Prostaglandins are powerful inducers of NGF and BDNF production in mouse astrocyte cultures

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Abstract We found that prostaglandin (PG) D_2 and PGE_2, which are major PGs in the brain of mammals, powerfully induced the secretion of nerve growth factor (NGF) from cultured mouse astrocytes; PGE_2 or PGD_2 induced an approximately 12- or 19-fold increase in NGF secretion after a 24-h incubation, respectively. Moreover, it was found that the sequential metabolites of PGD_2, PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2, induced the NGF secretion to the culture medium strikingly (60–98-fold of the control after a 24-h incubation). NGF secretion induced by the J_2 series of PGs was accompanied by the increased expression of NGF mRNA. These PGs also stimulated the secretion/synthesis of brain-derived neurotrophic factor (BDNF). Our findings suggest that PGs play a neuroprotective role by inducing NGF and BDNF production in the central nervous system.

Key words: Prostaglandin; Neurotrophin; Astrocyte; Neuroprotection; Nervous system

1. Introduction

Prostaglandins (PGs) are the major lipid mediators produced by cyclooxygenase activity, and they play important modulatory roles in various cells throughout the whole body. In the central nervous system (CNS), PGs and other bioactive lipids regulate various vital aspects of neural membrane biology, including protein–lipid interactions as well as trans-membrane and trans-synaptic signalings [1]. Some current reports raised a possibility that brain PGs are key players at the early stages of acute neural trauma and brain-cell damage associated with chronic neurodegenerative diseases such as Alzheimer's disease (AD) [2,3].

Nerve growth factor (NGF) is well known to be essential to preserve the cholinergic neurons of the basal nucleus of Meynert in culture, and it has been considered a potential therapeutic agent for the treatment of AD [4,5]. Brain-derived neurotrophic factor (BDNF) has clear neurorestorative and neuroprotective effects on dopaminergic neurons [6–8], and it is considered to contribute to neuroprotection in Parkinson's disease.

However, the effects of PGs on the production of neurotrophins have not yet been defined. Therefore, we investigated the effects of PGs on the synthesis/secretion of the above-mentioned neurotrophins by cultured mouse astrocytes.

2. Materials and methods

2.1. Materials

Anti-mouse β-NGF monoclonal antibody and anti-BDNF monoclonal antibody were purchased from Roche Molecular Biochemicals (Mannheim, Germany) and R&D systems (Minneapolis, MN, USA), respectively. Human recombinant BDNF and anti-BDNF polyclonal antibody were from Promega (Madison, WI, USA). $PGF_{2\alpha}$, PGI_2, PGE_2, PGD_2, PGA_2, PGB_2, PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}PGJ_2$ (15d-PGJ_2) were from Cayman (Ann Arbor, MI, USA). Lipopolysaccharide (LPS) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Astrocyte culture

Cortical astrocytes were derived from 2-day-old mice (ICR) as described previously [9] with minor modifications. After removal of the meninges, the cerebral cortex was cut into small pieces, and washed in Leibowitz L-15 medium. The pieces were then treated with 0.25% trypsin at 37°C for 25 min. After the trypsin had been neutralized in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) containing 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD, USA) and penicillin–streptomycin solution, the cells were rinsed and seeded into 25-cm^2 tissue culture flasks. The culture became almost confluent 4–5 days after the seeding, and the cells were then trypsinized and recultured in new flasks. This procedure was repeated twice. Finally, the cells were seeded at a density of 10^5 cells/cm^2 in 48-well plates or in 6-cm dishes. The confluent cells were exposed for about 10 days to FBS-free DMEM containing 0.5% bovine serum albumin, and then incubated with different concentrations of PGs for 24 h. Finally, the culture medium and cells were separately collected and used for the measurement of neurotrophin content.

2.3. Enzyme immunoassay of NGF

NGF content in the cell supernatant was measured by using a sensitive two-site enzyme-linked immunosorbent assay (ELISA) system as previously described [10] with slight modifications. Briefly, polystyrene microtiter plates (Corning, Corning, NY, USA) were coated for 1 h at room temperature with 50 μl of 0.2 μg/ml anti-β-NGF monoclonal antibody that had been diluted with 10 mM sodium carbonate–bicarbonate buffer, pH 9.3. After the plates had been washed three times with washing buffer (50 mM Tris–HCl, 200 mM NaCl, 10 mM $CaCl_2$, 0.1% Triton X-100; pH 7.0), non-specific binding sites were saturated with 10% Block Ace (Dainippon Pharmaceu-

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Abbreviations: PG, prostaglandin; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; AD, Alzheimer's disease; FBS, fetal bovine serum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide

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tical, Osaka, Japan), and the plates were then washed three times again with the buffer. Subsequently, 50 μl sample or β-NGF standard solution was added to each well of the plates, which were then incubated overnight at 4°C. Each well was next washed three times with the washing buffer, 50 μl of biotinylated anti-β-NGF monoclonal antibody conjugate was added, and incubation was continued for 1 h at 37°C. After the plates had been washed again three times, streptavidin-linked horseradish peroxidase (HRP) (Amgen, Thousand Oaks, CA, USA) was added to each well; then incubation was done for 1 h at room temperature, followed by five washes. The enzyme reaction was started by the addition of 100 μl of TMB-H_2O_2 solution (Kirkegaard and Perry, Gaithersburg, MD, USA), and stopped by the addition of 100 μl of 1 M H_3PO_4. The absorbance of the plates was read at a wavelength of 450 nm.

2.4. Enzyme immunoassay of BDNF

BDNF content in the cell supernatant was measured by the method described previously [11] with slight modifications. Briefly, the microtiter plates were coated with 50 μl of 1 μg/ml anti-BDNF monoclonal antibody. Human recombinant BDNF was used as a standard solution. Anti-BDNF polyclonal antibody was biotinylated for use as the secondary antibody. The bound antibody complex was measured with streptavidin-linked HRP by a method similar to that for NGF.

2.5. Semi-quantitative polymerase chain reaction (PCR)

After treatment of the astrocyte cultures with PGs for a desired period of time, the total RNA of the cells was extracted by using an RNeasy RNA extraction kit (Qiagen, Valencia, CA, USA), and then treated with DNase I (Invitrogen, San Diego, CA, USA). Reverse transcription (RT) was carried out with a Transcription Kit (Invitrogen). Aliquots of the cDNA were amplified in 25 μl of PCR cocktail containing each specific primer and KOD Dash DNA polymerase (Toyobo, Osaka, Japan). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) transcripts were used as a positive control. Based on the preliminary experiments conducted to establish the optimal conditions for the amplification of each primer pair, the target cDNAs of NGF, BDNF, and G3PDH cDNA were amplified for 32, 31, and 26 cycles, respectively. The following primer pairs were used: for NGF, 5′-AGTGAACATGCTGTGCCTCAAGCC-3′ (sense) and 5′-GGCAAGTCAGCCTCTTCTTGTAGC-3′ (antisense); for BDNF, 5′-AGGTGAGAAGAGTGATGACCATCC-3′ (sense) and 5′-CAACATAAATCCACTATCTTCCCC-3′ (antisense); and for G3PDH, 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ (sense) and 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (antisense). The expected product sizes were 937 bp for NGF, 777 bp for BDNF, and 983 bp for G3PDH. The PCR products were electrophoresed on an agarose gel containing ethidium bromide, and photographed under UV light. Intensity of the amplified bands was analyzed by using Scion Image software.

2.6. Statistical analysis

Data are presented as mean ± S.D. Significance was determined using one-way analysis of variance followed by a post-hoc test with statistical analysis software (SPSS, LEAD Technologies).

3. Results

We examined the effects of various natural PGs on the NGF secretion from cultured mouse astrocytes by ELISA. Fig. 1 shows the biosynthetic pathways of the PGs used. As shown in Fig. 2, $PGF_{2\alpha}$ and PGI_2 slightly stimulated the NGF secretion, whereas PGE_2 and PGD_2 stimulated it significantly. Galve-Roperh et al. reported that some lipid mediators such as ceramide [12] and LPS [13] stimulated NGF secretion from cultured astrocytes. They indicated the maximum level of NGF secretion after 24 h of treatment with 25 μM C_2-ceramide or 2 μg/ml LPS to be nine- or eight-fold, respectively, of the control. In our cultures, LPS similarly stimulated NGF secretion, and the maximum levels of NGF secretion elicited by 0.5 μg/ml LPS were about seven-fold of the control after 24 h (Fig. 1). In this study, the

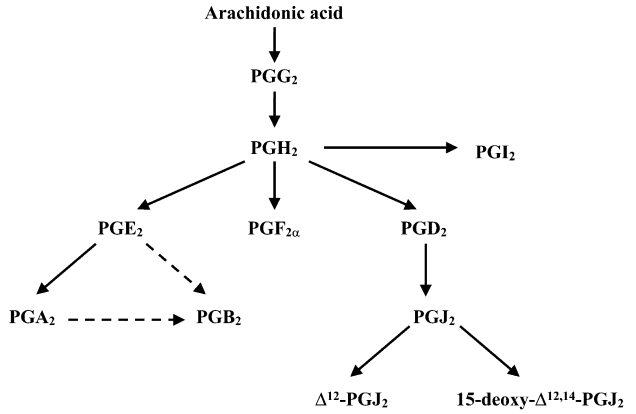

Fig. 1. The prostaglandin biosynthetic pathway. Arachidonic acid is first converted to the central prostanoid precursor PGH_2 via the action of the bifunctional enzyme PGH synthetase. PGH_2 is then converted to one of several related products, including PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2, through the action of specific PG synthetases. PGD_2 readily undergoes dehydration non-enzymatically to yield PGJ_2. PGJ_2 is further converted to Δ^{12}-PGJ_2 or 15d-PGJ_2; the formation of the former from PGJ_2 is accelerated by serum albumin. PGA_2 is the dehydration product of PGE_2, and under alkaline conditions, PGE_2 and PGA_2 are degraded to PGB_2.

maximum level of NGF secretion stimulated by PGE_2 or PGD_2 treatment was two- or seven-fold greater than the maximum level obtained with LPS.

PGE_2 is known to rapidly undergo non-enzymatic dehydration in culture medium containing serum albumin to produce PGA_2 [14]; and under alkaline conditions, PGE_2 and PGA_2 are degraded to PGB_2 [15]. So, we examined also the effects of PGA_2 and PGB_2. PGA_2 had a greater effect on NGF secretion from astrocytes than PGE_2, whereas PGB_2 had a weaker effect than PGE_2 (Fig. 3A). On the other hand, PGs of the J_2 series (PGJ_2, Δ^{12}-PGJ_2, and 15d-PGJ_2), naturally occurring derivatives of PGD_2 [16–18], showed a dramatically greater effect than PGD_2 on the NGF secretion from cultured astrocytes. As shown in Fig. 3B, PGD_2 induced an approximately 19-fold increase in NGF secretion, while PGJ_2 (30 μM), Δ^{12}-PGJ_2 (20 μM), and 15d-PGJ_2 (75 μM) caused an increase up to higher levels of 60-, 74-, and 98-fold, respectively, of the control.

To examine whether the stimulatory effects of PGD_2 and its metabolites on NGF secretion were accompanied by de novo synthesis of NGF protein, we determined the time dependence of their effects on NGF mRNA expression. Total RNA from the astrocytes treated with PGD_2, PGJ_2, Δ^{12}-PGJ_2, or 15d-PGJ_2 was analyzed by the RT-PCR method. As shown in Fig. 4, NGF mRNA expression began to be induced 3 h after the addition of these PGs and did not return to the basal level even after 48 h. The effects of PGD_2 and its metabolites on the NGF synthesis/secretion in cultured astrocytes were thus long-lasting.

BDNF mRNA expression level was also investigated, and we found that PGD_2 and its metabolites stimulated the expression of BDNF mRNA as well. Fig. 4B shows time courses of the level of BDNF mRNA after treatment of PGD_2 and its metabolites. The maximum levels of the BDNF mRNA were 2–2.8-fold of the control at around 12 h after the start of treatment with the PGs, and after 24 h they returned to the basal level almost completely. Unlike the case of NGF syn-

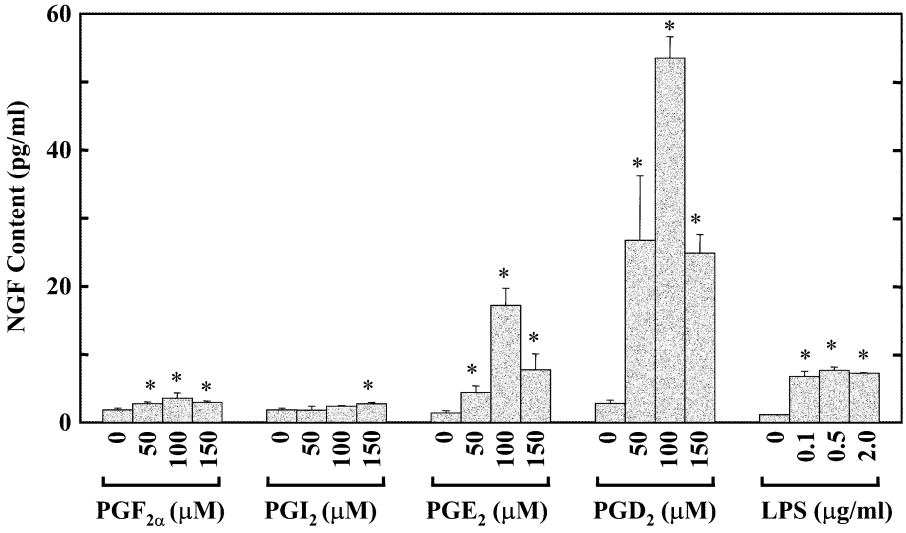

Fig. 2. Effects of PGs and LPS on the NGF secretion from mouse astrocytes. The cells were incubated with the indicated concentrations of PGs or LPS for 24 h. Results are the mean ± S.D. of three independent experiments. Statistically different data are indicated by asterisks ($P < 0.05$).

thesis, the BDNF synthesis induced by PGD_2 and its metabolites seemed to be comparatively transient. The amount of BDNF secreted into the culture medium was also examined. As shown in Fig. 5, even at 12 h after the start of treatment with these PGs, it was found that the BDNF secretion was stimulated, although the level of stimulation was lower than that for NGF. These levels of NGF and BDNF secretion are in good agreement with those of mRNA expression shown in Fig. 4.

4. Discussion

PGs are regarded as modulators of cellular functions in various physiological processes. As PGs have various functions in the CNS, they have recently been considered to serve as mediators of glial and neuronal functions [19,20].

Here we showed that several natural PGs induced NGF secretion markedly. The result suggests that PGs in the CNS act as potent inducers of NGF and thus would have neuroprotective effects. Especially, PGD_2 metabolites, i.e. cyclopentenone PGs of the J_2 series, stimulated the NGF secretion/synthesis quite potently and persistently. Also, these PGs stimulated the secretion/synthesis of BDNF in cultured astrocytes as well, although the levels were lower than those for NGF, and the stimulatory effect was comparatively transient. These differences in stimulation degree and term between NGF and BDNF may be due to some difference in the mechanisms for up-regulation by these PGs between these two neurotrophins.

It has been reported that PGD_2 is spontaneously dehydrated in aqueous solutions to yield PGJ_2 [16,17]. PGJ_2 is further converted to 15d-PGJ_2 in an albumin-independent manner or, alternatively, to Δ^{12}-PGJ_2 in an albumin-dependent manner [18]. PGE_2 is also known to be rapidly dehydrated to PGA_2 in culture medium containing serum albumin [14]. Actually, when PGD_2 or PGE_2 was incubated overnight

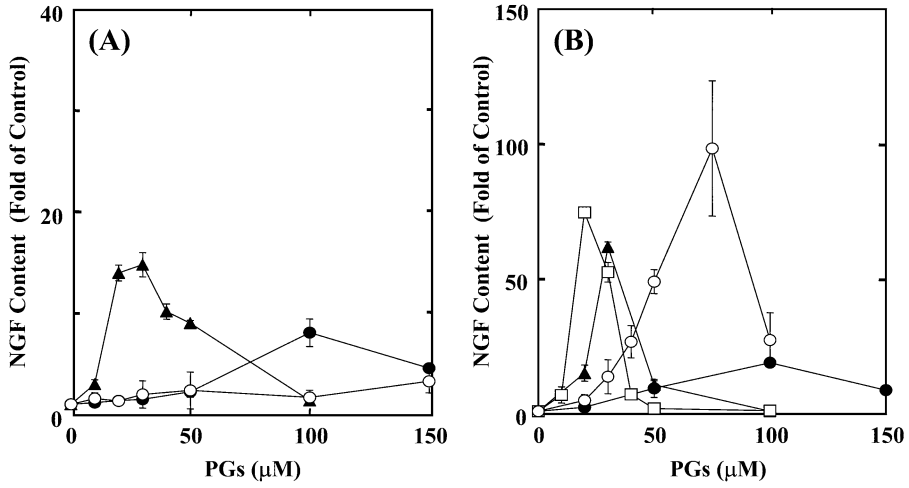

Fig. 3. Effects of PGE_2 and PGD_2 metabolites on the NGF secretion from mouse astrocytes. The cells were incubated with different concentrations of PGs for 24 h. A: PGE_2 (●) and its metabolites, PGA_2 (▲) and PGB_2 (○). B: PGD_2 (●) and its metabolites, PGJ_2 (▲), Δ^{12}-PGJ_2 (□), and 15d-PGJ_2 (○). Results are the mean ± S.D. of three independent experiments.

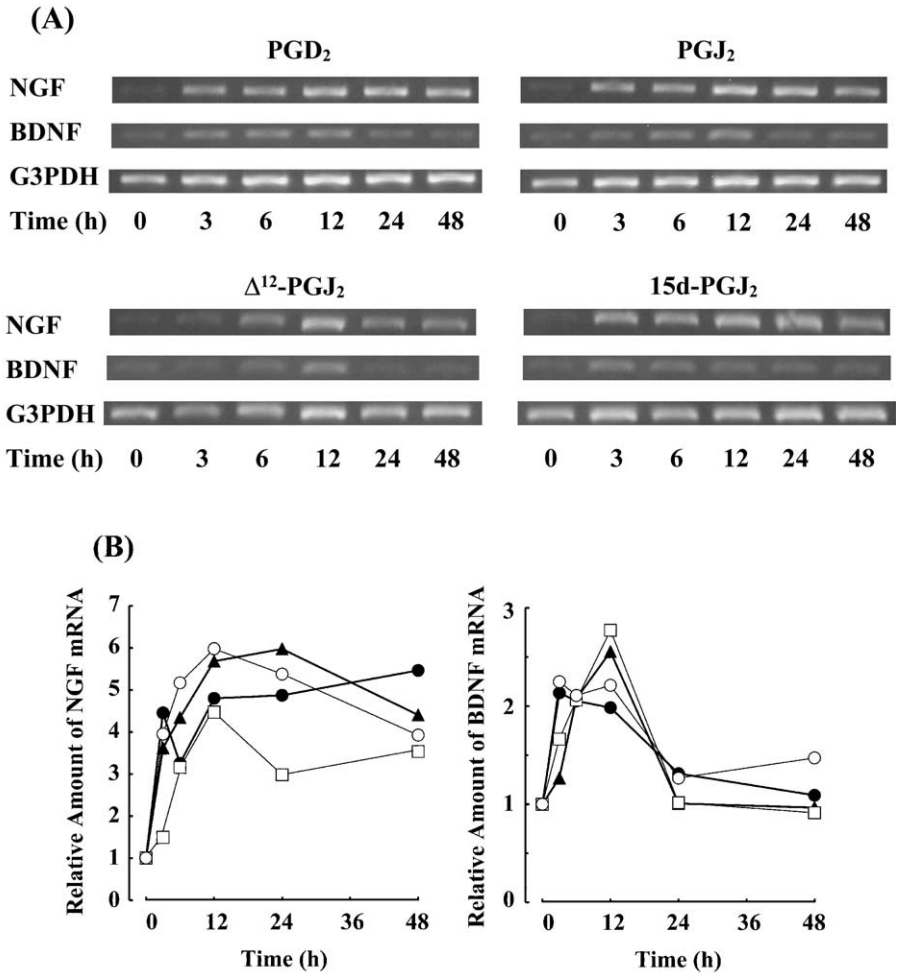

Fig. 4. Effects of PGD_2 and its metabolites on the mRNA levels of NGF, BDNF, and G3PDH. A: Total RNA from untreated astrocytes (0 h) or from those treated with 100 μM PGD_2, 30 μM PGJ_2, 20 μM Δ^{12}-PGJ_2, or 75 μM 15d-PGJ_2 for 3, 6, 12, 24, and 48 h was analyzed by the RT-PCR method. B: Time course of the mRNA expressions of NGF (left) and BDNF (right). The NGF, BDNF, and G3PDH intensities relative to those of control were calculated, and the ratios (relative NGF)/(relative G3PDH) and (relative BDNF)/(relative G3PDH) were used to indicate the relative amounts of NGF and BDNF gene expression, respectively. ●, PGD_2; ▲, PGJ_2; □, Δ^{12}-PGJ_2; ○, 15d-PGJ_2. Similar results were obtained in two independent experiments.

in our culture medium, we observed by thin-layer chromatography that almost all PGD_2 was converted to the J_2 series of PGs, and almost all PGE_2 to PGA_2. The J_2 series of PGs showed a dramatically greater effect on NGF secretion than PGD_2. Likewise, PGA_2 showed a greater effect than PGE_2. Therefore, the effect of PGD_2 or PGE_2 is likely to be mediated by their respective metabolic products.

At any rate, PGD_2 and its metabolites were found to be inducers of NGF and BDNF in cultured mouse astrocytes. PGD_2 is one of the most abundant PGs produced in the brain [21,22], and acts to induce physiological sleep [23–26]. As described above, PGD_2 is readily dehydrated to produce PGJ_2 and metabolized further to yield Δ^{12}-PGJ_2 or 15d-PGJ_2. It is clear that PGD_2 is converted to its metabolites in vivo as well as in vitro [18]. Consequently, it is conceivable that PGD_2 synthesized by PGD_2 synthase not only promotes sleep, but also, through its metabolism, may act on astrocytes in brain to stimulate potently the production of neurotrophins. It is known that neurotrophins not only prevent neuronal death but also promote regeneration of neural networks in the CNS. This hypothesis should provoke further research as to the roles of PGD_2, the most abundant prostanoid in the CNS. A variety of brain cells such as astrocytes, microglial cells, and neurons can synthesize PGs [27–29]. Since these PGs may have autocrine/paracrine actions, PGs produced in the brain may induce the synthesis of neurotrophins in astrocytes.

It has already been reported that LPS is an inducer of NGF secretion from astrocytes. But the details of the mechanism are not clear. On the other hand, it has been reported that LPS stimulates the release of PGE_2 from astrocytes in culture [30]. As we found in this study that PGE_2 stimulated NGF secretion from astrocytes, the effect of LPS on NGF secretion may be through the production of PGE_2 stimulated by the LPS treatment.

Recently, the association of low BDNF levels with major depressive disorders was suggested [31–33]. In animals subjected to forced swimming [34] or chronic immobilization stress [35,36], BDNF mRNA levels were significantly suppressed. Moreover, it was reported that the reduced BDNF levels in depressed patients recovered to basal levels after antidepressant treatment [37]. Our experiments revealed that PGD_2 and its metabolites stimulated the BDNF secretion/synthesis by mouse astrocytes. This is suggestive of correlation between depression and these PGs in the brain.

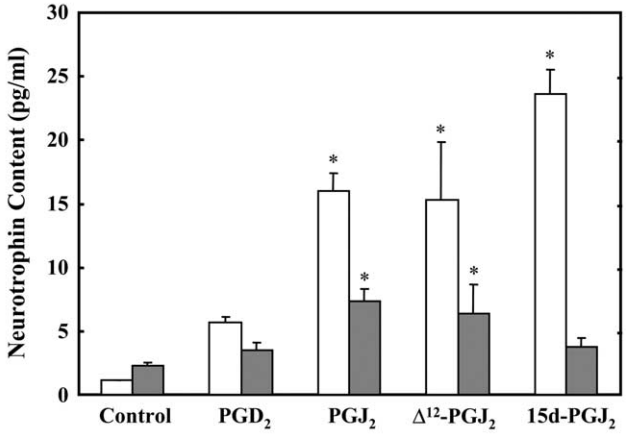

Fig. 5. The secretion levels of NGF and BDNF from mouse astrocytes after a 12-h incubation with PGD_2 and its metabolites. The cells were incubated for 12 h with 100 μM PGD_2, 30 μM PGJ_2, 20 μM Δ^{12}-PGJ_2, or 75 μM 15d-PGJ_2. NGF content (open bars) and BDNF content (solid bars) in the medium were measured by specific immunoassays as described in Section 2. Results are mean ± S.D. of two experiments. Statistically different data are indicated by asterisks ($P < 0.05$).

In conclusion, it is now certain that several natural PGs are powerful inducers of NGF and BDNF production in cultured mouse astrocytes. This fact suggests that PGs in the CNS may act neuroprotectively to induce the production/release of these neurotrophins by astrocytes.

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