A possible role of microglia-derived nitric oxide by lipopolysaccharide in activation of astroglial pentose-phosphate pathway via the Keap1/Nrf2 system

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Abstract

Background: Toll-like receptor 4 (TLR4) plays a pivotal role in the pathophysiology of stroke-induced inflammation. Both astroglia and microglia express TLR4, and endogenous ligands produced in the ischemic brain induce inflammatory responses. Reactive oxygen species (ROS), nitric oxide (NO), and inflammatory cytokines produced by TLR4 activation play harmful roles in neuronal damage after stroke. Although astroglia exhibit pro-inflammatory responses upon TLR4 stimulation by lipopolysaccharide (LPS), they may also play cytoprotective roles via the activation of the pentose phosphate pathway (PPP), reducing oxidative stress by glutathione peroxidase. We investigated the mechanisms by which astroglia reduce oxidative stress via the activation of PPP, using TLR4 stimulation and hypoxia in concert with microglia.

Methods: In vitro experiments were performed using cells prepared from Sprague–Dawley rats. Coexisting microglia in the astroglial culture were chemically eliminated using L-leucine methyl ester (LME). Cells were exposed to LPS (0.01 μg/mL) or hypoxia (1 % O2) for 12–15 h. PPP activity was measured using [1-14C]glucose and [6-14C]glucose. ROS and NO production were measured using 2′,7′-dichlorodihydrofluorescein diacetate and diaminofluorescein-FM diacetate, respectively. The involvement of nuclear factor-erythroid-2-related factor 2 (Nrf2), a cardinal transcriptional factor under stress conditions that regulates glucose 6-phosphate dehydrogenase, the rate-limiting enzyme of PPP, was evaluated using immunohistochemistry.

Results: Cultured astroglia exposed to LPS elicited 20 % increases in PPP flux, and these actions of astroglia appeared to involve Nrf2. However, the chemical depletion of coexisting microglia eliminated both increases in PPP and astroglial nuclear translocation of Nrf2. LPS induced ROS and NO production in the astroglial culture containing microglia but not in the microglia-depleted astroglial culture. LPS enhanced astroglial ROS production after glutathione depletion. U0126, an upstream inhibitor of mitogen-activated protein kinase, eliminated LPS-induced NO production, whereas ROS production was unaffected. U0126 also eliminated LPS-induced PPP activation in astroglial–microglial culture, indicating that microglia-derived NO mediated astroglial PPP activation. Hypoxia induced astroglial PPP activation independent of the microglia–NO pathway. Elimination of ROS and NO production by sulforaphane, a natural Nrf2 activator, confirmed the astroglial protective mechanism.

Conclusions: Astroglia in concert with microglia may play a cytoprotective role for countering oxidative stress in stroke.

Keywords: Ara-C (cytosine β-D-arabinofuranoside hydrochloride), Astrocyte, LME (L-leucine methyl ester), RNS (reactive nitrogen species), ROS (reactive oxygen species), TLR4 (Toll-like receptor 4)

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Background
Although multiple mechanisms underlie the pathophysiology of ischemic stroke, there is increasing evidence that inflammation accounts for its progression [1, 2]. A robust inflammatory reaction characterized by peripheral leukocyte influx into the cerebral parenchyma and the activation of endogenous microglia follows focal cerebral ischemia [3–7]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play pivotal roles in neuronal damage. ROS and/or RNS are, at least in part, produced by cerebral parenchymal cells, including neurons and glial cells, as well as peripheral inflammatory cells such as leukocytes. The origin of ROS appears to be from activated NADPH oxidase (NOX), and ROS, in turn, reacts with nitric oxide (NO) produced by activated inducible nitric oxide synthase (iNOS), leading to peroxynitrite generation, which is a highly toxic reactive species that can induce lipid peroxidation and protein oxidative damage [8]. Thus, the intrinsic protective mechanism that eliminates ROS may play an important role in countering post-stroke inflammation.

Toll-like receptors (TLRs) mediate innate immune response, and recent findings suggest that they also mediate post-stroke inflammatory responses [9]. There are at least 11 TLR proteins in mammals [10], and TLR4 is implicated in the pathogenesis of stroke-related inflammation. TLR4 is predominantly expressed in both microglia and astroglia, and also in neurons [11]. Although lipopolysaccharide (LPS), a component of bacterial cell walls, is a well-known classical ligand of TLR4, extracellular matrix and dysfunctional mitochondria released from damaged cells have been reported to be endogenous ligands of TLR4 [12]. TLR4 stimulation of microglia plays a harmful role in stroke through downstream signaling pathways: myeloid differentiation protein 88 (MyD88), mitogen-activated protein kinase (MAPK), also called extracellular signal-regulated kinase 1/2, and Janus kinases, which are signal transducers and activators of transcription. In particular, the activation of NFκB induces NOX and iNOS, which enhance ROS and NO production by microglia.

Astroglia express TLR4 even in the human brain, and the stimulation of TLR4 by LPS in cultured astroglia induces pro-inflammatory responses [13]. In contrast, LPS may also induce an astrogial anti-inflammatory, protective function via the pentose phosphate pathway (PPP) [14]. PPP is a shunt pathway of glycolysis and produces NADPH, which recycles the oxidized form of glutathione (GSH) back to the reduced form, leading to the removal of ROS by GSH peroxidase. Thus, PPP activity is an index of endogenous neuroprotective function. In fact, astroglial PPP activity is much higher than that of neurons [15], and PPP activation plays an important role in countering oxygen stress. The activation of astroglial TLR4 by LPS activates astrogial PPP through NO [14, 16, 17]. The exact mechanism by which NO activates PPP remains to be elucidated.

The rate-limiting enzyme of PPP, glucose 6-phosphate dehydrogenase (G6PDH), is regulated both allosterically and transcriptionally. Under stress conditions, the master regulator of the anti-stress response, the Kelch-like ECH-associated protein 1 (Keap1)/nuclear factorerythroid-2-related factor 2 (Nrf2) system induces various enzymes via transcriptional regulation mediated by Nrf2. In fact, G6PDH also harbors an antioxidant response element (ARE) to which Nrf2 binds and initiates the transcription of G6PDH, leading to PPP activation [18]. Nrf2 is bound to its adaptor protein Keap1 and is constantly degraded by the ubiquitin–proteasome system in the cytosol. However, several signals can affect the cysteine residues of Keap1 to induce a conformational change for releasing Nrf2. Free Nrf2 translocates into the nucleus and initiates transcription. Numerous reports show that both ROS and/or NO can induce Keap1 conformational change and Nrf2 activation [19–22]. Thus, TLR4-mediated production of ROS, NO, or both by astroglia is a potential mediator.

Another important issue that should be clarified is the cellular origin of ROS or NO. There is no doubt that LPS induces ROS and NO production in microglia. However, whether astrogial ROS and NO production are induced by LPS remains to be established. Most studies describing the astrogial response to LPS have been based on cell culture experiments [23]. Usually, an astrogial culture contains a small (<10%) population of microglia. If LPS acts on microglia, even on <10% of the total cells, microglia can produce sufficient ROS and NO in response to LPS.

The present study used a conventional secondary astrogial culture prepared from pups of Sprague–Dawley rats. To eliminate microglial cells, the conventional astrogial culture was treated with cytosine β-D-arabinofuranoside hydrochloride (Ara-C), followed by L-leucine methyl ester (LME) [24, 25]. This procedure completely eliminates Iba1-positive cells. We used both microgliapresent and microglia-absent astrogial cultures and evaluated PPP activity, as well as ROS and NO production in response to LPS.

Methods
Animals
Timed-pregnant Sprague–Dawley rats were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Male C57BL/6 (wild-type (WT)) mice (Japan SLC, Inc.) and Nrf2 gene knockout (KO) mice (on C57BL/6 background, originally generated by Dr. Masayuki Yamamoto) were used in this study. Nrf2-KO mice were genotyped by PCR amplification of genomic DNA extracted from tail snips.
All animal procedures were performed in accordance with The Animal Experimentation Guidelines of Keio University School of Medicine and were approved by the Committee on Animal Care and Use, Keio University (08097-[15]).

**Chemicals**

Chemicals and materials were obtained from the following sources: Insta-Fluor Plus and Hyamine hydroxide 10-X from Perkin-Elmer Life Sciences (Boston, MA, USA); D-[1-14C]glucose (specific activity, 2.035 GBq/mmol) and D-[6-14C]glucose (specific activity, 2.035 GBq/mmol) from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA); normal goat serum from Jackson ImmunoResearch (West Grove, PA, USA); rabbit Iba1 antibody from Santa Cruz Biochemistry (Delaware, CA, USA); 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126), a selective inhibitor of MAPK/ERK kinase1/2 (MEK1/2) from Cell Signaling Technology (Danvers, MA, USA); mouse monoclonal antibody against anti-β actin from Abcam (Cambridge, UK); rabbit anti-Nrf2 (H-300) polyclonal antibody, rhodamine-conjugated goat anti-mouse (for GFAP), anti-rabbit (for Nrf2) IgG antibody, and fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit (for Iba1) antibody from Santa Cruz Biochemistry; Dulbecco’s modified Eagle media (DMEM) with or without glucose, penicillin, and streptomycin from Life Technologies (Grand Island, NY, USA); defined fetal bovine serum from HyClone Laboratories (Logan, UT, USA); 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) and monochlorobimane (MCB) from Molecular Probe Inc. (Eugene, OR, USA); dianminofluorescein-FM diacetate (DA-FM DA) from Sekisui Medical (Chuo-ku, Tokyo, Japan); and all other chemicals were obtained from Sigma (St. Louis, MO, USA).

**Preparation of cells**

Primary astroglial cultures were prepared from the striatum of fetal rats on embryonic day 16, as previously described [26]. Mechanically dissociated cells were placed in 12-well culture plates (0.8 mL/well, respectively) or 25-cm² culture flasks (5 mL/flask) coated with poly-L-lysine (5 µg/mL). For the neuronal cultures, viable cells (1.5 × 10⁶ cells/mL) that excluded trypan blue were placed in the cultures, and Ara-C (10 µmol/L) was added 72 h later to induce mitotic arrest of the astroglia. The cells were cultured in a glucose medium (final concentration, 12 mmol/L D-glucose) at 37 °C in humidified air containing 21 % O₂ and 7 % CO₂. In vitro assays were performed using cultures that were 7 or 8 days old. The nutrient medium remained untouched until the experiments were initiated.

Microglial cells were obtained by collecting floating or loosely adherent cells in primary astroglial culture and were placed in 12-well culture plates or glass-bottomed dishes precoated with poly-L-lysine [27].

**Depletion of microglia from astroglial cultures**

Usually, secondary astroglial cultures contain only small amounts (<10 % of total cells) of microglia, as previously described [27, 28]. To eliminate all possible metabolic effects of microglial contamination, the treatment with a combination of Ara-C and LME was applied according to the method described by Pont-Lezica et al. [25]. Briefly, 10 µmol/L (final concentration in nutrient medium) of Ara-C was added to the astroglial culture 3 days prior to the assay. After 48 h, cells were further incubated with 50 mmol/L of LME (pH adjusted to ~7.4) for 60 min. Cells were then washed with phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺ and further cultured with a regular nutrient medium for 24 h. This treatment destroyed microglia by osmotic effects and...
yielded almost 100 % pure astroglial cell culture. The exact mechanism by which LME eliminates microglia remains to be elucidated. It has been speculated that LME is internalized by microglia, wherein it causes lysosomal disruption and subsequent apoptosis. In fact, macrophages and microglia are characteristically rich in lysosomes, causing them to be particularly vulnerable upon exposure to LME treatment [29, 30].

**Experimental protocol**

To assess the effects of LPS on astroglial PPP activation, ROS/NO production, and immunohistochemistry, the nutrient medium (12 mmol/L) was removed and fresh nutrient medium containing LPS (0.01, 0.1, or 1 μg/mL) was added and kept in a CO₂ incubator for 15 h. For neuronal cultures, a medium change per se damages cells via glutamate toxicity [31], LPS was directly added to the culture media of cells to obtain final concentrations of 0.01, 0.1, or 1 μg/mL, as described above. When astroglial cells were exposed to 1-isothiocyanato-(4R,S)-(methylsulfinyl)butane (sulforaphane), an Nrf2 activator [32–34] or S-nitroso-N-acetyl-DL-penicillamine (SNAP), an NO donor [35], the nutrient medium was replaced with a fresh medium containing the drug or vehicle (dimethyl sulfoxide (DMSO)), and cells were incubated for 15 h. When the effects of sulforaphane (10 μmol/L), U0126 (10 μmol/L), L-buthionine-sulfoximine (BSO; 50 μmol/L)—an inhibitor of GSH synthesis [36]—on LPS-induced ROS/NO production were assessed, cells were pre-incubated with these drugs for 12 h before LPS treatment and further incubated for 12 h with LPS, followed by the measurement of ROS and NO.

We next assessed the effects of hypoxia because it is also well recognized that hypoxic conditions per se shift the cellular metabolism from oxidative to glycolytic in various cells. However, whether hypoxia...
enhances PPP flux has not been addressed. An early study revealed that chronic hypoxia induced PPP activity in C6 glioma cells [37], but the mechanism that conveys the hypoxic signal for PPP activation is undetermined. In a recent study [38], hypoxia and reperfusion induced PPP activation in astroglia. Although the underlying mechanism was not described, the reperfusion-induced massive production of ROS in association with NO might exert a strong influence on PPP activation. Moreover, the effect of LPS or LPS in combination with hypoxia on astroglial PPP activity has not been reported. In the present study, we examined the effect of LPS stimulation in combination with hypoxia on PPP flux in astroglia. To assess the effect of hypoxia on PPP activation, we placed cells for 12 or 24 h in a hypoxic chamber (multi-gas incubator APM-30DR, ASTEC, Fukuoka-ken, Japan) at 37 °C in humidified air containing 1 % O₂ and 7 % CO₂. We assessed the combined effects of LPS (0.01 μg/mL) and hypoxia (1 % O₂) for 15 h in some neuronal and astroglial cells.

Measurement of the rates of D-[1-14C]glucose and D-[6-14C]glucose oxidation to 14CO₂

The rate of [14C]glucose oxidation to 14CO₂ was measured using a modification of a previously described method [39] after cells cultured in 25-cm² culture flasks were washed twice with PBS containing no glucose. The assay solutions consisted of Dulbecco’s balanced salt solution (DBSS) containing (mmol/L) 110 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgSO₄, 0.9 NaH₂PO₄, and 44 NaHCO₃, in addition to 2 mmol/L of D-glucose labeled with 1.0 μL/mL of D-[1-14C]glucose or D-[6-14C]glucose (original concentrations, 3.7 MBq/mL), were added, and the flasks were capped with rubber stoppers containing a center well and incubated at 37 °C for 60 min. The 14CO₂ produced was trapped by a cotton ball placed in the center well containing 100 μL of Hyamine hydroxide 10-X. The reactions were terminated by the injection of 250 μL of perchloric acid (2 mol/L) through the rubber stopper, and the flasks were kept at 4 °C overnight to trap the 14CO₂. The center wells were then transferred to 20-mL glass scintillation counter vials,
Fig. 3 (See legend on next page.)
The effects of chemical depletion of microglia. Chemical depletion of microglia eliminated LPS-induced production of ROS and NO and astroglial PPP activation. The rate of ROS/NO production was expressed as percent increase in the fluorescent signal at 60 min compared with that at 0 min. The rate of ROS production in the secondary astroglial culture exposed to LPS (0.01 μg/mL) for 12 h (a, ROS), and the rate of NO production in the secondary astroglial culture exposed to LPS (0.01 μg/mL) for 12 h (b, NO). Open columns, untreated astroglia. Striped columns, Ara-C/LME-treated astroglia. Values are the means ± SD of six wells. n.s., not significant, ***P < 0.001 versus control (grouped t test). PPP flux in untreated astroglia (a, PPP) and in microglia-depleted astroglia (b, PPP) after 15 h exposure to various concentrations of LPS as indicated in the figure. PPP flux increased in untreated astroglia at LPS of 0.01 μg/mL, but not in microglia-depleted astroglia. Values are the means ± SD of four flasks. n.s., not significant, **P < 0.01 versus control (ANOVA followed by Dunnett’s test). Representative photographs showing immunofluorescent staining with DAPI (blue), GFAP (green), or Nrf2 (red) in the secondary astroglial cultures. LPS (0.01 μg/mL) for 12 h induced the nuclear translocation of Nrf2 in microglia-containing astroglia (e), but did not in microglia-depleted astroglia (f). Scale bar, 50 μm.

Measurement of ROS and NO production

The production of ROS, mainly H₂O₂, and NO in cells was assessed by H₂DCFDA [42] and DAF-FM DA [43] using semiquantitative fluorometric measurement. Immediately prior to the assay, the nutrient medium was removed and the cells were washed twice with PBS without glucose. Then, DBSS containing 10 μmol/L of H₂DCFDA or DAF-FM DA dissolved in DMSO (final volume of DMSO 0.1 %) supplemented with 2 mmol/L glucose were added, and the cells were incubated at 37 °C in humidified air with 7 % CO₂ for 30 min. After the H₂DCFDA or DAF-FM DA were loaded for 30 min, the cells were washed twice again with PBS without glucose and DBSS containing 2 mmol/L D-glucose. The cells were further incubated for 60 min, and the fluorescence level indicating intracellular ROS or NO production was measured at 0 and 60 min using a fluorescent microplate reader (Infinite 200 PRO; Tecan Japan, Kanagawa, Japan) with excitation at 485 nm and emission at 530 nm. As the fluorescent signals linearly increased for up to 60 min (data not shown), the results were expressed as the percent increase in fluorescent signal at 60 min compared with that at 0 min.

Measurement of total glutathione content using a fluorometric method

The intracellular content of total glutathione in astroglia was assessed using MCB which forms an adduct with glutathione via an enzymatic reaction catalyzed by glutathione transferases [44]. Cells were incubated with MCB (50 μmol/l) for 30 min. The intracellular formation of the GSH–MCB adduct was assessed using a microplate reader with excitation at 360 nm and emission at 465 nm. The results were expressed as the fluorescence signal standardized by cellular protein content.

Immunohistochemistry

The translocation of Nrf2 from the cytosol to the nucleus was assessed using immunohistochemistry. Astroglial cells grown in a glass-bottomed 35-mm dish were fixed with 4 % paraformaldehyde for 10 min on ice. The cells were permeabilized with 0.5 % Triton X-100 for 10 min at room temperature, followed by post-fixation with 4 % paraformaldehyde for 5 min on ice. For immunostaining, the cells were washed twice with PBS containing MgCl₂ and CaCl₂, and nonspecific IgG binding sites were blocked by the incubation of cells in PBS containing 3 % bovine serum albumin and 3 % normal goat serum for 30 min at room temperature. Cells were incubated with primary antibodies (anti-GFAP [1:200], anti-Iba1 [1:200], and anti-Nrf2 polyclonal antibody [1:100]) for 2 h at room temperature.
Fig. 4 (See legend on next page.)
They were then incubated with the secondary antibodies (rhodamine-conjugated goat anti-rabbit IgG antibody or FITC-conjugated goat anti-mouse IgG antibody) with 4′,6-diamino-2-phenylindole (DAPI) for nuclear staining for 1 h. The cells were examined using a laser confocal microscopy system (Leica TCS SP5; Leica Microsystems, Exton, PA, USA).

**Western blotting**
Cells were homogenized in an ice-cold lysis buffer; radioimmunoprecipitation assay buffer, 0.5 % sodium dodecyl sulfate, ~0.1 M phenylmethanesulfonyl fluoride, and protease inhibitor cocktail. The concentration of total protein in the supernatant was determined using the BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA). The proteins were separated using 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA, USA). After treatment for 1 h with a blocking solution containing 5 % nonfat dry milk, the membrane was incubated with anti-TLR4 antibody (1:200), and anti-β actin antibody (1:5000). After washing, the membranes were reacted with the secondary antibodies, the membrane was incubated with horse-radish peroxidase-conjugated anti-goat (Santa Cruz), or anti-mouse antibody (Jackson ImmunoResearch) and the immunoreactive bands were visualized by enhanced chemiluminescence and detected with a lumino-image analyzer (Las-4000; Fujifilm, Tokyo, Japan).

**Evaluation of Nrf2 activation by quantitative reverse transcription PCR (qRT-PCR) analysis for mRNA of heme oxygenase-1 (HO-1)**
Total RNA from cultured cells was isolated using RNeasy mini kit (Qiagen, Valencia, CA) with a DNase (Qiagen, Valencia, CA) treatment. First-stranded cDNA was synthesized using PrimeScript RT reagent Kit (TaKaRa) according to the manufacturer’s instructions. qRT-PCR was performed using predesigned gene-specific TaqMan Gene Expression Assays (Applied Biosystems) with the StepOnePlus real-time PCR system (Applied Biosystems). Rat HO-1 TaqMan probe and primers (cat# Rn00561387_m1) and rat GAPDH TaqMan probe and primers (cat# Rn01775763_g1) were from Applied Biosystems. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization. The fold difference between levels of transcripts was calculated using the ΔΔCT method.

**Quantification of metabolites by capillary-electrophoresis/electrospray ionization (CE/ESI) MS**
The PPP flux and other metabolic pathway of glucose were analyzed using CE/ESI MS (Additional file 1).

**Statistical analyses**
Statistical comparisons among the values obtained for each group were performed using grouped t tests or one-way analysis of variance followed by the Dunnett test for multiple comparisons. A P value of <0.05 was considered statistically significant.

**Results**

**Ara-C/LME treatment eliminated microglia in the secondary astroglial culture**
As shown in Fig. 1a, b, the secondary astroglial culture mainly consists of GFAP-positive astrocytes (red). However, immunostaining with Iba1 (green) showed a small number of microglia coexisting with the astroglia (Fig. 1a). After Ara-C/LME-treatment, Iba1-positive cells were completely removed (Fig. 1b). The Iba1-positive cells were counted and shown as % of the total number of cells counted with DAPI (Fig. 1c). The proportion of microglia in the normal astroglial culture was approximately 10 %, as previously reported, but was reduced to zero after treatment with Ara-C/LME. Western blot analysis (Fig. 1d), however, showed TLR4 expression in the microglia-depleted astroglial culture as well as microglia-containing astroglia, indicating that cultured astroglial cells indeed harbor TLR4 and can potentially respond to the LPS stimulation.

**Microglia-enriched culture produced both ROS and NO in response to LPS**
We cultured primary microglial cells from primary astroglial cultures. As shown in Fig. 2a, Iba1-positive cells without GFAP-positive cells were obtained. Ara-C/LME treatment eliminated almost all Iba1-positive cells,
Fig. 5 (See legend on next page.)

A  HO-1

B  PPP

C  PPP
as expected (Fig. 2b). LPS (0.01 μg/mL for 12 h) induced both ROS and NO production in these Iba1-positive cells (Fig. 2c, d).

LPS-induced production of ROS and NO disappeared after chemical depletion of microglia

Astroglial cells containing microglia showed marked elevation in the rates of ROS (Fig. 3a) and NO (Fig. 3b) production after exposure to LPS (0.01 μg/mL) for 12 h. However, these increases were eliminated after microglial depletion (Fig. 3a, b).

LPS induced increases in PPP flux in microglia-containing astroglia, but not in microglia-depleted astroglia

As shown in Fig. 3c, the effect of LPS on increasing PPP flux in astroglia was evident only in the presence of microglia. LPS did not induce PPP activation after the elimination of microglia (Fig. 3d).

LPS induced nuclear translocation of Nrf2 in astroglia in the presence of microglia

Immunohistochemistry showed that LPS induced Nrf2 translocation into the nuclei of astroglial cells in the presence of microglia (Fig. 3e) but not after microglia depletion (Fig. 3f).

Inhibition of MAPK eliminated microglial NO production and astroglial PPP activation, indicating that MAPK involvement in LPS-induced PPP activation in astroglia is mediated by microglial NO production

As shown in Fig. 4, NO production presumably by microglia in response to LPS was eliminated by U0126, a selective inhibitor of MEK1/2 (Fig. 4b), whereas ROS production induced by LPS was not affected (Fig. 4a). Basal production of ROS, but not NO was reduced by U0126 (Fig. 4a). Similarly, U0126 eliminated NO production by LPS in microglia (Fig. 4d), while ROS production was not affected by U0126 (Fig. 4c). We examined if U0126 treatment affects basal production of ROS or NO in astroglia or microglia. Neither ROS nor NO production in astroglia was affected by U0126, while microglial ROS, but not NO was reduced (Fig. 4e, f).

NO directly induced Nrf2 activation, leading to PPP activation in astroglia

Next, we examined whether NO directly induces the activation of Keap1/Nrf2 system. HO-1 is a representative gene whose transcription is governed by Nrf2. Sulforaphane, a potent Nrf2 activator, was used as a positive control. As shown in Fig. 5a, both sulforaphane and SNAP, an NO donor, induced significant increases in HO-1 gene expression in microglia-free astroglia as assessed by qRT-PCR. Importantly, U0126 did not affect HO-1 gene expression by sulforaphane or SNAP, indicating direct action on Nrf2 activation independent of MAPK. Moreover, NO donor induced PPP activation in astroglia devoid of microglia (Fig. 5b), and these NO-induced PPP activation was not affected by U0126 (Fig. 5b). As shown above, PPP activation in astroglial–microglial culture by LPS was also dependent on the presence of microglia (Fig. 3c, d). We accordingly investigated whether microglia-derived NO or ROS or both induced the activation of astroglial PPP flux. On addition of U0126, which eliminated NO production from microglia (Fig. 4d), the PPP activation in astroglia also disappeared (Fig. 5c), indicating that NO but not ROS is involved in this microglia–astroglia axis.

Depletion of GSH synthesis induced ROS production in astroglia devoid of microglia in response to LPS

As shown in Fig. 1d, astroglia express TLR4. Thus, TLR4-mediated ROS/NO production should be induced in astroglia in response to LPS. We speculated that high astroglial ability to erase ROS masked ROS production. When we inhibited GSH synthesis in astroglia by adding BSO, an inhibitor of GSH synthesis (Fig. 6a), LPS did induce ROS production (Fig. 6b), but not NO production (Fig. 6c) in astroglial cell devoid of microglia.

Hypoxia enhanced PPP flux in astroglia in both presence and absence of microglia

In contrast to LPS stimulation, hypoxia induced increases in PPP flux in both untreated control astroglia (with microglia; Fig. 7a) and Ara-C/LME-treated astroglia (without microglia; Fig. 7b), indicating that hypoxia-induced PPP activation in astroglia is not dependent on microglia.
Hypoxia-induced PPP activation in astroglia was not associated with NO production

Hypoxia-induced PPP activation appeared microglia-independent, given that hypoxia enhanced PPP flux in microglia-depleted astroglia. Hypoxia also did not induce NO production in astroglial cells devoid of microglia (Fig. 7d). NO production by microglia-containing astroglia was not enhanced under hypoxia (Fig. 7c, d), indicating that PPP activation under hypoxia was not NO dependent. We speculated that
PPP is activated in association with activated glycolysis in response to hypoxia.

Hypoxia and TLR4 activation by LPS increased astroglial PPP flux in an additive manner

To investigate whether LPS-induced PPP increase is preserved in hypoxic conditions, we assessed the effect of combined hypoxia and LPS on PPP flux. As shown in Fig. 8, hypoxia and LPS enhanced PPP flux in an additive manner, confirming that the two pathways are independent.

Activation of PPP by sulforaphane, an activator of Nrf2, eliminated LPS-induced ROS and NO production in astroglia containing microglia

LPS activates TLR4 in microglia and induces inflammatory responses. In particular, ROS and NO produced by microglia lead to neuronal damage during ischemic stroke. As we have shown, NO derived from microglia enhances PPP activity in astroglia, which may play a role in reducing ROS and NO, thereby protecting neurons. To confirm astroglial protective roles, we measured ROS and NO production in astroglia with microglia after the activation of astroglial PPP. Sulforaphane, a potent activator of Nrf2, activated astroglial PPP and basal ROS production in astroglia [45]. In the present study, we confirmed that sulforaphane induced HO-1 gene expression in astroglia (Fig. 5a). Furthermore, sulforaphane did, indeed, induce increases in glutathione content in astroglia devoid of microglia (Fig. 9a). Consequently, as shown in Fig. 9b, c, LPS-induced robust increases in ROS and NO were completely eliminated. Again, the effects of sulforaphane on PPP activation that was observed in WT mouse (Fig. 10a) were eliminated in...
astroglia prepared from Nrf2-KO mouse (Fig. 10b). However, hypoxia-induced PPP activation (Fig. 10a) was preserved in astroglia deficient of Nrf2 (Fig. 10b).

Quantification of metabolites in astroglial pentose-phosphate pathway by capillary-electrophoresis/electrospray ionization (CE/ESI) MS

The metabolites of PPP were increased by hypoxia (1 % O₂ for 12 h) in astroglia prepared from WT and Nrf2-KO mouse. These results are in accordance with those obtained by the [1-¹⁴C]/[6-¹⁴C]glucose method. Namely, the difference of the CO₂ production from [1-¹⁴C]glucose and [6-¹⁴C]glucose is a good indication of the cellular PPP flux. Moreover, hypoxia-induced PPP enhancement was independent of Nrf2 (Additional file 2).

Neurons do not respond to either LPS stimulation or hypoxia by increasing PPP flux

In contrast to astroglia, neurons did not respond to LPS (Fig. 11a) or hypoxia (Fig. 11b) by enhancing PPP flux, indicating that neuronal cells are susceptible to oxidative stress.

Discussion

Inflammatory responses by neural cells (neurons and glial cells) as well as blood cells have been implicated in the underlying mechanisms of ischemic cell damage [46–53]. TLRs, in particular TLR4, play important roles in the initiation and progression of inflammatory response by intrinsic brain cells, given that brain ischemia induces neuronal release or the increased production of endogenous ligands of TLR4 (damage-associated molecular patterns such as high-mobility group box 1 and peroxiredoxin) in a relatively early stage of an ischemic event, i.e., within 24 h [9, 54, 55]. There is accumulating evidence that not only immune cells in the brain parenchyma, such as microglia, but also astroglia and neurons harbor TLR4 [56–58]. It is likely that microglia respond to these endogenous TLR4 ligands by producing inflammatory cytokines and ROS/NO, leading to further neuronal damage [59]. However, the full role of TLR4 in non-immune cells has not been elucidated. In the present study, we confirmed the presence of TLR4 in astroglia by Western blotting using an astroglial culture devoid of microglia. However, the role of astroglial TLR4 per se does not appear relevant.

The roles of astroglia in ischemic stroke are disputed. Astroglial TLR4 may play either a detrimental or beneficial role or both [60]. Gorina et al. reported that TLR4 stimulation by LPS even at a low concentration (0.01 µg/mL) induced the release of inflammatory cytokines from astroglia, suggestive of a harmful role [13]. For identifying the role of TLR4 in astroglia, cultured cells are useful tools [61–64]. However, usually microglial cells are also present...
and may affect the results obtained from astroglial cultures [65–68]. In the present study, we obtained a pure astroglial culture using Ara-C/LME-treatment. Although we did not detect cytokine release using this culture, ROS/NO production was not altered by the addition of LPS to the pure astroglial culture, whereas it showed drastic increases in the microglia-containing astroglial culture.

In contrast, Bolaños et al. reported the astroglial activation of PPP flux as a cytoprotective response induced by LPS (1 μg/mL) through NO [16]. PPP is a cytoprotective metabolic pathway of glucose, given that PPP activation induces NADPH production, which is necessary to recycle the oxidized form of GSH back to the reduced form, leading to removal of ROS by GSH peroxidase [69–71]. Importantly, in ischemic stroke, ischemia–reperfusion enhances ROS production in the mitochondria of neurons, leading to further neuronal damage [72, 73]. Thus, PPP activation after ischemia–reperfusion could be an important player in preventing cellular damage during ischemic stroke. Furthermore, they found that NO has a key role in activating PPP in astroglia in response to LPS stimulation [16]. The present study partly supported these beneficial effects of LPS on astroglia. We identified two important findings using two different cultures of astroglia with or without microglia. First, the origin of NO and ROS production in response to LPS stimulation was coexisting microglia. NO elimination by MAPK inhibition eliminated increases in PPP in astroglia, supporting the PPP-activating role of NO. Second, this NO-induced astroglial PPP activation was induced via the Keap1/Nrf2 system in astroglia. Interestingly, MAPK does not seem to be involved in the basal production of NO in microglia. In contrast, basal production of ROS in microglia decreased by MAPK inhibition, indicating different roles of MAPK in microglial production of NO and ROS.

Hypoxia per se induced PPP activation in astroglia by a completely different mechanism. Hypoxia could potentially activate PPP flux, given that the PPP is a branch pathway of glycolysis that is well known to be activated under hypoxic conditions [74, 75]. However, the effects of hypoxia on PPP flux of astroglial cells have been extensively examined [75, 76]. Instead, inflammatory cells such as macrophages alter their metabolic character to increase glycolysis in response to inflammation, which is associated with hypoxic environments. With respect to neurons, oxidative metabolism of glucose is required to produce sufficient ATP to maintain neuronal action potentials [77, 78]. In contrast, astroglial energy metabolism is more dependent on glycolysis [78–80]. We have reported that astroglial glycolysis and its branch pathway, the PPP, are important for protection of neurons against oxygen stress [45]. In the present study, we found that hypoxia-induced activation of the PPP in astroglia is mediated by a different pathway from that mediating NO-induced activation of the PPP and that these two pathways act in an additive manner.

Astroglial cultures always contain a small but appreciable number of microglia [81–83]. The present study showed that a normal astroglial culture contains as much as 10% of Iba1-positive cells. Elimination of
microglia from astroglia is important for distinguishing the astroglial response to TLR4 stimulation. Ara-C combined with LME eliminates microglia effectively [24, 25]. The mechanism by which LME destroys microglia has not been fully established. The lysosomal enzyme, which is abundant in microglia, is involved [29, 30]. In the present study, we confirmed that this treatment succeeded in completely eliminating microglia and that LPS-induced but not hypoxia-induced PPP activation occurred only in the presence of microglia. Interestingly, however, TLR4 expression in pure astroglia devoid of microglia was unaltered, indicating that astroglia per se express TLR4. Thus, LPS stimulation could possibly induce astroglial response through TLR4 activation, whether pro- or anti-inflammatory. We speculated that astroglial PPP activity is high enough to mask ROS production in response to LPS. In fact, LPS did induce increases in ROS production in astroglia in the absence of microglia after GSH depletion. We do not know why LPS did not induce NO production in astroglia.

PPP is a cytoprotective metabolic pathway of glucose, given that PPP activation induces the NADPH production that is necessary to recycle the oxidized form of GSH back to the reduced form, leading to the removal of ROS by GSH peroxidase [84]. The rate-limiting enzyme of PPP, G6PDH, is transcriptionally regulated: the transcriptional factor Nrf2 binds to the ARE located upstream of the G6PDH gene [18, 85]. Nrf2 is released from its adaptor protein Keap1 under stress imposed by ROS and RNS [19]. Thus, ROS or NO can elicit Nrf2 activation, leading to PPP activation. Bolaños et al. indicated that NO induces astroglial PPP activation via mitochondrial inhibition and resulting activation of AMP-activated protein kinase (AMPK) [16]. The results of the present study supported it, since the TCA flux as assessed by [6,14C]glucose oxidation decreased by NO. However, increases in PPP flux did not seem to be explained merely by a decreased flux to the TCA cycle. In the present study, we confirmed that NO per se induced Nrf2 activation and increases in PPP flux, and after the inhibition of NO production from microglia by U0126, LPS failed to induce PPP activation, even though ROS production was maintained. NO reportedly activates the Keap1/Nrf2 system by S-nitrosylation of the Keap1 protein, facilitating the release of Nrf2 [21]. ROS may also act as an activator of the Keap1/Nrf2 system through the modification of thiol residues of Keap1 [86–89]. The present study, however, did not support a major role of ROS in activating the Keap1/Nrf2 system.

Another possibility is astroglial TLR4-mediated response via MAPK-associated phosphorylation of Nrf2. The mechanisms by which Nrf2 is activated are multiple and the phosphorylation of Nrf2 at serine 40 is one of them [90–93]. Various kinases may phosphorylate Nrf2 [20]. However, our results showed that nuclear translocation of Nrf2 was induced only when microglia were also present. Thus, the direct involvement of MAPK-induced phosphorylation of Nrf2 in astroglia is unlikely.

TLR4 activation is an important mechanism in ischemic stroke, given that ischemia-induced neuronal damage triggers the release of endogenous ligands for TLR4 [9, 53, 94–96]. In the present study, we also examined the effects of hypoxia per se, given that hypoxia induces
Fig. 11 The effects of LPS or hypoxia on PPP flux in neurons. Neither LPS nor hypoxia induce the enhancement of PPP flux in neurons. Neurons were treated with Ara-C when prepared, but not with LME. Both LPS (0.01 μg/mL) (a PPP) and hypoxia (1 % O₂) (b PPP) decreased PPP flux in neurons. Values are means ± SD of four flasks. **P < 0.01 versus normoxia (grouped t test). Values are mean ± SD of four flasks. *P < 0.05, **P < 0.01, ***P < 0.001 versus controls (grouped t test).
marked glycolysis. If glycolysis is activated, PPP, a branch pathway of glycolysis, is simultaneously activated. In fact, PPP in astroglia was activated irrespective of the presence of microglia. LPS and hypoxia showed additive effects on PPP activation in astroglia containing microglia, indicating that hypoxia and TLR4 activation act independently. Hypoxia induced the activation of both glycolysis and PPP. Similarly, LPS induced PPP activation as well as glycolysis. In a recent study, hypoxia induced PPP activation coupled with glycolytic activation [97]. In this context, PPP activation is merely a consequence of glycolytic activation induced by hypoxia. Whether or not LPS-induced activation of glycolysis is dependent on the Nrf2 system is unclear from the results of the present study. These pathways are probably independent, given that LPS-induced increases in PPP flux were preserved under hypoxia. However, in brain ischemia, primary hypoxia causes cellular damage, and endogenous TLR4 ligands are released from the damaged cells. It is reasonable to speculate that hypoxia and TLR4 activation work independently to protect the living body against oxygen stress, given that ROS exerts deleterious effects in the progression of neuronal damage, especially when reperfusion of the occluded artery occurs. The protective mechanism of astroglia may play important roles, and its enhancement may lead to a novel therapeutic strategy for acute ischemic stroke.

Finally, we treated astroglia with sulforaphane to induce Nrf2 activation as evidenced by HO-1 expression. By the pretreatment of astroglia with sulforaphane, which had been confirmed to activate the PPP, ROS and NO production were completely blocked. Thus, the activation of PPP by the enhancement of Nrf2 in astroglia may lead to a novel therapeutic strategy for stroke.

Conclusions
Astroglia in concert with microglia may play a cytoprotective role to counter oxidative stress in stroke via the activation of PPP. The present study supported the role of NO, which seemed to be derived from microglia upon TLR4 stimulation, in increasing flux in astroglial PPP.

Additional files

Additional file 1: Quantification of metabolites in astroglial pentose-phosphate pathway by capillary-electrophoresis/electrospray ionization (CE/ESI) MS. (DOCX 20 kb)

Additional file 2: The effects of hypoxia on astroglial glucose metabolism of glycolysis, pentose-phosphate pathway, and TCA cycle. (DOCX 212 kb)

Abbreviations
ANOVA: analysis of variance; Ara-C: cytosine B-D-arabinofuranoside hydrochloride; ARE: antioxidant response element; BCA: bicinchoninic acid; BSO: L-buthionine-sulfoximine; DAF-FM DA: dianmofluorescein-FM diacetate; DAMPs: damage-associated molecular patterns; DAPI: 4’-6-diamidino-2-phenylindole; DBSS: Dulbecco’s balanced salt solution; DMEM: Dulbecco’s modified Eagle media; DMSO: dimethyl sulfoxide; ERIK1/2: extracellular s signal-regulated kinase 1/2; FITC: fluorescein-isothiocyanate; G6PDH: glucose 6-phosphate dehydrogenase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GFAP: glial fibrillary acidic protein; GSH: reduced form of glutathione; H2DCFDA: 2’、“-dichlorodihydrofluorescein diacetate; HMG1: high-mobility group box 1; HIF: hypoxia-inducible factor 1α; HO-1: heme oxygenase-1; INOS: inducible nitric oxide synthase; JAK2/STAT: Janus kinases/signaling transducers and activators of transcription; Keap1: Kelch-like ECH-associated protein 1; NO: nitric oxide; LPS: Lipo polysaccharide; LME: L-leucine methyl ester; MEK1/2: MAPK/ERK kinase 1/2; MAPK: mitogen-activated protein kinase; Myd88: myeloid differentiation protein 88; NOX: NADPH oxidase; NO: nitric oxide; NF2: nuclear factor-x-mycoid protein 2-related factor 2; NFκB: nuclear factor-kappa B; PPP: pentose-phosphate pathway; PBS: phosphate-buffered saline without Ca2+ and Mg2++; RIPA: radioimmunoprecipitation assay; RNS: reactive nitrogen species; ROS: reactive oxygen species; SOD-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; SNAP: S-nitroso-Acetyl-DL-penicillamine; Sulforaphane: 1-isothiocyanato-(4R)-methylsulfinylbutane; TCA: tricarboxylic acid; TLRs: Toll-like receptors; U0126: 1,4-diamin-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene; WT: wild type.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
ST designed experiments. ST, TI, KM, KM, YI, TH, and MK performed experiments. ST, TA, MS, and NS analyzed data. ST and TI wrote the manuscript. All authors read and approved the final manuscript.

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