Effects of ketamine and propofol on inflammatory responses of primary glial cell cultures stimulated with lipopolysaccharide

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Background. Ketamine has been reported to exert anti-inflammatory effects on macrophages stimulated with lipopolysaccharide (LPS) in vitro and in vivo. Several studies have reported conflicting results regarding the effects of propofol on cytokine production from immune cells. However, there have been no reports of the effects of these agents on inflammatory responses in glial cells. We investigated the effects of ketamine and propofol on LPS-induced production of nitric oxide, tumour necrosis factor-α (TNF-α) and prostaglandin E₂ (PGE₂) from primary cultures of rat glial cells in vitro.

Methods. Glial cells were stimulated with LPS in the absence and presence of various concentrations of ketamine (30–1000 μM) or propofol (30 and 300 μM). Nitric oxide released into the culture media was determined by measuring nitrite using the Griess reaction, and concentrations of TNF-α and PGE₂ were measured by enzyme-linked immunosorbent assay (ELISA).

Results. Ketamine reduced LPS-induced TNF-α production without significant inhibition of nitrite release in mixed glial cells, astrocyte cultures and microglial cultures. Ketamine also inhibited LPS-induced production of PGE₂ in astrocyte cultures. In contrast, propofol had no effect on LPS-induced nitrite or TNF-α production in mixed glial cells.

Conclusions. The data demonstrate that ketamine inhibited some of the inflammatory responses of both astrocytes and microglial cells treated with LPS without causing major change in nitric oxide release. Propofol had no effect on the production of nitric oxide or TNF-α from LPS-stimulated glial cells.


Keywords: anaesthetics i.v., ketamine; anaesthetics i.v., propofol; immune response; pharmacology, ketamine; pharmacology, propofol

Accepted for publication: August 16, 2005

Glial cells consisting of astrocytes and microglia are the major components mediating immune responses and inflammation in the central nervous system (CNS).¹,² The inflammatory responses of glial cells play roles in many pathological conditions, including neurodegenerative diseases, stroke, traumatic brain injury, infectious diseases and pathological pain.³–⁵ Glial cells can produce cytokines, reactive oxygen radicals and nitric oxide in response to ischaemic, traumatic and infectious insults, leading to exaggeration of the disease processes.⁶ It has been shown that suppression of inflammatory responses of glial cells mitigates some of these pathological conditions.⁷–⁸

Ketamine shows anti-inflammatory actions in various immune cells, such as macrophages and peripheral leucocytes, stimulated with lipopolysaccharide (LPS) in vitro and in vivo.⁹–¹¹ There have been several studies of the effects of propofol (2,6-diisopropylphenol) on cytokine release from LPS-stimulated immune cells; however, conflicting results demonstrating both inhibition and augmentation have been reported.¹²–¹⁴ The effects of these agents on the inflammatory responses of native glial cells have yet to be clarified. It is known that there are differences in the regulation of LPS-induced inflammatory responses between macrophages and glial cells.¹⁵–¹⁷ Nitric oxide and tumour necrosis factor-α (TNF-α) play key roles in acute and chronic neurodegenerative processes and their LPS-stimulated production in macrophages and leucocytes has been shown to be suppressed by ketamine.⁹–¹¹ We measured
changes in these mediators to compare the effects of ketamine on the inflammatory responses in glial and other immune cells. Prostaglandins released from glial cells are shown to be involved in the pathogenesis of neurological disorders related to inflammation.18 We chose prostaglandin E2 (PGE2) because this molecule plays an important role in pathological pain at the spinal level19 and mediates glia–glia and glia–neuron communication in various pathological conditions, including inflammation, by stimulating glutamate release from astrocytes.20 In this study, we investigated the effects of ketamine and propofol on LPS-induced production of nitric oxide, TNF-α and PGE2 in primary cultures of rat glial cells in vitro.

Methods

This study was approved by our institutional Animal Care And Use Committee.

Reagents

Racemic ketamine hydrochloride, LPS (serotype 055B5), d(-)-2-amino-5-phosphonopentanoic acid (D-AP5) and aminoguanidine were obtained from Sigma (St Louis, MO). Propofol was purchased from Aldrich (Oakville, Canada) and was dissolved in dimethyl sulfoxide (DMSO) shortly before application to make a 200 mM solution. Dulbecco’s modified Eagle’s medium (DMEM), L-15 medium and trypsin were purchased from Gibco (Grand Island, NY), and fetal bovine serum (FBS) was obtained from Wako (Osaka, Japan).

Primary culture of mixed glial cells

Cultures were prepared from whole brains of 2-day-old Wistar rats using the procedure described previously21 22 with some modifications. The meninges and blood vessels were carefully removed, and the tissue was minced with a mesh bag (300 μm) and trypsinized (trypsin–EDTA 2.5% and DNase 0.1% in L-15 medium). After centrifuging for 10 min at 450g and for 5 min at 120 g, the tissues were resuspended in DMEM containing FBS 10%, penicillin and streptomycin 100 μg ml⁻¹ and LPS. The culture media were collected after 24 h and centrifuged, and the supernatants were subjected to the assays described below.

Secondary cultures of microglia

Microglial cells were harvested from mixed glial cultures in 75 cm² flasks by shaking at 150 r.p.m. for 120 min at 37°C. Detached cells were collected by centrifugation (120 g for 10 min) and seeded at 4x10⁴ cells cm⁻². After incubation for 10 min at 37°C, non-adherent or weakly adherent cells were removed by gentle shaking and washed out. The remaining cells were cultured for 24 h and used as microglial cultures.

Immunocytochemistry

The cultures were fixed with paraformaldehyde 4% in phosphate-buffered saline (PBS) 0.1 M for 2 h and rinsed three times with PBS 0.1 M. Non-specific binding was blocked with 10% bovine serum albumin (BSA) for 5 h at room temperature. Subsequently, cultures were incubated with mouse monoclonal antibodies (1:200 dilution) to the specific markers of microglia (OX-42 against CD 11b surface antigen) or astrocytes (glial fibrillary acidic protein [GFAP]) in the blocking buffer for 24 h at 4°C. After washing three times with PBS, the cells were incubated with the secondary antibody (Alexa Fluor 594 goat anti-mouse IgG, 1:1000 dilution [Molecular Probes, Eugene, OR]) for 2 h at room temperature and rinsed. The preparations were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined using an inverted microscope equipped with fluorescence optics. We calculated the percentage of positively immunostained cells (in 200 cells) in each of three separate culture preparations obtained on different days.

Treatment of cultures

Glia cells were preincubated with ketamine (30, 100, 300 or 1000 μM) or propofol (30 or 300 μM) for 15 min, and then LPS was added at final concentrations of 0.5–10 μg ml⁻¹ for 24 h in the continuous presence of ketamine or propofol. In the preliminary experiments we found that the concentrations of 0.5–1.0 μg ml⁻¹ of LPS are saturating doses for nitric oxide release in mixed glial cells and astrocytes. These concentrations have been employed in many other studies measuring LPS-induced nitric oxide and TNF-α production in primary glial cultures.23 24 Thus we stimulated mixed glial cells and astrocytes with 0.5 or 1.0 μg ml⁻¹ of LPS. For microglia, we used 10 μg ml⁻¹ of LPS because we found that 1.0 μg ml⁻¹ was not sufficient to produce distinct increases in nitrite. In some experiments, cells were pretreated with aminoguanidine, a blocker for inducible nitric oxide synthetase (iNOS), or D-AP5, an N-methyl-D-aspartate (NMDA) receptor antagonist,25 before stimulation with LPS. The culture media were collected after 24 h and centrifuged, and the supernatants were subjected to the assays described below.
Measurement of released nitrite, TNF-α and PGE₂

The amount of nitric oxide released from glial cells was determined by assaying nitrite, a relatively stable metabolite of nitric oxide. Nitrite concentrations in the supernatants were measured using the Griess reaction as described previously. The optical density of assay samples was measured spectrophotometrically at 570 nm. Nitrite concentrations were determined using a standard curve constructed using known concentrations of sodium nitrite. The concentrations of TNF-α and PGE₂ in the supernatants of the culture media were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MO) according to the manufacturer’s instructions. The measurement sensitivities were 125 pmol for nitrite, 5 pg ml⁻¹ for TNF-α and 36.2 pg ml⁻¹ for PGE₂.

Assessment of the number of viable cells

The number of viable cells in each well after LPS treatment was assessed using a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The optical density of the reaction media was determined at 550 nm.

Statistics

All samples were assayed in duplicate and the values were averaged. Data are expressed as median (IQR). The Mann–Whitney test was used for comparison between two groups. A P-value <0.05 was considered significant. Concentration–inhibition curves were fitted to the Hill equation

\[ R = 1 - \frac{C^n}{C^n + IC_{50}^n} \]

where R is the relative concentration normalized to the values of the control group, C is the concentration of ketamine, n is a Hill coefficient and IC₅₀ is the concentration for 50% inhibition. Data for n and IC₅₀ are presented as mean (SD).

Results

Effects of ketamine on nitrite and TNF-α release from mixed glial cultures

The exposure of mixed glial cultures to LPS for 24 h increased nitrite concentration in the culture media from 0.76 (0.67–0.81) μM in the absence of LPS to 11.27 (9.96–12.63) μM and 13.49 (13.14–13.65) μM in the presence of LPS 0.5 μg ml⁻¹ and 1.0 μg ml⁻¹, respectively. Nitrite release induced by LPS 0.5 μg ml⁻¹ of LPS was slightly inhibited by ketamine 1000 μM, but the changes induced by ketamine did not reach statistical significance (Fig. 1A). Nitrite release elicited by LPS 1.0 μg ml⁻¹ was not affected by ketamine. The addition of 300 and 1000 μM of aminoguanidine, a blocker for iNOS, strongly inhibited LPS-induced nitrite production from mixed glial cells (Fig. 1B), indicating that LPS-induced nitrite production is mediated mainly by iNOS.

When mixed glial cells were exposed to LPS 0.5 μg ml⁻¹ and LPS 1.0 μg ml⁻¹ for 24 h, TNF-α concentration was markedly increased to 1122 (1059–1193) pg ml⁻¹ and 1082 (959–1133) pg ml⁻¹, respectively, whereas it was below the limits of detection without stimulation. LPS-induced TNF-α production was significantly suppressed by ketamine in a dose-dependent manner from 100 μM, the lowest dose tested (Fig. 1C). The addition of ketamine 100 μM decreased LPS-induced TNF-α production to 62.9 (49–65.2)% of that for the control group receiving LPS 0.5 μg ml⁻¹ only. Treatment with D-AP5 50 μM did not influence the increase in LPS-induced nitric oxide, whereas it caused a small but significant decrease in LPS-stimulated TNF-α production. However, the decrease in the TNF-α concentration accounted for only 17.3% of the value for the control group (Fig. 1D). In the absence of LPS stimulation, ketamine at 100, 300 or 1000 μM did not affect nitrite concentrations, and the TNF-α concentrations remained below detectable levels even when cultures were treated with ketamine (data not shown). As judged by the MTT assay, ketamine did not affect the number of viable cells in any of the experiments (Table 1).

Effects of propofol on nitrite and TNF-α release from mixed glial cultures

The concentration of the solvent DMSO used for the propofol stock solution was adjusted to 0.15% in the wells assigned for both 30 and 300 μM of propofol. We confirmed that this concentration of DMSO did not affect nitrite or TNF-α levels (Fig. 2). Propofol did not induce any significant changes in nitrite or TNF-α release from mixed glial cells stimulated with LPS 0.5 mg ml⁻¹. In the absence of LPS stimulation, the nitrite concentrations did not change and TNF-α levels remained undetectable in the presence of propofol 30 μM or 100 μM.

Effects of ketamine on nitrite and TNF-α release from microglial cultures

To identify the cell types on which ketamine acts, we isolated astrocytes and microglia from mixed glial cultures. The purities of the astrocyte and microglial cultures were 90% (87%, 94% 89%; average 90%; n=200 each) and 92% (88%, 92%, 96%; average 92%; n=200 each), respectively, as determined by immunocytochemistry (Fig. 3).

The exposure of microglial cultures to LPS 10 μg ml⁻¹ for 24 h increased the nitrite concentration in the culture supernatants from 1.41 (1.38–1.53) to 5.4 (4.94–5.85) μM and the TNF-α concentration from undetectable to 3295 (3249–3718) pg ml⁻¹. Although ketamine (30–1000 μM) did not affect LPS-induced nitrite release from microglia, it significantly suppressed LPS-induced TNF-α production in a concentration-dependent manner from 100 μM, as shown...
in Figure 4. The IC_{50} value of ketamine for the inhibition of TNF-α release was 485 (78) μM. Treatment with 100 μM ketamine decreased LPS-induced TNF-α production to 72.8 (70.1–90.1)% of that in the control group which received LPS alone.

**Effect of ketamine on nitrite, TNF-α and PGE_{2} release from astrocyte cultures**

Incubation with LPS 1.0 μg ml^{-1} for 24 h markedly stimulated nitrite, TNF-α and PGE_{2} production from astrocyte cultures, resulting in an increase from undetectable to 13.98 (11.54–16.15) μM for nitrite, 1137 (1046–1142) pg ml^{-1} for TNF-α and 36077 (35630–40881) pg ml^{-1} for PGE_{2}. Although ketamine (30–1000 μM) did not influence nitrite release from astrocytes (Fig. 5A), it significantly inhibited LPS-stimulated TNF-α production from astrocytes in a concentration-dependent manner from 30 μM, as shown in Fig. 5b. The IC_{50} value of ketamine was 82.1 (9.2) μM. The addition of ketamine 100 μM decreased the LPS-induced TNF-α level to 51.3 (46.2–62.3)% of the control value. Ketamine at 30 and 300 μM significantly reduced LPS-induced PGE_{2} release to 64.1 (62.7–69.2)% and 64.9 (61.7–67.4)% of the control, respectively, as shown in Figure 5c.

**Discussion**

To our knowledge, this is the first study to report the effects of anaesthetics on inflammatory responses of primary
cultures of rat glial cells stimulated with LPS. We found that ketamine reduced LPS-stimulated production of TNF-α in astrocytes and microglia without affecting nitric oxide release as estimated by nitrite measurements. Ketamine was also found to inhibit LPS-induced PGE2 production in astrocytes. Significant effects on astrocytes were observed at a ketamine concentration of 30 μM in media supplemented with serum. This concentration is comparable with the plasma concentrations shortly after i.v. injection of an anaesthetic dose (20 mg kg−1) in rats.28 29 Total plasma levels of ketamine are reportedly in the range of 33–94 μM immediately after 2.0–2.2 mg kg−1 i.v. administration in humans.30 Therefore we considered 30–100 μM as the higher range of clinically achievable concentrations.

Although ketamine has been shown to exert anti-inflammatory actions on a variety of immune cells, the exact mechanisms responsible for these actions are not well understood.8–11 We have shown that high doses of ketamine caused no significant changes in the number of viable cells estimated by MTT reduction assay. This finding excluded the possibility that the release of the inflammatory mediators is inhibited by the cytotoxic actions of ketamine. Our finding that ketamine reduced production of TNF-α and

Table 1 Effects of ketamine and propofol on glial cell viability. The number of viable cells was estimated using the MTT assay. Data are expressed as median (IQR). Neither ketamine nor propofol modified viability. Seven experiments using ketamine were performed for mixed glial cells, and six experiments were performed for all other conditions

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Optical density at 550 nm</th>
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<tr>
<td></td>
<td>Mixed glia (LPS 0.5 μg ml−1)</td>
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<tr>
<td>Ketamine</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.46 (0.45–0.48)</td>
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<tr>
<td>30</td>
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<tr>
<td>100</td>
<td>0.48 (0.47–0.49)</td>
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<tr>
<td>300</td>
<td>0.47 (0.47–0.50)</td>
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<tr>
<td>1000</td>
<td>0.51 (0.50–0.52)</td>
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<tr>
<td>Propofol</td>
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<tr>
<td>0</td>
<td>0.31 (0.31–0.33)</td>
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<tr>
<td>30</td>
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<td>300</td>
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Fig 2 The effect of propofol on LPS-induced (a) nitrite and (b) TNF-α release from mixed glial cultures. Mixed glial cultures were exposed to LPS 0.5 μg ml−1 with or without propofol for 24 h. The box and whisker plots show medians with 25th–75th and 5th–95th percentiles. Propofol or DMSO caused no significant changes in LPS-stimulated nitrite (n=6) or TNF-α (n=5) release.

Fig 3 Representative photomicrographs of secondary cultures of (a) astrocytes and (b) microglial cells subjected to immunocytochemical analysis. Left panels show phase-contrast micrographs and right panels show fluorescence micrographs. (a) The majority of cells in astrocyte cultures were positively stained with an antibody to glial fibrillary acidic protein (GFAP). (b) The majority of cells in microglial cultures were immunostained with OX-42. Scale bar, 100 μM.
PGE2 without affecting nitric oxide release is intriguing but inconsistent with earlier studies reporting inhibitory effects of ketamine on the release of both TNF-α and nitric oxide from a macrophage-like cell line and alveolar macrophages in response to LPS.10,31 The mechanisms for the differential effects on nitric oxide release from different cells remain to be clarified. It is known that LPS stimulation causes activation and nuclear translocation of nuclear factor κB (NF-κB) and activator protein 1 (AP-1), leading to transcriptional activation of proinflammatory genes, such as those encoding iNOS, TNF-α and cyclooxygenase-2, in glial cells as well as macrophages.2 However, a number of studies have revealed differences in the regulation of LPS-induced expression of

**Fig 4** The effect of ketamine on LPS-induced nitrite and TNF-α release from microglial cultures. Microglial cultures were exposed to LPS 10 µg ml⁻¹ with or without ketamine for 24 h. The data shown are medians with 25th–75th and 5th–95th percentiles. *P<0.01 compared with the corresponding values in the absence of the test compounds. (A) LPS-induced nitrite release was not affected by ketamine (n=6). (B) LPS-induced TNF-α release was significantly suppressed by ketamine in a concentration-dependent manner from 100 µM (n=5). The inset shows the concentration–inhibition relationship fitted to a Hill equation with IC₅₀=484.7 (77.7) µM and a Hill coefficient of 0.79 (0.12) (r=0.99).

**Fig 5** The effect of ketamine on LPS-induced nitrite, TNF-α and PGE2 release from astrocyte cultures. Astrocyte cultures were exposed to LPS 1.0 µg ml⁻¹ with or without ketamine for 24 h. The box and whisker plots show medians with 25th–75th and 5th–95th percentiles. *P<0.01 compared with the corresponding values in the absence of the test compounds. (A) Ketamine induced no significant changes in LPS-elicited nitrite release (n=6). (B) LPS-induced TNF-α release was significantly suppressed by ketamine in a concentration-dependent manner from 30 µM, the lowest concentration tested (n=6). The inset shows the concentration–inhibition relationship fitted to a Hill equation with IC₅₀=82.1 (9.2) µM and a Hill coefficient of 0.41 (0.03) (r=0.99). (C) LPS-induced PGE2 release was suppressed by ketamine at 30 and 300 µM (n=6).
iNOS and inflammatory cytokines between macrophages and glial cells. Certain intracellular signalling molecules, such as cyclic AMP and protein phosphatases, are known to regulate LPS-stimulated iNOS expression in opposite directions in macrophages and astrocytes.\textsuperscript{15,16} The mitogen-activated protein kinases ERK-1 and ERK-2 have different roles in LPS-induced signalling in macrophages and microglia.\textsuperscript{17} These cell-type-specific signalling pathways are likely to contribute to the different effects of ketamine observed in macrophages and glial cells. In another study which investigated the effects of ketamine in macrophages,\textsuperscript{10} different stimulatory conditions may have contributed to the discrepancy.

Regarding the molecular targets for ketamine-induced inhibition of TNF-\(\alpha\) and PGE\(_2\) production in glial cells, involvement of NMDA receptors in these effects is unlikely for the following reasons. First, D-AP5 failed to mimic the effects of ketamine, exhibiting only a minor inhibition of TNF-\(\alpha\) production. Secondly, the blocking action of ketamine on NMDA receptors should be saturated at much lower concentrations than those used in our study.\textsuperscript{32} Thirdly, NMDA receptors are not considered to be expressed on most astrocytes or microglia.\textsuperscript{33,34} It is possible that NMDA receptors play minor roles in LPS-induced TNF-\(\alpha\) production; however, these receptors do not seem to be the primary sites responsible for the observed effects of ketamine. The exact mechanism of the action of ketamine action on glial cells remains to be determined.

To examine the effects of propofol, we chose 30 \(\mu\)M as the clinical concentration and 300 \(\mu\)M as the pharmacological concentration, because the 95\% effective concentration of propofol for loss of consciousness in patients is reportedly around 30 \(\mu\)M in the total fraction combining free and plasma binding fractions.\textsuperscript{35} We found that propofol had no effect on LPS-induced nitric oxide or TNF-\(\alpha\) production in mixed glial cells at either the clinically relevant concentration or a concentration 10 times greater. We did not examine the effects of propofol on each cell type, because the results in mixed glial cells were negative. Our results indicate that TNF-\(\alpha\) release from primary cultures of glial cells is differentially modulated by ketamine and propofol. Whereas propofol at a concentration of 157 \(\mu\)M reportedly increased LPS-stimulated TNF-\(\alpha\) production in human whole blood,\textsuperscript{12} it was shown to inhibit nitric oxide and TNF-\(\alpha\) release from alveolar macrophages in an endotoxin-induced lung injury model\textsuperscript{14} and to suppress nitric oxide and iNOS expression in LPS-stimulated macrophages at 25–100 \(\mu\)M.\textsuperscript{36} A number of factors could account for the different results obtained in different experiments including cell-specific differences in the regulation of inflammatory responses mentioned above, differences in culture conditions (i.e. whole-blood culture containing heparin vs the usual culture system), and differences in \textit{in vivo} and \textit{in vitro} experiments. Further study is required to clarify the reasons for the difference in propofol action in different cells.

Quantification of nitrite by the Griess reaction has some limitations. It measures nitrite, the major product of nitric oxide, but not nitrate. It is possible that the nitrite concentrations may be a fraction of total nitric oxide released. Another method, such as converting nitrate to nitrite or measuring iNOS expression, could be used to circumvent this problem, but the Griess reaction has been widely used to monitor nitric oxide production in biological fluids. Because other studies reporting inhibitory effects of ketamine and propofol on nitric oxide release from immune cells also used the Griess reaction to estimate nitric oxide release,\textsuperscript{10,36} the different findings between earlier and current reports cannot be explained by limitations of this method.

This study simulates infection of the CNS with gram-negative bacteria, and our results suggest that ketamine may attenuate some of the inflammatory responses of glial cells in this pathological condition. Inflammatory responses of glial cells also develop in various forms of brain injury, including stroke and trauma. Spinal glial inflammation is also believed to play a role in exaggerated pain states.\textsuperscript{5} Glial inflammatory responses are relatively stereotypic\textsuperscript{1} and relevant to patients suffering from these various insults to the CNS. In these situations, the proinflammatory cytokines released, such as TNF-\(\alpha\) and interleukin 1\(\beta\), also activate glial cells via toll-like receptors and downstream signalling pathways partially shared by LPS-stimulated signalling.\textsuperscript{2} This study raised the possibility that ketamine might modulate some of the inflammatory processes in these pathological conditions. However, further studies are needed to clarify whether ketamine attenuates the response of glial cells to proinflammatory cytokines and whether it exerts anti-inflammatory effects on glial cells \textit{in vivo}.

In conclusion, we found that ketamine inhibited some of the inflammatory responses of both astrocytes and microglial cells treated with LPS without causing major changes in nitric oxide release. In contrast, propofol did not affect LPS-induced TNF-\(\alpha\) or nitric oxide release from glial cells.

Acknowledgements

This work was supported financially by the Yokohama Foundation for Advancement of Medical Science, Yokohama, Japan.

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