Prostaglandin D₂ Inhibits Inducible Nitric Oxide Synthase Expression in Rat Vascular Smooth Muscle Cells

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Abstract—Vascular smooth muscle cells (VSMCs) as well as macrophages have been shown to generate a substantial amount of NO in inflammatory vascular lesions. Prostaglandin (PG) D₂ (PGD₂) is produced by inflammatory cells, including mast cells and macrophages. We investigated whether PGD₂ modulates NO metabolism in rat VSMCs. PGD₂ at a concentration of 10⁻⁷ mol/L or greater dose-dependently inhibited nitrite accumulation in the medium of cultured VSMCs stimulated with interleukin 1β (IL-1β). In a dose-response analysis of IL-1β and nitrite accumulation, PGD₂ was seen to decrease the maximal ability of VSMCs to generate NO, arguing against competition by PGD₂ at cytokine receptors. Northern analysis showed that PGD₂ suppresses induction of inducible NO synthase (iNOS) mRNA in IL-1β–stimulated VSMCs, with consequent inhibition of iNOS protein expression in Western analysis. A thromboxane A₂ (TXA₂) analogue, U46619 (10⁻⁵ mol/L), produced less inhibition of NO generation than did PGD₂. Neither the PGI₂ analog carbasprostacyclin nor PGE₁ showed any inhibition. PGD₂ dose-dependently inhibited NO generation despite the addition of the TXA₂ antagonist SQ29548. PGJ₂, Δ¹₂-PGJ₂, and 15-deoxy-Δ¹₂,1⁴-PGJ₂, all metabolites of PGD₂, were as potent as or slightly stronger than PGD₂ in the inhibition of NO generation. These data suggest that PGD₂ suppresses NO generation in VSMCs by inhibiting iNOS mRNA expression, most likely through the cascade of the PGJ₂ series rather than through the TXA₂ receptor or cAMP upregulation. Such action makes it likely that PGD₂ regulates NO metabolism in vascular lesions. (Circ Res. 1998;82:204-209.)

Key Words: inducible nitric oxide synthase ■ prostaglandin D₂ ■ vascular smooth muscle cells ■ expression inhibition ■ prostaglandin J₂

Nitric oxide has been highlighted as a potent chemical mediator of vascular, immune, and neural function.¹ Enhanced expression of iNOS and increased NO generation have also been reported with various inflammatory states, including atherosclerosis,²,³ vasculopathy in septic shock,⁴ and postangioplastic vascular injury.⁵ In atherosclerosis, inflammatory cells such as macrophages and lymphocytes infiltrate into the lesions,⁶ releasing various mediators including cytokines.⁷ Recent studies have found NO generation to be enhanced in atherosclerotic lesions.⁵ In addition, VSMCs may be more important than macrophages as a source of NO in atherosclerotic lesions.³

PGD₂ is a metabolite of arachidonic acid derived from PGH₂, a common precursor in the synthesis of most PGs. PGD₂ is predominant among prostanoids in many organs, including liver, spleen, central nerve system, and intestines.⁸ PGD₂ inhibits platelet aggregation through activation of adenylate cyclase,⁹ relaxes or constricts arterial vessels according to viability of the endothelium,¹⁰ and acts as a neurotransmitter in the brain, mediating sleep.¹¹ PGD₂ and its metabolites also are known to inhibit proliferation of various cell lines.¹² More interestingly, PGD₂ is generated in arteries by endothelial cells,¹³ platelets,¹⁴ macrophages,¹⁵ and mast cells,¹⁶,¹⁷ which are the component cells of atheromatous lesions.⁶,¹⁸

To the best of our knowledge, however, few studies have directly addressed the roles of PGD₂ in the pathogenesis of vascular lesions. Hypothesizing that an interaction between PGD₂ and NO exists in vessel walls, we investigated the effect of PGD₂ on NO metabolism in cultured rat VSMCs.

Materials and Methods

Materials
PGD₂, Δ¹₂-PGJ₂, and PGE₁ were gifts from Ono Pharmaceutical (Osaka, Japan). All other prostanoids and related reagents were purchased from Cayman Chemical. Recombinant murine IL-1β and TNF-α were purchased from R&D Systems. Reduced NADPH was obtained from Sigma. cDNA probes for murine β-actin and GAPDH were purchased from Wako and the American Type Culture Collection (ATCC No. 57091), respectively. Moloney murine leukemia virus reverse transcriptase was obtained from GIBCO-BRL, and recombinant Taq DNA polymerase was obtained from TaKaRa Biomedicals. Deoxyxycytidine 5'-[α-³²P]triphosphate and 1-[(4,5-³H]leucine were purchased from DuPont-NEN. Other materials and reagents were obtained from commercial sources.

Cell Culture and Incubation
VSMCs were isolated from the thoracic aorta of 10- to 12-week-old male Wistar-Kyoto rats (Doken, Shimodate, Japan) by a standard explant method of Ross et al¹⁹ and grown in DMEM supplemented with 10% FBS.
with 10% fetal calf serum (Bioserum) and antibiotics. Culture purity was assayed by immunofluorescence staining with a monoclonal antibody specific for smooth muscle α-actin. Confluent cells between the 10th and 20th passages were used for all experiments. Incubation of cultured cells was performed at 37°C in a humidified atmosphere of 95% air/5% CO2. Solutions of cytokines were prepared with DMEM of cultured cells. After incubation periods with respective test compounds, VSMCs in 24-well culture clusters, aliquots of the culture were harvested and sonicated in ice-cold Tris buffer containing 50 mmol/L Tris-HCl (pH 7.5), 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 1 mmol/L leupeptin, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L dithiothreitol. The homogenate was centrifuged at 100 000 g for 60 minutes at 4°C. The supernatant containing cell-free enzyme was subjected to 12% SDS-PAGE and gel-fuged at 100 000 g for 60 minutes at 4°C.

Measurement of NO Production

NO production was measured as nitrite accumulation in medium from cultured VSMCs. After incubation of the cells in serum-free DMEM with the respective test compounds in 24-well culture clusters, aliquots of the supernatant containing NO synthase was partially purified enzyme was subjected to 12% SDS-PAGE and gel-fuged at 100 000 g for 60 minutes at 4°C. The supernatant containing NO synthase was subjected to 12% SDS-PAGE and gel-fuged at 100 000 g for 60 minutes at 4°C. The supernatant containing NO synthase was ligated into pCR II cloning method. The nucleotide sequence of the subcloned cDNA was determined by the diodeoxyxynucleotide chain-termination method using an autosequencer (373 DNA sequencing system, Perkin-Elmer). The sequence coincided with that of iNOS reported previously with 98.5% similarity. An insert of BosX restriction fragment of the iNOS plasmid was used as a probe for Northern blotting.

Selected Abbreviations and Acronyms

IL-1β = interleukin 1β
iNOS = inducible NO synthase
PCR = polymerase chain reaction
PG = prostaglandin
PKC = protein kinase C
TCA = trichloroacetic acid
TNF-α = tumor necrosis factor α
TX = thromboxane
VSMC = vascular smooth muscle cell

Measurement of Total Protein Synthesis

VSMCs grown in 24-well culture clusters were incubated for 24 hours in serum-free DMEM containing 1 μCi/ml [3H]leucine and various concentrations of PGD2. After harvesting the cells, cellular protein was precipitated with ice-cold 10% TCA and redissolved in 0.2N NaOH. The homogenate was centrifuged at 100 000 g for 60 minutes at 4°C. The supernatant containing protein was incubated with 10 mmol/L SDS and was autoradiographed on radiographic film at 80°C.

Measurement of NO Production

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Western Analysis

Polyclonal anti-iNOS antibody was raised as reported previously. After incubation periods with respective test compounds, VSMCs in culture were harvested and sonicated in ice-cold Tris buffer containing 50 mmol/L Tris-HCl (pH 7.5), 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 1 mmol/L leupeptin, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L dithiothreitol. The homogenate was centrifuged at 100 000 g for 60 minutes at 4°C. The supernatant containing 5 ng cytosolic protein was incubated at 4°C for 30 minutes with ADP Sophorose gel (Pharmacia) with gentle agitation. NO synthase was dose-dependently inhibited by N⁰-nonmonomethyl-L-arginine, an action reversed by l-arginine (data not shown).

Preparation of cDNA Probe for iNOS

Total RNA was extracted from IL-1β-stimulated VSMCs by an acid-agonadimium-thiocyanate method and subjected to reverse-transcription PCR. The cDNA was amplified by PCR with primers for rat iNOS (upper, 5'CATGGCTTCCCCGGTCAGAG 3'; lower, 5'TCCAG-CACCTCCAGGAACT 3') corresponding to nucleotide sequences 1587 to 1606 and 2642 to 2623, respectively; with 32 cycles of denaturing at 94°C for 1 minute, annealing at 56°C for 1 minute, and extension at 72°C for 1.5 minutes. PCR product of the expected size was

Figure 1. Effect of PGD2 on NO generation in cytokine-stimulated VSMCs. VSMCs were incubated for 24 hours with 20 U/mL IL-1β (A) or 30 ng/mL TNF-α (B) with the indicated concentrations of PGD2 for measurement of nitrite. Columns and bars represent mean±SEM (n=5), (¢) indicates without cytokine. *P<.01 vs 0 mol/L.

Statistical Analysis

Values are expressed as mean±SEM. Statistical analysis was performed by unpaired Student’s t test or by ANOVA followed by Scheffe’s F (multiple-comparison) test. Values of P<.05 were considered statistically significant.

Results

Effect of PGD2 on Cytokine-Stimulated NO Production in VSMCs

We incubated VSMCs in culture with 20 U/mL IL-1β or 30 ng/mL TNF-α in the presence of various concentrations of PGD2 (Fig 1). Nitrite accumulation in the culture medium over a 24-hour period was measured. Stimulation of VSMCs with IL-1β or TNF-α caused remarkable accumulation of nitrite. This was dose-dependently inhibited by simultaneous treatment of the cells with PGD2 at doses of 10−12 mol/L or greater.

We next examined the time course of nitrite accumulation in the culture medium, stimulating the cells with a cytokine cocktail (20 U/mL IL-1β and 30 ng/mL TNF-α) in the presence or absence of 10−5 mol/L PGD2 for various intervals of time (Fig 2A). Nitrite accumulation increased with time, over 24 hours, an effect suppressed by PGD2.

In a dose-response curve of IL-1β concentration and nitrite accumulation (Fig 2B), treatment with PGD2 mainly shifted the curve downward; i.e., PGD2 lessened the maximal response (nmol/10⁶ cells per 24 hours: 35.8±0.41 at 0 mol/L,
22.2±0.11 at 10⁻⁶ mol/L, and 11.4±0.15 at 10⁻⁵ mol/L PGD₂). PGD₂ also shifted the curve slightly to the right (ED₅₀ in U/mL: 3.85 at 0 mol/L, 7.58 at 10⁻⁶ mol/L, and 9.89 at 10⁻⁵ mol/L PGD₂). These data suggest that the inhibitory effect of PGD₂ is not due to antagonism at cytokine receptors.

In a chronological analysis of the inhibitory action of PGD₂ (Fig 2C), cultured VSMCs were incubated for 24 hours with the cytokine cocktail, and PGD₂ (3×10⁻⁵ mol/L) was added into the culture medium at various intervals after the start of incubation with cytokines. Nitrite accumulation in the culture medium over the 24-hour incubation period was measured. The addition of PGD₂ within 6 hours from the start effectively inhibited NO generation, whereas the inhibition was strikingly lessened when PGD₂ addition was delayed over 6 hours, which may represent a critical time point for PGD₂ inhibition of NO synthesis.

**Effect of PGD₂ on iNOS mRNA and Protein Expression**

We next examined whether PGD₂ influences cytokine-induced expression of iNOS mRNA or iNOS protein (Fig 3). VSMCs in culture were incubated for 24 hours with 20 U/mL IL-1β or its vehicle in the presence of various concentrations of PGD₂, after which total RNA or partially purified iNOS protein was extracted. In the Northern analysis (Fig 3A), PGD₂ dose-dependently inhibited IL-1β-stimulated induction of iNOS mRNA. PGD₂ at a concentration of 10⁻⁶ mol/L or greater effectively decreased iNOS mRNA expression. IL-1β suppressed β-actin mRNA expression, whereas PGD₂ restored it, arguing against an overall cytotoxic effect of PGD₂. In Western analysis (Fig 3B), PGD₂ dose-dependently inhibited a cytokine-stimulated increase in iNOS protein.

**Effect of PGD₂ on Total Protein Synthesis**

PGD₂, at concentrations up to 10⁻⁴ mol/L, did not significantly affect total protein synthesis in VSMCs (Table), providing further evidence that the reduction of iNOS expression by PGD₂ is not due to cytotoxicity.

**Effects of Related Prostanoids on NO Generation in VSMCs**

We compared the effect of PGD₂ with those of related prostanoids (Fig 4A). VSMCs were incubated for 24 hours with 20 U/mL IL-1β in the presence of various concentrations of PGD₂, U46619 (a stable TXA₂ analogue), carbaprostacyclin (a stable PGI₂ analogue), or PGE₁, after which nitrite accumulation in the medium was measured. PGD₂ reportedly acts as an agonist at TXA₂ receptors. In the present study, U46619 at 10⁻⁵ mol/L significantly, but less potently than PGD₂, reduced NO
generation in VSMCs. Both carboprostacyclin and PGE₁, which upregulate intracellular cAMP in VSMCs, 24–26 slightly increased NO production by VSMCs.

We next examined the effect of PGD₂ on NO generation in VSMCs stimulated for 24 hours with 20 U/mL IL-1β in the presence of the TXA₂ receptor antagonist SQ29548 (Fig 4B). In the present assay system, 10⁻⁴ mol/L of SQ29548 reversed the inhibitory effect of 10⁻⁵ mol/L U46619 by 70% (data not shown). SQ29548 did not alter the pattern of inhibition by PGD₂ (Fig 4B), although it slightly reversed the effect of PGD₂ (P<.01 by two-way ANOVA for 10⁻⁷ to 10⁻⁴ mol/L PGD₂).

Effects of PGs of the J₂ Series on NO Production in VSMCs

Since PGD₂ is converted to PGJ₂ and its metabolites (Fig 5A), 27–30 we next examined the effects of these PGD₂ metabolites on NO production by VSMCs (Fig 5B). VSMCs were incubated for 24 hours with 20 U/mL IL-1β and various concentrations of PGD₂, PGJ₂, Δ¹₂-PGJ₂, or 15-deoxy-Δ¹₂,14-PGJ₂, after which nitrite accumulation in the medium was measured. PGJ₂, Δ¹₂-PGJ₂, and 15-deoxy-Δ¹₂,14-PGJ₂ were equipotent with or slightly stronger than PGD₂ in inhibition. The calculated doses of the PGs (expressed as negative log mol/L) evoking 50% inhibition were 6.03 (15-deoxy-Δ¹₂,14-PGJ₂), 5.53 (Δ¹₂-PGJ₂), 5.47 (PGJ₂), and 5.43 (PGD₂), respectively.

We also verified the inhibitory effect of 15-deoxy-Δ¹₂,14-PGJ₂ on the expression of iNOS mRNA in VSMCs (Fig 6).

Discussion

We investigated whether PGD₂ modulates NO synthesis in VSMCs. To the best of our knowledge, this is the first report demonstrating a relationship between PGD₂ and NO synthase expression. In the present study, PGD₂ at ≥10⁻⁷ mol/L dose-dependently inhibited cytokine-induced NO production in cultured VSMCs (Fig 1). Stimulation of VSMCs by the cytokine cocktail resulted in time-dependent accumulation of nitrite over 24 hours, which PGD₂ suppressed (Fig 2A). Downward shift of the dose-response curve of IL-1β and
nitrite accumulation ruled out antagonism by PGD₂ at cytokine receptors (Fig 2B). The chronological analysis indicated the existence of a critical time point of PGD₂ addition for inhibition, suggesting impairment of iNOS expression by PGD₂ (Fig 2C). PGD₂ was shown to inhibit expression of the iNOS mRNA and the iNOS protein (Fig 3). TXA₂ analogue U46619 slightly, and less effectively than PGD₂, reduced NO production by VSMCs, whereas neither the PGI₂ analogue carprofen nor PGE₁ had an inhibitory effect (Fig 4A). Even under blockade of TXA₂ receptors by SQ29548, PGD₂ dose-dependently inhibited the NO generation (Fig 4B). PGJ₂, Δ₁₂-PGJ₂, and 15-deoxy-Δ₁₂,14-PGJ₂ were at least as potent as PGD₂ in inhibiting NO generation (Fig 5). Finally, 15-deoxy-Δ₁₂,14-PGJ₂ inhibited iNOS mRNA expression in VSMCs (Fig 6). These data suggest that PGD₂ inhibits iNOS mRNA expression in VSMCs most likely through the PGJ₂ cascade.

Recent studies have demonstrated that NO production is enhanced in inflammatory vascular lesions such as atherosclerosis, where inflammatory cells (including macrophages, lymphocytes, platelets, and mast cells) infiltrate the thickened intima. In such circumstances, large amounts of cytokines are generated, which are likely to cause massive NO generation through induction of iNOS. Furthermore, some reports have suggested that VSMCs may be the primary source of NO in atherosclerotic lesions. On the other hand, PGD₂ reportedly is produced by endothelial cells, platelets, macrophages, and mast cells. Mast cells in particular play a pivotal role in formation of atheromatous lesions and, in addition, have a potent capacity to produce substantial amounts of PGD₂, releasing secretory granules that contain cyclooxygenase and are able to generate PGD₂. Moreover, granules phagocytosed by macrophages or VSMCs might release considerable amounts of PGD₂ into the intracellular space of, or the extracellular space near, NO-producing cells. Accordingly, we think it important to address the interaction between PGD₂ and NO in vascular lesions.

The present data suggest that PGD₂ may act as a local mediator suppressing iNOS induction and consequent massive NO generation. Some steroids also have been demonstrated to inhibit iNOS expression in vascular cells, consistent with their use in treating septic shock. In cultured rat VSMCs, 10⁻⁷ mol/L of dexamethasone is sufficient to suppress iNOS expression. In the present study, concentrations of PGD₂ or its metabolites one to two orders higher were required to equal the degree of inhibition by steroids. Steroids, however, are circulating hormones, whereas prostanoids generally behave as local tissue mediators. Much higher concentrations of the PGs are attainable in limited local areas, where they are generated by inflammatory or vascular cells. PGD₂ actually has been found abundantly in homogenates from various tissues, including rat spleen, where its concentrations in extracts are >10⁻⁵ mol/L. Furthermore, PGD₂ and its J₃ metabolites have been demonstrated to promote adipocyte differentiation with EC₅₀ values in excess of 10⁻⁵ mol/L, similar to those values for inhibition of NO generation in VSMCs. These findings, combined with the present data, support the pathophysiological involvement of PGD₂ at these high concentrations in NO metabolism.

PGD₂ increases intracellular cAMP via adenylyl cyclase activation in platelets. The cell-surface PGD receptor is G protein-coupled and transmits a signal leading to cAMP upregulation. In cultured VSMCs and mesangial cells, cAMP-elevating agents, including PGI₂ and PGE₁, enhance iNOS expression and NO generation. PGD₂, like dexamethasone, is sufficient to suppress iNOS expression. Angiotensin II also inhibits iNOS expression in VSMCs, at least in part through upregulation of PKC. PGD₂ also can act as an agonist of the vasocostritor TXA₂, which elevates PKC levels in VSMCs. However, we observed only a slight inhibitory effect of the TXA₂ analogue on NO generation in VSMCs, failing to adequately explain the mechanism of the effect of PGD₂ in this manner. In fact, PGD₂ addition dose-dependently inhibited the NO generation even under blockade of TXA₂ receptors, indicating that a pathway other than via TXA₂ receptors is predominantly involved in the effect of PGD₂.

PGD₂ readily undergoes conversion in vivo and in vitro to yield additional, biologically active PGs of the J₃ series. Incubation of PGD₂ in the presence of plasma or serum albumin results in the rapid accumulation of several major degradation and isomerization products, including Δ₁₂-PGJ₂ and 15-deoxy-Δ₁₂,14-PGJ₂. Some of these products, including Δ₁₂-PGJ₂, have antiproliferative effects, presumably involving their direct binding to nuclear proteins. Furthermore, 15-deoxy-Δ₁₂,14-PGJ₂, a PGJ₂ derivative, recently has been demonstrated to be a natural ligand for an intracellular receptor promoting adipocyte differentiation. In the present study, the four PGs showed doses producing 50% inhibition (expressed as negative log mol/L) in the following order: 15-deoxy-Δ₁₂,14-PGJ₂>Δ₁₂-PGJ₂>PGE₁>PGD₂, which may reflect the delay involved in conversion of these products. These results suggest that the inhibitory effect of PGD₂ on NO generation is exhibited most likely through the PGJ₂ cascade. Therefore, although the inhibitory effect of PGD₂ on iNOS induction may be partially mediated by TXA₂ receptors, it appears to result mainly from a direct action of PGD₂, or its J₃ metabolites on intranuclear components rather than via the adenylyl cyclase–cAMP or inositol phospholipid pathway.
id–PKC pathway. The need for relatively high doses of the PGs also argues for this, because it would require much higher doses in the culture media to attain concentrations high enough to act on intracellular receptors than cell-surface ones. An intracrine mechanism might cause considerably high intracellular concentrations. The subcellular pathway downstream of the PGJ2 cascade remains to be investigated.

In conclusion, we demonstrated that PGD2 suppresses NO generation in VSMCs, which is due to inhibition of iNOS mRNA expression most likely operating mainly through a novel PGD2 signaling pathway, the PGJ2 cascade. These results suggest the possibility of involvement of PGD2 in NO metabolism in the pathogenesis of vascular diseases.

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