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Norepinephrine Increases IκBα Expression in Astrocytes*

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The neurotransmitter norepinephrine (NE) can inhibit inflammatory gene expression in glial cells; however, the mechanisms involved are not clear. In primary astrocytes, NE dose-dependently increased the expression of inhibitory IκBα protein accompanied by an increase in steady state levels of IκBα mRNA. Maximal increases were observed at 30–60 min for the mRNA and at 4 h for protein, and these effects were mediated by NE binding to β-adrenergic receptors. NE activated a 1.3-kilobase IκBα promoter transfected into astrocytes or C6 glioma cells, and this activation was prevented by a β-antagonist and by protein kinase A inhibitors but not by an NFκB inhibitor. NE increased IκBα protein in both the cytosolic and the nuclear fractions, suggesting an increase in nuclear uptake of IκBα. IκBα was detected in the frontal cortex of normal adult rats, and its levels were reduced if central NE levels were depleted by lesion of the locus ceruleus. The reduction of brain IκBα levels was paralleled by increased inflammatory responses to lipopolysaccharide. These results demonstrate that IκBα expression is regulated by NE at both transcriptional and post-transcriptional levels, which could contribute to the observed anti-inflammatory properties of NE *in vitro* and *in vivo*.

The activation of inflammatory responses in brain is normally under tight regulation that prevents the accumulation of potentially cytotoxic mediators including cytokines and reactive oxygen species (1–3). It has therefore been suggested that intrinsic mechanisms exist that maintain the brain in a refractory state of inflammatory activation. In primary cultures of rat astrocytes, we showed that neurotransmitter norepinephrine (NE)[1] prevents induction of the inducible form of nitric oxide synthase (NOS2) (4, 5) by bacterial endotoxin lipopolysaccharide (LPS) or by a combination of proinflammatory cytokines (interleukin 1β, tumor necrosis factor α, and interferon γ). Similarly, others show that NE reduces glial expression of pro-inflammatory cytokines including interleukin 1β and tumor necrosis factor α (6–9) and of cell adhesion molecules (13). A similar role for NE in regulating inflammatory events in brain is supported by our recent findings that experimental depletion of brain NE levels by chemical lesion of the locus ceruleus (LC) increases the cortical inflammatory responses to injection of aggregated amyloid β (14). The fact that LC neurons are lost in Alzheimer's disease (15) and that levels of β_2-adrenergic receptor (βARs) are reduced in astrocytes in multiple sclerosis patients (16, 17) suggests that diminished NE levels or perturbations of the NE-signaling system contribute to the neuroinflammation that occurs in these diseases.

The mechanism(s) by which NE reduces inflammatory gene expression is not yet well defined. In astrocytes, we found that NE induced protein binding to a 27-bp region of the rat NOS2 promoter, which is located immediately upstream of a NFκB binding site located at bp position −107 to −96 (18). This 27-bp region contains several potential binding sites for regulatory transcription factors, including CREB and C/EBP, and consistent with this we found that the NE-dependent protein binding to this region was reduced by preincubating nuclear extracts with an antibody to CREB. Those results suggested that the NE-dependent binding of CREB or a closely related protein to this region of the NOS2 promoter suppresses the NFκB-dependent transcriptional activity. However, the means by which transcription factor binding to the 27-bp region can attenuate NFκB-dependent transcription occurring downstream, and whether interactions with inhibitory IκB proteins are involved is not yet known.

The processes leading to NFκB activation are now well characterized (19, 20). In brief, a heterodimeric NFκB complex is maintained in the cytoplasm due to association with an inhibitory IκB protein. The IκB family of proteins contain ankyrin repeats in their carboxyl termini, which bind to and mask the nuclear localization sequence present in the NFκB subunits. After the appropriate cellular stimulation (by cytokines, UV radiation, infection), activation of protein kinases leads to eventual activation of the IκB kinase complex, which in turn phosphorylates IκB proteins at their amino termini. This modification converts IκB into a substrate for ubiquination and subsequent targeting for degradation by the 26 S proteasome. IκB breakdown or dissociation from NFκB reveals the nuclear localization sequence, allowing for nuclear uptake of active NFκB. In our studies using astrocytes, we found that although NE did not reduce NFκB activation (as assessed by nuclear uptake of the p65 subunit and by electrophoretic mobility shift assay), NE prevented the rapid degradation of IκBα that normally occurs upon incubation with LPS or cytokines (18). Furthermore, treatment with NE alone increased basal IκBα pro-

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[1] The abbreviations used are: NE, norepinephrine; C/EBP, CCAAT/enhancer-binding proteins; CREB, cAMP-responsive element binding; DSP4, *N*-(2-chloroethyl)-*N*-ethyl-2 bromobenzylamine; IκB, inhibitor of NFκB; LC, locus ceruleus; LPS, lipopolysaccharide; NOS, NO synthase; NOS2, the inducible form of NOS; NFκB, nuclear factor κB; PKA, protein kinase A; AR, adrenergic receptor; kb, kilobase; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; RT, reverse transcriptase.

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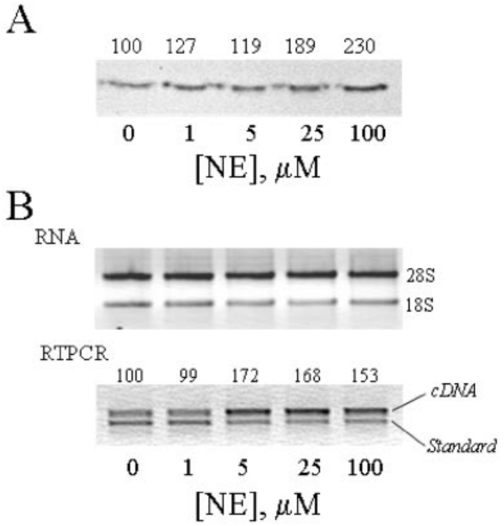

FIG. 1. **NE dose-dependently increases astroglial IκBα expression.** Primary rat astrocytes were incubated in the indicated concentrations of NE. *A*, whole cell lysates were prepared after 24 h of incubation and used for Western blot analysis of IκBα protein. The blot shown is representative of two other experiments, and the average band densities (relative to 100 for non-treated cells) is shown *above* the *lanes*. *B*, cytosolic RNA was prepared after a 2-h incubation and used for RT-PCR analysis of IκBα mRNA. PCR was carried out in the presence of 20 fg of a lower molecular weight internal competitive standard. The gel shown is representative of three separate experiments, and the average ratio of IκBα cDNA to the internal standard product density is shown *above* the *lanes*. The average ratio of cDNA:standard in the control samples was 1.09, which is normalized to 100.

tein expression, suggesting that NE might have direct effects on IκBα expression. Although an increase in IκBα levels might be expected to reduce nuclear uptake of NFκB, the fact that IκB proteins can complex with and inhibit NFκB while associated with DNA within the nucleus (21) suggests that the NE-dependent increase of IκBα exerts inhibitory effects while bound to the NOS2 promoter.

In the present report, we show that in astrocytes NE alone increases IκBα protein and mRNA levels and increases the activation of the IκBα promoter. As found for suppression of NOS2 by NE, these increases were mediated by binding to βARs and blocked by protein kinase A (PKA) inhibitors. Increases in IκBα protein levels were found both in the cytosol as well as in the nucleus, consistent with the possibility that IκBα inhibits NOS2 by binding to NFκB on the NOS2 promoter. Finally, we show that in rats in which brain NE levels were depleted (by chemical lesion of the LC), the cortical levels of IκBα are decreased, and the subsequent induction of inflammatory gene expression is increased. Together, these findings provide evidence that NE (most likely acting via increases in cAMP) directly activates IκBα gene expression and provides a working model to help explain how NE restricts inflammatory events in brain.

MATERIALS AND METHODS

Reagents—Cell culture reagents (Dulbecco's modified Eagle's medium, antibiotics, and LPS (*Salmonella typhimurium*) were from Sigma. Fetal calf serum was from Atlanta Biological (Norcross, GA). Human recombinant interleukin 1β (4×10^6 unit/mg) was obtained from the NIH AIDS reagents program. Recombinant rat interferon γ (4×10^6 unit/mg) and synthetic oligonucleotides were from Invitrogen. Anti-IκBα (SC-371) rabbit polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); horseradish peroxidase-conjugated goat anti-rabbit IgG(H+L) antibodies (#4050-05) were from Fisher. *Taq* polymerase and cDNA reagents were from Promega (Madison, WI) and Invitrogen. A 1.3-kb human IκBα promoter cloned into pGL3 basic (Promega) was a kind gift of Dr. Hector Wong (University of Pittsburgh, PA).

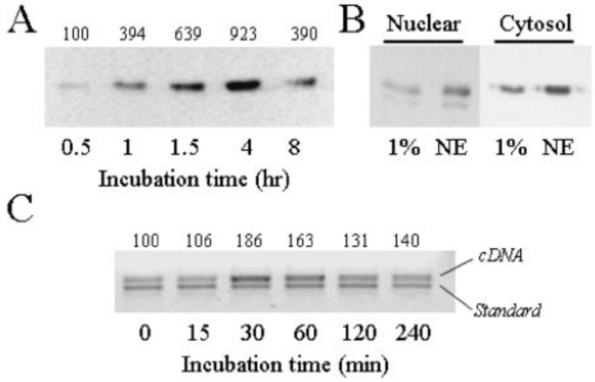

FIG. 2. **Kinetics and subcellular localization of increased IκBα expression.** Astrocytes were incubated with 25 μM NE for the indicated times. *A*, whole cell lysates were prepared, and aliquots were examined by Western blot analysis for levels of IκBα. The blot shown is representative of two other experiments, and the average band densities (relative to 100 for non-treated cells) is shown *above* the *lanes*. *B*, astrocytes were incubated for 24 h with 25 μM NE, after which nuclear and cytosolic fractions were prepared, and equal amounts of protein were analyzed by Western blots for IκBα levels. Similar results were obtained in one other experiment. *C*, RNA was prepared at the indicated times after incubation with 25 μM NE and used for competitive RT-PCR analysis of IκBα mRNA levels. The ratios of the cDNA to the internal standard band densities are presented *above* the *lanes* (and are normalized to 100 for controls, where the ratio was 0.70), and the gel shown is representative of 3 different experiments.

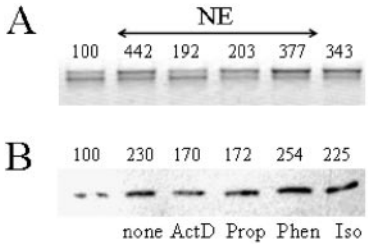

FIG. 3. **Effects of actinomycin D and adrenergic receptor ligands on IκBα expression.** Astrocytes were incubated in 25 μM NE alone, NE with actinomycin D (*ActD*, 5 μg/ml), the βAR antagonist propranolol (*Prop*, 25 μM), or the αAR antagonist phenoxybenzamine (*Phen*, 25 μM), or with the βAR agonist isoproterenol (*Iso*, 25 μM) alone. *A*, RNA samples were prepared after a 4-h incubation and used for competitive RT-PCR analysis of IκBα mRNA levels. The gel shown is representative of two different experiments. The ratios of cDNA to internal standard are shown above and normalized to the control ratio 0.61, which is set to 100. *B*, whole cell lysates were analyzed after a 24-h incubation for levels of IκBα protein. The blot shown is representative of two different experiments, and average band intensities are shown *above* the *lanes*.

Cell Culture—C6 glioma cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics (penicillin and streptomycin). Cells were passaged once a week and used after 3–4 days at which point they were 90–95% confluent. Primary astrocytes were from cerebral cortices of post-natal day 1 Sprague-Dawley rats as previously described (22). Media was changed every 3 days. After 2 weeks of growth in complete media (Dulbecco's modified Eagle's medium with 10% fetal calf serum) the cultures consisted of 95–98% astrocytes and 2–3% microglia.

Production of Stably Transfected Rat C6 Cell Lines—C6 cells or astrocytes were grown until ~40% confluent. Cells were transfected with a 1.3-kb fragment of the human IκBα promoter driving luciferase reporter expression with LipofectAMINE (Invitrogen), according to the manufacturer's recommendations. C6 cells were cotransfected with pSV2Neo vector (Stratagene, La Jolla, CA), containing the neomycin resistance gene, and after 2 days growth, stable cells were selected by growth in 0.8 mg/ml of antibiotic G418.

LC Depletion and Inflammatory Activation—The LC was chemically lesioned as previously described (14). In brief, adult female Sprague-Dawley rats received two intraperitoneal injections (1 week apart) of either *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP4, 5 mg/kg) dissolved in PBS or PBS alone. Four weeks after the second treatment,

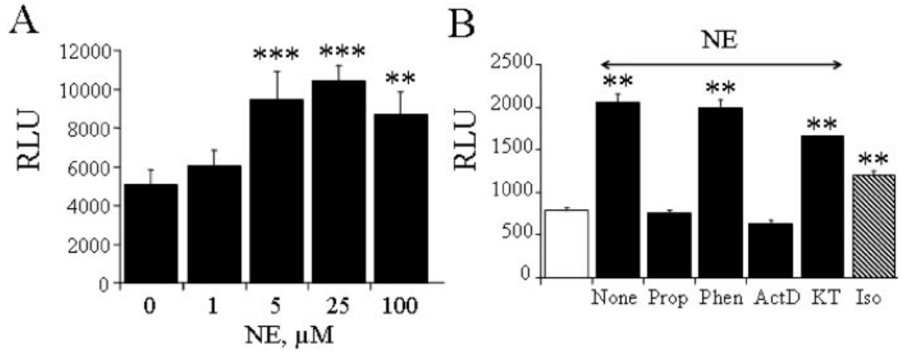

FIG. 4. **Effect of NE on the activation of the IκBα promoter in astrocytes.** Primary astrocytes were transiently transfected with a 1.3-kb fragment of the human IκBα promoter driving luciferase reporter gene expression. One day later, the cells were incubated for 4 h with the indicated concentrations of NE (*A*) or with 25 μM NE alone or together with actinomycin D (*ActD*, 5 μg/ml), the βAR antagonist propranolol (*Prop*, 25 μM), the αAR antagonist phenoxybenzamine (*Phen*, 25 μM), or the PKA inhibitor KT5720 (*KT*, 100 nM) or with isoproterenol alone (*Iso*, 25 μM). The data shown are relative light units (*RLU*) and is the mean ± S.E. of at 3–5 different experiments. **, $p < 0.001$; ***, $p < 0.0001$ *versus* no NE.

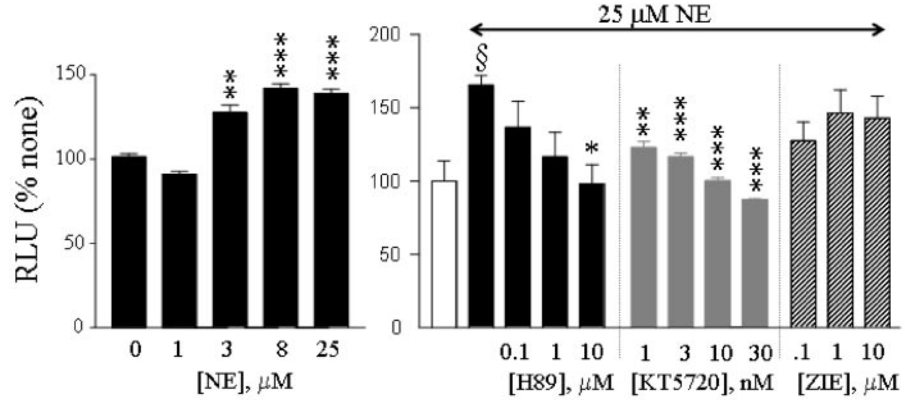

FIG. 5. **Effect of NE on the activation of IκBα promoter in C6 cells.** C6 cells stably transfected with a 1.3-kb fragment of the human IκBα promoter driving luciferase reporter gene expression were incubated with the indicated concentrations of NE for 4 h (*left panel*) or with 25 μM NE in the presence of the indicated concentrations of the PKA inhibitor H89, KT5720, or the NFκB inhibitor ZIE (*right panel*). The data shown are relative light units (*RLU*) produced compared with that of the control cells (no NE) and are the mean ± S.E. of 3–6 independent experiments. In *A*: **, $p < 0.001$; ***, $p < 0.0001$ *versus* no NE; in *B*: *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$ *versus* NE alone; §, $p < 0.0001$ *versus* no NE.

brain inflammation was induced as described (23) by giving animals an intraperitoneal injection of LPS (0 or 1 mg/kg), and 24 h later, protein and RNA samples were prepared from frontal cortices.

Western Blotting Analysis—Cells or tissue samples were lysed in 10 volumes of 8 M urea containing protease inhibitors (Sigma) and sonicated briefly. Aliquots were either frozen at −80 °C or mixed with 5× gel sample buffer (0.5% SDS, 200 mM Tris-HCl, 50 mM EDTA, 50% glycerol) and boiled for 10 min. Protein samples (10 μg) were separated through 10% polyacrylamide gel containing SDS and transferred onto polyvinylidene difluoride membranes by semi-dry electrophoretic transfer. The membranes were blocked with PBS containing 5% dry milk for 1 h. Then the membranes were rinsed with PBS and incubated with primary antibodies in PBS containing 0.05% Tween 20 (PBST) and 0.2% bovine serum albumin overnight at 4 °C. The primary antibody solution was removed, membranes were washed 4 times in PBST, and 0.1 μg/ml peroxidase-labeled goat secondary antibodies was added for 2 h. After 4 washes with PBST, bands were visualized by incubation in enhanced chemiluminescence reagents (Pierce) and exposure to x-ray film.

Preparation of Cell Extracts for Luciferase Measurements—Cells were lysed by addition of CHAPS buffer (10 mM CHAPS, 10 mM Tris, pH 7.4), and the plate was 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 (10–20 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 (Rosys-Anthos).

Preparation of Cytosolic and Nuclear Fractions—Cells were washed in cold PBS, pelleted, and resuspended in hypotonic buffer (10 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol) and protease inhibitors (Sigma). After 15 min on ice, Nonidet P-40 was added to a final concentration of 0.6%, and the lysates were incubated a further 5 min and then centrifuged for 15 min at 12,000 × *g* to pellet the nuclei. The supernatant (cytosolic fraction) and pellets (nuclear fraction) were mixed with SDS loading buffer and boiled for Western blot analysis.

RT-PCR Analysis—Total cytoplasmic RNA was prepared from cells and tissues using TRIzol reagent (Invitrogen), and mRNA levels were estimated by RT-PCR (18). The primers used for NOS2 detection were 1704F (5′-CTG CAT GGA ACA GTA TAA GGC AAA C-3′), corresponding to bases 1704–1728, and 1933R (5′-CAG ACA GTT TCT GGT CGA TGT CAT GA-3′), complementary to bases 1908–1933 of rat NOS2 cDNA sequence, which yield a 230-bp product. The primers used for IκBα were 299F (5′-CAT GAA GAG AAG ACA CTG ACC ATG GAA-3′) and 627R (5′-TGG ATA GAG GCT AAG TGT AGA CAC G-3′), which yield a 328-bp product. The primers used for glyceraldehyde-3-phosphate dehydrogenase detection were 796F (5′-GCC AAG TAT GAT GAC ATC AAG AAG) and 1059R (5′-TCC AGG GGT TTC TTA CTC CTT GGA), which yield a 264-bp product. Quantitative estimates of IκBα mRNA levels were obtained by carrying out competitive RT-PCR as previously described (14, 18, 39), in which a known amount of a smaller (by 50 bp) competitive internal standard is included in the PCR reaction along with the cDNA template. The internal standard contains the same primer binding sites and is amplified with the same efficiency as the IκBα cDNA. Comparison of PCR product band intensities (derived from cDNA and from internal standard) at the end of the reaction allows estimation of the initial starting amount of IκBα cDNA relative to the amount of standard added. PCRs were initiated by a hot start method, and conditions were 35 cycles of denaturation at 93 °C for 15s, annealing at 63 °C for 20s, and extension at 72 °C for 20s followed by 5 min at 72 °C in a Hybaid Thermoreactor (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 Infotech 2000 imaging system. In some experiments, changes in mRNA levels were also estimated by real time PCR using similar cycling conditions in the presence of SYBR Green (1:10,000 dilution of stock solution from Molecular Probes, Eugene, OR), carried out in a 20-μl reaction in a Corbett Rotor-Gene (Corbett Research, Sydney, Australia). Relative mRNA concentrations were calculated from the relative take-off point of the PCR reactions (the point at which fluorescence signal was above background levels) using the manufacturer's software included in the unit.

Data Analysis—All enzymatic experiments were done at least in triplicate, and means ± S.E. were determined. Statistical significance was assessed by one way analysis of variance followed by Fisher's *post hoc* tests, and *p* values < 0.05 were considered significant.

RESULTS

NE Increases IκBα Expression—Incubation of primary rat astrocytes with NE caused a dose-dependent increase in total cellular levels of the IκBα protein (Fig. 1*A*). Increases were observed beginning between 1 and 5 μM NE and were maximal (up to 230% control values) between 25 and 100 μM NE. The increases in protein levels were paralleled by an increase in the steady state levels of the IκBα mRNA (Fig. 1*B*), with the greatest increase (almost 2-fold control values assessed by competitive RT-PCR) observed between 5 and 25 μM NE. Limited analyses using real time PCR (data not shown) confirmed that induction of IκBα mRNA was maximal at 25 μM NE and that competitive RT-PCR underestimated the actual magnitude of the increase (which was estimated to be up to 9-fold compared with control values by real time PCR).

Examination of IκBα protein levels after different incubation times with NE showed that maximal increases occurred after 4 h of incubation, reaching 9–10-fold control values (Fig. 2*A*). In other experiments (not shown) we observed a subsequent gradual decrease in IκBα protein levels occurring after 8 h and returning to control values by 24 h. Increases in IκBα protein levels were detected in both the cytosolic as well as the nuclear fraction (Fig. 2*B*), suggesting that NE not only increases IκBα expression but also influences its subcellular localization.

The effects of NE on IκBα mRNA (Fig. 2*C*) was also time-dependent, with maximal increases (roughly 2-fold control values) occurring sooner than that seen with proteins, namely between 30 and 60 min of incubation. The IκBα mRNA levels measured after 4 h showed little further decrease over the next 24 h. Similar analyses using real time PCR (not shown) confirmed that maximal induction of IκBα mRNA occurred between 60 and 90 min of incubation, with a maximal increase of ~7-fold observed. The effect of NE on IκBα protein and mRNA levels were mediated by binding to βARs (Fig. 3), since co-incubation with a βAR antagonist (propranolol) prevented the increase by NE, whereas co-incubation with an αAR antagonist (phenoxybenzamine) had no effect. In addition, the effects of NE were replicated by the βAR agonist isoproterenol.

NE Increases IκBα Promoter Activation—The actions of NE were blocked by co-incubation with the transcriptional inhibitor actinomycin D (Fig. 3), suggesting that the increased mRNA levels were due to increased IκBα gene transcription and not to increased mRNA stability. To test this possibility, we examined the effects of NE on the activation of a 1.3-kb fragment of the human IκBα promoter (Fig. 4*A*). As found for IκBα mRNA levels, activation of the IκBα promoter in astrocytes was dose-dependently increased by NE, with significant increases observed at 5 μM and higher. This activation was blocked by actinomycin D or propranolol, not effected by phenoxybenzamine, and increased by incubation with isoproterenol alone (Fig. 4*B*). The effects of NE were also reduced (by roughly 20%) upon incubation with the selective PKA inhibitor KT5720. These results demonstrate that NE can directly activate IκBα gene transcription, most likely via βAR-dependent increases in cAMP and possibly involving PKA activation.

To further explore the mechanisms mediating NE effects, we selected rat C6 glioma cells for stable expression of the IκBα promoter (Fig. 5). In these cells, incubation with NE also led to a dose-dependent promoter activation, although the maximal induction was ~50% over control values (as compared with the roughly 2-fold induction observed in the primary astrocytes). Co-incubation with the PKA inhibitors H89 or KT5720 significantly and potently blocked the activation due to NE, whereas co-incubation with the NFκB inhibitor ZIE had no effect on promoter activation. In the absence of NE, these drugs only slightly reduced the basal activity of the IκBα promoter.

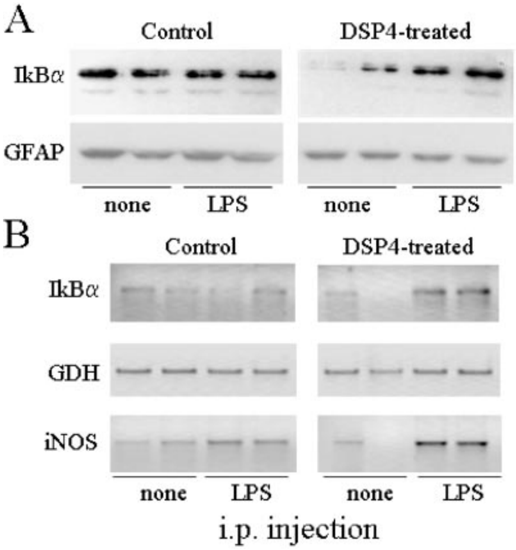

FIG. 6. **Effects of LC lesion on IκBα expression and brain inflammation.** Adult rats were treated with DSP4 to lesion the LC then injected intraperitoneally with LPS to induce an inflammatory response. After 24 h, samples prepared from frontal cortices were analyzed by Western blots for levels of IκBα protein and glial fibrillary acidic protein (*A*), and RNA samples were analyzed by RT-PCR for levels of IκBα, NOS2, and glyceraldehyde-3-phosphate dehydrogenase (*GDH*) mRNAs (*B*). *Control*, non-DSP4-treated animals; *DSP4*, DSP4-treated animals; *none*, animals injected intraperitoneally (*i.p.*) with saline; *LPS*, animals injected intraperitoneally with LPS. For each condition, samples from two different animals are shown, and similar results were obtained in a second series of experiments.

LC Lesions Reduce IκBα Expression in Vivo—Chemical lesion of noradrenergic LC neurons results in diminished cortical NE levels and an increased inflammatory response to Aβ (14). To determine whether changes in IκBα could be involved, we examined protein lysates prepared from frontal cortices of control and DSP4-treated rats (Fig. 6*A*). IκBα was detected at high levels in control animals, and this expression was not appreciably modified at 24 h after peripheral LPS injection. In contrast, IκBα levels in DSP4-treated animals were significantly decreased compared with non-DSP4 treated animals. Furthermore, peripheral LPS injection into those animals resulted in a pronounced increase in IκBα levels, which, due to the presence of an NFκB site within its promoter, is often used as an index of inflammatory gene activation, therefore suggesting an overall greater inflammatory response than what occurred in non-DSP4-treated animals. Glial fibrillary acidic protein levels measured in the same protein samples showed only slight changes due to either DSP4 treatment or LPS injection.

The effects of DSP4 treatment on cortical inflammatory response was examined by RT-PCR analysis of cortical mRNA samples (Fig. 6*B*). Measurements of IκBα mRNA were consistent with the results of Western blot analysis and indicate a decrease in basal levels due to DSP4 treatment and a large increase due to LPS injection in the DSP4-treated animals (over 10-fold) compared with no apparent increase in the control animals. The levels of the NOS2 mRNA were low in non-

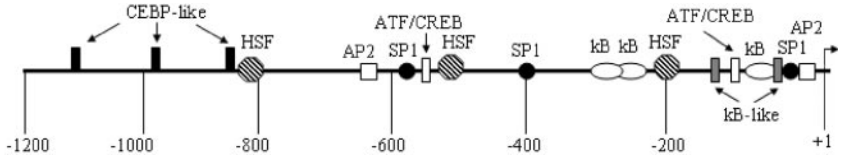

FIG. 7. **Schematic representation of potential transcription factor binding sites in the IκBα promoter.** Several previously characterized sites (*κB*, *κB-like*, *Sp1*, *AP2*) are shown as well as potential binding sites for heat shock factor (*HSF*) and C/EBP and activating transcription factor (*ATF*)/CREB (identified using MatInpsector software).

LPS-injected animals, were increased ~40% by LPS in the control animals, and were increased ~3–4-fold in the DSP4-treated animals. In control animals, levels of glyceraldehyde-3-phosphate dehydrogenase mRNA were not increased by peripheral LPS, but slightly decreased (to 80% of control values), whereas in DSP4-treated animals, LPS slightly increased glyceraldehyde-3-phosphate dehydrogenase levels (~30% over control values). These results suggest that NE normally maintains IκBα expression at levels that limit the brain inflammatory response to peripheral LPS injection (or other inflammatory stimuli).

DISCUSSION

We previously showed in primary astrocytes and in C6 cells that incubation with NE partially blocked the rapid decrease in IκBα protein levels that occurs upon incubation with LPS and cytokines (18). However, those results did not address whether NE reduced degradation of pre-existing IκBα or increased *de novo* synthesis of new IκBα. However, we also observed that NE alone increased IκBα levels *versus* control cells, suggesting that NE could directly increase IκBα expression, perhaps by increasing transcription and/or translation. In the present study we provide evidence that NE directly increases transcription of the IκBα gene, leading to increased IκBα mRNA levels, and thereby reducing the overall loss of IκBα that occurs upon inflammatory stimulation. These findings provide a molecular mechanism to help explain previous reports which demonstrate that increases in cAMP by β-agonists (24, 27, 28), by peptides including α-melanocyte-stimulating hormone (25) or vasoactive intestinal peptide (26), or by use of cAMP mimetics (24) increase IκBα mRNA levels.

Evidence that cAMP can increase IκBα gene expression has been reported several times. In rat Kupffer cells (27), LPS induced NOS2 expression that was reduced by treatment with forskolin, dibutyryl cyclic AMP, cholera toxin, or isoproterenol. In these cells NFκB activation (assessed by nuclear uptake of p65 subunit) was also blocked by forskolin, as was the LPS-dependent IκBα degradation. The authors showed that forskolin potentiated the normal increase in IκBα mRNA that occurs after LPS treatment and, furthermore, that forskolin alone increased steady state IκBα mRNA levels. Similarly, in human pancreatic cancer cells, the induction of macrophage colony stimulating factor by interleukin 1α was blocked by increases in cAMP as was activation of NFκB (28). In these cells, degradation of IκBα was prevented by cAMP, and levels of IκBα mRNA were increased. Because IκBα mRNA stability was not effected, the authors concluded that cAMP increased IκBα gene transcription. In human THP1 monocytic cells (24), the LPS-induced tumor necrosis factor α and interleukin 8 production was inhibited by βAR agonists as was nuclear uptake of NFκB. In these cells, the rapid (by 30 min) loss of IκBα due to LPS was not significantly reduced by βAR agonists, but there was a delayed increase in cytoplasmic IκBα protein levels occurring between 1 and 3 h after treatment with LPS. Importantly, the addition of isoproterenol alone did not increase IκBα protein levels, indicating that the addition of an inflammatory stimuli was needed. By carrying out studies in the presence of LPS together with the protein synthesis inhibitor cycloheximide, it was found that isoproterenol increased the IκBα protein half-life (from 20 to 60 min).

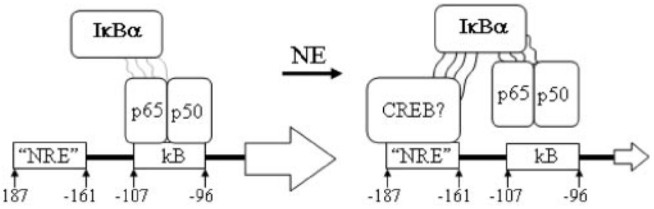

FIG. 8. **Schematic showing how NE regulates IκBα expression leading to NOS2 inhibition.** The NOS2 promoter contains an essential κB site located at positions −107 to −96 relative to the transcriptional start site. Immediately upstream is a 27-bp NE-responsive region (*NRE*, bases −187 to −161) containing binding sites for CREB and C/EBP, whose presence is necessary to observe inhibition by NE (see Ref. 18). Upon inflammatory stimulation, activated NFκB (consisting of p50·p65 heterodimers) enters the nucleus, binds to the proximal κB site, and initiates transcription. Although IκBα is present in the nucleus, its association with DNA-bound NFκB is weak. NE, via increasing cAMP and activating protein kinase A, activates CREB (or a CREB-related protein), which binds to the NRE region, where it can interact (either directly or indirectly through other proteins, perhaps CBP/P300 or the PKA catalytic subunit) with IκBα, stabilizing IκBα·NFκB complex formation and preventing transcription.

The signaling pathways by which NE, via increases in cAMP, activates IκBα promoter expression are not yet known. The IκBα promoter has been sequenced from several species, and most attention has focused on the presence of three κB and two κB-like sequences that confer NFκB inducibility onto IκBα expression (29, 30). It has been shown that the κB1 site at position −63 to −53 as well as the κB-like site at position −34 to −24 are required for induction by inflammatory agents and are, therefore, responsible for autoregulation of NFκB activation since newly synthesized IκBα will rapidly complex with and inactivate NFκB. In several cell types, cAMP has been shown to activate NFκB (31–33), and in previous studies we observed that NE alone could activate a truncated NOS2 promoter which still contained the proximal NFκB binding site (18). However, in those studies NE did not lead to detectable levels of NFκB activation (as assessed by electrophoretic mobility shift assay or by nuclear staining for the p65 subunit), suggesting instead that cAMP-inducible factors were responsible for activation of the NOS2 promoter. However, it remains possible that in the current studies, IκBα induction by NE is due in part to low levels of cAMP-dependent NFκB activation.

Additional potential transcription factor binding sites have been identified in the IκBα gene promoter that could confer induction by cAMP, including SP1 and AP2 as well as two potential activating transcription factor-CREB sites and several near consensus C/EBP binding sites (see Fig. 7). Evidence that Sp1-dependent genes can be modulated through cAMP is indicated since Sp1-dependent reporter gene activity and DNA binding of recombinant Sp1 was stimulated by PKA (34), and cAMP-dependent activation of a phosphodiesterase 5A2 promoter was blocked when the Sp1 binding sequences were specifically mutated (35). Similarly, AP-2 dependent gene transcription was activated by cAMP (36) including that of 2′3′-

phosphodiesterase expression in C6 astrocytoma cells (37). Activation of Sp1 and AP2 sites, alone on in concert with low levels of NFκB activation, could therefore contribute to cAMP-dependent induction of IκBα expression. Whether the potential activating transcription factor-CREB or C/EBP-like sites play a role in NE effects remains to be determined.

Alternatively, there is accumulating evidence that IκBα (38) as well as IκBβ (39) is a heat shock protein since the IκBα promoter contains near consensus binding sites for the heat shock transcription factor 1 (Ref. 40 and see Fig. 7) and since its expression is increased after the induction of a heat shock response. The ability of NE to induce a heat shock response has been reported several times, although the signaling mechanism is not clear, being reported to be due to activation of αARs (41, 42), βARs (43), or increased release of NO (44). In astrocytes, it has been shown that NE can increase expression of small heat shock proteins (45, 46). Therefore, it is possible that NE increases the astroglial heat shock response, which together with effects on other transcription factors as mentioned above, can cause IκBα gene expression.

The means by which increased IκBα can reduce astroglial NOS2 expression is also not fully understood. We have shown using NOS2 promoter constructs, that a 27-bp region located immediately upstream is necessary to confer inhibition by NE, since removal of this area abolished any inhibitor action of NE on NOS2 promoter transcription (18). Because NE did not modify nuclear uptake or DNA binding of NFκB yet increased IκB expression and nuclear localization, we propose that normally, nuclear levels of inhibitory IκB proteins do not form stable complexes with DNA-bound NFκB and, therefore, do not inhibit NFκB-dependent transcription. We suggest that induction of a factor binding to a site located in the 27-bp region by NE stabilizes the association of IκBα with NFκB, resulting in inhibition of transcription (Fig. 8). The ability of nuclear-located IκBα to interact with and inhibit DNA bound NFκB has previously been reported (21).

The *in vivo* relevance of noradrenergic regulation of IκBα expression and, thus, inflammatory gene expression is suggested by observations that the noradrenergic neurons of the LC are damaged or lost in Alzheimer's disease (15), leading to a loss (or at least a transient loss) in noradrenergic signaling within projection areas. A possible perturbation in noradrenergic signaling is also implicated in multiple sclerosis, since it has been shown that treatment with βAR agonists (47, 48) can provide protection in the animal model experimental autoimmune encephalomyelitis, and more recently that the levels of β_2ARs in astrocytes were decreased in multiple sclerosis patients as compared with healthy controls (16). We have recently tested this hypotheses by examining the effects of LC depletion on the inflammatory response to cortical injection of aggregated β-amyloid (14). We observed that both the magnitude as well as the duration of the inflammatory responses measured (NOS2 and interleukin 1β expression) were increased if cortical NE levels were first depleted. In the current study, we show that the same DSP4 treatment led to a dramatic decrease in cortical levels of the IκBα protein, consistent with the idea that NE normally keeps the IκBα gene transcriptionally active. The consequences of having diminished IκBα levels are exemplified by the fact that after peripheral injection of LPS, a well characterized method that has been shown to induce brain inflammatory gene expression (23), the levels of the IκBα protein and mRNA are more strongly increased in the DSP4-treated animals than in the controls. Likewise, in control animals, peripheral LPS induced low levels of the NOS2 mRNA, whereas in DSP4 treated animals the magnitude of NOS2 induction (relative to non-LPS injected animals) was much greater.

In summary, our data demonstrate the NE, via activation of βARs, increases in cAMP, and activation of PKA can directly increase IκBα gene expression in astrocytes. Whether the same holds true in other cell types is not clear. Increased IκBα levels can reduce overall levels of NFκB activation either by maintaining NFκB in the cytoplasm or, as we postulate in astrocytes, by binding to NFκB within the nucleus. Our data suggest that certain anti-inflammatory drugs may act in part by counteracting the loss of IκB expression due to perturbation of NE levels or NE-signaling system. In this respect, the findings that non-steroidal anti-inflammatory drugs and agonists of the peroxisome proliferator-activated receptor γ can increase IκB expression (10–12) suggests a common mode of action for these therapeutic interventions.

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