Protective effects of agmatine on lipopolysaccharide-injured microglia and inducible nitric oxide synthase activity

Soo Kyung Ahn, Samin Hong, Yu Mi Park, Ja Yong Choi, Won Taek Lee, Kyung Ah Park, Jong Eun Lee

Aims: Proinflammatory factors released from activated microglia contribute to maintaining homeostasis against various noxious stimuli in the central nervous system. If excessive, however, they may initiate a pathologic neuroinflammatory process. In this investigation, we evaluated whether agmatine, a primary polyamine known to protect neurons, reduces lipopolysaccharide (LPS)-induced damage to microglia in vitro and in vivo.

Main methods: For in vitro study, BV2-immortalized murine microglia were exposed to LPS with agmatine treatment. After 24 hours, cell viability and the amount of nitrite generated were determined. For in vivo study, LPS was microinjected into the corpus callosum of adult male albino mice. Agmatine was intraperitoneally administered at the time of injury. Brains were evaluated 24 hours after LPS microinjection to check for immunoreactivity with a microglial marker of ionized calcium binding adaptor molecule 1 (Iba1) and inducible nitric oxide synthase (iNOS). Using western blot analysis, protein expression of iNOS as well as that of the proinflammatory cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-1β, was determined.

Key findings: Agmatine significantly reduced the LPS-induced BV2 microglial cytotoxicity from over 80% to less than 60% (p < 0.001), as determined by lactate dehydrogenase assay. It suppressed the nitrite production from 16.4±3.14 μM to 5.5±1.27 μM (p < 0.001), as measured using the Griess reaction. Agmatine also decreased the activities of microglia and iNOS induced by LPS microinjection into corpus callosum.

Significance: Our findings reveal that agmatine attenuates LPS-induced microglial damage and suggest that agmatine may serve as a novel therapeutic strategy for neuroinflammatory diseases.

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systems (Kim et al., 2000; Liu et al., 2000; Huo et al., 2011). The effects of agmatine on LPS-induced cytotoxicity and the generation of nitrite (NO₂⁻), the stable metabolite of NO, were determined. In addition, using an intracerebral LPS microinjection mouse model, the effects of agmatine were assessed on microglial activation and the expression of inducible NOS (iNOS) in vivo.

Materials and methods

Materials

Agmatine sulfate, LPS (O55:B5), and modified Griess reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin were purchased from Thermo Scientific HyClone (Logan, UT, USA). Primary antibodies against Iba1 and iNOS were obtained from Abcam (Cambridge, MA, USA) and Millipore (Billerica, MA, USA), respectively. Primary antibodies against TNF-α, IL-1β, and β-actin were ordered from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The ABC Kit, Vector® NovaRED Substrate Kit, and Vector® SG Peroxidase Substrate Kit were purchased from Vector Laboratories (Burlingame, CA, USA).

Cell culture and lipopolysaccharide-induced cytotoxicity

A BV2-immortalized murine microglia cell line exhibiting both the phenotypic and functional properties of reactive microglial cells (Bocchini et al., 1992) was grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were plated on 24-well plates at a density of 4 × 10⁵ cells/well and cultured at 37 °C in 5% CO₂.

To study LPS-induced cytotoxic injury, BV2 cells were washed twice with serum-free DMEM and exposed to 1 μg/mL of LPS in serum-free DMEM for 24 hours. In the agmatine treatment group, agmatine was added to the culture medium at the start of injury. Our preliminary studies using the LDH assay tested agmatine concentrations from 10 to 300 μM. Since cell death was significantly reduced at 100 μM and greater concentrations of agmatine (data not shown), we used 100 μM agmatine for subsequent experiments. In control group, cells were not exposed to LPS. At least three different experiments performed using separate cell preparations, and triplicate determinations were performed for each experiment.

Lactate dehydrogenase assay

LPS-induced cytotoxicity was quantified by measuring the amounts of LDH released into the culture medium from injured cells (Hong et al., 2007, 2009; Ahn et al., 2011). LDH release (cytotoxicity %) was calculated by dividing the value at the experimental time point by the maximum value. The maximum LDH release was measured after freezing each culture at −70 °C overnight, followed by rapid thawing, which induced nearly complete cell damage.

Measurement of nitrite production

Nitrite generation was determined using the Griess reaction (Han et al., 2002; Ahn et al., 2011). Duplicate 100 μL aliquots of culture medium collected from each culture were added to a 96-well plate and mixed with 100 μL of modified Griess reagent. The plate was incubated in a dark room for 15 minutes at room temperature, and the absorbance of the reaction product was measured at 540 nm on a microplate reader.

Lipopolysaccharide microinjection into the corpus callosum

A total of 18 adult male ICR mice (6 weeks old, 30 and 35 g) were purchased from Samtako, Inc. (Osan, Korea). Six animals were used for each study group (no treatment control group, LPS injection only group, and agmatine treatment group). They were treated according to a protocol approved by the Yonsei University Animal Care and Use Committee and the NIH guidelines, and efforts were made to minimize animal suffering and the number of animals sacrificed. Animals were maintained in controlled conditions with a 12:12 light/dark cycle and standard food and water provided ad libitum. Animals in the agmatine treatment group received an intraperitoneal injection of 100 mg/kg agmatine at the time of injury. Mice in the LPS injection only group received an equivalent volume of normal saline at the same time point.

To induce the neuroinflammation, LPS was microinjected into the corpus callosum (Lee et al., 2005; Yao et al., 2010). Neuroinflammation primarily involves the activation of glial cells including microglia. And LPS, a bacterial endotoxin, has been widely utilized as a potent inflammation inducer in various experimental systems (Dutta et al., 2008). Briefly, animals were anesthetized with an intraperitoneal injection of ketamine and xylazine. The depth of anesthesia was assessed by a toe pinch every 15 minutes. Rectal temperature, respiration, and heart rate were monitored and maintained in the physiologic range throughout the procedures. Mice were positioned in a small-animal stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). LPS (5 μg/5 μL) was microinjected into the corpus callosum using a 32-gauge needle through a dentist’s burr hole. Injection into the corpus callosum was performed according to the following coordinates: 0.1 mm posterior from Bregma, 1.2 mm lateral from the sagittal suture, and 2.2 mm below the dura mater. Twenty-four hours after injection, animals were sacrificed and decapitated for analysis. At least three different experiments were performed using separate animal preparations each for immunohistochemistry and western immunoblots.

Histological analysis

The forebrains were cut into 3-mm-thick coronal slices using a mouse brain matrix (Ted Pella, Inc., Redding, CA, USA) and fixed with 4% paraformaldehyde (pH 7.4) in 0.1 M phosphate buffer for 24 hours. Paraffin-embedded, 5-μm-thick sections were rehydrated and immunostained with primary antibodies against Iba1 (1:1000) followed by biotinylated secondary antibodies. Sections were visualized using the ABC Kit and Vector® NovaRED Substrate Kit. Subsequently, sections were also reacted with primary antibodies against iNOS (1:1000) followed by biotinylated secondary antibodies. Sections were visualized using the ABC Kit and Vector® SG Peroxidase Substrate Kit.

In a coronal section, the immunoreactive cells for Iba1/iNOS were visualized using the ABC Kit and Vector® SG Peroxidase Substrate Kit. In a coronal section, the immunoreactive cells for Iba1/iNOS were visualized using the ABC Kit and Vector® SG Peroxidase Substrate Kit.

Western immunoblots

For protein extraction, tissues were homogenized as previously described (Cui et al., 2012). Cell homogenates were centrifuged at 13,000g for 15 minutes at 4 °C. Protein concentrations in the resultant supernatants were determined using the BCA protein assay (Thermo Fisher Scientific, Rockford, IL, USA), and equal amounts of protein (50 μg) from each sample were boiled in Laemmli sample buffer and resolved by SDS-PAGE on 8% gels. Proteins were transferred to polyvinylidene fluoride membranes (Millipore), and the membranes were probed overnight at 4 °C with antibodies against iNOS, TNF-α, IL-4, or β-actin. Immunoreactive bands were detected with horseradish peroxidase–conjugated secondary antibodies and were visualized by enhanced chemiluminescence.
Statistical analysis

Data are expressed as the mean ± standard error of the mean (S.E.M.). Statistical tests to determine differences between groups were performed with Kruskal–Wallis test post hoc using the MedCalc program for Windows, version 12.2.1 (MedCalc Software, Mariakerke, Belgium). $p$-Values less than 0.05 were considered significant.

Results

Agmatine attenuates lipopolysaccharide-induced cell death in BV2 microglia

LPS-induced cytotoxicity in BV2 microglia was confirmed using a lactate dehydrogenase (LDH) assay (Fig. 1). Baseline LDH release (%) was about 20%, and it reached more than 80% 24 hours after exposure to LPS. This LPS-induced cytotoxicity decreased to less than 60% when cells were treated with 100 μM agmatine ($p<0.001$, Kruskal–Wallis test; $p<0.05$, all pairs with post-hoc analysis).

Agmatine reduces nitrite generation in lipopolysaccharide-injured BV2 cells

To investigate the putative neuroprotective mechanism of agmatine in LPS-injured BV2 microglial cells, nitrite production was assessed (Fig. 2). After a 24-hour exposure to LPS, the concentration of nitrite in the culture media increased to 16.4 ± 3.14 μM, but agmatine treatment significantly suppressed it to 5.5 ± 1.27 μM ($p<0.001$, Kruskal–Wallis test; $p<0.05$, all pairs with post-hoc analysis).

Agmatine decreases lipopolysaccharide-induced activation of microglia and their expression of inducible nitric oxide synthase

In vivo activation of microglia was assessed by expression of ionized calcium binding adaptor molecule 1 (Iba1). Compared to the LPS injection only group, the number of Iba1-positive cells significantly decreased in the agmatine treatment group (Fig. 3 and Table 1). In the ipsilateral cortex and striatum, the number of Iba1-positive cells was 243.00 ± 6.11 for the no treatment control group. It increased to 413.00 ± 34.70 for the LPS injection only group, but decreased to 326.00 ± 19.86 for the agmatine treatment group ($p=0.039$, Kruskal–Wallis test; $p<0.05$, all pairs with post-hoc analysis). Regarding the number of iNOS-positive cells, it was 310.33 ± 6.94 for the no treatment control group. It increased to 445.67 ± 24.36 for the agmatine treatment group ($p=0.027$, Kruskal–Wallis test; $p<0.05$, all pairs with post-hoc analysis). Consequently, the number of Iba1 and iNOS double-positive cells was 126.00 ± 1.38 for the LPS injection only group and 154.67 ± 6.33 for the agmatine treatment group ($p=0.027$, Kruskal–Wallis test; $p<0.05$, all pairs with post-hoc analysis).

Agmatine suppresses the expression of inducible nitric oxide synthase induced by lipopolysaccharide microinjection into the corpus callosum

To verify the effect of agmatine on iNOS activity in the LPS-microinjected mouse cerebral cortex, the expression of iNOS was assessed in the ipsilateral cortex using western immunoblots (Fig. 4). The iNOS expression significantly increased in the LPS microinjection only group, but it was reduced in the agmatine treatment group. Relative band intensity to the no treatment control group was 1.38 ± 0.03 and 1.14 ± 0.03 for the LPS microinjection and the agmatine treatment group, respectively ($p=0.024$, Kruskal–Wallis test; $p<0.05$, all pairs with post-hoc analysis). This tendency was similar for the two representative proinflammatory molecules of TNF-α and IL-1β.

Discussion

In the present study, we investigated agmatine’s in vitro and in vivo effects on injured microglia. BV2-immortalized murine microglial cells were exposed to LPS for 24 hours; agmatine significantly reduced both cytotoxicity and NO production. Our in vitro data are similar to those of Abe et al. (2000), who showed that agmatine suppressed LPS-induced NO production in cultured microglia. And we applied agmatine to injured microglia in an animal model using adult mice. It was determined that intracerebrally injected LPS damaged and activated microglial cells. Agmatine treatment decreased the activation of microglia and attenuated the expression of iNOS as well as that of TNF-α and IL-1β. Although Abe et al. (2000) reported that agmatine had no effect on the expression of iNOS in cultured microglia, we found that agmatine directly influences the expression of iNOS in vivo. This discrepancy might be caused by differences between cellular and animal experiments. In addition, it may be because we used adult mice while they used cells from two-day-old rat neonates. While we did identify discrepancies in iNOS activity, NO production from our in vivo and in vitro data is similar to their in vitro data; agmatine suppresses LPS-induced NO generation. Due to the fact that microglia are the resident macrophages in the CNS and migrate into the damaged area, LPS microinjection recruited...
Fig. 3. Representative immunohistochemical staining of ionized calcium binding adaptor molecule 1 (Iba1) and inducible nitric oxide synthase (iNOS) in the ipsilateral cortex and striatum 24 hours after lipopolysaccharide (LPS) microinjection into the corpus callosum. In the agmatine treatment group, 100 mg/kg of agmatine was intraperitoneally injected at the time of injury. Blue/gray-labeled cells represent Iba1-positive cells and red labeled cells represent iNOS positive cells. Three animals were used for each group. The scale bar is 100 μm (A) and 20 μm (B).
Table 1
Number of immunoreactive cells for ionized calcium binding adaptor molecule 1 (Iba1) and inducible nitric oxide synthase (iNOS) in the cerebral cortex and striatum after lipopolysaccharide (LPS) microinjection into corpus callosum.

<table>
<thead>
<tr>
<th></th>
<th>No treatment control (A)</th>
<th>LPS injection (B)</th>
<th>LPS injection + agmatine (C)</th>
<th>p-Value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Post-hoc analysis&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=3)</td>
<td></td>
<td>A and B</td>
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<tr>
<td>Ipsilateral</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iba1 (+)</td>
<td>243.00±6.11</td>
<td>413.00±34.70</td>
<td>326.00±19.86</td>
<td>0.039</td>
<td>p&lt;0.05</td>
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<tr>
<td>iNOS (+)</td>
<td>310.33±6.94</td>
<td>614.67±35.67</td>
<td>445.67±24.36</td>
<td>0.027</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Double (+)</td>
<td>126.00±1.53</td>
<td>191.67±14.44</td>
<td>154.67±6.33</td>
<td>0.027</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Contralateral</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iba1 (+)</td>
<td>244.00±7.73</td>
<td>335.00±40.22</td>
<td>287.67±20.21</td>
<td>0.061</td>
<td>–</td>
</tr>
<tr>
<td>iNOS (+)</td>
<td>307.67±4.81</td>
<td>483.33±19.10</td>
<td>387.33±22.18</td>
<td>0.027</td>
<td>p&lt;0.05</td>
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<tr>
<td>Double (+)</td>
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<td>172.67±11.86</td>
<td>145.33±3.71</td>
<td>0.027</td>
<td>p&lt;0.05</td>
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Data are expressed as the mean±SEM.

<sup>a</sup> Kruskal-Wallis test.

the microglia and increased the number of them in vivo. On the contrary, because the total number of cells is fixed in vitro, LPS treatment distinctly showed the cytotoxicity of microglia. Activated microglia release various neurotoxic mediators such as TNF-α, IL-1β, glutamate, and free radicals. Because LPS is a potent microglial activator (Kim et al., 2000; Liu et al., 2000) and triggers iNOS in microglia (Boje and Arora, 1992; Kitamura et al., 1996), we used it as a source for microglial injury. iNOS produces large amounts of NO, and microglia-derived NO functions as a neurotoxin as well as a neurotransmitter (Banati et al., 1993; Murphy et al., 1996).

Agmatine has been reported to rescue neuronal cells from various injuries in vitro and in vivo. Currently, its neuroprotective effects are thought to be due to its affinity for the α2-adrenergic, imidazoline, and NMDA receptors. Recently, however, several reports have shown that agmatine also acts as an inhibitor of iNOS and reduces the excessive generation of NO (Feng et al., 2002). The neuroprotective effects of agmatine also seem to be associated with its NO modulating properties. We previously reported that agmatine rescues microglia from oxidative stress in vitro and in vivo, and its protective effect may be associated with iNOS activity (Ahn et al., 2011). The present study provides evidence that agmatine has a microglial protective effect against neuroinflammatory damage as well as ischemic injury and that its effect may be through modulation of NO signaling.

The precise molecular mechanisms underlying the way in which agmatine suppresses the activity and expression of NOS is not fully understood. However, both NOS and ADC use the same source of the amino acid l-arginine to make NO and agmatine. NO regulates the activity and expression of NOS using negative feedback (Kopincová et al., 2011). It directly inhibits the activity of NOS and indirectly inhibits the expression of NOS by suppressing the activation of nuclear factor-kappa B (NF-kB). We previously reported that agmatine can reduce phosphorylation of NF-kB in cultured retinal neuronal cells (Hong et al., 2007). If NO and agmatine share some chemical features as different products of l-arginine it is possible that either agmatine competes with NO in binding to NOS or agmatine downregulates NOS via NF-kB signaling.

Conclusions

Our data indicated that agmatine protects microglia from LPS-induced injury and that the neuroprotective and anti-inflammatory effects of agmatine may be associated with suppression of NO production from damaged microglia. Agmatine may serve as a novel therapeutic strategy for neuroinflammatory diseases as well as cerebral ischemic and/or traumatic insults.

Conflict of interest statement

The authors declared no conflict of interest.

Acknowledgments

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References


