes, both ZIE and lactacystin inhibited C6 cell NOS2 expression in a dose-dependent manner. C6 nitrite production was significantly reduced versus control cells at ZIE concentrations greater than 40 nM and at lactacystin concentrations greater than 3.4 μM. At lower doses of either of these inhibitors there was an increase in nitrite production compared with control cells. The maximum increase in nitrite production over control levels was seen at 7.5 nM ZIE (40–60% increase) and between 100 and 800 nM lactacystin (approximately 60–70% increase versus control cells). These results confirm that low doses of proteasome inhibitors cause a paradoxical increase rather than decrease in NOS2 expression.

To confirm that these effects were not species-specific, we tested the effects of lactacystin on NOS2 expression in human astrocytoma U172 cells (Fig. 1C). In these cells, NOS2 is maximally induced by the cytokine combination of IFN and IL-1β (64). As found for both primary astrocytes and C6 cells, cytokine-dependent nitrite accumulation was potentiated in these cells at the lower doses (80–300 nM) lactacystin, and inhibited at higher doses.

To determine if lactacystin needed to be added simultaneously with NOS2 inducers, we incubated C6 cells with inhibitory amounts of lactacystin either at the same time or up to 6 h after addition of the NOS2 inducers (Fig. 2). Delaying lactacystin addition until 3 h after LPS and IFN greatly decreased its inhibitory capacity, while delaying until 6 h after NOS2 inducers reversed its effects from inhibitory to stimulatory. These results suggest that the inhibitory action of lactacystin is mediated at an early event during NOS2 induction, whereas the potentiating effect involves later events.

**Effect of Lactacystin on NOS2 Expression**—To determine if changes in nitrite production were accompanied by changes in NOS2 expression, we measured NOS2 mRNA levels following incubation with lactacystin (Fig. 3). As previously shown (17), incubation of C6 cells with LPS and IFN for 4 h led to the
appearance of NOS2 mRNA. After 24 h of incubation in LPS and IFN, NOS2 mRNA levels were reduced versus levels observed at 4 h. Estimation of mRNA levels by comparison to an internal competitive standard revealed a 4–5-fold reduction in NOS2 mRNA levels occurring between 4 and 24 h. In the presence of 1 μM lactacystin, NOS2 mRNA levels at 4 h were much lower than control levels; however, those levels were only slightly reduced (10–20%) after 24 h of incubation. Incubation with 10 μM lactacystin greatly reduced the LPS/cytokine-dependent NOS2 mRNA accumulation at 4 h, and this was further reduced after 24 h. In the same samples levels of glyceraldehyde-3-phosphate dehydrogenase mRNA were not reduced by lactacystin after 4 h and only slightly (less than 10%) reduced after 24 h. These results demonstrate that lactacystin inhibits initial NOS2 mRNA accumulation and that low, but not high, doses prevent the subsequent decrease in mRNA levels normally seen. However, whether this is due to increased NOS2 mRNA stability and/or increased NOS2 gene transcription is not yet known (but see below, Fig. 4).

Since changes in NOS2 mRNA levels could be due to alteration of NOS2 promoter activity, we tested the effects of lactacystin on activation of a 2.2-kb fragment of the rat NOS2 promoter attached to the luciferase reporter gene and stably transfected into C6 cells (C6–2.2 cells, Fig. 4). In these cells, the basal level of luciferase activity was increased 4-fold after 6 h with LPS plus IFN. The presence of lactacystin during this 6-h period caused a dose-dependent decrease in luciferase activity, indicating that lactacystin decreases initial LPS plus IFN-dependent NOS2 promoter activation, with similar potency to the inhibition of nitrite production seen in astrocytes or C6 cells. After 20 h incubation with LPS plus IFN, the NOS2 promoter was activated roughly 3-fold compared with basal activity (i.e. 20 h in the presence of media alone). In contrast to results obtained after 6 h, co-incubation with lactacystin during the 20-h incubation did not inhibit promoter activation except at the highest concentration tested (27 μM). At lower concentrations, there was a tendency to increase promoter activation, with the increases at 3.4 and 6.8 μM being statistically different from control values. These results suggest that increased nitrite production at low doses of lactacystin are due to a delayed increase in NOS2 promoter activation occurring at least 6 h after initial addition of the NOS2 inducers.

**Biphasic Effects of Lactacystin on NFκB Activation**—We have previously shown that in glial cells, as in other cell types, transcription from the NOS2 promoter is mediated by activation of transcription factor NFκB (33, 51). We used EMSAs to determine if changes in NFκB activation correlated with the observed dual effects of lactacystin (Fig. 5). A 5-h incubation with LPS and IFN led to appearance of two major DNA-protein complexes (labeled III and IV), and the levels of both were decreased, but still present, after 24 h of incubation (Fig. 5A). In the presence of 1 μM lactacystin, the levels of both these complexes were decreased at 5 h compared with control cells. However, after 24 h of incubation the level of complex III was greatly increased, whereas complex IV was further decreased. At a higher concentration of lactacystin (10 μM), formation of complex III was completely abrogated after 5 h of incubation, whereas levels of complex IV were reduced but still present. After 24 h of incubation, complex III was once again detectable. The composition of NFκB present in complexes III and IV were determined by supershift EMSAs (Fig. 5B). Incubation with LPS and IFN for 5 h led to the appearance of complexes III and IV, as well as several minor bands (complexes I, II, and V), the formation of all could be blocked by co-incubation with excess κB oligonucleotide but not mutated κB oligonucleotide. Supershift EMSAs indicated that complex III contained both p50 and p65 subunits, whereas complex IV did not contain p50 but did contain p65. These results demonstrate that lactacystin inhibi-
tracts were prepared from C6 cells incubated with 1 μg/ml LPS plus 20 units/ml IFN-γ and the indicated concentrations of lactacystin. Aliquots (containing 2 μg of protein) were examined by EMSA for formation of NFκB DNA-protein complexes. A, nuclear extracts were prepared after 0-, 1-, 5-, or 24-h incubations. Two major complexes, III and IV, were detected. The gel shown is representative of two separate experiments. B, the NFκB subunits present in complexes III and IV were determined by supershift EMSA. Extracts were prepared from cells treated for 5 h with LPS and IFN and preincubated with antibodies specific to NFκB subunits p50 or p65. Specific and nonspecific complex formation was determined by incubation with 100-fold excess of sense or mutant NFκB oligonucleotides. Additional minor complexes (I, II, and V) are indicated.

**Fig. 5. Effect of lactacystin on NFκB activation.** Nuclear extracts were prepared from C6 cells incubated with 1 μg/ml LPS plus 20 units/ml IFN-γ and the indicated concentrations of lactacystin. Aliquots (containing 2 μg of protein) were examined by EMSA for formation of NFκB DNA-protein complexes. A, nuclear extracts were prepared after 0-, 1-, 5-, or 24-h incubations. Two major complexes, III and IV, were detected. The gel shown is representative of two separate experiments. B, the NFκB subunits present in complexes III and IV were determined by supershift EMSA. Extracts were prepared from cells treated for 5 h with LPS and IFN and preincubated with antibodies specific to NFκB subunits p50 or p65. Specific and nonspecific complex formation was determined by incubation with 100-fold excess of sense or mutant NFκB oligonucleotides. Additional minor complexes (I, II, and V) are indicated.

Effects of Lactacystin on NFκB Levels—Since NFκB activation requires degradation of inhibitory IκB proteins, we examined the effects of lactacystin on IκB-α and IκB-β protein levels (Fig. 6). C6 cells displayed a basal level of both IκB-α and -β. After a 1-h incubation with LPS and IFN, the levels of both isoforms were decreased compared with basal levels. In the presence of lactacystin, the loss of both isoforms was reduced when measured at this time point. Re-expression of both IκB proteins was detected at 6 h of incubation with LPS and IFN. However, in the presence of lactacystin the reappearance of IκB-α was reduced, whereas the reappearance of IκB-β was greatly increased. After 24 h of incubation in LPS and IFN, the levels of IκB-β were equal to or greater than initial levels, whereas levels of IκB-α were still reduced compared with zero time values. The presence of lactacystin did not modify IκB-α levels when measured at 24 h, whereas IκB-β levels were increased by lactacystin. These results indicate that lactacystin delayed the degradation of pre-existing IκB proteins. Additionally, incubation with lactacystin led to an increase in IκB-β, but not IκB-α, levels at later times.

The above results demonstrate that lactacystin selectively increased IκB-β expression. Since increases in the levels of hypophosphorylated IκB-β can increase and prolong NFκB activation, we determined the effects of lactacystin on the phosphorylation state of IκB-β (Fig. 7). As previously shown (39), alkaline phosphatase treatment of cell extracts to remove phosphoryl groups resulted in increased electrophoretic mobility of IκB-β, confirming that non-phosphorylated IκB-β migrates faster than phosphorylated forms and indicating that in quiescent C6 cells the majority of IκB-β was phosphorylated. Following incubation with LPS and IFN, the levels of IκB-β decreased in a time-dependent manner, and by 4 h a hypophosphorylated form having increased mobility was apparent. This form was still present at 6 h but not increased versus 4 h. In the presence of lactacystin, the loss of IκB-β was attenuated, and a hypophosphorylated, faster migrating species could be detected as early as 1 h after incubation. Between 2 and 6 h of incubation, the IκB-β in the lactacytin-treated cells was primarily non-phosphorylated, and overall levels were greater than those seen in the non-treated cells. These results indicate that lactacystin promoted the re-expression of hypophosphorylated IκB-β at earlier times and at greater levels than did LPS plus IFN alone.

To determine if overexpression of IκB-β could reverse the effects of lactacystin, we transfected C6 cells with a cDNA encoding full-length rat IκB-β or a truncated IκB-β lacking the carboxyl-terminal PEST site and casein kinase II phosphoryl-
NOS2 expression and examine further the mechanism by which lactacystin modifies the increase in mobility of the reacting proteins (gested that lactacystin might be directly increasing and IFN-γ-nated and IFN-γ promoter induction due to co-incubation with lactacystin incubation with LPS and IFN. Similarly, the augmentation of pendently inhibited the induction of the NOS2 promoter upon incubation with cells with empty vector alone (up to 1 μg/ml) had no effect on NOS2 promoter activation. After 24 h, 1 μg/ml LPS plus 20 units/ml IFN-γ was added to the cells, and NOS2 promoter activation was measured 24 h later. The fold activation by LPS and IFN-γ was 2.0-fold over basal levels (slightly less than the 3-fold shown in Fig. 4) and is given as 100%. The results shown are from one of three independent experiments, each done in duplicate or triplicate, and the variability between experiments was less than 10%.

In extracts from the same cells, levels of B- were increased by lactacystin, as well as by the combination of LPS/IFN and casein kinase II phosphorylation sites. In Fig. 10, HSP70 was detected at early times may contribute to inhibition by lactacystin after 6 and 24 h of incubation but not after 1 h. Higher migrating forms of B- can be seen after 4 h in the upper panel and after 2 h in the bottom panel. The presence of an intermediate migrating form after 1 h in the bottom panel may reflect an additional phosphorylated state of B-. As in A, the mobilities of minor cross-reacting proteins (arrowheads) did not increase with time, confirming the increase in mobility of the B- bands.

To examine further the mechanism by which lactacystin modifies NOS2 expression and B- expression, we tested lactacystin effects on heat shock protein (HSP) expression, since it has been shown that proteasome inhibitors can promote HSP expression (45, 52-54) and since HSP70 can suppress NOS2 expression (22, 51, 55, 56). C6 cells were incubated with an inhibitory dose of lactacystin (6.5 μM), and cell lysates were analyzed by Western blots for the presence of HSP70 after different periods of incubation (Fig. 10). HSP70 was detected at low levels in control cells, as well as in cells incubated for up to 1 h with lactacystin. However, after 6 h of incubation, cells treated with lactacystin expressed easily detectable HSP70 levels. Incubation with NOS2 inducers alone did not induce HSP70 expression, although the combination of lactacystin with LPS/IFN induced a higher level of HSP70 than lactacystin alone. After 20 h incubation, HSP70 levels were further increased by lactacystin, as well as by the combination of LPS/IFN plus lactacystin. These results demonstrate that lactacystin induces HSP70 expression in glial cells and suggests that HSP70 expression at early times may contribute to inhibition...
of NOS2 expression. IxB-β is a Heat Shock Protein—The observation that lactacystin induces a HSR in glial cells, as well as induces IxB-β expression, suggested to us that IxB-β could be responding to the HSR. To test this hypothesis, we examined IxB-α and IxB-β levels in C6 cells after thermal induction of a HSR (Fig. 11). As previously reported (51), 20 min of hyperthermia induced IxB-α expression when measured 4 h after heat shock. In the same cells, we found that a 20-min heat shock also increased IxB-β expression. These results demonstrate that IxB-β, as is the case for IxB-α, is induced by the HSR suggesting that IxB-β is also a stress protein.

**DISCUSSION**

Although NOS2 expression in astrocytes and glial cells requires NFκB activation, the precise role of distinct members of the IκB family in mediating glial NFκB activation and thus NOS2 expression has not been fully characterized. In the current study we have extended our previous results showing that inhibition of the 26 S proteasome inhibits astroglial and C6 cell NOS2 expression (22). We have found that incubation with low doses of the highly specific proteasome inhibitor lactacystin led to a paradoxical increase, rather than decrease, in NOS2 expression. This conclusion is supported by our observations that low doses of lactacystin increased nitrite accumulation, NOS2 mRNA levels, and NFκB activation when measured after 24 h of incubation. That the potentiating effect is mediated by a delayed event, and not by an early event in NOS2 expression, is further supported by the observation that high doses of lactacystin added after NOS2 inducers also led to an increase in nitrite accumulation. Finally, examination of the degradation and re-expression patterns of IxB-α and IxB-β suggests that lactacystin may be potentiating NOS2 expression due to a reduction in the re-expression of IxB-α and/or an increase in the re-expression of IxB-β (Fig. 6), both of which could contribute to an overall increase in the levels of sustained NFκB activity. The finding (Fig. 7) that lactacystin increased expression of a hypophosphorylated IxB-β suggests that non-phosphorylated IxB-β contributes to increased NFκB activation and NOS2 expression. These findings suggest that changes in the relative levels of IxB proteins dictate the overall level and duration of NFκB activation, which can result in either inhibitory or stimulatory effects, and point out that using proteasome inhibitors to block inflammatory gene activation could, if added subsequent to initial inflammatory activation, result in stimulatory rather than inhibitory actions.

Our results showing NOS2 promoter activation (Fig. 4) and NFκB activation (Fig. 5B) at 20–24 h after the addition of inflammatory stimuli indicate that stimulation of glial cells with LPS and IFN leads to long lasting activation of NFκB. Sustained NFκB activation has previously been suggested to be necessary for macrophage NOS2 activation since inhibition of delayed but not initial NFκB activation decreased LPS-dependent NOS2 expression (26). These observations are consistent with the findings that NFκB activation due to stimulation with LPS (or IL1-β), in contrast to other inducers such as tumor necrosis factor–α or phorbol 12-myristate 13-acetate, results in a persistent activation rather than a rapid transient response (29, 39). The kinetics of rapid, transient NFκB activation are such that by 3–5 h after inducer addition, there remains little or no active NFκB in the nucleus as determined by electrophoretic mobility shift assays (EMSA). In contrast, incubation of cells with LPS or IL1-β results in “persistent” NFκB activation, characterized by a slow, gradual diminution of active NFκB over the course of 24–48 h. We therefore conclude that glial NOS2 induction involves the sustained activation of NFκB.

Until recently, it has not been clear how the known regulation of IxB-α could account for sustained NFκB activation. Following initial NFκB activation, newly synthesized IxB-α translocates to the nucleus and binds to existing NFκB-DNA complexes causing repression of ongoing transcription, dissociation of NFκB from DNA, and export of inactive NFκB/IκB complex back to the cytoplasm. The net effect is rapid abrogation of transcriptional activity. Observations made in cells that are characterized by constitutive NFκB activation (B-cells, human immunodeficiency virus-infected cells, and cancer cells) suggested that differences in the levels and stability among IκB family members could strongly influence the overall response to inflammatory stimuli, and suggested the IxB-β protein as a dual positive/negative regulator of NFκB activity. Following cloning of IxB-β (29), it was discovered that during persistent but not transient NFκB activation, there is re-expression of IxB-β. Whereas in some cases newly IxB-β is rapidly phosphorylated at its carboxyl terminus, and functions like IxB-α to inhibit NFκB activation (57), further studies demonstrated that during persistent activation, newly synthesized IxB-β is not phosphorylated. Instead, hypophosphorylated IxB-β associates with NFκB (and prevents NFκB association with inhibitory IxB-α) and acts as a chaperone to bring NFκB to the nucleus where it is transcriptionally active (29, 37, 38, 40, 57). Thus, the phosphorylation state of IxB-β dictates its role as an inhibitory versus a stimulatory molecule. Our results are consistent with this model, since treatment of cells with LPS and IFN led to a re-expression of predominantly hypophosphorylated IxB-β, whose expression was further increased by lactacystin, providing evidence that glial NOS2 expression involves persistent NFκB activation.

It is not yet clear what are the signals causing re-expression of IxB-β, since in contrast to IxB-α, IxB-β does not respond to NFκB activation. However, several reports indicate that LPS and certain cytokines, which induce IxB-β expression, induce a HSR both in vivo and in vitro (58, 59), suggesting that IxB-β may be responding as a stress protein. In our studies, incubation of cells with lactacystin, in the absence of LPS or cytokines, induced the expression of HSP70, consistent with previous observations that proteasome inhibitors induce a HSR (42, 45, 52–54). In the same cells, we also observed that lactacystin alone induced IxB-β expression. In view of the findings that IxB-α is an HSP (47), the above considerations suggest that IxB-β is also a stress protein and that increased IxB-β expression by lactacystin is due to establishment of an HSR. Our findings that brief hyperthermia-induced IxB-β expression is consistent with the possibility that IxB-β is an HSP. However it is not yet known whether or not the promoter region of IxB-β contains a heat shock element, which can be identified in the promoter region of IxB-α (60). We suggest that in cells stimulated with LPS and cytokines, lactacystin enhances the HSR.
initiated by LPS thus leading to an increase in IkB–β expression. The mechanism by which lactacystin, or other proteasome inhibitors, can induce an HSR is not clear; however, it has been suggested that the proteasome constitutively degrades a protein which otherwise would activate heat shock factors (61).

Our results suggest that the re-expression of both IkB–α and IkB–β is critical to determine the duration and magnitude of NO2S expression, and that inhibition of the proteasome by lactacystin exerts multiple effects that can lead to suppression or augmentation of overall NFκB activation and NO2S expression. To explain the dual effects, we propose the following model. In the absence of lactacystin, incubation of C6 cells with a combination of LPS plus IFN causes the rapid loss of pre-expressed NFκB mediated by increases in IkB which would be administered to an already existing inflammatory condition that could result in exacerbation, rather than attenuation, of the response.

Acknowledgment—We thank Dr. Elena Galea for critical reading of this manuscript.

REFERENCES

1. Licinio, J., Prolo, P., McCann, S. M., and Wong, M. L. (1999) Mol. Med. Today 5, 225–232
2. DeGroot, C. A., Ruuls, S. R., Theeuwes, J. M., Dijkstra, C. D., and VanderValk, P. (1997) J. Neurophysiol. Exp. Neurol. 56, 10–20
3. Bagasra, O., Michaels, P. H., Zheng, Y. M., Hoberniki, L. E., Spitsin, S. V., Fu, Z. F., Tawadrois, R., and Koprowski, H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12041–12045
4. Be L., Dawson, T. M., Wesselingh, S., Molk, S., Cot, J., Kong, P. A., Hanley, and Trapp, B. D. (1994) Ann. Neurol. 36, 778–786
5. Endoh, M., Maiese, K., and Wagner, J. (1994) Brain Res. 651, 92–100
6. Wallace, M. N., and bisland, S. K. (1994) Neurology (Baltimore) 49, 905–919
7. Wu, K., Park, J. K., Cho, C. M., Winnie, H. M., and Kim, Y. S. (1998) J. Neurosci. 4, 445–450
8. Wada, K., Chatzizanetakis, K., Kravdieh, S., Busto, R., and Dietrich, W. D. (1998) Neurology (Baltimore) 43, 1427–1436
9. Wallace, M. N., Geddes, J. G., Purpasis, D. A., and Masson, M. R. (1997) Exp. Neurol. 144, 266–272
10. Hori, K., Burd, P. R., Furuke, K., Kuts, J., Wei, H. A., and Clouse, K. A. (1999) Blood 93, 1843–1850
11. Cross, A. H., Micco, T. P., Lin, R. F., Hickey, W. F., Trotter, J. L., and Tilton, R. G. (1994) J. Clin. Invest. 93, 2684–2690
12. Iadecola, C. (1997 Trends Neurosci. 20, 132–139
13. Gaies, K., Feinstein, D. L., and Reis, D. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10945–10949
14. Simmons, M. L., and Murphy, S. (1993) Eur. J. Neurosci. 5, 825–831
15. Doorn, S., Corbett, J. A., McDaniel, M. L., and Choi, D. W. (1999) Neurosci. Lett. 164, 229–232
16. Lee, S. C., Dickson, D. W., Liu, W., and Bronson, C. F. (1993) J. Neuroimmunol. 46, 19–24
17. Feinstein, D. L., Galea, E., Roberts, S., Berquist, H., Wang, H., and Reis, D. J. (1994) J. Neurochem. 62, 315–321
18. Simmons, M. L., and Murphy, S. (1992) J. Neurochem. 59, 897–905
19. Baereue, P. A., and Henkel, T. (1996) Adv. Inmunol. 12, 141–179
20. Aman, K. T., Albanese, C., Petelli, D. R., and Van ESh, L. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5795–5800
21. Chau, C. C., Lokkenjord, J. R., Sheng, W. S., Hu, S., and Peterson, P. K. (1997) Neuroreport 8, 3163–3166
22. Feinstein, D. L., Galea, E., and Reis, D. J. (1997) Nitric Oxide 1, 167–176
23. Grisieavage, J. M., Wilk, S., and Ignarro, L. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3308–3312
24. Pahan, K., Raymond, J. R., and Singh, I. (1999) J. Biol. Chem. 274, 7528–7536
25. Murphy, W. J., Muroi, M., Zhang, C. X., Suzuki, T., and Russell, S. W. (1996) J. Endotoxin Res. 3, 381–393
26. Xie, Q. W., Whinast, R., and Nathan, C. (1993) J. Exp. Med. 177, 1779–1784
27. Henkel, T., Machleidte, T., Alkalay, I., Kronke, M., Ben-Neriah, Y., and Baereue, P. A. (1993) Nature 365, 182–185
28. Alkalay, I., Yaron, A., Hatzbaul, A., Orian, A., Chechanoever, A., and Ben-Neriah, Y. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10599–10603
29. Thompson, J. E., Phillips, R. J., Erijdumjndromage, H., Tempst P., and Ghosh, S. (1995) Cell 80, 573–582
30. Traenckner, E. B., Pahl, R., Henkel, T., Schmidt, K. N., Wilk, S., and Baereue, P. A. (1995) EMBO J 14, 2876–2883
31. Scherer, D. C., Brockman, J. A., Chen, Z., Maniatis, T., and Ballard, D. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11259–11263
32. Simenonius, S., Staufer, D. W., Hendrickson, W. A., and Thonos, D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 49–54
33. Feinstein, D. L. (1998) J. Neurochem. 70, 1484–1496
34. Kemler, I., and Fontana, A. (1999) Oit 26, 212–220
35. Fiebich, B. L., Hofer, T. J., Lieb, K. H., Butcher, R. D., Schumann, G., Schulze-Osthoff, K., and Bauer, J. (1999) Neuropharmacology 38, 1325–1333
36. Berg, A. A., and Baldwin, A. S., Jr. (1993) Genes Dev. 7, 2064–2070
37. Attar, R. M., Macdonald-Bravo, H., Ravetos-Suarez, C., Durham, S. K., and Bravo, R. (1998) Mol. Cell. Biol. 18, 477–487
38. Delacasa, C., Petapoulos, L., Zmeureanu, D., and Hiscott, J. (1999) J. Biol. Chem. 274, 13010–13016
39. SuYang, H., Phillips, R., Douglas, I., and Ghosh, S. (1996) Mol. Cell. Biol. 16, 5444–5449
40. Johnson, D. R., Douglas, I., Jahnke, A., Ghosh, S., and Pober, J. S. (1996) J. Biol. Chem. 271, 16137–16132
41. Chu, Z. L., Kinsey, T. A., Liu, L., Qi, X., and Ballard, D. W. (1996) Mol. Cell. Biol. 16, 5974–5984
42. Zhou, M., Xu, W., and Gibson, H. N. (1996) J. Biol. Chem. 271, 24769–24775
43. Mellgren, R. L. (1997) J. Biol. Chem. 272, 29899–29903
44. Fenteany, G., and Schreiber, R. L. (1998) J. Biol. Chem. 273, 8545–8548
45. Kim, D., Kim, S. H., and Li, G. C. (1999) Biochem. Biophys. Res. Commun. 254, 264–268
46. Wong, H. R., Ryan, M., and Wispe, J. R. (1997) Biochem. Biophys. Res. Commun. 231, 257–263
47. Wong, H. R., Ryan, M., and Wispe, J. R. (1999) Cell Stress Chaperones 4, 1–7
48. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnick, J. S., and Tannpahnbaum, S. R. (1982) Anal. Biochem. 126, 131–138
49. Zhang, H., Chen, X., Teng, X., Snead, C., and Catravas, J. D. (1998) Biochem. Pharmacol. 55, 1873–1880
50. Galea, E., Reis, D. J., and Feinstein, D. L. (1994) J. Neurosci. Res. 37, 406–414
51. Feinstein, D. L., Galea, E., Aquino, D. A., Li, G. C., Xu, H., and Reis, D. J. (1996) J. Biol. Chem. 271, 17724–17732
52. Rossi, A., Elia, G., and Santoro, M. G. (1998) J. Biol. Chem. 273, 16446–16452
53. Bush, K. T., Goldberg, A. L., and Nigam, S. K. (1997) J. Biol. Chem. 272, 9986–9992
54. Lee, D. H., and Goldberg, A. L. (1998) Trends Cell Biol. 8, 397–403
55. de Vera, M. E., Wong, J. M., Zhou, J. Y., Tseng, E., Wong, H. R., Billiar, T. R., and Geller, D. A. (1996) Surgery 120, 144–149
56. Scarim, A. L., Heitmeier, M. R., and Corbett, J. A. (1998) Endocrinology 139, 5050–5057
57. Velasco, M., Díaz-Guerra, M. J., Martin-Sanz, P., Alvarez, A., and Bosca, L. (1997) J. Biol. Chem. 272, 23025–23030
58. Hao, W., Myhre, A. P., and Palmer, J. P. (1999) Autoimmunity 29, 93–101
59. Meng, X., Brown, J. M., Ao, L., Nordeen, S. K., Franklin, W., Harken, A. H., and Banerjee, A. (1996) Am. J. Physiol. 271, C1316–C1324
60. DeMeester, S. L., Buchman, T. G., Qiu, Y., Jacob, A. K., Dunnigan, K., Hotchkiss, R. S., Karl, I., and Cobb, J. P. (1997) Arch. Surg. 132, 1283–1287
61. Mathew, A., Mathur, S. K., and Morimoto, R. I. (1998) Mol. Cell. Biol. 18, 5091–5098
62. Lin, K. I., Baraban, J. M., and Ratan, R. R. (1998) Cell Death Differ. 5, 577–583
63. Kloetzel, P. M. (1998) Gene Ther. 5, 1297–1298
64. Fujisawa, H., Ogura, T., Hokari, A., Weisz, A., Yamashita, J., and Esumi, H. (1995) J. Neurochem. 64, 85–91