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Effect of Phosphodiesterase Inhibitors on Nitric Oxide Production by Glial Cells

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YOSHIKAWA, M., SUZUMURA, A., ITO, A., TAMARU, T. and TAKAYANAGI, T. *Effect of Phosphodiesterase Inhibitors on Nitric Oxide Production by Glial Cells.* Tohoku J. Exp. Med., 2002, **196** (3), 167-177 —— Nitric oxide (NO) is considered to play a crucial role in the development of various pathological processes in the CNS, such as neuronal degeneration, inflammation and demyelination. In order to search for the agents which suppress NO production in the CNS, we examined the effects of one of the agents which elevate cyclic AMP production, phosphodiesterase inhibitors (PDEIs), on NO production by glial cells in vitro. All the types of PDEIs, from type I- to V-specific and non-specific, suppressed the production of NO by mouse microglia and astrocytes stimulated with lipopolysaccharide, in a dose-dependent manner. Suppression of inducible NO synthase by PDEIs was confirmed by the expression of mRNA by RT-PCR. Although it required 10 μM or higher concentration to effectively suppress NO production in vitro, certain combinations of three different PDEIs synergistically suppressed NO production by astrocytes at 1 μM which could be obtained in vivo at usual therapeutic doses. Similary, combinations of three PDEIs at 1 μM synergistically increased intracellular cAMP in astrocytes. The suppressive effects of PDEIs on NO production were abolished by addition of tumor necrosis factor α (TNFα). Thus, the main suppression mechanism of NO might be indirect through suppression of TNFα. Since some PDEIs are reported to pass through the blood-brain-barrier, the combination of three PDEIs may be worth trying in neurological diseases, such as multiple sclerosis, human immunodeficiency virus-related neurological diseases and other neurodegenerative disorders in which NO may play a crucial role. ——— nitric oxide; astrocytemicroglia; tumor necrosis factor α; phosphodiesterase inhibitor

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Nitric oxide (NO) produced by glial cells has been implicated to play a role in the pathogenesis of various CNS diseases such as multiple sclerosis (MS) (Sherman et al. 1992), acquired immunodeficiency syndrome (AIDS)-related neurological disorders (Dickman et al. 1994), Parkinson disease (Youdin et al. 1994) and Altzheimer's disease (Rogers et al. 1988). Microglia stimulated in vitro with lipopolysaccharide (LPS) and interferon-γ (IFNγ) secrete NO. Activated microglia was reported to kill oligodendrocytes through production of NO, suggesting a possible role of NO in the development of demyelinating lesions in MS (Merrill et al. 1993). Astrocytes also express inducible NO synthase (iNOS) mRNA and produce NO when stimulated in vitro or in mice with experimental allergic encephalomyelitis (EAE) (Tran et al. 1997). In MS brain, the levels of iNOS mRNA were markedly elevated in reactive astrocytes in demyelinating lesions, suggesting the possible role of astrocyte-derived NO in the pathogenesis of demyelination (Bö et al. 1994). Therefore, suppression of NO production with glial cells may be an useful strategy in the future treatment for inflammatory and demyelinating diseases in the CNS, such as MS. It has been shown that inhibitors of iNOS inhibited induction of NO production by a murine macrophage cell line (Cross et al. 1994), and treatment with inhibitors of NO suppressed development of clinical signs of EAE (Ruuls et al. 1996).

Tumor necrosis factor α (TNFα), an inflammatory cytokine, also play a critical role in the development of various inflammatory diseases or neurodegenerative diseases in the CNS, either directly or indirectly via induction of NO. Recent studies have shown that phosphodiesterase inhibitors (PDEIs) suppressed TNFα production by mouse macrophages via elevation of intracellular cyclic 3′, 5′-adenosine monophosphate (cAMP) concentration (Renz et al. 1988; Strieter et al. 1988). PDEIs also successfully suppressed TNFα production with a variety of cells (Nataf et al. 1993; Sommer et al. 1995).

There are several types of PDEIs, nonspecific and at least five types of isotype-specific PDEIs (Asano et al. 1977; Hidaka et al. 1979). We have shown recently that all the types of PDEIs suppressed the production of TNFα by murine microglia and astrocytes. We also found that certain combinations of three different types of PDEIs at very low concentration synergistically suppressed production of TNFα and effectively suppressed the development of EAE (Tamaru and Suzumura 1998; Yoshikawa et al. 1999), while treatment with each single PDEI only slightly delayed the onset of EAE. Since TNFα is a potent inducer of NO, suppression of TNFα may result in suppression of NO. However, the effect of PDEIs on NO production by glial cells has not been clarified.

In the present study, we examined whether PDEIs suppressed NO production by microglia and astrocytes, and whether the combination of three different types of PDEIs at low doses synergistically suppressed NO production by these cells. We also examined whether suppression of NO by PDEIs was mediated by the suppression of inflammatory cytokines, such as TNFα, interleukin (IL)-1 or IL-6.

MATERIALS AND METHODS

Reagents

Recombinant murine IL-1β, TNFα, human IL-6 and polyclonal anti-TNFα antibody were obtained from Genzyme (Boston, MA, USA). The source of each type of PDEI used in this study and the maximum serum concentrations

Abbreviations: NO, nitric oxide; PDEIs, phosphodiesterase inhibitors; LPS, lipopolysaccharide; BBB, blood brain barrier; MS, multiple sclerosis; AIDS, acquired immunodeficiency syndrome; IFN γ, interferon γ; TNFα, tumor necrosis factor α; iNOS, inducible NO synthase; EAE, experimental allergic encephalomyelitis; IL, interleukin.

TABLE 1. *Souce and concentration of phosphodiesterase inhibitors*

Type	Name	Source	Max. serum conc.	
Type I	Vinpocetine	Takeda Chemical Industries	2.33 μM	15 mg/day p.o.
Type II	None			
Type III	Pentoxifylline	HOECHST MARION ROUSSEL AG	1.8 μM	40 mg/kg p.o.
	Cilostazol	Otsuka Pharmaceutical	2.1 μM	100 mg p.o.
	Ibudilast	Kyorin Pharmaceutical	0.3 μM	10 mg p.o.
Type IV	Rolipram	Meiji Seika Kaisha	NA	NA
Type V	Dipyridamole	Yamanouchi Pharmaceutical	1.2 μM	25 mg p.o.
Non-specific	Papaverine HCl	Dainippon Pharmaceutical	5.3 μM	80 mg i.v.
	Theophylline	Eisai	47.9 μM	400 mg p.o.

NA, not available.

in the clinical trials are summarized in Table 1. LPS was obtained from Difco (Detroit, MI, USA). The ELISA kit for assessment of cytoplasmic cAMP was purchased from amersham pharmacia biotech (Buckinghamshire, UK).

Cell culture

Primary mixed glial cell cultures were prepared from newborn C57 BL/ 6 mice (SLC, Shizuoka) as described previously (Suzumura et al. 1984). In brief, after the meninges were carefully removed, the brain was dissociated by passing it through a 320-mm-pore nylon mesh. The cell suspension was washed twice with Hanks' balanced salt solution, triturated and placed in 75-cm^2 culture flasks (Falcon 3024, Beckton-Dickinson, Lincoln Park, NJ, USA) at a density equivalent of two brains per flask in 10 ml Eagle's minimum essential medium supplemented with 10% fetal calf serum, 5 mg/ml bovine insulin, and 0.2% glucose. Microglia were isolated on the 14th day with the "shaking off" method as previously described (Suzumura et al. 1987). The purity of the culture was 97-100% as determined by non-specific Fc-receptor staining. Astrocyte-enriched cultures were prepared from the primary mixed glial cell cultures by repetitive exposure to trypsin and replating (Suzumura et al. 1988). The purity of the cultures exceeded 95% was determined by indirect immunofluorescence staining with antibodies to glial fibrillary acidic protein (Suzumura et al. 1988).

Measurement of nitrite

Nitrite (NO_2^-) contents in the supernatants from purified microglia and astrocyte cultures after addition of stimuli and PDEIs were assayed by the Griess method. Measurement of nitrite by Griess reagent has been shown to reflect NO generation (Green et al. 1982). A standard curve was established using nitrite in a range between 1 and 100 μM. Griess reagent consists of equal volumes of 0.1% naphthylethylene diamine dihydrochloride in distilled water and a mixture of 1% sulfanilamide plus 5% H_3PO_4. An equal volume of samples, or standard, and Griess reagent was mixed and incubated for 10 minutes at room temperature, and the optical density of the reaction mixture was measured in a microplate reader at 550 nm, against culture medium as blanks.

Effect of PDEIs on nitrite production by microglia and astrocyte

Microglia and astrocyte-enriched cultures were plated in 24 well flat- bottom microwell plates (Falcon 3001 Beckton Dickinson, Lincoln Park, NJ, USA) at a density of 5×10^5/ml. They were cultured for 72 hours with or without LPS (10 μg/ml in astrocyte culture and 1 μg/

ml in microglia culture) in the presence of graded concentrations of PDEIs (1 to 100 μM). Microglia were treated with vinpocetine, milrinone, ibudilast, dipyridamole, papaverine and theophylline. Astrocytes were treated with vinpocetine, cilostazole, pentoxifylline, rolipram, ibudilast, dipyridamole, papaverine and theophylline (see Table 1). The culture supernatants were then collected and assayed for their nitrite contents.

Effect of combinations of different three PDEIs at 1 mM

Astrocytes were stimulated for 72 hours with 10 μg/ml LPS in the presense of various combinations of three different PDEIs at 1 μM. Then, the nitrite contents of the supernatants were assayed as above. PDEIs used in combination treatment included vinpocetine (type I-specific), pentoxifylline, cilostazole (type III-specific), ibudilast (type I, III-specific) and theophylline (non-specific)

Effect of other inflammatory cytokines

TNFα (40-4000 U/ml), IL-1 (0.2-20 U/ml), IL-6 (200 U/ml) and anti-TNFα antibody ($1:10^2$) were added in astrocyte culture treated with combination of vinpocetine, ibudilast, and cilostazole at 1 μM in the presence of LPS and incubated for 72 hours.

Measurement of intracellular cAMP

Astrocyte-enriched cultures (100 μl volumes) were plated in a 96 well microtiter plates at the concentrations of 1×10^5 cells/ml. They were cultured at 37°C for three days with each PDEI at 1 μM or combinations of three PDEIs (vinpocetine, ibudilast and theophylline) at 1 μM in the presence of 10 μg/ml LPS. Then, cells were lysed by addition of 0.25% dodecyltrimethylammonium bromide solution in assay buffer (0.05 M acetate buffer pH 5.8 containing 0.02% bovine serum albumin and 0.01% preservative). The intracellular cAMP levels of lysed cells were measured with a mouse cAMP ELISA kit.

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Astrocyte-enriched cultures at a concentration of 1×10^5 cells/ml were stimulated for 24 hours with 10 μg/ml LPS in the presence or absence of combinations of three PDEIs at 1 μM. In some experiments, TNFα (4000 U/ml) was added to the culture stimulated above.

Total cellular RNA was then extracted by the guanidium thiocyanate method (Chomczynski and Hu 1994). PDEIs used in combination treatment included vinpocetine (type I-specific), cilostazole (type III-specific), ibudilast (type I, III-specific) and theophylline (non-specific). The amount of RNA was determined spectrophotometrically. After 1 μg of total RNA was denatured for 5 minutes at 70°C, the RT reaction was performed by incubating at 37°C for 60 minutes in 20 μl reaction solution containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs, 40 ng random primer *p* (dN) 6, 6 U ribonuclease inhibitor, and 40 U M-MLV Reverse transcriptase (Gibco BRL, Gland Island, NY, USA), followed by heating at 70°C for 10 minutes. The reaction mixtures were diluted five-fold with double-distilled water and used as the cDNA solution. cDNA samples were stored at 4°C until polymerase chain reaction (PCR) analysis. Specific primers for mouse iNOS, TNFα and β actin were as follows.

iNOs: sense, 5′-CCCTTCCGAAGTTTCTGGCAGCAGC,

iNOs: antisense, 5′-GGCTGTCAGAGCCTCGTGGCTTTGG,

TNFα: sense, 5′-ATGAGCACAGAAAGCATGATCCGC,

TNFα: antisense, 5′-CCAAAGTAGACCTGCCCGGACTC,

β actin: sense, 5′-GTGGGCCGCTCTAGGCACCAA,

β actin: antisense, 5′-CTCTTTGATGTCACGGACGATTTC.

The PCR reaction mixture contained 1× PCR buffer, 0.4 μM dNTPs, 0.4 μM sense and antisense primers, respectively, 0.005 U Ampli Tag DNA polymerase (Perkin Elmer, Norwalk, CT, USA), and 5 μl cDNA solution in a 10 ml volume. Five microtiter of the PCR product was analyzed on 1.5% agarose gel in the presence of ethidium bromide.

Statistical analysis

All the experiments were performed at least triplicates in a experiment. Data were presented as means±standard error of the mean (S.E.M.). Statistical analysis of the data was performed by one-way analysis of variance (ANOVA). When ANOVA indicated differences among the group, the Bonferroni correction for multiple comparisons was applied. Analysis were performed using the statistical software package StatView 5.0 (SAS Institute Inc, Cary, NC, USA).

RESULTS

Unstimulated microglia did not produce a detectable amount of nitrite in culture supernatants as measured by the Griess method. When stimulated with LPS for 72 hours, production of nitrite was detected in the culture supernatants, 15.6±4.7 μM nitrite. LPS-induced nitrite production by microglia was suppressed by all the PDEIs, from type I to V and nonspecific PDEIs, in a dose dependent manner (Fig. 1). All the PDEIs significantly suppressed LPS-induced nitrite production at concentrations higher than 10 μM. However, those at 1 μM did not significantly suppressed nitrite production by microglia. Similar results were obtained in astrocytes. Although, unstimulated astrocyte did not produce a detectable amount of nitrite in culture supernatant, stimulated astrocyte with 10 μg/ml LPS produced 19.8±4.9 μM nitrite. All the

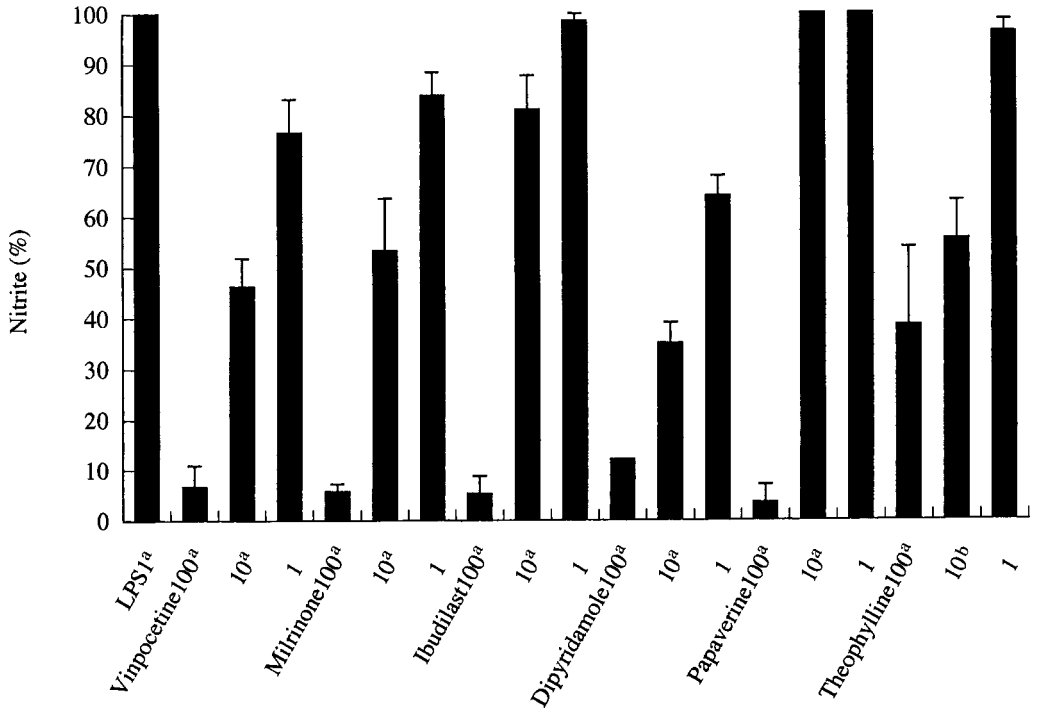

Fig. 1. The effects of PDEIs on nitrite production by microglia.
Each column represents the % of 1 mg/ml LPS-stimulated values. Results are the mean±S.E.M. of three experiment. LPS-stimulated concentrations of nitrite was 15.6±4.6 μM.
[a] $p<0.01$. [b] $p<0.1$.

PDEIs at higher than 10 μM significantly suppressed nitrite production by astrocytes, while those at 1 μM did not significantly suppress nitrite production except for rolipram (Fig. 2).

Certain combinations of three different PDEIs at 1 μM synergistically suppressed nitrite production by astrocytes (Fig. 3) as effective as 100 μM of each PDEI. The combination including ibudilast and pentoxifylline, or ibudilast and cilostazole suppressed LPS induced nitrite production by 70-80%. The combination of ibudilast, dipyridamole and theophylline also gave favorable results while other combinations suppressed production by 20-40%. Intracellular cAMP levels in astrocyte culture remained unchanged after incubation with LPS alone (4.6 ± 2.0 pmol/10^6 astrocytes). Addition of 1μM of vinpocetine, ibudilast or theophylline increased cAMP concentration from 1.2 to 1.6 fold. When treated with the combination of these three PDEIs at 1 μM, intracellular cAMP increased by about 2 fold (Fig. 4), suggesting that the combination of different types of PDEIs functions additively to elevate cAMP.

Addition of recombinant TNFα in astrocyte culture treated with the combination of vinpocetine, ibudilast and cilostazole at 1 μM significantly diminished suppressive effects of PDEIs on LPS-induced nitrite production by astrocytes in a dose-dependent manner (Fig. 5), indicating that the suppression of nitrite production by astrocytes treated with PDEIs was mediated by suppression of TNFα production by these cells. Addition of IL-1, IL-6 and anti-TNFα antibody had no significant effect on suppression of nitrite production by PDEIs.

The suppression of LPS-induced iNOS expression was confirmed at a mRNA level examined by RT-PCR. Treatment with the combinations of vinpocetine, ibudilast and cilostazole at 1 μM most significantly down

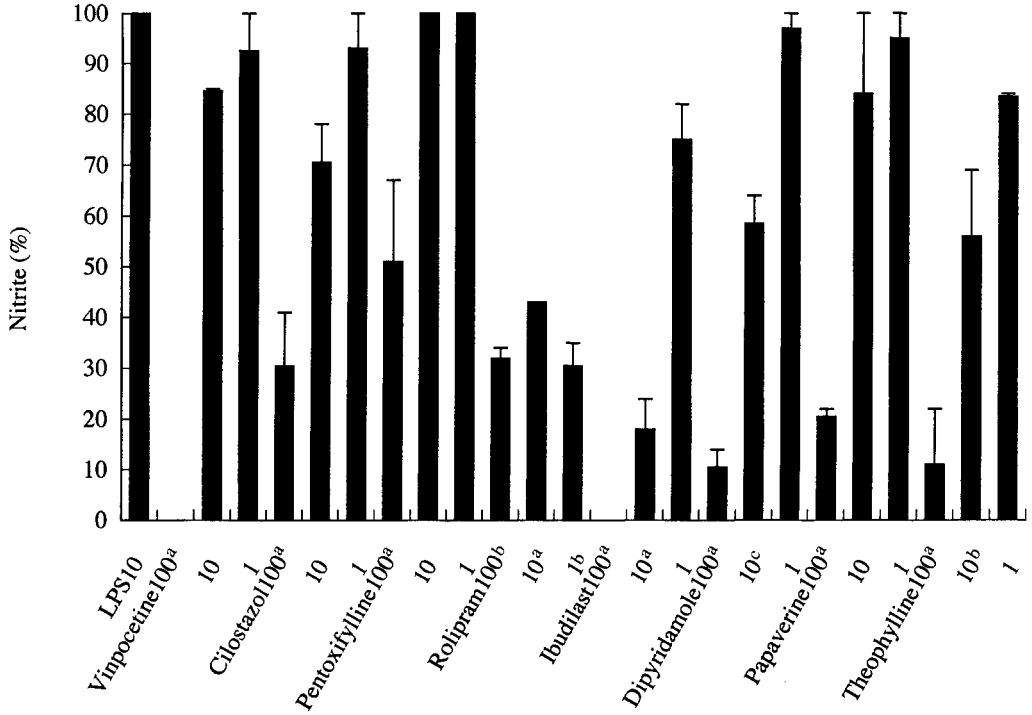

Fig. 2. The effects of PDEIs on nitrite production by astrocyte.
Each column represents % of 10 μg/ml LPS-stimulated values. Results are the mean$\pm$S.E.M. of three experiment. LPS-stimulated concentrations of nitrite was 19.8 ± 5.0 μM.
[a]$p<0.01$. [b]$p<0.1$. [c]$p<0.5$.

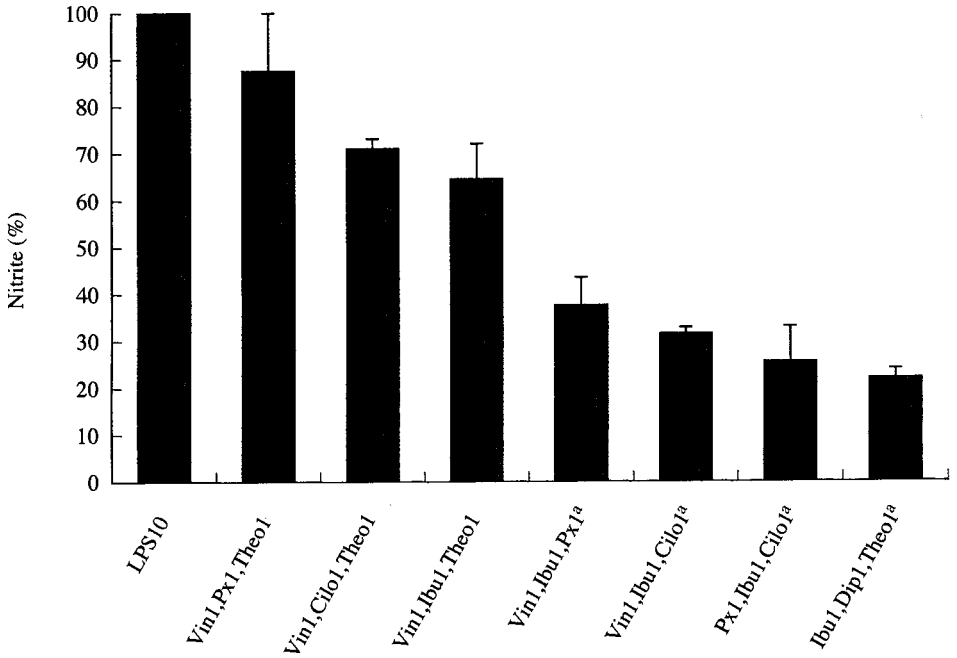

Fig. 3. The effects of the combination of three different PDEIs at 1 μM on nitrite production by astrocytes. Each column represents % of 10 μg/ml LPS-stimulated values. PDEIs used are type I-specific vinpocetine (Vin), type III-specific pentoxifylline (Px) and cilostazole (Cilo), type I and III-specific ibudilast (Ibu), type V-specific dipyridamole (Dip) and non-specific theophylline (Theo). Results are the mean$\pm$S.E.M. of three experiment.
[a] $p<0.01$.

regulated the expression of mRNA encoding TNFα and iNOS (Fig. 6, lane 4). Addition of recombinant TNFα abolished the suppressive effects of these PDEIs (Fig. 6, lane 7). Other combinations of the three PDEIs slightly down regulated the expression of TNFα and iNOS mRNA expression which was also slightly restored with addition of TNFα (Fig. 6).

DISCUSSION

In the present study, we have shown that all the types of PDEIs suppressed the nitrite production by mouse microglia and astrocytes stimulated with LPS in a dose-dependent manner. Although most PDEIs did not significantly suppress at 1 μM, certain combinations of three PDEIs at 1 μM synergistically suppressed nitrite production by these cells as effectively as 100 μM for each PDEI. The combination of three PDEIs also effectively suppressed iNOS mRNA in astrocytes when examined by RT-PCR.

There are several conflicting reports about the effect of PDEIs on NO production. Rolipram, type IV PDEI, was reported to augment spontaneous and LPS/IFNγ-induced NO production by macrophages (Jung et al. 1996). Alonzo et al. (1995) also showed induction of NO in rat peritoneal macrophage with high concentrations of rolipram (300 μM) and cAMP analogue, though other cAMP elevating agents such as IBMX and forskolin had no such effects. In contrast, Oda et al. (1997) showed that endothelin which increase intracellular-cAMP, dibutyryl cAMP and forskolin suppressed iNOS expression in rat glial cells stimulated with TNFα and IL-1β. Németh et al. (1997) also reported that amrinone, type III specific PDEI, inhibited NO production at the range of 10–100 μM in primary rat peritoneal macrophages. Another study reported that 50 μM of forskolin slightly inhibited NO release by IFN-γ-

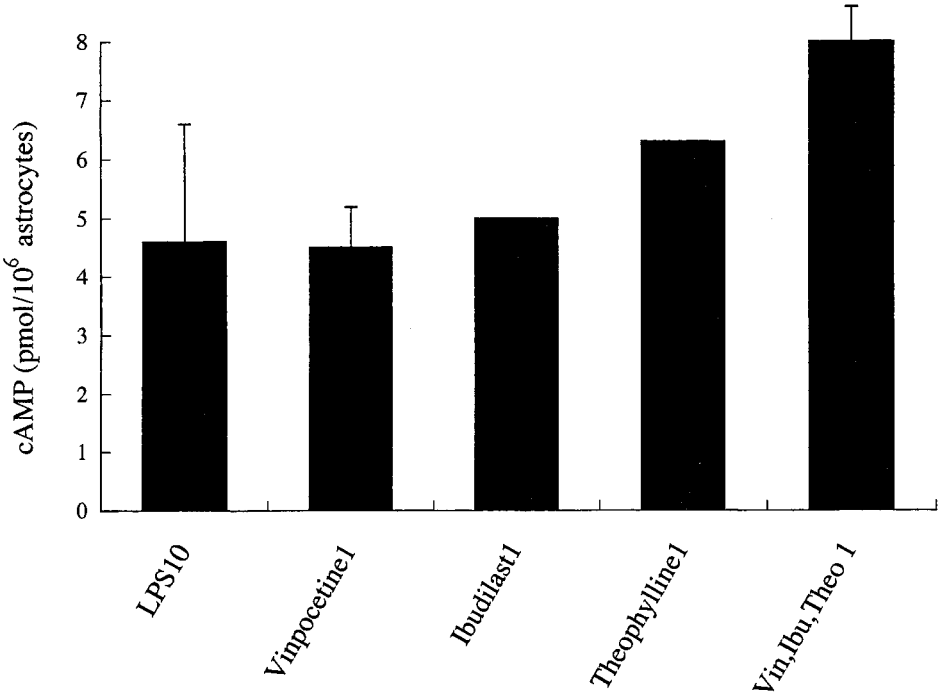

Fig. 4. Increase in intracellular cAMP in astrocytes stimulated with 10 μg/ml LPS and treated with PDEIs. PDEIs used are 1 μM of vinpocetine, ibudilast, theophylline and combination of those three PDEIs. Each column represents cAMP concentration in 1×10^5 cells. Results are the mean$\pm$S.E.M. of two experiment. LPS-stimulated concentration of cAMP was 4.6 ± 2.0 pmol/10^6 astrocytes

stimulated astrocytes, while it augmented NO release by microglia stimulated by either IFN-γ or IFN-γ+ TNFα (Hellendall and Ting 1997). Our data showed cAMP elevating agent, PDEIs suppressed iNOs gene expression. This suggested that cAMP decreased the iNOs gene transcription. One of the transcription factor during the induction of iNOs by LPS in macrophage is NF-$\varkappa$B. Forskolin, cAMP elevating agent, inhibited the LPS-stimulated nuclear translocation of NF-$\varkappa$B and decreased transcription of iNOs gene and decreased iNOs mRNA (Shamimunisa et al. 1998). Our study do not investigates the mechanism of cAMP to the iNOs, though their study would support our results. The reason for the conflicting results about the effect of PDEIs on NO production is possible that glial cells have different sensitivity from macrophage to PDEIs. It is also possible that NO production vary upon different stimuli. Alternatively, since elevation of intracellular cAMP by PDEIs is transient (Knudsen et al. 1986), differences may come from different time of assays. We have examined the time course of LPS-induced nirite production (data not shown) and assays have been conducted under optimal conditions at 72 hours.

Furthermore, since the addition of TNFα, but not IL-1, IL-6 or anti-TNFα antibody, diminished the suppressive effects of PDEIs on nitrite production in a dose-dependent manner, another suppression mechanism might be indirect through suppression of TNFα. Although both IL-1 and IL-6 were NO inducing agents, TNFα may be the most potent inducer of NO production by glial cells, as in other cell types. Since we have shown that all the PDEIs used in this study suppressed TNFα production by glial cells (Yoshikawa et al. 1999), PDEIs may exert potent anti-inflammatory effects by down regulating both

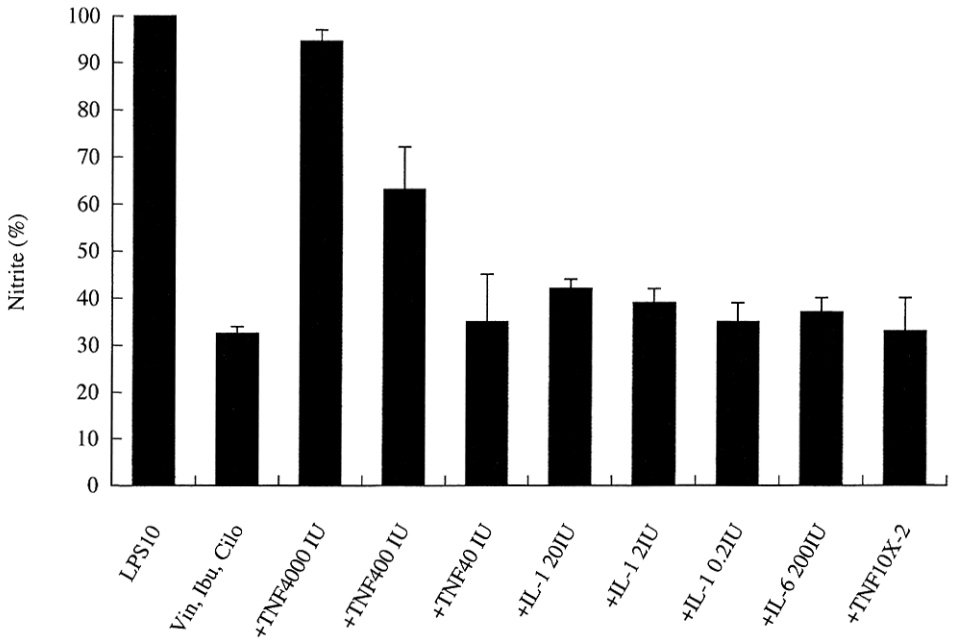

Fig. 5. Effect of cytokines on nitrite production by astrocytes treated with combination of vinpocetine, ibudilast and cilostazole at 1 μM. Graded concentration of TNFα, IL-1, IL-6 and TNFα antibody were added. Each column represents % of 10 μg/ml LPS-stimulated values.

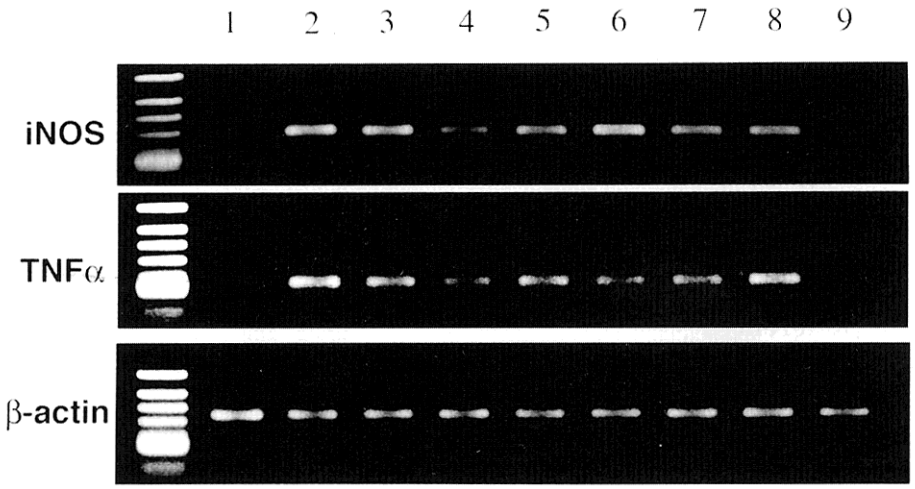

Fig. 6. RT-PCR analyses for the expression of iNOs, TNFα mRNA and β-actin in astrocyte unstimulated (1), treated with 10 μg/ml LPS (2), +Vin, Ibu, and Theo at 1 μM (3), +Vin, Ibu and Cilo at 1 μM (4), +Ibu, Dip and Theo at 1 μM (5), +Vin, Ibu, and Theo at 1 μM+4000 IU of TNFα (6), +Vin, Ibu and Cilo at 1 μM+4000 IU of TNFα (7), +Ibu, Dip and Theo at 1 μM+4000 IU of TNFα (8), 100 μM of cAMP.

production of TNFα and NO release. Similarly, combination of PDEIs at 1 μM synergistically increased intracellular cAMP in astrocytes. This result supports the mechanism of suppressive effects of PDEIs on NO production is mediated by increasing intracellular cAMP.

All the PDEIs used in this study are now widely and safely used in Japan to treat asthma or strokes. The serum concentrations of most PDEIs in usual therapeutic doses are less than

10 μM (Table 1). Therefore, a single PDEI would not suppress nitrite production in vivo. However, as we have shown in this study, a combination of three PDEIs at 1 μM synergistically suppressed nitrite production as potent as 100 μM of each PDEI. It is possible that higher concentrations of PDEI would be necessary to effectively suppress NO production in the CNS. However, several PDEIs have been shown to pass through blood-brain-barrier (BBB), and it has been also shown that the chemicals can enter the brain rather smoothly though the damaged BBB in the case of inflammation or other pathological conditions.

Taking together, usual therapeutic dose of PDEIs, when used in combination, may suppress TNFα and NO production by glial cells in vivo. It is possible that the combination therapy is a useful strategy for future treatment of intractable neurological diseases in which NO may play a causative role.

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