UP-REGULATION OF MICROGLIAL CD11B EXPRESSION BY NITRIC OXIDE
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Running title: NO increases CD11b in microglia

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Increased expression of CD11b, the beta-integrin marker of microglia, represents microglial activation during neurodegenerative inflammation. However, the molecular mechanism behind increased microglial CD11b expression is poorly understood. The present study was undertaken to explore the role of nitric oxide (NO) in the expression of CD11b in microglial cells. Bacterial lipopolysaccharide (LPS) induced the production of NO and increased the expression of CD11b in mouse BV-2 microglial cells and primary microglia. Either a scavenger of NO (PTIO) or an inhibitor of iNOS (L-NIL) blocked this increase in microglial CD11b expression. Furthermore, co-microinjection of PTIO with LPS was also able to suppress LPS-mediated expression of CD11b, loss of dopaminergic neuronal fibers and neurotransmitters in striatum in vivo. Similarly, other inducers of NO production such as interferon-γ (IFN-γ), interleukin-1β (IL-1β), HIV-1 gp120, and double-stranded RNA (poly IC) also increased the expression of CD11b in microglia through NO. The role of NO in the expression of CD11b was corroborated further by the expression of microglial CD11b by GSNO, an NO donor. Because NO transduces many intracellular signals via guanylate cyclase (GC), we investigated the role of GC, cyclic GMP (cGMP) and cGMP-activated protein kinase (PKG) in microglial expression of CD11b. Inhibition of LPS- and GSNO-mediated up-regulation of CD11b either by NS2028 (a specific inhibitor of GC) or by KT5823 and Rp-8-Br-cGMP (specific inhibitors of PKG), and increase in CD11b expression either by 8-Br cGMP or by MY-5445 (a specific inhibitor of cGMP phosphodiesterase) alone suggest that NO increases microglial expression of CD11b via GC-cGMP-PKG. In addition, GSNO induced the activation of cAMP response element-binding protein (CREB) via PKG that was involved in the up-regulation of CD11b. This study illustrates a novel biological role of NO in regulating the expression of CD11b in microglia through GC-cGMP-PKG-CREB pathway that may participate in the pathogenesis of devastating neurodegenerative disorders.

Microglia are considered as CNS-resident professional macrophages that function as the principal immune effector cells of the CNS responding to any pathological event. Activation of microglia has been implicated in the pathogenesis of a variety of neurodegenerative diseases, including Alzheimer’s disease (AD), Parkinson’s disease (PD), Creutzfeld-Jacob disease, HIV-associated dementia (HAD), stroke, and multiple sclerosis (MS) (1). It has been found that activated microglia accumulate at sites of injury or plaques in neurodegenerative CNS (1-6). Although activated microglia scavenge dead cells from the CNS and secrete different neurotrophic factors for neuronal survival (1,5,6), it is believed that severe activation causes various autoimmune responses leading to neuronal death and brain injury (1-6). During severe activation microglia not only secrete various neurotoxic molecules but also express different proteins and surface markers. Among different surface markers, CD11b is the most potential one with immense biological significance (6,7). It acts as a binding protein for intracellular cell adhesion molecule-1 (ICAM-1) and complement C3bi (8). It is reported that in various neuroinflammatory diseases, the increased Cd11b expression corresponds to the severity of microglial activation (1,6,7). Morphologically, microglial activation is associated with intense ramification and cytoskeletal rearrangement in which changes in shape and motility correlate with increased expression of CD11b (1,6,7). During this activation process, the cytoplasmic domain of
CD11b is believed to interact increasingly with cytoskeletal protein (1). However, the mechanism by which microglial expression of CD11b is increased in neurodegenerative CNS remains unclear.

Because activated microglia also express inducible nitric oxide synthase (iNOS) to produce excessive amount of NO, a molecule implicated virtually in all reported neurodegenerative and neuroinflammatory conditions (9,10), we were prompted to investigate if NO plays a role in microglial expression of CD11b. Here we report that NO is instrumental in increasing the expression of CD11b in microglia. Different inducers of NO production such as LPS, IFN-γ, IL-1β, HIV-1 gp120, and poly IC stimulated microglial expression of CD11b via NO. Furthermore, we also demonstrate that NO employed the guanylate cyclase (GC) – cyclic GMP (cGMP) – cGMP-activated protein kinase (PKG) – cAMP response element-binding protein (CREB) pathway to up-regulate the expression of CD11b in microglia.

MATERIALS AND METHODS

Reagents: Fetal bovine serum and DMEM/F-12 were from Mediatech Inc. LPS (Escherichia coli), actinomycin D and poly IC were purchased from Sigma. HIV-1 coat protein gp120 was obtained from US Biological. L-N6-(1-Iminoethyl)-lysine (L-NIL), NS-2028 (an inhibitor of guanylate cyclase), 8-Br cGMP, MY-5445 (an inhibitor of cGMP phosphodiesterase), and KT5823 (an inhibitor of PKG) were obtained from Biomol. Recombinant mouse IFN-γ and IL-1β were obtained from R&D.

Isolation of Mouse Microglia: Microglial cells were isolated from mixed glial cultures according to the procedure of Giulian and Baker (11). Briefly, on day 7 to 9 the mixed glial cultures were washed 3 times with DMEM/F-12 and subjected to a shake at 240 rpm for 2 h at 37°C on a rotary shaker. The floating cells were washed and seeded on to plastic tissue culture flasks and incubated at 37°C for 2 h. The attached cells were removed by trypsinization and seeded on to new plates for further studies. Ninety to ninety-five percent of this preparation was found to be positive for Mac-1 surface antigen.

Mouse BV-2 microglial cells (kind gift from Virginia Bocchini of University of Perugia) were also maintained and induced as indicated above.

Assay for NO synthesis: Synthesis of NO was determined by assay of culture supernatants for nitrite, a stable reaction product of NO with molecular oxygen, using Griess reagent as described (12,13).

Flow cytometry: Surface expression of CD11b on BV-2 microglial cells was checked by flow cytometry as described earlier (14,15). Briefly, 1 X 10⁶ cells suspended in RPMI 1640-FBS were incubated in the dark with appropriately diluted FITC-labeled antibodies to CD11b (Mac-1 Integrin) (BD Pharmingen) at 4°C for 30 min. Following incubation, cell suspension was centrifuged, washed thrice and resuspended in 500 µl of RPMI-FBS. The cells were then analyzed through FACS (Beckton Dickinson). A minimum of 10,000 cells was accepted for FACS analysis. Cells were gated based on morphological characteristics. Apoptotic and necrotic cells were not accepted for FACS analysis.

Immunofluorescence analysis: It was performed as described earlier (16). Briefly, cover slips containing 100-200 cells/mm² were fixed with 4% paraformaldehyde for 20 min followed by treatment with cold ethanol (-20°C) for 5 min and 2 rinses in PBS. Samples were blocked with 3% BSA in PBST for 30 min and incubated in PBST containing 1% BSA and rabbit anti-CD11b (1:50). After three washes in PBST (15 min each), slides were further incubated with Cy2 (Jackson ImmunoResearch Laboratories, Inc.). For negative controls, a set of culture slides was incubated under similar conditions without the primary antibodies. The samples were mounted and observed under a BioRad MRC1024ES confocal laser-scanning microscope.

Microinjection of LPS into the striatum of C57BL/6 mice: Male C57BL/6 mice (8-10 week old) were anesthetized with ketamine and xylazine and underwent cerebellar operations in a Kopf small animal stereotaxic instrument (David Kopf, CA). Briefly, the animal was mounted in a stereotaxic frame on a heating blanket. Body temperature was maintained at 37 ± 0.5°C during the time of surgery. A midsagittal incision was made to expose the cranium and a hole <0.5 mm in diameter was drilled with a dental drill over the cerebrum according to the following coordinates: 5 mm anterior to lambda, lateral (L) 2.2 mm, ventral (V) 3.5 (as shown in Fig. 6A). Four microgram of
LPS in the presence or absence of L-NIL (10 μg) dissolved in a 3 μl saline was injected using a 5 μl syringe (Hamilton, Reno, Nevada) over a period of 2 min and the needle was held in place for another min before withdrawing it from the skull to prevent reflux up the needle tract. Similarly control mice received 3 μl saline. The incision was closed with surgical staples and covered with a mixture of Bacitracin and Hurricane (20% benzocaine).

**Tyrosine hydroxylase immunostaining:** Five days after microinjection, mice were perfused with 4% paraformaldehyde and their brains were processed for immunohistochemical studies. Sections (10 μm) were incubated with a polyclonal anti-tyrosine hydroxylase (TH; 1,000 dilution; Calbiochem) for 24 h at 4°C. Biotinylated secondary antibodies followed by avidin-biotin complex were used. Immunoreactivity was visualized by Vectastatin Elite ABC kit (Vector Laboratories, Inc.). Striatal optical density of TH immunostaining, measured by SpotDenso Analysis Tools in Fluorochem 8800 Imaging System, was used as an index of striatal density of TH innervation.

**Measurement of dopamine and its metabolite levels in striatal tissues:** After 5 d of microinjection, mice were sacrificed, and their striata were dissected out and stored at –80°C until analysis. On the day of the assay, striatal tissues were sonicated in 0.2 M perchloric acid (0.5 ml/100 mg tissue) containing isoproterenol (100 ng/100 mg tissue) as internal standard. After centrifugation at 20,000 g for 15 minutes at 4°C, the pH of supernatants was adjusted to pH 3.0 with 1 M sodium acetate. After filtration, 10 μl of supernatant was injected onto an Eicompak SC-3ODS column (Complete Stand-Alone HPLC-ECD System EICOM HTEC-500 from JM Science Inc., Grand Island, NY) and analyzed for dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) following manufacturer’s protocol. Briefly, the mobile phase consisted of 20% methanol and 80% 0.1M Citrate-Acetate buffer (pH 3.5) with 220 mg/l sodium octane sulfate (SOS) and 5 mg/l disodium EDTA. The flow rate was maintained at 340-400 μl/min.

**Semi-quantitative RT-PCR analysis:** Total RNA was isolated from BV-2 microglial cells, primary microglia or striatal tissues surrounding the point of microinjection using Ultraspec-II RNA reagent (Biotech Laboratories, Inc.) following manufacturer’s protocol. To remove any contaminating genomic DNA, total RNA was digested with DNase. Semi-quantitative RT-PCR was carried out as described earlier (17) using a RT-PCR kit from Clontech. Briefly, 1 μg of total RNA was reverse transcribed using oligo(dT)_{12,18} as primer and MMLV reverse transcriptase (Clontech) in a 20 μl reaction mixture. The resulting cDNA was appropriately-diluted, and diluted cDNA was amplified using Titanium Taq DNA polymerase and following primers. Amplified products were electrophoresed on a 1.8% agarose gels and visualized by ethidium bromide staining.

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**CD11b:**

**Sense:** 5’- CAGATCAACAATGTGACCGTATGGG-3’

**Antisense:** 5’- CATCATGTCTTGTACTGCCGCTTG-3’

**IL-1β:**

**Sense:** 5’- CTCCATGAGCTTTGTACAAGG-3’

**Antisense:** 5’- TGCTGTAGTACCAGTTGGG-3’

**GAPDH:**

**Sense:** 5’- GTGAAGGTCGGTGTGAACG-3’

**Antisense:** 5’- TTGGCTCCACCCTCAAATG-3’

The relative expression of either CD11b or IL-1β (CD11b or IL-1β/GAPDH) was measured after scanning the bands with a Fluor Chem 8800 Imaging System (Alpha Innotech Corporation).

**Real-time PCR analysis for CD11b mRNA:** It was performed using the ABI-Prism7700 sequence detection system (Applied Biosystems) as described earlier (17). Briefly, reactions were performed in a 96-well optical reaction plates on cDNA equivalent to 50 ng DNase-digested RNA in a volume of 25 μl, containing 12.5 μl TaqMan Universal Master mix and optimized concentrations of FAM-labeled probe, forward and reverse primers following manufacturer’s protocol. All primers and FAM-labeled probes for mouse CD11b and GAPDH were obtained from Applied Biosystems. The mRNA expression of CD11b was normalized to the level of GAPDH mRNA. Data were processed by the ABI Sequence Detection System 1.6 software and analyzed by ANOVA.

**Electrophoretic mobility shift assay (EMSA):** Nuclear extract preparation and EMSA was
performed as described previously with some modifications (15,18). Briefly, oligonucleotides containing the consensus binding sequence for CREB (5’-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3’) (Promega) was radiolabeled with [γ-32P]ATP using polynucleotide T4 kinase. Labeled probe was purified with chroma spin column (BD Biosciences). Six-microgram nuclear extract was incubated with binding buffer and non-specific oligonucleotides for 15 minutes in ice prior to incubation with labeled probe for another 15 minutes. Subsequently, samples were separated on a 6% polyacrylamide gel in 0.25X TBE buffer, which were then dried and exposed to generate autoradiograms.

RESULTS

Bacterial lipopolysaccharides (LPS) increase the expression of CD11b in mouse BV-2 microglial cells via NO: Microglia express CD11b, however, during microglial activation, the expression of CD11b is markedly increased. Because microglial activation is also associated with the production of NO, we investigated the role of NO in microglial expression of CD11b. Mouse BV-2 microglial cells were stimulated with different concentrations of LPS, the prototype inducer of different immune cells including CNS microglia (18,19). It is clear from figure 1A that LPS dose-dependently increased the expression of CD11b in BV-2 cells with maximum increase observed at 0.75 or 1.0 µg/ml of LPS. Assay of nitrite in culture supernatants shows that the increase in CD11b expression was associated with the production of NO (Fig. 1B). Next during time-dependent analysis of LPS-mediated up-regulation of CD11b expression, we observed that LPS was unable to increase the mRNA expression of CD11b within 6 h of stimulation (Fig. 1C). However, the increase in CD11b expression was visible at 12 h with the maximum up-regulation observed at 24 h of stimulation (Fig. 1C). When we measured time-dependent production of nitrite in response to LPS, we observed that the induction of NO production began at 12 h and reached the maximum at 24 h (Fig. 1D). In fact, the expression of CD11b paralleled to the production of NO suggesting a possible involvement of NO in the increase in CD11b expression.

We then compared the expression pattern of another inducible gene in microglia. In contrast to the expression pattern of CD11b, marked induction of IL-1β mRNA was observed within 6 h of stimulation with LPS (Fig. 1C). Because the expression of IL-1β was observed before the increase in CD11b expression, we also investigated if IL-1β was playing a role in LPS-induced expression of CD11b. The time-course of IL-1β-induced CD11b expression shows that IL-1β was unable to stimulate the expression of CD11b within 12 h of stimulation (Fig. 1E). However, at 24 h of stimulation, the up-regulation of CD11b was clearly visible (Fig. 1E) suggesting that IL-1β may not be involved in LPS-induced expression of CD11b.

Therefore, to investigate the role of NO in LPS-mediated up-regulation of CD11b, we examined the effect of L-NIL (an inhibitor of NOS) and carboxy PTIO (a scavenger of NO) on LPS-mediated increase in CD11b mRNA expression in BV-2 glial cells. It is clearly evident from semi-quantitative RT-PCR analysis that both L-NIL and PTIO markedly inhibited LPS-mediated expression of CD11b (Fig. 1F & 1G). Quantitative real-time PCR analysis also reveals marked inhibition of LPS-mediated expression of CD11b mRNA by L-NIL and PTIO (Fig. 1H). Next we investigated the effect of L-NIL and PTIO on the expression of CD11b protein in LPS-stimulated cells. Because CD11b is a surface protein, we analyzed its expression by FACS using FITC-labeled antibodies against CD11b. Figure 2A represents auto-fluorescence as this was observed in unconjugated normal BV-2 glial cells. As areas under M1 and M2 in figure 2A-E represent auto-fluorescence and fluorescence due to CD11b respectively, there was some expression of CD11b on the surface of normal BV-2 glial cells (Fig. 2B) in contrast to marked increase in CD11b expression on the surface of LPS-stimulated cells (Fig. 2C). Consistent to the inhibition of CD11b mRNA expression, both L-NIL and PTIO markedly inhibited LPS-mediated stimulation of CD11b protein expression (Fig. 2D & E). Immunofluorescence analysis of CD11b in BV-2 microglial cells also shows that LPS stimulation increased the expression of CD11b and that L-NIL and PTIO attenuated LPS-mediated CD11b expression (Fig. 2F). Taken together, these studies
suggest that LPS up-regulates the expression of CD11b in BV-2 microglial cells via NO.

**LPS increases the expression of CD11b in mouse primary microglia via NO:** To understand if NO is required for the increase in CD11b expression by LPS in primary cells, we examined the effect of L-NIL and PTIO on LPS-mediated expression of CD11b in mouse primary microglia (Fig. 3). Consistent to the induction of NO production and increase in CD11b mRNA expression in BV-2 microglial cells, LPS induced the production of NO (Fig. 3A) and increased the expression of CD11b mRNA as revealed by semi-quantitative RT-PCR (Fig. 3B) and quantitative real-time PCR analyses (Fig. 3C) in mouse primary microglia. However, either blocking the production of NO by L-NIL or scavenging NO by PTIO (Fig. 3A) markedly suppressed LPS-mediated stimulation of CD11b mRNA expression (Fig. 3B&C) in primary microglia. Apart from CD11b, microglia express many other surface markers such as, CD18, CD11a, CD11c etc. In addition, CD11b pairs with CD18 to exhibit functional activity, therefore, we wondered if NO was also regulating the expression of other surface markers including CD18. In mouse primary microglia, LPS increased the expression of CD18, CD11a and CD11c compared to control (Fig. 3D). Interestingly, similar to the regulation of CD11b, both L-NIL and PTIO suppressed LPS-mediated increase in CD18, CD11c and CD11a expression in primary microglia (Fig. 3D) suggesting that NO is capable of regulating the expression of various surface markers of microglia associated with its activation.

**Involvement of NO in proinflammatory cytokine-, double-stranded RNA (dsRNA)- and HIV-1 gp120-mediated increase in CD11b expression in BV-2 microglial cells:** Microglia are activated under various pathological conditions, such as inflammation, viral infection etc (1,20,21). Because LPS increased the expression of CD11b in microglia through NO, we investigated whether other inducers of microglial activation also increase CD11b expression via NO. Therefore, BV-2 microglial cells were stimulated with proinflammatory cytokines (IL-1β and IFN-γ), HIV-1 coat protein gp120 (22) and double-stranded RNA in the form of polyinosinic-polycytidilic acid (polyIC) (23) in the presence or absence of L-NIL and PTIO. All four stimuli (IFN-γ, IL-1β, gp120, and polyIC) induced the production of NO and increased the expression of CD11b in BV-2 microglial cells (Fig. 4). Similar to the inhibition of LPS-mediated expression of CD11b, either blocking the production of NO by L-NIL or scavenging NO by PTIO knocked down IFN-γ-, IL-1β-, gp120-, and polyIC-mediated increase in CD11b mRNA expression (Fig. 4A-D) suggesting that different neuroinflammatory and neurodegenerative stimuli also up-regulate the expression of CD11b in microglia via NO.

**LPS-mediated increase in CD11b expression in vivo in the striatum depends on NO:** Using various approaches, the studies presented above have shown that different neurotoxins increase the expression of CD11b in microglial cells or cultured primary microglia via NO. However, these studies do not indicate whether NO may have the capacity to influence the expression of CD11b in vivo in the CNS. It is increasingly becoming clear that microglial activation plays an important role in the loss of dopaminergic neurons in striatum and nigra of patients with Parkinson’s disease (3-5). Therefore, we were prompted to investigate if NO is also involved in the up-regulation of CD11b expression in vivo in the striatum. As expected, microinjection of LPS but not saline into the striatum of 8- to 10-week old male C57BL/6 mice induced the expression of iNOS (Fig. 5B) and CD11b (Fig. 5C&D). Next to analyze the role of NO in LPS-mediated expression of CD11b, PTIO was microinjected together with LPS. As revealed by semi-quantitative RT-PCR (Fig. 5C) and quantitative real-time PCR (Fig. 5D), PTIO knocked down LPS-mediated increase in CD11b expression in vivo in the striatum (Fig. 5C&D). This has been further supported by immunofluorescence analysis. Increased number of CD11b+ microglia was observed in LPS-microinjected brain sections compared to that in saline-microinjected sections (Fig. 5E). However, co-microinjection of PTIO markedly inhibited LPS-mediated increase in CD11b+ microglia (Fig. 5E) suggesting the requirement of NO in LPS-induced microgliosis in vivo in the brain.

**LPS-mediated loss of dopaminergic neuronal fibers and neurotransmitters in vivo in the striatum also depends on NO:** Because NO is involved in microglial expression of CD11b, and microglial activation plays an important role in the loss of dopaminergic neurons in midbrain (3-5), we investigated if mitigation of microglial activation
by PTIO had any effect on the integrity of striatal dopaminergic fibers in LPS-intoxicated striatum. We stained fibers of dopaminergic neurons in striatum by tyrosine hydroxylase (TH) immunoreactivity. As evident from figure 6A&B, TH+ neuronal fibers are present in saline-microinjected striatum. However, microinjection of LPS caused marked loss of TH+ fibers in striatum (Fig. 7A&B). On the other hand, PTIO exhibited almost complete protection against LPS-mediated loss of TH+ fibers in striatum (Fig. 6A&B) suggesting the possible involvement of NO in inflammation-coupled striatal loss of TH-containing fibers. To further corroborate these results, we measured the levels of dopamine (DA) and its metabolites – dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in vivo in the striatum as a functional index of dopaminergic system. In LPS-microinjected mice that did not receive any PTIO, there was marked reduction of DA and DOPAC but not HVA compared with vehicle control (Table-1). However, in LPS-microinjected mice that did receive PTIO, the levels of DA and DOPAC were all significantly higher than those in LPS-microinjected mice that did not receive PTIO (Table-1). Taken together, these results suggest that attenuation of NO production in vivo in the striatum is capable of protecting LPS-mediated loss of TH+ fibers and neurotransmitter levels probably by reducing microglial activation.

_S-nitroso glutathione (GSNO) alone increases the expression of CD11b in BV-2 microglial cells:_ Because LPS and other inducers of iNOS increased the expression of CD11b via NO, next we investigated if NO alone was capable of increasing the expression of CD11b in microglia. Therefore, we examined the effect of GSNO, a NO donor, on CD11b expression in BV-2 microglial cells. As evident from semi-quantitative RT-PCR analysis (Fig. 7A) and quantitative real-time PCR analysis (Fig. 7B), GSNO alone dose-dependently increased the expression of CD11b mRNA with maximum increase observed at 200 or 500 μM. These results suggest that NO alone is sufficient to increase the expression of CD11b in microglia. Because we are considering NO as a possible mediator of LPS-induced increased expression of CD11b, we performed a time-course experiment for GSNO-mediated increase in CD11b expression. While LPS-mediated up-regulation of CD11b was visible after 12 h or 18 h of stimulation (Fig. 1C), GSNO was capable of increasing the expression of CD11b within 60 min of stimulation (Fig. 7C) suggesting that NO functions downstream to LPS in regulating the expression of CD11b in microglia. Next we investigated the possibility whether NO regulates the expression of CD11b at the level of transcription or post-transcription. Inhibition of GSNO-mediated increase in CD11b expression by actinomycin D (an inhibitor of RNA synthesis) (Fig. 7D) suggests that NO-mediated up-regulation of CD11b is possibly a transcriptional event.

_Involvement of guanylate cyclase (GC) in LPS- and GSNO-mediated increase in CD11b expression in microglia:_ Next we investigated mechanisms by which NO may up-regulate the expression of CD11b in microglia. Because GC is intimately coupled to NO-induced downstream signaling events (24,25), we attempted to explore the role of GC in LPS- and GSNO-mediated increase in CD11b expression in microglial cells. We examined the effect of NS-2028, a very specific inhibitor of GC (26), on LPS- and GSNO-mediated expression of CD11b in BV-2 microglial cells. As evident from semi-quantitative RT-PCR analyses (Fig. 8A&C) and real-time PCR analysis (Fig. 8B&D), NS-2028 markedly inhibited LPS- and GSNO-mediated increase in CD11b expression in microglial cells. Time-course experiment showed that 8-Br cGMP alone was capable of increasing the expression of CD11b within 60 min of stimulation (Fig. 9B). Next the effect of MY5445 on the expression of CD11b was tested in BV-2 microglial cells. As expected, MY5445 alone also markedly increased the expression of CD11b (Fig. 9C). These results suggest that LPS increases microglial expression of CD11b via NO-GC-cGMP pathway.
Role of cGMP-activated protein kinase (PKG) in LPS- and GSNO-mediated up-regulation of CD11b mRNA expression in microglia: Because cGMP transduces many of its downstream signaling pathways via PKG (27), we were prompted to investigate if PKG was involved in LPS- and NO-mediated increase in CD11b expression in microglia. It is clearly evident from figure 10A&B that KT5823, a specific cell-permeable inhibitor of PKG (28), dose-dependently attenuated LPS-mediated up-regulation of CD11b mRNA with maximum inhibition observed at 25 or 50 μM. Similarly, semi-quantitative RT-PCR (Fig. 10C) and quantitative real-time PCR (Fig. 10D) analyses also reveal marked inhibition of GSNO-mediated expression of CD11b mRNA by KT5823 in BV-2 microglial cells. To confirm the involvement of PKG further, we examined the effect of Rp-8-Br-cGMP (another specific inhibitor of PKG) on NO-mediated increase in CD11b expression. As evident from figure 10E, Rp-8-Br-cGMP strongly inhibited GSNO-mediated increase in CD11b expression. These results suggest that LPS increases microglial expression of CD11b via NO-GC-cGMP-PKG pathway.

Role of cAMP-response element-binding protein (CREB) in NO-mediated up-regulation of CD11b mRNA expression in microglia: Next we decided to find out mechanism(s) that possibly couple NO-GC-cGMP-PKG pathway to the expression of CD11b. Using the MatInspector V2.2 search machinery; we have found the presence of six CREs in the promoter of mouse CD11b gene. Therefore, we hypothesized if CREB was involved in NO-mediated up-regulation of CD11b downstream of GC-cGMP-PKG. Activation of CREB was monitored by DNA-binding activity that was evaluated by the formation of a distinct and specific complex in a gel shift DNA-binding assay. Treatment of BV-2 microglial cells with 200 μM GSNO resulted in time-dependent induction of DNA binding activity of CREB (Fig. 11A). This gel shift assay detected a specific band in response to GSNO that was competed off by an unlabeled probe suggesting that GSNO induces the DNA-binding activity of CREB. Consistently, GSNO (200 μM) alone also induced the transcriptional activity of CREB as monitored by a 3.3±0.4 fold increase in luciferase activity from pCRE-Luc (CREB-dependent reporter construct; Stratagene) by GSNO over the control (data not shown). Because PKG functions downstream to NO in the regulation of CD11b expression, we then tested if NO was inducing the activation of CREB via PKG. Inhibition of GSNO-induced activation of CREB by KT5823 (Fig. 11B) suggests that NO is inducing the activation of CREB via PKG. Next to investigate if CREB is involved in NO-mediated up-regulation of CD11b, we used antisense oligonucleotides to knockdown CREB. Following antisense (ASO) and scrambled (ScO) oligonucleotides were used for this study. CREB ASO: 5’-GTC TGC TCC AGA TTC-3’ CREB ScO: 5’-GAT CCC GAT TCG TCT-3’ As shown in figure 11C, ASO but not ScO against CREB inhibited the expression of CREB mRNA. Interestingly, ASO but not ScO against CREB abrogated GSNO-mediated up-regulation of CD11b mRNA (Fig. 11D). These results suggest that LPS increases microglial expression of CD11b via NO-GC-cGMP-PKG-CREB pathway.

DISCUSSION

Although the substantia niagra has an extremely high density of resting microglia (29), in general, microglia comprise of only 2 to 5% of total brain cells in healthy human being (1). However, after any degenerative injury or insult, microglial number increases dramatically and microglial population may represent up to 12% of total brain cells (1,21). Therefore, common pathological hallmarks of several neurodegenerative diseases include the loss of invaluable neurons associated with or followed by massive activation of microglia (1,21,30). Although microglial activation has an important repairing function, once microglia become activated in neurodegenerating microenvironment, it always goes beyond control and eventually detrimental effects override beneficial effects. Therefore, understanding mechanisms that regulate microglial activation is an important area of investigation that may enhance the possibility of finding a primary or an adjunct therapeutic approach against incurable neurodegenerative disorders. Microglial activation is represented by increased expression of CD11b. Several lines of evidence presented in this manuscript clearly demonstrate that NO plays a key role in microglial expression of CD11b. First, in LPS-stimulated microglial cells, the increase in CD11b expression began after the production of NO. Second, LPS and other inducers of iNOS
(IFN-γ, IL-1β, HIV-1 gp120, and poly IC) were unable to stimulate the expression of CD11b in microglia where either NO was scavenged by PTIO or NO production was inhibited by L-NIL. Similarly, microinjection of LPS into the striatum caused marked up-regulation of CD11b. Consistent to the effect observed in isolated microglia, L-NIL and PTIO strongly inhibited LPS-mediated increase in striatal CD11b expression in vivo. Microglial activation is believed to play a pivotal role in the loss of dopaminergic neurons in striatum and nigra of patients with Parkinson’s disease. This loss of dopaminergic neurons then leads to deficiency in dopamine (DA), a chemical substance that enables people to move normally and smoothly. Interestingly, attenuation of LPS-mediated increase in striatal CD11b expression by PTIO was also associated with protection against LPS-induced deficits of striatal dopaminergic system as observed by restoration of TH-positive fibers and neurotransmitters. Third, NO alone was also capable of stimulating the expression of CD11b in microglial cells. Microglia in the healthy brain usually do not express iNOS but following ischemic, traumatic, neurotoxic, or inflammatory damage, microglia express iNOS and produce excessive amount of NO in mouse, rat, and human (14,18,19,21,30,31). Our results clearly indicate that the up-regulation of CD11b expression in reactive microglia follows NO production. Apart from CD11b, up-regulation of various other surface markers such as, CD18, CD11a and CD11c is observed in reactive microglia. Interestingly, NO is also involved in the up-regulation of these surface markers in microglia.

The signaling mechanisms for the increase in CD11b expression in microglia are not known. Because the up-regulation of microglial CD11b depends on NO, and among many downstream targets of NO, activation of guanylate cyclase (GC) represents the most important one due to its involvement in many physiological processes including vasodilation, modulation of synaptic transmission and inhibition of platelet aggregation (24,25), we were prompted to explore the role of GC in microglial expression of CD11b. Interestingly, our results demonstrate that NO empolys GC to stimulate the expression of CD11b in microglia. Our conclusion is based on the following. First, LPS as well as GSNO, a NO donor, was unable to increase the expression of CD11b in microglia that was treated with NS-2028, an inhibitor of GC. Second, cell-permeable cGMP analog (8-Br cGMP) and MY-5445, an inhibitor of cGMP phosphodiesterase, were also capable of stimulating microglial expression of CD11b. Third, cGMP is known to execute its function via cGMP-activated protein kinase (PKG). Consistently, KT5823, a specific inhibitor of PKG, attenuated LPS- as well as GSNO-mediated increase in CD11b expression. Fourth, the promoter of mouse CD11b contains six cAMP response elements (CRE). GSNO induced the activation of CRE-binding protein (CREB) via PKG in microglia and earlier Chan et al (32) have reported possible phosphorylation of CREB by PKG in nucleus tractus solitarii. Interestingly, antisense knockdown of CREB abrogated GSNO-mediated up-regulation of microglial CD11b suggesting that NO up-regulates the expression of CD11b through GC-cGMP-PKG-mediated activation of CREB. Because microglial activation is involved in the pathophysiology of several neurodegenerative disorders, our results suggest that NO-coupled GC-cGMP-PKG-CREB signaling pathway may also be an important player under neurodegenerative conditions.

NO, a short-lived and diffusible free radical, plays many roles as a signaling and effector molecule in diverse biological systems; it is a neuronal messenger and is involved in vasodilation as well as in antimicrobial and antitumor activities (33). On the other hand, NO has also been implicated in several CNS disorders, including inflammatory, infectious, traumatic, and degenerative diseases (34-38). There are considerable evidences for the transcriptional induction of iNOS (the high-output isoform of NOS) in the CNS that is associated with autoimmune reactions, acute infection, and degenerative brain injury (34-38). NO is potentially toxic to neurons and oligodendrocytes that may mediate toxicity through the formation of iron-NO complexes of iron-containing enzyme systems (39), oxidation of protein sulphhydryl groups (40), nitration of proteins, and nitrosylation of nucleic acids and DNA strand breaks (41). Here we demonstrate that NO is a key player in microglial activation as well, in which NO increases microglial expression of CD11b. Therefore, specific targeting of NO either by iNOS inhibitors or NO scavengers may be an important
step for the attenuation of microglial activation.

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FOOTNOTES
This study was supported by grants from National Institutes of Health (NS39940 and NS48923), National Multiple Sclerosis Society (RG3422A1/1) and Michael J. Fox Foundation for Parkinson’s Research.

Table-1. Levels of dopamine and its metabolites in striatal tissues

| Treatments       | DA  | DOPAC | HVA  |
|------------------|-----|-------|------|
| Control/Vehicle  | 11.2| 0.82  | 1.2  |
| LPS              | 5.7 | 0.45  | 1.02 |
| LPS + L-NIL      | 12.8| 0.76  | 1.12 |
| LPS + PTIO       | 18.9| 1.05  | 1.32 |

Five days after microinjection, mice were sacrificed, and levels of dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured in striatum as described under “Materials and Methods”. Data represents mean ± SD of six mice per group. * p < 0.001 compared with the LPS-treated group without L-NIL and PTIO.

FIGURE LEGENDS

Fig. 1. Role of NO in LPS-mediated up-regulation of CD11b expression in mouse BV-2 microglial cells. Cells were stimulated with different concentrations of LPS under serum-free condition. After 24 h of stimulation, the expression of CD11b was analyzed in cells by semi-quantitative RT-PCR (A) and concentration of nitrite was measured in supernatants (B) by ‘Griess’ reagent as described under “Materials and Methods”. Next cells were stimulated with 0.75 µg/ml of LPS under serum-free condition for different time period followed by analysis of CD11b and IL-1β mRNA expression in cells (C) and assay of nitrite (D) in supernatants. Cells were stimulated with 10 ng/ml of IL-1β for different time period followed by analysis of CD11b mRNA expression by RT-PCR (E). Cells preincubated with different concentrations of L-NIL (F) and PTIO (G) for 1 h were stimulated with 0.75 µg/ml of LPS. After 24 h of stimulation, the expression of CD11b mRNA was analyzed in cells by semi-quantitative RT-PCR. Quantitative real-time PCR (H) was also employed to further clarify the mRNA expression of CD11b. Results are mean ± S.D. of three different experiments. * p < 0.001 vs LPS.

Fig. 2. Effect of L-NIL and PTIO on LPS-mediated up-regulation of CD11b protein expression in mouse BV-2 microglial cells. Cells preincubated with L-NIL (75 µM) and PTIO (75 µM) for 1 h were stimulated with 0.75 µg/ml of LPS. After 24 h of stimulation, cells were also analyzed for surface expression of CD11b protein by FACS (A, unconjugated control; B, conjugated control; C, LPS; D, LPS
Effect of L-NIL and PTIO on LPS-mediated increase in CD11b and other surface marker expression in mouse primary microglia. Cells preincubated with L-NIL (75 μM) and PTIO (75 μM) for 1 h were stimulated with 0.75 μg/ml of LPS. After 24 h of stimulation, the production of nitrite was assayed in supernatants (A), and the expression of CD11b mRNA was analyzed in cells by semi-quantitative RT-PCR (B) and quantitative real-time PCR (C). Results are mean ± S.D. of three different experiments. *p < 0.001 vs LPS. D) The expression of CD18, CD11c and CD11a was also analyzed by semi-quantitative RT-PCR.

Fig. 4. Effect of L-NIL and PTIO on IFN-γ-, IL-1β-, HIV-1 gp120-, and poly IC-mediated increase in CD11b expression in mouse BV-2 microglial cells. Cells preincubated with L-NIL (75 μM) and PTIO (75 μM) for 1 h were stimulated with 6.25 U/ml of IFN-γ (A), 10 ng/ml of IL-1β (B), 200 pg/ml of gp120 (C), or 100 μg/ml of poly IC (D). After 24 h of stimulation, the expression of CD11b mRNA was analyzed in cells by semi-quantitative RT-PCR and concentration of nitrite was measured in supernatants. Results are mean ± S.D. of three different experiments.

Fig. 5. Effect of PTIO on LPS-mediated up-regulation of CD11b expression in vivo in mouse striatum. Four micrograms of LPS in the presence or absence of PTIO (10 μg) dissolved in 3 μl saline was stereotaxically injected into striatum (A) of C57/BL6 mice. After 24 h of microinjection, the expression of iNOS protein was examined by immunofluorescence analysis (B) and the expression of CD11b mRNA was analyzed by semi-quantitative RT-PCR (C) and quantitative real-time PCR (D). Results are mean ± S.D. of three different experiments. *p < 0.001 vs LPS. Brain sections were also immunostained for the expression of CD11b protein (E).

Fig. 6. Effect of PTIO on LPS-induced loss of TH-positive fibers in vivo in the striatum. Four micrograms of LPS in the presence or absence of PTIO (10 μg) dissolved in 3 μl saline was stereotaxically injected into striatum of C57/BL6 mice. After 5 d of microinjection, TH immunostaining (A) and optical density of TH+ fibers (B) were performed as described under “Materials and Methods”. Results are mean ± S.D. of five different mice. *p < 0.001 vs saline control; **p < 0.001 vs LPS.

Fig. 7. GSNO increases the expression of CD11b in mouse BV-2 microglial cells. Cells were stimulated with different concentrations of GSNO under serum-free condition. After 24 h of stimulation, the expression of CD11b was analyzed in cells by semi-quantitative RT-PCR (A) and quantitative real-time PCR (B). Results are mean ± S.D. of three different experiments. *p < 0.001 vs control. C) Cells were stimulated with 200 μM GSNO for different time period followed by analysis of CD11b mRNA expression. D) Cells preincubated with different concentrations of actinomycin D for 15 min were stimulated with 200 μM GSNO. After 1 h of stimulation, the expression of CD11b mRNA was analyzed by semi-quantitative RT-PCR.

Fig. 8. Effect of NS-2028 on LPS- and GSNO-mediated increase in CD11b expression in mouse BV-2 microglial cells. Cells preincubated with different concentrations of NS-2028 for 1 h were stimulated with either 0.75 μg/ml LPS (A&B) or 200 μM GSNO (C&D) under serum-free condition. After 24 h of stimulation, the expression of CD11b was analyzed in cells by semi-quantitative RT-PCR (A&C) and quantitative real-time PCR (B&D). Results are mean ± S.D. of three different experiments. *p < 0.001 vs LPS. **p < 0.001 vs GSNO.

Fig. 9. 8-Br-cGMP and MY-5445 increase the expression of CD11b in mouse BV-2 microglial cells. A) Cells were stimulated with different concentrations of 8-Br cGMP and after 24 h, the expression of CD11b was analyzed by semi-quantitative RT-PCR. B) Cells were stimulated with 100 μM 8-Br cGMP for different time period followed by analysis of CD11b mRNA expression. C) Cells were stimulated with different concentrations of MY-5445 for 24 h followed by analysis of CD11b mRNA expression.

Fig. 10. Effect of inhibitors of protein kinase G (PKG) on NO-mediated increase in CD11b expression in mouse BV-2 microglial cells. Cells preincubated with different concentrations of KT5823 for 1 h were stimulated with either 0.75 μg/ml LPS (A&B) or 200 μM GSNO (C&D) under serum-free condition. After 24 h of stimulation, the expression of CD11b was analyzed in cells by semi-quantitative
RT-PCR (A&C) and quantitative real-time PCR (B&D). Results are mean ± S.D. of three different experiments. a $p < 0.001$ vs LPS. b $p < 0.001$ vs GSNO. E) Cells preincubated with different concentrations of Rp-8-Br-cGMP (Rp) for 1 h were stimulated with 200 μM GSNO for 24 h followed by analysis of CD11b mRNA expression by semi-quantitative RT-PCR.

Fig. 11. **Involvement of CREB in NO-mediated increase in CD11b expression in mouse BV-2 microglial cells.** A) Cells were stimulated with 200 μM GSNO under serum-free condition and at different minute intervals, the DNA-binding activity of CREB was analyzed by EMSA. In the last lane, the DNA-binding activity of nuclear extract (90 min) was competed out by 50-fold excess unlabeled probe. The upper and lower arrows indicate CREB DNA-binding and unbound probe respectively. B) Cells preincubated with different concentrations of KT5823 for 1 h were stimulated with 200 μM GSNO under serum-free condition. After 1 h of stimulation, the DNA-binding activity of CREB was analyzed by EMSA. Figures represent three independent experiments. C) Cells received 1 μM of either antisense (ASO) or scrambled oligonucleotides (ScO) against CREB. After 42 h of incubation, cells were analyzed for the expression of CREB mRNA by semi-quantitative RT-PCR. D) Cells preincubated with 1 μM ASO or ScO against CREB for 42 h received 200 μM GSNO. After 1 h of stimulation, the expression of CD11b was analyzed by semi-quantitative RT-PCR.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
**Fig. 7**

**A**

| GSNO (µM) | 0 | 100 | 200 | 500 |
|-----------|---|-----|-----|-----|
| **CD11b** |   |     |     |     |
| **GAPDH** |   |     |     |     |

**B**

![Bar graph showing relative expression](image)

**C**

| Time (min) | 0 | 30 | 60 | 90 | 120 |
|------------|---|----|----|----|-----|
| **CD11b**  |   |    |    |    |     |
| **GAPDH**  |   |    |    |    |     |

**D**

| GSNO (200 µM) | - | + | + | + |
|---------------|---|---|---|---|
| Actinomycin D (µM) | 0 | 0 | 5 | 10 |
| **CD11b**     |   |   |   |   |
| **GAPDH**     |   |   |   |   |
Fig. 8
Fig. 9

8-Br cGMP (μM) → 0  25  50  100  200

A

| Time (min) | 0 | 30 | 60 | 90 | 120 |
|------------|---|----|----|----|-----|

B

MY-5445 (μM) → 0  1  2  5

C

CD11b

GAPDH

CD11b

GAPDH
**Fig. 11**

Unlabeled probe

Time (min) → 0 30 60 90 120 90

GSNO (200 μM) → - + + +

KT5823 (μM) → 0 0 10 25

Control CREB AsO CREB ScO

C

CREB

GAPDH

Control GSNO (200 μM) GSNO + CREB AsO GSNO + CREB ScO

D

CD11b

GAPDH
Up-regulation of microglial CD11b expression by nitric oxide
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J. Biol. Chem. published online March 20, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M600236200

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