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Reactive oxygen species up-regulate CD11b in microglia via nitric oxide: Implications for neurodegenerative diseases

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Abstract

Microglial activation is considered as a hallmark of several neurodegenerative disorders. During microglial activation, the expression of CD11b, the beta-integrin marker of microglia, is increased. However, the molecular mechanism behind increased microglial CD11b expression is poorly understood. The present study was undertaken to explore the role of reactive oxygen species (ROS) in the expression of CD11b in microglial cells. Bacterial lipopolysaccharide (LPS) stimulated the expression of CD11b in mouse BV-2 microglial cells and primary microglia, the effect that was blocked by antioxidants such as *N*-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC). Furthermore, comicroinjection of either NAC or PDTC with LPS was also able to suppress LPS-stimulated expression of CD11b in striatum in vivo. Similarly, other neurotoxic molecules, such as interleukin-1 β (IL-1 β), IL-12 p40₂, fibrillar amyloid- β (A β) peptides, HIV-1 gp120, and double-stranded RNA (poly(IC)), also stimulated the expression of CD11b in microglia through the involvement of ROS. Complete inhibition of LPS-stimulated expression of CD11b by catalase, induction of CD11b expression by H₂O₂ alone, and inhibition of superoxide-stimulated CD11b expression by catalase suggest that H₂O₂, but not superoxide, is in fact involved in the expression of CD11b. Interestingly, we also demonstrate that ROS stimulated the expression of CD11b after the induction of nitric oxide (NO) production and failed to stimulate CD11b when NO production was inhibited by either 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) or L-N⁶-(1-iminoethyl)-L-lysine (L-NIL). Taken together, these studies suggest that the up-regulation of CD11b in microglia is redox sensitive and that ROS up-regulates CD11b via NO.

Keywords

Microglial activation; CD11b; Redox; Nitric oxide; Neurodegeneration

Introduction

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 [2–4]. Although activated microglia scavenge dead cells from the CNS and secrete different neurotrophic factors for neuronal survival [5,6], it is believed that severe activation causes inflammatory responses leading to neuronal death and brain injury [7,8]. During activation, microglia secrete various neurotoxic molecules and express different proteins and surface markers. Among different microglial surface markers, CD11b is the most important one with biological significance. It acts as a binding protein for intracellular cell adhesion molecule-1 (ICAM-1) and complement C3b [9,10]. It is reported that in various neuroinflammatory diseases, the increased CD11b expression corresponds to the severity of microglial activation [11,12]. Microglial activation is usually associated with rounded and amoeboid cells with increased expression of CD11b. During this activation process, the cytoplasmic domain of CD11b is believed to interact increasingly with cytoskeletal protein [13]. However, the mechanism by which microglial expression of CD11b is increased in neurodegenerative CNS remains unclear.

Previously, we reported that nitric oxide (NO) stimulates the expression of CD11b via the sGC-cGMP-PKG-CREB pathway [14]. Because activated microglia also produce reactive oxygen species (ROS) [15,16] and diffusible ROS are implicated virtually in all reported neurodegenerative and neuroinflammatory conditions, we were prompted to investigate if ROS play a role in microglial expression of CD11b. Here we report that ROS is instrumental in regulating the expression of CD11b in microglia. Different pathogenic stimuli such as lipopolysaccharide (LPS), IL-1 β , fibrillar amyloid- β , HIV-1 gp120, and poly(IC) increased microglial expression of CD11b via ROS. Furthermore, we also demonstrate that ROS up-regulated CD11b expression via NO. Interestingly, ROS failed to stimulate microglial CD11b when either NO was scavenged by PTIO or the activity of inducible nitric oxide synthase (iNOS) was inhibited by L-NIL. This study illustrates a novel role of ROS in increasing the expression of CD11b in microglia via NO that may participate in the pathogenesis of neurodegenerative disorders.

Materials and methods

Reagents

Fetal bovine serum and DMEM/F-12 were from Mediatech Inc. L-NIL and carboxy-PTIO were purchased from Biomol. LPS (*Escherichia coli*), poly(IC), *N*-acetylcysteine (NAC), pyrrolidine dithiocarbamate (PDTC), diamide, hypoxanthine, xanthine oxidase, aminotriazole, 7-nitroindazole (7-NINA), and 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT) were purchased from Sigma. HIV-1 coat protein gp120 was obtained from US Biologicals. Recombinant mouse IL-12 p40 homodimer (p40₂) and human IL-1 β were obtained from R and D. SIN-1 (3-morpholinosydnonimine), catalase (bovine liver), and superoxide dismutase (bovine liver) were purchased from Calbiochem.

Preparation of fibrillar A β

Fibrillar A β 1–42 and control reverse peptide A β 42-1 (Bachem Bioscience, CA) were prepared by incubating freshly solubilized peptides at 50 μ M in sterile distilled water at 37 °C for 5 days as described earlier [17].

Isolation of mouse primary microglia

Microglia were isolated from mixed glial cultures following the procedure of Giulian and Baker [18] as described earlier [19–21]. Briefly, on Days 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 onto new plates for further studies. Ninety to 95% of this preparation was found to be positive for Mac-1 surface antigen. Twenty-four hours after plating, some of these cells showed a well-spread amoeboid morphology and the rest exhibited an elongated processed morphology. Mouse BV-2 microglial cells (kind gift from Virginia Bocchini of University of Perugia) were also maintained in the same media.

Stimulation of microglia

Cells plated at 70 to 80% confluence were challenged with LPS (0.5 µg/ml), amyloid-β 1–42 (1 µM), gp120 (200 pg/ml), poly(IC) (100 µg/ml), IL-1β (10 ng/ml), or IL-12 p40₂ (20 ng/ml) for different time periods under serum-free conditions.

Treatment of microglia with antisense oligonucleotides

The following antisense (ASO) and scrambled oligonucleotides (ScO) were used to knock down p22 phox (a subunit of NADPH oxidase): p22phox: ASO, 5'-CGCCAGCGCCTGCTCGTTGGC-3'; and ScO: 5'-GGCCTTCCCGGTTAGCCCCGG-3'. Cells were treated with 1 µg/ml ASO and ScO against p22phox for 36 h in serum-containing media followed by stimulation with LPS under serum-free conditions as described earlier [22].

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, as described earlier [23–25]. Briefly, supernatants were centrifuged to remove cells, and 400 µl of each supernatant was allowed to react with 200 µl of Griess reagent and incubated at room temperature for 15 min. The optical density of the assay samples was measured spectrophotometrically at 570 nm. Fresh culture media served as the blank. Nitrite concentrations were calculated from a standard curve derived from the reaction of NaNO₂ in the assay.

Immunofluorescence analysis

Immunofluorescence analysis was performed as described earlier [14,26]. Briefly, cells cultured in 8-well chamber slides (Lab-Tek II) 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 an Olympus IX81 fluorescent microscope.

Monitoring ROS production in microglial cells

Cells, cultured in 8-well chamber slides, were treated with LPS and other ROS inducers under serum-free conditions. At different time points of stimulation, supernatants were removed and cells were washed with prewarmed HBSS followed by the addition of 100 µl of 25 M carboxy-H₂DCFDA to each well for 30 min of incubation. During the last 5 min of incubation, Hoechst 33342 was added to each well at a dilution of 1:1000 for staining nuclei. Then cells were washed with HBSS, mounted with DPX mounting media, and observed under an Olympus IX81 fluorescent microscope.

Flow cytometry

Surface expression of CD11b on BV-2 microglial cells was checked by two-color flow cytometry as described earlier [27]. Briefly, 1×10^6 cells suspended in RPMI 1640-FBS were incubated in the dark with appropriately diluted FITC-labeled antibodies to CD11b (Mac-1 Integrin) (Chemicon) and PE-labeled annexin V (for monitoring cell death) (Abcam) at 4 °C for 30 min. Following incubation, the cell suspension was centrifuged, washed thrice, and resuspended in 500 μ l 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.

Microinjection of LPS into the striatum of C57BL/6 mice

Male C57BL/6 mice (8–10 weeks old) were anesthetized by 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: anteroposterior (AP) 5 mm, lateral (L) 2.2 mm, ventral (V) 3.5 mm relative to bregma (Fig. 5A). Four micrograms of LPS in the presence or absence of NAC (100 μ g) and PDTTC (6.25 μ g) dissolved in a 3 μ l saline was injected using a 5- μ l syringe (Hamilton, Reno, NV) over a period of 3 min and the needle was held in place for another minute before withdrawing it from the skull to prevent reflux up the needle tract. Similarly control mice received 3 μ l saline. The incision was closed with surgical staples and covered with a mixture of Bacitracin and Hurricane (20% benzocaine).

Semiquantitative 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 (Biotecx Laboratories, Inc.) following the manufacturer's protocol. To remove any contaminating genomic DNA, total RNA was digested with DNase. Semiquantitative RT-PCR was carried out as described earlier [14,28] using a RT-PCR kit from Clontech. Briefly, 1 μ g of total RNA was reverse-transcribed using oligo (dT)_{12–18} as primer and MMLV reverse transcriptase (Clontech) in a 20- μ l reaction mixture. The resulting cDNA was appropriately diluted, and diluted cDNA was amplified using Titanium Taq DNA polymerase and the following primers. Amplified products were electrophoresed on a 1.8% agarose gels and visualized by ethidium bromide staining. CD11b: sense, 5'-CAGATCAACAATGTGACCGTATGGG-3', and antisense, 5'-CAT-CATGTCCTTGTAAGTCCGCTTG-3'; GAPDH: sense, 5'-GGTGAAGGTCGGTGTGAACG-3', and antisense, 5'-TTGGCTCCACCCTTCAAGTG-3'. The relative expression of CD11b (CD11b/GAPDH) was measured after scanning the bands with a Fluor Chem 8800 Imaging System (Alpha Innotech Corporation).

Real-time PCR analysis for CD11b mRNA

Real-time PCR analysis for CD11b mRNA was performed using the ABI-Prism7700 sequence detection system (Applied Biosystems) as described earlier [14,28]. 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 μ l, containing 12.5 μ l TaqMan Universal Master mix and optimized concentrations of FAM-labeled probe, forward and reverse primers following the 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.

Cell viability measurement

Mitochondrial activity was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma).

Results

Bacterial lipopolysaccharides stimulate the expression of CD11b in mouse BV-2 microglial cells via a redox-sensitive pathway

Microglial activation is associated with marked increase in CD11b expression. Because activation of microglia is also involved with the production of ROS, we investigated the role of ROS in microglial expression of CD11b. In our previous study, we have shown that LPS stimulates the expression of microglial CD11b with increasing dose and time [14]. LPS is known to induce various ROS molecules in microglia [29–31]. We also examined if LPS induced the generation of intracellular ROS in BV-2 microglial cells using a cell-permeant fluorescent probe. It is clear from Fig. 1A that the generation of ROS was evident as early as 15 min, exhibiting an apparent peak at 30 min of stimulation by LPS. Next to study the involvement of ROS in LPS-mediated up-regulation of CD11b, we used antioxidants, *N*-acetylcysteine and pyrrolidine dithiocarbamate. It is clearly evident from semiquantitative RT-PCR analysis that both NAC and PDTC were capable of inhibiting LPS-stimulated expression of CD11b mRNA (Figs. 1B and 1D). We also observed the inhibitory effect of NAC and PDTC on LPS-stimulated CD11b mRNA expression by quantitative real-time PCR analysis (Figs. 1C and 1E). Similarly, another antioxidant ascorbate also suppressed the LPS-mediated increase in CD11b mRNA expression (Fig. 1F). It is clear from MTT results in Fig. 1G that the inhibition of the expression of CD11b by antioxidants was not due to any change in the viability of the cells.

Next we investigated the effect of NAC and PDTC on the expression of CD11b protein in LPS-stimulated cells. Because CD11b is a surface protein, we analyzed its expression by flow cytometry using FITC-labeled antibodies against CD11b. To monitor cell viability under various treatment conditions, we also included annexin V-PE. As expected, LPS treatment markedly increased the surface expression of CD11b protein in microglial cells (Fig. 2A). Consistent with the inhibition of CD11b mRNA expression, FACS analysis revealed that both NAC and PDTC markedly inhibited LPS-stimulated expression of CD11b protein on the surface of microglia (Fig. 2A) without altering cell viability. Next we confirmed these results by immunofluorescence. BV-2 microglial cells were incubated with either 0.5mM NAC or 25 μ M PDTC for 1 h prior to the treatment with 0.5 μ g/ml LPS. As evident from Fig. 2B, both NAC and PDTC significantly inhibited the LPS-up-regulated protein expression of CD11b. Taken together, these studies suggest that LPS induces CD11b expression in BV-2 microglial cells via redox-sensitive mechanisms.

LPS also stimulates the expression of CD11b in mouse primary microglia via ROS

Each and every process is not identical between a transformed cell line and corresponding primary cells. Therefore, it is very important that the observations made in a cell line be verified in corresponding primary cells. We investigated if LPS-mediated up-regulation of CD11b was redox sensitive in mouse primary microglia. Cells pretreated with NAC and PDTC were challenged with LPS. Similar to BV-2 microglial cells, both NAC and PDTC markedly inhibited CD11b mRNA expression in LPS-stimulated primary microglia (Fig. 3A). We confirmed the inhibitory effect of NAC and PDTC on LPS-stimulated CD11b mRNA expression by quantitative PCR analysis (Fig. 3B). Consistent with the RT-PCR result, both NAC and PDTC suppressed the expression of CD11b mRNA in LPS-stimulated cells. However, NAC and PDTC had no effect on cell viability in LPS-stimulated primary microglia

(Fig. 3C). These results suggest that LPS stimulates the expression of CD11b in primary microglia via alteration of cellular redox.

Involvement of ROS in amyloid- β -, double-stranded RNA (dsRNA)-, HIV-1 coat protein gp120-, and proinflammatory cytokine-induced expression of CD11b in BV-2 microglial cells

Activated microglia are considered to play an important role in various pathological conditions associated with viral encephalopathy, Alzheimer's disease, Parkinson disease, HIV-associated dementia, multiple sclerosis, etc. [32–34]. Because ROS are involved in LPS-mediated up-regulation of CD11b in microglia, we were prompted to investigate if antioxidants were also capable of negating the up-regulation of CD11b in microglial cells stimulated with etiological reagents of various neurological disorders. BV-2 microglial cells were challenged with fibrillar A β peptides (etiological reagent for AD) [35], HIV-1 coat protein gp120 (one of the etiological reagents for HAD) [36,37], double-stranded RNA in the form of poly(IC) (one of the etiological reagents for viral encephalopathy) [38,39], and proinflammatory cytokines IL-1 β and IL-12 p40₂ (etiological reagents for neuroinflammatory disorders) followed by analysis of CD11b mRNA by semiquantitative RT-PCR (upper panels in Fig. 4) and quantitative real-time PCR (lower panel in Fig. 4). As expected, all these neurotoxins increased the mRNA expression of CD11b in microglia (Fig. 4). However, similar to the regulation of LPS-mediated expression of CD11b, both NAC and PDTC knocked down A β -, gp120-, poly(IC)-, IL-1 β -, and p40₂-mediated up-regulation of microglial CD11b mRNA expression (Figs. 4A, 4B, 4C, 4D, and 4E). MTT results indicated that the suppressive effect of NAC and PDTC was not influenced by cell viability (data not shown). Taken together, these results suggest that etiological reagents of various neurological disorders increase microglial expression of CD11b via redox-sensitive pathway.

LPS-stimulated expression of CD11b in the striatum in vivo is also controlled by ROS

However, these studies do not indicate whether ROS may have the capacity to influence the expression of CD11b mRNA in vivo in the CNS. According to the recent studies, it is clear that microglial activation plays a significant role in the loss of striatal dopaminergic neurons [40,41]. Therefore, we investigated if microglial up-regulation of CD11b in vivo in the striatum was redox sensitive. LPS, alone or together with NAC or PDTC, was microinjected into the striatum (Fig. 5A) of 8- to 10-week-old male C57BL/6 mice. Both semiquantitative RT-PCR (Fig. 5B) and quantitative real-time PCR analysis (Fig. 5C) revealed that NAC and PDTC were capable of reducing LPS-stimulated expression of CD11b in mouse striatum. These results indicate that microglial up-regulation of CD11b in vivo in the CNS is also redox-sensitive and that microglial activation could be reduced by antioxidants.

ROS alone was able to up-regulate the expression of CD11b in BV-2 microglial cells

Because LPS and other proinflammatory disease state inducers stimulated the expression of CD11b via ROS, we investigated whether ROS alone was sufficient to induce microglial CD11b expression. Therefore, we stimulated microglial cells with different prooxidants and oxidants (aminotriazole, diamide, hypoxanthine/xanthine oxidase, and H₂O₂) and examined the mRNA expression of CD11b by semiquantitative RT-PCR (upper panels of Fig. 6) and quantitative real-time PCR (lower panel of Fig. 6). Interestingly, exogenously added hypoxanthine (H) and xanthine oxidase (XO) that is known to produce superoxide radicals increased the expression of CD11b (Fig. 6A). Similarly, hydrogen peroxide dose dependently stimulated the expression of CD11b mRNA with the maximum stimulation observed at 200 μ M or higher concentration of H₂O₂ (Fig. 6B). Aminotriazole, a cell-permeable catalase inhibitor, is capable of increasing the cellular level of H₂O₂. It is clearly evident from Fig. 6C that aminotriazole alone also increased the expression of CD11b. Diamide is known as a thiol group-specific prooxidant that scavenges protons from reduced glutathione and thereby

stimulates oxidative stress by increasing the level of oxidized glutathione. Consistent with other oxidants and prooxidants, diamide alone also stimulated the expression of CD11b in microglia (Fig. 6D).

To understand if ROS were indeed formed endogenously in microglial cells on prooxidant treatment, cells were treated with diamide and the formation of ROS was detected at different time points. As evidenced in Fig. 6E, diamide markedly induced the formation of ROS at different time points of stimulation. Although the production of ROS was not evident at 15 min of stimulation, a significant amount of ROS was visible at 30 min of stimulation (Fig. 6E). However, an intracellular level of ROS was almost at the control level after 2 h of stimulation with diamide (Fig. 6E). Similarly, ATZ treatment also increased the level of intracellular ROS in microglial cells (data not shown). Next to confirm that these prooxidants increased the expression of CD11b via ROS, we examined the effect of NAC and PDTC on prooxidant-mediated increase in CD11b in microglia. It is clearly evident from semiquantitative (upper panels) and real-time PCR (lower panel) analysis in Figs. 7A, 7B, 7C, and 7D that both NAC and PDTC eliminated (H+XO)-, H₂O₂-, diamide-, and aminotriazole-induced expression of CD11b in microglial cells. MTT results show that this inhibitory effect of NAC and PDTC was not influenced by cell viability (data not shown). These results suggest that ROS alone are sufficient to up-regulate microglial CD11b expression.

Involvement of NADPH oxidase in LPS-stimulated expression of CD11b in BV-2 microglia

Next, we attempted to identify the ROS-producing molecule that couples LPS to CD11b in microglia. Because NADPH oxidase is capable of producing ROS (superoxide radicals) in response to different stimuli [29,42–44], we were prompted to investigate if NADPH oxidase was involved in LPS-mediated up-regulation of CD11b in microglia. To verify the involvement of NADPH oxidase, we used antisense oligonucleotides (ASO) against p22phox. At first, we tested the specificity of these ASO. Western blot analysis against p22phox and actin shows that ASO, but not scrambled oligonucleotides (ScO), knocked down the expression of p22phox protein without altering the expression of actin protein in microglial cells (Figs. 8A and 8B). It is clearly evident from semiquantitative RT-PCR analysis in Fig. 8C that ASO, but not ScO, against p22phox markedly reduced LPS-mediated up-regulation of microglial CD11b. These results indicate the possible involvement of NADPH oxidase-generated ROS in LPS-mediated up-regulation of microglial CD11b.

Hydrogen peroxide, but not superoxide, is directly involved in the up-regulation of microglial CD11b

NADPH oxidase catalyzes the formation of both superoxide and hydrogen peroxide. Furthermore, superoxide radicals are also not very stable. Therefore, we decided to investigate which of two ROS was involved in microglial up-regulation of CD11b. We used SOD and catalase to neutralize superoxide and H₂O₂ respectively in the extracellular milieu. It is apparent from quantitative real-time PCR data in Fig. 9A that both SOD and catalase were able to inhibit LPS-induced microglial expression of CD11b. However, catalase was much more potent than SOD in abrogating LPS-mediated increase in CD11b expression (Fig. 9A), indicating the direct involvement of H₂O₂ in LPS-mediated up-regulation CD11b. We investigated the possibility if the commercial preparation of catalase was contaminated with SOD. However, using the SOD activity assay kit (Chemicon, CA) we could not detect any SOD in the catalase preparation (data not shown), suggesting that the effect was due to catalase but not contaminated SOD. To further confirm, we examined the effect of catalase on superoxide-mediated up-regulation of CD11b. Interestingly, semiquantitative RT-PCR (Fig. 9B) and quantitative real-time PCR (Fig. 9C) results clearly show that catalase alone was capable of abrogating the expression of CD11b mRNA in (hypoxanthine/xanthine oxidase)-stimulated microglia. Because this is an important finding, we tried to verify it further by FACS

analysis. As expected, treatment of microglial cells with the mixture of hypoxanthine and xanthine oxidase led to an increase in surface expression of CD11b without altering cell viability (Figs. 9D and 9E). However, consistent with CD11b mRNA expression, catalase alone markedly suppressed the surface expression of CD11b protein in (hypoxanthine+xanthine oxidase)-stimulated microglia (Fig. 9F). On the other hand, SOD had a very weak inhibitory effect on superoxide-mediated increase in CD11b protein expression (Fig. 9G). Taken together, these results suggest that hydrogen peroxide, but not superoxide, is involved in the up-regulation of CD11b in microglia.

Involvement of NO, but not peroxynitrite, in microglial up-regulation of CD11b

In our earlier study [14], we have shown that LPS, proinflammatory cytokines and etiological reagents of various neurodegenerative disorders stimulate the expression of CD11b in microglia via nitric oxide. Therefore, we wondered if NO was playing a role in ROS-mediated increase in CD11b expression in microglia. At first, we performed a time-course experiment to monitor the expression of inducible nitric oxide synthase, the production of NO, and the expression of CD11b in LPS-stimulated microglia. Although LPS induced the expression of iNOS within 6 h of stimulation, the increase in CD11b expression was visible from 18 h of LPS stimulation (Fig. 10B), the time point marked by enhanced production of NO (Fig. 10A). Because NAC inhibited LPS-mediated up-regulation of CD11b (Fig. 1 and Fig. 2), we examined if this antioxidant suppresses LPS-induced expression of iNOS mRNA in microglial cells. Consistent with our hypothesis, NAC markedly suppressed the expression of iNOS in LPS-stimulated cells (Fig. 10C). Therefore, these results further emphasize an important role of NO in microglial expression of CD11b. Both constitutive NOS (cNOS or NOS1) and iNOS (NOS2) produce NO from L-arginine and NO produced from both NOS could be involved in the up-regulation of microglial CD11b. To understand whether cNOS and/or iNOS are/is involved in the expression of CD11b, microglial cells pretreated with different concentrations of AMT (a specific inhibitor of iNOS) and 7-NINA (a specific inhibitor of cNOS) were stimulated with LPS. Consistent with our results, AMT (Fig. 10D), but not 7-NINA (Fig. 10E), suppressed the up-regulation of CD11b in LPS-stimulated microglial cells, suggesting that iNOS-derived NO plays a role in LPS-mediated up-regulation of microglial CD11b.

Because NADPH oxidase is also involved in LPS-mediated increase in CD11b expression and NADPH oxidase-derived superoxide spontaneously reacts with NO to produce peroxynitrite, one may foresee a possible role of this highly reactive intermediate in microglial expression of CD11b. Therefore, to identify or exclude the role of peroxynitrite, we examined the effect of SIN-1, a donor of peroxynitrite, on the expression of CD11b in microglial cells. As evident from Fig. 10F (upper panels), SIN-1 markedly induced the mRNA expression of CD11b at different time points of stimulation ranging from 2 to 24 h. Although NAC suppressed the expression of CD11b in LPS-and other proinflammatory stimuli-challenged microglial cells, this antioxidant had no effect on SIN-1-mediated increase in CD11b (Fig. 10F, middle panels). This is probably because NAC does not interfere with the production of NO by SIN-1. However, scavenging of NO by PTIO markedly abrogated SIN-1-mediated microglial up-regulation of CD11b (Fig. 10F, lower panels). These results suggest that peroxynitrite may not be involved in the up-regulation of microglial CD11b. To confirm these results further, we treated microglial cells with DETA-nonoate, a NO donor, in the presence or absence of NAC, (H+XO), and PTIO. Consistent with our previous work [14], DETA-nonoate alone increased the expression of CD11b mRNA in microglial cells (Fig. 10G). As expected, NAC was unable to modulate DETA-nonoate-mediated increase in CD11b mRNA expression (Fig. 10H, upper panels). On the other hand, although the combination of H and XO did not completely abrogate the expression of CD11b, this combination had an inhibitory effect on the up-regulation of CD11b mRNA in DETA-nonoate-treated microglial cells (Fig. 10H, middle panels). These results suggest that the observed inhibition is due to possible trapping of NO by superoxide

produced from (H+XO) and that NO, but not peroxynitrite, plays a role in microglial up-regulation of CD11b. Consistently, scavenging of NO by PTIO deleted the ability of DETA-nonoate of increasing the expression of CD11b mRNA in microglial cells (Fig. 10H, lower panels).

How does ROS up-regulate CD11b in microglia?

We performed a time-course experiment to monitor CD11b expression and nitrite release in response to H₂O₂. Assay of nitrite in culture supernatants shows that the increase in CD11b expression (Fig. 11B) was associated with the production of NO (Fig. 11A). H₂O₂ was unable to increase the mRNA expression of CD11b within 6 h of stimulation when there was very little NO production (Fig. 11B). However, the increase in CD11b expression (Fig. 11B) was visible at 18 h when there was a significant amount of NO production (Fig. 11A). In fact, the expression of CD11b paralleled the production of NO, suggesting a possible involvement of NO in H₂O₂-mediated increase in microglial CD11b expression.

To further confirm the role of NO in H₂O₂-mediated up-regulation of CD11b, we examined the effect of L-NIL (an inhibitor of NOS) and carboxy-PTIO (a scavenger of NO) on H₂O₂-mediated increase in CD11b mRNA expression in BV-2 microglial cells. Interestingly, both L-NIL and PTIO reduced the level of nitrite (Fig. 11C) and knocked down the expression of CD11b mRNA in H₂O₂-treated microglia as evidenced by semiquantitative RT-PCR (Fig. 11D) and quantitative real-time PCR (Fig. 11E). Diamide, another prooxidant, alone also induced the production of nitrite (Fig. 11C) and the expression of CD11b (Figs. 11D and 11E) in microglial cells. However, similar to H₂O₂, diamide also failed to induce the expression of CD11b in the presence of L-NIL and carboxy-PTIO (Figs. 11D and 11E). Similarly, a time-course experiment shows that the combination of H and XO up-regulated the mRNA expression of CD11b after 18 h of stimulation and PTIO treatment abrogated such ability of (H+XO) (Fig. 11F). Taken together, these studies suggest that ROS up-regulates microglial expression of CD11b via NO.

Discussion

Although microglia populate only 2 to 3% of total brain cells in a healthy human being, the number increases up to 12 to 15% during different neurodegenerative diseases. Microglial activation is always associated with neuronal inflammation and ultimately neuronal apoptosis [45,46]. Although microglial activation may not be always bad as it has an important repairing function as well, once microglia become activated in neurodegenerating microenvironment, it always goes beyond control and eventually detrimental effects override beneficial effects. Therefore, microglial activation is a hallmark of different neurodegenerative diseases and understanding underlying mechanisms for microglial activation is an important area of study. Microglial activation is represented by increased expression of CD11b. This is a surface integrin of microglia that acts like a bridge between cytoskeletal proteins and cell membrane, thereby probably helping microglia during shape change and ramification.

Several lines of evidence presented here clearly demonstrate that ROS play a key role in microglial expression of CD11b. *First*, LPS, a prototype inducer of inflammation in many cell types including microglia, up-regulated CD11b in microglia. However, antioxidants like NAC, PDTC, and ascorbate attenuated LPS-induced CD11b expression. *Second*, microinjection of LPS into the striatum caused marked up-regulation of CD11b. Consistent with the effect observed in isolated microglia, both NAC and PDTC strongly inhibited LPS-mediated increase in striatal CD11b expression in vivo. *Third*, we extended the study beyond LPS and examined if ROS are involved in microglial expression of CD11b induced by other neurotoxins and etiological reagents of various neurodegenerative disorders. It is important to know that both NAC and PDTC inhibited microglial expression of CD11b mRNA induced by various

neurotoxins such as A β , poly(IC), gp120, IL-1 β , and IL-12 p40₂. *Fourth*, antisense knockdown of p22phox, a subunit of NADPH oxidase complex, reduced the expression of CD11b in LPS-stimulated microglia. Because NADPH oxidase is known to produce superoxide and peroxide radicals in many cell types including microglia, the inhibition of CD11b expression with antisense knockdown of NADPH oxidase clearly indicates the involvement of ROS in microglial increase in CD11b expression. *Fifth*, ROS alone was also capable of inducing CD11b expression in microglia as confirmed by the treatment with H₂O₂ and the combination of H and XO. Consistently, either depletion of endogenous glutathione by diamide or increase in intracellular H₂O₂ through aminotriazole-mediated inhibition of catalase also increased the expression of CD11b in microglia.

During activation, microglia produce a wide variety of ROS. However, among different ROS, hydrogen peroxide was found to be directly involved in the microglial CD11b expression. This finding was established by quantitative PCR analysis in which SOD inhibited the expression of CD11b by about 50% and catalase inhibited by over 95%. While SOD dismutates superoxide radical to hydrogen peroxide and oxygen, catalase, on the other hand, converts hydrogen peroxide to H₂O and O₂, and thereby reducing the burden of H₂O₂ in microglia. We hypothesized that SOD-mediated partial inhibition of CD11b expression was due to its ability to reduce the burden of ROS from microglia by converting 2 mol of superoxide (O₂⁻) to 1 mol of peroxide (O₂²⁻) and 1 mol of valuable molecular oxygen. Therefore, although both peroxide and superoxide participate in microglial expression of CD11b, it is H₂O₂ that plays a more direct role than O₂⁻ in microglial up-regulation of CD11b.

Intracellular signaling mechanisms for the increase in CD11b expression in microglia are poorly understood. Recently, we have reported that LPS induces the expression of CD11b via NO [14]. Because LPS and other neurotoxins stimulate the production of ROS much before the production of NO, and the expression of CD11b peaks at 24 h of stimulation, we were prompted to investigate whether NO was involved in ROS-mediated up-regulation of CD11b. Interestingly, our results demonstrate a unique example in which ROS employs NO to stimulate the expression of CD11b in microglia. Our conclusion is based on the following. *First*, in H₂O₂-stimulated microglial cells, the increase in CD11b expression began after the production of NO. H₂O₂ stimulated the expression of CD11b after 18 h of stimulation when there was significant amount of NO production (Fig. 11). On the other hand, DETA-nonoate, a nitrite donor, induced the expression of CD11b within 2 h of stimulation (Fig. 10). *Second*, H₂O₂ and diamide 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, (H+XO) also stimulated the expression of CD11b after 18 h of stimulation and PTIO disabled (H+XO) from doing so. Recently we have characterized mechanisms downstream of NO that are involved in the up-regulation of CD11b in microglia [14]. Although the guanylate cyclase (GC)–cyclic GMP (cGMP) pathway plays an important role in maintaining various physiological processes, our studies have shown that NO up-regulates CD11b expression in microglia via activation of sGC-cGMP-protein kinase G (PKG) pathway [14]. Therefore, we may extend our conclusion that ROS up-regulate the expression of CD11b in microglia via NO-sGC-cGMP-PKG pathway.

ROS are short-lived and diffusible free radicals that play many roles as a signaling and effector molecule in physiology and pathophysiology. Microglia, resident macrophage of the CNS, have been shown to produce different ROS in response to LPS [47], gp120 [48], and beta-amyloid [49]. It has been shown that microglia-derived ROS contributes to the direct killing of hippocampal CA1 neurons [43,45] in AD and dopaminergic neurons [50] in PD. Despite its direct apoptotic effect in neurons, here we demonstrate that ROS is a key player in microglial activation as well in which ROS increases microglial expression of CD11b via NO. During different neurodegenerative diseases, microglia grows by size and number and ramifies to the

distant region of CNS, implicating severe and global neuroinflammation. Because CD11b, the surface integrin of microglia, helps to coordinate cytoskeletal rearrangement with ramified membrane structure, increased expression of CD11b is expected to be associated with microglial ramification during microgliosis. Although the in vitro situation of mouse microglia in culture and its treatment with etiological reagents of AD, HAD, and viral encephalopathy may not truly resemble the in vivo situation of microglia in the brain of patients with these neurodegenerative disorders, our results suggest that specific targeting of either ROS or NO may be an important step for the attenuation of microglial activation in these neurodegenerative disorders.

Abbreviations

AD, Alzheimer's disease
 AMT, 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine
 carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
 CNS, central nervous system
 HAD, HIV-associated dementia
 ICAM-1, intracellular cell adhesion molecule-1
 iNOS, inducible nitric oxide synthase
 L-NIL, L-N⁶-(1-iminoethyl)-L-lysine
 LPS, lipopolysaccharide
 MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
 7-NINA, 7-nitroindazole
 NAC, *N*-acetylcysteine
 NO, nitric oxide
 PD, Parkinson's disease
 PDTC, pyrrolidine dithiocarbamate
 PKG, protein kinase G
 ROS, reactive oxygen species
 RT-PCR, reverse transcriptase-polymerase chain reaction

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Fig. 1. Effect of NAC and PDTC on LPS-mediated increase in CD11b mRNA expression in mouse BV-2 microglial cells

Cells were stimulated with 0.5 μ g/ml LPS under serum-free conditions. At different time point of stimulation, the generation of ROS was monitored by carboxy- H_2 DCFDA (A). Cells preincubated with different concentrations of NAC (B and C) and PDTC (D and E) for 1 h were stimulated with 0.5 μ g/ml LPS. After 24 h of stimulation, the expression of CD11b mRNA was analyzed in cells by semiquantitative RT-PCR (B and D). Quantitative real-time PCR (C and E) was also employed to further clarify the mRNA expression of CD11b. Cells preincubated with different concentrations of sodium ascorbate for 1 h were stimulated with 0.5 μ g/ml LPS. After 24 h of stimulation, the expression of CD11b mRNA was analyzed in cells by

semiquantitative RT-PCR (F). Cell viability was examined by MTT assay (G). Results are mean $\pm$ SD of three different experiments. ^a $P < 0.001$ vs LPS.

Fig. 2. Effect of NAC and PDTC on LPS-mediated increase in CD11b protein expression in mouse BV-2 microglial cells

Cells preincubated with either NAC (0.5mM) or PDTC (25 μ M) for 1 h were stimulated with 0.5 μ g/ml of LPS under serum-free conditions. After 24 h of stimulation, the surface expression of CD11b was monitored by two-color FACS analysis (A). Percentage of cells in various quadrants has been noted. Cells were immunostained with CD11b (B) as described under Materials and methods. Results represent three independent experiments.

Fig. 3. Effect of NAC and PDTC on LPS-stimulated expression of CD11b in mouse primary microglia

Cells preincubated with NAC (0.5 mM) and PDTC (25 μ M) for 1 h were stimulated with 0.5 μ g/ml of LPS under serum-free conditions. After 24 h of stimulation, the expression of CD11b mRNA was analyzed in cells by semiquantitative RT-PCR (A) and quantitative real-time PCR (B). Cell viability was examined by MTT assay (C). Results are mean $\pm$ SD of three different experiments. ^a $P < 0.001$ vs LPS.

Fig. 4. Effect of NAC and PDTC on amyloid- β -, HIV-1 gp120-, poly(IC)-, IL-1 β -, and IL-12p40₂-stimulated expression of CD11b in mouse BV-2 microglial cells

Cells preincubated with NAC (0.5 mM) and PDTC (25 μ M) for 1 h were stimulated with 1 μ M amyloid- β 1–42 (A), 200 pg/ml of gp120 (B), 100 g/ml of poly(IC) (C), 10 ng/ml of IL-1 β (D), or 20 ng/ml p40₂ (E) under serum-free conditions. After 24 h of stimulation, the expression of CD11b mRNA was analyzed in cells by semiquantitative RT-PCR (upper panels) and quantitative real-time PCR (lower panel). Results are mean $\pm$ SD of three different experiments. ^a P <0.001 vs A β ; ^b P <0.001 vs gp120; ^c P <0.001 vs poly(IC); ^d P <0.001 vs IL-1 β ; ^e P <0.001 vs p40₂.

Fig. 5. LPS-stimulated expression of CD11b in vivo in mouse striatum is redox sensitive
 Four micrograms of LPS in the presence or absence of NAC (100 μ g) and PDTC (6.25 μ g) dissolved in 3 μ l saline was stereotactically injected into striatum (A) of C57/BL6 mice. After 24 h of microinjection, the expression of CD11b mRNA was analyzed in striatum by semiquantitative RT-PCR (B) and quantitative real-time PCR (C). Results are mean $\pm$ SD of three different experiments. ^a P <0.001 vs LPS.

Fig. 6. ROS alone induce the expression of CD11b in mouse BV-2 microglial cells

Cells were stimulated with the combination of different concentrations of hypoxanthine (H) and xanthine oxidase (XO) (A), H_2O_2 (B), aminotriazole (ATZ) (C), and diamide (D) under serum-free conditions. After 24 h of stimulation, the mRNA expression of CD11b was analyzed in cells by semiquantitative RT-PCR (upper panels) and quantitative real-time PCR (lower panel). Results are mean $\pm$ SD of three different experiments. $^aP < 0.001$ vs untreated control. (E) Cells were stimulated with 25 μ M diamide under serum-free conditions. At different time points of stimulation, the level of ROS was monitored using carboxy- H_2 DCFDA. Results represent three independent experiments.

Fig. 7. Effect of NAC and PDTC on oxidant-stimulated expression of CD11b in mouse BV-2 microglial cells

Cells preincubated with NAC (0.5 mM) and PDTC (25 M) for 1 h were stimulated with the combination of 1 μM H and 0.5 mU/ml XO (A), 200 μM H₂O₂ (B), 25 μM diamide (C), and 25 μM ATZ (D) under serum-free conditions. After 24 h of stimulation, the expression of CD11b mRNA was analyzed in cells by semiquantitative RT-PCR (upper panels) and quantitative real-time PCR (lower panel). Results are mean ±SD of three different experiments. ^a*P*<0.001 vs (H+XO); ^b*P*<0.001 vs H₂O₂; ^c*P*<0.001 vs diamide; ^d*P*<0.001 vs ATZ.

Fig. 8. Effect of NADPH oxidase (p22 phox) on the expression of CD11b in BV-2 microglial cells (A) Cells were incubated with 1 μ M antisense (ASO) and scrambled oligonucleotides (ScO) against p22phox for 48 h followed by analysis of p22phox protein by Western blot. Cells were also immunoblotted with antibodies against actin as control (lower panel). (B) Bands were scanned and relative density of p22phox (p22/actin) was shown. Results are mean $\pm$ SD of three different experiments. ^a P <0.01 vs control. (C) Cells preincubated with ASO and ScO against p22phox for 36 h were stimulated with LPS (0.5 μ g/ml) under serum-free conditions. After 24 h of stimulation, the mRNA expression of CD11b was analyzed in cells by semiquantitative RT-PCR. Results represent three independent experiments.

Fig. 9. Effect of catalase and superoxide dismutase on the expression of CD11b in BV-2 microglial cells

(A) Cells preincubated with either catalase or SOD (25 and 50 U/ml) for 1 h were stimulated with LPS (0.5 μ g/ml). After 24 h of stimulation, the expression of CD11b mRNA was analyzed by quantitative real-time PCR. Cells preincubated with catalase for 1 h were stimulated with the mixture of 5 μ M H and 0.5 mU/ml XO under serum-free conditions. After 24 h of stimulation, the expression of CD11b mRNA was analyzed by semiquantitative RT-PCR (B) and quantitative real-time PCR (C). Results are mean $\pm$ SD of three different experiments. ^a $P < 0.001$ vs LPS; ^b $P < 0.001$ vs H+XO. Cells preincubated with either catalase or SOD (25 and 50 U/ml) for 1 h were stimulated with a mixture of 5 μ M H and 0.5 mU/ml XO under serum-free conditions. After 24 h of stimulation, the surface expression of CD11b protein was analyzed by two-color FACS analysis (D, vehicle-control; E, H+XO; F, H+XO+ catalase; G, H+XO+SOD). Percentage of cells in lower right quadrants has been noted. Results represent two independent experiments.

Fig. 10. Role of nitric oxide and peroxynitrite in the expression of CD11b in BV-2 microglial cells (A) Cells were treated with 0.5 μ g/ml LPS under serum-free conditions. At different hours of stimulation, supernatants were analyzed for nitrite production (A) and cells were analyzed for the mRNA expression of iNOS and CD11b (B). Results are mean $\pm$ SD of three different experiments. ^a $P < 0.001$ vs control (0 h). Cells pretreated with 1 mM NAC for 1 h were stimulated with 0.5 μ g/ml LPS. At different hours of stimulation, cells were analyzed for the mRNA expression of iNOS (C). Cells pretreated with different concentrations of either AMT (D) or 7-NINA (E) for 1 h were stimulated with 0.5 μ g/ml LPS. After 24 h, cells were analyzed for the mRNA expression of CD11b. Cells pretreated with 1 mM NAC or 50 μ M PTIO for 1 h were stimulated with 50 μ M SIN-1. At different hours of stimulation, cells were analyzed for the mRNA expression of CD11b (F). Cells were stimulated with 25 μ M DETA-nonoate. At different hours of stimulation, cells were analyzed for the mRNA expression of CD11b (G). Cells were stimulated with 25 μ M DETA-nonoate in the presence or absence of NAC, (H+XO), and PTIO. At different hours of stimulation, cells were analyzed for the mRNA expression of CD11b (H). Results represent three independent experiments.

Fig. 11. The role of nitric oxide in ROS-stimulated expression of CD11b in BV-2 microglial cells
Cells were treated with 300 μM H₂O₂ under serum-free conditions. At different hours of stimulation, supernatants were analyzed for nitrite production (A) and cells were analyzed for the mRNA expression of CD11b (B). Cells were treated with L-NIL (25 and 50 μM) and PTIO (25 and 50 μM) 1 h before the treatment of either 300 μM H₂O₂ or 25 μM of diamide. After 24 h of stimulation, supernatants were analyzed for nitrite production (C) and the mRNA expression of CD11b was analyzed in cells by RT-PCR (D) and quantitative real-time PCR (E). Cells pretreated with 50 μM PTIO for 1 h were stimulated with the combination of H and XO. At different hours of stimulation, cells were analyzed for the mRNA expression of CD11b (F). Results are mean ±SD of three different experiments. ^a *P*<0.001 vs control (0 h) for

NO; ^b $P < 0.001$ vs H₂O₂ for NO; ^c $P < 0.001$ vs diamide for NO; ^d $P < 0.001$ vs H₂O₂ for mRNA; ^e $P < 0.001$ vs diamide for mRNA.