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Microglia Proliferation Is Regulated by Hydrogen Peroxide from NADPH Oxidase

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Microglia are resident brain macrophages that become activated and proliferate following brain damage or stimulation by immune mediators, such as IL-1β or TNF-α. We investigated the mechanisms by which microglial proliferation is regulated in primary cultures of rat glia. We found that basal proliferation of microglia was stimulated by proinflammatory cytokines IL-1β or TNF-α, and this proliferation was completely inhibited by catalase, implicating hydrogen peroxide as a mediator of proliferation. In addition, inhibitors of NADPH oxidase (diphenylene iodonium or apocynin) also prevented microglia proliferation, suggesting that this may be the source of hydrogen peroxide. IL-1β and TNF-α rapidly stimulated the rate of hydrogen peroxide produced by isolated microglia, and this was inhibited by diphenylene iodonium, implying that the cytokines were acting directly on microglia to stimulate the NADPH oxidase. Low concentrations of PMA or arachidonic acid (known activators of NADPH oxidase) or xanthine/xanthine oxidase or glucose oxidase (generating hydrogen peroxide) also increased microglia proliferation and this was blocked by catalase, showing that NADPH oxidase activation or hydrogen peroxide was sufficient to stimulate microglia proliferation. In contrast to microglia, the proliferation of astrocytes was unaffected by the presence of catalase. In conclusion, these findings indicate that microglial proliferation in response to IL-1β or TNF-α is mediated by hydrogen peroxide from NADPH oxidase. The Journal of Immunology, 2006, 176: 1046–1052.

Materials and Methods

Materials

Apocynin was purchased from Calbiochem; all other reagents were purchased from Sigma-Aldrich.

Gliarial cultures

Primary mixed cultures of astrocytes and microglia were prepared from the cerebral cortices of 7-day-old Wistar rats. Meninges were removed from the cerebral hemispheres and then dissociated using a solution of Earle’s balanced salt solution containing 0.3% BSA, 0.004% DNase I, and 0.025% trypsin. Cells were plated at 0.1 × 10^6 cells/cm^2 in 24-well plates coated with 0.0005% poly-l-lysine. Cultures were maintained in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin. Cells were kept at 37°C in a humidified atmosphere of 5% CO2/95% air.

At confluence, glial cultures were used to isolate microglial cells by gently shaking/tapping the mixed glial cultures to dislodge microglia loosely attached to astrocytes. Medium from the mixed glial cultures containing microglia was removed and centrifuged (135 × g) for 5 min, and the supernatant was discarded before cells were resuspended in the relevant buffer.

Assessment of microglia proliferation

Gliacl cultures were used at 4 days in vitro in microglia proliferation experiments (mean percentage of microglia in glial cultures at the start of experiments was 23 ± 5%). Cultures were treated with inflammatory mediators (10 ng/ml IL-1β; 10 ng/ml TNF-α; 10 ng/ml IFN-γ; 3 μM AA), inhibitors (1 mM apocynin; 10 μM DPI), or scavengers (128 U/ml superoxide dismutase (SOD); 765 U/ml catalase) or NADPH oxidase activator (10 pg/ml PMA) or hydrogen peroxide generators (100 μM xanthine and 1.1 mM xanthine oxidase) at the start of the experiment (time = 0).
Microglia cells were identified using isolectin B4 (from *Griffonia simplicifolia*; Molecular Probes), which has a strong affinity for microglia but not astrocytes (25). An Alexa Fluor 488 conjugate of isolectin B4 (10 ng/ml) was added to cultures and incubated for 15 min at 37°C. Cultures were stained initially (time = 0) and sister cultures at 24, 48, and 72 h. Stained cells (microglia) were visualized and counted by viewing under a fluorescence microscope (excitation 488 nm, emission 530 nm). We used this method to assess microglial proliferation because it does not require washing of the cell cultures, which tends to displace the weakly attached microglia, leading to an underestimate of microglia numbers. We found that isolectin B4 specifically stained microglia regardless of their activation status, and this is in agreement with other studies (26).

**Assessment of cell viability**

The viability of cells was assessed either by propidium iodide (PI) staining, trypan blue staining, or MTT assay. In mixed glial cultures, necrosis of microglia was quantified by addition to the cultures for 15 min at 37°C of PI (2 μg/ml, to stain necrotic cells) and isolectin B4 (10 ng/ml, to stain microglia), and PI staining was assessed using a fluorescence microscope (Axiovert S-100; Zeiss) and filters for excitation at 365 nm and emission at 420 nm. Cells were counted in three microscopic fields in each well (three wells per treatment) and expressed as a percentage of the total number of cells. Each treatment was repeated at least three times.

In pure microglial cultures, necrosis was assessed by adding trypan blue (0.2%) to the cells and visualized by light microscopy. In addition, in pure microglial cultures we measured the MTT-to-formazan-converting activity of cells, which is considered to be a measure of cell viability. Microglial cells were incubated for 72 h with 1 mM apocynin, or 10 μM DPI, or 765 U/ml catalase, or 128 U/ml SOD, or no treatment. Then cells were removed, centrifuged, and resuspended in PBS (pH 7.4) at a density of 100,000 trypan blue-negative cells/ml and incubated with 0.5 mg/ml MTT for 30 min, 37°C. Afterward, the converted dye was liberated from the cells and solubilized by addition of acidic isopropanol (0.04 M HCl in absolute isopropanol), and the absorbance intensity of λ = 570 nm light (proportional to formazan concentration in the solution) was measured. The proportionality of the absorbance increase to number of live cells was confirmed with 1, 2, and 3 × 10^5 cells/ml (0.19, 0.39, and 0.56 OD units/30 min, respectively), and we also confirmed that dead cells gave a low absorbance increase (0.03, 0.04, and 0.05 OD units/30 min with 1, 2, and 3 × 10^5 cells/ml, respectively).

**Measurement of hydrogen peroxide production**

The rate of hydrogen peroxide formation by isolated microglia was measured in a continuous fluorometric assay. The reaction mixture contained 1 μM Amplex Red (Molecular Probes), 10 U/ml HRP, and 3.5 × 10^-3 M microglia/ml resuspended in HBSS without glucose. The rate of hydrogen peroxide production was measured in a stirred cuvette by a fluorometer (RF-1501; Shimadzu) at excitation 560 nm and emission 587 nm.

**BrdU incorporation**

Microglia were isolated from mixed glial cultures as described above. Microglia were plated on poly-L-lysine-coated 96-well plates at a density of 1 × 10^5 microglia/cm^2 in 1:1 astrocyte conditioned DMEM and fresh DMEM supplemented with 10% FCS. Wells were then treated with stimulators (10 ng/ml IL-1β; 10 ng/ml TNF-α; 3 μM AA) alone or along with catalase (765 U/ml). Cells were assessed for new DNA synthesis by using a BrdU cell proliferation ELISA kit (Roche). BrdU (10 μM) was added to the plate for 24 h and then cells were fixed according to the manufacturer’s protocol. BrdU incorporation was detected by the addition of an anti-BrdU Ab with peroxidase activity. Substrate solution was added and the resultant color detected by a plate reader (Fluostar Optima; BMG) at an absorbance wavelength of 370 nm.

**Assessment of astrocyte proliferation**

Microglia were removed from confluent glial cultures as described above. The remaining cells (astrocytes) were washed twice with PBS and then gently trypsinized (0.1% in Tris-EDTA buffer). The cells were spun down and resuspended in DMEM supplemented with 10% FCS. Astrocytes were plated in black 96-well poly-L-lysine-coated plates at a density of 6 × 10^3/cm^2. To assay for cell number, 1 mg/ml PI and 40 mg/ml digitonin was added to wells and the fluorescence was measured by a plate reader (Fluostar Optima; BMG) at excitation of 550 nm and emission of 630 nm. A range of cell densities was assayed and the relationship between relative fluorescence units and cell number was found to be linear (data not shown).

**Statistical analysis**

Each experiment was repeated on cells from at least three separate cell cultures, and for each cell culture each determination was made in at least triplicate, from which a mean for that culture was determined. Data are expressed as mean on these means ± SD of the means (SDM). The data were analyzed for significance using ANOVA.

**Results**

**Stimulation of microglia proliferation by inflammatory mediators is due to NADPH oxidase activation and mediated by hydrogen peroxide**

The mechanism of proinflammatory cytokine (IL-1β and TNF-α) stimulation of microglia proliferation was investigated in glial (astrocyte and microglia) cultures. Microglia were counted as cells stained by fluorescent isolectin B4. We found that microglia in untreated cultures showed a 2-fold increase in microglia over 72 h (mean number of microglia per field ± SDM at t = 0: 21 ± 16; 72 h: 52 ± 18). Microglia proliferation was enhanced in the presence of 10 ng/ml IL-1β or 10 ng/ml TNF-α (mean number of microglia per field ± SDM at 72 h: IL-1β: 122 ± 31, TNF-α: 108 ± 6). A combination of IL-1β with IFN-γ, however, did not stimulate microglial proliferation, suggesting that IFN-γ may block IL-1β-induced proliferation (mean number of microglia per field ± SDM at 72 h: 10 ng/ml IL-1β±10 ng/ml IFN-γ: 78 ± 27).

The mechanism of this stimulated proliferation was then investigated. We found that catalase (which breaks down hydrogen peroxide) but not SOD (which breaks down superoxide to hydrogen peroxide) blocked proliferation of microglia following stimulation by IL-1β (Fig. 1b) or TNF-α (Fig. 1c), as well as basal proliferation (Fig. 1a). These data implicate hydrogen peroxide as the mediator of proliferation.

One potential source of extracellular hydrogen peroxide might be the NADPH oxidase expressed by microglia. Inhibitors of NADPH oxidase (apocynin and diphenylene iodonium (DPI)) were found to completely block proliferation in both basal and stimulated conditions (Fig. 1), suggesting that NADPH oxidase mediates microglial proliferation. Apocynin, DPI, catalase, or SOD were not toxic to the glial cultures (Table I).

The previous experiments above used mixed glial cultures, which contain both microglia and astrocytes. We investigated whether pure cultures of microglia would also proliferate in response to activation of the NADPH oxidase and subsequent hydrogen peroxide. We measured the incorporation of BrdU using a colorimetric assay in the presence of catalase and/or IL-1β. We found significantly higher levels of BrdU incorporation in microglia following IL-1β treatment than in untreated cultures over 24 h, which was completely abolished in the presence of catalase (mean absorbance units ± SDM of three or more separate cultures; control: 0.41 ± 0.07; catalase: 0.09 ± 0.04; IL-1β: 0.61 ± 0.08; IL-1β + catalase: 0.06 ± 0.04; concentrations as in Fig. 1). Additionally the proliferation of microglia in pure cultures was stimulated in the presence of IL-1β over 48 h (mean cell numbers ± SDM of three separate cultures at t = 0: 45 ± 7; at t = 48: control: 59 ± 5; IL-1β (10 ng/ml): 76 ± 4).

We also investigated whether catalase affected the proliferation of astrocytes. Using a fluorescence assay for cell number, we found that astrocyte proliferation was unaffected in the presence of catalase over a period of 72 h (mean cell numbers ± SDM of three separate cultures; t = 0: 5750 ± 1733; t = 24: control: 7386 ± 754, catalase (765 U/ml): 7478 ± 308; t = 48: control: 15315 ± 843, catalase: 13745 ± 592; t = 72: control: 16393 ± 385; catalase:17717 ± 309 astrocytes/cm²).
Activation of the NADPH oxidase or generation of hydrogen peroxide induces microglia proliferation

The previous results implicated NADPH oxidase stimulation in the mechanism of microglia proliferation. We therefore tested whether stimulation of the NADPH oxidase using a known activator would also stimulate microglia proliferation. PMA has been extensively used to activate NADPH oxidase via protein kinase C. We found that low concentrations (10 pg/ml) of PMA stimulated microglia proliferation (Fig. 2), which was blocked by inhibitors of the NADPH oxidase (DPI or apocynin) or by breaking down hydrogen peroxide (catalase). However, higher concentrations of PMA (1 ng/ml) blocked microglial proliferation (data not shown). AA has been implicated in a number of pathologies

FIGURE 1. Inhibition of microglia proliferation by blocking NADPH oxidase or removal of hydrogen peroxide. Apocynin (1 mM), DPI (10 μM), and catalase (765 U/ml; alone or with 128 U/ml SOD) inhibited basal (Control) microglia proliferation at 72 h. Similarly, stimulation of microglia proliferation by 10 ng/ml IL-1β (b) or 10 ng/ml TNF-α (c) was also completely prevented by inhibitors of the NADPH oxidase (DPI or apocynin) or by breaking down hydrogen peroxide (catalase). Statistical differences were established using ANOVA at **, p < 0.01 and ###, p < 0.001. The * denotes significant differences in comparison to t = 0 (□) and the # replaces the * when comparing to the control, IL-1β, or TNF-α alone (■) in a, b, or c, respectively, and NS denotes no significant difference (p > 0.05). Error bars indicate SDM, n = 3 or more.
and is a known activator of the oxidase. We found that AA also stimulated microglia proliferation (Fig. 3), and this effect was completely abrogated by in the presence of hydrogen peroxide scavenger (catalase) or by blocking the NADPH oxidase (apocynin or DPI).

To test whether superoxide/hydrogen peroxide production alone could stimulate proliferation, we used xanthine/xanthine oxidase or glucose oxidase to generate superoxide/hydrogen peroxide. We found that both xanthine/xanthine oxidase and glucose oxidase stimulated microglia proliferation and this was prevented by catalase (Fig. 4). However, the addition of 1 μM hydrogen peroxide did not affect microglia proliferation, suggesting that the continuous generation of hydrogen peroxide is required for the mitogenic effects.

Rapid stimulation of microglial NADPH oxidase by IL-1β or TNF-α

The above data indicate that microglia proliferation, assessed in mixed glial cultures, is mediated by hydrogen peroxide from NADPH oxidase. We tested whether the proinflammatory molecules were acting directly on the microglia to induce hydrogen peroxide production. Using HRP oxidation of Amplex Red to fluorescent resorufin, we continuously measured hydrogen peroxide production by isolated microglia. A suspension of microglia treated with either IL-1β, TNF-α, PMA, or AA showed a significant increase in the rate of hydrogen peroxide production in comparison to unstimulated microglia (Fig. 5). This increased rate was completely prevented by the addition of DPI (data not shown), confirming that the increased rate of hydrogen peroxide production was due to stimulation of the microglial NADPH oxidase.

GM-CSF is a known stimulator of microglia proliferation. We tested whether GM-CSF also activated the NADPH oxidase. Using a suspension of isolated microglia, we found that the addition of 100 ng/ml GM-CSF did not acutely (up to 90 min) stimulate ROS production (data expressed are mean picomoles H2O2/minute per 1 × 10^5 microglia ± SDM of three separate cultures; control: 6 ± 1; 100 ng/ml GM-CSF: 7 ± 1). The proliferation of microglia in pure cultures was stimulated by GM-CSF and this proliferation was sensitive to catalase (mean number of microglia per field ± SDM at t = 0 h: 23 ± 5; t = 24; control: 34 ± 10, GM-CSF: 53 ± 10, catalase (765 U/ml) + GM-CSF: 31 ± 12; t = 48; control: 47 ± 9, GM-CSF: 78 ± 19, catalase + GM-CSF: 30 ± 3).

Discussion

IL-1β and TNF-α are two cytokines released by microglia in response to infection, trauma, or neuronal damage in various CNS pathologies (10). It has been previously reported that IL-1β and TNF-α can stimulate proliferation of microglia (11, 27). Since both IL-1β and TNF-α have been shown to induce ROS production in other cells and tissues (13, 14), we aimed to investigate whether these cytokines could also stimulate ROS production by microglia and whether this mediated microglia proliferation. IL-1β and TNF-α did indeed rapidly increase the rate of hydrogen peroxide production by isolated microglia, and this increase was prevented by an inhibitor of NADPH oxidase (DPI), indicating that the cytokines act directly on microglia to stimulate hydrogen peroxide production from the NADPH oxidase. We found that IL-1β or TNF-α stimulated the proliferation of microglia in mixed glial cultures, and IL-1β increased the incorporation of BrdU in pure microglia cultures. In all cases (including unstimulated), microglia proliferation was prevented by catalase, an enzyme that breaks down hydrogen peroxide to water. SOD alone did not have any inhibitory effects on microglial proliferation, but together with catalase completely blocked proliferation, suggesting that hydrogen peroxide and not superoxide mediates proliferation. The source of

### Table I. Apocynin, DPI, catalase, and SOD are nontoxic to microglia in pure or mixed cultures

<table>
<thead>
<tr>
<th>Parameter/Sample</th>
<th>Control</th>
<th>Apocynin</th>
<th>DPI</th>
<th>Catalase</th>
<th>SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTT activity, OD units</td>
<td>0.19 ± 0.07</td>
<td>0.19 ± 0.04</td>
<td>0.22 ± 0.08</td>
<td>0.20 ± 0.06</td>
<td>0.19 ± 0.05</td>
</tr>
<tr>
<td>PI-positive microglia (%)</td>
<td>6 ± 1</td>
<td>7 ± 4</td>
<td>10 ± 5</td>
<td>9 ± 4</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

*Microglial cells were incubated for 72 h with 1 mM apocynin, or 10 μM DPI, or 765 U/ml catalase, or 128 U/ml SOD, then cell viability was measured by MTT assay and trypan blue exclusion as indicated in Materials and Methods. MTT-reducing activity is expressed as the increase in OD units at 570 nm over the 30-min incubation with MTT of 100,000 trypan blue-negative cells/ml. Trypan blue-positive cells are expressed as the percentage of total microglia. Microglial necrosis (expressed as a percentage of all microglia) was also measured in the mixed (astrocytes plus microglia) cultures of Fig 1 at the end of the 72-h incubations. Results are mean ± SDM of three separate cultures.

**FIGURE 2.** Activation of NADPH oxidase stimulates microglia proliferation. PMA (10 pg/ml) stimulated microglia proliferation over a period of 72 h. This PMA-induced proliferation was blocked by inhibitors of NADPH oxidase (10 μM DPI or 1 mM apocynin) or by catalase (765 U/ml). Statistical differences were established using ANOVA at **, p < 0.01 and ***, p < 0.001. Significant differences are in comparison to the number of microglia at 72 h in untreated cultures. The # replaces the *, when comparing the effects of DPI/apocynin/catalase on the PMA-treated culture at 72 h. Error bars indicate SDM, n = 3.
mitogenic hydrogen peroxide appeared to be NADPH oxidase, as inhibitors (DPI or apocynin) also blocked proliferation.

To test the hypothesis that hydrogen peroxide production from NADPH oxidase may stimulate microglia proliferation, we used a low concentration of PMA to activate the NADPH oxidase to the same extent as that induced by IL-1β and TNF-α (Fig. 5). We found that PMA significantly stimulated microglia proliferation over 72 h and this proliferation was completely prevented by inhibitors of NADPH oxidase (DPI, apocynin) or by catalase. We tested whether AA may also stimulate proliferation of microglia, since it is well known to stimulate the ROS production of other cells via activating NADPH oxidase (19). AA did indeed stimulate microglia proliferation, and this was also blocked by NADPH oxidase inhibitors or catalase. AA has been implicated in a range of CNS pathologies, such as Alzheimer’s disease (15), cerebral HIV infection (16), and ischemia (17). AA activation of microglia proliferation might contribute to these pathologies. It is possible that downstream products of AA may also affect AA-induced stimulation of microglia proliferation, since prostaglandin E₂ has been found to have mitogenic effects on astrocytes, which may indirectly enhance microglia proliferation (28).

We also demonstrated that the continuous production of hydrogen peroxide from xanthine/xanthine oxidase or glucose oxidase stimulated microglia proliferation, which was blocked by catalase, whereas a single addition of hydrogen peroxide did not affect proliferation. These results indicate that hydrogen peroxide, as a result of NADPH oxidase activation or other sources, can stimulate proliferation of microglia.

We have shown here, and others have previously found (11), that the proliferation of microglia is greater in the presence of astrocytes. It is therefore possible that inhibitors and/or enzymatic scavengers (such as catalase) inhibit microglial proliferation through an effect on astrocytes. However, we did not observe any effect of catalase on astrocyte proliferation, indicating that hydrogen peroxide regulation of proliferation is specific to microglia rather than astrocytes.

GM-CSF is a strong mitogenic factor for microglia. We found that this stimulation of microglia proliferation was catalase sensitive, but GM-CSF did not stimulate ROS production by isolated microglia. TGF-β is a known suppressor of GM-CSF-induced and other cytokine-induced stimulation of microglia proliferation. It
has previously been reported that TGF-β is able to inhibit superoxide production from microglia (29) and this may be one mechanism by which TGF-β acts as a negative regulator of microglia proliferation.

Hydrogen peroxide and the NADPH oxidase have been shown to stimulate proliferation in a number of different cell types (22–24). One mechanism for the mitogenic effects of hydrogen peroxide is due to the inhibition of protein tyrosine phosphatases (30). It has recently been shown that the activation of CD45 (a transmembrane tyrosine phosphatase expressed in cells of monocytic lineage) blocks GM-CSF-induced microglia proliferation (9). It has also been reported that hydrogen peroxide can inhibit CD45 activity (31). Hydrogen peroxide has been shown to oxidize critical sulfhydryl groups in tyrosine phosphatases (30), which results in increased tyrosine phosphorylation and prolongation of mitogenic signaling (9, 32). Thus, CD45 might be one potential target for hydrogen peroxide in regulating microglial proliferation.

Microglia have been proposed to play a detrimental role in most neuropathologies (3, 33, 34). The findings in this report suggest that ROS may not only be directly neurotoxic but can also act to enhance the inflammatory response by microglia. We have recently shown that the NADPH oxidase and microglia play a crucial role in neuronal death induced by inflammatory-activated glia (35). In summary, our work has shown that proliferation of microglia can be stimulated by several proinflammatory mediators that are able to directly stimulate microglial NADPH oxidase, leading to subsequent hydrogen peroxide production, which acts as a mitogenic signal for microglia.

Disclosures
The authors have no financial conflict of interest.

References


