Prostaglandin D$_2$ 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) D$_2$ (PGD$_2$) is produced by inflammatory cells, including mast cells and macrophages. We investigated whether PGD$_2$ modulates NO metabolism in rat VSMCs. PGD$_2$ 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, PGD$_2$ was seen to decrease the maximal ability of VSMCs to generate NO, arguing against competition by PGD$_2$ at cytokine receptors. Northern analysis showed that PGD$_2$ 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 A$_2$ (TXA$_2$) analogue, U46619 (10$^{-5}$ mol/L), produced less inhibition of NO generation than did PGD$_2$. Neither the PGI$_2$ analog carboxaprostacyclin nor PGE$_1$ showed any inhibition. PGD$_2$ dose-dependently inhibited NO generation despite the addition of the TXA$_2$ antagonist SQ29548. PGJ$_2$, 12,14-PGJ$_2$, and 15-deoxy-Δ$^{12,14}$-PGJ$_2$, all metabolites of PGD$_2$, were as potent as or slightly stronger than PGD$_2$ in the inhibition of NO generation. These data suggest that PGD$_2$ suppresses NO generation in VSMCs by inhibiting iNOS mRNA expression, most likely through the cascade of the PGJ$_2$ series rather than through the TX receptor or CAMP upregulation. Such action makes it likely that PGD$_2$ regulates NO metabolism in vascular lesions. (Circ Res. 1998;82:204-209.)

Key Words: inducible nitric oxide synthase ■ prostaglandin D$_2$ ■ vascular smooth muscle cells ■ expression inhibition ■ prostaglandin J$_2$

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

PGD$_2$ is a metabolite of arachidonic acid derived from PGH$_2$, a common precursor in the synthesis of most PGs. PGD$_2$ is predominant among prostanooids in many organs, including liver, spleen, central nerve system, and intestines. PGD$_2$ inhibits platelet aggregation through activation of adenylate cyclase, relaxes or constricts arterial vessels according to viability of the endothelium, and acts as a neurotransmitter in the brain, mediating sleep. PGD$_2$ and its metabolites also are known to inhibit proliferation of various cell lines. More interestingly, PGD$_2$ is generated in arteries by endothelial cells, platelets, macrophages, and mast cells, which are the component cells of atheromatous lesions.

To the best of our knowledge, however, few studies have directly addressed the roles of PGD$_2$ in the pathogenesis of vascular lesions. Hypothesizing that an interaction between PGD$_2$ and NO exists in vessel walls, we investigated the effect of PGD$_2$ on NO metabolism in cultured rat VSMCs.

Materials and Methods

Materials

PGD$_2$, 12-PGJ$_2$, and PGE$_1$ were gifts from Ono Pharmaceutical (Osaka, Japan). All other prostanooids 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. Deoxyxytidine 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 al and grown in DMEM supplemented...
with 10% fetal calf serum (Bioserum) and antibiotics. Culture purity was assessed 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 containing 0.1% bovine serum albumin.

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 media were mixed with an equal volume of Griess reagent [1% sulfanilamide/0.1% N-(1-naphthyl)ethylenediamine dihydrochloride/5% phosphoric acid]. Optical absorption was measured spectrophotometrically at a wavelength of 540 nm, using sodium nitrite as the standard. Medium incubated in a cell-free well was used to obtain a baseline value. In the present assay system, nitrite accumulation induced by cytokines was dose-dependently inhibited by N⁵-monomethyl-L-arginine, an action reversed by l-arginine (data not shown).

Western Analysis
Polyclonal anti-iNOS antibody was raised as reported previously. Twenty micrograms of total RNA was separated by formaldehyde/1.0% agarose gel electrophoresis and transferred to a nylon membrane (Hybond N⁺, Amersham). Nitrite synthase protein was detected with anti-iNOS antibody using peroxidase-labeled anti-rabbit IgG as the second antibody (Hibond N⁺, Millipore). NO synthase protein was detected with anti-iNOS antibody using peroxidase-labeled anti-rabbit IgG as the second antibody and 4-chloro-1-naphthol as the substrate.

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. After neutralization with HCl, TCA again was added, and the radioactivity of [³H]leucine incorporated into the TCA-insoluble cellular fraction was measured by liquid scintillation counting.

Preparation of cDNA Probe for iNOS
Total RNA was extracted from IL-1β-stimulated VSMCs by an acid-guanidinium-thiocyanate method and subjected to reverse-transcription PCR. The cDNA was amplified by PCR with primers for rat iNOS (upper, 5’CATGCTTCCCCGTCAGAG 3’; lower, 5’TCCAGCACCCTCCAGGAAGT 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 ligated into pCR II plasmid vector (Invitrogen) by a T/A cloning method. The nucleotide sequence of the subcloned cDNA was determined by the deoxyoligonucleotide 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 BstXI restriction fragment of the iNOS plasmid was used as a probe for Northern blotting.

Northern Analysis
After respective incubation periods of cultured VSMCs, total RNA was extracted by the acid-guanidinium-thiocyanate method. Thirty micrograms of total RNA was separated by 1% agarose gel electrophoresis and transferred to a nylon membrane (Hibond N⁺, Amersham) in 20× SSC. After ultraviolet wave cross-linking, RNA immobilized on the membrane was hybridized for 16 hours at 65°C with ³²P-labeled cDNA probe for rat iNOS, murine β-actin, or human GAPDH in hybridization buffer (Amersham). The membrane was washed finally at 65°C in 0.1× SSC containing 0.1% SDS and was autoradiographed on radiographic film at −80°C.

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<0.05 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 10⁻⁴ 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 nitrate accumulation (Fig 2B), treatment with PGD₂ mainly shifted the curve downward; i.e., PGD₂ lessened the maximal response (mmol/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 PGD_2). PGD_2 also shifted the curve slightly to the right (ED_{50} in U/mL: 3.85 at 0 mol/L, 7.58 at 10^{-4} mol/L, and 9.89 at 10^{-5} mol/L PGD_2). These data suggest that the inhibitory effect of PGD_2 is not due to antagonism at cytokine receptors.

In a chronological analysis of the inhibitory action of PGD_2 (Fig 2C), cultured VSMCs were incubated for 24 hours with the cytokine cocktail, and PGD_2 (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 PGD_2 within 6 hours from the start effectively inhibited NO generation, whereas the inhibition was strikingly lessened when PGD_2 addition was delayed over 6 hours, which may represent a critical time point for PGD_2 inhibition of NO synthesis.

**Effect of PGD_2 on iNOS mRNA and Protein Expression**

We next examined whether PGD_2 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 PGD_2, after which total RNA or partially purified iNOS protein was extracted. In the Northern analysis (Fig 3A), PGD_2 dose-dependently inhibited IL-1β-stimulated induction of iNOS mRNA. PGD_2 at a concentration of 10^{-6} mol/L or greater effectively decreased iNOS mRNA expression. IL-1β suppressed β-actin mRNA expression, whereas PGD_2 restored it, arguing against an overall cytotoxic effect of PGD_2.

In Western analysis (Fig 3B), PGD_2 dose-dependently inhibited a cytokine-stimulated increase in iNOS protein.

**Effect of PGD_2 on Total Protein Synthesis**

PGD_2, 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 PGD_2 is not due to cytotoxicity.

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

We compared the effect of PGD_2 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 PGD_2, U46619 (a stable TXA_2 analogue), carbaprostacyclin (a stable PGI_2 analogue), or PGE_1, after which nitrite accumulation in the medium was measured. PGD_2 reportedly acts as an agonist at TXA_2 receptors. In the present study, U46619 at 10^{-5} mol/L significantly, but less potently than PGD_2, reduced NO
generation in VSMCs. Both carbaprostacyclin 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
Prostaglandin D₂ and Nitric Oxide

nitrile accumulation ruled out antagonism by PGD₂ at cyto-
kiné 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 PG₁ analogue
carbaprostacyclin 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 atheroscle-
sis,1,2 where inflammatory cells (including macrophages, lympho-
cytes, platelets, and mast cells) infiltrate the thickened intima.6,18
In such circumstances, large amounts of cytokines are generated,7
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.1 On the other hand, PGD₂ reportedly is produced by
endothelial cells,7 platelets,1 macrophages,19 and mast cells.20
Mast cells in particular play a pivotal role in formation of
atheromatous lesions21 and, in addition, have a potent capacity
to produce substantial amounts of PGD₂, releasing secretory granules
that contain cyclooxygenase and are able to generate PGD₂.15,17
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.15
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 gener-
ation. Some steroids also have been demonstrated to inhibit iNOS
expression in vascular cells,22,23 consistent with their use in treating
septic shock.4 In cultured rat VSMCs, 10⁻⁷ mol/L of dexameth-
sone is sufficient to suppress iNOS expression.32 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
prostaglandins 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 >10⁻⁴ mol/L.24 Furthermore, PGD₂ and its J₂ metab-
olites have been demonstrated to promote adipocyte differentia-
tion 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 pathophys-
iological involvement of PGD₂ at these high concentrations in
NO metabolism.

PGD₂ increases intracellular cAMP via adenylate cyclase
activation in platelets.7 The cell-surface PGD receptor is G
protein-coupled and transmits a signal leading to cAMP upregu-
lation.25 In cultured VSMCs and mesangial cells, cAMP-elevating agents, including PG₁ and PGE₁, enhance
iNOS expression and NO generation.24–26 In the present study,
both the PG₁ analogue and PGE₁, at a dose of 10⁻⁵ mol/L,
again increased NO generation, although to relatively small
degrees (Fig 4A), probably because the cells had been almost
maximally stimulated by the cytokine. In contrast, PGD₂
decreased NO generation in VSMCs, arguing against signifi-
cant involvement of cAMP upregulation in the effect of PGD₂
on iNOS expression. Angiotensin II also inhibits iNOS ex-
pression in VSMCs, at least in part through upregulation of
PKC.26 PGD₂ also can act as an agonist of the vasoconstrictor
TXA₃,23 which elevates PKC levels in VSMCs.27 However, we
observed only a slight inhibitory effect of the TXA₃ analogue
on NO generation in VSMCs, failing to adequately explain the
mechanism of the effect of PGD₂ in this manner (Fig 4A). In
fact, PGD₂ addition dose-dependently inhibited the NO
generation even under blockade of TXA₃ receptors (Fig 4B),
indicating that a pathway other than via TXA₃ 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 J₂ series.28–30 (Fig
5A). Incubation of PGD₂ in the presence of plasma or serum
albumin results in the rapid accumulation of several major
dehydration and isomerization products, including Δ₁₂-PGJ₂
and 15-deoxy-Δ₁₂,14-PGJ₂.27,28,30 Some of these products, in-
cluding Δ₁₂-PGJ₂, have antiproliferative effects, presumably
involving their direct binding to nuclear proteins.12,38 Fur-
thermore, 15-deoxy-Δ₁₂,14-PGJ₂, a PGJ₂ derivative, recently has
been demonstrated to be a natural ligand for an intracellular
receptor promoting adipocyte differentiation.29,30 In the
present study, the four PGs showed doses producing 50% inhibi-
tion (expressed as negative log mol/L) in the following order:
15-deoxy-Δ₁₂,14-PGJ₂,Δ₁₂-PGJ₂,PGJ₂,PGD₂ (Fig 5B), 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 J₂ metabolites on intracellular components rather
than via the adenylate cyclase–cAMP or inositol phospholi-
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 PGJ_2 cascade remains to be investigated.

In conclusion, we demonstrated that PGD_2 suppresses NO generation in VSMCs, which is due to inhibition of iNOS mRNA expression most likely operating mainly through a novel PGD_2 signaling pathway, the PGJ_2 cascade. These results suggest the possibility of involvement of PGD_2 in NO metabolism in the pathogenesis of vascular diseases.

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References


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