Signaling Events During Induction of Plasminogen Activator Inhibitor-1 Expression by Sphingosylphosphorylcholine in Cultured Human Dermal Fibroblasts

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Sphingosylphosphorylcholine (SPC) is a bioactive sphingolipid metabolite that can enhance wound healing. In a search for effectors downstream of SPC in the wound-healing process, we found that the expression of the gene for plasminogen activator inhibitor-1 (PAI-1) was significantly affected. ELISA and western blot analyses showed that SPC markedly induced PAI-1 production in human dermal fibroblasts cultured *in vitro*. Inhibition by pre-treatment with pertussis toxin (PTx), but not by tyrphostin A47 (a receptor tyrosine kinase inhibitor), indicated that PTx-sensitive G proteins were involved in SPC-induced PAI-1 expression. SPC elicited a rapid and transient increase in intracellular calcium levels ($[Ca^{2+}]_i$), measured using laser scanning confocal microscopy, which was partly mediated through PTx-sensitive G proteins. Pre-treatment with thapsigargin, but not with EGTA, abolished SPC-induced PAI-1 expression, indicating the importance of Ca^{2+} release from internal stores. Phorbol-12-myristate-13-acetate (PMA) induced the expression of PAI-1, and pre-treatment with Ro 31-8220 (a PKC inhibitor) markedly suppressed SPC-induced PAI-1 expression. SPC-induced PAI-1 expression was also significantly suppressed by PD98059 (a specific MAPK kinase 1/2 inhibitor). Consistent with this result, SPC stimulated the phosphorylation of p42/44 extracellular signal-regulated kinase (ERK). Together, these results suggest that SPC induces PAI-1 production through a G protein-coupled calcium increase and downstream kinase signaling events in cultured human dermal fibroblasts.

Key words: calcium/mitogen-activated protein kinase/pertussis toxin-sensitive g protein/plasminogen activator inhibitor-1/protein kinase c/sphingosylphosphorylcholine
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Sphingolipid metabolites, such as sphingosine, sphingosine-1-phosphate (S1P), sphingosylphosphorylcholine (SPC), and lysophosphatidic acid (LPA), are important signaling molecules in intracellular and/or extracellular communications (Liscovitch and Cantley, 1994). SPC has been shown to accelerate cutaneous wound healing, and effect believed to be mainly due to its mitogenic potential for many target cells, including keratinocytes, fibroblasts, and endothelial cells (Sun *et al*, 1996; Wakita *et al*, 1998). In addition, several lines of evidence suggest that SPC has other important functions in the wound-healing process; examples include the stimulation of endothelial cell migration and morphogenesis, the enhancement of fibroblast contraction, and the induction of extracellular matrix (ECM) component (Boguslawski *et al*, 2000; Suhr *et al*, 2000, 2003). Despite accumulating evidence for various functional roles, the precise mechanism underlying the stimulation of wound healing by SPC remains to be elucidated. In an effort to find downstream effectors of SPC, we have performed intensive *in vivo* and *in vitro* screening tests. We found that the expression of plasminogen activator inhibitor-1 (PAI-1) was greatly influenced by SPC treatment.

PAI-1, a member of the serine protease inhibitor (serpin) superfamily, is known to have a pivotal role in the fibrinolytic system. During fibrinolysis, tissue-type plasminogen activator (tPA) converts the proenzyme plasminogen into active plasmin, a broad-spectrum proteolytic enzyme that degrades the fibrin clot. By binding to its active site, PAI-1 neutralizes tPA activity and thereby regulates the production of plasmin (Huber, 2001). In addition to this critical role in the fibrinolytic system, there is evidence supporting the involvement of PAI-1 in several other biological phenomena, including wound healing (Romer *et al*, 1991; Jensen and Lavker, 1996; Irigoyen *et al*, 1999; Providence *et al*, 2000). Interestingly, it has been reported that PAI-1 level is significantly elevated under conditions associated with tissue fibrosis and excessive fibrin accumulation, such as sclerosis and keloid formation, supporting its role as an important regulator of proteolytic environment in tissue remodeling (Tuan *et al*, 1996; Higgins *et al*, 1999; Tuan *et al*,

Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+}; MAPK, mitogen-activated protein kinase; PAI-1, plasminogen activator inhibitor-1; PKC, protein kinase C; PTx, pertussis toxin; S1P, sphingosine-1-phosphate; SPC, sphingosylphosphorylcholine; uPA, urokinase-type plasminogen activator

2003). The function of PAI-1 in wound repair is thought to be tightly regulated in a spatiotemporal manner. Temporal changes in the expression and/or the localization of PAI-1 are particularly likely to affect the stability of cell-to-ECM adhesive complexes; increased synthesis or availability of PAI-1 may inhibit cell detachment from substrates, contributing to the stabilization of cell-to-matrix contact sites (Ciambrone and McKeown-Longo, 1990; Germer *et al*, 1998). The involvement of PAI-1 in SPC-stimulated wound healing raises the possibility that PAI-1 plays such a role during this process. Although independently, both SPC and PAI-1 have been clearly implicated in wound healing; the relationship between these two molecules has not been characterized. At present, limited data are available to support the hypothesis that plasminogen activator systems are involved in an SPC-stimulated wound-healing process. For instance, SPC upregulates the cell-surface plasminogen activity, and at the same time increases the cell surface expression of both urokinase-type plasminogen activator (uPA) and its receptor in keratinocytes (Wakita *et al*, 1998).

In this study, we demonstrate for the first time that SPC induces the expression of PAI-1 in cultured human dermal fibroblast, and that a G protein-coupled signaling cascade is coordinately involved in this process.

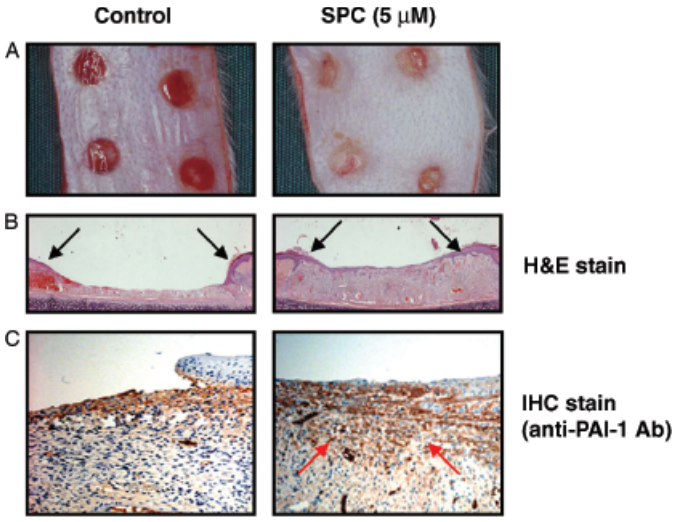

Figure 1
PAI-1 induction by SPC in healing wounds. (*A*) The inner ears of rabbit were wounded using a biopsy punch at size of about 6 mm. Wounded areas were covered with a plastic film, then injected daily with 5 μM SPC solution for 4 d. (*B*) Sections were prepared with paraffin, and stained with Hematoxylin–Eosin (H&E stain). (*C*) Immunohistochemical staining (IHC stain) was carried out with monoclonal anti-PAI-1 antibody. After washing in PBS, sections were incubated sequentially with biotinylated rabbit anti-mouse IgG and with peroxidase-conjugated avidin. Black arrows indicate the wound edges. Red arrows represent the PAI-1 staining in the dermis.

Results

PAI-1 induction by SPC in healing wounds The healing effects of several sphingolipid metabolites and related derivatives (S1P, SPC, LPA, *N*-acetylphytosphingosine (NAPS) and tetraacetylphytosphingosine (TAPS)) were tested using a well-established rabbit ear wound model. Of these, SPC demonstrated the most powerful promotion of wound healing. Control wounds healed slowly, showing incomplete closure of wounded areas at 4 d after injury, whereas almost all SPC-treated areas were closed (Fig 1*A* and *B*). These results were consistent with those of previous reports (Sun *et al*, 1996; Wakita *et al*, 1998). Immunohistochemical examination showed that treatment with SPC significantly increased granulation tissue, and induced PAI-1 production in the dermis (Fig 1*C*).

Induction of PAI-1 expression by SPC in human dermal fibroblasts To investigate the effect on PAI-1 production, SPC was added to the fibroblast cells in serum-free cultures. As PAI-1 is a secretary protein, we first measured PAI-1 in conditioned media using an ELISA technique. As shown in Fig 2*A*, SPC significantly induced the release of PAI-1 in cultured human dermal fibroblast cells in a time-dependent manner. In addition, SPC also induced the release of uPA, the regulation of which is closely related to that of PAI-1 production (Irigoyen *et al*, 1999). The levels of PAI-1 were, however, several-fold higher than those of uPA. Consistent with this observation, western blot analysis showed that SPC treatment resulted in high-level induction of intracellular PAI-1 protein, reaching a maximum at 12 h and declining slightly by 24 h (Fig 2*B*, *upper panel*). SPC-induced PAI-1 expression was also confirmed by reverse fibrin autography, which revealed that PAI-1 activity also increased concomitantly (Fig 2*B*, *lower panel*). As shown in Fig 2*C* and *D*, SPC significantly increased release, cellular content and enzyme activity of PAI-1 in a concentration-dependent manner. Northern blot analysis revealed a time-dependent increase in PAI-1 mRNA level in response to SPC (Fig 3). These results clearly demonstrated that SPC induces the PAI-1 expression, at both the transcriptional and translational levels, in cultured human dermal fibroblasts.

Involvement of G protein-coupled calcium increase in SPC-induced PAI-1 expression In other systems, SPC is known to affect downstream molecular events through G protein-coupled signaling pathway (Seufferlein and Rozengurt, 1995; Zhu *et al*, 2001; Xu, 2002). These findings led us to investigate whether the G protein-coupled signaling events were also involved in SPC-induced PAI-1 expression in cultured human dermal fibroblasts. After pre-incubation with pertussis toxin (PTx), an inhibitor for $G_{i/o}$ subfamily, a marked inhibition of SPC-induced PAI-1 expression was observed (Fig 4*A*). On the other hand, pre-treatment of tyrphostin A47, a receptor tyrosine kinase inhibitor, had no effect on PAI-1 induction by SPC (Fig 4*B*). As SPC is known to affect the intracellular calcium level ($[Ca^{2+}]_i$) *via* a G protein-dependent pathway (Okajima and Kondo, 1995; Chin and Chueh, 1998), we measured $[Ca^{2+}]_i$ by laser scanning confocal microscopy. Treatment with SPC led to a robust $[Ca^{2+}]_i$ increase, peaking at 7 s then decreasing to 50% of the maximum level at 15 s (Fig 5*A*). As anticipated, this response was significantly diminished by pre-incubation with PTx (Fig 5*B*), indicating that Ca^{2+} mobilization by SPC was partly mediated through PTx-sensitive G proteins. The rapid and transient nature of the Ca^{2+} increase

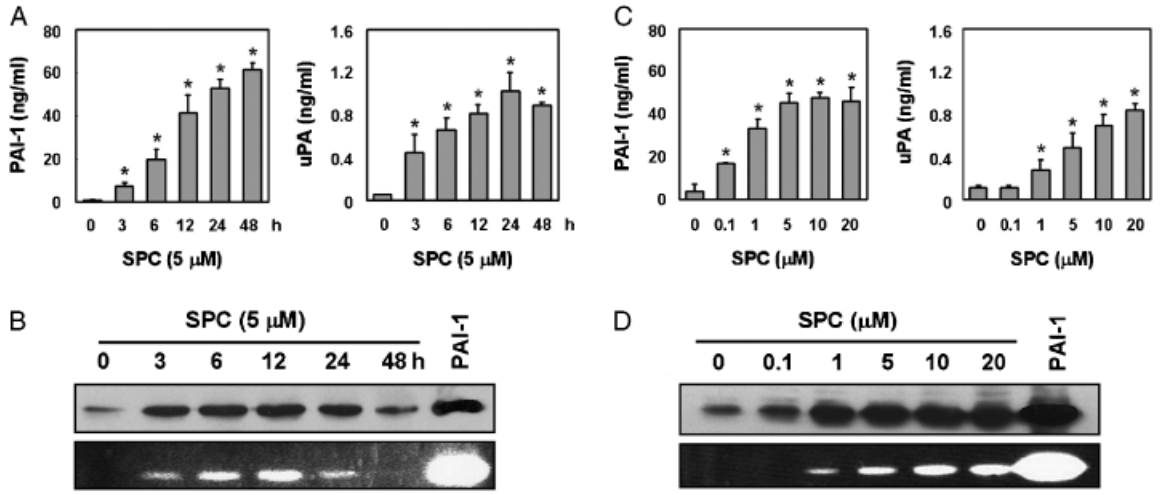

Figure 2
SPC-induced PAI-1 expression in cultured human dermal fibroblasts. (*A*) Cells were treated with 5 μM SPC at the indicated time points. Plasminogen activator inhibitor-1 (PAI-1) and urokinase-type plasminogen activator (uPA) in conditioned media were quantified using ELISA kits. Values represent the means and SEM from three independent measurements (*$p < 0.01$ *vs* control). (*B*) PAI-1 content of cellular extracts was analyzed by western blotting (*upper panel*), and enzyme activity was determined by reverse fibrin autography (*lower panel*). Cells were treated with 5 μM SPC at the indicated time points, and extracted in lysis buffer. Proteins (100 μg per lane) were separated on 10% polyacrylamide gels. PAI-1 denotes the purified PAI-1 positive control, which was obtained commercially. All bands migrated to a location corresponding to a molecular mass of 50 kDa. (*C*) Concentration-dependence of the effect of SPC on PAI-1 expression. Cells were treated with SPC at the indicated concentrations and cultured for 12 h. The released PAI-1 and uPA were measured as in (*A*). (*D*) Cells were treated with SPC at the indicated concentrations for 12 h. PAI-1 content and enzyme activity were assayed as in (*B*).

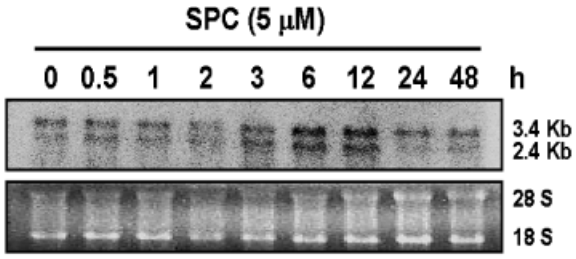

Figure 3
Northern blot analysis of PAI-1 expression in cultured human dermal fibroblasts. Cells were treated with 5 μM SPC at the indicated time points, and total RNA was isolated. Aliquots of 10 μg of total RNA were loaded in each lane, and the ethidium bromide-stained gel was photographed as a loading control.

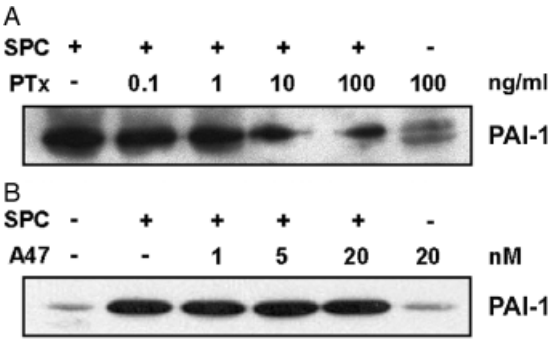

Figure 4
Effects of pertussis toxin (PTx) and tyrphostin A47 (A47) on SPC-induced PAI-1 expression. Cells were preincubated for 4 h with the G protein inhibitor PTx (*A*), or the receptor tyrosine kinase inhibitor A47 (*B*) at the indicated concentrations. After the addition of 5 μM SPC, incubation was continued for a further 12 h. Cellular content of PAI-1 was assessed by western blot analysis. Each lane contained 100 μg of protein extracts.

suggested release from intracellular Ca^{2+} stores, rather than influx from extracellular sources. To test this assumption, cells were pre-treated with thapsigargin (an intracellular Ca^{2+} chelator) or EGTA (to chelate extracellular Ca^{2+}). As shown in Fig 6*A*, pre-treatment with thapsigargin, but not with EGTA, completely inhibited SPC-induced PAI-1 expression. The calcium ionophore A23187 also induced PAI-1, and SPC interacted synergistically to enhance PAI-1 induction over a longer period than was seen with A23187 alone (Fig 6*B*).

Effect of PKC activation on SPC-induced PAI-1 expression It has been well established that an increase in $[Ca^{2+}]_i$ led to the activation of protein kinase C (PKC) in many systems (Desai *et al*, 1993; Bitar and Yamada, 1995; Seufferlein and Rozengurt, 1995). Cells were treated with phorbol-12-myristate-13-acetate (PMA) for 30 min (PKC activation) or for 4 h (PKC depletion) prior to SPC treatment. PKC activation alone induced the PAI-1 production, which was not further enhanced by SPC addition (Fig 7*A*). Pre-treatment with PMA for a longer period had no effect on PAI-I expression. Pre-treatment with Ro 31-8220, a PKC inhibitor, however, resulted in marked suppression of SPC-induced PAI-1 expression, indicating the involvement of PKC activation in the process (Fig 7*B*).

Involvement of MAPK pathway in SPC-induced PAI-1 expression Cells were pre-treated with PD98059, a specific MAPK kinase 1/2 inhibitor. As shown in Fig 8*A*, PD98059 significantly suppressed PAI-1 induction by SPC. SPC was also shown to induce the rapid phosphorylation of p42/44 extracellular signal-regulated kinase (ERK) (Fig 8*B*).

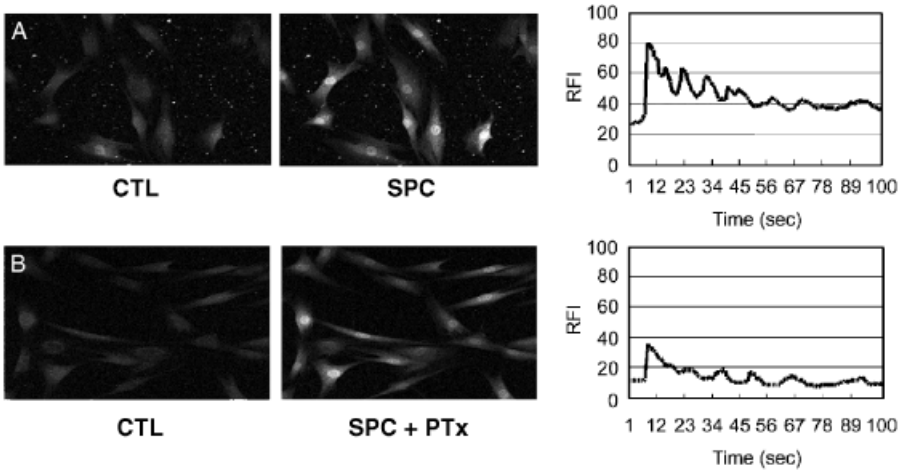

Figure 5
Measurements of intracellular Ca^{2+} level. Cells were loaded with 4 μM Fluo-3, AM for 40 min, and then subjected to confocal laser scanning microscopy. SPC (5 μM) was added alone (*A*) or following pre-treatment with 100 ng per mL pertussis toxin (PTx) (*B*). Results are presented as relative fluorescence intensity (RFI).

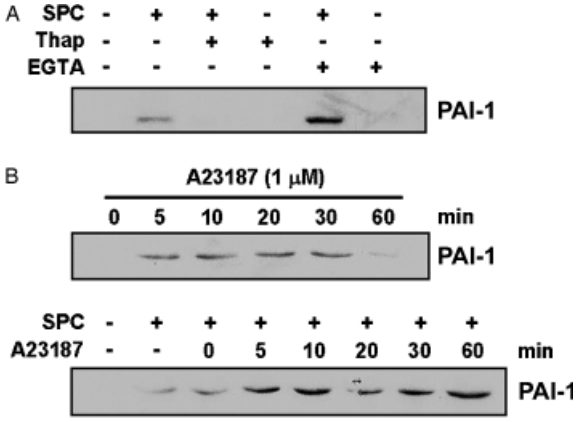

Figure 6
Effects of Ca^{2+} on SPC-induced PAI-1 expression. (*A*) Fibroblasts were pre-treated with the intracellular Ca^{2+} chelator thapsigargin (Thap) at 30 nM or with the extracellular chelator EGTA (1 mM) for 30 min. SPC (5 μM) was then added and incubation was continued for 12 h. (*B*) The calcium ionophore A23187 (1 μM) was added to cultures, and cellular extracts were prepared at the indicated time points (*upper panel*). In cotreatment experiments, SPC (5 μM) was added 12 h before the treatment of A23187 (1 μM) (*lower panel*). Western blot analyses were performed with 100 μg of cellular protein per lane.

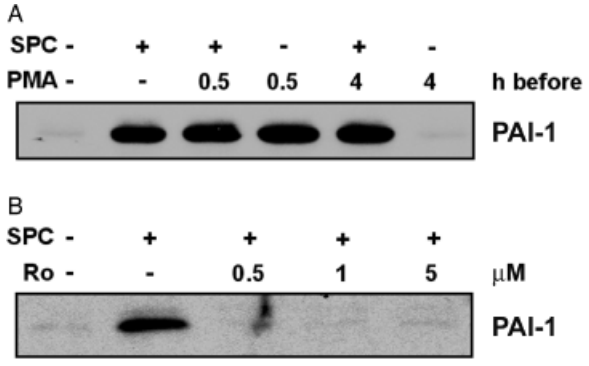

Figure 7
Effects of protein kinase C (PKC) activation on SPC-induced PAI-1 expression. (*A*) Fibroblasts were pre-treated with 100 nM phorbol-12-myristate-13-acetate (PMA) for 0.5 or 4 h, followed by the addition of 5 μM SPC. Cells were further incubated for 12 h, and extracts were subjected to western blotting for PAI-1. (*B*) Cells were pre-treated with the PKC inhibitor Ro 31-8220 (Ro) at the indicated concentrations for 30 min, followed by SPC treatment as described above. Each lane contained 100 μg of protein.

These results demonstrated that an MAPK signaling pathway is involved in SPC-induced PAI-1 expression.

Discussion

In this study, we demonstrated that SPC, which stimulates wound healing, induces PAI-1 production in human dermal fibroblasts. Although SPC is a well-known potent mitogen for a number of target cells, the effect of SPC is unlikely to be explained simply by its impact on cell proliferation. Wound healing is a multistep process, in which distinct yet interrelated phases overlap. These include the inflammatory phase, the proliferative phase, and the regeneration phase (Chan *et al*, 2001). There is accumulating evidence that components of the fibrinolytic system are indispensable to one or more of these phases. For instance, plasminogen-deficient mice display delayed wound healing after skin injury, which is related to impaired keratinocyte migration (Bugge *et al*, 1996; Romer *et al*, 1996). It has also been reported that downregulation of PAI-1 synthesis, in HaCaT cells transfected with an inducible antisense vector, markedly impairs both the rate and extent of wound closure (Li *et al*, 2000). Additional study has revealed that plasmin induces the degradation of collagen, thereby regulating fibroblast-mediated tissue remodeling during wound healing (Pins *et al*, 2000). These results clearly indicate that the function of the fibrinolytic system is fundamental to proper wound healing. Our results strengthen the notion that PAI-1 is one important downstream effector in SPC stimulation of the wound-healing process.

The sphingolipid metabolite S1P has been shown to act as an intracellular second messenger (Olivera and Spiegel, 1993; Mattie *et al*, 1994; Spiegel, 1999). Many investigations, however, have revealed effects of S1P that are sensitive to PTx, suggesting the presence of cognate G protein-coupled receptors (Goodemote *et al*, 1995; Wu *et al*, 1995; Zhu *et al*, 2001). Indeed, S1P was demonstrated to activate the heterotrimeric G protein-coupled orphan receptor Edg-1, which was originally cloned as an en-

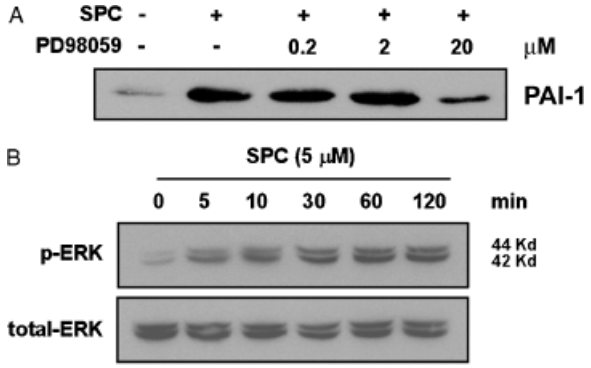

Figure 8
Involvement of a mitogen-activated protein kinase (MAPK) pathway in SPC-induced PAI-1 expression. (*A*) Cells were incubated with the specific MAPK kinase 1/2 inhibitor PD98059 at the indicated concentrations for 30 min, and then stimulated with 5 μM SPC for 12 h. Cellular PAI-1 was assayed by western blot analysis. (*B*) Cells were treated with 5 μM SPC at the indicated time points. Cellular extracts were separated on 8% polyacrylamide gels and subjected to western blot analyses for phospho-p42/44 ERK (p-ERK) or p42/44 ERK (total-ERK).

dothelial differentiation gene (Lee *et al*, 1998). Several groups have identified other members of a closely related family of G protein-coupled orphan receptors that are functional receptors for sphingolipid metabolites (Masana *et al*, 1995; Yamaguchi *et al*, 1996; Ancellin and Hla, 1999; Gonda *et al*, 1999). Thus, it is now believed that sphingolipid metabolites exert their actions from the extracellular environment *via* G protein-coupled receptors. Recently, the specific high-affinity receptors for SPC, OGR1, and GPR4, have been identified (Xu *et al*, 2000; Zhu *et al*, 2001). OGR1 and GPR4 are highly homologous, and have similar high affinities for SPC. It has been suggested, however, that they act through different G protein pathways. SPC induced the ERK activation in a PTx-insensitive manner in OGR1-transfected cells, whereas the activation of ERK was sensitive to PTx in GPR4-transfected cells. Our results suggest that GPR4 may medicate SPC-induced PAI-1 expression in cultured dermal fibroblasts. The presence and involvement of this type of receptor, however, remain to be demonstrated.

Evidences in other systems have indicated that SPC induces increases in $[Ca^{2+}]_i$, thereby affecting cell proliferation (Seufferlein and Rozengurt, 1995; Chin and Chueh, 1998). We hypothesized that an increase in $[Ca^{2+}]_i$ was also involved in SPC-induced PAI-1 expression. As expected, our confocal laser scanning microscopy data clearly showed that SPC induced a rapid and transient increase in $[Ca^{2+}]_i$. The involvement of $[Ca^{2+}]_i$ was further supported by experiments showing both PAI-1 induction by calcium ionophore and complete suppression of PAI-1 induction following intracellular Ca^{2+} depletion by thapsigargin. Interestingly, pre-treatment with PTx could not completely suppress either the increase in $[Ca^{2+}]_i$ or the induction of PAI-1 expression. These results raise the possibility that another pathway, together with PTx-sensitive G protein, may be involved in SPC effect. One candidate is Rho signaling pathway. In a preliminary study, PTx and C3-exoenzyme (a specific inhibitor of Rho) each independently caused partial inhibition of the induction of PAI-1 by SPC. Marked synergistic enhancement of suppression was seen when they were used together (data not shown). We are currently investigating the possible involvement of the Rho signaling pathway in SPC-induced PAI-1 expression.

Increased intracellular Ca^{2+} levels can activate numerous downstream signaling targets, including PKC and MAPK pathway. For example, SPC-induced MAPK activation was shown to be critically dependent on increases in $[Ca^{2+}]_i$ in porcine aortic smooth muscle cells, and PKC activation was also shown to be involved in this process (Chin and Chueh, 1998). In this study, we demonstrated that the activations of PKC and MAPK pathway were involved in SPC-induced PAI-1 expression. Together with previous reports, our results raise the possibility that the activation of PKC and MAPK pathway may be functional downstream signaling events following SPC-induced increases in $[Ca^{2+}]_i$. The putative relationship between these signaling cascades, however, must be investigated further.

In summary, we have demonstrated that SPC promotes both wound healing and PAI-1 production, and that a G protein-coupled Ca^{2+} increase and additional downstream signaling events are involved in this process.

Materials and Methods

Materials SPC was purchased from Matreya (Pleasant, California). PAI-1 and uPA ELISA kits, monoclonal PAI-1 antibody, and purified PAI-1 were obtained from American Diagnostica (Greenwich, Connecticut). Pertussis toxin, tyrphostin A47, thapsigargin, EGTA, A23187, PMA, Ro 31-8220, and PD 98059 were obtained from Calbiochem (La Jolla, California). FBS, penicillin/streptomycin solution, trypsin/EDTA, and DMEM were from Gibco BRL (Rockville, Maryland), and plasminogen and thrombin were from Sigma (St Louis, Missouri). Anti-phospho-p42/44 ERK antibody was purchased from New England BioLabs (Beverly, Massachusetts). Fluo-3, AM was supplied by Molecular Probes (Eugene, Oregon).

***In vivo* testing** Five Netherlands rabbits, between 10 and 12 wk of age, were anesthetized with phenobarbital. All tests were approved by IRB (Institute of Medical Research, College of Medicine, Chungnam National University). Full-thickness wounds, approximately 6 mm in diameter that did not cross the muscle fascia were made in the inner surface of the ear using a biopsy punch. The wounds were not sutured, but were dressed with a transparent plastic film. Experimental groups received daily injections of 5 μM SPC solution into the wounded areas, which were then re-covered with plastic film. Rabbits were euthanized after 4 d of treatment, and the wounded areas were dissected out and embedded in paraffin. Sections were stained with hematoxylin and eosin to examine cell migration and remodeling. Immunohistochemical staining was performed as follows. After brief washing in PBS, sections were blocked in PBS containing 0.5% skim milk, and incubated for 2 h at room temperature with a 1:200 dilution (5 μg per mL) of monoclonal anti-PAI-1 antibody. After further washing in PBS, sections were incubated sequentially with biotinylated rabbit anti-mouse IgG and with peroxidase-conjugated avidin.

Cell culture Normal human skin samples were obtained from circumcisions, in accordance with the ethical committee approval process of Chungnam National University Hospital. Specimens were briefly sterilized in 70% ethanol, minced, and then incubated in DMEM supplemented with 10% FBS and antibiotics. Dermal fibroblasts normally outgrew from the explants after 5–7 d. At confluence, cells were routinely passaged using a 1:4 split ratio. Cells were used between passages 4 and 16. For treatment with SPC, approximately 1×10^6 cells were seeded on 100 mm culture

dishes and grown to confluence. Cells were starved of serum for 24 h, then treated with SPC in serum-free medium.

PAI-1 and uPA ELISAs Levels of PAI-1 and uPA in conditioned media were quantified using ELISA kits according to the manufacturer's recommended protocols. Measurements were repeated at least three times, with independent cell batches obtained from three different donors.

PAI-1 Western blotting Cell extracts were prepared in lysis buffer containing 62.5 mM Tris, pH 6.8, 50 mM DTT, 2% SDS, 10% glycerol, and proteinase inhibitors. Total protein was measured using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, California). Samples were run on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and incubated with anti-PAI-1 antibody (1:200 dilution in 2.5% skim milk in PBS-T) for either 2 h at room temperature or overnight at 4°C with gentle agitation. Blots were then incubated with peroxidase-conjugated anti-mouse IgG (Amersham, Buckinghamshire, UK) at a dilution of 1:2000 in 2.5% skim milk in PBS-T, and were developed by enhanced chemiluminescence (Amersham).

Reverse fibrin autography The PAI-1 activity in cell lysates was assayed according to a slight modification of the method reported previously (Nordt *et al*, 1995). Samples of cell extracts (100 μg) were run on 10% SDS-polyacrylamide gels, under non-reducing conditions. Following electrophoresis, SDS was removed by washing with 2.5% Triton X-100 for 90 min at room temperature. Gels were placed on an agarose indicator containing thrombin (0.06 U per mL), purified plasminogen (5 μg per mL), fibrinogen (2 mg per mL), and tPA (0.05 U per mL). Gels were incubated overnight at 4°C to allow penetration of proteins into the fibrin-agarose, and then incubated for 3–4 h at 37°C to induce enzyme activity. PAI-1 activity was visualized by the appearance of an opaque band.

Northern blot analysis Total RNA samples (10 μg) were electrophoresed on 1% agarose gels containing formaldehyde and transferred onto Hybond-N^+ membranes (Amersham). Blots were prehybridized for 30 min at 62°C, then hybridized overnight at 62°C in the same solution containing ^{32}P-labeled probe. Following hybridization, blots were washed, and then analyzed using a Fuji BAS 2500 phosphoimager (Fuji Film, Tokyo, Japan).

Measurement of $[Ca^{2+}]_i$ Cells were grown on coverslips for 24 h, under serum-free conditions, then incubated with 4 μM Fluo-3, AM for 40 min. After three washes with serum-free medium, coverslips were mounted in perfusion chambers of our own design and subjected to confocal laser scanning microscopy (Zeiss LSM 410; Carl Zeiss, MicroImaging, Thornwood, New York). Scanning was performed at 1 s intervals with 488 nm excitation argon laser and 515 nm long-pass emission filter. SPC was added to the cells using an automatic pump system. All images were processed to analyze the changes in Ca^{2+} concentration at the cellular level. Results are expressed as relative fluorescence intensity.

Statistical analysis Data for PAI-1 and uPA ELISAs were statistically evaluated using Student's *t* test. Statistical significance was set at $p < 0.01$.

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