Effect of Enhanced Prostacyclin Synthesis by Adenovirus-Mediated Transfer on Lipopolysaccharide Stimulation in Neuron-Glia Cultures

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ABSTRACT: Prostacyclin (PGI2) is known as a short-lived, potent vasodilator and platelet anti-aggregatory eicosanoid. This work attempts to selectively augment PGI2 synthesis in neuron-glia cultures by adenoviral (Ad) gene transfer of PGI synthase (PGIS) or bicistronic cyclooxygenase 1 (COX-1)/PGIS and examines whether PGI2 confers protection against lipopolysaccharide (LPS) stimulation. Cultures released low levels of eicosanoids. Upon Ad-PGIS or Ad-COX-1/PGIS infection, cultures selectively increased prostacyclin release. Both PGIS- and COX-1/PGIS–overexpressed cultures contained fewer microglial numbers. Further, they significantly attenuated LPS-induced iNOS expression and lactate, nitric oxide, and TNF-α production. Taken together, enhanced prostacyclin synthesis in neuron-glial cultures reduced microglia number and suppressed LPS stimulation.

KEYWORDS: prostaglandin; inflammation; neuroprotection; gene transfer; cyclooxygenase; prostacyclin synthase

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INTRODUCTION

Inflammation is an important contributor to neuronal damage in neurodegenerative diseases. Microglia, the resident immune cells in the brain, play a pivotal role in inflammatory reaction. Several lines of evidence have shown that lipopolysaccharide (LPS) treatment elicits neurotoxicity through microglial activation.\(^1-3\) Activated microglia produce a wide array of factors, including cytokine, nitric oxide, and eicosanoids.\(^4,5\) The accumulated impact of these factors on neurons can lead to neuronal death.\(^3\)

Prostacyclin (prostaglandin I\(_2\); PGI\(_2\)) is synthesized by the sequential action of cyclooxygenase (COX) 1 or 2 and prostacyclin synthase (PGIS). It is rapidly produced following tissue injury or inflammation\(^6\) and is a paracrine mediator with two main effects: vasodilation and inhibition of platelet aggregation.\(^7\) PGI\(_2\) is cytoprotective at the blood–brain barrier and in cerebral circulation.\(^8,9\) Because of its instability in vivo, several PGI\(_2\) analogs have been developed and demonstrated to reduce ischemic brain damage.\(^8,10,11\) In contrast, PGE\(_2\) and PGD\(_2\) have been reported as functional important products of COX in the brain, involving induction of astrogliosis.\(^12-14\) PGE\(_2\) is the most abundant prostaglandin in the brain. Despite its classic role as a proinflammatory molecule, much in vivo and in vitro evidence has demonstrated the anti-inflammatory effects of PGE\(_2\).\(^15\)

We constructed recombinant adenovirus (Ad) vectors to encode for green fluorescence protein (GFP), COX-1, COX-2, PGIS, and bicistronic COX-1/PGIS, allowing for stable delivery of these genes to infected cells. By the combined infection of Ad-COX-1 or -2 and Ad-PGIS, we selectively enhanced PGI\(_2\) production in endothelial cells.\(^16,17\) This work attempts to selectively augment PGI\(_2\) synthesis in primary neuron-glia culture by direct adenoviral gene transfer of PGIS or bicistronic COX-1/PGIS and examines whether it confers protection against damage induced by LPS. LPS, a cell wall component of gram-negative bacteria, is a powerful immune challenge associated with an increase in the circulatory levels of numerous cytokines.

MATERIALS AND METHODS

Reagents

LPS (E. coli 0111:B4) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich Co. \(^{[1-14C]}\) arachidonic acid and radioisotopic prostanoids were purchased from Amersham Biosciences (Buckinghamshire, UK). Acetonitrile and other organic solvents were obtained from Merck (Darmstadt, Germany).

Recombinant Adenovirus Production

Replication-defective adenoviruses containing phosphoglycerate kinase (PGK) (containing only the promoter but not the encoding genes), GFP (expressing the green fluorescence protein of jelly fish), COX-1, PGIS, and bicistronic COX-1/PGIS were produced as previously described.\(^16\) Ad-GFP, Ad-PGIS, and Ad-COX-1/PGIS used PGK as a driving promoter. The viral titers of the purified Ads were determined by a plaque-forming assay.
Neuron-Glial Mixed Culture

Mixed neuron-glia cultures were prepared from cerebrocortical regions of embryonic Sprague-Dawley rat fetus at gestation day 15, as described by Hung et al.18 Briefly, cells were dissociated with mixtures of papain/protease/deoxyribonuclease I (0.1%:0.1%:0.03%) and plated onto polylysine-coated multiwell plates. Cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented either with 10% fetal bovine serum (FBS) as serum-containing medium or with N2 (Gibco BRL, Grand Island, NY) as serum-free medium. During LPS challenge, the culture medium was changed to DMEM supplemented with 2% FBS and 2% horse serum.

Tranaducing Cells with Ads

Cells were fed with maintaining medium. Ad was added to each well with a multiplicity of infection (MOI, pfu/cell) of 50. Ad-GFP was used for optimizing the infection, and Ad-PGK was used as vector (mock) control. Two days after Ad-GFP infection, cells were processed for immunohistochemical staining. Ad-GFP–infected cells in serum-containing medium were double-immunostained with cell markers and GFP. Several cell markers were used: MAP-2 or βIII tubulin (Chemicon, Temecula, CA) for neurons, glial fibrillary acidic protein (GFAP; Chemicon) for astrocytes, Griffonia simplicifolia lectin isoelectin B4 (GSA) or ED-1 (Sertec, Oxford, UK) for microglia, and Reca-1 (Sertec) for endothelial cells. Ad-PGK, Ad-PGIS, or Ad-COX-1/PGIS, each at MOI 50, was added to cultured cells in serum-containing medium (DMEM plus 10% FBS). Two days later, cells were treated with LPS at a dose of 100 ng/mL for 2 days. The culture medium was then assayed for lactate dehydrogenase (LDH) release and for lactate, nitric oxide (NO), and tumor-necrosis factor a (TNF-α) measures, while cells were processed for immunocytochemistry.

Chemical Assays

LDH activity in the medium was measured spectrophotometrically as the oxidation of NADH in the presence of pyruvate at 340 nm.19 Lactate release in the medium was assayed spectrophotometrically at 340 nm as NADH reduction via a sequential reaction of LDH and alanine aminotransferase.20 The production of NO was assayed as accumulation of nitrite in medium using colorimetric reaction with Griess reagent.21 Briefly, after 2 days of LPS treatment, the culture supernatants (150 mL) were collected and mixed with 50 mL of Griess reagent (1% sulfanilamide/0.1% naphthyl ethylene diamine dihydrochloride/2% phosphoric acid) and incubated at room temperature for 10 min. The absorbance was measured at 540 nm. Sodium nitrite (NaNO2) was used as the standard to calculate nitrogen dioxide (NO2−) concentrations. TNF-α production in medium was measured by using an ELISA kit from R&D Systems (Minneapolis, MN) according to the manufacturer’s instruction.

Extraction and Analysis of Arachidonic Acid Metabolites

Cultured cells after Ad infection were incubated in DMEM (serum-free) containing 10 mM [1-14C] arachidonic acid (AA) at 37°C for 10 min. The cells were saved for Western blot analysis, and the media, containing released eicosanoids, were ex-
tracted by Sep-Pak C\textsubscript{18} Cartridge (Waters Associates, Milford, MA) as previously described\textsuperscript{22}. In brief, extracted \textsuperscript{14}C-labeled AA metabolites were analyzed by reverse-phase HPLC column chromatography equipped with an on-line radioisotopic detector (Packard 150-TP). The stationary phase was Inertsil 7 ODS-3 (4.6 × 150 mm; Vercopak, Taiwan). The mobile phase consisted of gradient elution between solvent A (acetonitrile) and solvent B (0.1% acetic acid, pH 3.7) under the following conditions at flow rate of 1 mL/min: 34% B for 10 min, 34–40% B within 4 min, 40–50% B within 1 min, 50% B for 5 min, 50–75% B within 10 min, 75–100% B within 10 min, and 100% B for 10 min. The eicosanoids were identified by their retention times with the authentic radioisotopic standards.

**Western Blot Analysis**

Cultured neurons were washed twice with phosphate-buffered saline (PBS) and solubilized in 350 mL lysis buffer containing PBS, 1% NP-40, and a protease inhibitor kit (BM, Germany). The supernatant was collected after centrifugation. Protein was quantified by BCA assay (Pierce, Rockford, IL); protein (5 µg) of the cell lysate was analyzed by Western blot (SDS-PAGE), using 8% or 12% gels, as previously described\textsuperscript{16}. Many primary antibodies were used, including anti-COX-1 (Cayman Chemical, Ann Arbor, MI), anti-COX-2 (Chemicon), anti-iNOS (BD Biosciences, Caribbean, CA), anti-PGIS (customer-ordered synthesis), and anti-β-actin (Santa Cruz Biotech, Santa Cruz, CA).

**RESULTS AND DISCUSSION**

**Adenovirus Preferentially Infected Non-Neuronal Cells**

To optimize the infection condition and characterize the cell-type infective pattern, mixed neuron-glial cultures maintained in serum-free or serum-containing medium were infected with 50 MOI Ad-GFP. Two days after infection, cells were fixed and processed for immunohistochemistry. As shown in Figure 1A and B, Ad-GFP predominantly transfected non-neuronal cells and neurons were sparsely infected. No neurons were found to express GFP in serum-containing medium (Fig. 1B), whereas few neurons (<5% of MAP-2–positive cells) possessed GFP(+) in serum-free medium (Fig. 1A). This may be due to the presence of serum proteins, such as serum albumin, which attenuate adenoviral infectivity by nonspecific binding to adenovirus\textsuperscript{23,24}. We performed double-immunostaining of GFP with various cell markers in serum-containing Ad-GFP–infected cultures. The results showed that among GFP(+) cells, about 30% of non-neuronal cells were GFAP(+) astrocytes (Fig. 1C), 50% of cells were ED-1(+) microglia (Fig. 1D), and <5% cells each were Reca-1(+) endothelial cells (data not shown).

**Overexpression of PGIS or COX-1/PGIS–Enhanced Prostacyclin Synthesis**

Mixed neuron-glia cultures were each infected with mock control (Ad-PGK), Ad-PGIS, and Ad-COX-1/PGIS at 50 MOI. Two days later, cells either were treated with LPS or were harvested to measure AA metabolic activity and protein expression. Figure 2A demonstrates that mixed neuron-glial cultures produced low basal levels
FIGURE 1. Low neuronal infection efficiency with Ad-GFP in neuron-glia cultures. Cultures were infected by Ad-GFP (at 50 MOI) for 2 days and processed for morphological analysis. (A) Cells were maintained in serum-free medium during Ad-GFP infection. *Left*, MAP-2 positive; *right*, GFP positive. (B–D) Cells were maintained in serum-supplemented medium during Ad-GFP infection. *Left panels* in B, C, and D are MAP-2, GFAP, and ED-1 positive, respectively; *right panels* in B–D are GFP positive. Arrow indicates colocalization of two staining antibodies. Images presented are from one experiment and are representative of at least three independent experiments.
of eicosanoids. Upon Ad-PGIS or Ad-COX-1/PGIS infection, the level of $^{14}$C-labeled 6-keto PGF$_{1a}$ (6KP), a degradation product of PGI$_2$, was enhanced. Ad-PGIS–infected cells produced more PGI$_2$ than that of control or mock control (Ad-PGK only). Furthermore, $^{14}$C-labeled AA metabolites were efficiently shunted through $^{14}$C-labeled 6KP synthesis in bicistronic Ad-COX-1/PGIS–infected cells. The overexpressed COX-1 actively converted $^{14}$C-labeled AA to PGH$_2$, which was hydrolyzed to PGE$_2$ and was further converted to $^{14}$C-labeled 6KP by the overexpressed PGIS in the same cell. This is consistent with the results of our previous study, which found that the optimal ratios for achieving large PGI$_2$ augmentation without overproduction of other prostanoids were 1 or 2 parts of Ad-COX-1 to 1 part of Ad-PGIS. $^{17}$ Western blot analysis of these Ad-infected cells are shown in Figure 2B. Significantly enhanced PGIS and/or COX-1 proteins were detected in Ad-PGIS– and Ad-COX-1/PGIS–infected cells, respectively. This indicates that the transduced enzymes are functionally active in producing prostacyclin from AA. Because Ad-GFP predominantly infected microglia or astroglia, Ad-PGIS– or Ad-COX-1/PGIS–infected cells would increase synthesis of prostacyclin, which was then released and

**FIGURE 2.** Adenovirus-mediated overexpression of COX-1 and PGIS and the resultant augmentation of 6KP synthesis in neuron-glia cultures. (A) Analysis of eicosanoids generated by Ad-infected cultures in response to [1-$^{14}$C] AA treatment. 6KP is a degradation product of prostacyclin. Peaks of the first 5-min fractions are nonspecific. Each prostanoid peak was verified by co-elution with an authentic radiolabeled prostanoid. (B) Western blot analysis of COX-1 and PGIS protein levels in cultured neurons, each infected with 50 MOI of Ad. Both figures are representative of two experiments with similar results.
FIGURE 3. NO synthesis and expressions of iNOS, COX-2, and PGIS in Ad-PGK−, Ad-PGIS−, and Ad-COX-1/PGIS−infected neuron-glia cultures before and after LPS challenge. (A) Western blot analysis of protein levels of PGIS, COX-1, COX-2, and iNOS in neuron-glia cultures receiving Ad-PGK, Ad-PGIS, or Ad-COX-1/PGIS infection. After 2 days of Ad infection, cultures were treated with LPS (100 ng/mL) for 2 days and harvested. Equal amounts of protein (5 μg/lane) were analyzed by Western blot using anti-PGIS, anti-COX-1, anti-COX-2, and anti-iNOS antibodies. Protein bands were visualized using horseradish peroxidase-conjugated secondary antibodies and electrochemiluminescence (ECL). One experiment, which represents two experiments, is shown with similar results. (B) Inhibition of LPS-induced NO
affected microglia. Prostacyclin subsequently bound to IP receptors in cells exerting downstream signal transduction.

**Overexpression of PGIS or COX-1/PGIS–Reduced LPS Stimulation in Mixed Neuron-Glia Cultures**

LPS treatment can induce microglial activation and release a variety of cytokines, NO, and eicosanoids. Whether Ad infection can affect LPS stimulation is not clear. To address this question, mixed neuron-glia cultures were infected with each of Ad-PGK, Ad-PGIS, and Ad-COX-1/PGIS. Two days later, cells were further treated with LPS at a dose of 100 ng/mL for 2 days. As shown in Figure 3A, Ad-PGIS or Ad-COX-1/PGIS infection enhanced the expression of PGIS or COX-1/PGIS, whereas iNOS and COX-2 levels were not detectable. By contrast, LPS treatment induced both iNOS and COX-2 expression in control and Ad-PGK–infected cells. Ad-PGIS or Ad-COX-1/PGIS infection effectively reduced LPS-induced COX-2 and iNOS levels. Concurrently, LPS-stimulated nitrite releases were significantly inhibited in Ad-PGIS– and Ad-COX-1/PGIS–infected cells (Fig. 3B) compared to control and Ad-PGK–infected cells. This indicates that enhanced prostacyclin synthesis in PGIS– or COX-1/PGIS–overexpressed cells significantly inhibits iNOS expression and NO production. This inhibitory effect on NO production seems to be due to a reduction in GSA(+) microglial numbers (Fig. 4A) and/or a reduction in iNOS protein expression (Fig. 3A). Cell viabilities, measured as LDH release in medium, were not affected by Ad infection or LPS treatment. Furthermore, GFAP(+) astrocytes were not affected by either Ad infection or LPS treatment (data not shown).

We also measured TNF-α levels and lactate release in the medium. Increased levels of lactate, a product of glycolysis, is a sensitive index for impairment in energy metabolism. As shown in Figure 4B and C, significantly lower TNF-α levels were found in Ad-PGIS– and Ad-COX-1–infected cells compared to those of the control. LPS, at a dose of 100 ng/mL, significantly enhanced TNF-α and lactate levels in the control and Ad-PGK-infected cells (Fig. 4B and C). This LPS-stimulating effect was not seen in Ad-PGIS– or Ad-COX-1–infected cells. This indicates that overexpression of PGIS or COX-1/PGIS with enhanced prostacyclin synthesis in culture significantly inhibited LPS-induced lactate and TNF-α release. The lactate levels induced by LPS treatment could be due to the enhanced production of NO and TNF-α, which impairs oxidative phosphorylation.

Results from this study demonstrate that PGI2 synthesis can be augmented by cultured neurons infected with either Ad-PGIS only or with bicistronic Ad-COX-1/PGIS. The mechanism by which Ad-PGIS– or Ad-COX-1/PGIS–infected cells reduces microglial numbers has not yet been clarified and is subject to hypotheses at present. In the brain, at least two distinct prostacyclin receptors, designated as IP1 and IP2, were reported.25 The IP2 receptor was found only in the central nervous sys-

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production, as nitrite release, in the medium by Ad-PGK, Ad-PGIS, or Ad-COX-1/PGIS infection in neuron-glia cultures. Control on x-axis, no viral infection of naive cultures; filled bar in chart, unstimulated cultures. Data were expressed as means ± SEM from four independent experiments done in triplicate. Asterisks (**) indicate significant differences between no stimulation and LPS stimulation (P < 0.01 by ANOVA and t-test) within each Ad-infected cell.
FIGURE 4. Effect of Ad-PGIS or Ad-COX-1/PGIS infection on microglial numbers, lactate, and TNF-α levels in neuron-glia cultures before and after LPS stimulation. (A) Lectin-reactive microglia cells expressed as GSA(+) field (magnification ×100). (B) Lactate levels in the medium. (C) TNF-α level in the medium. Control on x-axis, no viral infection of naive cul-
tem and therefore termed as central-type prostacyclin receptor.\textsuperscript{25} The IP\textsubscript{1} receptor is mainly coupled to G protein-coupled receptor (Gs); hence stimulation of the receptor results in cAMP production.\textsuperscript{26} IP\textsubscript{2}, however, had no effect on cAMP production. A selective ligand for IP\textsubscript{2} attenuated neuronal damage in cerebral ischemia, whereas treatment with iloprost, a selective IP\textsubscript{1} ligand, did not show a protective effect.\textsuperscript{27} In the present study, microglial numbers were significantly reduced in Ad-PGIS– and Ad-COX-1/PGIS–infected cells. It remains to be determined which receptor subtype (IP\textsubscript{1} or IP\textsubscript{2}) is involved in this effect. Further studies are needed to elucidate the role played by downstream molecules in the inhibition of microglial activation.

Understanding the role of prostaglandin \textit{in vivo} is difficult because the activated COX cascade is often accompanied by the generation of a broad range of other active molecules, including cytokines, NO and the prostanooid precursor arachidonic acid itself and its metabolites. Furthermore, individual prostaglandins may also exert opposite or synergetic effects on common targets. Thus, the final contribution of prostaglandins to tissue damage or repair seems to depend on the balance between the different prostaglandins released by non-neuronal or neuronal cells in different circumstances. Here we show that enhanced prostacyclin synthesis effectively reduced microglial numbers and LPS stimulation. Inhibition of the microglial response by enhanced prostacyclin synthesis could be an effective therapeutic approach to alleviating the progression of diseases. We suggest that prostacyclin may have a neuroprotective role in modulating the immune response at the injured site.

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**REFERENCES**


\textsuperscript{a}Significant difference between control and Ad-infected cells ($P < 0.05$ or 0.01 by ANOVA and $t$-test).
\textsuperscript{b}Significant difference between LPS-stimulated and LPS plus Ad-infected cells ($P < 0.05$ by ANOVA and $t$-test).
\textsuperscript{c}Significant difference between control and LPS within each Ad-infected cell. Data were expressed as means ± SEM from three or four independent experiments done in triplicate.