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Effects of Noradrenaline on Neuronal NOS2 Expression and Viability

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ABSTRACT

The authors previously showed that conditioned media (CM) from activated microglia increased inducible nitric oxide synthase (NOS2) in cortical neurons. Here they examined the ability of noradrenaline (NA) to reduce neuronal NOS2 or cell death. Primary mouse cortical neurons were activated using CM from microglia incubated with lipopolysaccharide (LPS). Neuronal NOS2 was assessed by increases in nitrite accumulation, and increases in NOS2 mRNA levels and fluorescence of the NO-sensitive probe DAF-2 DA. NOS2 induction was associated with an increase in neuronal LDH release. When NA was added during microglial activation, neuronal NOS2 was significantly reduced (by approximately 70%); in contrast if NA was added to the neurons along with CM, there was less reduction (about 35% decrease) in NOS2 expression. NA added to either microglia or to neurons reduced neuronal LDH release comparably. Pretreatment of CM with blocking antibody to TNFα, alone or with IL1-receptor antagonist, partially reduced neuronal cell death and NOS2. Incubation of neurons with NA increased IkBα, which could reduce NOS2. These results demonstrate that NA modulates neuronal NOS2 expression and damage, and that these effects are primarily due to inhibition of microglia released factors. Perturbations of NA could exacerbate neuronal damage by allowing for increased inflammatory responses. *Antioxid. Redox Signal.* 8, 885–892.

INTRODUCTION

It is now accepted that the inducible form of nitric oxide synthase (NOS2) can be expressed in most neural cell types including glial cells (see review by Saha and Pahan; this issue), endothelial cells, oligodendrocytes (see review by Boullerne and Benjamins; this issue, pages 967–980), and neurons (12), however, neuronal NOS2 (nNOS2) expression has been least well characterized. *In vitro* induction of nNOS2 has been described following incubation with bacterial endotoxin lipopolysaccharide (LPS), proinflammatory cytokines, and some growth factors. *In vivo*, nNOS2 is expressed after a variety of stimuli including certain types of stress (23), oxidative insults (29), and proinflammatory factors (1). However, since neighboring glial cells can release substances that in turn stimulate neurons, it is not clear if factors that activate nNOS2 act directly on neurons or indirectly to promote release of glial substances.

In previous studies, we showed that intraparenchymal injection of LPS and interferon-γ (IFNγ) into adult cerebellum induced nNOS2 in granule cells and neuronal death (16). In contrast, similar injections into the striatum resulted in primarily glial NOS2 expression with little or no neuronal induction (11). *In vitro* studies using glial cells have demonstrated that the neurotransmitter noradrenaline (NA) potently blocks glial inflammatory activation (5, 7), reducing cytokine production as well as NOS2 expression. Since nNOS2 expression has been observed in brains of Alzheimer's disease patients (18, 20, 38), we hypothesized that NA normally restricts nNOS2 in brain but that loss of noradrenergic locus ceruleus (LC) neurons (24) and dysregulation of NA levels (26) is permissive for increased inflammatory gene expres-

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sion. Indeed, chemical lesion of LC neurons in mice or rats greatly exacerbates the inflammatory response to Aβ, and converts the primary cellular site of NOS2 from glial to neuronal (14). However, the question remains as to whether the effects of NA loss are mediated directly on neurons (for example, by decreasing levels of antiinflammatory molecules) or due to increased release of inflammatory molecules from neighboring glial cells.

To address these questions, we recently showed that conditioned media (CM) from LPS-stimulated microglia could induce NOS2 in highly enriched cultures of cortical neurons prepared from embryonic day-15 rats (22). We demonstrated that IL-1β contributes to, but is not necessary for nNOS2 expression, and that its induction was attenuated if microglia were treated with NA, suggesting an effect of NA on the release of microglial factors (3). In the current study, we further characterize NOS2 expression in highly enriched neuronal cultures, demonstrate that LPS is insufficient to induce nNOS2, and show that NA does not significantly reduce nNOS2 if added directly to the neurons despite a strong induction of inhibitory IκBα expression that blocks NOS2 in glial cells.

METHODS

Materials

Cell culture reagents (DMEM, and antibiotics) were from Cellgro Mediatech (Herndon, VA). Fetal calf serum (FCS), neurobasal medium (NBM), B27 supplements, and DMEM-F12 were from GIBCO Life Technologies (Carlsbad, CA). LPS (*Salmonella typhimurium*), NA, and the NOS2 inhibitor 2-amino-dihydro-6-methyl-4H-1,3-thiazine (AMT) were from Sigma (St. Louis, MO). The IL1-receptor antagonist (IL-1ra) and 4,5-diaminofluorescein diacetate (DAF-2 DA) were from Calbiochem (San Diego, CA). Taq polymerase, cDNA synthesis reagents and Alexa Fluor 488-labeled LPS were from Invitrogen (Carlsbad, CA). The antibody against TNFα was from Preprotech (Rocky Hill, NJ). Anti-IκBα (Sc-371) rabbit polyclonal antibody was from Santa Cruz Biotechnologies, (Santa Cruz, CA). DAPI was from Vector Laboratories (Burlingame, CA).

Cells

Rat cortical microglial cells were obtained as previously described (37). Briefly, 1-day-old Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) were used. Enriched cultures of microglia were prepared from primary mixed cultures of rat cortical glial cells, plated in T-75 cm^2 flasks in DMEM containing 10% FCS and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin; Sigma), and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Microglia were detached from the astrocyte monolayer by gentle shaking 11–13 days after the dissection and again after one week from the first shaking. The cells were plated in 96-well plates at a density of 3×10^5 cells/cm^2 using 100 μl/well of DMEM-F12 (10% FCS and antibiotics). Under these conditions, the cultures were 95%–98% OX42-positive. All experiments were carried out in 10% FCS DMEM-F12.

Primary cultures of cortical neurons were performed as described (29), with some modifications. Brains were removed from embryonic day 16 Sprague–Dawley rats, and the cortical area was dissected. Neurons were mechanically dissociated in 80% BME containing 33 mmol/L glucose, 2 mmol/L glutamine, 16 mg/L gentamycin, 10% horse serum, and 10% FCS, and plated at 2×10^5 cells/cm^2 in poly-L-lysine-precoated 6-, 12-, or 96-multiwell plates. Two types of neuronal preparations were established. For mixed neuron–microglia cultures the medium was changed on day 4 with fresh growth medium containing cytosine arabinoside (10 μmol/L) and lacking FCS. These cultures consist of 80 ± 20% NeuN positive cells, and experiments were performed after 9 days *in vitro* using fresh medium lacking both FCS and cytosine arabinoside. Highly enriched neuronal cultures were obtained by replacing the medium 24 h after initial plating with serum free NBM supplemented with 0.5 mmol/L glutamine and complete B27, and after 4 days 50% of the medium was replaced with fresh NBM. These cultures consist of 98 ± 2% NeuN positive cells, and experiments were performed after 9–10 days *in vitro* using NBM supplemented with 0.5 mmol/L glutamine and B27 without antioxidants.

NOS2 activity assay

NOS2 was induced in neuronal cultures using conditioned media (CM) harvested from microglia incubated for 24 h in plain medium (CM–Control) or medium containing 1 μg/ml of LPS (CM–LPS) alone or with NA (as indicated). Neurons were further incubated for 48 h and NO production was measured indirectly by nitrite accumulation in the cell culture media (9). An aliquot of the cell culture media (80 μl) was mixed with 40 μl of Griess reagent and the absorbance measured at 550 nm. For each experiment, the amount of nitrites present in the CM due to microglial activation was determined and subtracted from the values obtained after incubation of neurons.

Cell viability

At the end of each experiment neuronal viability was assessed by measurement of the released lactate dehydrogenase (LDH), using the CytoTox-96 kit from Promega (Madison, WI), according to manufacturer's instructions.

mRNA analysis

Total cytoplasmic RNA was prepared from cells using TRIZOL reagent (Invitrogen); aliquots were converted to cDNA using random hexamer primers, and mRNA levels estimated by RT–PCR. The primers used were: IκBα 586F (5′-GCC TGG CCA GTG TAG CAG TCT T-3′) and 720R (5′-CAG CAC CCA AAC TCA CCA AGT G-3′), which yield a 136 bp product; NOS2, 26F (5′-CAC AGT GTC GCT GGT TTG AA-3′) and 457R (5′- GTG AAG GGT GTC GTG AAA AAT C-3′), which yield a 431 bp product; CD14, 669F (5′-GAT CTG TCT GAC AAC CCT GAG T-3′) and 935R (5′-GTG CTC CTG CCC AGT GAA AGA T-3′), which yield a 267 bp product; TLR4, 2873F (5′-CAG GCA CAA GGA AGT AGC AAT-3′) and 3119R, 5′-TAG AAA AAG GGA

AAG GAA GGA AAC A-3′, which yield a 347 bp product; NOS1, 2630F (5′-CTT GCG TTT CTT TCC CCG TAA-3′) and 2937R (5′-TCC CCC TCC CTC ATC TTC AG-3′), which yield a 307 bp product; and glyceraldehyde 3- phosphate dehydrogenase (GDH), 796F (5′-GCC AAG TAT GAT GAC ATC AAG AAG-3′) and 1059R (5′-TCC AGG GGT TTC TTA CTC CTT GGA-3′), which yield a 264 bp product. PCR conditions were 35 cycles at 95°C for 10 s, annealing at 58°C for 15 s, and extension at 72°C for 30 s, followed by 5 min at 72°C in an Eppendorf Thermoreactor (Hamburg, Germany). PCR products were separated by electrophoresis through 1.5% agarose gels containing 0.1 μg/ml ethidium bromide.

Protein analysis

After desired incubations, neurons were washed twice in ice-cold phosphate buffered saline (PBS) and lysed using 8 *M* urea. The protein content in each sample was determined using bovine serum albumin as standard and Bradford reagent. Ten μg of proteins were mixed 1:3 with 3X gel sample buffer (150 m*M* Tris-HCl pH 6.8, 7.5% SDS, 45% glycerol, 7.5% of bromophenol blue, 15% β-mercaptoethanol), boiled for 5 min and separated through 10% polyacrylamide SDS gels. Apparent molecular weights were estimated by comparison to colored molecular weight markers (Sigma). After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes by semi-dry electrophoretic transfer. The membranes were blocked with 10% (w/v) low-fat milk in TBST (10 m*M* Tris, 150 m*M* NaCl, 0.1% (w/v) Tween-20, pH 7.6) for 1 h at room temperature, and incubated in the presence of anti-IκBα antibody (at 1:1,500 dilution) overnight with gentle shaking at 4°C. The primary antibody was removed, membranes washed three times in TBST, and further incubated for 1 h at room temperature in the presence of anti-rabbit IgG-HRP secondary antibody, diluted 1:8,000. Following three washes in TBST, bands were visualized by incubation in ECL reagents for 1 min and exposure to X-ray film for 10 min. Membranes were washed three times in TBST, blocked with 10% (w/v) low-fat milk in TBST for 1 h at room temperature, and used for β-actin immunoblot (incubating with anti-β-actin antibody at 1:1,000 dilution, overnight with gentle shaking at 4°C and anti-mouse IgG-HRP secondary antibody, diluted 1:10,000).

LPS staining

Cells were incubated with 10 μg/ml Alexa Fluor 488-labeled LPS for 1 h in a humidified atmosphere containing 5% CO_2. After this, cells were washed with PBS and mounted for visualization.

DAF-2 DA staining

NO was detected in the neurons with DAF-2 DA, which binds specifically to NO resulting in enhanced fluorescence (2). Cells grown on chamber slides were incubated for 4 h at 37°C with microglial CM-C or CM-LPS in combination with 20 μ*M* DAF-2 DA. Nuclei were stained using DAPI (400 ng/ml in PBS for 3 min) and slides covered using Vectashield mounting fluid (Vector Laboratories Inc., Burlingame, CA). Images were obtained on a Zeiss Axioplan2 fluorescence microscope equipped with an Axiocam MRm digital camera and Axiovision (Thornwood, NY) 4.2 imaging software.

Data analysis

All experiments were done at least in triplicate. Data are analyzed by one-way ANOVA followed by Dunnett's multiple comparison or Bonferroni post hoc tests and p values < 0.05 were considered significant.

RESULTS

Cultures of primary cortical neurons that were maintained in BME could be labeled (approximately 20% of the cells were positive) using fluorescent Alexa-tagged LPS (Fig. 1A). These cultures also expressed the LPS receptor CD14 and its membrane associated signaling protein TLR4 (Fig. 1C) suggesting the presence of an intact LPS signaling system. Mixed neuronal:glial cultures produced significant levels of nitrites (approximately 10-fold background values) after 48 h treatment with bacterial endotoxin LPS (1 μg/ml), which was abolished if the cells were pretreated with LME to reduce microglial content (Fig. 1B). In contrast, neuronal cultures grown in NBM did not show a significant nitrite production after 48 h incubation with LPS alone, and only a modest production (2.6 μ*M* nitrite per 48 h) due to the cytokine combination of TNFα, IL-1β, and IFNγ. These cultures had only slight (about 2% of the cells) binding of Alexa-LPS, and had lower levels of CD14 and TLR4 mRNAs. Staining of these cultures for the presence of microglial marker CD11b also revealed about 2%–3% microglial cells (data not shown). These results show that neurons prepared in NBM are approximately 98% pure, and that neither LPS nor a typical proinflammatory cytokine mixture can induce robust NOS2 expression in these cultures.

Since neurons can express NOS2 *in vivo* where activation of neighboring glial cells can influence neuronal expression, we tested if conditioned media (CM) from glial cells could provide necessary factors for nNOS2 expression. NBM neurons incubated with CM from microglia that had been activated with LPS (CM–LPS) produced significant amounts of nitrites (8 μ*M* nitrites per 48 h, Fig. 2). These nitrites were due to activity of NOS2 since co-treatment with the selective NOS2 inhibitor AMT abolished nitrite production (Fig. 3). The production of NO from neurons was confirmed using the fluorescent probe DAF-2 DA, which showed increased fluorescence in neuronal cell bodies following treatment with CM-LPS (Fig. 2B). CM–LPS increased levels of the NOS2 mRNA in these cells, while levels of the neuronal isoform NOS1 were not altered (Fig. 4). These data confirm that highly enriched neuronal cultures can express functional NOS2 dependent on microglial derived factors.

Consistent with previous studies (22), activation of microglial cells with LPS and NA generated a media (CM–LPS–NA), which induced about 70% less nitrite accumulation than did the CM–LPS (Fig. 3A) and caused a smaller increase in nNOS2 mRNA levels (Fig. 4). Incubation with CM–LPS–NA

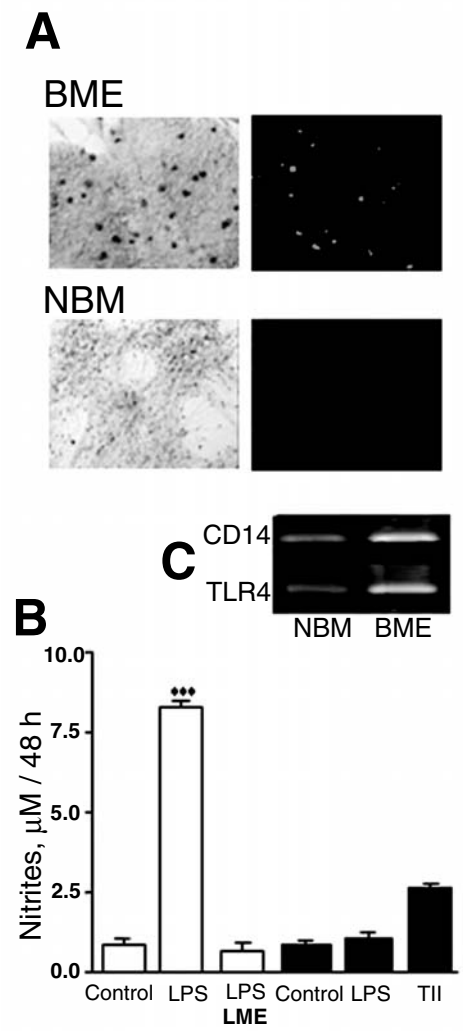

FIG. 1. Characterization of neuronal cultures. (**A**) Phase contrast (*left panels*) and fluorescence images (*right panels*) of neuron–microglia mixed cultures (BME) and purified neuronal cultures (NBM) incubated for 1 h with 10 μg/ml Alexa Fluor 488-labeled LPS. (**B**) Nitrite levels measured in neuron–microglia mixed cultures (*white bars*) and purified neuronal cultures (*black bars*) after 48 h incubation with LPS (1 μg/ml) or TII (TNFα, 50 ng/ml; IFNγ, 100 U/ml; IL-1β, 10 ng/ml). Some mixed cultures were also treated with 10 m*M* leucine methyl ester (LME) for 24 h to reduce microglial content before LPS was added. ***$p < 0.001$ vs. control. (**C**) Representative gel of PCR analysis for CD14 and TLR4 in mixed and pu-

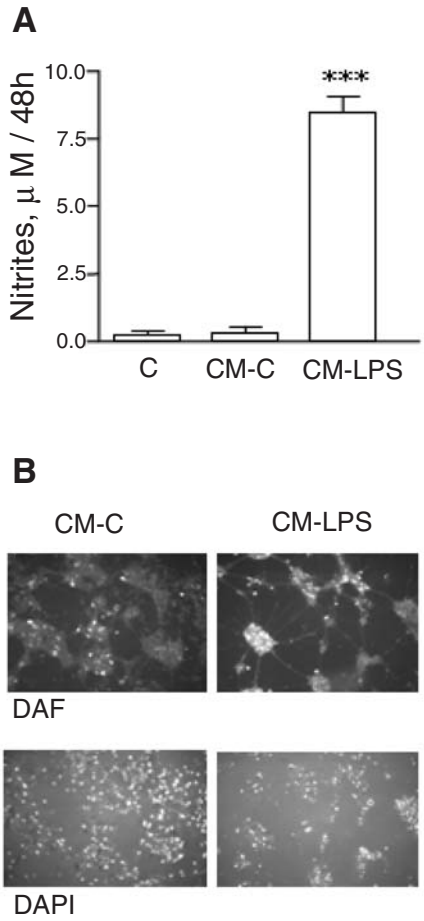

FIG. 2. Effects of microglial conditioned media on neuronal NOS2. (**A**) Nitrite levels were measured in neuronal media after 48 h incubation with CM from resting microglia (CM–C) or microglia treated with LPS 1 μg/ml (CM–LPS) diluted at 50% in neuronal media. Data are expressed as μ*M* of nitrites and are means ± standard error of $n = 4$ replicates. The results are analyzed by one-way ANOVA. ***$p < 0.001$ vs. control. (**B**) Representative images corresponding to neurons incubated for 4 h with DAF-2 DA in combination with CM from resting microglia (CM–C) or microglia treated for 24 h with LPS 1 μg/ml (CM–LPS). The images shown are representative of experiments done on three separate neuronal preparations.

also reduced neuronal LDH release about 20% compared to CM–LPS (Fig. 3B). In contrast, when NA was added directly to neurons during activation with CM–LPS, the reduction in nitrite production was less (about 35% decrease) although the effect on LDH was comparable. Interestingly, despite that incubation with AMT completely inhibited neuronal nitrite production, it had only a modest effect on reducing LDH release (86% of that due to CM–LPS). These results indicate that the effects of NA on nNOS2 are mainly due to suppression of factors released from microglial cells; and that nNOS2 expression is not the sole cause of neuronal damage.

We previously showed that IL-1β blockade partially reduced the ability of CM–LPS to induce neuronal LDH release and nNOS2 (22). Similarly, treatment of neurons with CM–LPS in the presence of a blocking antibody against TNFα, either alone or in combination with the IL1-ra, reduced LDH release to a similar extent as did incubation with IL1-ra alone (Fig. 5A). However, in contrast to incubation with IL1-ra, which reduced nitrite production by about 45% (22), incubation with the TNFα blocking antibody did not significantly reduce neuronal nitrite production (Fig. 5B). These findings suggest that TNFα alone is not the primary inducer of nNOS2, and again that nNOS2 is not the only cause of neuronal damage.

Since the suppressive effects of NA in glial cells involve increased expression of inhibitory IκB proteins (15), we ex-

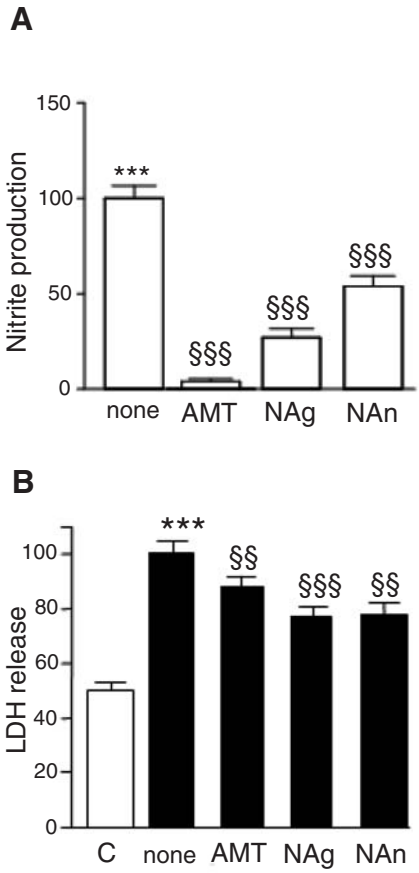

FIG. 3. Effects of NA on neuronal viability and nitrite production. (**A**) Nitrite levels were measured in neuronal media after 48 h incubation with CM from microglia treated with LPS (1 μg/ml) alone (none) or LPS plus NA 10 μ*M* (NAg). In the indicated groups, CM–LPS was added in combination with NA 10 μ*M* (NAn) or AMT (100 μ*M*). CM was diluted at 50% in neuronal media. Data are expressed as percent of nitrite release from control cells and are means ± standard error of $n = 4$ replicates. The results are analyzed by one-way ANOVA. ***$p <$ 0.001 vs. control, §§§$p < 0.001$ vs. CM–LPS. (**B**) Neurons were incubated as described in (**A**) and lactate dehydrogenase (LDH) levels were measured in neuronal media after 48 h. Data are expressed as percentage of LDH released in the CM–LPS treated group (none), and are means ± standard error of $n = 4$ replicates (control = 20.2 ± 1.4 % of total LDH release). The results are analyzed by one-way ANOVA. ***$p < 0.001$ vs. control, §§§$p <$ 0.001 vs. CM–LPS, §§$p < 0.01$ vs. CM–LPS.

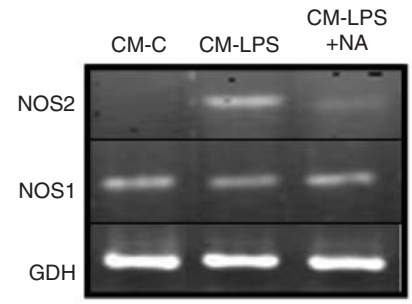

FIG. 4. Effects of microglial condition media on neuronal NOS mRNA levels. Neurons were incubated for 6 h with media from resting microglia (CM–C), or microglia treated for 24 h with LPS (1 μg/ml) or LPS + NA (10 μ*M*) diluted at 50% in neuronal media. RNA samples were isolated, converted to cDNA, and analyzed by RT–PCR for levels of NOS2, NOS1, and GDH mRNAs. Representative gels are shown.

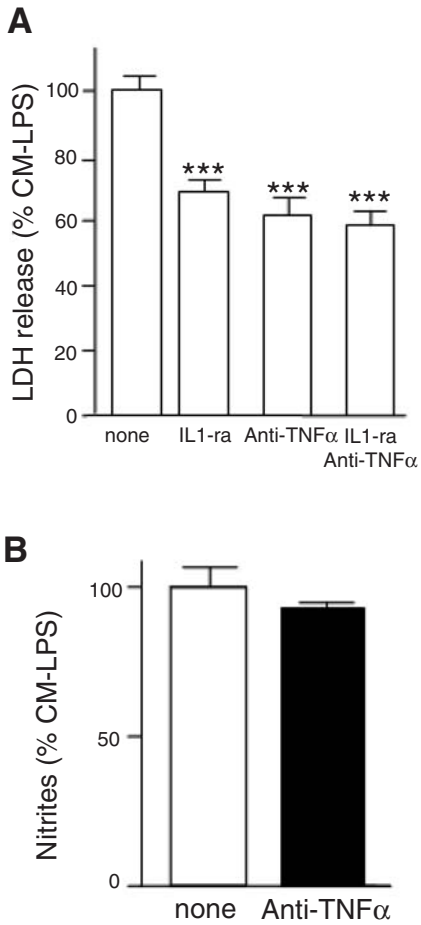

FIG. 5. Role of IL-1β and TNFα in neuronal toxicity. Neurons were incubated as described in Figure 3 and (**A**) lactate dehydrogenase (LDH) and (**B**) nitrite levels were measured in neuronal media after 48 h. Where indicated, CM–LPS was added in combination with IL-1 receptor antagonist (IL-1ra) (100 ng/ml), blocking antibody against TNFα (20 μg/ml) or both. LDH data are expressed as percentage of total LDH release and are means ± standard error of $n = 4$ replicates. The results are analyzed by one-way ANOVA. ***$p < 0.001$ vs. CM–LPS. Nitrite data are mean ± S.E. of three replicates.

amined IκBα levels in NBM neurons (Fig. 6). Both RT–PCR and Western blot analysis showed that NA increased IκBα expression. Since NA only partially reduced nNOS2 expression, these data suggest that the NA-dependent increase in neuronal IκBα is not sufficient to prevent nNOS2, and thus point to involvement of non-NFκB pathways in nNOS2 expression.

DISCUSSION

In the current study we extend recent findings to show that although highly enriched cultures of cortical neurons have the

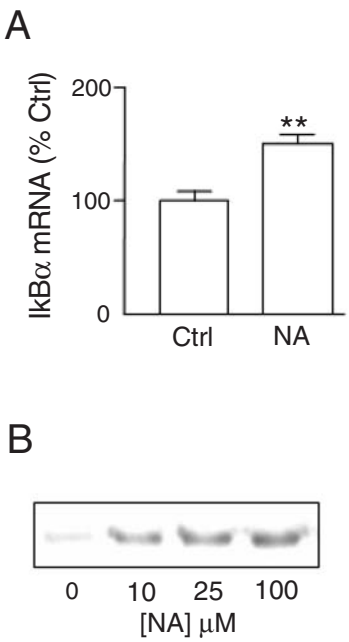

FIG. 6. Effect of NA on IκBα expression. (**A**) Neurons were incubated for 1 h with NA (25 μ*M*), RNA samples were extracted, converted to cDNA and analyzed by Q-PCR for levels of IκBα mRNA. Data are expressed as percentage of control (100%). ***$p < 0.001$ vs. control. (**B**) Neurons were incubated for 4 h with NA (10–100 μ*M*), and cytosolic lysates were examined for the presence of IκBα protein by Western blot analysis. The gel shown is representative of experiments done on three separate neuronal preparations.

capacity to express NOS2, they do not respond to treatment with LPS or cytokines alone but instead require additional glial-released factors. The exact identity of these factors remains to be determined, but our data suggest that molecules other than TNFα and IL-1β are involved. We also demonstrate that although NA upregulates IκBα expression in neurons, which is a potent inhibitor of NOS2 (4, 8), NA has only a modest inhibitory effect on neuronal NOS2, suggesting that NFκB-independent pathways contribute to neuronal NOS2 expression. The inhibitory effects of NA on neuronal NOS2 expression (both *in vitro* as well as *in vivo*) are therefore likely to be due to suppression of microglial activation.

Our inability to detect NOS2 induction in cortical neurons by LPS *in vitro* contrasts with several previous studies. Those studies used differentiated PC12 cells, a transformed cell line that may have distinct properties from neurons (17, 39), cerebellar granule cells that are prepared from postnatal day 8 pups (13, 27, 32), or injections of LPS into adult cerebellum (16, 28). In a recent study, cortical neurons in culture were shown to respond to LPS alone (35). In that study, neurons were prepared from postnatal day 1 pups rather than embryonic day 15 or 16, which may have resulted in a different neuronal phenotype. Additionally, although those authors did not observe any GFAP staining (thus minimal astrocyte presence), they did not report staining for microglial cells, thus it remains possible that in those cultures LPS induced NOS2 in microglial cells, or caused release of microglial factors that subsequently led to neuronal NOS2 expression. An earlier study reported that the proinflammatory cytokine IL-1β could activate NOS2 in hippocampal neurons; however the purity of those cultures was not described nor was immunohistochemical localization carried out to confirm neuronal localization (34).

We ascertained the purity of our neuronal cultures by several methods. We used the binding of fluorescently tagged LPS to assess the microglial content of our cultures, and determined that neurons prepared in NBM contain <2% microglial cells. We also carried out RT–PCR for the LPS receptor CD14 and its signaling partner TLR4. LPS normally binds to the serum protein LPS-binding protein (LBP), which facilitates transfer of LPS to CD14, a glycosylphosphatidylinositol (GPI)-anchored protein (36). The binding of this complex to transmembrane protein TLR4 initiates a cascade of intracellular events eventually responsible for LPS-regulated signal transduction and in many cases activation of NFκB dependent gene expression. Neither CD14 nor TLR4 expression has been reported on neurons (21); and microglia have been shown to be the major cells in the CNS expressing TLR4. The basis for our inability to detect nNOS2 versus other studies may therefore be due in part to the purity of our neuronal population.

In contrast to LPS, incubation of neurons with microglial CM–LPS resulted in NOS2 expression as determined by nitrite production, inhibition by AMT, accumulation of NOS2 mRNA, and increased DAF-2 fluorescence that could be localized to neurons. In our studies, we found that NA had only a modest effect on nNOS2 if added directly to the neurons, however co-treating microglial cells with NA during the time of LPS activation yielded CM with greatly reduced ability to induce nNOS2. The inability of NA to directly reduce nNOS2 was not due to lack of inhibitory IκBα expression, since NA increased neuronal levels of both IκBα mRNA and protein. Instead, these findings indicate that NA primarily acts by reducing the levels of a microglial derived factor necessary for robust nNOS2 expression. The identity of this factor(s) remains to be determined, since even simultaneous blockage of both IL-1β and TNFα only partially reduced nNOS2 expression.

Our findings are consistent with a recent report that CM from Aβ-stimulated microglia can induce nNOS2 in mouse cortical neurons (6). Those authors identified critical factors for nNOS2 as being TNFα and glutamate, since blocking TNFα with a specific antibody, or using an NMDA receptor antagonist completely abolished nNOS2 and neuronal damage. The ability of glutamate to activate NFkB in cerebellar cultures has been described several times (10, 19, 31, 33). It is therefore possible that LPS increases microglial release (or decreases uptake) of an excitatory amino acid required for optimal nNOS2 induction. Interestingly, it has been shown that binding to kB DNA elements and activation of κB-dependent gene expression in neurons can occur independent of the canonical NFκB dimeric proteins, and instead activation can be achieved by transcription factor Sp1 related proteins (25) whose levels can be altered by glutamate. This could account for the lack of inhibition by NA despite increased IκBα expression.

We observed that while NA significantly blocked nNOS2 if added to microglial cells; and partially blocked nNOS2 when added to neurons, there was only a small reduction in neu-

ronal LDH release. This points to the presence of additional factors that are not sensitive to inhibition by NA, which contribute to neuronal damage. Likewise, co-treatment to reduce both IL-1β and TNFα only partially reduced neuronal LDH release indicating the importance of other damaging factors. Since microglia release glutamate upon activation (30), this may also account for neuronal damage; however whether microglial glutamate release (or uptake) is modulated by NA is not yet established.

ACKNOWLEDGMENTS

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ABBREVIATIONS

Aβ, amyloid-beta; AMT, 2-amino-dihydro-6-methyl-4H-1,3-thiazine; BME, basal medium Eagle; CM, conditioned medium; DAF-2 DA, 4,5 diaminofluorescein diacetate; DMEM, Dulbecco's modified eagle medium; ECL, enhanced chemiluminescence; FCS, fetal calf serum; HRP, horse radish peroxidase; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; IκBα, inhibitor of κBα; IFN, interferon; IL, interleukin; LC, locus ceruleus; NA, noradrenaline; NBM, neurobasal medium; LME, leucine methyl ester; NOS2, nitric oxide synthase type 2; SDS, sodium sodecyl sulfate; TBST, tris-buffered saline tween-20; TLR4, toll-like receptor 4; TNF, tumor necrosis factor.

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