



# Transglutaminase 2 expression induced by lipopolysaccharide stimulation together with NO synthase induction in cultured astrocytes

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## TRANSGLUTAMINASE 2 EXPRESSION INDUCED BY LIPOPOLYSACCHARIDE STIMULATION TOGETHER WITH NO SYNTHASE INDUCTION IN CULTURED ASTROCYTES

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**Abstract**—Activation of glia has been observed in neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), multiple sclerosis and brain ischemia. Excessive production of nitric oxide (NO), as a consequence of increased inducible NO synthase (iNOS) in glia, contributes to neurodegeneration. Transglutaminase 2 (TG2) is a cross-linking enzyme, which is activated in neurodegenerative diseases such as PD, AD and Huntington's diseases. However, mechanisms contributing to the increased TG activity in neurodegenerative diseases remain to be clarified. In the present study, we examined the expression of TG2 in cultured rat hippocampal astrocytes activated with lipopolysaccharide (LPS), which is generally used for a stimulant of iNOS induction. The expressions of TG2 mRNA and protein were increased by stimulation with LPS in a dose-dependent manner. The LPS-induced TG2 expression was diminished by ammonium pyrrolidine-1-carbodithioate; an inhibitor for nuclear factor (NF)- $\kappa$ B activation, suggesting the factors involved. Both expressions of TG2 and iNOS induced by LPS stimulation were suppressed by an antioxidant, ethyl pyruvate, in a dose-dependent manner. Furthermore, they were also suppressed by cystamine, an inhibitor of TG activity. These results suggest that the level of TG2 expression is regulated by oxidative stress and the activity of TG itself, and that the induction of iNOS and NO production are closely associated with TG2 expression in LPS-stimulated activation of astrocytes.

**Keywords:** astrocyte, transglutaminase 2, NO production, iNOS, nuclear factor- $\kappa$ B, cystamine, ethyl pyruvate

*Running Title: Increased expression of TG2 in LPS-activated astrocytes.*

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*Abbreviations:* AD, Alzheimer's disease; APDC, ammonium pyrrolidine-1-carbodithioate; DAN, 2,3-diaminonaphthalene; DMEM, Dulbecco's modified Eagle medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; I- $\kappa$ B $\alpha$ , inhibitory subunit  $\alpha$  of NF- $\kappa$ B; IL, interleukin; iNOS, inducible NO synthase; LPS, lipopolysaccharide; NF- $\kappa$ B, nuclear factor-kappa B; NO, nitric oxide; PD, Parkinson's disease; PBS, phosphate-buffered saline; TG, transglutaminase; TNF, tumor necrosis factor; TLR4, toll-like receptor-4.

Transglutaminase 2 (TG2; protein-glutamine  $\gamma$ -glutamyltransferase) belongs to a family of  $\text{Ca}^{2+}$ -dependent enzymes that catalyzes  $N^{\epsilon}$ -( $\gamma$ -L-glutamyl)-L-lysine isopeptide bond formation between lysine and glutamine residues in protein(s) and/or primary amines (Folk, 1980; Folk and Chung, 1985; Griffin et al., 2002; Jeitner et al., 2009; Zhang et al., 1998).  $N^{\epsilon}$ -( $\gamma$ -L-glutamyl)-L-lysine cross-linking stabilizes intra- and extra-cellular proteins as macromolecular assemblies that are used for a variety of essential physiological purposes, such as barrier function in epithelia, apoptosis and extracellular matrix formation (Aeschlimann and Thomazy, 2000; Fesus and Piacentini, 2002). TG2 is normally expressed at low levels in many different tissues and inappropriately activated under a variety of pathological conditions (Ientile et al., 2007; Kim et al., 2002). However, the roles of TG2 are unclear of which specifically play in disease etiology.

Several studies have shown that TGs are aberrantly-activated in neurodegenerative diseases (Wilhelmus et al., 2008) such as stroke (Tolentino et al., 2004), Alzheimer's disease (AD) (Citron et al., 2001; Kim et al., 1999; Yamada et al., 1998), Parkinson's disease (PD) (Junn et al., 2003) and Huntington's disease (HD) (Lesort et al., 1999). Increased TG activity may contribute to the formation of insoluble deposits in AD brain, insoluble Lewy bodies in PD brain and insoluble nuclear inclusion bodies in HD brain (Johnson et al., 1997; Lesort et al., 2000). However, mechanisms contributing to increased TG activity in neurodegenerative diseases remain to be clarified.

Functional changes of glial cells are involved in neurodegenerative processes in various CNS diseases. For example, inducible nitric oxide synthase (iNOS) is induced in astrocytes and microglia activated under brain ischemia through nuclear factor-kappaB (NF- $\kappa$ B) signaling pathway together with proinflammatory cytokines, such as interleukin (IL)-1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$  (Nomura, 2001). Glial function is important to neuronal function and survival (Benarroch, 2005; Chadi et al., 2009; Schipper et al., 2009; Tsacopoulos et al., 1997; Varvel et al., 2009).

TG activity has been detected in cultured rat astrocytes as well as neurons (Campisi et al., 1992; Monsonogo et al., 1997). Interestingly, TG2 expression in astrocytes is induced by inflammation-associated cytokines such as IL-1 $\beta$  and TNF- $\alpha$  (Monsonogo et al., 1997). On the other hand, lipopolysaccharide (LPS) is well known to activate cultured astrocytes inducing iNOS expression (Murakami et al., 2003) and proinflammatory cytokines (Faure et al., 2001; Martin et al., 2006).

In the present study, we examined whether TG2 expression increases in cultured rat astrocytes activated by LPS stimulation. We found that LPS stimulation could increase in TG2 expression and that some reagents inhibited the expression. We also compared the effect of the reagents on TG2 expression with that on iNOS expression.

## EXPERIMENTAL PROCEDURES

### Materials

LPS from *Salmonella enteritidis*, DNase I (DN-25) and anti- $\beta$ -actin antibody were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Dulbecco's modified Eagle medium (DMEM) was obtained from Gibco BRL (Grand Island, NY, U.S.A.). Fetal bovine serum was obtained from PAA Laboratories GmbH (Pasching, Austria). Anti-TG2 antibody was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.). Horseradish peroxidase conjugated goat anti-mouse IgG (H+L) antibody was purchased from Bio-Rad Laboratories Inc. (Hercules, CA, U.S.A.). Cystamine dihydrochloride and ethyl pyruvate were

purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 2,3-Diaminonaphthalene (DAN) and ammonium pyrrolidine-1-carbodithioate (APDC) were obtained from Dojindo (Kumamoto, Japan).

### **Preparation of astrocytes culture**

This study was carried out in compliance with the Guideline for Animal Experimentation at Osaka Prefecture University, with an effort to minimize the number of animals used and their suffering. Astrocytes were prepared as described previously (Murakami et al., 2003; Si et al., 1997). In brief, hippocampus from 19 or 20-day-old embryos, which were taken out from pregnant Wistar rats deeply anesthetized, were cleared of meninges, cut into about 1-mm<sup>3</sup> blocks, and treated with 0.25% trypsin in Ca<sup>2+</sup>, Mg<sup>2+</sup>-free phosphate-buffered saline containing 5.5 mM glucose for 20 min at 37°C with gentle shaking. An equal volume of horse serum supplemented with 0.1 mg/ml of DNase I was added to the medium to inactivate the trypsin. Then, the tissues were centrifuged at 350 x g for 5 min. The tissue sediments were triturated through a pipette with DMEM containing 10% fetal bovine serum, 100 µg/ml streptomycin and 50 unit/ml penicillin. After filtering cell suspensions through a lens-cleaning paper (Fujifilm Co., Tokyo, Japan), the cells were plated on polyethyleneimine-coated 100-mm-diameter plastic dishes (Iwaki, Asahi Glass Co., Tokyo, Japan) at a density of 0.8-1.3 x 10<sup>5</sup> cells/cm<sup>2</sup>. Cultures were maintained in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C with changing medium every 3 days. After one week, astrocytes were replated to remove neurons. On days 12-14, they were replated onto 96-well plates (MS-8096F; for tissue culture, Sumitomo, Tokyo, Japan) using an ordinary trypsin-treatment technique at a density of 4 x 10<sup>4</sup> cells/well and stabilized for 1 day, then we used for experiments. Cultured cells were replated onto proper plates or dishes for each experiment.

More than 90% of the cells were immunoreactively positive to an astrocyte marker, glial fibrillary acidic protein; using the antibody (Sigma) and FITC-conjugated anti-rabbit IgG antibody. Less than 10% of the cells were positive to a microglial marker, lectin from *Bandeiraea simplicifolia* bs-I isolectin b4 (peroxidase-labeled, Sigma).

### **Nitrite assay**

Nitric oxide (NO) production by astrocytes was determined by the assay of nitrite, a relatively stable metabolite of NO. The nitrite concentration was assayed by a fluorometric method using DAN reagent as previously described (Si et al., 1997). The supernatants (100 µl) of the cultured astrocytes were collected after 24 h stimulation with 1 µg/ml LPS, and mixed with 20 µl of freshly prepared DAN solution (0.05 mg/ml in 0.62 M HCl) at room temperature. After 10-20 min, 100 µl of 0.28 M NaOH was added and formation of 2,3-naphthotriazole, the fluorescent product, was measured using a microplate reader (ARVO MX 1420, Wallac, Turku, Finland) with excitation at 355 nm and emission at 460 nm. A standard curve of NaNO<sub>2</sub> was established in an identical fashion in each assay.

### **Cell viability**

To evaluate cell viability, we assayed total mitochondrial activity with Cell Counting Kit-8 (Dojindo). After the cells were stimulated usually for 24 h, one-tenth volume of the reagent was added and incubated for 20 min at 37°C, and then the color development was measured at 460 nm.

### **Western blotting**

Cultured astrocytes were homogenized in 20 mM Tris-HCl (pH 7.5) buffer containing 1 mM EDTA and protease inhibitor cocktail (Sigma P8340). Each homogenate was added at a

volume ratio of 4:1 to 50 mM Tris-HCl buffer (pH 6.8) containing 50% glycerol, 10% sodium dodecyl sulfate, 0.05% bromophenol blue and 25% 2-mercaptoethanol, followed by mixing and boiling at 100°C for 5 min. Each aliquot of 20 µg proteins was loaded on a 10% polyacrylamide gel for electrophoresis at a constant voltage of 120 V for 2 h at room temperature and subsequent blotting to a polyvinylidene fluoride membrane previously treated with 100% methanol. After blocking by 5% skimmed milk dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween 20, the membrane was reacted with antibodies against TG2, iNOS or β-actin followed by a reaction with anti-mouse IgG antibody conjugated with peroxidase. Proteins reactive with those antibodies were detected with the aid of ECL detection reagents through exposure to X-ray films. Laser densitometric analysis was performed to standardize the results of Western blotting as described previously (Hori et al., 2004). The graphs showed TG2/β-actin or iNOS/β-actin ratio of the density of detection bands. The densitometric value when the cells were stimulated with 1 µg/ml LPS was taken as 100% in each cell preparation, then the values of different cell preparations were analyzed statistically.

Protein concentrations were determined by the method of Bradford using CBB color solution (Nacalai Tesque, Kyoto, Japan), according to the manufacturer's protocol, with bovine serum albumin (BSA) as the standard.

### Reverse transcription-polymerase chain reaction (RT-PCR)

Cultured astrocytes were washed with PBS, followed by extraction of mRNA using RNeasy mini kit (Qiagen, Hilden, Germany) and subsequent synthesis of complementary DNA (cDNA) with oligo dT primers, mixture of dNTP (deoxyribonucleotide triphosphate), RNase inhibitor, Buffer RT and Omniscript Reverse Transcriptase (Omniscript Reverse Transcription Kit; Qiagen). Reverse transcriptase reaction was run at 37°C for 60 min, followed by inactivation of the enzyme at 94°C for 5 min, and an aliquot of synthesized cDNA was directly used for PCR. PCR was performed in buffer containing Taq PCR Master Mix (Qiagen) and each primer for the corresponding TG2, iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

PCR was performed with primers specific for the TG2 (Tolentino et al., 2004), iNOS and GAPDH described below. Each reaction for TG2 and GAPDH were carried out for appropriate cycles to semi-quantitative analysis. The conditions of each PCR cycles for these primers were as follows: denaturation at 95 °C for 45 sec; annealing at 60°C for 45 sec; and extension at 72°C for 1 min. Electrophoresis was run for an aliquot of PCR amplification products on a 2% agarose gel, followed by detection of DNA with ethidium bromide. The results showed TG2/GAPDH ratio of the density of detection bands using Scion image (Scion Corporation).

*Specific primers;*

TG2:	sense	5'-ACTTTGACGTGTTTGCCCAC-3'
	antisense	5'-CAATATCAGTCGGGAACAGGTC-3'
GAPDH:	sense	5'-TGCTGAGTATGTCGTGGAGTCT-3'
	antisense	5'-AATGGGAGTTGCTGTTGAAGTC-3'

### Data analysis

For statistical analysis of the data, one-way ANOVA followed by Scheffe's multiple comparison procedure was used. Differences between treatments were considered statistically significant when  $p < 0.05$ .

## RESULTS

### LPS-stimulated TG expression

*Expression of TG2 increased in cultured astrocytes after LPS stimulation.* To examine the expression of TG2 in astrocytes, we used cultured astrocytes prepared from hippocampus of E19-20 rat brains. Cultured astrocytes were stimulated with various concentrations of LPS for 24 h, and then TG2 expression was analyzed by RT-PCR and Western blotting. The level of TG2 mRNA was increased by LPS stimulation, in a dose-dependent manner (Fig. 1A). Similarly, the expression level of TG2 protein increased more than ten-fold, dose-dependently; the half effective concentration of LPS was about 1 ng/ml (Fig. 1B, C).

*Inhibition of LPS-induced TG2 expression by an NF- $\kappa$ B inhibitor.* Activation of NF- $\kappa$ B is known to be involved in the intracellular signaling pathway of LPS stimulation. We examined the effect of an NF- $\kappa$ B inhibitor, APDC, on TG2 expression in cultured astrocyte. The cells were stimulated by 1  $\mu$ g/ml LPS for 24 h with various concentrations of APDC, and then the expression of TG2 was analyzed. Enhanced expression of TG2 protein induced by LPS stimulation decreased with APDC dose-dependently, and was significantly suppressed with 300-1000  $\mu$ M APDC (Fig. 2). Cell viability was not affected significantly by the stimulation with LPS and/or APDC (data not shown).

*Inhibition of LPS-induced TG2 expression by ethyl pyruvate.* Ethyl pyruvate is a stable derivative of pyruvate and plays a role as a potent scavenger for reactive oxygen species (ROS) (Kim et al., 2005). We examined the effect of ethyl pyruvate on TG2 expression in cultured astrocytes. The cells were stimulated by 1  $\mu$ g/ml LPS for 24 h with various concentrations of ethyl pyruvate, and then the expression of TG2 was analyzed. Enhanced expression of TG2 protein induced by LPS stimulation was significantly suppressed with 10 mM ethyl pyruvate (Fig. 3).

*Inhibition of LPS-induced TG2 expression by cystamine.* To assess whether transamidation is involved in the pathway of LPS-induced TG2 expression, we examined the effect of cystamine, an inhibitor of transglutaminase, in cultured astrocytes. The cells were stimulated by 1  $\mu$ g/ml LPS for 24 h with various concentrations of cystamine, and then the expression of TG2 was analyzed. Enhanced expression of TG2 protein induced by LPS stimulation was significantly suppressed with 500  $\mu$ M cystamine (Fig. 4). In the absence of LPS, cystamine treatment showed no significant effects on TG2 expression (Fig. 4). Cell viability was not affected by LPS and/or cystamine treatment within the concentrations used in these experiments (data not shown).

### iNOS induction and NO production

*Inhibition of LPS-induced iNOS expression and NO production by APDC.* It is well known that LPS stimulation in cultured astrocytes induces iNOS expression and NO production through NF- $\kappa$ B activation. We confirmed the effect of APDC on LPS-induced iNOS expression and NO production in order to compare with the effect on TG2 expression described above. Cultured astrocytes were stimulated with 1  $\mu$ g/ml LPS in the presence of various concentrations of APDC for 24 h, and then iNOS expression was detected and NO production in the medium was analyzed. APDC (1 mM) significantly inhibited LPS-induced iNOS expression (Fig. 5A). APDC also decreased LPS-induced NO production in a

dose-dependent manner (Fig. 5B).

*Inhibition of LPS-induced iNOS expression and NO production by ethyl pyruvate.* Similarly, we assessed the effect of ethyl pyruvate on LPS-induced iNOS expression and NO production in order to compare with the effect on TG2 expression. The cells were stimulated by 1  $\mu\text{g/ml}$  LPS for 24 h in the presence of various concentrations of ethyl pyruvate, and then iNOS expression and NO production were assessed. Ethyl pyruvate (10 mM) considerably inhibited LPS-induced iNOS expression (Fig. 6A). Ethyl pyruvate, moreover, diminished LPS-induced NO production in cultured astrocytes in a dose-dependent manner (Fig. 6B).

*Inhibition of LPS-induced iNOS expression and NO production by cystamine.* We examined the effect of an inhibitor of TG, cystamine, on the LPS-stimulated iNOS expression and NO production. The cells were incubated with 1  $\mu\text{g/ml}$  LPS in the presence of various concentrations of cystamine for 24 h, and then iNOS expression and NO production were evaluated. Cystamine blocked LPS-induced iNOS expression and NO production in a dose-dependent manner (Fig. 7A, B).

## DISCUSSION

In this study, we demonstrated that TG2 expressions, both of mRNA and protein, were induced when the cells were activated by LPS stimulation in cultured rat hippocampal astrocytes. The LPS-induced TG2 expression was inhibited by three different types of reagents; an inhibitor of NF- $\kappa$ B activation, an ROS scavenger and an inhibitor of TG activity. Furthermore, interestingly, these three reagents also inhibited LPS-induced iNOS expression and NO production as well as the TG2 expression. It is suggested that increased TG2 expression may enhance LPS-induced iNOS expression and NO production. Inversely, it has been reported that released NO may cause S-nitrosylation of thiol group of TG2 protein and inhibit TG activity (Catani et al., 1998; Lai et al., 2001; Rossi et al., 2000). It is likely that the induction mechanisms of TG2 and iNOS are tightly related with each other (Fig. 8).

Exposure to LPS has been believed to stimulate intracellular signaling pathway through NF- $\kappa$ B activation. The pathways are triggered by activation of toll-like receptor-4 (TLR4) expressed on the cell membrane of various types of cells except neurons in the CNS (Lee et al., 2005; Palsson-McDermott and O'Neill, 2004). In our hands, for example, LPS markedly facilitates the production of NO in cultured rat brain astrocytes (Murakami et al., 2003; Takano et al., 2005) and microglia (Takano et al., 2003) through expression of iNOS. Moreover, activation of TLR4 by LPS induces productions of a variety of cytokines, such as TNF- $\alpha$  (Martin et al., 2006) and interferon- $\gamma$  (Faure et al., 2001) also through NF- $\kappa$ B signaling pathway (Frantz et al., 1999). In the present study, we demonstrated that LPS induced TG2 expression in cultured astrocytes, as well as iNOS. An NF- $\kappa$ B inhibitor, APDC, decreased the LPS-stimulated induction of TG2, suggesting that NF- $\kappa$ B might be associated with that induction. The fact that the NF- $\kappa$ B binding site is identified on the TG2 promoter region (Caccamo et al., 2005a, 2005b; Campici et al., 2003, 2004) gives further support to the involvement of NF- $\kappa$ B signaling pathways in mechanisms underlying the up-regulation of TG2 mRNA and protein by LPS stimulation in cultured astrocytes.

Moreover, these reports also suggest the involvement of ROS in glial activation; glutamate induces TG2 up-regulation, through redox state alteration or increase of intracellular ROS followed by NF- $\kappa$ B activation (Caccamo et al., 2005a, 2005b; Campici et

al., 2003, 2004). Lee et al. (2003) indicated that lysophosphatidic acid and transforming growth factor- $\beta$  induced the increase of intracellular ROS followed by activation of TG2, which was blocked by ROS scavengers, *N*-acetyl-L-cysteine and catalase. In the present study, ethyl pyruvate, an ROS scavenger, inhibited LPS-induced TG2 expression. It is also shown that ethyl pyruvate has anti-inflammatory effect (Kim et al., 2005). ROS are important mediators of inflammation (Raha and Robinson, 2000). These facts suggest that LPS could induce redox state alteration or oxidative stress followed by NF- $\kappa$ B activation, and then up-regulate TG2 expression. Indeed, there are several reports that LPS induces intracellular ROS (Lee et al., 2006; Qin et al., 2004; Wang et al., 2004) and that oxidative stress activates NF- $\kappa$ B (Pawate et al., 2004) in microglia and astrocytes.

An inhibitor of TG, cystamine, on the other hand, decreased the LPS-induced iNOS expression and NO production. These results suggest that TG activity might augment the pathway of NF- $\kappa$ B activation in a self-enhancing manner to induce iNOS expression and NO production after LPS triggering. It is consistent with some reports in microglial cell line, BV-2, in which TG activity is increased by LPS exposure and inhibitors of TG activity reduce LPS-induced NO production (Park et al., 2004; Lee et al., 2004). The authors of these reports proposed that TG2 could activate NF- $\kappa$ B *via* a novel pathway; rather than stimulating phosphorylation and degradation of the inhibitory subunit  $\alpha$  of NF- $\kappa$ B (I- $\kappa$ B $\alpha$ ), but the polymerization of I- $\kappa$ B $\alpha$  by TG activity to regulate iNOS transcription (Lee et al., 2004). Other than I- $\kappa$ B $\alpha$ ,  $\alpha$ -synuclein was also reported to be polymerized in TG2 over-expressed cells, leading to the formation of its aggregates (Junn et al., 2003).

It is reported that cystamine prolongs survival of Huntington's disease model mice due to inhibition of TG activity (Karpuj, 2002). In our present study, it is likely that cystamine suppress an aberrant activation of astrocytes at the step of NF- $\kappa$ B activation leading to the iNOS induction. However, the other possibilities have not been excluded; cystamine may inhibit other thiol-dependent enzymes in addition to TG, may affect as an anti-oxidative property and may inhibit caspase activity (Ientile et al., 2003; Lesort et al., 2003). In our preliminary experiment, monodancylcadaverine, another TG inhibitor, also suppressed LPS-induced NO production in cultured astrocytes (data not shown). Therefore, the target of cystamine is plausible to be TG, although the further investigation should be necessary.

It is reported that TG2 expression in rat astrocytes is induced by inflammation-associated cytokines such as IL-1 $\beta$  and TNF- $\alpha$  (Monsonogo et al., 1997). As described above, LPS can induce the productions of these cytokines in addition to the iNOS induction. Therefore, these self-promoting cytokines also might be involved in the TG induction mechanism by LPS stimulation in cultured astrocytes.

In summary, LPS increased TG2 expression *via* NF- $\kappa$ B activation in cultured astrocytes. When astrocyte's activation is triggered by certain stimulators, TG2 induction may play an important role for the further activation of cellular functions. The regulation of TG activity in glial cells might be an important target in treatment of CNS diseases.

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Fig. 1 Takano, et al.

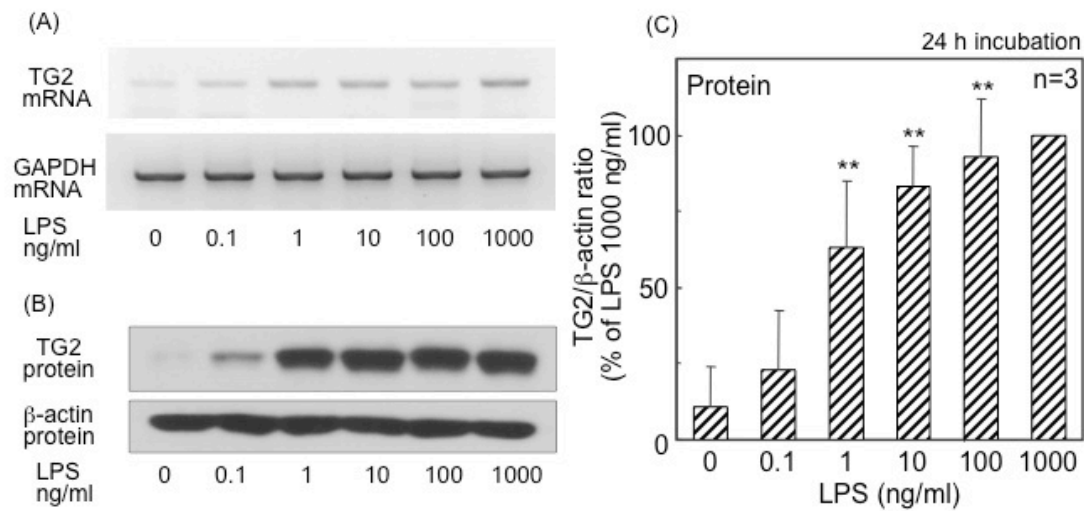


Fig. 1 Expression of TG2 mRNA and protein in activated astrocytes by LPS stimulation. Cultured astrocytes were stimulated by various concentrations of LPS for 24 h. (A) The expression of mRNAs of TG2 and GAPDH were detected by RT-PCR procedure. Typical bands of RT-PCR for TG2 and GAPDH mRNAs were shown in the photograph. (B) The expression of TG2 protein was detected by Western blotting. Typical bands of Western blotting for TG2 and  $\beta$ -actin proteins were shown in the photograph. (C) The graph shows TG2/ $\beta$ -actin ratio of the density of detection bands. Data are mean  $\pm$  S.D. of three samples from different cell preparations. \*\* $P < 0.01$ , significantly different from control.

Fig. 2 Takano, et al.

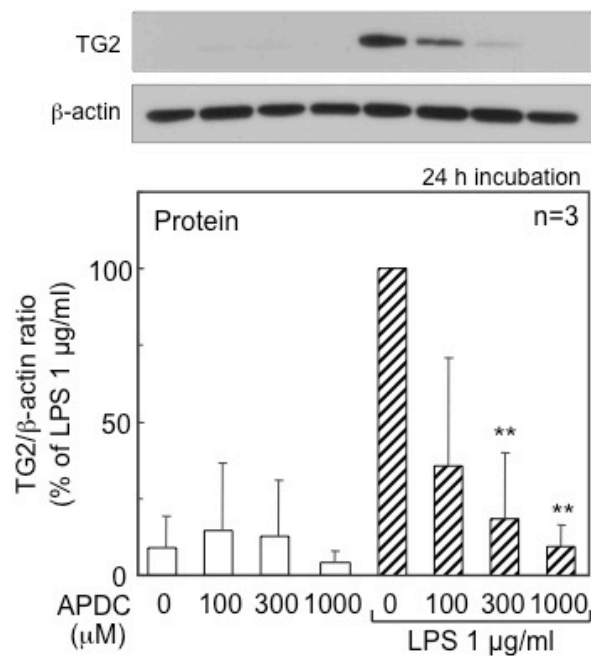


Fig. 2 Inhibition of LPS-stimulated TG2 expression by APDC. Cultured astrocytes were stimulated by 1 µg/ml LPS for 24 h in the presence of various concentrations of APDC. The expression of TG2 protein was detected by Western blotting. The results are shown in photograph and graph as similar to Figs. 1B, C. Data are mean ± S.D. of three samples from different cell preparations. \*\* $P < 0.01$ , significantly different from LPS 1 µg/ml.

Fig. 3 Takano, et al.

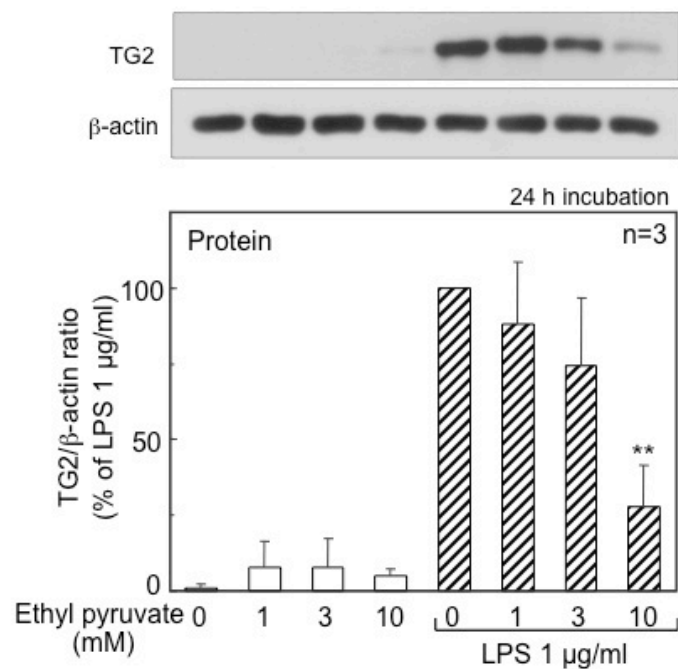


Fig. 3 Inhibition of LPS-stimulated TG2 expression by ethyl pyruvate. Cultured astrocytes were stimulated by 1  $\mu$ g/ml LPS for 24 h in the presence of various concentrations of ethyl pyruvate. The expression of TG2 protein was detected by Western blotting. The results are shown in photograph and graph as similar to Figs. 1B, C. Data are mean  $\pm$  S.D. of three samples from different cell preparations. \*\* $P < 0.01$ , significantly different from LPS 1  $\mu$ g/ml.

Fig. 4 Takano, et al.

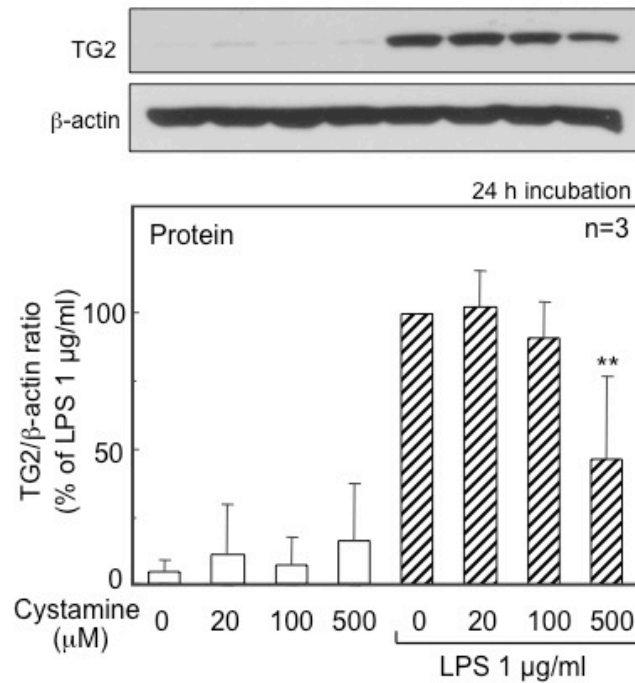


Fig. 4 Inhibition of LPS-stimulated TG2 expression by cystamine. Cultured astrocytes were stimulated by 1  $\mu$ g/ml LPS for 24 h in the presence of various concentrations of cystamine. The expression of TG2 protein was detected by Western blotting. The results are shown in photograph and graph as similar to Figs. 1B, C. Data are mean  $\pm$  S.D. of three samples from different cell preparations. \*\* $P < 0.01$ , significantly different from LPS 1  $\mu$ g/ml.

Fig. 5 Takano, et al.

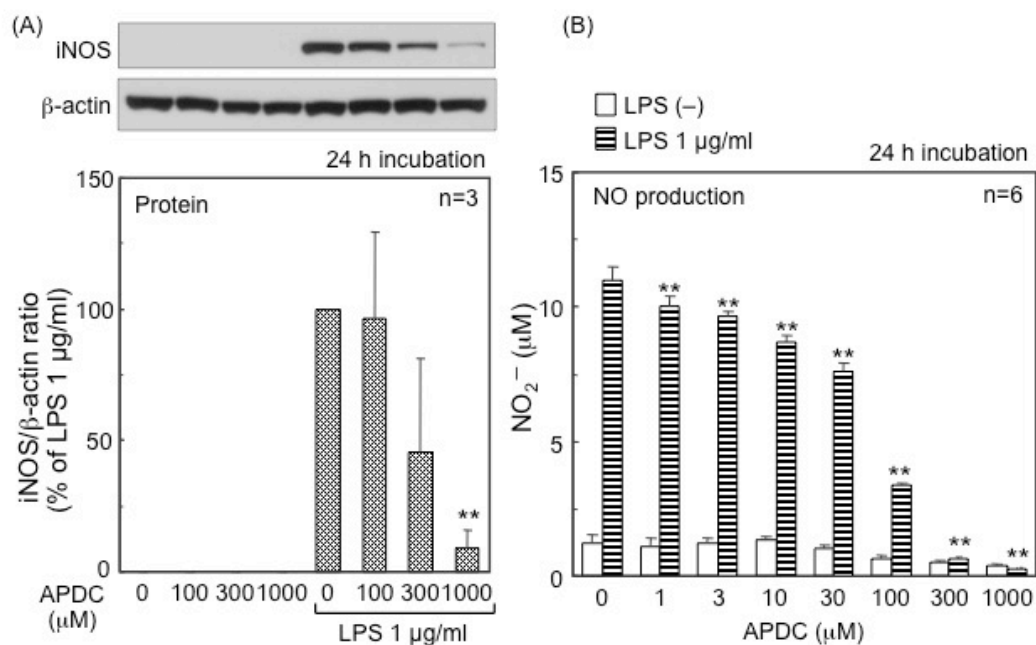


Fig. 5 Inhibition of LPS-induced iNOS expression and NO production by APDC. Cultured astrocytes were stimulated by 1 μg/ml LPS for 24 h in the presence of various concentrations of APDC. (A) The expression of iNOS protein was detected by Western blotting. A typical band of Western blotting for iNOS protein was shown in the photograph. The graph shows iNOS/β-actin ratio of the density of detection bands. Data are mean ± S.D. of three samples from different cell preparations. (B) The concentration of nitrite in the medium was measured by fluorescent assay with DAN reagent. Data are mean ± S.D. of six samples. This result is representative of three replicate experiments. \*\* $P < 0.01$ , significantly different from LPS 1 μg/ml.



Fig. 6 Takano, et al.

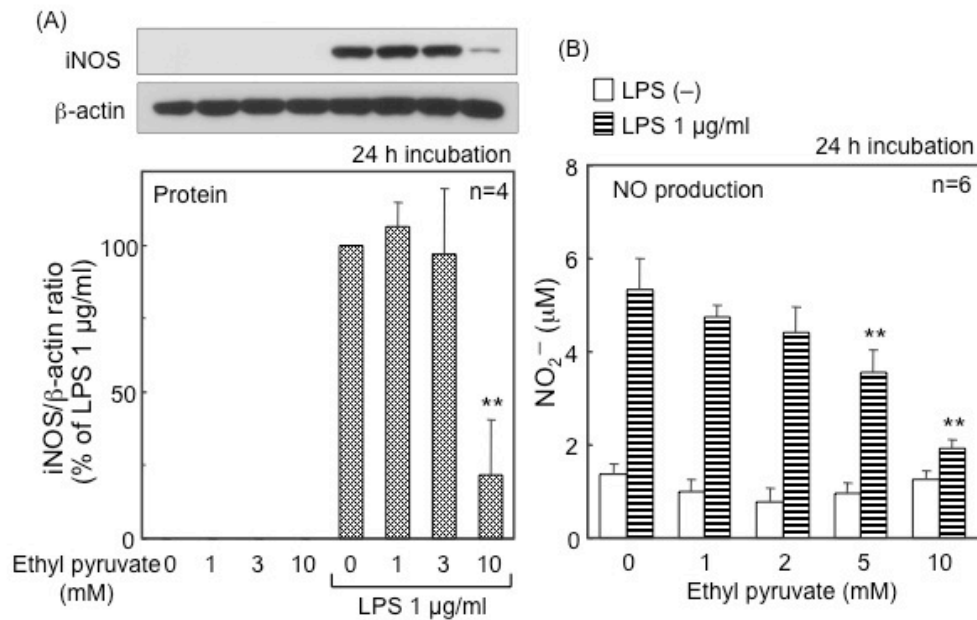


Fig. 6 Inhibition of LPS-induced iNOS expression and NO production by ethyl pyruvate. Cultured astrocytes were stimulated by 1  $\mu$ g/ml LPS for 24 h in the presence of various concentrations of ethyl pyruvate. (A) The expression of iNOS protein was detected by Western blotting. The results are shown in photograph and graph as similar to Fig. 5A. Data are mean  $\pm$  S.D. of four samples from different cell preparations. (B) The concentration of nitrite in the medium was measured. Data are mean  $\pm$  S.D. of six samples. This result is representative of three replicate experiments. \*\* $P < 0.01$ , significantly different from LPS 1  $\mu$ g/ml.

Fig. 7 Takano, et al.

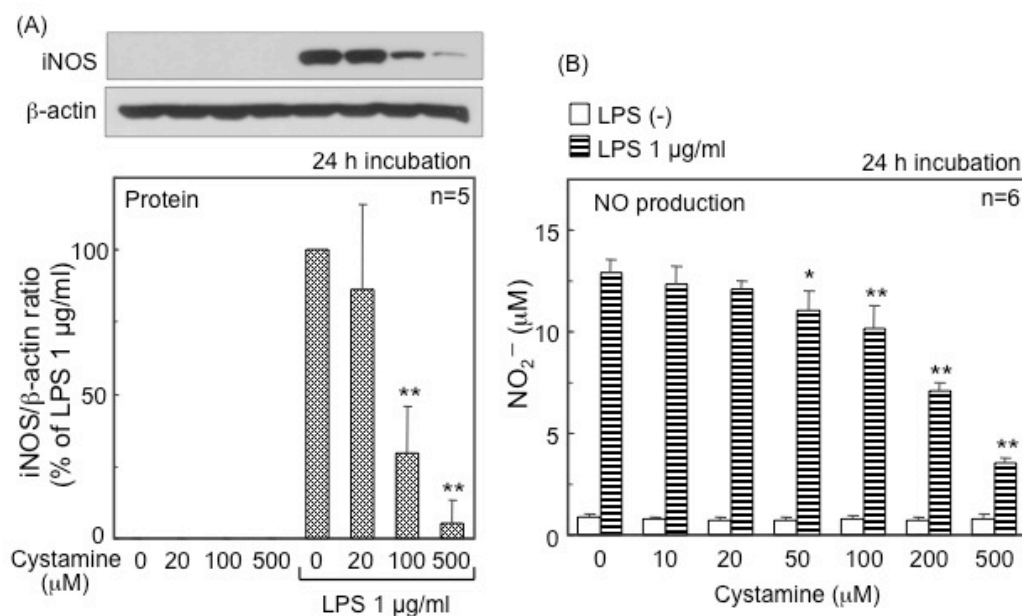


Fig. 7 Inhibition of LPS-induced iNOS expression and NO production by cystamine. Cultured astrocytes were stimulated by 1  $\mu$ g/ml LPS for 24 h in the presence of various concentrations of cystamine. (A) The expression of iNOS protein was detected by Western blotting. The results are shown in photograph and graph as similar to Fig. 5A. Data are mean  $\pm$  S.D. of four samples from different cell preparations. (B) The concentration of nitrite in the medium was measured. Data are mean  $\pm$  S.D. of six samples. This result is representative of three replicate experiments. \*\* $P < 0.01$ , significantly different from LPS 1  $\mu$ g/ml.

Fig. 8 Takano, et al.

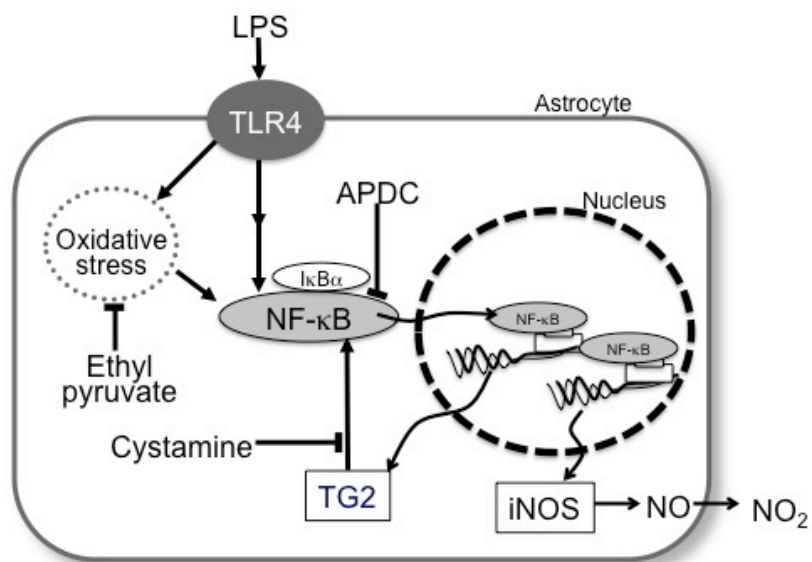


Fig. 8 Schematic possible mechanism for TG2 and iNOS inductions in activated astrocytes by LPS stimulation. See text.