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Physiological concentration of prostaglandin E₂ exerts anti-inflammatory effects by inhibiting microglial production of superoxide through a novel pathway

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Abstract

This study investigated the physiological regulation of brain immune homeostasis in rat primary neuron-glial cultures by sub-nanomolar concentrations of prostaglandin E₂ (PGE₂). We demonstrated that 0.01 to 10 nM PGE₂ protected dopaminergic neurons against LPS-induced neurotoxicity through a reduction of microglial release of pro-inflammatory factors in a dose-dependent manner. Mechanistically, neuroprotective effects elicited by PGE₂ were mediated by the inhibition of microglial NOX2, a major superoxide-producing enzyme. This conclusion was supported by: 1) the close relationship between inhibition of superoxide and PGE₂-induced neuroprotective effects; 2) the mediation of PGE₂-induced reduction of superoxide and neuroprotection via direct inhibition of the catalytic subunit of NOX2, gp91^{phox}, rather than

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Conflicts of interests

The authors have declared that no conflict of interest exists.

through the inhibition of conventional prostaglandin E2 receptors; 3) abolishment of the neuroprotective effect of PGE₂ in NOX2-deficient cultures. In summary, this study revealed a potential physiological role of PGE₂ in maintaining brain immune homeostasis and protecting neurons via an EP receptor-independent mechanism.

Keywords

Prostaglandin E₂; microglia; NOX2; neuroprotection; neuroinflammation

Introduction

Prostaglandin E₂ (PGE₂) exhibits diverse hormone-like effects in animals, acting through a wide range of physiological targets and mechanisms, such as neurons, smooth muscles, secretory cells, and immune function etc. [1–3]. Immune function of PGE₂ in modulating neuro-inflammation has been an active area of research, but the roles of PGE₂ as a pro-inflammatory or anti-inflammatory mediator are far from clear. Under normal conditions, a modest level of PGE₂ is produced in neurons, microglia, and astroglia; however, a large amount of PGE₂ is produced mainly by microglia during neuroinflammation [4]. Bifunctional effects of PGE₂ on inflammatory conditions have been documented. In pathological conditions, PGE₂ regulates multiple processes in the nervous system, including fever generation, sickness behavior, pro-inflammatory cytokines production, such as IL-6 and IL-1 β , and also contributes to neuropathic pain and neurodegenerative disorders [5–13]. Conversely, PGE₂ also exhibits anti-inflammatory functions [14,15]. Several lines of evidence suggest that PGE₂ generated during periods of inflammation is subject to negative feedback in order to down-regulate other pro-inflammatory factors [16,17]. Moreover, accumulating *in vivo* and *in vitro* studies reveal that exogenous PGE₂ is able to inhibit lipopolysaccharide (LPS)-induced microglial activation and the production of pro-inflammatory factors, including TNF- α , IL-1 β , iNOS, IL-6 and IL12 [18–22].

Numerous studies reveal that PGE₂ displays functionally-opposing actions in distinct cell types or disease models, including pro- and anti-inflammatory effects, or toxic and protective effects to neurons [23,24,11,25,14,26,27]. The reason for these opposing reports is not clear. We speculated that the difference in concentration of PGE₂ used in various reports could contribute to the controversial conclusions. Most pharmacological studies involve pathological PGE₂ concentrations ranging from 100 nM to 10 μ M, which is 100- to 1000-fold higher than physiological concentrations of PGE₂ in the central nervous system (CNS) (< 10 nM), while very few reports use low concentrations of PGE₂ [28–33]. The purpose of this study was to gain a further understanding of the physiological function of PGE₂ in the modulation of brain immune homeostasis by focusing on the effects of sub-nanomolar concentrations of the prostaglandin. In the present study, we investigated whether low concentrations of PGE₂ (0.01 to 10 nM) were capable of eliciting a neuroprotective effect through mediation of pro-inflammatory factors, especially superoxide free radicals produced by the enzyme NADPH oxidase (NOX2), in an LPS-induced neuro-inflammation model. We found that low concentrations of PGE₂ displayed both anti-inflammatory and neuroprotective functions, and further investigation revealed that anti-inflammatory effects

were independent of prostaglandin E2 receptors (EP). Additionally, we discovered that inhibition of NOX2-generated superoxide production from microglia was the key mechanism underlying the anti-inflammatory function of sub-nanomolar concentrations of PGE₂.

Materials and Methods

Animals

Timed-pregnant (gestational day 14) female Fisher 344 rats were purchased from Charles River Laboratories (Raleigh, NC, USA). Timed-pregnant C57BL/6J and B6.129S-Cybb^{tm1Din}/J (gp91 deficient) mice were generated by our institute's animal husbandry staff using breeders obtained from Jackson Laboratories (Bar Harbor, ME, USA). EP2 knockout mice were kindly gifted from Dr. Robert Langenbach at National Institute of Environmental Health Sciences. Rat and mouse dams were housed in polycarbonate cages in animal facilities with controlled environment conditions with a 12 h artificial light-dark cycle and provided fresh deionized water and NIH 31 chow *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee and conducted in strict accordance with the National Institutes of Health animal care and use guidelines.

Reagents—Xanthine, xanthine oxidase, SQ 22,536, L798106, poly-D-lysine, superoxide dismutase (SOD), mazindol, 3,3'-diaminobenzidine and urea-hydrogen peroxide tablets were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water-soluble tetrazolium salt-1 (WST-1) was purchased from Dojindo (Rockville, MD, USA). Lipopolysaccharide (LPS; *E. coli* strain O111: B4) was purchased from Calbiochem (San Diego, CA, USA). Cell culture ingredients were obtained from Life Technologies (Grand Island, NY, USA). Prostaglandin E₂ (PGE₂), 17-phenyl trinor prostaglandin E₂, butaprost, CAY-10598, SC-19220, AH-6809 and AH-23848 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-tyrosine hydroxylase (TH) was purchased from Chemicon (Billerica, MA, USA) and antibody diluent was purchased from DAKO (Carpinteria, CA, USA). TNF- α ELISA kit was purchased from R&D Systems (Minneapolis, MN, USA). Direct cAMP ELISA kit was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Anti-p47^{phox} antibody was purchased from Millipore (Temecula, CA, USA). Anti-gp91^{phox} antibody was purchased from BD Biosciences (San Jose, CA, USA). Anti-GAPDH antibody was purchased from Abcam (Cambridge, MA, USA). Alexa Fluor 488 goat anti-rabbit IgG was purchased from Invitrogen (Carlsbad, CA, USA). Goat anti-rabbit biotinylated secondary antibody was purchased from Vector Laboratory (Burlingame, CA, USA).

Mesencephalic neuron-glia culture—Rat mesencephalic neuron-glia cultures were prepared following protocols described previously [34,35]. Briefly, midbrain tissues were dissected from day 14 embryos, and then gently triturated into single cell suspension. Cells were then seeded (5×10^5 cells/well) in poly-D-lysine (20 μ g/ml) pre-coated 24-well plates. The cultures were incubated at 37°C in 5% CO₂ for three days and then replenished with 500 μ l of fresh maintenance media. Cultures were treated 7 days after seeding.

Primary cortical mixed glial culture

Primary cortical mixed glial cultures were prepared from rat or mouse pup brains at postnatal day 1–3, as previously described [35,34]. Briefly, the cortices were isolated, the meninges and blood vessels removed, the tissue gently dissociated through trituration and the single cell suspension plated on either 24-well plates or 96-well plates pre-coated in poly-D-lysine (20 µg/ml) at 1×10^5 cells/well or 5×10^4 cells/well, respectively. Cells were maintained in DMEM-F12 (1:1) media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µM non-essential amino acids, 50 U/ml penicillin, and 50 µg/ml streptomycin. Media was refreshed every 3 days until they were experimentally treated 7 days after seeding.

Microglia-enriched cultures

Microglia-enriched cultures were prepared from primary mixed glial cultures as previously described [35,34]. Briefly, mixed glia cultures were plated on 150 cm³ flasks pre-coated in poly-D-lysine (20 µg/ml) at 5×10^7 cells/flask, and were maintained in DMEM-F12 media changed every three days for two weeks. At two weeks, microglia were shaken off at 180 rpm for 40 minutes and re-plated either on 6-well plates pre-coated in poly-D-lysine (20 µg/ml) at 1×10^6 cells/well for cAMP assay.

Cell Line Culture

Mycoplasma-free rat HAPI microglial cell line was maintained in DMEM supplemented with 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin. Cells were split into T75 flasks and used for experiments at passages 3–5, where they were seeded in 24-well plates at 1×10^5 cells/well and treated 24 h later.

Wild-type and transfected monkey kidney COS7 cells were gifts from Dr. Mary Dinanuer (Indiana University, IN, USA) and cultured as described previously [36,37]. Briefly, wild-type COS7 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 µg/ml streptomycin. COS7 cells stably expressed with gp91^{phox}, p22^{phox}, p47^{phox}, and p67^{phox} (COS-NOX2 cells) were maintained in DMEM as above containing 0.8 mg/ml Geneticin, 2 µg/ml puromycin, and 0.35 mg/ml hygromycin B.

Dopamine uptake assay—The [³H] Dopamine (DA) uptake assay was performed as described previously [38]. Briefly, the rate of uptake of radiolabeled DA by DANergic neuron cultures was measured for 21 minutes at 37°C. Cells were washed and lysed to release internalized radiolabeled DA and quantified with a liquid scintillation counter (Tri-Carb 4000; Packard, Meriden, CT, USA). Nonspecific [³H] DA uptake was accounted for by competitively inhibiting DA uptake with 10 µM of mazindol.

Immunocytochemical and immunofluorescence staining

For immunocytochemical staining, neuron-glia cultures were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 20 minutes; fixed cultures were treated with 1 % hydrogen peroxide for 10 minutes and incubated for 20 min with blocking solution (BSA 1%/Triton X-100 0.4%/Normal Goat Serum 4% in PBS). Cells were immunostained overnight at 4°C with rabbit polyclonal antibody against tyrosine hydroxylase (TH; 1:5000)

in Antibody Diluent. Cultures were washed for 10 minutes in PBS (three times) and incubated for 2 h with PBS containing 0.3% Triton X-100 and a biotinylated secondary antibody (goat anti-rabbit antibody, 1:227; Vector Laboratory, Burlingame, CA). After washing (three times) with PBS, the cultures were incubated for 1 h with the Vectastain ABC reagents (Vector Laboratory, Burlingame, CA) diluted in PBS containing 0.3% Triton X-100. To visualize the signal, the cultures were incubated with 3,3'-diaminobenzidine and urea-hydrogen peroxide tablets dissolved in water. TH-positive cells were manually counted under a microscope (Nikon, model DIAPHOT, Garden City, NY, USA) by at least two investigators and the results were averaged.

For immunofluorescence staining, microglia-enriched cultures were fixed with 3.7% formaldehyde in PBS for 20 minutes and incubated for 20 min in blocking solution (BSA 1%/Triton X-100 0.4%/Normal Goat Serum 4% in PBS) to block non-specific binding. Cells were immunostained overnight at 4°C with rabbit polyclonal antibody against p47^{phox} (1:1000) diluted in Antibody Diluent. The antibody was detected and visualized using Alexa Fluor 488 goat anti-rabbit IgG (1:750) secondary antibody. The images were acquired using multiphoton laser-scanning microscope Zeiss 710.

Peritoneal macrophage isolation—Macrophages were obtained from peritoneal cavities of mice using protocols described previously [39]. Briefly, mice were injected with 1 ml of 3% Brewer thioglycollate medium into the peritoneal cavity. After 4 days, mice were euthanized, and the peritoneal cavity was flushed with 5ml ice-cold RPMI medium 1640. After being washed twice in RPMI, the cells were preincubated in the no-serum medium for 1 h. The cells were then washed twice to remove non-adherent cells. Adherent macrophages were cultured in DMEM containing 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

[³H]-labeled PGE₂ binding assay—The binding assay was performed according to protocols described previously [40]. Briefly, macrophages from C57BL/6J and CYBB (gp91 deficient) mice, wild-type COS7 cells and COS7 cells stably expressing gp91^{phox}, p47^{phox} and p67^{phox} (COS7-NOX2) (2×10⁶ cells) were incubated with 0.3 nM of ³H-labeled PGE₂ in binding buffer (HBSS + 10% fetal bovine serum) for 2 hours at 37°C. After binding, the cells were transferred to glass fiber filter with 1 µm pore size and washed three times using warm HBSS. Then the filters were put into vials containing 15 ml of Ultima Gold scintillation fluid (PerkinElmer, Waltham, MA, USA) and counted for radioactivity. The results are expressed as a percentage of total binding observed with corresponding COS7-WT or C57BL/6 group.

Saturation curves of [³H]-PGE₂ binding were performed using COS7-WT and COS7-NOX2 cells. Total binding was measured by the binding of [³H]-labeled PGE₂ ranging from 0.03 to 3 nM while nonspecific binding was determined in the presence of additional 3 µM of PGE₂. Specific binding was calculated by the difference between total binding and nonspecific binding.

TNF-α, MCP-1 and nitrite measurement—The release of TNF-α and MCP-1 were measured from culture supernatant with a commercial enzyme-linked immunosorbent assay

(ELISA) kit from R&D Systems, in accordance with the manufacturer's instructions. NO was assessed by measuring nitrite in 50 μ l of supernatants using Griess reagent as described previously [41]. The culture supernatant was collected at 3 hours after treatment for TNF- α and 24 hours after treatment for NO and MCP-1 assay. The results were quantified using an SPECTAmax PLUS 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

cAMP assay—Primary microglia cells were seeded in 6-well plates (1×10^6 cells/well) and then treated with vehicle, LPS, or PGE₂ (1, 10, 50, 100 and 1000 nM) for 30 minutes. Cells were lysed in 0.1 M HCl for 10 min, and the supernatants were used for cAMP assays following the manufacturer's protocol of cAMP Enzyme Immunoassay Kit. The absorbance was measured at 405 nm using a colorimetric 96-well plate reader. Data were expressed as picomoles cAMP per 1 million cells.

Superoxide assay—The production of extracellular superoxide was determined by measuring the superoxide dismutase (SOD)-inhibitable reduction of water-soluble tetrazolium salt-1 (WST-1)[42,43]. Mixed glial or neuron-glia cultures in 96-well culture plates were incubated at 37°C for 30 minutes with vehicle or PGE₂. Then the cultures were washed twice with HBSS without phenol red. After washing, the cultures were treated with LPS or LPS+PGE₂ in 150 μ l phenol red-free, to which 50 μ l of WST-1 (1 mM) with and without 800 U/ml SOD was immediately added. The absorbance at 450 nm was read with a SpectraMax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) and the data at 8–14 min post-treatment were analyzed. The difference in absorbance observed in the presence and absence of SOD was considered to be the amount of superoxide produced, and results were expressed as the percentage of vehicle-treated control cultures. For figure 3D, the cultures were pretreated with EP1 to EP4 receptor antagonists for 30 minutes, and then the steps described above were followed.

Xanthine/xanthine oxidase reaction—Xanthine/xanthine oxidase (X/XO) assays were conducted in the presence of indicated concentrations of PGE₂, 0.01 U xanthine oxidase, 50 μ M xanthine, and 250 μ M partially acetylated WST-1 in 50 mM potassium phosphate buffer (pH 7.6) in a 96-well plate (100 μ L/well final volume). Xanthine was added to initiate the reaction, and the absorbance at 450 nm was continuously monitored for 20 minutes using an SPECTAmax PLUS 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) and the data at 8–14 min post-treatment were analyzed. Superoxide dismutase (SOD) added in the X/XO system served as a positive control.

Plasma membrane preparation and Western blot analysis—Plasma membrane of HAPI was isolated following a published protocol [44]. Briefly, cells were suspended in isolation buffer (10 mM Tris-Cl, pH 8.0, 0.25 M sucrose, 1 mM EDTA, and protease inhibitor cocktails), and cell membranes were broken by Dounce homogenization. The cell lysates were centrifuged at 6000 xg for 10 min at 4 °C to remove unbroken cells, cell debris, and mitochondria; afterward, pellets of membranes were obtained by ultracentrifugation at 100,000 xg for 1 h at 4 °C. After being washed with 1 M KCl, membrane pellets were either used fresh or stored at –80 °C. The pellets were solubilized in 1% Nonidet P-40 hypotonic lysis buffer. Equal amounts of protein were separated by 4% to 12% Bis-Tris Nu-PAGE gel

and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% non-fat milk and incubated with antibodies against p47^{phox}, gp91^{phox} (1:1,000 dilution) or GAPDH (1:2,500 dilution). The protein bands were developed by incubating with horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) and an enhanced chemiluminescence substrate kit (Millipore, Billerica, MA, USA). The results were quantified by AlphaImager 3400 software (Alpha Innotech, Ltd., San Leandro, CA, USA).

Statistics

Data are presented as the mean $\pm$ SEM. Comparisons between more than two groups was conducted using one-way ANOVA, followed by Bonferroni's *post hoc* multiple comparison test. Comparisons between more than two parameters were conducted by two-way ANOVA analysis, followed by Bonferroni's *post hoc* multiple comparison test. Data were analyzed using Prism (v6.00, GraphPad, San Diego, CA). P-values less than or equal to 0.05 were considered statistically significant.

Results

Low nanomolar concentrations of PGE₂ protect dopaminergic neurons against LPS-induced neurotoxicity

To determine the immune regulatory function of PGE₂ in concentrations close to those found under normal physiological conditions, mesencephalic neuron-glia cultures were pretreated with PGE₂ ranging from 0.01 nM to 10 nM for 30 minutes followed by LPS treatment (15 ng/ml). After seven days, the viability of dopaminergic (DA) neurons was determined by a [³H] DA uptake assay, tyrosine hydroxylase (TH)-positive neuron counting, and morphological analysis. [³H] DA uptake showed that pre-treatment of PGE₂ protected neurons against LPS-induced neurotoxicity ($F_{(5, 33)} = 30$, $P < 0.0001$) in a dose-dependent manner. *Post hoc* analysis revealed that exposure of LPS reduced DA uptake capacity by 60% compared to vehicle-treated cultures ($t = 11.1$, $P < 0.0001$). Pretreatment with PGE₂ significantly blocked this reduction at 1 nM ($t = 5.028$, $P < 0.0001$) and 10 nM ($t = 7.022$, $P < 0.0001$), when compared to DA uptake in LPS-only treatments (Fig. 1A). Consistent with DA uptake results, TH-positive neuron count showed a similar protective effect of PGE₂ ($F_{(5, 36)} = 11.2$, $P < 0.0001$) (Fig. 1B). *Post hoc* analysis showed that neuroprotective effects of PGE₂ were observed in concentrations as low as 0.1 nM (0.1 nM: $t = 3.245$, $P < 0.05$; 1nM: $t = 4.275$, $P < 0.01$; 10 nM: $t = 4.838$, $P < 0.001$), compared to the LPS-only treatments. Morphological analysis showed that the neurites of TH-positive neurons in LPS-treated cultures were short and fragmented compared to those in the control cultures. The neurites in cultures pretreated with PGE₂ were less affected, compared with neurites in LPS-treated cultures (Fig. 1C). Together, these results indicate that nanomolar concentrations of PGE₂ exert potent neuroprotective effects against LPS-induced neurotoxicity.

PGE₂ displays varying potency in inhibiting LPS-induced production of pro-inflammatory factors in mixed glial cultures

Accumulating evidence indicates that neuro-inflammation caused by over-activated microglia is a critical contributor to neuronal loss in neuro-degeneration. Our previous

primary neuron-glia culture studies indicate that microglia are the major source of LPS-stimulated production of pro-inflammatory factors (e.g., cytokines, chemokines, and free radicals), which mediate neuro-inflammation and cause neurotoxicity[45]. To investigate the mechanisms of neuroprotection elicited by nanomolar concentrations of PGE₂, we investigated the release of pro-inflammatory factors in rat mixed glial cultures (containing 80% astroglia and 20% microglia). The reason why mixed glia cultures were used in lieu of enriched microglia cultures in this study was microglia can survive longer when cultured together with either neurons or astroglia [46,47]. The results revealed that PGE₂ reduced the production of superoxide, TNF- α , NO, and MCP-1 (superoxide, $F_{(5, 18)} = 69.35$, $P < 0.0001$; TNF- α , $F_{(7, 79)} = 340.8$, $P < 0.0001$; NO, $F_{(7, 67)} = 55.78$, $P < 0.0001$; MCP-1, $F_{(5, 12)} = 34.87$, $P < 0.0001$) in a dose-dependent manner (Figure 2). However, it is interesting to note that the inhibitory potency of PGE₂ varies greatly: the half-maximal inhibitory concentration (IC₅₀) value is 0.13 ± 0.08 nM for inhibition of superoxide, 10.14 ± 0.18 nM for TNF- α , and more than 100 nM for both NO and MCP-1 production. These results suggest that PGE₂ inhibited production of these pro-inflammatory factors through different mechanisms. Moreover, the similar IC₅₀ values of PGE₂ in inhibiting neurotoxicity (0.14 nM) and superoxide production (0.13 nM) suggest a strong likelihood that the inhibition of superoxide production is critical in mediating PGE₂-elicited neuroprotective effects. Thus, more detailed studies were conducted to further understand the molecular mechanisms underlying the regulation of superoxide production by nanomolar concentrations of PGE₂.

Inhibition of superoxide production is not due to the scavenging effect of PGE₂ and is not mediated through EP receptors

One of the mechanisms of suppressing superoxide production is scavenging of superoxide by agents of interest. To investigate this possibility, we determined whether nanomolar concentrations of PGE₂ possess the ability to scavenge superoxide free radicals. We measured the effect of PGE₂ on superoxide production using a xanthine/xanthine oxidase superoxide-producing system. Superoxide production was dramatically inhibited by superoxide dismutase (SOD), but not by PGE₂ with different concentrations (0.01–10 nM) (Supplementary Fig. 1).

The lack of a superoxide-scavenging effect of PGE₂ prompted us to investigate the potential signaling pathway mediating the inhibitory effects of PGE₂ on superoxide production. Using a pharmacological approach, we determined the potency of different agonists selective for EP receptor subtypes in inhibiting LPS-induced superoxide production. Rat mixed glial cultures were pretreated with various agonists, 17-phenyltrinor PGE₂ (EP1/3 agonist), Butaprost (EP2 agonist), and CAY10598 (EP4 agonist) with concentrations ranging from 0.01 to 10 nM for 30 minutes prior to LPS (20 ng/ml) stimulation. The results showed that all EP agonists mimicked the inhibitory effects of PGE₂ on superoxide production, with IC₅₀ values similar to that of PGE₂ (17-phenyl trinor PGE₂, $F_{(5, 12)} = 60.07$, $P < 0.0001$; Butaprost, $F_{(5, 24)} = 187.9$, $P < 0.0001$; CAY10598, $F_{(5, 12)} = 37.93$, $P < 0.0001$) (Fig. 3A–3C). Moreover, simultaneously blocking all four EP receptors by adding receptor antagonists to mixed glial cultures failed to disrupt the inhibitory effect of PGE₂ (Fig. 3D). The EP receptor-independent effect of PGE₂ was further supported by using EP receptor-deficient mixed glial cultures. Here we used mixed glial cultures prepared from EP2 receptor

knockout mice because the EP2 receptor has been previously implicated to be critical in mediating the anti-inflammatory effect of micromolar concentrations of PGE₂ [48–50]. PGE₂-induced inhibition of superoxide production did not differ between EP2-deficient and control mixed glia cultures, indicating that the EP2 receptor was not involved in mediating nanomolar PGE₂-induced inhibition of superoxide (Fig. 3E). Additionally, the EP2 receptor agonist butaprost was still capable of inhibiting superoxide production in EP2-deficient mixed glia cultures (Fig. 3F). Since different signaling pathways mediate each EP receptor subtype and cause distinct cellular effects [3], the similar potencies displayed by these EP receptor agonists suggest that their inhibitory properties may be mediated by a novel, EP receptor-independent mechanism. Taken together, these results demonstrate that inhibition of LPS-induced superoxide is not due to the superoxide scavenging capacity of PGE₂ at low concentrations and is instead mediated through a novel EP receptor-independent mechanism.

NOX2 plays a pivotal role in the neuroprotective effects of low concentrations of PGE₂

A key superoxide-producing enzyme in activated phagocytes such as microglia, NADPH oxidase (NOX2) is composed of two membrane subunits (gp91^{phox} and p22^{phox}) and several cytosolic subunits (p47^{phox}, p67^{phox}, p40^{phox}, Rac1, and Rap1A). Previous reports indicate that activation of microglial NOX2 is key to the pathogenesis of inflammation-related neurodegeneration, and its gp91^{phox} subunit is a target for several anti-inflammatory agents [51,52]. To further investigate whether the neuroprotective effect of low doses of PGE₂ is mediated through NOX2, we determined the viability of DA neurons in both wild-type and NOX2-deficient neuron-glia cultures. Genetic ablation of gp91^{phox} abolished neuroprotection by PGE₂ (Figure. 4), indicating that NOX2 plays a critical role in mediating the anti-inflammatory and neuroprotective effects of low concentrations of PGE₂.

PGE₂ directly interacts with NADPH oxidase subunit gp91^{phox}

To investigate the possibility that PGE₂ suppressed LPS-induced superoxide production by inhibiting NOX2 through binding to the gp91^{phox} subunit, we conducted a binding assay using tritium labeled PGE₂. We compared the binding capacity of PGE₂ in wild-type COS7 cells and COS7 cells transfected with NOX2 membrane subunits (gp91^{phox}/p22^{phox}) and cytosolic subunits (p47^{phox} and p67^{phox}) (COS7-NOX2). The reason for the co-transfection of gp91^{phox} and p22^{phox} is that p22^{phox} facilitates stable expression of gp91^{phox} in the plasma membrane of COS7 cells [53]. The [³H]-labeled PGE₂ binding assay showed that COS7-NOX2 cells have a binding capacity to [³H]-PGE₂ that is two-fold higher than that of wild-type COS7 cells ($P<0.0001$, Fig 5A). We also compared the PGE₂ binding capacity in macrophages isolated from wild-type and gp91^{phox}^{-/-} mice. We used peritoneal macrophages instead of microglia in this study for the following reasons: 1) microglia are considered to be the macrophages of the brain, as both cells are derived from bone marrow monocytes, and the protein structure of gp91^{phox} of these two cell types is identical; 2) macrophages have high levels of gp91^{phox}, and it was easy to generate large numbers of primary macrophages required for this study. The [³H]-PGE₂ binding capacity was significantly reduced in macrophages from gp91^{phox}^{-/-} mice than in that from wild-type mice ($P<0.01$, Fig. 5B). The saturation study was performed using wild-type COS7-WT and transfected COS7-NOX2 cells. The [³H]-PGE₂ binding was saturable with a binding affinity at a low nanomolar concentration range (Fig. 5C and 5D). The calculated K_d value was 0.83

± 0.04 nM, which is close to the IC_{50} concentrations for the inhibition of both LPS-induced superoxide production (0.13 nM) and neurotoxicity (0.14 nM). These results give credence to the possibility that PGE_2 inhibits LPS-induced superoxide production and subsequent neurotoxicity by binding to the gp91^{phox} subunit.

PGE_2 inhibits the LPS-induced increase in translocation of the cytosolic subunit p47^{phox} to the cell membrane

The activation of NOX2 requires the translocation of phosphorylated cytosolic subunits (p47^{phox}, p67^{phox}, and p40^{phox}) to the cell membrane to form the active enzyme complex by binding to the membrane subunits gp91^{phox} and p22^{phox}. We previously reported that several anti-inflammatory agents reduced the production of superoxide by changing the conformation of the membrane subunit gp91^{phox} to reduce the binding affinity for the cytosolic subunits of NOX2 and prevent their translocation to the membrane[52]. To obtain additional evidence supporting the possibility that NOX2 acts through a novel signaling pathway that mediates the anti-inflammatory action of PGE_2 , we examined the membrane translocation of the cytosolic subunit p47^{phox} by determining the level of p47^{phox} in cytosol and membrane. Primary microglia were pretreated with/without 10 nM of PGE_2 for 30 minutes following by LPS stimulation for 15 minutes. Staining results showed that LPS significantly increased the immunofluorescence intensity in the membrane, indicating translocation of the cytosolic subunit p47^{phox} to the membrane. Pre-treatment with PGE_2 alone did not differ from the control while when combined with LPS, it almost completely prevented the LPS-induced translocation of p47^{phox} to the membrane (Fig. 6A). We also conducted Western blot analysis to verify the result of immunofluorescence staining. In this study, rat microglia cell line HAPI cells were used, due to the large number of high purity cells of this type that can be harvested for Western blot analysis. Consistent with immunostaining results, LPS stimulation significantly increased the band intensity of p47^{phox} in the membrane fraction (Control VS. LPS, $t = 10.86$, $P < 0.001$). PGE_2 pre-treatment completely prevented these changes (LPS VS. PGE_2 + LPS, $t = 10.47$, $P < 0.001$) (Fig. 6B and 6C). Taken together, these results suggest that PGE_2 can bind to the subunit of NOX2 gp91^{phox} to inhibit the LPS-induced translocation of cytosolic p47^{phox} and subsequent superoxide production.

Discussion

The anti-inflammatory effects of PGE_2 have been extensively studied under both pharmacological and pathological conditions. However, concentrations of PGE_2 used in such studies are in the micromolar range (0.1–10 μ M) and are rarely observed under normal physiological conditions. The present study demonstrated that low concentrations of PGE_2 (0.01 to 10 nM), which are close to normal brain extracellular concentrations (< 10 nM)[28–33], exert potent protection of LPS-induced DA neuron damage via a reduction in the release of pro-inflammatory factors, especially the release of reactive oxygen species (ROS) from microglia. Further, the mechanistic studies conducted here reveal that the anti-inflammatory and neuroprotective effects of low concentrations of PGE_2 are not mediated through the conventional EP receptors (EP1-EP4). Instead, we found that PGE_2 could directly inhibit the activation of microglial NOX2 by binding to its catalytic membrane subunit gp91^{phox}. Our

study also indicates that a reduction in superoxide production plays a major role in PGE₂-induced anti-inflammatory neuroprotective effects. These findings revealed a receptor-independent novel mechanism by which physiological concentrations of PGE₂ function to maintain brain immune homeostasis.

PGE₂ has been reported to exert both pro- and anti-inflammatory effects; however, the reasons behind these opposing effects remain unclear. Use of different concentrations and experimental models could be responsible for some of these bi-functional effects of PGE₂. We hypothesized that the difference in concentrations could play a role in these opposite effects of PGE₂ on immune function. Therefore, this study focused on elucidating the mechanism(s) mediating the action of PGE₂ in low concentrations. The LPS-induced release of pro-inflammatory factors, including TNF- α , MCP-1, nitric oxide, and superoxide-related ROS from microglia, was found to decline in the presence of PGE₂ in a dose-dependent manner. However, the IC₅₀ concentrations of PGE₂ necessary to suppress these pro-inflammatory factors varied greatly. Among these immune factors, superoxide production was the most critical factor related to the anti-inflammatory and neuroprotective effects of PGE₂; 1.0 nM of PGE₂ completely suppressed LPS-induced production of superoxide and achieved total neuroprotection. In contrast, higher concentrations of PGE₂ were needed to inhibit LPS-induced production of TNF- α , MCP-1, and nitric oxide to exert a neuroprotective effect. These findings prompted us to search in further detail for the mechanism(s) behind inhibition of superoxide production in the presence of low concentrations of PGE₂.

The most salient finding of this study is the EP receptor-independent inhibition of superoxide production by low concentrations of PGE₂. Most of our knowledge regarding the inhibition of superoxide production by PGE₂ arrived from studies on peripheral phagocytes using micromolar concentrations of PGE₂ [48–50], which indicate that the EP2/cAMP signaling pathway plays a pivotal role in inhibition of superoxide production inhibition by PGE₂. In contrast, the lack of suppression of superoxide by either low concentrations of PGE₂ or the EP2 agonist butaprost in EP2 receptor-deficient mixed glia cultures, along with a lack of effect of combined pretreatment with EP1-4 receptor antagonists on PGE₂-induced inhibition of superoxide, imply the existence of an EP receptor-independent regulatory mechanism in superoxide inhibition by PGE₂ in microglia. Moreover, we found that cAMP was undetectable in primary microglial cells treated with 10 nM or less of PGE₂ (Supplementary Fig. 2), further supporting the EP receptor-independent effect of PGE₂.

Previous studies have shown that NOX2, the main superoxide-producing enzyme on microglia cell membranes, is an important source of ROS[51,54,55]. ROS, including the superoxide anion, hydroxyl radical, lipid hydroperoxides, and hydrogen peroxide, are generated from activated microglia and play important roles in amplifying the expression of pro-inflammatory genes in microglia and subsequent neuro-degeneration[54]. Various *in vitro* and *in vivo* studies indicate that deficiency in NOX2 or pharmacological inhibition of this enzyme's activity can significantly reduce the production of pro-inflammatory factors, as well as subsequent neuro-degeneration[51,56,57]. Such studies prompted us to speculate that NOX2 could be the target of physiological concentrations of PGE₂, and the following evidence strongly supports this hypothesis: 1) all the specific agonists for the different EP

receptor subtypes, such as 17-phenyltrilor PGE₂ (EP1 and EP3), butaprost (EP2) and CAY10598 (EP4), displayed similar potencies as PGE₂ in inhibiting superoxide production (Fig. 2A, 3A–3C). In contrast, EP receptor antagonists or lack of an EP2 receptor failed to influence the inhibitory effects of PGE₂. These results further indicate that a superoxide-generating enzyme, rather than any of the EP receptors, is a common target for the different PGE₂ analogs; 2) the ligand binding studies indicated that PGE₂ bound to the membrane catalytic subunit gp91^{phox} with a high affinity (i.e., a K_d value around 10^{−10} M), which is close to the K_i value for inhibition of superoxide production (Fig. 5C and 5D); and 3) PGE₂ failed to reduce superoxide produced by the xanthine/xanthine oxidase system, indicating that the inhibition of superoxide production by PGE₂ is not due to its superoxide free radical-scavenging effect (Supplementary Fig. 1). Together, the evidence indicates that NOX2 is a novel target mediating the anti-inflammatory action of nanomolar concentrations of PGE₂.

To further understand the nature how PGE₂ interacts with the catalytic subunit gp91^{phox}, we conducted a pharmacophore analysis by comparing PGE₂ with a series of conventional NOX2 inhibitors. Despite the differences in chemical structures between PGE₂ and its analogs used in this study and some of the known NOX2 inhibitors (e.g., diphenyleneiodonium (DPI), apocynin or naloxone[52]), the analysis showed that several chemical features shared between PGE₂, EP receptor agonists, and NOX2 inhibitors, as deduced by the program Molecular Operating Environment (MOE, Chemical Computing Group, Inc., Montreal, Canada), include hydrophobic centroids, hydrogen bond acceptors, and hydrogen bond donors (Supplementary figure 3). Thus, it is likely that PGE₂ and EP receptor agonists might share similar binding properties to microglial NOX2 as DPI and naloxone, hence eliciting similar biological effects.

Although we have identified microglial gp91^{phox} as a novel binding site for PGE₂ in eliciting its anti-inflammatory effect, the underlying mechanism(s) into how PGE₂ inhibits NOX2 activation remains to be determined. Our previous study indicated that after binding, the NOX2 inhibitor, may alter the conformation of gp91^{phox} and hinder the binding of cytosolic subunits, including p67^{phox} and p47^{phox}, thus preventing formation of an active enzyme complex[52]. Since the crystal structure of gp91^{phox} is still unavailable, we were not able to demonstrate this actual conformational change after treatment with PGE₂. Thus, we used an alternative approach by determining the translocation of cytosolic subunits to the cell membrane and demonstrated that PGE₂ reduces the translocation of p47^{phox} to the cell membrane (Fig. 6).

In summary, this study demonstrated that physiological concentrations of PGE₂ elicited potent anti-inflammatory/neuroprotective effects. These actions are mediated through a novel EP receptor-independent mechanism by inhibiting activation of microglial NOX2 via direct binding to its catalytic subunit gp91^{phox}. These results provide new insights into the potential physiological function of PGE₂ in maintaining CNS immune-homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Reduction of LPS-induced dopamine neuron toxicity in midbrain neuron-glia cultures after 7 days, as seen by (A) [³H] DA uptake, (B) TH-positive neuron count, and (C) immunostaining. Scale bar, 50 μm. Primary rat midbrain neuron-glia cultures were pretreated with PGE₂ for 30 minutes followed by LPS treatment (15 ng/ml). Values are mean ± SEM from three independent experiments, with duplicates for each experiment. Data were analyzed with one-way ANOVA followed by Bonferroni's *post hoc* multiple comparison test. * $P < 0.05$, ** $P < 0.01$, ### and *** $P < 0.0001$ Bonferroni's *t*-test compared to vehicle-treated group and LPS-treated group, respectively.

Figure 2.

Dose-dependent inhibitory effects of PGE₂ on LPS-stimulated production of pro-inflammatory factors (A) superoxide, (B) TNF- α , (C) NO and (D) MCP-1 with different IC₅₀ values. Rat mixed glia cultures were pretreated with various concentrations of PGE₂ for 30 minutes followed by LPS treatment. Values are mean $\pm$ SEM from three independent experiments, with duplicates for each experiment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ Bonferroni's t -test compared to LPS-only group.

Figure 3.

Rat mixed glia cultures were pretreated with various concentrations of (A) 17-phenyltrilor PGE₂, (B) butaprost, and (C) CAY10598 for 30 minutes, and then exposed to 20 ng/ml of LPS. (D) Rat mixed glia cultures were pretreated with a combination of 4 EP receptor subtype antagonists, SC-19220 (EP1, 3 μ M), AH-6809 (EP2, 3 μ M), L-798106 (EP3, 1 μ M) and AH-23848 (EP4, 1 μ M), for 30 minutes, followed by 10 nM of PGE₂ for additional 30 minutes, and then exposed to 20 ng/ml of LPS. (E) Wild-type (C57BL/6) and EP2 receptor KO mixed glia cultures were treated with various concentrations of PGE₂ for 30 minutes, and then exposed to 20 ng/ml of LPS. (F) EP2 receptor KO mixed glia cultures were treated with various concentrations of butaprost for 30 minutes, and then exposed to 20 ng/ml of LPS. The generation of superoxide was measured by monitoring the SOD-inhibitable reduction of WST-1. Values are mean $\pm$ SEM from four independent experiments, with duplicates. ** $P < 0.01$, *** $P < 0.0001$ Bonferroni's t -test compared to LPS-only group.

Figure 4.

Midbrain neuron-glia cultures from (A) wild-type and (B) gp91^{phox} KO mice were pre-treated with PGE₂ (10 nM) for 30 minutes and then exposed to LPS (15 ng/ml). After 7 days, the cultures were subjected to DA uptake assay. Values are mean $\pm$ SEM from three independent experiments, with triplicates. ** $P < 0.01$, Bonferroni's t -test compared to LPS-only group.

Figure 5.

(A) Wild-type COS7 cells and COS7 cells stably expressing gp91^{phox} and p22^{phox}, p47^{phox} and p67^{phox} (COS7-NOX2) cells were used to conduct the [³H]-labeled PGE₂ binding assay. (B) Wild-type (gp91^{phox+/+}) and gp91^{phox}-deficient (CYBB) macrophages were used to conduct the [³H]-labeled PGE₂ binding assay. Data are shown as a percentage of the wild-type control. Results are presented as means ± SEM from three independent experiments in duplicate. *** $P < 0.0001$ and ** $P < 0.01$, Paired t -test. Saturation curves of [³H]-PGE₂ binding were conducted using COS7-WT (C) and COS7-NOX2 cells (D). Total binding was measured by the binding of [³H]-labeled PGE₂ ranging from 0.03 to 3 nM while nonspecific binding was determined in the presence of additional 3 μM of PGE₂. Saturation isotherms were analyzed in GraphPad Prism 6. Specific binding was calculated by the difference between total binding and nonspecific binding. This experiment was conducted three times with similar results.

Figure 6.

(A) Primary microglial cells were pre-treated with PGE₂ or vehicle for 30 minutes and then stimulated with LPS (15 ng/ml) for 15 minutes. Cells were immunostained with a rabbit polyclonal antibody against p47^{phox} (green) and DAPI (blue). Scale bar, 10 μm. (B) and (C) HAPI cells (rat microglia cell line) were pretreated with PGE₂ (10 nM) or vehicle for 30 minutes, followed by LPS stimulation (15 ng/ml). After LPS stimulation for 15 minutes, subcellular fractions were isolated to conduct Western blot analysis in order to determine the level of p47^{phox} in the membrane fractions. gp91^{phox} was used as membrane internal control. Values are mean ± SEM from three independent experiments. *** $P < 0.0001$ Bonferroni's t -test compared to vehicle-treated group; ### $P < 0.0001$ Bonferroni's t -test compared to LPS-treated group.