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Regulatory mechanism of calcium-dependent glutamate release from astrocytes by cytokines via the nitric oxide signal pathway

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Osaka Prefecture University

2010
Abstract

Cytokines are chemical mediators that were originally investigated as essential mediators in the immune and inflammatory systems. Some cytokines are produced in the central nervous system (CNS) and exhibit various effects on neurons, microglia, and astrocytes. Astrocytes, which are essential for maintaining homeostasis in the normal CNS, can release chemical transmitters, including glutamate, in a calcium-dependent manner. These chemical transmitters, which are referred to as “gliotransmitter,” may mediate communication between neurons and astrocytes. However, to date, no studies have been conducted on the effects of cytokines on calcium-dependent glutamate release from astrocytes. Therefore, in the present study, I investigated the regulatory mechanisms of cytokine-induced calcium-dependent glutamate release.

My findings indicate that cytokines enhance calcium-dependent glutamate release and induce the expression of inducible nitric oxide synthase (iNOS) and the production of nitric oxide (NO). The inhibition of iNOS eliminated the cytokine-induced enhancement of glutamate release, and treatment with an NO donor increased glutamate release even in the absence of cytokines. Additionally, cytokines enhanced calcium-dependent glutamate release from rat glioma cell line C6 cells serving as astrocytes models through NO production as well as astrocytes. Thus,
cytokines enhance glutamate release from astrocytes, and this enhancement is mediated by NO.

The main NO signal pathway, which is referred to as the NO-cGMP pathway, is mediated by soluble guanylate cyclase (sGC), leading to the production of a second messenger, cyclic 3′,5′-guanosine monophosphate (cGMP). The cGMP messenger activates cGMP-dependent protein kinase (PKG) and facilitates the phosphorylation of various proteins. There has been little research regarding the involvement of the NO-cGMP pathway in glutamate release from astrocytes, although many reports have indicated that this pathway regulates neurotransmitter release. I investigated the effects of the NO-cGMP pathway on the enhancement of glutamate release from astrocytes treated with NO donor and found that there was no significant difference in glutamate release between the cGMP analog-treated cells and nontreated cells. Although the inhibition of PKG did not affect NO-enhanced glutamate release, the inhibition of sGC attenuated the NO-enhanced glutamate release. These results demonstrate that the activation of sGC participates in NO-enhanced glutamate release, whereas cGMP alone does not. These conflicting results suggest that a novel signal transduction pathway, different from the classical NO-cGMP pathway, is involved in NO-enhanced glutamate release.
Recently, Sawa et al. reported the formation as well as the chemical and biological functions of a nitrated cyclic nucleotide 8-nitro-cGMP (a novel derivative of cGMP), in NO-mediated signal transduction in macrophage cells. Thus far, no studies have been conducted on the formation of 8-nitro-cGMP in astrocytes, and its effect on glutamate release from astrocytes remains unclear. The present study identified the NO-dependent formation of 8-nitro-cGMP in astrocytes by performing immunocytochemistry and quantitative analysis of its accumulation using liquid chromatography (LC)-tandem mass spectrometry (MS/MS). Immunocytochemistry demonstrated marked 8-nitro-cGMP production in astrocytes in an exogenous NO concentration and time-dependent manner. Furthermore, the strong immunostaining for 8-nitro-cGMP observed in astrocytes treated with lipopolysaccharide (LPS) and cytokine was attenuated by iNOS or sGC inhibitor. These results show that the formation of 8-nitro-cGMP is mediated by NO activation of sGC. A quantitative analysis using LC-MS/MS indicated that after a rapid and transient increase in cGMP, the amount of 8-nitro-cGMP increased linearly to a level approximately one tenth of the peak concentration of cGMP. This 8-nitro-cGMP level was maintained after the cGMP level decreased.

I hypothesized that 8-nitro-cGMP plays a key role in explaining the
contradictory regulatory mechanism of NO-enhanced glutamate release that can not be explained by the NO-cGMP pathway alone. In my study on the effects of 8-nitro-cGMP on NO-enhanced glutamate release, I found that 8-nitro-cGMP enhanced calcium-dependent glutamate release. Furthermore, 8-nitro-cGMP rescued the inhibition of glutamate release by sGC inhibitor. These results indicate that 8-nitro-cGMP can elucidate an otherwise inexplicable contradiction of enhanced glutamate release from NO-treated astrocytes.

In this study, I report that cytokines induced the expression of iNOS and produced NO in astrocytes, which then enhanced calcium-dependent glutamate release through the formation of 8-nitro-cGMP mediated by NO production and sGC activation. This study may reveal that cytokines regulate the tripartite synapse through the enhancement of calcium-dependent glutamate release, which is mediated by NO and 8-nitro-cGMP as a novel signal modulator. Thus, cytokines may participate in the communication between neurons and astrocytes via the production of NO and 8-nitro-cGMP, which serve as modulators.
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Abbreviations

A23187; 5-(Methylamino)-2-[[2R,3R,6S,8S,9R,11R)-3,9,11-trimethyl-8-[(1S)-1-methyl-2-oxo-2-(1H-pyrrol-2-yl)-ethyl]-1,7-dioxaspiro[5.5]undec-2-yl][methyl]-4-benzoazolocarboxylic acid

NOC7; 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propinamine

L-NMMA; N<sup>G</sup>-monomethyl-L-arginine

8-Br-cGMP; 8-bromoguanosine 3', 5' cyclic monophosphate

1400W; N-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide, dihydrochloride

NS 2028; 4H-8-Bromo-1,2,4-oxadiazolo(3,4-d)benz(b)(1,4)oxazin-1-one

KT 5823; (9S,10R,12R)-2,3,9,10,11,12-Hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester
Chapter 1

Introduction

Cytokines have long been characterized as modulators of the immune system; however, they have since been found to mediate a diverse array of functions in nonimmune tissues, including the central nervous system (CNS). Moreover, cytokines produced from glia cells are implicated in a variety of neurological disorders such as Parkinson’s disease, Alzheimer’s disease, bacterial infection, and ischemic injury [1-4].

Glia cells are classified into two groups: macroglia and microglia. Macroglia are subdivided into ependymal cells, Schwann cells, oligodendroglia, and astroglia. The astrocyte is a subtype of astroglia, which outnumbers all other glia cells and are the most abundant neural cells in the brain. Astrocytes, which were traditionally thought to support neuronal activity by acting as “brain glue” express numerous receptors that enable them to respond to virtually all known neuroactive compounds, including neurotransmitters, neuropeptides, growth factors, and cytokines. The well-accepted roles of astrocytes comprise housekeeping functions to maintain neuronal function: providing metabolic support for neurons, taking up extracellular ions such as $K^+$ and neurotransmitters such as glutamate and gamma-aminobutyric acid (GABA),
synaptogenesis via release of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), angiogenesis, and blood-brain barrier (BBB) maintenance [5-11].

Previously, it was reported that astrocytes treated with cytokines may also exacerbate tissue damage as they can release proinflammatory cytokines such as tumor necrosis factor (TNF-α), as well as nitric oxide (NO) and reactive oxygen species (ROS) that can adversely affect cell survival after injury [12-14]. On the other hand, recent study has revealed that cytokine-activated astrocytes generate an adaptive postinjury response to promote CNS repair [5]. Cytokine-activated astrocytes increase cytosolic antioxidant proteins, promote revascularization, restore the BBB, promote remyelination and subsequent remission from a demyelinating event, enhance neuronal survival through the release of neurotrophic factors and promote the formation of new synapses and neurons [5]. Much is known about the role of astrocytes in the CNS: however, some aspects remain to be determined.

Cytokines induce the expression of inducible NO synthase (iNOS) in astrocytes [5]. NO produced by NOS has diverse physiological functions such as vascular and neuronal signal transduction, host defense, cell death regulation, and modulation of neurotransmitter release. The main NO pathway is mediated by soluble guanylate cyclase (sGC), leading to the production of a second messenger, cyclic 3′, 5′-guanosine
monophosphate (cGMP) [15]. The neuronal and vascular signaling mediated by NO is typically driven by the NO-cGMP pathway [16, 17]. However, the existence of NO-related biological phenomena that are not necessarily affected by cGMP has been suggested. For example, NO function seems to be modified by different chemical reactions such as S-nitrosylation and nitration of nucleotides and proteins that are induced by NO-derived reactive nitrogen oxides including nitrogen dioxide (NO$_2$) and peroxynitrite [18–20]. Recently, Sawa et al. confirmed the presence of a novel nitrated derivative of cGMP (8-nitro-cGMP) and indicated that 8-nitro-cGMP acts as not only a cGMP analog, activating cGMP-dependent protein kinase (PKG), but also makes it possible to form protein Cys-cGMP adducts as a new posttranslational modification, called “protein S-guanylation” [21]. Furthermore, Sawa et al. identified a unique chemical property of 8-nitro-cGMP, i.e., the potential to generate superoxide anions via the activation of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) reductase-like enzymes, including three NOS isoforms and P450 reductase, resulting in phosphodiesterase resistance and membrane permeability [21].

Astrocytes are now attracting greater attention because these cells have been shown to have important roles in the regulation of synaptic transmission. Astrocytes detect neuronal activity and can release chemical transmitters (“gliotransmitters”),
which in turn control synaptic activity [22-24]. This new understanding has led to the idea that astrocytes are intimately involved in the regulation of neuronal network function in vivo and thus are crucial determinants of higher brain functions and, consequently, of behavior [22-24]. Astrocytes have also been suggested to define extracellular neurotransmitter levels, buffer the extracellular milieu, and propagate Ca\(^{2+}\) signals [25]. Furthermore, astrocytes contain machinery for vesicular-mediated release of gliotransmitters, including glutamate, adenosine triphosphate (ATP), and D-serine, which modulate synaptic function [26-28]. Synaptic transmission is potentiated and spontaneous postsynaptic current activity is increased when synaptic astrocytes are functionally involved. These findings lead to the establishment of the ‘tripartite synapse’ concept (Fig. 1-1), which is based on the notion that astrocyte processes surrounding a synapse comprise a third functional component in addition to the presynaptic bouton and the postsynaptic terminal [30]. It was thought that the mechanisms of glutamate release from astrocytes involve the reversal of uptake by glutamate transporters, anion channels opened by cell swelling, Ca\(^{2+}\)-dependent exocytosis, diffusional release through ionotropic purinergic receptors (P2XRs) and functional “hemichannels” or unpaired half-gap junction channels (or connexons) on the cell surface [31-35]. However, recent research has shown that the main mechanism of
glutamate release from astrocytes is mediated by exocytosis associated with the soluble 
\( N \)-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) 
complex consisting of vesicle-associated membrane protein 2 (VAMP2), 
synaptosome-associated protein of 23 kDa (SNAP-23), a homolog of neuronal 
synaptosome-associated protein of 25 kDa (SNAP-25), and syntaxin [36-39]. Araque 
et al. have shown that glutamate released from astrocytes could excite postsynaptic 
\( N \)-methyl- \( D \)-aspartic acid (NMDA) receptors to modulate synaptic memory [40]. 
Fiacco and McCarthy reported that in CA1 pyramidal cells, astrocytes released 
glutamate that could increase the frequency of \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) spontaneous 
excitatory postsynaptic currents (EPSCs) to modulate synaptic plasticity [41]. These 
reports support the hypothesis that astrocytes affect plasticity through release of 
glutamate to stimulate the NMDA receptor.

To date, there have been no studies on the effects of inflammatory cytokines on 
calcium-dependent glutamate release from astrocytes. Therefore, the aim of this study 
is to investigate the effects of cytokines and the contribution of NO and NO-cGMP 
pathway-related substances on calcium-dependent glutamate release from astrocytes. 
Even though the effects of cytokines on glutamate release from astrocytes have not been
studied several reports have shown that cytokines modulate astrocyte activity [42]. In Chapter 2, I will discuss my investigation of the effects of cytokines on calcium-dependent glutamate release from astrocytes. During this study, I found that a cytokine mixture enhanced calcium-dependent glutamate release from astrocytes and that this enhancement of glutamate release is mediated via NO produced by iNOS expression. Furthermore, in Chapter 3, I will shown that the novel nitrated nucleic acid 8-nitro-cGMP is produced in astrocytes and in C6 cells via sGC activation by the exogenous and endogenous NO that is produced through cytokine-induced iNOS expression. Finally, I will describe the effect of the NO-cGMP pathway and of 8-nitro-cGMP on glutamate release from astrocytes.
Fig. 1-1. Tripartite synapse.
Signal transduction between pre-synaptic neuron - post-synaptic neuron, pre-synaptic neuron - astrocyte, and astrocyte - postsynaptic neuron mediated by gliotransmitter. Astrocytes that received chemical transmitters release "gliotransmitters" including glutamate, ATP, and D-serine in a calcium-dependent manner. Astrocytes modulate neuronal transmission via gliotransmitter release. To date, there is no study on the effect of cytokines on calcium-dependent glutamate release.

Fig. 1-2. Simplified scheme of the NO-cGMP pathway and chemical modification.
NO-cGMP pathway is mediated by soluble guanylate cyclase (sGC), leading to the production of a second messenger, namely, cyclic 3',5'-guanosine monophosphate (cGMP). The cGMP acts as a second messenger, activating cGMP-dependent protein kinase (PKG), and facilitates the phosphorylation of various proteins. On the other hand, NO chemically modify by different chemical reactions, such as S-nitrosylation and nitration of nucleotide, protein, and lipid. Nitration is induced by NO-derived reactive nitrogen oxides such as nitrogen dioxide (NO₂) and peroxynitrite. Further chemical modification of cGMP by NO gives rise to formation of the nitrated derivative, 8-nitro-cGMP.
Chapter 2

Cytokine-induced enhancement of calcium-dependent glutamate release from astrocytes mediated by nitric oxide

Introduction

Cytokines are chemical mediators that were originally investigated as essential mediators in the immune and inflammatory systems. There is considerable evidence that cytokines are also produced in the CNS and that they exhibit various effects on neural cells [1, 4, 43]. Astrocytes are essential for maintaining homeostasis throughout the normal CNS. They express numerous receptors that enable them to respond to virtually all known neuroactive compounds including neurotransmitters, neuropeptides, growth factors, and cytokines. These receptors enable astrocytes to function not only as sentinels, but also to participate in signal processing [5]. Astrocytes treated with cytokines release TNF-α, arachidonic acid metabolites, NO, and ROS [5]. An excess amount of these factors might result in the development of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases [1, 2]. On the other hand, cytokine-activated astrocytes can promote the recovery of CNS function [5].
Cytokine-activated astrocytes produce energy substrates and trophic factors for neurons and oligodendrocytes, act as free radical and excess glutamate scavengers, actively restore the BBB, promote remyelination, and stimulate neurogenesis from neural stem cells [5]. Although much is known about the role of astrocytes in the CNS, some aspects remain to be elucidated. Cytokines induce the expression of iNOS in astrocytes, resulting in the production of NO [44, 45]. NO is a physiologic messenger involved in several neural functions, such as synaptic plasticity that includes long-term potentiation (LTP) and long-term depression (LTD), neuroendocrine secretion, sensory processing, and cerebral blood flow [46, 47].

The main NO signal pathway, also referred to as the “NO-cGMP pathway” is mediated by sGC leading to the production of a second messenger, namely, cGMP. This cGMP activates PKG and facilitates the phosphorylation of various proteins. The neuronal and vascular signaling mediated by NO is typically driven by the NO-cGMP pathway [15-17]. Although the pathophysiological role of the NO produced by astrocytes has been actively investigated, it is not yet fully understood.

Since astrocytes have been shown to have important roles in the regulation of synaptic transmission, these cells are now attracting as much attention as neurons. Astrocytes detect neuronal activity and can release chemical transmitters including
glutamate, ATP, and D-serine, which in turn control synaptic activity [48]. Astrocytes release these neurotransmitters in a calcium-dependent manner through an exocytotic pathway and express the proteins necessary for exocytosis. This new understanding has led to the idea that astrocytes are intimately involved in the regulation of neuronal network function. The astrocyte-derived signals act both presynaptically and postsynaptically to regulate synaptic transmission [30]. Astrocytes express a plethora of metabotropic receptors including metabotropic glutamate and ATP receptors; these receptors are then able to couple with second messenger systems [52, 53]. This signaling has been shown to elevate the intracellular calcium level in astrocytes, thus enabling neurons and astrocytes to communicate with each other [54]. This finding has resulted in the novel concept of a tripartite synapse consisting of a presynaptic neuron, a postsynaptic neuron, and astrocytes [48].

Calcium-dependent glutamate release from astrocytes is regulated by ATP, prostaglandin, and ammonia [49, 55, 56]. However, because there have been no studies on the effects of inflammatory cytokines on the calcium-dependent glutamate release from astrocytes, the regulation mechanism of calcium-dependent glutamate release from astrocytes is not fully understood. I hypothesized that cytokines may affect calcium-dependent glutamate release from astrocytes. In this chapter, I
demonstrated the effects of inflammatory cytokines and the contribution of NO on calcium-dependent glutamate release from astrocytes and from rat glioma cell line C6 cells as an astrocyte model cells. Furthermore, I investigated whether the NO-cGMP pathway participates in the glutamate release from NO donor-treated C6 cells.

**Material and Methods**

**Cell culture**

Preparation of astrocytes was carried out in compliance with the Guide for Animal Experimentation at Osaka Prefecture University. Primary astrocytes were prepared from Wistar rats using a modification of a technique described previously [57]. Briefly, brain cortices from 20-day-old rat embryos were cleaned of their meninges, cut into blocks, and dissociated with 0.25% trypsin (Sigma, St Louis, MO, USA). An equal volume of horse serum (Gibco, Carlsbad, CA, USA) supplemented with DNase I (0.1 mg/ml, Sigma) was added to the medium to inactivate the trypsin, and the tissues were centrifuged at 300 × g for 5 min. The tissue sediments were resuspended in DMEM (Sigma) containing 10% fetal calf serum (Gibco), penicillin (1 × 10^5 units/l, Sigma), and streptomycin (100 mg/l, Sigma). The cells were plated on 100-mm diameter polyethyleneimine-coated plastic dishes at a density of
(0.8–1.3) × 10^5 cells/cm^2. Cultures were maintained at 37 °C in 5% CO_2 and 95% air, and the medium was changed every 3 days. After 1 week, astrocytes were replated to remove the neurons using a standard trypsin treatment technique. On days 12–14, the cells were replated again onto a 96-well plate, a 24-well plate, or a 60-mm dish at a density of 1.0 × 10^5, 2.0 × 10^5, and 1.0 × 10^6 cells/well in each plate or dish, respectively, and stabilized for 1 day before the experiments.

Rat glioma cell line C6 cells, most commonly used as a astrocyte model cells, were cultured in DMEM containing 10% fetal calf serum (Gibco), penicillin (1 × 10^5 units/l, Sigma), 100 μg/ml streptomycin, and 100 units/ml penicillin under a humidified 5% CO_2 atmosphere at 37 °C.

**Treatment of astrocytes with reagents**

Astrocytes and C6 cells were treated with cytokines, as will be explained below. The culture medium was changed to DMEM containing 1% fetal calf serum, penicillin (1 × 10^5 units/l), streptomycin (100 mg/l), and l-arginine (100 μM) with or without a cytokine mixture (3 ng/ml each of interleukin-1β (IL-1β, Wako Chemicals, Osaka, Japan), TNF-α (Wako Chemicals), and interferon-γ (IFN-γ, Wako Chemicals) and incubated for 12 h at 37 °C. For iNOS inhibition, S-isopropyl-isothiourea (10 μM,
Dojindo, Kumamoto, Japan) was added together with the cytokines. For sGC inhibitor, NS 2028 (10 μM, Cayman Chemical Company, Michigan, USA) and PKG inhibitor, KT 5823 (5 μM, Almone Lab Ltd, Jerusalem, Israel) was added before NO donor treatment. NOC7 (0.1 mM, Dojindo), an NO donor and Br-cGMP (0.1 mM, BIOLOG Life Science Institute, Bremen, Germany) was added 30 min before calcium stimulation.

Nitrite Analysis

NO synthesis was determined by assaying nitrite, a stable reaction product of NO with molecular oxygen, in the culture supernatants. In brief, 100 μl of culture supernatant was allowed to react with 100 μl of Griess reagent [58] and was incubated at room temperature for 15 min. The optical density of the assay samples was measured spectrophotometrically at 540 nm. Fresh culture media served as the blank in all experiments. Nitrite concentrations were calculated from a standard curve derived from the reaction of NaNO₂ in the assay.

Glutamate release assays

Glutamate release experiments and determination of their concentrations were performed according to the modified method described for neurons [59, 60]. The
astrocytes were washed briefly with prewarmed Ringer's solution (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 2.5 mM CaCl$_2$, 1.2 mM MgSO$_4$, 11 mM glucose, and 15 mM HEPES–NaOH; pH 7.4) and were incubated in the same solution. The solution was renewed 4 times at intervals of 2 min. After changing Ringer's solution, the cultures were challenged with Ringer's solution containing the calcium ionophore A23187 (10 μM, Merck, Darmstad, Germany) with or without CaCl$_2$ at intervals of 2 min. Fractions were collected for measuring the released glutamate. Glutamate content was determined by reverse-phase high performance liquid chromatography (HPLC) on a Mightysil-C18 column (4.6 mm × 150 mm, Kanto Chemical, Tokyo, Japan), using precolumn derivatization with o-phthalaldehyde and fluorescence detection.

**Fluorescence immunocytochemistry**

For fluorescence immunocytochemistry, the cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 20 min followed by treatment with methanol for 3 min. After washing with PBS, the Cy3 conjugated anti-glial fibrillary acidic protein (GFAP) antibody (0.5 μg/ml, Sigma), anti-MAP2 antibody (1 μg/ml, Sigma), or the iNOS specific antibody i2G4 [57]
conjugated with Cy5 (1 μg/ml) prepared by using the Fluorolink mAb Cy labeling kit (GE Healthcare Biosciences Corp., Piscataway, NJ, USA), according to the manufacturer's instructions, were added and incubated for 30 min. Following the washing, for MAP2, the anti-rabbit IgG secondary antibody conjugated with Cy5 (0.1 μg/ml, GE Healthcare Biosciences Corp.) was added to the cells and incubated for 30 min. Nuclei were stained with Hoechst 33258 (Dojindo). The cells were mounted using fluorescent mounting media and observed under the fluorescence microscope Axioplan 2 (Carl Zeiss, Jena, Germany). Images were processed to reduce noise using the deconvolution software AutoDeblur (Lexi, Tokyo, Japan).

**Immunoblotting**

The level of iNOS, GFAP, and MAP2 expression was determined by immunoblotting analysis. The cells cultured in 100-mm dishes were treated with various reagents. After washing with PBS, the cells were homogenized with Tris–HCl (10 mM pH 7.5) containing 1% NP-40 and protease inhibitor cocktail (Sigma). The homogenates were centrifuged at 12,000 × g for 15 min. The soluble fractions were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10%) and subsequent blotting onto nitrocellulose membranes. The membrane was blocked
with Blocking One (Nacalai Tesque, Kyoto, Japan) and reacted with HRP-conjugated iNOS specific antibody i2G4 [57] (1 μg/ml), anti-actin antibody (0.5 μg/ml, Sigma), anti-GFAP antibody (1 μg/ml, Sigma), and anti-MAP2 antibody (1 μg/ml,). The membrane that reacted with the anti-actin and anti-GFAP antibodies was further incubated for 1 h with HRP-labeled anti-rabbit IgG secondary antibody (0.1 μg/ml, GE heatlcare); the membrane that reacted with anti-MAP2 was further incubated for 1 h with HRP-labeled anti-mouse IgG secondary antibody (0.1 μg/ml, GE Healthcare Biosciences Corp). The reactive bands were visualized using the SuperSignal chemiluminescent reagent (Pierce, Rockford, IL, USA). Signal intensities were quantified using a LAS-1000 imaging system (Fujifilm, Tokyo, Japan).

**Statistical analysis**

For statistical analysis of the data, one-way analysis of variance (ANOVA) followed by Student's $t$-test was used. Differences between treatments were considered statistically significant for $p < 0.05$. 
Results

Calcium-dependent glutamate release from astrocytes

Immunohistochemical analysis of astrocytes revealed that more than 96% of the cells were immunopositive for the astrocyte marker GFAP but not for the neuron marker MAP2 (data not shown). Absence of neuron contamination in the astrocyte culture was also confirmed by immunoblotting, in which immunoreactivity with the anti-MAP2 antibody was observed to be less than the detection limit (data not shown). Fig. 2-1 shows the ionophore-evoked calcium-dependent glutamate release from cultured astrocytes. Extracellular glutamate was measured with reverse-phase high-performance liquid chromatography (HPLC) every 2 min in the presence or absence of calcium. The addition of the calcium ionophore A23182 (10 μM) caused an increase in extracellular glutamate from astrocytes in the presence of calcium but not in the absence of extracellular calcium. C6 cells also released glutamate in a calcium-dependent manner similar to that of astrocytes (data not shown). This result indicated that the release of glutamate from astrocytes and C6 cells was dependent on extracellular calcium.
Effect of cytokines on calcium-dependent glutamate release from astrocytes

Fig. 2-2 shows the effect of cytokines on the calcium-dependent glutamate release from astrocytes. The addition of the calcium ionophore A23187 (10 μM) caused an increase in extracellular glutamate from the astrocytes in the presence of calcium but not in the absence of extracellular calcium as well as showed Fig. 2-1. In order to determine the effect of cytokines on the calcium-dependent glutamate release, cells were pretreated with a mixture of 3 cytokines (IL-1β, TNF-α, and IFN-γ) because this cytokine mixture induces a higher level of iNOS expression in astrocytes [58]. The pretreatment of the cytokines enhanced the calcium-dependent glutamate release. There was a 50% increase over baseline levels.
Induction of iNOS expression and NO production by cytokines in astrocytes

Cytokines are also known as inducers of NO production. Therefore, I hypothesized that a relationship existed between NO production and the enhancement of glutamate release by cytokines. In order to confirm this hypothesis, I first examined the induction of iNOS expression and NO production by a cytokine mixture (Fig. 2-3). Immunoblotting showed that iNOS protein expression was induced by cytokine treatment (Fig. 2-3A). Immunofluorescence analysis showed that iNOS is expressed in the cytosol of GFAP-positive cells after treatment with the cytokines (Fig. 2-3B). A small amount of GFAP-negative and iNOS-positive cells are most likely to be microglia.

Fig. 2-2. Effect of cytokines on calcium-dependent glutamate release from astrocytes.

Astrocytes were preincubated in the medium containing a cytokine mixture (IL-1β, TNF-α, and IFN-γ) for 12 h. After washing, the cells were stimulated for 2 min with the calcium ionophore A23187 in the presence or absence of calcium. The glutamate contents in the extracellular solution were determined by reverse-phase HPLC. The glutamate release from control cells in the presence of calcium was used to normalize the percentage of each sample. Each bar graph represents the mean ± S.E. of four experiments. *Comparison with the release from control cells in the presence of calcium, \( P < 0.05 \), one-way ANOVA test.
NO production was also induced by the treatment, but the increase was diminished by the addition of an iNOS inhibitor (Fig. 2-3C).

Fig. 2-3. Induction of iNOS expression and NO production by cytokines in astrocytes.
A. Immunoblotting of astrocytes treated with cytokines. The expression of iNOS in astrocytes treated with cytokines was analyzed by immunoblotting using the HRP-labeled i2G4 mAb. B. Immunocytochemistry of iNOS and GFAP in astrocytes. Astrocytes treated with cytokines were fixed with paraformaldehyde-methanol. After washing with PBS, anti-GFAP conjugated with Cy3 and i2G4 conjugated with Cy5 were added and incubated for 30 min. Nuclei were stained with Hoechst 33258. Upper and lower panels show control and cytokines-treated astrocytes, respectively. Left panels: GFAP stain (green), center panels: iNOS stain (red), and right panels: GFAP and iNOS merge. Scale bars = 50 μm. C. Production of nitrite from astrocytes treated with cytokines. The supernatant of the astrocyte culture treated with cytokines with or without an iNOS inhibitor was assayed for nitrite content by the Griess reaction. Each bar graph represents the mean ± S.E. of four experiments. * Comparison with the nitrite from control cells $P < 0.05$, one-way ANOVA test.
Effects of iNOS inhibitor and NO donor on the cytokines-induced enhancement of calcium-dependent glutamate release from astrocytes

I hypothesized that a relationship existed between NO production and the enhancement of glutamate release by cytokines. As shown in Fig. 2-3, cytokines induced iNOS expression in astrocytes. Next, I examined the contribution of NO to the enhancement of glutamate release from astrocytes and C6 cells by cytokines. Fig. 2-4 shows the effect of the iNOS inhibitor and the NO donor on the enhancement of calcium-dependent glutamate release from the astrocytes and C6 cells treated with cytokines. The iNOS inhibitor attenuated the cytokine-induced enhancement of glutamate release from astrocytes. Furthermore, pretreatment of the cells with the exogenous addition of an NO donor enhanced the glutamate release from astrocytes even in the absence of cytokines (Fig. 2-4A). Similarly, the iNOS inhibitor attenuated the cytokine-induced enhancement of glutamate release from C6 cells. NO donor enhanced the glutamate release from C6 cells even in the absence of cytokines as well as astrocytes (Fig. 2-4B).
Effect of the NO-cGMP pathway on NO-enhanced calcium-dependent glutamate release

In order to examine the role of the NO-cGMP pathway, which is well known to be a target of NO, the effects of an sGC inhibitor, NS 2028, a PKG inhibitor, KT 5823, and the cGMP analog 8-Br-cGMP on glutamate release from C6 cells were investigated. As shown in Fig. 2-5A, neither the sGC inhibitor nor the PKG inhibitor affected glutamate release from C6 cells treated with NO donor for 0.5 h. Furthermore,
treatment with cGMP analog for 0.5 h and 18 h did not enhance glutamate release compared with control cells (Fig. 2-5A, B). On the other hand, although the PKG inhibitor did not affect glutamate release from C6 cells treated with NO donor for 18 h, the sGC inhibitor attenuated the enhancement of glutamate release from C6 cells treated with NO donor for 18 h. These results suggest that two different processes could regulate the NO-enhanced calcium-dependent glutamate release from astrocytes. The immediate potentiation mechanisms of glutamate release caused by treatment with NO donor for a short period may be regulated by direct effects of NO that are uninvolved in the NO-cGMP pathway. Alternatively, the delayed potentiation mechanism of glutamate release may be regulated by unaccountable effects in the NO-cGMP pathway alone. Thus, our results suggested that a novel NO signal transmission mechanism, different from the NO-cGMP pathway, may be involved in NO-enhanced glutamate release from astrocytes.
Fig. 2-5. Effects of NO-cGMP pathway on NO-mediated enhancement of glutamate release.

C6 cells were pretreated with NS 2028 (10 μM), KT 5823 (5 μM), and NOC7 (0.1 mM) or 8-Br-cGMP (0.1mM) for 0.5 h (A) and 18 h (B), and glutamate release was induced. After washing, the cells were stimulated for 2 min with the calcium ionophore A23187, and the glutamate contents of the extracellular solution were determined by reverse-phase HPLC. Each bar graph represents the mean ± S.E. of four experiments. * Comparison with the release from control cells, P < 0.05, one-way ANOVA test.
Discussion

The study in this chapter revealed that cytokines enhance the calcium-dependent glutamate release from astrocytes. To our knowledge, this is the first report to describe the modulation of the calcium-dependent glutamate release from astrocytes by cytokines. The results of this investigation also indicate that NO mediates cytokine-induced enhancement of the calcium-dependent glutamate release. Furthermore, these findings suggest that NO-enhanced calcium-dependent glutamate release from astrocytes is regulated by two different processes consistent with the direct effects of NO and a novel signal transduction that is unexplained by the classical NO-cGMP pathway alone.

Calcium-dependent glutamate release from astrocytes occurs through an exocytotic pathway [30, 36]. Astrocytes and neurons express synaptic vesicle proteins and members of the synaptic soluble NSF attachment receptor (SNARE) complex [39]. In neurons, exocytosis requires SNARE proteins consisting of synaptobrevin 2, syntaxin, and synaptosome-associated protein of 25 k Da (SNAP25). Astrocytes also express the SNARE proteins consisted of synaptobrevin 1 and 2, syntaxin 1A and 4, and SNAP23 homologus to those of neurons [36-39]. Moreover, vesicular glutamate transporters (VGLUT) 1 and 2 are expressed in astrocytes [61]. Glutamate release
from neuronal cells by exocytosis is inhibited by treatment with the *Clostridium botulinum* neurotoxin (BoNT) that cleaves the SNARE complex [54]. Araque, A, et.al. demonstrated that glutamate release from astrocytes is inhibited by treatment with BoNT [62]. Thus, the molecular mechanism of glial exocytosis is similar to that of neuronal exocytosis.

The immediate potentiation mechanisms of glutamate release caused by NO may be regulated by the direct effects of NO that are uninvolved in the NO-cGMP pathway. On the other hand, it is possible that the delayed potentiation mechanism of glutamate release is regulated by unaccountable effects independent of the downstream factors of the NO-cGMP pathway. Regrettably, it was not possible to elucidate a more detailed mechanism of NO-enhanced calcium-dependent glutamate release, although it is clear that NO regulates calcium-dependent neurotransmitter release in a highly complex manner. It is generally accepted that NO directly interacts with synaptic vesicle proteins or members of the synaptic SNARE complex to modulate exocytosis [63, 64]. In addition, it has been reported that SNAP-25 and Nunc-18 of the synaptosome are nitrated by peroxynitrite and that these modifications increase the formation of sodium dodecyl sulfate (SDS)-resistant SNARE complexes and vesicle release [66]. The NO-enhanced glutamate release from astrocytes may result from the
modification of these proteins. Additional research concerning the regulatory mechanism of the glutamate release from astrocytes by NO is required.

The glutamate released from astrocytes acts both presynaptically and postsynaptically to regulate synaptic transmission [30]. Presynaptically, the glutamate from astrocytes can access metabotropic glutamate receptors and kainate receptors to enhance synaptic transmission. Postsynaptically, the glutamate from astrocytes can act on extrasynaptic NMDA receptors to depolarize the neuronal membrane and promote neuronal synchrony of NMDA receptors, which can regulate synaptic plasticity [30]. Therefore, it is presumed that an alteration of glutamate release from astrocytes can modulate the synaptic transmission between pre- and postsynaptic neurons; thus, an alteration may influence the tripartite synapse. In the present study, I showed that cytokines promote NO synthesis, which induces the enhancement of astroglial glutamate release. This study raises the possibility that cytokines might modulate the synaptic transmission between pre- and postsynaptic neurons. Nevertheless the role of cytokine-induced enhancement of glutamate release from astrocytes in the CNS remains to be determined. Inflammatory cytokines are responsible not only for neurodegeneration but also for neuroprotection [5]. It is possible that cytokines affect the tripartite synapse and strength of the trisynaptic connection under pathological and
physiological conditions by modulating glutamate release from astrocytes; this may be considered to be both detrimental and beneficial for correct synaptic plasticity. Further studies are required to clarify these points.
Chapter 3

Formation of the novel second messenger 8-nitro-cGMP in astrocytes and modulation of glutamate release by 8-nitro-cGMP from astrocytes

Introduction

At the present, NO constitutes a unique class of mediator that plays important roles in maintaining the homeostasis of biological systems [67]. However, a complete picture of the mechanisms by which NO regulates homeostasis remains unknown. NO plays diverse physiological roles in vascular regulation, neuronal transmission, inflammation, and host defense against microbial pathogens [68]. The NO-cGMP pathway, which is the main NO pathway, is mediated by sGC leading to the production of a second messenger, namely, cGMP [15]. The cGMP messenger activates PKG and facilitates the phosphorylation of various proteins [16]. However, the existence of cGMP-independent phenomena has been suggested. For example, NO function seems to be exerted by different chemical reactions such as S-nitrosylation and nitration of amino acids and proteins, which are induced by NO-derived reactive nitrogen oxides such as nitrogen dioxide (NO₂) and peroxynitrite [18, 19].

NO-derived reactive nitrogen oxide species (RNOS) cause nitration of nucleic
acids, amino acids, and proteins. Akaike et al. previously found that nitrated guanine derivatives, including 8-nitroguanine and 8-nitroguanosine, formed in cultured cells and in tissues derived from murine viral pneumonia and human lung disease [22, 69, 70]. Recently, Sawa et al. discovered that a novel nitrated cyclic nucleotide, 8-nitro-cGMP, is generated after NO production in a macrophage cell line (RAW 264.7 cells) and in human hepatoma cells (HepG2) [21, 71]. However there have been no reports of 8-nitro-cGMP formation in the CNS. The nucleotide 8-nitro-cGMP not only acts as a cGMP analog, activating PKG, but it also enable the formation of protein Cys-cGMP adducts as a new posttranslational modification, known as protein S-guanylation [21]. Furthermore, Sawa et al. identified a unique chemical property of 8-nitro-cGMP, i.e., the potential to generate superoxide anions via the activation of NADPH reductase-like enzymes, including three NOS isoforms and P450 reductase, resistance to phosphodiesterase, and membrane permeability [21].

In the nervous system, NO is involved in several neural functions such as synaptic plasticity that includes long-term potentiation (LTP) and long-term depression (LTD) [46]. However, the effects of NO on gliotransmitter release are unknown. The regulation of neurotransmitter release mediated by NO is reported to be a cGMP-dependent/independent mechanism in which NO S-nitrosylates the cysteine
residue of the SNAP-25 and N-ethylmaleimide-sensitive-factor protein (NSF), peroxynitrite that is formed by the reaction of NO, and superoxide anion nitrates the tyrosine of the SNARE protein [72-75]. As described in Chapter 2, NO regulates the cytokine-induced enhancement of the calcium-dependent glutamate release. Furthermore, as indicated previously, the NO-mediated enhancement of glutamate release is regulated a complex mechanism that cannot be explained by the NO-cGMP pathway alone.

8-nitro-cGMP expected to act diverse functions in immune cells [71]. However, no information is available concerning of the function of 8-nitro-cGMP as a novel second messenger in CNS. Consequently, I hypothesized that 8-nitro-cGMP may affect the regulation of gliotransmitter release as a novel modulator. In this study, I demonstrated that the detailed mechanism of 8-nitro-cGMP formation and its intracellular content. Furthermore, I demonstrated 8-nitro-cGMP affects calcium-dependent glutamate release from C6 cells.
Materials and Methods

Materials

The monoclonal anti-8-nitro-cGMP antibody 1G6 prepared as previously described [21] was a kind gift from Dr. Takaaki Akaike, Kumamoto University.

Synthesis of 8-nitroguanosine 3’,5’-cyclic monophosphate (8-nitro-cGMP)

8-nitro-cGMP was synthesized by the following 2 reactions as previously described [21]: (i) bromination at the C8 position of the guanosine moiety, and (ii) nucleophilic substitution of the bromo group with NO\textsubscript{2}⁻. In brief, cGMP (150 mg, 0.4 mmole) was reacted with bromine (0.5 ml) in 5 ml of formamide on ice for 30 min. The reaction was terminated by adding 2 ml of aniline. 8-Bromoguanosine 3’,5’-cyclic monophosphate (8-Br-cGMP) was washed 3 times with diethyl ether, purified using a C18 solid-phase column (Waters, 35 ml), and eluted with 0.02% trifluoroacetic acid plus 20% methanol. 8-Br-cGMP (35 mg, 0.12 mmole) was reacted with sodium nitrite (0.5 mmole) in 1.5 ml of dimethyl sulfoxide containing 23 mM HCl at 70 °C for 5 days. 8-nitro-cGMP was purified using a C18 solid-phase column in a manner similar to that used for 8-Br-cGMP purification. In addition, 8-nitro-cGMP was purified by high performance liquid chromatography (HPLC) with a preparative reverse-phase column.
(CAPCELL PAK C18 20 mm × 150 mm; Shiseido, Tokyo, Japan), eluted with 3.0 ml/min of solvent A (10 mM borate, 50 mM NaCl, pH 9.0) and methanol in a linear gradient from solvent A to 30% methanol for 30 min, and monitored at 254 and 397 nm. 8-nitro-cGMP was applied to a solid-phase column and eluted with 0.02% trifluoroacetic acid plus 20% methanol.

**Cell treatment**

C6 cells, rat glioma cell line, were cultured as described in chapter 2. Cells were plated at a density of 1.4 x 10^6 cells per well in 6-well plates for preparation of cell lysate, at 2 x 10^5 cells per chamber in BD Falcon Culture Slides (BD Biosciences, San Jose, CA) for immunocytochemistry, and at 2 x 10^6 cells per 100 mm dish for LC-MS/MS.

Astrocytes were obtained from rat cerebral cortex as described chapter 2 and were plated to 2 x 10^5 cells per polyethylenimine-coated chamber in BD Falcon Culture Slides slide for immunocytochemistry.

**Treatment of cells with reagents**

To study 8-nitro-cGMP formation, cells were treated with 50 μM
S-nitroso-N-acetylpenicillamine (SNAP, Cayman Chemical, Ann Arbor, MI) for various time periods (indicated in figure legends). For induction of inducible NO synthase (iNOS) expression, cells were treated with 1 or 10 µg/ml lipopolysaccharide (LPS, from Escherichia coli; L8274; Sigma-Aldrich Corporation, St. Louis, MO), 100 U/ml interferon-γ (IFN-γ), 100 U/ml tumor necrosis factor α (TNFα), and 10 ng/ml interleukin-1β (IL-1β) (all cytokines from R&D Systems, Inc., Minneapolis, MN). In some experiments, cells were stimulated in the presence of 1 mM N^G^-monomethyl-L-arginine (L-NMMA, Sigma-Aldrich) or 10 µM 1400W to inhibit NO synthase activity and 1 µM NS 2028 to inhibit sGC activity. To examine the effect of glutathione depletion on 8-nitro-cGMP formation, cells were treated with 1 mM buthionine sulfoximine (BSO) for 8 h and then with SNAP or LPS plus cytokines in the presence of 1 mM BSO. To study the effects of 8-nitro-cGMP on calcium-dependent glutamate release from C6 cells, C6 cells were treated with 0.1 mM 8-nitro-cGMP was added for 0.5 or 18 h before calcium stimulation.

**Fluorescence immunocytochemistry**

Formation of 8-nitro-cGMP was analyzed by the method described previously [21]. In brief, C6 cells, plated on Culture Slides (BD Biosciences) and treated as
described above, were fixed with Zamboni solution (4% paraformaldehyde and 10 mM picric acid in 0.1 M phosphate buffer, pH 7.4) at 4 °C for 7 h. After permeabilization with 0.5% Triton X-100 at room temperature for 15 min, cells were incubated with BlockAce (Dainippon Pharmaceutical, Osaka, Japan) overnight at 4 °C to block nonspecific antigenic sites. Cells were then incubated overnight at 4 °C with anti-8-nitro-cGMP monoclonal antibody 1G6 (1 μg/ml) (10) in Can Get Signal Immunoreaction Enhancer Solution 1 (TOYOBO, Osaka, Japan). After three rinses with PBS, cells were incubated with Cy3-labeled goat anti-mouse IgG antibody (GE Healthcare, Piscataway, NJ). The cells were mounted using fluorescent mounting media and observed under the fluorescence microscope (Axioplan 2, Carl Zeiss, Jena, Germany). equipped with an AxioCam MRm camera (Carl Zeiss).

Sample preparation for quantitative analysis of 8-nitro-cGMP by liquid chromatography (LC)-MS/MS and high-performance LC-electrochemical detection (HPLC-ECD)

Intracellular levels of 8-nitro-cGMP and cGMP were measured by LC-electrospray ionization (ESI)-MS/MS and HPLC-ECD. C6 cells were plated on 100-mm dishes at a density of 3 x 10^6 cells per dish. After treatment with 50 μM
SNAP or with a mixture of LPS (1 or 10 μg), INF-γ (100 U/ml), TNFα (100 U/ml), and IL-1β (5 ng/ml) in 10 ml of Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum for various times, cells were washed twice with ice-cold PBS and homogenized in 3 ml of methanol containing 5 mM N-ethylmaleimide (NEM, Sigma-Aldrich). After the homogenate samples were centrifuged at 5,000 g at 4 °C, their resultant supernatant was dried in vacuo and then redissolved in distilled water. After the samples were mixed with hexane, the aqueous phase was obtained. This fraction was dried in vacuo and reconstituted with 100 μl of water. This sample was used for LC-ESI-MS/MS and HPLC-ECD analyses.

**LC-ESI-MS/MS**

LC-ESI-MS/MS was performed with a Varian 1200L triple-quadrupole mass spectrometer (Varian, Inc., Palo Alto, CA), after reverse-phase HPLC on a Mightysil RP-18 column (50 × 2.0 mm i.d.; Kanto Chemical) with a linear 0-100% CH₃CN gradient for 5 min in 0.1% formic acid at 40 °C. Total flow rate was 0.15 ml/min, and injection volume was 20 μl. The column effluent was introduced directly into the mass spectrometer operated in positive mode under following conditions: collision gas (argon) pressure 2.2 mTorr, drying gas (nitrogen) pressure 19 psi at 300 °C, nebulising
gas (nitrogen) pressure 54 psi, scan time 1 s, needle voltage 5000 V, shield voltage 600 V, capillary voltage 100 V, and detector voltage 2000 V. The LC-MS/MS scanning was performed under the multiple reaction monitoring mode with the scanning parameters shown in Table 1. These parameters were determined with the Automated MS/MS Breakdown software (Varian) using a stock solution of 8-nitro-cGMP and cGMP (10 μg/ml) in 50% CH$_3$CN containing 0.05% formic acid. HPLC-ECD analysis for 8-nitro-cGMP formation was performed as described previously [21].

<table>
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<td><strong>LC-MS/MS scanning parameters</strong></td>
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**Glutamate release assays**

Glutamate release experiments and determination of their concentrations were performed as described in chapter 2. C6 cells were washed briefly with prewarmed Ringer's solution (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 2.5 mM CaCl$_2$, 1.2 mM MgSO$_4$, 11 mM glucose, and 15 mM HEPES–NaOH; pH 7.4) and were incubated in the same solution. The solution was renewed 4 times at intervals of 2 min. After changing Ringer's solution, the cultures were challenged with Ringer's solution
containing 10 μM A23187 calcium ionophore with or without CaCl₂ at intervals of 2 min. Fractions were collected for measuring the released glutamate. Glutamate content was determined by reverse-phase HPLC on a Mightysil-C18 column (4.6 mm × 150 mm, Kanto Chemical), using pre-column derivatization with o-phthalaldehyde and fluorescence detection.
Results

NO-dependent formation of 8-nitro-cGMP in astrocytes and C6 cells

To elucidate the roles of 8-nitro-cGMP in the signal transduction pathway downstream of NO, I first examined the formation of 8-nitro-cGMP in C6 cells in response to NO. I studied 8-nitro-cGMP by means of immunocytochemistry utilizing the 1G6 monoclonal antibody against 8-nitro-cGMP [21]. Immunostaining for 8-nitro-cGMP was markedly increased in C6 cells after treatment with the NO donor SNAP in a time and NO-dependent manner (Fig. 3-1A, B). This 8-nitro-cGMP increase was further enhanced by BSO, an inhibitor of glutathione biosynthesis, which suggests that glutathione levels may affect 8-nitro-cGMP formation. Similarly, 8-nitro-cGMP levels markedly increased in C6 cells expressing increased amounts of iNOS after stimulation with LPS plus a mixture of the proinflammatory cytokines IFN-γ, TNFα, and IL-1β (Fig. 3-1C). Treatment of cells with L-NMMA, an inhibitor of NO synthases almost completely nullified this elevated 8-nitro-cGMP immunoreactivity. In addition, the present immunostaining of C6 cells was totally nullified by the treatment of an inhibitor of soluble guanylate cyclase (sGC) NS 2028 (Fig. 3-1C). These immunocytochemical results thus strongly support the conjecture that 8-nitro-cGMP would remain near sites of its generation in cells, or in the molecular
region where putative targets for 8-nitro-cGMP may reside. These data also indicate that the antibody used in this study indeed specifically recognized the 8-nitro-cGMP rather than macromolecules containing molecular structures similar to 8-nitro-cGMP. These multiple lines of evidence thus demonstrate that formation of 8-nitro-cGMP depends on NO production in the cells. Next, I examined the formation of 8-nitro-cGMP in astrocytes in response to exogenous and endogenous NO (Fig. 3-2). Immunostaining for 8-nitro-cGMP was markedly increased in astrocytes after treatment with the NO donor SNAP in a time and NO-dependent manner as well as C6 cells (Fig. 3-2A). Similarly, 8-nitro-cGMP levels markedly increased in astrocytes expressing increased amounts of iNOS after stimulation with LPS plus IFN-γ (Fig. 3-2B). Treatment of cells with 1400W, an inhibitor of NO synthases, and NS 2028, an inhibition of sGC activation, almost completely nullified this elevated 8-nitro-cGMP immunoreactivity (Fig. 3-2B).
Fig. 3-1. Immunocytochemical analysis of NO-dependent 8-nitro-cGMP formation in C6 cells. A, C6 cells were pretreated with 1 mM BSO for 8 h or were untreated, followed by treatment with 50 μM SNAP for the times indicated. B, C6 cells were pretreatment with 1 mM BSO for 8 h or were untreated, followed by treatment with 20, 50, 100 μM SNAP for the NO concentration indicated. C, C6 cells pretreated with 1 mM BSO were stimulated with a mixture of LPS (1 μg/ml) and the cytokines IFN-γ (100 U/ml), TNF-α (50U/ml), and IL-1β (30 ng/ml) for 12 or 24 h in the absence of 1 mM L-NMMA or 1 μM NS 2028. Immunostaining was performed with anti-8-nitro-cGMP monoclonal antibody 1G6. Scale bars indicate 25 μm.
Fig. 3-2. Immunocytochemical analysis of NO-dependent 8-nitro-cGMP formation in astrocytes.

A, Astrocytes were pretreated with 1 mM BSO for 8 h followed by treatment with 12, 25, 50 μM SNAP for 12 and 24 h for NO concentration and times indicated. B, Astrocytes were pretreated with 1 mM BSO for 8 h followed by stimulation with a mixture of LPS (1 μg/ml) and IFN-γ (100 U/ml) for 12 or 24 h in the absence of 10 μM 1400W or 1 μM NS 2028. Immunostaining was performed with anti-8-nitro-cGMP monoclonal antibody 1G6. Scale bars indicate 25 μm.
Quantitative analysis of 8-nitro-cGMP

I then investigated generation of 8-nitro-cGMP by utilizing LC-ESI-MS/MS and HPLC-ECD. I quantified 8-nitro-cGMP in C6 cell extracts obtained at various time points after SNAP treatment (Fig. 3-3) and after treatment with LPS plus cytokines (Fig. 3-5). Both analytical methods revealed accumulation of 8-nitro-cGMP in cells treated with SNAP, and both methods produced a similar time course of accumulation (Fig. 3-3B). I also examined the change in cGMP levels according to time. Compared with cGMP, which showed a rapid and transient elevation and a peak level within a few hours after SNAP treatment (Fig. 3-3C), 8-nitro-cGMP became detectable during the peak and decline in the cGMP level and increased linearly for another several hours, even after cGMP turned down to a plateau (Fig. 3-3B). The amount of 8-nitro-cGMP eventually reached a value that was one tenth of the peak value of cGMP. It is notable that 8-nitro-cGMP production was sustained, in contrast with the quick decline in cGMP.
Fig. 3-3. Quantitative analysis, by LC-ESI-MS/MS and HPLC-ECD, of 8-nitro-cGMP and cGMP formation in SNAP-treated C6 cells.

C6 cells were treated with 50 μM SNAP for various times in the presence of 1 mM BSO, and cell extracts were prepared as described in material and methods. A, LC-MS/MS chromatograms of 8-nitro-cGMP and cGMP. B, change in intracellular 8-nitro-cGMP concentration after SNAP treatment determined with LC-MS/MS and HPLC-ECD. C, change in intracellular cGMP concentration after SNAP treatment determined with LC-MS/MS.
More important, from the LC-ESI-MS/MS analysis for LPS/cytokine-activated C6 cells, significant amount of 8-nitro-cGMP was unequivocally identified with the cells after stimulation with LPS and cytokines (IFN-γ, TNFα, and IL-1β) to induce iNOS and endogenous NO production (Fig. 3-4 and 3-5).

The apparent intracellular concentration of 8-nitro-cGMP formed in the iNOS-expressing cells was comparable to that in SNAP-treated cells. Although exact data is not shown here, only about 10% of 8-nitro-cGMP appears to be recovered from the methanol homogenate, when it was added exogenously to the cell homogenates. I therefore expect from the present apparent 8-nitro-cGMP values determined via LC-MS/MS (Fig. 3-3 and 3-5) that at least low micromolar concentrations of 8-nitro-cGMP could be formed from NO generated in the cells.
Fig. 3-4. iNOS expression and nitrite production in C6 cells stimulated with LPS plus cytokines.

A, expression of iNOS in C6 cells treated with LPS and cytokines. C6 cells were pretreated with BSO (1 mM) for 8 h, after which they were stimulated with a mixture of LPS (1 or 10 µg/ml), IFN-γ (100 U/ml), TNFα (100 U/ml), and IL-1β (5 ng/ml) for indicated time periods in the presence of 1 mM BSO, in the same manner as in Fig. 3-1. Cell lysates (10 µg of protein) were analyzed via the Western blotting with an anti-iNOS antibody (Santa Cruz Biotechnology). B, immunocytochemistry of iNOS in C6 cells. C6 cells were stimulated with LPS (1 µg/ml) and cytokines (same as in A) for 12 h in the presence of 1 mM BSO. iNOS (green) was detected as described previously (3). Nuclei were stained with Hoechst 33258 (blue). Scale bars indicate 25 µm. C, nitrite concentrations in culture supernatants of C6 cells. C6 cells were treated with the same mixture of LPS and cytokines as in A. Nitrite concentrations in culture supernatants were analyzed by means of the Griess reagent assay. Data represent means ± s.d. (n = 3).
Effect of 8-nitro-cGMP on calcium-dependent glutamate release from astrocytes

In chapter 2, I showed that NO regulated calcium-dependent glutamate release.
from astrocytes by a complex mechanism that cannot be explained by the classical NO-cGMP pathway. The detailed mechanism of NO-mediated enhancement of glutamate release from astrocytes remains unknown. In this chapter, I investigated the effects of 8-nitro-cGMP, which exerts various functions as a novel second messenger, on the glutamate release from C6 cells. Glutamate release from C6 cells pretreated with 0.1 mM 8-nitro-cGMP for 0.5 and 18 h was higher than that from control cells (Fig. 3-6A). Although the enhancement by NO was canceled by the sGC inhibitor, 8-nitro-cGMP restored the cancellation (Fig. 3-6B). These results indicate that the NO-induced enhancement of calcium-dependent glutamate release is mediated by 8-nitro-cGMP. These findings may explain the discrepancy between the effect of sGC inhibitor and cGMP analogue as shown in Fig. 2-5B.
Fig. 3-6. Effects of 8-nitro-cGMP on the NO-mediated enhancement of calcium-dependent glutamate release from C6 cells.

A, C6 cells were treated with 0.1 mM 8-nitro-cGMP for 0.5 and 18 h. B, C6 cells were pretreated with NS 2028 (10 μM) for 0.5 h, and NOC7 (0.1 mM) for 18 h, and glutamate release was induced. After washing, the cells were stimulated for 2 min with the calcium ionophore A23187, and the glutamate contents of the extracellular solution were determined by reverse-phase HPLC. The glutamate release from control cells in the presence of calcium was used to normalized the percentage of each sample. Each bar graph represents the mean ± S.E. of four experiments. *

Comparison with the release from control cells, \( P < 0.05 \), one-way ANOVA test.
Discussion

Chemical nitration of biological molecules by NO-derived RNOS, such as peroxynitrite and NO₂, has been well documented [18-20]. Of the various nitrated compounds identified to date, 8-nitro-cGMP has several unique features [21, 79, 80].

My rigorous immunocytochemical and MS/MS analyses unequivocally identified 8-nitro-cGMP formation in C6 cells and astrocytes. This is the first report that clarifies the formation of 8-nitro-cGMP in the CNS by the chemical analysis. I used C6 cells for these quantitative analyses because they express relatively high levels of sGC, which is the sole enzyme responsible for effective cGMP formation on stimulation with NO. Also, C6 cells are easily activated by proinflammatory cytokines to express iNOS. In fact, C6 cells readily respond to stimulation with NO and produce an appreciable quantity of cGMP, as shown by LC-MS/MS (Fig. 3-3C). In addition, the time course of intracellular cGMP formation was typical, in that formation increased quickly after stimulation with NO and thereafter declined to insignificant levels within hours. The sequential increase in cGMP and 8-nitro-cGMP levels, with a prolonged linear increase in 8-nitro-cGMP, may support our contention that 8-nitro-cGMP derives from cGMP, which is initially produced by sGC activated by NO.

Although in vivo mechanisms of 8-nitro-cGMP formation are not yet clear,
NO-dependent guanine nitration mediated through RNOS must be involved in 8-nitro-cGMP formation within cells [21]. The difference in time profiles of cGMP and 8-nitro-cGMP formation may be explained by the chemical nature of 8-nitro-cGMP, in as much as this compound resists degradation by phosphodiesterases [21]. It is also possible that guanine nitration may be taking place primarily on the guanine moieties of other nucleotides than cGMP (e.g., GMP, GDP, and GTP). This may be one of the feasible models for nitrated nucleotide-mediated electrophilic signaling occurring in vivo. In any event, an important conclusion here is that 8-nitro-cGMP and cGMP may have distinct roles in NO signal transduction.

In this chapter, I found that 8-nitro-cGMP regulate the calcium-dependent glutamate release as a novel mediator, which functions in downstream of NO. However, the molecular mechanism of 8-nitro-cGMP induced enhancement of glutamate release has been obscure. It was reported that NO modulates neurotransmitter release through S-nitrosylation of rat syntaxin 1 at Cys 145 and rat NSF at Cys264 [75, 81]. Additionally, it was reported that modification of SNAP25 and SNAP23 regulates exocytosis through palmitoylation of it’s cysteine residue [82, 83]. In this way, the modification of cysteine residues of exocytosis-related proteins is very important to regulate neurotransmitter release. 8-nitro-cGMP readily reacts with
sulfhydryl group of cysteine residues and formed a protein-S-cGMP adduct, named protein S-guanylation [21]. It was considered that the enhancement of calcium-dependent glutamate release from astrocytes mediated 8-nitro-cGMP may be caused by the modification of these exocytosis related proteins through S-guanylation by 8-nitro-cGMP. Furthermore, it was reported that hydrogen peroxide and superoxide regulate intracellular calcium concentration and NSF activity [84, 85]. Sawa et al. identified 8-nitro-cGMP generates superoxide anions via the activation of NADPH reductase-like enzymes, including 3 NOS isoforms and P450 reductase [21]. It was also considered that the enhancement of calcium-dependent glutamate release from C6 cells mediated 8-nitro-cGMP may be caused by the generation of superoxide mediated with 8-nitro-cGMP. Additional studies are required to investigate the regulatory mechanism of the glutamate release from astrocytes by 8-nitro-cGMP.

The present study showed that 8-nitro-cGMP was produced in a NO-dependent manner in astrocytes. The production of 8-nitro-cGMP was mediated through the activation of sGC in a NO and time-dependent manner. Furthermore, my results indicated that NO-induced enhancement of calcium-dependent glutamate release was regulated by 8-nitro-cGMP, but not by cGMP. Thus, the enhanced calcium-dependent glutamate release from astrocytes treated with 8-nitro-cGMP can explain the
discrepancies between the mechanism of NO-enhanced calcium-dependent glutamate release and the classical NO-cGMP pathway alone. As shown in chapter 2, my results suggest that NO-induced enhancement of calcium-dependent glutamate release is regulated by the 2 different processes; one of these is an immediate potentiation mechanisms of NO-induced glutamate release, and the other one is a delayed potentiation mechanism mediated by 8-nitro-cGMP. Thus, 8-nitro-cGMP may extend the duration of the effects of NO or the NO-cGMP pathway.
In this study, the role of cytokines in CNS as a regulatory mediator of gliotransmitter release from astrocytes was examined. In chapter 2, I investigated the effects of cytokines on calcium-dependent glutamate release from astrocytes. To date there are no studies regarding the effects of cytokines on gliotransmitter release from astrocytes. To examine whether cytokines affect on gliotransmitter release from astrocytes, I prepared rat cerebral cortex astrocytes. This astrocytes culture included more than 96% of the GFAP positive astrocytes and did not contain neuron (data not shown). Glutamate release from astrocytes was evoked by stimulus of calcium ionophore A23182 in calcium-dependent manner (Fig. 2-1). Cytokines mixture enhanced the calcium-dependent glutamate release from astrocytes (Fig. 2-2). Cytokines are also known as inducers of NO production. Therefore, I hypothesized that a relationship existed between NO production and the enhancement of glutamate release by cytokines. In order to confirm this hypothesis, I first examined the induction of iNOS expression and NO production by a cytokine mixture. Astrocytes treated with cytokine mixture expressed iNOS and produced a lot of NO. This NO
production was inhibited by iNOS inhibitor (Fig. 2-3). Next, I examined the contribution of NO to the enhancement of glutamate release from astrocytes treated with cytokines. The iNOS inhibitor attenuated the enhancement of glutamate release. Furthermore, pretreatment of the cells with the exogenous addition of an NO donor enhanced the glutamate release from astrocytes even in the absence of cytokine (Fig. 2-4). Cytokine also enhanced calcium-dependent glutamate release from C6 cells through the NO production as well as astrocytes. Finally, to investigate whether NO-cGMP pathway affects on this NO-enhanced glutamate release, I pretreated C6 cells with various NO-cGMP pathway related reagents for 0.5 and 18 h, including sGC and PKG inhibitor and cGMP analog. The PKG inhibitor did not attenuated the enhancement of the glutamate release from C6 cells treated with NO donor. Furthermore, cGMP analog did not enhance the glutamate release. On the other hand, sGC inhibitor attenuated NO enhanced calcium-dependent glutamate release from C6 cells treated with NO donor for 18 h, but not for 0.5 h (Fig. 2-5). These results could not explain with NO-cGMP pathway by itself. In this way, my results suggested that a novel NO signal transduction mechanism which is different from classical NO-cGMP pathway may be involved in NO-enhanced glutamate release from astrocytes.

In chapter 3, the formation of 8-nitro-cGMP in glia cells and the effects of
8-nitro-cGMP on calcium dependent glutamate release from C6 cells were reported. This study is the world’s first report described quantitative determination of 8-nitro-cGMP in CNS cells. Immunostaining for 8-nitro-cGMP was markedly increased in C6 cells after treatment with the NO donor SNAP in time and NO concentration-dependent manner (Fig. 3-1). Similarly, 8-nitro-cGMP levels markedly increased in C6 cells expressing increased amounts of iNOS after stimulation with LPS plus a mixture of the cytokines IFN-γ, TNFα, and IL-1β. L-NMMA, an inhibitor of NO synthases, and NS 2028, an inhibitor of sGC activity, almost completely nullified this elevated 8-nitro-cGMP immunoreactivity (Fig. 3-1). 8-nitro-cGMP was produced in astrocytes in a NO dependent manner as well as C6 cells (Fig. 3-2). These results indicate that 8-nitro-cGMP is formed in a NO and time-dependent manner, which is mediated by sGC activation. Next, I quantified 8-nitro-cGMP and cGMP in C6 cell extracts obtained at various time points after NO donor treatment and after treatment with LPS plus cytokines by utilizing LC-ESI-MS/MS. Compared with cGMP which showed a rapid and transient elevation and a peak level within a few hours after SNAP treatment, 8-nitro-cGMP became detectable during the peak and decline in the cGMP level and increased linearly for another several hours, even after cGMP turned down to a plateau. The amount of 8-nitro-cGMP eventually reached a value that was one tenth
of the peak value of cGMP (Fig. 3-3 and Fig. 3-5). Finally, I investigated whether 8-nitro-cGMP affects on calcium-dependent glutamate release from astrocytes. 8-nitro-cGMP enhanced calcium-dependent glutamate release from C6 cells. Furthermore, 8-nitro-cGMP counteracted the depression effect of NO-enhanced calcium-dependent glutamate release by sGC inhibitor. These results suggest that 8-nitro-cGMP produced by NO acts as a novel gliotransmitter release-regulatory modulator. These results indicated calcium-dependent glutamate release from astrocytes is regulated by self-effect of NO which perform immediately and effect of 8-nitro-cGMP which is produced through the sGC activation in a time-dependent manner. This study predicted that cytokines may affect the tripartite synapse and strength of the tripartite synaptic connection under pathological and physiological conditions by modulating glutamate release from astrocytes through the production of NO and 8-nitro-cGMP.
Fig. 4. Regulatory mechanism by cytokines via NO and 8-nitro-cGMP signal pathway of calcium-dependent glutamate release from astrocytes.

Cytokines enhance calcium-dependent glutamate release from astrocytes by NO and 8-nitroc-GMP through the iNOS expression and sGC activation. Cytokines may affect the tripartite synapse and strength of the tripartite synaptic connection under pathological and physiological conditions by modulating glutamate release from astrocytes through the production of NO and 8-nitro-cGMP.
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