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Platelet-Derived Growth Factor-Induced Arachidonic Acid Release for Enhancement of Prostaglandin E_2 Synthesis in Human Gingival Fibroblasts Pretreated with Interleukin-1β

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Abstract Platelet-derived growth factor (PDGF) is a biological mediator for connective tissue cells and plays a critical role in a wide variety of physiological and pathological processes. We here investigated the effect of PDGF on arachidonic acid release and prostaglandin E_2 (PGE_2) synthesis in human gingival fibroblasts (HGF). PDGF induced arachidonic acid release in a time- and dose-dependent manner, and simultaneously induced a transient increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), but less provoked PGE_2 release and cyclooxygenase-2 (COX-2) mRNA expression. When $[Ca^{2+}]_i$ was increased by Ca^{2+}-mobilizaing reagents, arachidonic acid release was increased. The PDGF-induced arachidonic acid release and increase in $[Ca^{2+}]_i$ were prevented by a tyrosine kinase inhibitor. On the other hand, in the HGF pre-stimulated with interleukin-1β (IL-1β), PDGF clearly increased PGE_2 release. The PDGF-induced PGE_2 release was inhibited by a tyrosine kinase inhibitor. In the HGF pretreated with IL-1β, arachidonic acid strongly enhanced PGE_2 release and COX-2 mRNA expression. These results suggest that PDGF stimulates arachidonic acid release by the increase in $[Ca^{2+}]_i$ via tyrosine kinase activation, and which contributes to PGE_2 production via COX-2 expression in HGF primed with IL-1β. J. Cell. Biochem. 92: 579–590, 2004.

Key words: platelet-derived growth factor; arachidonic acid; cyclooxygenase-2; prostaglandin E_2; interleukin-1β

Abbreviations used: PDGF, platelet-derived growth factor; PGE_2, prostaglandin E_2; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; HGF, human gingival fibroblasts; COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; α-MEM, α-minimum essential medium; FBS, fetal bovine serum.

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Prostaglandin E_2 (PGE_2) is widely distributed in various organs and exerts effects on various biological activities [Shimizu and Wolfe, 1990]. It is considered that PGE_2 is involved in the pathogenesis of periodontal disease, because elevated levels of PGE_2 were detected in the inflamed gingival tissues and crevicular fluids and were associated with an increased severity, progression of periodontal lesions, and bone loss [Offenbacher et al., 1984, 1986, 1992; Cutler et al., 2000]. In human gingival fibroblasts (HGF), chemical mediators of inflammation, such as bradykinin and histamine, and cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), have been demonstrated to provoke PGE_2 release [Lerner and Modéer, 1991; Modéer et al., 1993; Niisato et al., 1996; Nakao et al., 2000a, 2002]. Prostaglandin synthesis is regulated by

two successive metabolic steps, the release of arachidonic acid from membranous phospholipids and its conversion to prostanoids. Cyclooxygenase (COX) is a rate-limiting enzyme for the conversion of arachidonic acid to prostanoids. We have previously demonstrated that the expression of COX-2 mRNA, the inducible COX, was stimulated by IL-1β and TNF-α in HGF [Nakao et al., 2000b, 2001, 2002].

Platelet-derived growth factor (PDGF) is a biological mediator for connective tissue cells and has been implicated in a variety of physiological and pathological processes, including proliferation, wound healing, synthesis, and secretion of mediator of inflammation [Abboud, 1993]. Binding of PDGF to its cell surface receptors initiates a number of intracellular signaling events, such as phosphatidylinositol turnover, Ca^{2+} mobilization, tyrosine phosphorylation, and activation of transcription factors [Williams, 1989; Claesson-Welsh, 1994]. In periodontal tissues, PDGF has been considered to play a critical role in the normal turn over as well as in a repair and regeneration of periodontal tissues [Giannobile et al., 1996; Nishimura and Terranova, 1996], because it has been reported that PDGF reduces the inhibitory effects of lipopolysaccharide on the proliferation of HGF [Bartold et al., 1992]. However, the precise mechanism and role of PDGF on the regulation of signaling pathways in HGF remains unresolved.

In this study, we investigated the effect of PDGF on arachidonic acid release and PGE_2 synthesis to elucidate the role of PDGF in HGF.

MATERIALS AND METHODS

Materials

PDGF-BB and IL-1β were purchased from Genzyme (Cambridge, MA). Fura-2/AM was purchased from Dojindo Laboratory (Kumamoto, Japan), [^{3}H]Arachidonic acid was purchased from NEN Research Products (Boston, MA). α-Minimum essential medium (α-MEM) and One-Step RT-PCR with Platinum Taq were purchased from Invitrogen (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Sigma (St. Louis, MO). Rneasy was obtained from Qiagen (Tokyo, Japan). PGE_2 EIA Kit was obtained from Cayman (Ann Arbor, MI). Other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cell Culture

α-MEM supplemented with 10% FBS and 100 U/ml of penicillin and 100 μg/ml of streptomycin was used for cell culture. HGF were obtained from explants of healthy human gingival connective tissue according to the methods of Somerman et al. [1988]. When the cells surrounding the tissue explants were confluent, they were subcultured with 0.05% trypsin, 0.02% EDTA in Hanks balanced salt solution, and transferred to tissue culture flasks [Ogata et al., 1995].

Determination of Intracellular Ca^{2+} Concentration

Confluent HGF were loaded with 2 μM fura-2/AM in α-MEM for 30 min at 37°C and detached from flask by the solution containing 0.05% trypsin and 0.02% EDTA. Cells were washed and suspended in α-MEM until use and then resuspended in Krebs–Ringer–HEPES buffer [120 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.96 M $NaHPO_4$, 0.2% glucose, 0.1% BSA, 1 mM $CaCl_2$ (Ca^{2+} containing solution), or 1 mM EGTA (Ca^{2+}-free solution) and 10 mM HEPES, pH 7.4] for the determination of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [Nakao et al., 2001]. The cell suspension was continuously stirred using a magnetic stirring at 37°C. The fluorescence of fura-2-loaded HGF was monitored with CAF-110 spectrophotometer (Nihon Bunko, Japan) with excitation at 340 and 380 nm and emission at 500 nm. $[Ca^{2+}]_i$ was calculated from the measurement of the ratio of fluorescence intensities [Grynkiewicz et al., 1985].

Determination of [^{3}H]Arachidonic Acid Release

HGF were plated into 12-wells culture plate. The cells which reached near confluent were prelabeled with [^{3}H]arachidonic acid (0.25 μCi) in α-MEM containing 10% FBS for 18 h. After the incubation, the cells labeled with [^{3}H]arachidonic acid were washed twice with release buffer [134 mM NaCl, 4.7 mM KCl, 5 mM glucose, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $NaHCO_3$, 2 mM $CaCl_2$ (Ca^{2+} containing solution), or 1 mM EGTA (Ca^{2+}-free solution), 10 mM HEPES, pH 7.2, and 0.1% fatty acid-free BSA] and replaced with 1 ml of release buffer. After preincubation for 10 min at 37°C, the cells were stimulated with the various concentration of PDGF for the indicated times at 37°C. At the

end of incubation, the aliquots of release buffer were removed on ice and counted for radioactivity in a liquid scintillation counter.

RT-PCR

Total RNA was isolated using Rneasy. RT-PCR was performed using Super Script One-Step RT-PCR with Platinum Taq System. Primers were synthesized on the basis in the reported human cDNA sequence for COX-2 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) [Tokunaga et al., 1987; Hla and Neilson, 1992]. Sequence of primers used were as follow: COX-2 forward, 5′-ATGA-GATTGTGGAAAAATTGCT-3′; COX-2 reverse, 5-GATCATCTCTGCCTGAGTATC-3′; GAPDH forward, 5′-CCACCCATGGCAAATTCCATGG-CA; GAPDH reverse, 5′-TCTAGACGGCAGGT-CAGGTCCACC-3′. The PCR reactions were performed in a final volume of 50 μl, which contained 25 μl reaction mixture, 1 μg total RNA, 10 μM each of forward and reverse primers, and 1 μl Taq Mix. Three-step cycling program was used and consisted of the following: cDNA synthesis and predenaturation step at 50°C for 30 min and at 94°C for 2 min (1 cycle), denature at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s (22 cycles), and final extension at 72°C for 10 min [Nakao et al., 2000b, 2002]. After amplification, 10 μl of each reaction mixture was analyzed by 2% agarose gel electrophoresis (0.5× Tris-borate buffer) and visualized by ethidium bromide staining under UV light. The relative amount of PCR product was normalized to the corresponding amount of GAPDH PCR product. The PCR products for COX-2 and GAPDH were 300 and 598 bps, respectively.

Measurement of PGE_2

HGF were cultured in 24-well culture plates in α-MEM containing 10% FCS. When the cells were confluent, they were deprived of serum for 18 h, and PDGF and IL-1β were added at various time intervals and then medium was collected and amount of PGE_2 was determined by enzyme immunoassay using commercially available kit as previously described [Nakao et al., 2000a].

Statistical Analysis

Significant differences were determined using Student's *t* test. A P value <0.05 was considered to be statistically significant.

RESULTS

PDGF-Induced Arachidonic Acid Release

We determined the effect of PDGF on arachidonic acid release in HGF. Figure 1A shows that PDGF (50 ng/ml) provoked [^{3}H]arachidonic acid release from the HGF prelabeled with [^{3}H]arachidonic acid in a time-dependent manner. The effect was reached to a maximal level within 30 min. Figure 1B summarizes [^{3}H]arachidonic acid release induced by various concentrations of PDGF. When the cells were stimulated with 5–50 ng/ml PDGF for 30 min, PDGF induced [^{3}H]arachidonic acid release in a dose-dependent manner.

Increase in $[Ca^{2+}]_i$ Induced by PDGF

Because PDGF has been demonstrated to induce the increase in $[Ca^{2+}]_i$ in other kinds of fibroblasts [Lopez-Rivas et al., 1987; Tucker et al., 1990; Chow and Powis, 1993], we examined the effect of PDGF on $[Ca^{2+}]_i$ in fura-2-loaded HGF. PDGF (50 ng/ml) provoked an increase in $[Ca^{2+}]_i$ which consisted of a rapid transient and subsequent small sustained phase (Fig. 2A and Table I). The same PDGF-induced Ca^{2+} mobilization was shown even in the HGF attached on coverslips (data not shown). To elucidate the role of extracellular Ca^{2+} on PDGF-induced increase in $[Ca^{2+}]_i$, when HGF were stimulated with 50 ng/ml PDGF in the absence of extracellular Ca^{2+}, the transient phase was evoked, although the level decreased, whereas the sustained phase was abolished (Fig. 2A and Table I). These results suggest that the transient and sustained increases in $[Ca^{2+}]_i$ stimulated by PDGF are due to Ca^{2+} release from the intracellular Ca^{2+} stores and Ca^{2+} influx from extracellular sites, respectively.

Relationship Between PDGF-Induced Arachidonic Acid Release and Increase in $[Ca^{2+}]_i$

We next examined the relationship between PDGF-induced arachidonic acid release and increase in $[Ca^{2+}]_i$ in HGF. When HGF were stimulated with various concentrations of PDGF, which induced arachidonic acid release, elevation of $[Ca^{2+}]_i$ was elicited in a dose-dependent manner (Fig. 2B). Next, we examined effects of ionomycin and A23187, these drugs increase in $[Ca^{2+}]_i$ without receptor activation, on arachidonic acid release. As Table II shows, 2 μM ionomycin and 1 μM A23187 mimicked the

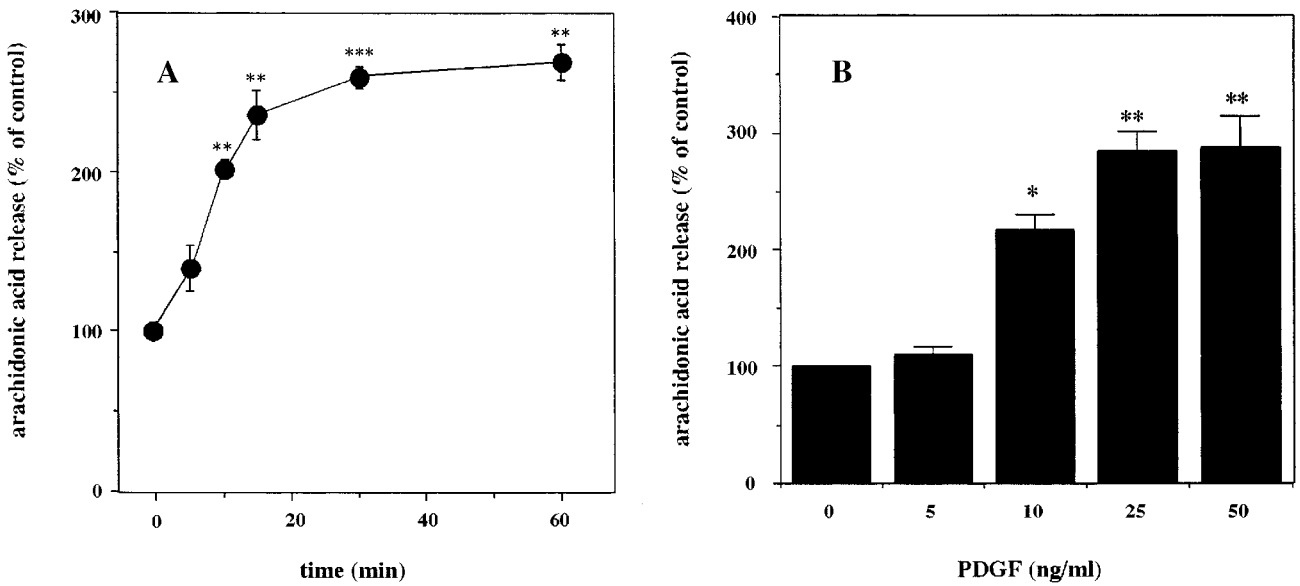

Fig. 1. Time- and dose-dependent PDGF-induced arachidonic acid release in HGF. **A**: The HGF prelabeled with [^{3}H]arachidonic acid were stimulated with PDGF (50 ng/ml). At indicated times, the media was collected and the radioactivity of [^{3}H]arachidonic acid released was determined. **B**: The HGF prelabeled with [^{3}H]arachidonic acid were stimulated with various concentrations of PDGF for 30 min. The media were collected and the radioactivity of [^{3}H]arachidonic acid released was determined. Results are means ± SE in three independent experiments. Significant differences from control: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

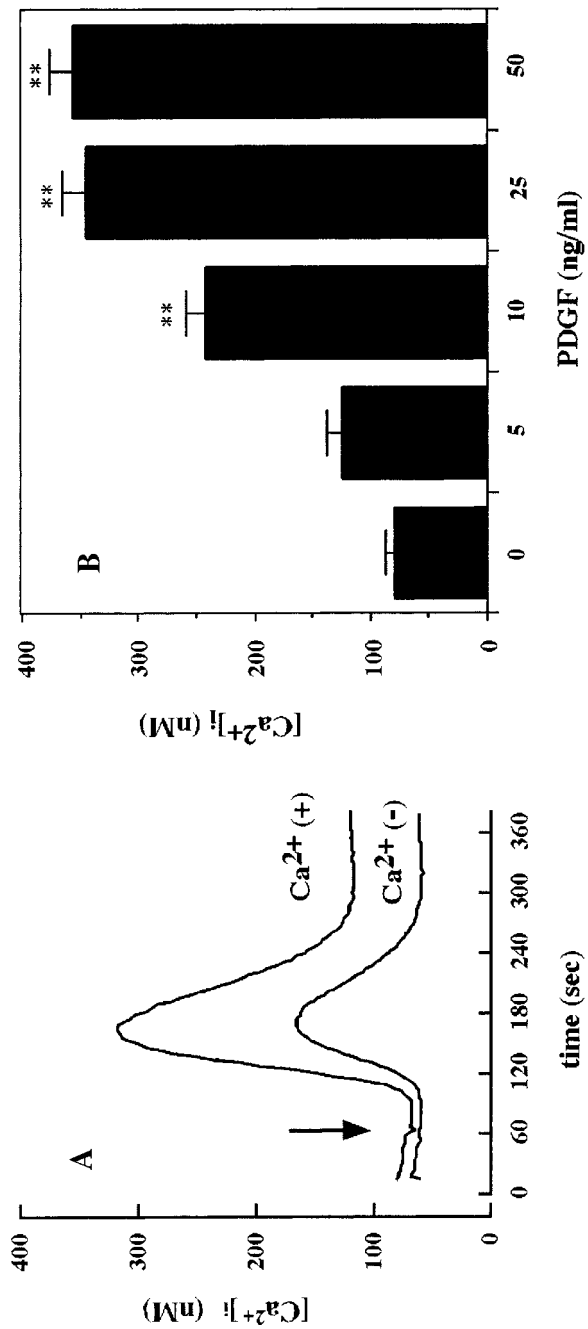

Fig. 2. PDGF-induced increase in $[Ca^{2+}]_i$. **A**: Fura-2-loaded HGF were stimulated with PDGF (50 ng/ml) in the presence $[Ca^{2+}\ (+)]$or absence $[Ca^{2+}\ (-)]$ of extracellular Ca^{2+}. PDGF was added in the medium as indicated by an arrow. Results are representative in four independent experiments. **B**: Dose-response of PDGF-induced increase in $[Ca^{2+}]_i$. Fura-2-loaded HGF were stimulated with various concentrations of PDGF, and the peak levels of $[Ca^{2+}]_i$ were determined. Results are means ± SE in four independent experiments. Significant differences from control: $^{**}P < 0.01$.

TABLE I. PDGF-Induced Increase in $[Ca^{2+}]_i$ in the Absence or Presence of Extracellular Ca^{2+}

Ca^{2+}	Control	Transient	Sustained
		$[Ca^{2+}]$i (nM)	
+	76.2 ± 6.0	359.7 ± 12.2[b]	130.5 ± 14.3[a]
−	51.6 ± 1.7	156.7 ± 12.0[b]	50.3 ± 3.2

Fura-2-loaded HGF were stimulated with PDGF (50 ng/ml) in the presence (+) or absence (−) of extracellular Ca^{2+}. The intracellular Ca^{2+} levels before stimulation (control), and of transient and sustained peaks induced by PDGF were described. The transient and sustained levels were determined at 120 and 240 s after PDGF stimulation, respectively. Results are means ± SE in four independent experiments. Significant differences from control.
[a]$P < 0.05$.
[b]$P < 0.01$.

TABLE III. Effect of Extracellular Ca^{2+} on PDGF-Induced Arachidonic Acid Release

PDGF	Ca^{2+}	Arachidonic acid release
		(% of Control)
−	+	100
+	+	257.0 ± 21.5[a]
−	−	94.3 ± 7.3
+	−	109.3 ± 6.1

The HGF prelabeled with [^{3}H]arachidonic acid were stimulated with PDGF (50 ng/ml) for 30 min in the presence (+) or absence (−) of extracellular Ca^{2+}. The media were collected and the radioactivity of [^{3}H]arachidonic acid released was determined. Results are means ± SE in three independent experiments. Significant differences from control.
[a]$P < 0.05$.

effect of PDGF. These observations suggest that arachidonic acid release is coupled to increase in $[Ca^{2+}]_i$. When HGF were stimulated with PDGF (50 ng/ml) in the absence of extracellular Ca^{2+}, arachidonic acid release was almost completely blocked (Table III). Therefore, it is likely that the effect of PDGF on arachidonic acid release is dependent on Ca^{2+} entry from extracellular sites.

Effect of Tyrosine Kinase Inhibitor on PDGF-Induced Arachidonic Acid Release and Increase in $[Ca^{2+}]_i$

It is well known that effects of growth factors including PDGF are coupled to tyrosine kinase activation [Seedoft, 1995]. Therefore, we examined the effects of herbimycin A, a tyrosine kinase inhibitor, on PDGF-mediated arachidonic acid release and Ca^{2+} mobilization. As Table IV shows, PDGF failed to induce arachidonic acid release in the HGF pretreated with 1 μM herbimycin A. In the HGF pretreated with herbimycin A, PDGF-induced increase in $[Ca^{2+}]_i$ was also completely inhibited (Table IV).

TABLE II. Inonomycin- and A23187-Induced Arachidonic Acid Release in HGF

Drug	Concentration	Arachidonic acid release
		(% of Control)
Vehicle		100
Ionomycin	2 μM	277.2 ± 29.2[a]
A23187	1 μM	263.0 ± 29.1[a]

The HGF prelabeled with [^{3}H]arachidonic acid were incubated with ionomycin (2 μM) or A23187 (1 μM) for 30 min. The media were collected and the radioactivity of [^{3}H]arachidonic acid released was determined. Results are means ± SE in three independent experiments. Significant differences from control.
[a]$P < 0.05$.

These results suggest that tyrosine kinase activation is involved in the induction of Ca^{2+} mobilization and arachidonic acid release by PDGF.

Effect of PDGF on PGE_2 Production

Because arachidonic acid from membranous phospholipids converts to prostanoids, effect of PDGF on PGE_2 release was examined. However, when PDGF (50 ng/ml) stimulated HGF for 30 min, no significant increase in PGE_2 release was shown (control, 0.104 ± 0.022 ng/well; PDGF, 0.141 ± 0.03 ng/well; n = 5). We have previously demonstrated that IL-1β and TNF-α stimulate the expression of COX-2 mRNA, a rate-limiting enzyme for the conversion of arachidonic acid to prostanoids [Nakao et al., 2000b, 2001, 2002]. Both cytokines

TABLE IV. Inhibitory Effect of Herbimycin A on PDGF-Induced Arachidonic Acid Release and Increase in $[Ca^{2+}]_i$

PDGF	Herbimycin A	Arachidonic acid release	$[Ca^{2+}]_i$
		(% of Control)	(nM)
−	−	100	74.6 ± 8.5
+	−	259.8 ± 22.1[a]	377.0 ± 30.3[a]
−	+	111.0 ± 11.3	82.0 ± 9.5
+	+	106.0 ± 3.1	87.2 ± 8.6

To determine arachidonic acid release, HGF were preincubated with [^{3}H]arachidonic acid in the absence or presence of herbimycin A (1 μM) for 18 h. The HGF prelabeled with [^{3}H]arachidonic acid were stimulated with PDGF (50 ng/ml) for 30 min. The media were collected and the radioactivity of [^{3}H]arachidonic acid released was determined. To determine $[Ca^{2+}]_i$, HGF were preincubated without or with herbimycin A (1 μM) for 17.5 h, and were further incubated with fura-2/AM for 0.5 h. The fura-2-loaded fibroblasts were stimulated with PDGF (50 ng/ml). Results are means ± SE in four independent experiments. Significant differences from control.
[a]$P < 0.01$.

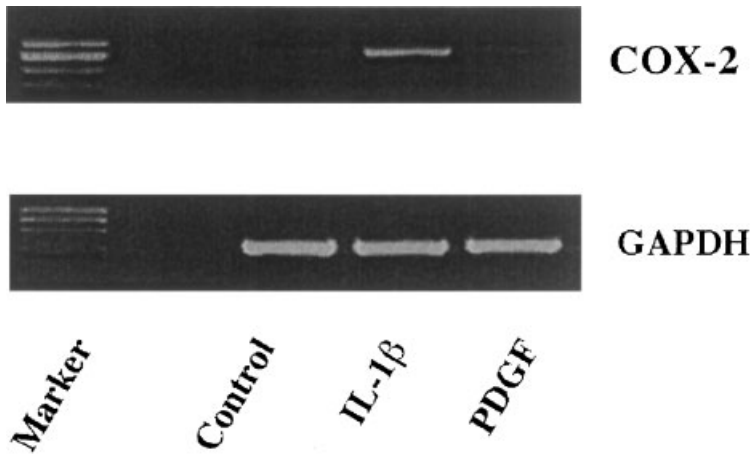

Fig. 3. No increase of COX-2 mRNA expression in PDGF-treated HGF. HGF were stimulated with PDGF (50 ng/ml) or IL-1β (500 pg/ml) for 3 h. The levels of COX-2 mRNA and GAPDH mRNA were assessed by RT-PCR (22 cycle). Molecular size markers (Hae III-digested øX-174) are shown in the **left lane**. COX-2 PCR products run near the 310-bp fragment. Results are representative in three independent experiments.

also increased COX-2 enzyme levels (data not shown), and consequently provoked PGE_2 release [Nakao et al., 2000b, 2002]. Then we determined the effect of PDGF on COX-2 mRNA expression. However, as Figure 3 shows, PDGF failed to induce the expression of COX-2 mRNA, whereas IL-1β stimulated the expression of COX-2 mRNA as previously demonstrated (Fig. 3). These observations suggest that PDGF does not directly contribute to PGE_2 production.

PGE_2 Production by PDGF in IL-1β-Pretreated HGF

We next investigated whether PDGF stimulates PGE_2 production in the HGF pretreated with IL-1β, because Ca^{2+} mobilizing drugs, such as bradykinin and ionomycin, stimulated PGE_2 release in the IL-1β-pretreated HGF [Nakao et al., 2000a]. When the HGF pretreated with IL-1β (500 pg/ml) for 6 h were stimulated with PDGF (50 ng/ml) for 30 min, PGE_2 release was clearly enhanced, as Figure 4A,B shows. The PDGF-induced PGE_2 release was completely abolished by the tyrosine kinase inhibitor 100 μM genistein (Fig. 4A), this drug can inhibit the effects of PDGF such as arachidonic acid release much faster than herbimycin A (Nakao and Sugiya, unpublished data), or by the chelation of extracellular Ca^{2+} by EGTA (Fig. 4B). These observations suggest that PDGF contributes to PGE_2 production via supply of arachidonic acid in the HGF pretreated with IL-1β.

Arachidonic Acid-Induced PGE_2 Synthesis

We then investigated the effect of arachidonic acid on PGE_2 release in the HGF pretreated with IL-1β (500 pg/ml) for 6 h. As Figure 5A shows, arachidonic acid (1 μM) clearly increased PGE_2 release in the IL-1β-pretreated HGF but barely in the HGF without the pretreatment by IL-1β. Arachidonic acid also clearly enhanced COX-2 mRNA expression in the HGF pretreated with IL-1β but not in the non-treated HGF (Fig. 5B), suggesting that arachidonic acid stimulates PGE_2 synthesis in the HGF pretreated with IL-1β.

DISCUSSION

We have demonstrated here that PDGF induces arachidonic acid release and simultaneous increase in $[Ca^{2+}]_i$ in HGF. The observations that the concentrations of PDGF evoked arachidonic acid release induced the increase in $[Ca^{2+}]_i$, and ionomycin and A23187, which increase $[Ca^{2+}]_i$ without receptor activation, stimulated arachidonic acid release, suggest that PDGF-induced arachidonic acid release is coupled to the increase in $[Ca^{2+}]_i$. The PDGF-induced arachidonic acid release and the increase in $[Ca^{2+}]_i$ were completely inhibited by the tyrosine kinase inhibitor herbimycin A, suggesting that the tyrosine kinase activation is necessary for the PDGF effect. PDGF receptor is a tyrosine kinase that directly phosphorylates and activates phospholipase C-γ [Rhee and

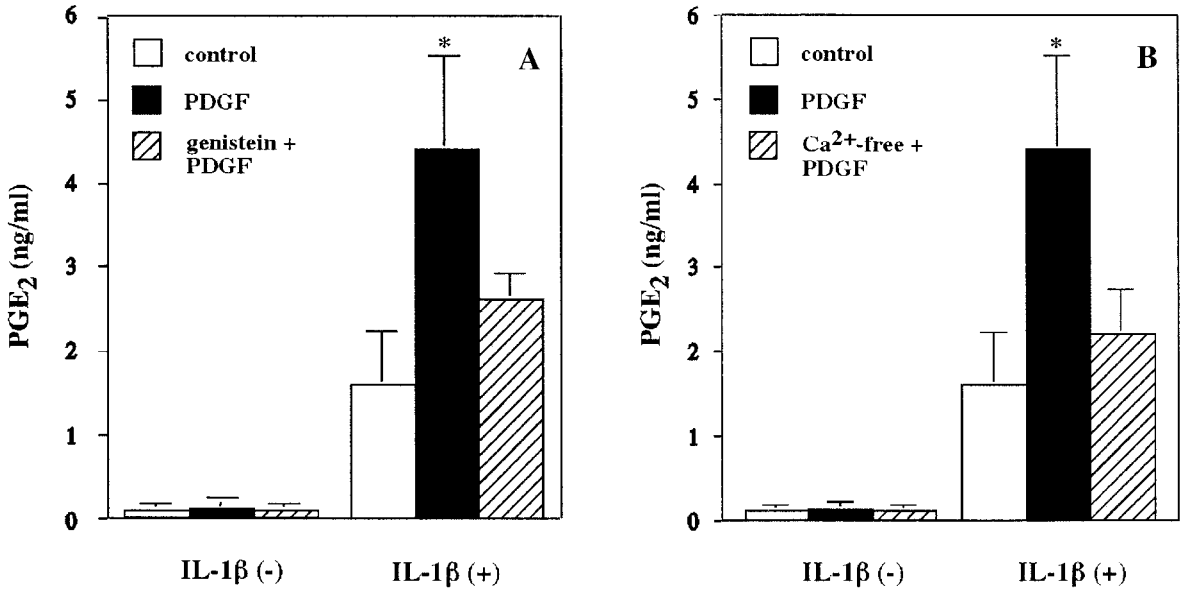

Fig. 4. Effects of a tyrosine kinase inhibitor and extracellular Ca^{2+} on PDGF-induced PGE_2 in the HGF pretreated with 1L-1β. HGF were pretreated with [1L-1β(+)] or without 500 pg/ml IL-1β [1L-1β(−)] for 6 h, and further incubated with vehicle (control) or PDGF (50 ng/ml) for 30 min. To examine the effects of a tyrosine kinase inhibitor (**A**) or extracellular Ca^{2+} (**B**), HGF were stimulated with PDGF in the presence of genistein (25 μM) or EGTA (3 mM), respectively. After stimulation, PGE_2 levels in the media were measured by enzyme immunoassay. Values are expressed as the means ± SE of five independent experiments. Significant differences from IL-1β treatment: $^*P < 0.05$.

Choi, 1992]. The activation of phospholipase C-γ catalyzes hydrolysis of phosphatydylinositol 4,5-bisphosphate and subsequently produces inositol 1,4,5-trisphosphate, which induces the release of Ca^{2+} from intracellular stores [Berridge, 1993]. Because the PDGF-induced increase in $[Ca^{2+}]_i$ was observed even in the absence of extracellular Ca^{2+}, and was completely inhibited by the tyrosine kinase inhibitor herbimycin A, it is most likely that the PDGF-induced increase in $[Ca^{2+}]_i$ is coupled to the activation of phospholipase C-γ activated by tyrosine kinase activation in HGF.

The increase in $[Ca^{2+}]_i$ induced by PDGF consisted of an initial transient and a subsequently sustained phases. The transient peak was remained in the absence of extracellular Ca^{2+}, suggesting that the initial and sustained increases in $[Ca^{2+}]_i$ are due to the release of intracellular Ca^{2+} pools and the Ca^{2+}entry of extracellular sites. Since PDGF-induced arachidonic acid release was inhibited in the absence of extracellular Ca^{2+}, it is probable that Ca^{2+} entry is important for the PDGF-induced arachidonic acid release, as demonstrated in Swiss 3T3 fibroblasts [Chow and Powis, 1993].

Arachidonic acid release has been well known to be catalyzed by the activation of phospholipase A_2 (PLA_2). Several structurally diverse forms of PLA_2 in mammalian cells have been demonstrated [Gijón and Leslie, 1999; Gijón et al., 2000]. Of those, it is well demonstrated that the activation of cytosolic PLA_2 ($cPLA_2$) is regulated by the levels of intracellular Ca^{2+}. The $cPLA_2$ has been shown to play an important role in agonist-induced arachidonic acid release in many systems [Gijón and Leslie, 1999; Gijón et al., 2000]. To confirm the contribution of $cPLA_2$ to the effect of PDGF in HGF, we examined the effects of PLA_2 inhibitors, such as $AACOCF_3$, *N*-(*p*-amylcinnamoyl)anthranillic acid, manoalide, ONO-RS-082, and $PACOCF_3$, on PDGF-induced arachidonic acid release. However, since all of these drugs resulted in the decrease of viability of HGF (data not shown), which appears to be side effects of the drugs, we failed to pharmacologically find out whether $cPLA_2$ contributes the PDGF-induced arachidonic acid release. However, the finding that arachidonic acid release was dependent on the increase in $[Ca^{2+}]_i$ suggests the effect of PDGF on arachidonic acid release is due to the result of the $cPLA_2$ activation.

Fig. 5. Arachidonic acid-induced PGE_2 release and COX-2 mRNA expression in the HGF pretreated with 1L-1β. **A**: HGF were pretreated with or without IL-1β (500 pg/ml) for 6 h, and further incubated with arachidonic acid (1 μM) for 30 min. After stimulation, PGE_2 levels in the media were measured by enzyme immunoassay. Values are expressed as the means ± SE of seven independent experiments. Significant differences between two treatments: $^{**}P < 0.01$. **B**: HGF were pretreated with or without IL-1β (500 pg/ml) for 6 h, and further incubated with arachidonic acid (1 μM) for 30 min. Total RNA was isolated from the cells, and RT-PCR was performed. The levels of COX-2 and GAPDH mRNAs were assessed by RT-PCR (22 cycle). Molecular size markers (Hae III-digested øX-174) are shown in the **left lane**. COX-2 PCR products run near the 310-bp fragment. Results are representative in three independent experiments.

It has been reported that PDGF stimulates production of prostanoids including PGE_2 in Swiss 3T3 cells [Habenicht et al., 1985; Domin and Rozengurt, 1992], NIH 3T3 cells [Kelner and Uglik, 1995], rheumatoid arthritis synovial cells [Goddard et al., 1992], 3T6 fibroblasts [Moreno, 1996, 2000], and mouse osteoblasts [Chen et al., 1997]. Prostaglandins are converted from arachidonic acid. The conversion is mediated by the constitutive enzyme COX-1 and the inducible enzyme COX-2 [DeWitt, 1991]. The expression of COX-1 was slightly detected in HGF [Nakao et al., 2001]. However, PDGF clearly evoked arachidonic acid release, but less induced PGE_2 release in HGF. These findings suggest that COX-1 less contributes to PGE_2 synthesis in HGF. COX-2 has been demonstrated to contribute to PGE_2 synthesis in HGF, because cytokines such as IL-1β and TNF-α stimulate COX-2 expression and provoke a large amount of PGE_2 production [Nakao et al., 2000a,b, 2001, 2002]. While PDGF failed to induce COX-2 mRNA expression, PGDF enhanced IL-1β-induced PGE_2 production in the HGF pretreated with IL-1β. The PDGF-induced PGE_2 production was inhibited by the tyrosine kinase inhibitor genistein or chelating extracellular Ca^{2+}, which inhibit PDGF signaling as described above. Because IL-1β did not stimulate Ca^{2+} mobilization [Lerner et al., 1992] and provoked less arachidonic acid release [Yucel-Lindberg et al., 1999], it is suggested that PDGF plays an important role in PGE_2 production by supply of arachidonic acid via Ca^{2+} mobilization in HGF primed with IL-1β. Furthermore, arachidonic acid directly enhanced PGE_2 synthesis via COX-2 expression in the HGF pretreated with IL-1β. These observations suggest that not only is arachidonic acid released by PDGF used as a substrate for COX-2, but it also functions like an agonist in the HGF primed with IL-1β. We have previously demonstrated that bradykinin enhanced PGE_2 production and COX-2 expression in the HGF pretreated with IL-1β [Nakao et al., 2000a,b, 2001]. Because bradykinin provokes arachidonic acid release in HGF [Modéer et al., 1990], it is most likely that arachidonic acid plays an important role for PGE_2 synthesis in the HGF primed with IL-1β.

In the human inflamed gingiva, the increase in PDGF levels has been demonstrated [Pinheiro et al., 2003], suggesting that PDGF is involved in the inflammation of periodontal disease. Bradykinin invoked a greater response in inflamed tissues than in non-inflamed tissues, implicating that the effect of bradykinin is potentiated in the cells primed by cytokines, and has been considered to play an important role in potentiating inflammatory responses [Bathon et al., 1989; O'Neill and Lewis, 1989]. As we demonstrated here, PDGF showed the same potentiating effect as bradykinin in the IL-1β-primed HGF. Therefore, PDGF appears to cause for potentiating inflammation of periodontal disease.

In conclusion, we have demonstrated that PDGF increases in $[Ca^{2+}]_i$ and provokes arachidonic acid release through tyrosine phosphorylation in HGF. Arachidonic acid directly contributes to COX-2 mRNA expression and PGE_2 synthesis in IL-1β-primed HGF. We are further studying with the mechanism of arachidonic acid-induced COX-2 mRNA expression.

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