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Research Paper

Synthesis of Prostaglandin E_1 Phosphate Derivatives and Their Encapsulation in Biodegradable Nanoparticles

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Purpose. Prostaglandin E_1 (PGE_1) is an effective treatment for peripheral vascular diseases. The encapsulation of PGE_1 in nanoparticles for its sustained-release would improve its therapeutic effect and quality of life (QOL) of patients.
Methods. In order to encapsulate PGE_1 in nanoparticles prepared with a poly(lactide) homopolymer (PLA) and monomethoxy poly(ethyleneglycol)-PLA block copolymer (PEG-PLA), we synthesized a series of PGE_1 phosphate derivatives and tested their efficacy.
Results. Among them, PGE_1 2-(phosphonooxy)ethyl ester sodium salt (C2) showed the most efficient hydrolysis to yield PGE_1 in human serum. An *in vitro* platelet aggregation assay showed that C2 inhibited aggregation only after pre-incubation in serum, suggesting that C2 is a prodrug of PGE_1. *In vivo*, intravenous administration of C2 caused increase in cutaneous blood flow. In the presence of zinc ions, all of the synthesized PGE_1 phosphate derivatives could be encapsulated in PLA-nanoparticles. Use of L-PLA instead of D,L-PLA, and high molecular weight PLA resulted in a slower release of C2 from the nanoparticles.
Conclusions. We consider that C2-encapsulated nanoparticles prepared with L-PLA and PEG-D,L-PLA have good sustained-release profile of PGE_1, which is useful clinically.

KEY WORDS: biodegradable nanoparticles; encapsulation; prostaglandin E_1; zinc.

INTRODUCTION

The number of patients with peripheral obstructive vascular diseases such as arteriosclerosis obliterans has increased in line with aging of the population and increases in the prevalence of diabetes and hyperlipidemia. The condition can result in amputation of lower limbs or even death in severely affected patients (1). Various clinical treatments such as vascular bypass surgery have been developed for these diseases; however, the prognosis is not still good. Furthermore, a large number of patients (about 5–8% of elderly) suffer from mild peripheral vascular diseases (such as intermittent claudication) (2), for which effective drug treatments have not been established.

Prostaglandin E_1 (PGE_1), which has various physiological actions such as vasodilation, angiogenesis and inhibition of platelet aggregation, may thus serve as an effective treatment for peripheral obstructive vascular diseases. Results from a number of clinical and animal studies support this notion (3–5). However, the range of activities of PGE_1 are also related to adverse effects (such as hypotension and diarrhea) due to its distribution throughout the body when administered systemically (4,6). Furthermore, in addition to its chemical instability (hydrolysis to PGA_1), PGE_1 is easily inactivated by 15-hydroxydehydrogenase during the passage through the lung (7–9). Therefore, a drug delivery system that enables the stabilization of PGE_1 and its targeting at the site

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ABBREVIATIONS: ADAM, 9-anthryldiazomethane; ADP, adenosine 5′-diphosphate; ALP, alkaline phosphatase; AS-013, Δ^8-9-*O*-butyryl prostaglandin F_1 butyl ester; $(BnO)_2$-PN$(CH(CH_3)_2)_2$, dibenzyl *N*,*N*-diisopropyl phosphoramidite; C*x* (*x*=2, 3, 4, 6, 12), PGE_1 *x*-(phosphonooxy)alkyl ester sodium salt; DEA, diethanolamine; DMAP, 4-dimethylaminopyridine; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; EtOAc, ethyl acetate; FAB, fast atom bombardment; EPR, enhanced permeability and retention; HCl, hydrochloric acid; ^{1}H-NMR, proton nuclear magnetic resonance; HPLC, high-performance liquid chromatography; *m*-CPBA, *m*-chloroperoxybenzoic acid; MPS, mononuclear phagocyte system; MQW, Milli-Q water; MS, mass spectra; Mw, molecular weight; PBS, phosphate-buffered saline; PEG, poly(ethyleneglycol)PGE1, prostaglandin E1; PLA, poly(lactide); PLE, porcine liver esterase; PPP, platelet-poor plasma; PRP, platelet-rich plasma; QOL, quality of life; S.E.M., standard error mean; Tris, tris(hydroxymethyl) aminomethane.

of vascular injury is important. With these points in mind, we developed lipo-PGE_1, a preparation incorporating PGE_1 into an oil-in-water lipid emulsion (lipid microspheres) consisting of a soybean oil core and lecithin surfactant with a diameter of approximately 200 nm (10–12). Incorporation of PGE_1 into lipid microspheres protects PGE_1 from inactivation in the lung and enables the selective delivery of PGE_1 to damaged blood vessels, resulting in enhanced therapeutic effects and reduced adverse effects (10,13,14). Lipo-PGE_1 is used clinically in Japan, South Korea and China for systemic administration and exhibits a more potent therapeutic effect for peripheral obstructive vascular diseases than does PGE_1 clathrated in cyclodextrin which is used clinically worldwide (12,15). We also synthesized a stable PGE_1 prodrug (Δ^8-9-*O*-butyryl prostaglandin F_1 butyl ester, AS-013) and lipo-AS-013 showed superior characteristics to lipo-PGE_1 in both animal and clinical studies (6,16,17). However, lipid microspheres cannot retain PGE_1 for a long period of time *in vivo* (16,18). Therefore, daily intravenous drip infusion is necessary for clinical treatment with lipo-PGE_1, which in turn requires patient hospitalization, resulting in a low quality of life (QOL). Encapsulation of PGE_1 in more stable nanoparticles that permit a longer-lasting therapeutic effect provided by the sustained-release of PGE_1 would consequently be of significant clinical benefit. Encapsulation of PGE_1 in nanoparticles with size of approximately 50–200 nm would enhance the selective delivery of PGE_1 to damaged blood vessels due to the enhanced permeability and retention (EPR) effect (14).

The encapsulation of drugs in biodegradable and biocompatible polymeric solid particles, such as poly(lactide) homopolymer (PLA)-particles is effective for achieving a sustained-release formulation of drugs (19–21). For example, encapsulation of luteinizing hormone-releasing hormone in microparticles prepared from PLA achieved a long-term therapeutic effect by enabling sustained-release of the hormone concomitant with the degradation of PLA, as has already been employed in clinical practice (22,23). Thus, PLA-nanoparticles (diameter 50–200 nm for the EPR effect) with sustained-release of PGE_1 may prove beneficial for the treatment of peripheral obstructive vascular diseases. One obstacle to the use of solid nanoparticles in the treatment of patients in clinical practice is the uptake of these particles by the mononuclear phagocyte system (MPS), or in other words by the reticuloendothelial system (21,24). Use of a monomethoxy poly (ethyleneglycol)-PLA block copolymer (PEG-PLA) enables the nanoparticles to escape from this uptake due to the steric barrier by which the PEG chain prevents interaction of the nanoparticles with opsonins and cells responsible for MPS, such as Kupffer cells (stealth effect) (18,21,24). Another obstacle is that relatively hydrophilic drugs, such as PGE_1 and betamethasone, are very hard to encapsulate in PLA-nanoparticles (25–27). Hydrophilic drugs can be encapsulated into nanoparticles using a double emulsion (*w*/*o*/*w*) process, however, the size of this type of particles is generally more than 400 nm diameter and may have less EPR effect. For betamethasone, we recently overcame this obstacle by using betamethasone phosphate. After insolubilization in the presence of zinc, betamethasone phosphate could be efficiently encapsulated in PLA-nanoparticles by the oil-in-water solvent diffusion method. Betamethasone phosphate released upon degradation of the nanoparticles could then be hydrolyzed to yield betamethasone both *in vitro* and *in vivo*, resulting in a long-lasting therapeutic effect (28,29).

In the present study, we synthesized a series of PGE_1 phosphate derivatives with different spacer (alkyl chain) length (PGE_1 *x*-(phosphonooxy)alkyl ester sodium salt (C*x*; *x*=2, 3, 4, 6, 12)) and evaluated their efficacy both *in vitro* and *in vivo*. All of these derivatives can be encapsulated in PLA-nanoparticles. Of the derivatives, C2 showed the most efficient hydrolysis to yield PGE_1 in human serum. C2 showed a potent inhibitory activity on platelet aggregation *in vitro* and increased cutaneous blood flow *in vivo*. C2-encapsulated nanoparticles prepared with L-PLA and PEG-D,L-PLA showed a good sustained-release profile of C2.

MATERIALS AND METHODS

Materials and Animals

D,L-PLA, zinc chloride and 1,4-dioxane were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). L-PLA was from Taki Chemical Co., Ltd. (Kakogawa, Japan). AS-013 was from our laboratory stock. PEG-D,L-PLA (average molecular weight of PEG and PLA are 5,600 and 9,400, respectively) was synthesized and evaluated as described previously (18,30). Porcine liver esterase (PLE) and human placenta alkaline phosphatase (ALP) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Wistar rats (6 weeks old, male) were from Kyudo Co., Ltd. (Kumamoto, Japan). The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health, and were approved by the Animal Care Committee of Kumamoto University.

Analysis of Synthesized Molecules

Low-resolution- and high-resolution-fast atom bombardment (FAB) mass spectra (MS) were measured on a JMS-700 instrument (JEOL Ltd., Tokyo, Japan). Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JNM AL-300 instrument (300 MHz) (JEOL Ltd., Tokyo, Japan), using tetramethylsilane as an internal standard. Analytical thin-layer chromatography was performed using silica gel glass plates (60 F_{254}) (Merck Ltd., Tokyo, Japan). Column chromatography was performed using Silica gel 60N (Kanto Chemical Co., Tokyo, Japan). Compound 4 (PE1) shown in Fig. 1 was obtained from Daiichi Fine Chemical Co., Ltd. (Takaoka, Japan)

Synthesis of PGE_1 Phosphate

The structures of PGE_1 phosphate derivatives and outlines of their synthesis are shown in Fig. 1. The recovery of each compound and analysis of NMR data are provided in the supplementary information.

Compounds (1a–e) (1.7 mmol) were mixed with 1*H*-tetrazole (2.5 mmol) and dibenzyl *N*.*N*-diisopropyl phosphoramidite ($(BnO)_2$-$PN(CH(CH_3)_2)_2$) (3.4 mmol) in dichloromethane (10 ml). After stirring at room temperature overnight, *m*-chloroperoxybenzoic acid (*m*-CPBA) (3.4 mmol)

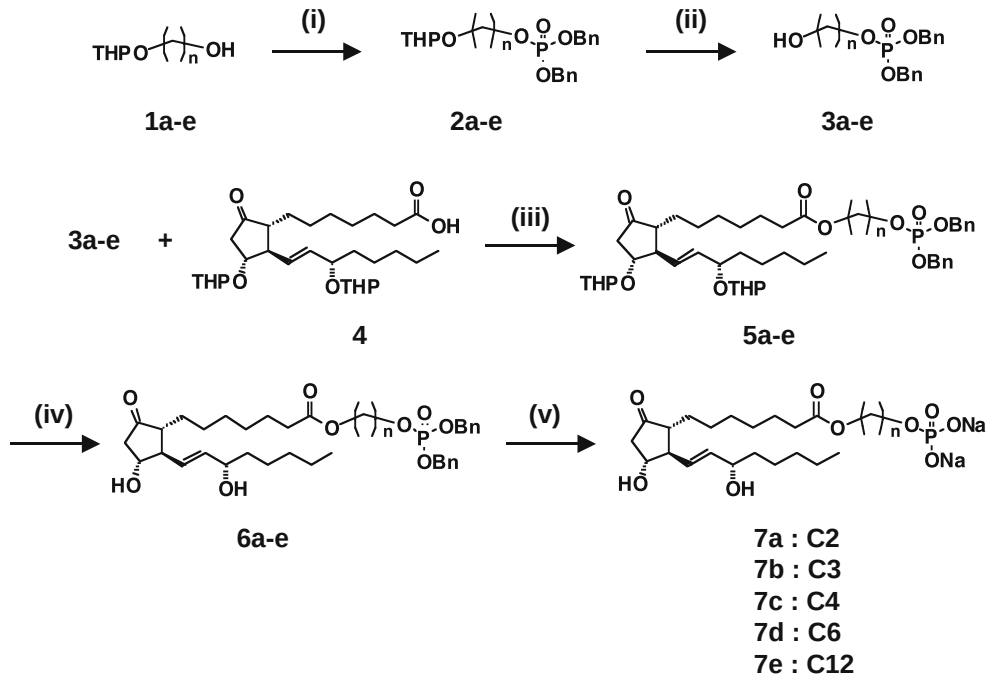

Fig. 1. Pathways for the synthesis of PGE_1 phosphate derivatives. Compounds: 1a–3a, 5a–7a (n=2); 1b–3b, 5b–7b (n=3); 1c–3c, 5c–7c (n=4); 1d–3d, 5d–7d (n=6); 1e–3e, 5e–7e (n=12). Reagents and solvents: *(i)* $(BnO)_2$-PN($CH(CH_3)_2)_2$, 1*H*-tetrazole, *m*-CPBA, dichloromethane; *(ii)* pyridinium *p*-toluene sulfonate, ethanol; *(iii)* EDC, DMAP, dichloromethane; *(iv)* acetic acid, tetrahydrofuran, MQW; *(v)* 1,4-cyclohexadiene, 10% palladium-carbon, acetic acid, sodium acetate, ethanol.

was added and stirred at room temperature for 30 min. The mixture was diluted with chloroform (30 ml), washed successively with saturated sodium hydrogen carbonate (10 ml×3) and saturated sodium chloride (10 ml×3). The organic layer was dried over sodium sulfate and concentrated in vacuo. The resulting residue was purified by silica gel column chromatography (from ethyl acetate (EtOAc)/hexane=1:1 to 100% EtOAc) to give compounds (2a–e) as a colourless oil.

Compounds (2a–e) (1.35 mmol) and pyridinium *p*-toluene sulfonate (0.3 mmol) in ethanol (5 ml) were stirred at 55°C for 3 h. After evaporation, the residue was purified by silica gel column chromatography (from EtOAc/hexane=3:2 to 100% EtOAc) to give compounds (3a–e) as a colourless oil.

Compounds (3a–e) (0.25 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (0.4 mmol), 4-dimethylaminopyridine (DMAP) (0.2 mmol) and compound 4 (PE1) (0.2 mmol) in dichloromethane (3 ml) were stirred at room temperature for 10 min. The mixture was diluted with chloroform (30 ml) and washed successively with saturated sodium hydrogen carbonate (10 ml×3) and saturated sodium chloride (10 ml×3). The organic layer was dried over sodium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc/hexane=1:1) to give compounds (5a–e) as a colourless oil.

Compounds (5a–e) (0.052 mmol) in acetic acid (1.8 ml)/tetrahydrofuran (0.45 ml)/Milli-Q water (MQW) (1.8 ml) were stirred at 35°C for 4 h and mixed with saturated sodium hydrogen carbonate (5 ml) at 0°C. The mixture was extracted with EtOAc (50 ml×3) and the combined organic layer was washed with saturated sodium chloride (10 ml×3) and dried over sodium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography (from EtOAc/hexane=1:1 to 100% EtOAc) to give compounds (6a–e) as a colourless oil.

Compounds (6a–e) (0.026 mmol) were mixed with 10% palladium-carbon (64 mg) in 1,4-cyclohexadiene (2.8 ml)/acetic acid (0.2 ml)/ethanol (5 ml). After stirring at room temperature for 2 h, sodium acetate (0.052 mmol) was added and 10% palladium-carbon was removed by filtration, followed by washing with ethanol. The combined filtrate was concentrated to give compounds (7a–e) as a yellowish paste.

Determination of PGE_1 and Its Derivatives

A Waters Alliance system, running Empower software (Milford, MA), was used for the high-performance liquid chromatography (HPLC) analysis. Samples were separated using a 4.6×100-mm TSKgel Super-ODS column (Tosoh Co., Tokyo, Japan).

For detection of PGE_1 phosphate derivatives, solvent A (acetonitrile) and solvent B (5 mM ammonium acetate) were used at a flow rate of 0.5 ml/min. After injection of sample (0 min), the mobile phase was changed as follows; 25% solvent A (1 min), a linear gradient of 25–60% solvent A (7 min), a linear gradient of 60–100% solvent A (5 min) and 100% solvent A (7 min). The detection was performed at 195 nm.

For detection of PGE_1, solvent A (acetonitrile) and solvent B (MQW) were used at a flow rate of 0.3 ml/min. Samples were incubated with 9-anthryldiazomethane (ADAM) (Funakoshi Co. Ltd., Tokyo, Japan) at 37°C for 8 h. After injection of the sample (0 min), the mobile phase was changed as follows; 65% solvent A (25 min), a linear gradient of 65–100% solvent A

(10 min) and 100% solvent A (10 min). Fluorescence at 412 nm (fluorescence peak wavelength of ADAM reagent) was detected using a 2475 Multi λ Fluorescence Detector.

Preparation and Characterization of Nanoparticles

Nanoparticles were prepared by the oil-in-water solvent diffusion method as described previously (30). L-PLA in 1,4-dioxane or D,L-PLA in acetone was mixed with PEG-D,L-PLA and diethanolamine (DEA) in acetone and zinc chloride and each PGE_1 phosphate derivative in MQW (the total amount of block copolymers and homopolymer was fixed at 25 mg and total volume was 0.8 ml). Samples were incubated for 10 min at room temperature. The mixture was added dropwise (at a rate of 48 ml/h) to 25 ml of MQW stirred at 1,000 rpm. After addition of 0.5 ml of 0.5 M sodium citrate (pH 7.0) and 12.5 μl of 200 mg/ml Tween80, nanoparticles were purified and concentrated by ultrafiltration (Centriprep YM-50, Millipore Co., Billerica, MA).

For determination of the PGE_1 phosphate derivative content in nanoparticles, the nanoparticle suspension was mixed with 0.01 M sodium citrate (pH 7.0) and centrifuged at 50,000×*g* for 30 min. The pellet was washed and suspended in MQW, freeze-dried and weighed. The PGE_1 phosphate derivative content was determined using HPLC, as described above. The drug content was defined as the ratio of PGE_1 phosphate derivative weight to the total weight of nanoparticles.

Particle size and distribution were determined by the dynamic light scattering method (ZETASIZER Nano-ZS, Malvern Instruments Ltd., Malvern, UK) and the average diameter was calculated by Marquadt's method.

Treatment of PGE_1 Phosphate Derivatives with Serum, Plasma and Enzyme

This assay was performed as described in (16) with some modifications. The PGE_1 phosphate derivative (1 mM) was incubated at 37°C in 0.1 ml of human serum, rat plasma, or 0.1 M tris(hydroxymethyl) aminomethane (Tris)/hydrochloric acid (HCl) (pH 7.4) containing 2.5 U PLE or 25 mU ALP. Samples were taken periodically and diluted with ice-cold methanol. After incubation on ice for 30 min, the mixtures were centrifuged at 16,100×*g* for 10 min. The supernatants were evaporated to dryness and PGE_1 or its derivative content was determined by HPLC as described above.

Assay of Inhibition of Platelet Aggregation

This assay was performed as described in (17) with some modifications. Venous blood was collected from healthy human volunteers using 3.8% sodium citrate as an anticoagulant. Samples were centrifuged for 15 min at 160×*g* to obtain the upper phase (platelet-rich plasma (PRP)), and the lower phase was further centrifuged for 10 min at 1,500×*g* to obtain platelet-poor plasma (PPP). PRP was pre-incubated with PGE_1 or its derivatives and then mixed with adenosine 5′-diphosphate (ADP) (2 μM at final concentration). Samples were further incubated for 3 min and the extent of aggregation was measured using an NKK hematracer (PAC-8S, Niko Bioscience Co., Ltd., Tokyo, Japan). PPP was used as control.

Measurement of Cutaneous Blood Flow

This was performed as described in (17) with some modifications. Wistar rats were anaesthetized and a blood flow meter probe (ALF21, Advance Co., Osaka, Japan) was attached to left planta pedis. PGE_1 or C2 was intravenously administered via the tail vein at a dose of 10 nmol/kg.

RESULTS

The structures of PGE_1 phosphate derivatives and outlines of their synthesis are shown in Fig. 1. Tetrahydropyran ethers (1a–e) (31) were reacted with $(BnO)_2$-PN$(CH(CH_3)_2)_2$ in the presence of 1*H*-tetrazole to give dibenzyl phosphites, which were then oxidized with *m*-CPBA to yield dibenzyl phosphates (2a–e). The tetrahydropyran-protected group of 2a–e was deblocked by treatment with pyridinium *p*-toluene sulfonate in ethanol to afford alcohols (3a–e). These alcohols were then coupled to compound 4 (PE1), producing esters (5a–e). Removal of the tetrahydropyran-protected groups in 5a–e with aqueous acetic acid gave 6a–e. Catalytic hydrogenation with 1,4-cyclohexadiene of 6a–e was followed by treatment with sodium acetate to provide the desired sodium salts (7a–e).

We prepared Cn (n=2, 3, 4, 6, 12)-encapsulated nanoparticles with D,L-PLA, PEG-D,L-PLA, zinc chloride and DEA by the solvent diffusion method using the same protocol as that used for preparation of betamethasone phosphate-encapsulated PLA-nanoparticles (30). The particle size was similar for the different PGE_1 phosphate derivatives (Fig. 2A). On the other hand, the efficiency of encapsulation (drug content of nanoparticles) increased as a function of the spacer (alkyl chain) length (Fig. 2B). As was the case for betamethasone phosphate, very little of each PGE_1 phosphate derivative (less than 0.1%) could be encapsulated in the nanoparticles prepared in the absence of zinc chloride, and PGE_1 could not be encapsulated in the nanoparticles even in the presence of zinc chloride (data not shown), suggesting that insolubilization due to the interaction between zinc ion and phosphate group is important for efficient encapsulation. These PEG-containing nanoparticles seem to have a "core-corona" structure, because the zeta potential value was much lower than that of PEG-non-containing nanoparticles (deta not shown).

The efficiency of each PGE_1 phosphate derivative for hydrolysis by PLE or ALP was compared. As shown in Fig. 3A, in addition to AS-013, the compounds C6 and C12 were gradually hydrolyzed to yield PGE_1 in the presence of PLE, while C2, C3 and C4 were not. On the other hand, all of the PGE_1 phosphate derivatives tested were hydrolyzed by ALP, although the efficiency was different for each one (Fig. 3B). We also compared the production of PGE_1 from each PGE_1 phosphate derivative in human serum. As shown in Fig. 3C, a clear-cut production was observed only with AS-013 and C2. Based on results in Fig. 3A–C, we hypothesized that C2 can be hydrolyzed by esterase if the phosphate group is removed by phosphatase. To test this notion, we examined the production of PGE_1 from C2 in the presence of both PLE and ALP. The efficient production of PGE_1 was observed in the presence of both enzymes, but not with PLE or ALP alone (Fig. 3A, D). We also examined the production of PGE_1 from C2 in rat plasma and found that this took place

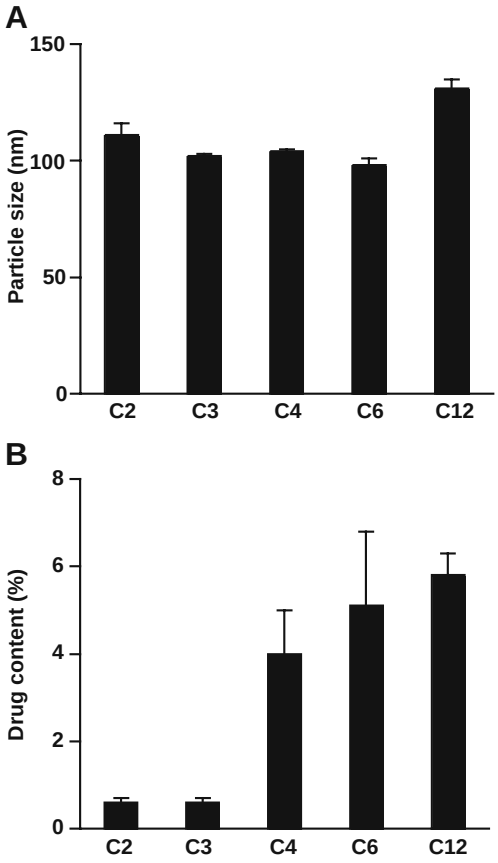

Fig. 2. Characterization of nanoparticles encapsulating PGE_1 phosphate derivatives. Nanoparticles encapsulating each PGE_1 phosphate derivative (5 mg) were prepared with 21 mg D,L-PLA and 4 mg PEG-D,L-PLA in the presence of 4.6 mg zinc chloride and 3.8 mg DEA by an oil-in-water solvent diffusion method. Particle size (**A**) and drug content (**B**) of the nanoparticles were determined as described in "MATERIALS AND METHODS." Values are mean ± S.E.M. (n=3).

within 30 min (Fig. 3E), which was more rapid than that in human serum (Fig. 3C). Based on the result in Fig. 3C, we selected C2 for further *in vitro* and *in vivo* analyses.

PGE_1 is chemically very unstable and the higher stability of AS-013 seems to be responsible for its relatively potent therapeutic effect (6,16,17). We compared the long-term chemical stability of C2 to that of AS-013 in water *in vitro*. As shown in Fig. 4A, C2 was more stable than AS-013 at 37°C. Stability at 4°C was indistinguishable between C2 and AS-013 (Fig. 4B).

We also examined the inhibitory effect of C2 on ADP-induced platelet aggregation *in vitro*, an established assay system for determining the anti-platelet aggregation activity of PGE_1 (16). We previously reported that, in contrast to PGE_1, pre-incubation in PRP is required for AS-013 to exert its inhibitory effect, because this step allows AS-013 to be hydrolyzed to PGE_1 (16). We reproduced this result here as shown in Table I. C2 showed no inhibitory effect on platelet aggregation without the pre-incubation step; however, it inhibited the aggregation as effectively as AS-013 and PGE_1 did after a 30 min pre-incubation (Table I). These results support the idea that C2 is efficiently hydrolyzed to yield PGE_1 in human blood.

We also evaluated the activity of C2 *in vivo* by monitoring its effect on cutaneous blood flow. Intravenous administration of PGE_1 induced a rapid and transient increase in blood flow (Fig. 5), as described previously (17). Compared to PGE_1, administration of C2 caused a slower-to-rise increase in blood flow, suggesting that C2 functions as a prodrug of PGE_1 and is gradually hydrolyzed in blood to yield PGE_1.

The *in vitro* release of C2 from different types of PLA-nanoparticles incubated at 37°C was examined in either phosphate-buffered saline (PBS) (Fig. 6C) or 50% fetal bovine serum (FBS) (Fig. 6D). Since the concentration of C2 in medium (released C2) was under the limitation of detection, the release was determined by measuring the C2 remaining in the particles. As shown in Fig. 6C, D, C2-encapsulated nanoparticles prepared with a blend of D,L-PLA (Mw=6,200) and PEG-D,L-PLA did not show good sustained-release profile in that more than half of encapsulated C2 was released from the nanoparticles within 6 h. We recently found that PLA-nanoparticles show better sustained-release profile when L-PLA is used instead of D,L-PLA (30), thus we applied this knowledge to the preparation of C2-encapsulated nanoparticles. As shown in Fig. 6C, D, C2-encapsulated nanoparticles prepared with L-PLA (Mw=5,500) released C2 slower than did those nanoparticles prepared using D,L-PLA (Mw=6,200). This modification of the PLA isomer did not clearly affect the particle size and the efficiency of C2 encapsulation (Fig. 6A, B).

As it has been suggested that higher molecular weight PLA results in nanoparticles with better sustained-release profile (32,33), we tested this idea for C2-encapsulated nanoparticles. The release of C2 from nanoparticles prepared with L-PLA (Mw=17,500) was much slower than that with L-PLA (Mw=5,500) (Fig. 6C, D). This modification of PLA molecular weight did not affect the particle size but did decrease the efficiency of C2 encapsulation (Fig. 6A, B). We determined the amount of Tween 80 in nanoparticles to about 6% of nanopartcles (*w*/*w*).

DISCUSSION

The incorporation of PGE_1 into lipid microspheres (lipo-PGE_1) that can then be administered via daily intravenous drip infusion is an effective means of treatment for patients with peripheral obstructive vascular diseases. However, daily drip infusion requires patient hospitalization, resulting in low QOL and prevents use of this drug for mild diseases, such as intermittent claudication. Thus, a new drug formulation that enables sustained-release of PGE_1 is therapeutically important. For this purpose, in this study, we have used the solvent diffusion method to prepare PGE_1 phosphate-encapsulated nanoparticles consisting of a blend of PEG-PLA and PLA. This idea is based on the following previous results: (1) PLA-nanoparticles slowly release the drug as the degradation of PLA proceeds (22,23,34); (2) a hydrophilic molecule with phosphate group (as in the case of betamethasone phosphate) becomes water-insoluble with zinc and can be encapsulated in PLA-nanoparticles (28,30); (3) incorporation of PEG-PLA in the PLA-nanoparticles prevents their uptake by MPS (18,21,24).

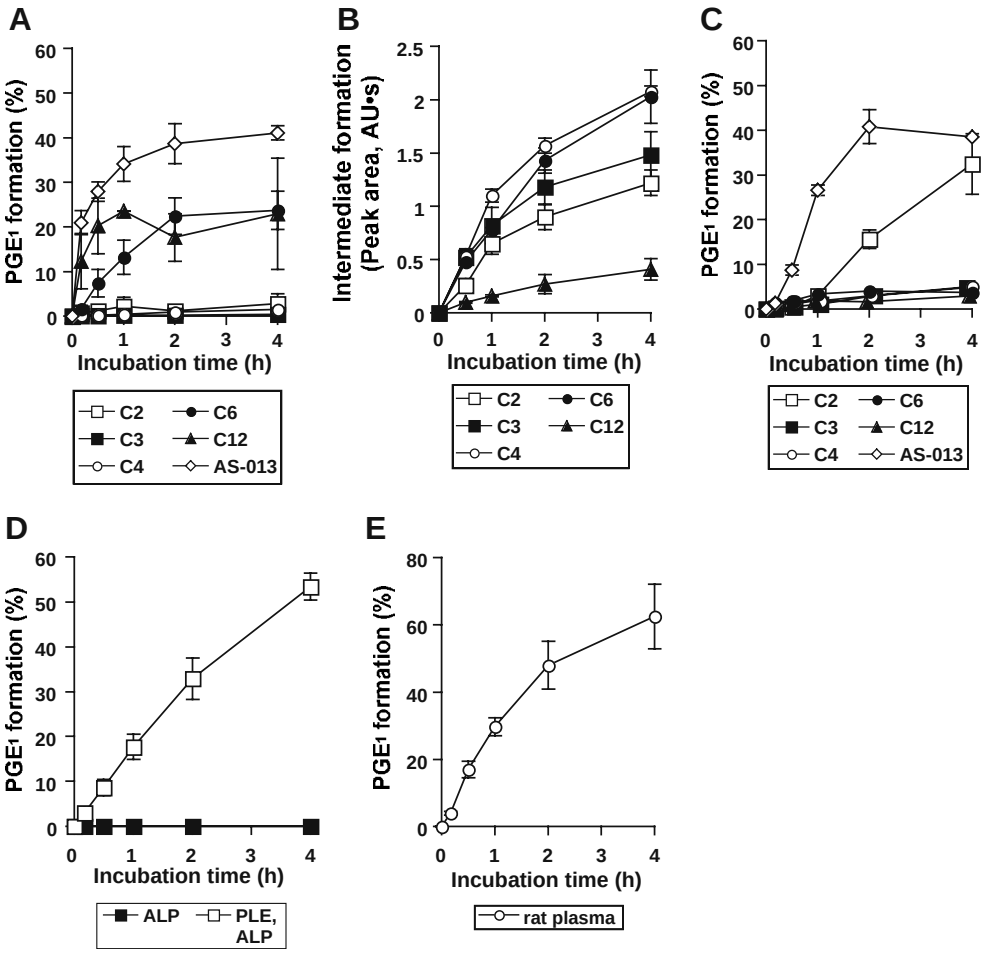

Fig. 3. Hydrolysis of PGE_1 phosphate derivatives. PGE_1 phosphate derivatives (**A**–**C**) or C2 only (**D**, **E**) were incubated at 37°C for indicated periods in the presence of PLE (**A**, **D**), ALP (**B**, **D**), human serum (**C**) or rat plasma (**E**). The amount of PGE_1 produced was determined by HPLC. The amount of each PGE_1 derivative before incubation was defined as the 100% value (**A**, **C**–**E**). The peak area corresponding to the dephosphorylated form of each derivative is shown in (**B**). Values are mean ± S.E.M. (n=3).

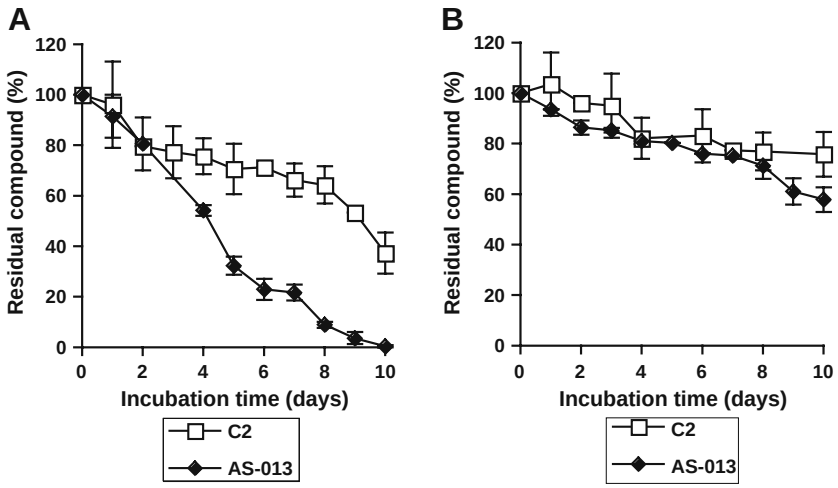

Fig. 4. Stability of C2 and AS-013. C2 or AS-013 (1 mM) was incubated at 37°C (**A**) or 4°C (**B**) in water for indicated periods. The amount of C2 or AS-013 remaining at specific time points was determined by HPLC. Values are mean ± S.E.M. (n=3).

Table I. Inhibitory Effect of PGE_1 and Its Derivatives on ADP-Induced Human Platelet Aggregation

Compound	ED50 (μM)			
	Incubation	0 min	15 min	30 min
PGE1		0.14±0.03	0.07±0.01	0.09±0.01
C2		>10	1.81±0.62	0.33±0.06
AS-013		4.92±1.11	0.81±0.06	0.73±0.07

Human PRP was pre-incubated with different concentrations of each PGE_1 derivative for the indicated periods at 37°C and platelet aggregation was induced by the addition of ADP. The concentration of each compound required for 50% inhibition of platelet aggregation (ED_{50}) is shown.

We synthesized a series of PGE_1 phosphate derivatives with different spacer (alkyl chain) length and established a protocol for their encapsulation in PLA-nanoparticles. The efficient encapsulation of each PGE_1 phosphate derivative in PLA-nanoparticles is dependent on the presence of zinc and DEA, i.e. zinc ion bridges between a carboxyl group of PLA and a phosphate group of the derivatives with a specific pH range obtained by DEA, seems to be important for the encapsulation process, as shown for the encapsulation of betamethasone phosphate (30). Among the derivatives, only C2 could be efficiently hydrolyzed to yield PGE_1 in human serum, showing that the spacer length is important for developing this type of prodrug. Surprisingly, C2 could be hydrolyzed to yield PGE_1 in the presence of PLE only after its dephosphorylation by ALP. The phosphate group of C2 may prevent esterase access to the ester bond, because PGE_1 phosphate derivatives with a longer spacer could be hydrolyzed in the presence of PLE. On the other hand, the more efficient hydrolysis of C2 than other derivatives in human serum suggests that de-phosphorylated C2 is better substrate for esterase in serum than that of other derivatives. Injectable nanoparticles that persist in the blood and contain other drugs could also offer a therapeutically important application

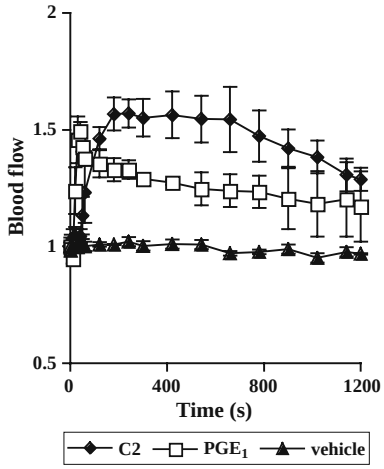

Fig. 5. Effect of C2 and PGE_1 on cutaneous blood flow. Wister rats were anaesthetized and C2, PGE_1 (10 nmol/kg) or vehicle was administered intravenously. The change of blood flow was monitored as a function of time. Values are mean ± S.E.M. (n=3).

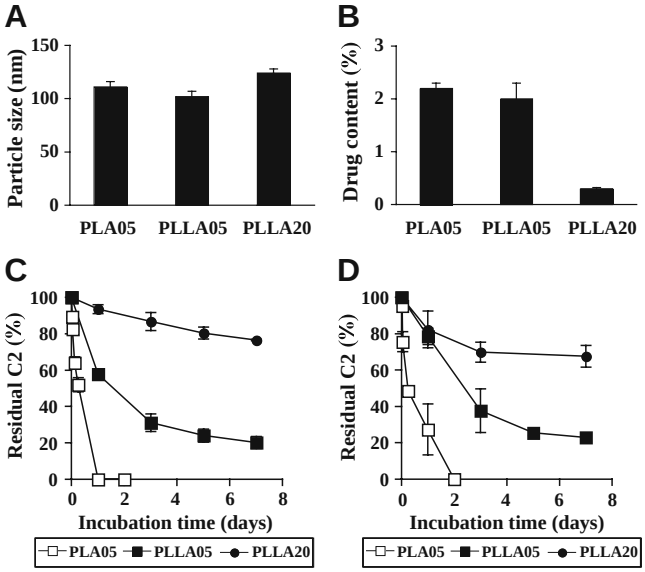

Fig. 6. Characterization of different nanoparticles encapsulating C2. Nanoparticles were prepared using D,L-PLA (Mw=6,200, 21 mg) (*PLA05*), L-PLA (Mw=5,500, 19 mg) (*PLLA05*) or L-PLA (Mw 17,500, 12.9 mg) (*PLLA20*) in the presence of zinc chloride (4.6, 2.7 or 2.7 mg for PLA05, PLLA05 or PLLA20, respectively) and DEA (1.9, 1.1 or 1.1 mg for PLA05, PLLA05 or PLLA20, respectively). Particle size (**A**) and drug content (**B**) were determined as described in the legend of Fig. 2. C2/PLA molar ratio in the formulation of PLA05, PLLA05 or PLLA20 was 0.16, 0.15 or 0.18, respectively. In (**C** and **D**), results are shown for experiments in which nanoparticles were dispersed in PBS (**C**) or 50% FBS (**D**) and incubated at 37°C for indicated periods. The C2 content remaining in the nanoparticles as a function of time was determined by HPLC. Values shown are mean ± S.E.M. (n=3).

because they enable site-specific and controlled-release delivery of the drug. The information obtained here for PGE_1 may be useful to develop similar approaches with respect to other prodrugs and their encapsulation in nanoparticles.

C2 showed good activity both *in vitro* and *in vivo*. The stability of C2 at 37°C in water was better than that of AS-013, which is much more stable than PGE_1 (16). In AS-013, the carbonyl group of PGE_1 at C-9 is acylated and a double bond between C-8 and C-9 is introduced to prevent the tautomerization (inactivation) of PGE_1 to its enol form (16,17). The phosphate group in C2 may stabilize PGE_1 in a different way. For example, acidic phosphate group in C2 may stabilize PGE_1 that is more stable under acidic conditions (35). Acylation at C-9 and the introduction of a double bond between C-8 and C-9 in C2 may result in better stability. An *in vitro* platelet aggregation assay showed that the activity of C2 is as potent as that of AS-013. Furthermore, the intravenous administration of C2 caused a slower-to-rise increase in blood flow, compared to PGE_1. This *in vivo* property may be due to the fact that C2 is more stable than PGE_1 and must be hydrolyzed to an active form. These results also point to the advantage of C2 not only as a molecule to be encapsulated in nanoparticles, but also for other clinical uses. For example, C2 in lipid microspheres (lipo-C2) may offer superior properties to those of lipo-PGE_1 and lipo-AS-013. We showed that the hydrolysis of C2 to yield PGE_1 took place more rapidly in rat plasma than in

human serum, even though it still took more than 30 min (Fig. 3E). On the other hand, blood flow increased very rapidly (within 5 min) after the intravenous administration of C2 (Fig. 5). This discrepancy was also observed for AS-013 (16) and may be due to the use of a very high concentration of C2 for the analysis in Fig. 3. The hydrolysis may occur rapidly *in vivo* where the drug concentration is much lower.

We achieved a good sustained-release profile of C2-encapsulated nanoparticles prepared using L-PLA, and in particular high molecular weight L-PLA. L-PLA has different physicochemical properties from D,L-PLA, such as thermodynamic stability and kinetic stability (36), which may be contribute to sustained-release profile of the nanoparticles. C2-containing nanoparticles prepared with L-PLA (Mw=17,500) were much better sustained-release profile than those prepared with L-PLA (Mw=5,500). Similar results have been reported for PLA-particles encapsulating other drugs (32,33).

Efficiency of C2 encapsulation in PLA-nanoparticles was very low. However, PGE_1 has high specific activities of vasodilation, angiogenesis and inhibition of platelet aggregation. For example, only 5 μg PGE_1 is clinically used per day for lipo-PGE_1. Therefore, even though the efficiency of C2 encapsulation is about 0.3% for nanoparticles prepared with L-PLA (Mw=17,500), the quantity of nanoparticles necessary to deliver an effective dose is calculated to be 16 mg of PLA-nanoparticle (50 μg of C2 corresponding to 35 (5 μg×7 days) μg of PGE_1) that is less than the amount of lipid microspheres (143 mg) administered with 5 μg PGE_1 as lipo-PGE_1.

We recently produced another type of PGE_1-encapsulated PLA-nanoparticles (18). In that report, PGE_1 itself was insolubilized with iron and encapsulated in PLA-nanoparticles. At present, we are not able to specify which formulation (PGE_1-iron or C2-zinc) would be more beneficial for clinical practice. The advantage of the PGE_1-iron formulation is that the safety and effectiveness of PGE_1 has already shown in clinical practice (C2 is a new compound and its safety needs to be tested more thoroughly). On the other hand, the advantage of the C2-zinc formulation is that C2 is more stable than PGE_1, which may result in a longer shelf life and easier mass production of the corresponding nanoparticles. Another advantage of the C2-zinc formulation is that the selective activation of C2 at sites of vascular injury and inflammation can be expected, since the alkaline phosphatase activity was suggested to be higher in peripheral tissues, particularly in inflamed tissue compared to blood (37,38). Furthermore, a number of beneficial activities of zinc, such as anti-atherosclerotic and anti-inflammatory activities, have been reported (39), which suggest that zinc in the C2-zinc PLA-nanoparticles may enhance the therapeutic effect of PGE_1. Further analyses of both formulations, especially of their pharmacological activity in animals, are necessary to determine which formulation is likely to be more suitable for clinical practice.

CONCLUSION

Based on results in this study, we consider that C2-encapsulated nanoparticles prepared with L-PLA and PEG-D,L-PLA have good sustained-release profile of PGE_1, which is useful clinically.

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