Core aldehydes of alkyl glycerophosphocholines in atheroma induce platelet aggregation and inhibit endothelium-dependent arterial relaxation

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Abstract **Plaque disruption with superimposed thrombosis is considered to be responsible for precipitating acute coronary syndrome. We identified *sn*-1-alkyl- and *sn*-1-acyl-type glycerophosphocholine (GroPCho) core aldehydes from human atheromas and demonstrated their activities on platelets and arteries. The naturally occurring core aldehydes were identified and quantified in relation to synthetic standards by high performance liquid chromatography with on-line electrospray mass spectrometry. 1-*O*-Hexadecyl-2-(5-oxovaleroyl)-*sn*-GroPCho (C_5 alkyl GroPCho core aldehyde), occurring in atheroma at less than 0.1% of total phosphatide, induced aggregation of washed rabbit platelets (50% effective dose was approximately 50 nM). Aggregations induced by C_5 alkyl GroPCho core aldehydes were completely inhibited by two different platelet-activating factor receptor antagonists. 1-Palmitoyl-2-(5-oxovaleroyl)-*sn*-GroPCho (C_5 acyl GroPCho core aldehyde) induced platelet shape change, but not aggregation. By contrast, 10 μM C_5 alkyl and C_5 acyl GroPCho core aldehydes both inhibited endothelium-dependent relaxation of rabbit artery by 50% (endothelium-independent relaxation was not affected). The present demonstration of platelet aggregation by physiologically relevant concentrations of alkyl GroPCho core aldehydes suggests that alkyl GroPCho core aldehyde generated in atheroma could be involved in precipitating acute coronary events, in which thrombus formation following lipid-rich plaque disruption plays an important role.**—Kamido, H., H. Eguchi, H. Ikeda, T. Imaizumi, K. Yamana, K. Hartvigsen, A. Ravandi, and A. Kuksis. **Core aldehydes of alkyl glycerophosphocholines in atheroma induce platelet aggregation and inhibit endothelium-dependent arterial relaxation.** ***J. Lipid Res.*** **2002.** 43: **158–166.**

Supplementary key words acute coronary syndrome • glycerophosphocholine • lipid peroxidation • mass spectrometry • phospholipid • platelet-activating factor

Atherosclerosis is considered to be a chronic inflammatory condition in which oxidized LDL plays important roles (1). The mechanism responsible for the sudden conversion of a stable disease (atherosclerosis without thrombosis) to life-threatening acute coronary syndrome is thought to be plaque disruption with superimposed thrombosis (2). Thrombus formation occurs when the thrombogenic lipid core becomes exposed to blood after plaque disruption (3, 4). The vulnerable plaque typically has a substantial lipid core.

We previously isolated the core aldehydes (aldehydes still esterified to parent molecules) of both surface (*sn*-1-acyl-type glycerophosphocholines, GroPCho) and interior lipid esters (cholesterol and acylglycerols) from extensively oxidized human LDL (5). Atherosclerotic lesions contain these core aldehydes, suggesting the presence of oxidized LDL in atheroma (6, 7). *sn*-1-Acyl-type C_5 GroPCho core aldehyde [1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine] is one of the three biologically active oxidized phospholipids in minimally oxidized LDL (6). Subbanagounder et al. (8) have claimed that phospholipid oxidation products and/or platelet-activating factor (PAF) play an important role in early atherogenesis. *sn*-1-Acyl-type C_5 GroPCho core aldehyde has a structure similar to that of PAF, which has proinflammatory and thrombogenic properties. The PAF receptor, however, is well known to show a strong preference for the *sn*-1 ether bond. Marathe et al. (9) reported the existence of 1-*O*-hexadecyl-2-(butanoyl or butenoyl)-*sn*-glycero-3-phosphocholines (C_4 PAF analogs) in oxidized LDL. 1-*O*-Hexadecyl-2-arachidonoyl-*sn*-GroPCho (precursor of PAF and C_4 PAF ana-

Abbreviations: C_5 acyl GroPCho core aldehyde, 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycerophosphocholine; C_5 alkyl GroPCho core aldehyde, 1-*O*-hexadecyl-2-(5-oxovaleroyl)-*sn*-glycerophosphocholine; DNPH, 2,4-dinitrophenylhydrazine; DNPH derivative, 2,4-dinitrophenylhydrazone derivate; ED_{50}, 50% effective dose; GroPCho, glycerophosphocholine; LC/ES/MS, reversed-phase HPLC with on-line electrospray mass spectrometry; PAF, 1-*O*-hexadecyl-2-acetyl-*sn*-glycerophosphocholine (platelet-activating factor); PtdCho, phosphatidylcholine.

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logs), however, occurs in plasma to a limited extent (10), whereas it is richly represented in the cell membranes of PAF-secreting macrophages (11). It would be anticipated that oxidation of 1-*O*-hexadecyl-2-arachidonoyl-*sn*-GroPCho (precursor membrane lipid of PAF) of the macrophages and foam cells of atheroma would be a rich source of 1-*O*-hexadecyl-2-(5-oxovaleroyl)-*sn*-GroPCho (C_5 alkyl GroPCho core aldehyde) (5, 12).

The purpose of this study was to confirm the presence of C_5 alkyl GroPCho core aldehydes in human atheroma, and to demonstrate that the core aldehydes generated from the same precursor membrane phospholipid as PAF have prothrombogenic properties. For this purpose, we semi-synthesized C_5 alkyl GroPCho core aldehydes and C_5 acyl GroPCho core aldehydes by subjecting the corresponding unsaturated GroPCho to ozonization and reduction with triphenylphosphine (13). Using these standards as reference compounds and carriers, we isolated *sn*-1 alkyl-type GroPCho core aldehydes as well as *sn*-1 acyl-type GroPCho core aldehydes from human atheromas and tested the effects of these core aldehydes on platelet aggregation and endothelium-dependent arterial relaxation.

MATERIALS AND METHODS

Chemicals and reagents

Cholesteryl oleate was obtained from Sigma (St. Louis, MO). 2,4-Dinitrophenylhydrazine (2,4-DNPH) was obtained from Aldrich (Milwaukee, WI). Acetonitrile and 2-propanol (HPLC grade) were from Wako Pure Chemical (Osaka, Japan) or Fisher Scientific (Toronto, Canada), whereas propionitrile was from Romil (Loughborough, England). All other chemicals and solvents were of reagent grade or better quality and were obtained from local suppliers.

Preparation of alkyl and acyl GroPCho core aldehydes

GroPCho core aldehydes (**Fig. 1**) were prepared by subjecting the corresponding unsaturated GroPCho to ozonization and reduction with triphenylphosphine as described previously (13). C_5 alkyl GroPCho core aldehyde was made from 1-*O*-hexadecyl-2-arachidonoyl-*sn*-GroPCho, whereas C_5 acyl GroPCho core aldehyde was made from 1-palmitoyl-2-arachidonoyl-*sn*-GroPCho. An ozone generator was purchased from Nigorigawa (Tokyo, Japan). GroPCho core aldehydes were purified by TLC and measured by gas-liquid chromatography as diglycerides after phospholipase C (*Bacillus cereus*) digestion as described previously (13).

Atheromas

Aortic plaques were removed, with the consent of the patients, from eight males and one female, between the ages of 41 and 79 years (mean, 69 years), who were undergoing endarterectomy for arteriosclerosis obliterans or aortic reconstruction for atherosclerotic aneurysm. Plaque materials were obtained in the operating room and processed immediately as described below. In addition, one atheroma sample was obtained postmortem from a 64-year-old woman, from whom samples of healthy arterial wall were also obtained.

Isolation of phospholipid core aldehydes from atheroma

The ester-bound fatty aldehydes were isolated by a method modified from Esterbauer et al. (5, 14). The atheroma sample was placed in 0.01 M phosphate-buffered saline, pH 7.4, containing EDTA (1 mg/ml) and butylated hydroxytoluene (200 μg/ml), washed, minced, and homogenized with a rotor/stator-type homogenizer (Ultra-Turrax; IKA-Werk, Staufen, Germany). Aldehydes in the homogenates were converted to the 2,4-DNPH derivatives, using a method described previously (5). DNPH derivatives of lipid ester core aldehydes were extracted with chloroform-methanol 2:1 (v/v). The DNPH derivatives were analyzed by HPLC with on-line electrospray mass spectrometry. The DNPH derivatives after phospholipase C digestion gave $[M-1]^-$ ions corresponding to 1-*O*-hexadecyl (1-*O*-octadecyl)-2-(9-oxo)nonanoyl-*sn*-glycerols and 1-*O*-hexadecyl (1-*O*-octadecyl)-2-(5-oxo)valeroyl-*sn*-glycerols.

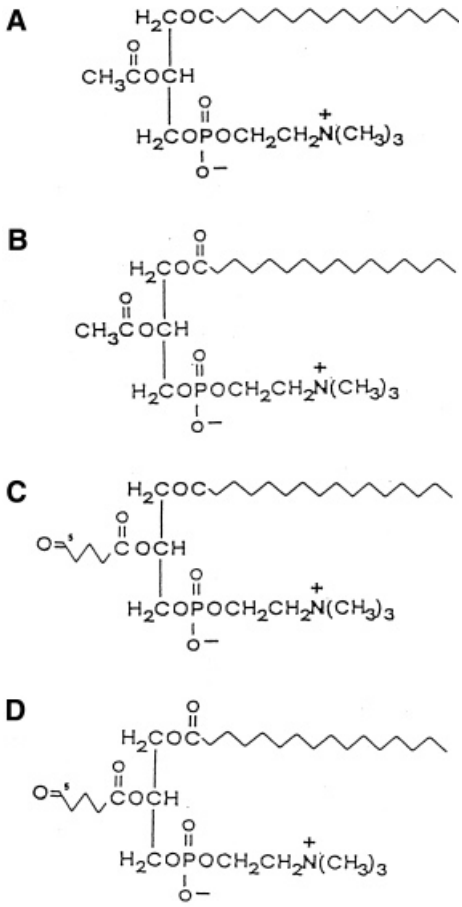

Fig. 1. Chemical structures of platelet-activating factors with an *sn*-1 alkyl or acyl group and of glycerophosphocholine core aldehydes with an *sn*-1 alkyl or acyl group. A: 1-*O*-Hexadecyl-2-acetyl-*sn*-glycerophosphocholine (PAF); B: 1-palmitoyl-2-acetyl-*sn*-glycerophosphocholine (acyl PAF); C: 1-*O*-hexadecyl-2-(5-oxovaleroyl)-*sn*-glycerophosphocholine (C_5 alkyl GroPCho core aldehyde); D: 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycerophosphocholine (C_5 acyl GroPCho core aldehyde).

TLC resolution of lipid ester core aldehydes

The DNPH-treated lipid extracts of the atheroma samples were applied to silica gel H plates and were subjected to TLC, using a double development with dichloromethane (to a height of 10 cm) and, after solvent evaporation, with toluene (to a height of 17 cm) (**Fig. 2**). The yellow zones corresponding to standard DNPH derivatives of 5-oxovaleroyl (R_f 0.34) and 9-oxononanoyl (R_f 0.41) cholesterol (zone 1) and the aldehyde esters of 7-ketocholesterol (R_f 0.10) (zone 2) were scraped off and eluted with chloroform-methanol. Similarly recovered were the DNPH derivatives of the phospholipid-bound aldehydes

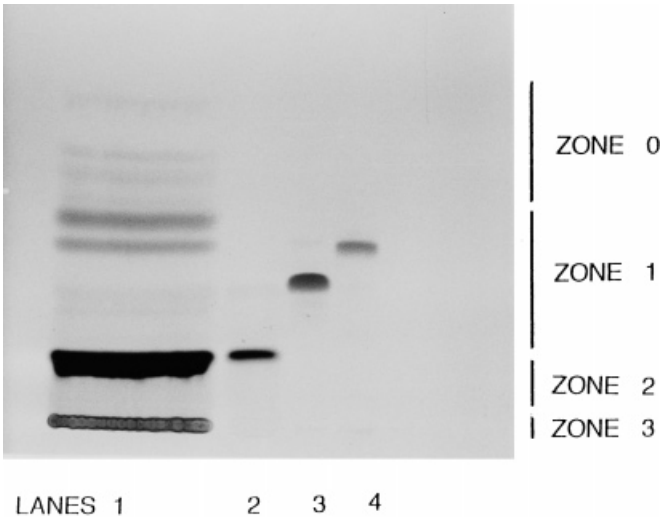

Fig. 2. Normal-phase thin-layer chromatogram of a total extract from a DNPH-treated atheroma sample. Zone 1, core aldehydes of cholesteryl esters; zone 2, core aldehydes of 7-ketocholesteryl esters; zone 3, core aldehydes of glycerophospholipids; zone 0, simple 16- to 18-carbon aldehydes. Lane 1, total lipid extract of DNPH-treated atheroma; lane 2, free DNPH reagent; lane 3, DNPH derivative of cholesteryl 5-oxovalerate; lane 4, DNPH derivative of cholesteryl 9-oxononanoate. TLC conditions as given in Materials and Methods. TLC bands are recognized by the yellow color of the DNPH reagent or the hydrazones. Sample: atheroma obtained from a 77-year-old male patient.

from the origin of the TLC plate (R_f 0.0–0.05) (zone 3), which are further described below.

Dephosphorylation with phospholipase C

Aliquots of lipid extracts obtained with acidified DNPH and the TLC fraction of the DNPH derivatives of the phospholipid-bound aldehydes were subjected to dephosphorylation with phospholipase C (*B. cereus*) for 2 h at 37°C as previously described (5).

Reversed-phase HPLC of DNPH-modified lipid extracts dephosphorylated with phospholipase C was performed on a reversed-phase C_{18} column, using a linear gradient of 10–30% 2-propanol in acetonitrile or 20–80% 2-propanol in methanol (30 min). The HPLC peaks were monitored at 358 nm (5). The negatively charged $[M]^-$ ions in the thermospray were also monitored (12).

Reversed-phase HPLC with direct liquid inlet interface mass spectrometry (LC/DIL/MS) of DNPH derivatives of diradylglycerols derived from oxophospholipid extracts of atheroma

HPLC was performed on Supelcosil LC-18 column (250 × 4.6 mm ID, Mississauga, Ontario) with a linear gradient of 30–90% propionitrile in acetonitrile. The column was installed in a Hewlett-Packard Model 1084 B liquid chromatograph and was run at 1–1.5 ml/min. About 1% of effluent was admitted to a Hewlett-Packard Model 5985B quadrupole mass spectrometer. Negative chemical ionization spectra were taken every 5 s in the mass range of 200–900 (5).

Normal-phase HPLC and LC/MS of total lipid extracts and TLC subfractions from atheromas

Normal-phase HPLC separations of phospholipids were performed on Spherisorb 3-μm columns [100 mm × 4.6 mm ID (Analtech, Deerfield, IL)] installed into a Hewlett-Packard (Palo Alto, CA) model 1090 liquid chromatograph connected to a Hewlett-Packard model 5988B quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray interface. The column was eluted with a linear gradient of 100% solvent A [chloroform-methanol-30% ammonium hydroxide 80:19.5:0.5 (v/v/v)] to 100% solvent B [chloroform-methanol-water-30% ammonium hydroxide 60:34:5:0.5(v/v/v/v)] in 14 min, then at 100% solvent B for 10 min. Both negative and positive ionization spectra were taken in the mass range 400–1,100. The molecular species of the various glycerophospholipids were identified on the basis of the molecular mass provided by the mass spectrometer, the knowledge of the fatty acid composition of the phospholipid class, and the relative elution order (the less polar long-chain species emerging ahead of the more polar short-chain species) of the phospholipids from the normal-phase column. Selected ion spectra were retrieved from the total ion spectra by computer (15, 16).

Preparation of washed rabbit platelets

Blood was drawn from a carotid artery by cannulation or from the heart by cardiocentesis of male Japanese white rabbits, using 3.8% sodium citrate as anticoagulant (9:1). Pentabarbiturate injection was used as anesthetic. Platelet-rich plasma was prepared by centrifugation of the blood at 225 *g* for 10 min. To the platelet-rich plasma, a 1:1,000 volume of prostacyclin (0.1 mg/ml) was added and centrifuged at 1,200 *g* for 10 min. The sedimented platelets were washed twice with citrate-buffered saline (pH 6.5). The final pellets were resuspended in HEPES-Tyrode buffer (pH 7.38) at a density of 3.0×10^8 platelets/ml.

Determination of platelet-aggregating activity

Test samples of C_5 alkyl core aldehyde or of C_5 and C_9 acyl GroPCho core aldehydes were dissolved in 99.5% ethanol, and a 4-μl aliquot of the solution was mixed with 400 μl of platelet suspension (3.0×10^8 platelets/ml) in HEPES-Tyrode buffer containing 1 mM $CaCl_2$ and fibrinogen (0.5 mg/ml) at 37°C. Platelet aggregation was monitored by continuous recording of light transmission, using a C550 whole blood aggregometer (Chrono-Log, Havertown, PA). In some experiments, the platelets were pretreated with different concentrations of CV-6209 or BN52021 for 1 min before the addition of test samples of C_5 alkyl GroPCho core aldehydes.

Shape change analysis of platelets

The light transmission of a suspension of asymmetric particles (discoid platelets) increases with stirring, whereas that of spherical, symmetric particles with or without uniformly distributed pseudopods is insensitive to stirring. On the basis of the light-scattering theory, the shape change parameter assay has been used to assess platelet responses to low dose or weak stimulants (17, 18). This method allows quantitative analysis of the change in shape. Test samples of C_5 acyl GroPCho core aldehydes were dissolved in 99.5% ethanol, and a 4-μl aliquot of the solution was mixed with 400 μl of platelet suspension (3.0×10^8 platelets/ml) in HEPES-Tyrode buffer containing 1 mM $CaCl_2$ and fibrinogen (0.5 mg/ml) at 37°C. Light transmission was monitored with a C550 whole blood aggregometer (Chrono-Log). The stirrer was turned on and off, alternately, every 30 s. In some experiments, the platelets were pretreated with different concentrations of CV-6209, staurosporine, and cytochalasin D for 3 min before the addition of test samples of C_5 acyl GroPCho core aldehydes. The shape change parameter was calculated as 1 − test deflection/control deflection.

Preparation of rabbit arteries

All animal experiments were conducted in accordance with the guidelines issued by the Animal Research Committee of the Kurume University School of Medicine. Japanese white rabbits

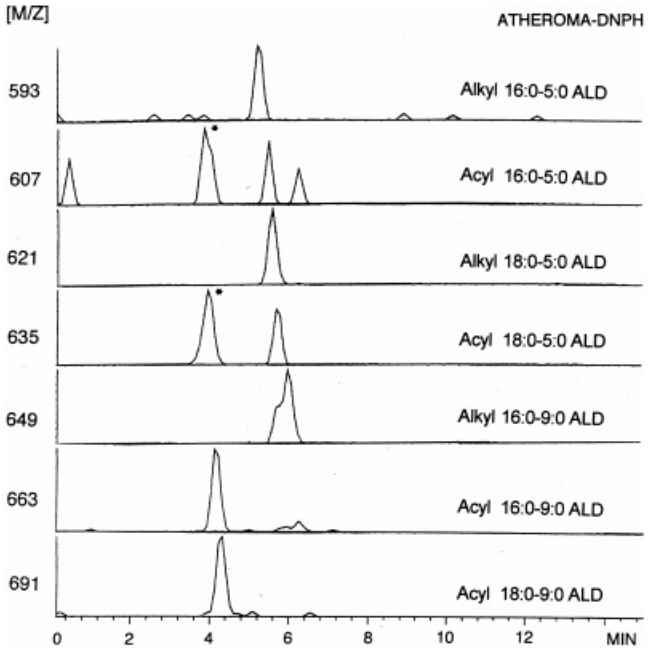

Fig. 3. Single-ion plots (*m/z*) for the DNPH derivatives of the major alkylacyl and diacylglycerol core aldehydes derived from glycerophospholipids of human atheroma. Peak identification: alkyl 16:0-5:0 ALD, 1-*O*-hexadecyl-2-(5-oxovaleroyl)-*sn*-GroPCho; acyl 16:0-5:0 ALD, 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-GroPCho; alkyl 18:0-5:0 ALD, 1-*O*-octadecyl-2-(5-oxovaleroyl)-*sn*-GroPCho; acyl 18:0-5:0 ALD, 1-stearoyl-2-(5-oxovaleroyl)-*sn*-GroPCho; alkyl 16:0-9:0 ALD, 1-*O*-hexadecyl-2-(9-oxononanoyl)-*sn*-GroPCho; acyl 16:0-9:0 ALD, 1-palmitoyl-2-(9-oxononanoyl)-*sn*-GroPCho; acyl 18:0-9:0 ALD, 1-stearoyl-2-(9-oxononanoyl)-*sn*-GroPCho. Sample: Atheroma obtained from a 77-year-old male patient.

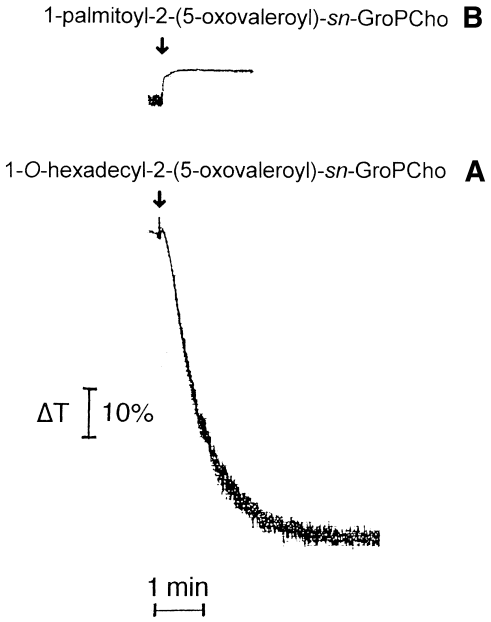

Fig. 4. Effects of (A) 1 μM 1-*O*-hexadecyl-2-(5-oxovaleroyl)-*sn*-GroPCho and (B) 5 μM 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-GroPCho on aggregations of washed rabbit platelet.

(2.5–3.5 kg) were anesthetized with sodium pentobarbital (30 mg/kg, intravenous) and the abdominal aorta was rapidly excised and placed in Krebs-Henseleit buffer oxygenated with a gas mixture of 95% O_2-5% CO_2 of the following composition (in mM): NaCl 118.3, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, and glucose 11.1 at pH 7.35 to 7.45. The isolated aorta was cleaned of periarterial tissue and was cut into rings 2 to 3 mm in length.

Organ chamber experiments

Pharmacological experiments with isolated rabbit arterial strips were performed as previously described (19). Rings were mounted on stainless steel hooks, suspended in 2-ml tissue baths, and connected to force-displacement transducers (UC-2; Kishimoto Industry, Shizuoka, Japan) to record changes in isometric force on an eight-channel recorder (Recti-Horiz-8K; Sanei, Osaka, Japan). The baths were filled with 2 ml of Krebs-Henseleit solution and aerated at 37°C with a gas mixture of 95% O_2-5% CO_2. Rings were initially stretched to give a preload of 2.0 *g* force and were equilibrated. To examine the effects of GroPCho core aldehydes, the rings were preincubated with 10 μM acyl or alkyl C5 GroPCho core aldehydes for 60 min. During this period, the Krebs-Henseleit solution with 10 μM acyl or alkyl C_5 GroPCho core aldehydes in the tissue bath was replaced every 20 min. GroPCho core aldehydes were dispersed with sonication for 3 min in Krebs-Henseleit buffer before use. Relaxations were examined during a contraction caused by 0.3 μM phenylephrine. Once a stable contraction with phenylephrine was obtained, an endothelium-dependent vasodilator, acetylcholine (Ach), was added to the bath in cumulative concentrations at 10^{-9} to 10^{-5} M, or an endothelium-independent vasodilator, acidified $NaNO_2$ (10^{-8} to 10^{-3} M), was added. In all experiments, indomethacin (10^{-5} M) was added to organ baths to exclude the effect of endogenous prostanoids. Data are expressed as percentage relaxation of the contractions made in response to phenylephrine.

RESULTS

Isolation and identification of GroPCho core aldehydes from human atheromas

Figure 2 shows a normal-phase TLC separation of the total lipid extract from a DNPH-treated atheroma sample. Only the hydrazone bands are visible. Zone 0 contains the DNPH derivatives of simple C_{16-18} aldehydes, zone 1 contains the core aldehyde esters of cholesterol, zone 2 contains the core aldehyde esters of 7-ketocholesterol, and zone 3 contains the core aldehyde esters of GroPCho. The hydrazones in the various TLC zones were identified by HPLC in relation to standards and by on-line mass spectrometry. **Figure 3** shows the single-ion plots for the DNPH derivatives of the major core aldehydes of the alkyl and acyl GroPCho recovered from the atheroma of a patient with abdominal aneurism. The mass chromatograms represent DNPH derivatives of the aldehyde-containing diacylglycerol moieties derived from DNPH derivatives of the core aldehydes of phosphatidylcholine (PtdCho) by hydrolysis with phospholipase C. The major core aldehydes were identified as the C_{16} alkyl (*m/z* 593) and C_{18} alkyl (*m/z* 621) GroPCho (5-oxo)valeroyl and C_{16} (*m/z* 649) and C_{18} (*m/z* 691) GroPCho (9-oxo)nonanoyl derivatives, along with the C_{16} acyl (*m/z* 607) and C_{18} acyl (*m/z* 635) GroPCho (5-oxo)valeroyl and C_{16} acyl (*m/z* 663) and C_{18} acyl (*m/z* 691) GroPCho (9-oxo)nonanoyl derivatives. The

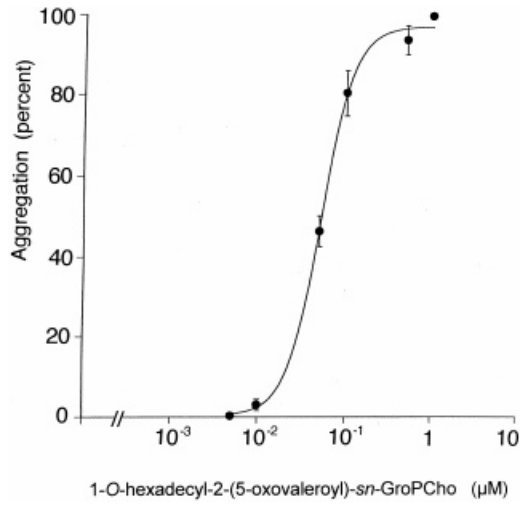

Fig. 5. Dose-response curve for platelet aggregation induced by 1-*O*-hexadecyl-2-(5-oxovaleroyl)-*sn*-GroPCho. Each point represents the mean ± SE of five separate experiments.

proportions of the alkyl and acyl C_5 GroPCho core aldehydes approximated the proportions of the alkyl and acyl *sn*-1-16:0(18:0)-2-20:4 GroPCho in the atheroma lipid extract and their content seldom exceeded 0.1% of the total PtdCho content (results not shown). The amounts of the core aldehydes varied considerably, probably reflecting differing degrees of maturity of the atheroma.

Effects of C_5 GroPCho core aldehydes on platelet aggregation

Figure 4 shows platelet-aggregating activities of 1 μM C_5 alkyl GroPCho core aldehyde on washed rabbit platelets.

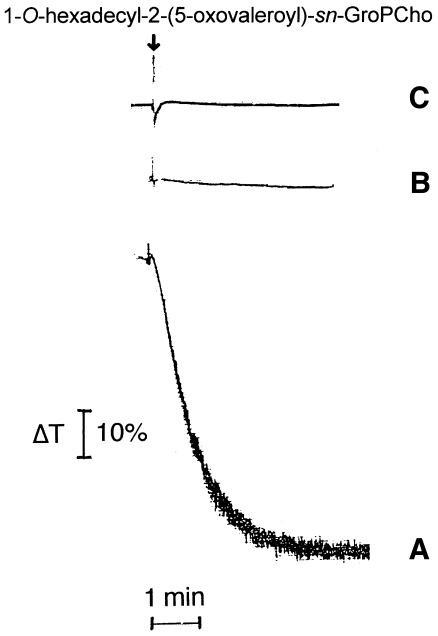

Fig. 6. Effects of CV-6209 and BN52021 on aggregations of washed rabbit platelets induced by 1 μM 1-*O*-hexadecyl-2-(5-oxovaleroyl)-*sn*-GroPCho. A: Control. B: Pretreated with 0.1 μM CV-6209. C: Pretreated with 50 μM BN52021 for 1 min at 37°C.

Percent aggregation induced by C_5 alkyl GroPCho core aldehyde was 100 ± 0% at 1 μM, 93.9 ± 3.7% at 0.5 μM, 80.5 ± 5.7% at 0.1 μM, 46.5 ± 3.9% at 0.05 μM, 3.0 ± 1.4% at 0.01 μM, and 0.3 ± 0.3% at 0.005 μM (mean ± SE; n = 5) (**Fig. 5**). The 50% effective dose (ED_{50}) for inducing platelet aggregation was about 50 nM. The aggregations induced by C_5 alkyl GroPCho core aldehyde (**Fig. 6A**) were completely suppressed in the presence of two structurally different PAF receptor antagonists: CV-6209 (Fig. 6B) or BN52021 (Fig. 6C).

In contrast to C_5 alkyl GroPCho core aldehyde, C_5 acyl GroPCho core aldehyde did not induce aggregation up to 10 μM. Higher concentrations than 10 μM tended to cause platelet lysis.

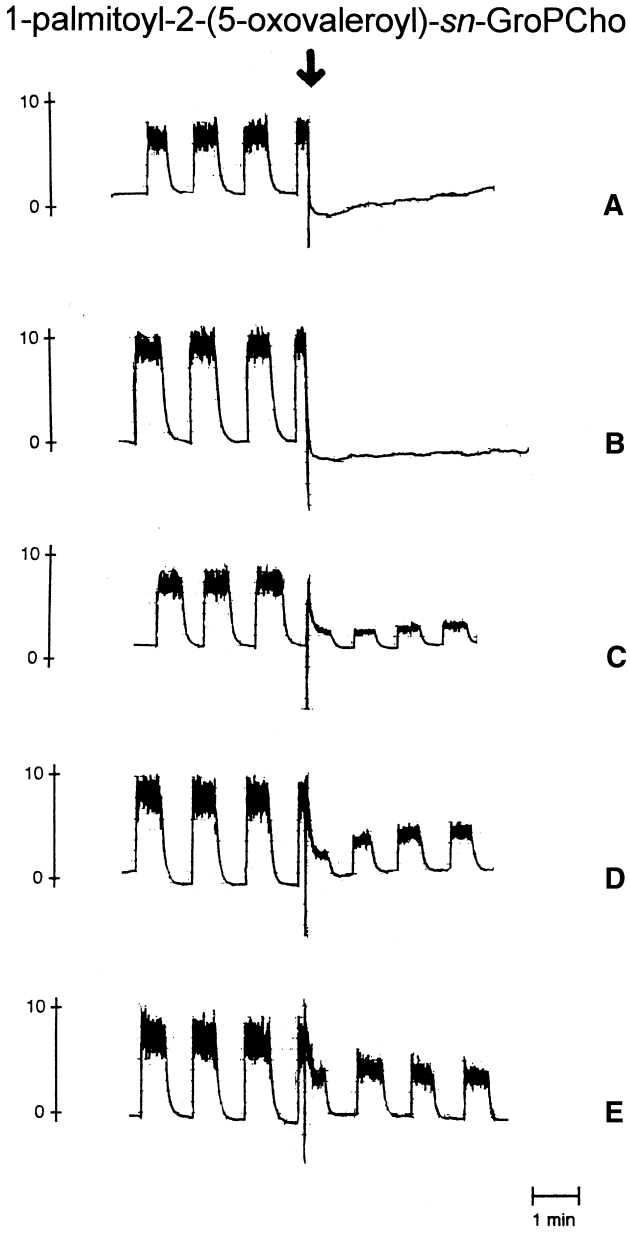

Fig. 7. Effects of (A) 10 μM, (B) 5 μM, (C) 1 μM, (D) 0.5 μM, and (E) 0.1 μM 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-GroPCho on shape changes of washed rabbit platelets. The stirrer was turned on and off every 30 s alternately. The ordinate represents light transmission in arbitrary units, and the abscissa indicates time.

Effect of C_5 acyl GroPCho core aldehyde on platelet shape change

C_5 acyl GroPCho core aldehyde failed to induce platelet aggregation. However, as is shown in Fig. 4B, the light transmission of a platelet suspension decreased immediately after addition of C_5 acyl GroPCho core aldehyde. This was regarded as an indicator of platelet disc-to-sphere shape change. The shape change parameter assay was performed to assess platelet responses to weak stimulants as previously described (17, 18). **Figure 7** illustrates the effects of (A) 10 μM, (B) 5 μM, (C) 1 μM, (D) 0.5 μM, and (E) 0.1 μM C_5 acyl GroPCho core aldehyde on shape changes. The values of the shape change parameter were 1 at 10 μM, 1 at 5 μM, 0.72 ± 0.03 at 1 μM, 0.59 ± 0.07 at 0.5 μM, and 0.54 ± 0.08 at 0.1 μM (mean ± SD; 10 μM, n = 1; 5 μM, n = 2; 1 μM, n = 6; 0.5 μM, n = 11; 0.1 μM, n = 3). The shape change induced by C_5 acyl GroPCho core aldehyde was not suppressed in the presence of PAF receptor antagonist (CV-6209) (**Fig. 8**). A 1 μM concentration of CV-6209 did not, by itself, induce shape changes (n = 4). The shape change was suppressed, however, in the presence of protein kinase inhibitor (staurosporine) or in the presence of inhibitor of actin polymerization (cytochalasin D), suggesting involvement of protein kinase activation and cytoskeletal change (Fig. 8).

Effects of C_5 GroPCho core aldehydes on endothelium-dependent arterial relaxation

Effects of C_5 alkyl or acyl GroPCho core aldehydes on endothelium-dependent arterial relaxation evoked by Ach were examined (**Fig. 9**). Incubations of rabbit artery rings with 10 μM concentrations of C_5 alkyl or C_5 acyl GroPCho core aldehydes exerted similar significant ($P < 0.001$) inhibitory effects on endothelium-dependent relaxation to cumulative concentrations of Ach (10^{-9} to 10^{-5} M) during a stable plateau contraction caused by 0.3 μM phenylephrine compared with time control artery rings (rings incubated in Krebs-Henseleit buffer). The endothelium-independent relaxation evoked by acidified $NaNO_2$ was not significantly affected by incubations with 10 μM C_5 alkyl GroPCho core aldehyde or C_5 acyl GroPCho core aldehyde (**Fig. 10**).

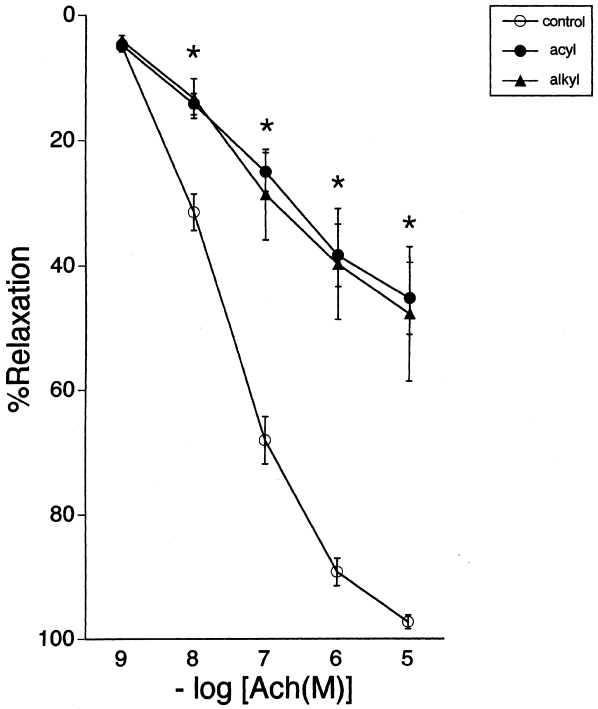

Fig. 9. Cumulative concentration-response curves demonstrating the effects of 1-*O*-hexadecyl-2-(5-oxovaleroyl)-*sn*-GroPCho and 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-GroPCho on endothelium-dependent relaxations in rabbit aortic rings. Each curve is shown from the rings relaxed with cumulative concentrations of acetylcholine after treatment for 60 min with Krebs-Henseleit buffer (control) or 10 μM 1-*O*-hexadecyl-2-(5-oxovaleroyl)-*sn*-GroPCho (alkyl) or 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-GroPCho (acyl) in Krebs-Henseleit buffer. Each point is the mean of 16 (control and acyl) and 8 (alkyl) observations. Vertical bars indicate the SE. * $P < 0.001$ as compared with controls.

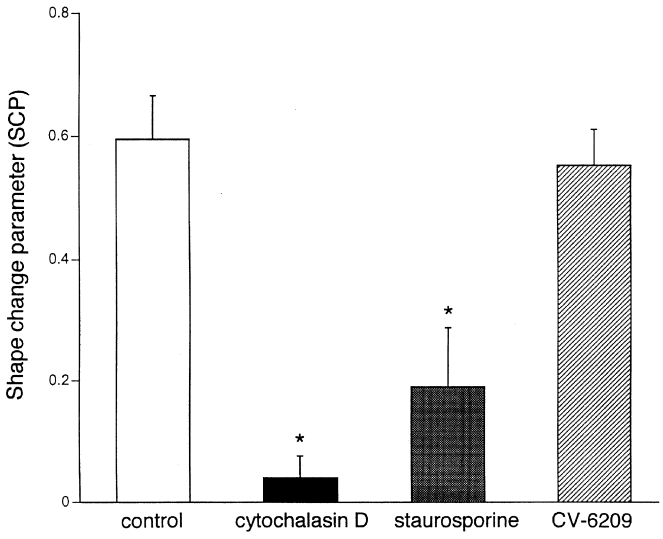

Fig. 8. Effects of cytochalasin D (0.01 μM), staurosporine (50 nM), and CV-6209 (1 μM) on the shape change parameter (SCP) of washed rabbit platelets induced by 0.5 μM 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-GroPCho. Data represent means ± SD of the experiments (control, n = 11; cytochalasin D, n = 3; staurosporine, n = 4; CV-6209, n = 4) * $P < 0.001$, Student's *t*-test. CV-6209 (1 μM) by itself did not induce shape changes (n = 4).

DISCUSSION

The C_5 alkyl GroPCho core aldehyde induced aggregation on washed rabbit platelets through the PAF receptor at ED_{50} of 50 nM. In contrast, C_5 acyl GroPCho core aldehyde did not induce aggregation up to a concentration of 10 μM. The results confirm the importance of the *sn*-1-alkyl ether group in inducing platelet aggregation through activation of the PAF receptor. The present results for C_5 GroPCho core aldehydes are consistent with those for GroPChos containing a short-chain monocarboxylate or dicarboxylate ester reported earlier (20–22). C_5 acyl GroPCho core aldehyde induced shape change. The abil-

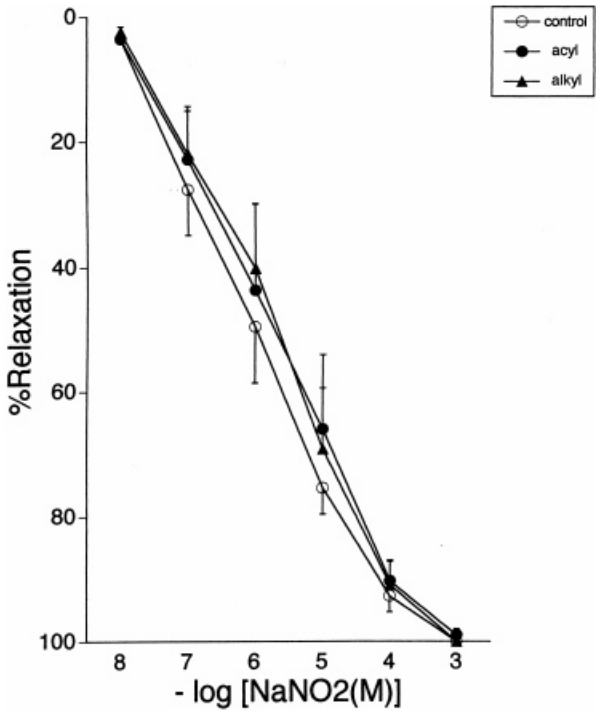

Fig. 10. Cumulative concentration-response curves demonstrating the effects of 1-*O*-hexadecyl-2-(5-oxovaleroyl)-*sn*-GroPCho and 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-GroPCho on endothelium-independent relaxations in rabbit aortic rings. Each curve is shown from the rings relaxed with cumulative concentrations of acidified $NaNO_2$ after treatment for 60 min with Krebs-Henseleit buffer (control) or 10 μM 1-*O*-hexadecyl-2-(5-oxovaleroyl)-*sn*-GroPCho (alkyl) or 1-palmitoyl-2-(5-oxovaleroyl)-sn-GroPCho (acyl) in Krebs-Henseleit buffer. Each point is the mean of four (control, acyl, and alkyl) observations. Vertical bars indicate the SE.

ity was not mediated by PAF receptor. The shape change was suppressed in the presence of staurosporine or cytochalasin D. Because staurosporine is a potent inhibitor of many tyrosine and serine/threonine protein kinases, including myosin light chain kinase (23), the actin polymerization through protein kinase activation by C_5 acyl GroPCho core aldehyde was considered to be involved in this ability.

In contrast to the effects on platelet activation through the PAF receptor, C_5 alkyl or acyl GroPCho core aldehydes exerted similar inhibitory effects on endothelium-dependent arterial relaxation. The results were similar to those of lyso-phospholipids (lysoPtdCho, lyso-PAF, and sphingomyelin) reported by Mangin et al. (24). Considering the high concentrations (10 μM) required by these lysophospholipids to exert inhibitory effects on endothelium-dependent arterial relaxation, the effects could be due, at least in part, to the nonspecific detergent effect of these lyso-phospholipids (25).

The existence of various oxidized acyl GroPChos (surface components of lipoprotein) has been previously reported in minimally to extensively oxidized lipoproteins. We reported the presence of 1-palmitoyl-(1-stearoyl) 2-(9-oxononanoyl)-, 1-palmitoyl-(1-stearoyl) 2-(8-oxooctanoyl)-, and 1-palmitoyl-(1-stearoyl) 2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholines in extensively oxidized LDL and HDL (5, 26). Watson et al. (6, 27) later showed that 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine, and 1-palmitoyl-2-(5, 6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphocholine were present in minimally or mildly oxidized LDL. Although these oxidized acyl GroPChos in the modified lipoproteins have structures similar to that of PAF, their biological activities are different because of differences in the fatty chains at the *sn*-2 position and the absence of an alkyl group at the *sn*-1 position (9, 28–30). O'Brien et al. (31) have reported that atheromas contain oxidation-specific epitopes not only of LDL proteins but also of cell-associated proteins.

It is well known that the PAF receptor shows a strong preference for the *sn*-1 ether bond (alkyl GroPCho). The present study shows that C_5 alkyl GroPCho core aldehyde present in atheromas can induce platelet aggregation via activation of the PAF receptor. C_5 acyl GroPCho core aldehyde can induce platelet shape change, but not aggregation. Siess et al. (32) reported that lysophosphatidic acid (LPA) present in atheromas can induce platelet shape change via activation of the LPA receptor. They did not demonstrate platelet aggregation by LPA. Zieseniss et al. (33) reported that oxidized phosphatidylethanolamines in oxidized LDL elicit a pronounced prothrombotic response by promoting platelet prothrombinase activity. However, to our knowledge, platelet aggregation via PAF receptor activation by the oxidized phospholipid present in atheromas has not been reported.

Mizuno et al. (34) reported that intracoronary thrombi observed in 10 of 14 patients with unstable angina were white thrombi, whereas in all 15 patients with acute myocardial infarction were red thrombi. White thrombi have been considered platelet-rich thrombi. The levels of PAF were reported to be higher in the coronary arteries from patients with advanced coronary artery disease (35). Aggregation of platelets via the PAF receptor by PAF and alkyl GroPCho core aldehydes in atheromas could contribute to the formation of white thrombi (platelet-rich thrombi) in unstable angina and to that of full-blown red thrombi (red blood cell and fibrin-rich thrombi) in acute myocardial infarction. Alkyl and acyl GroPCho core aldehydes in atheromas, in cooperation with other lyso-phospholipids, could contribute to the inhibition of endothelium-dependent arterial relaxation.

Figure 11 summarizes the demonstrated and postulated transformations of 1-*O*-hexadecyl-2-arachidonoyl GroPCho in the atherosclerotic tissue. Free arachidonic acid liberated by phospholipase A_2 from membrane lipid precursor (1-*O*-hexadecyl-2-arachidonoyl-*sn*-GroPCho) is oxidized enzymatically to generate eicosanoids. PAF is formed when the *sn*-2 position of lyso-1-*O*-hexadecyl-GroPCho is acetylated enzymatically. When arachidonic acid still bound to the membrane lipid (1-*O*-hexadecyl-2-arachidonoyl-*sn*-GroPCho) is oxidized nonenzymatically, C_5 alkyl GroPCho core aldehyde is generated as the main product (5, 12). Eicosanoids and PAF, which are generated enzymatically

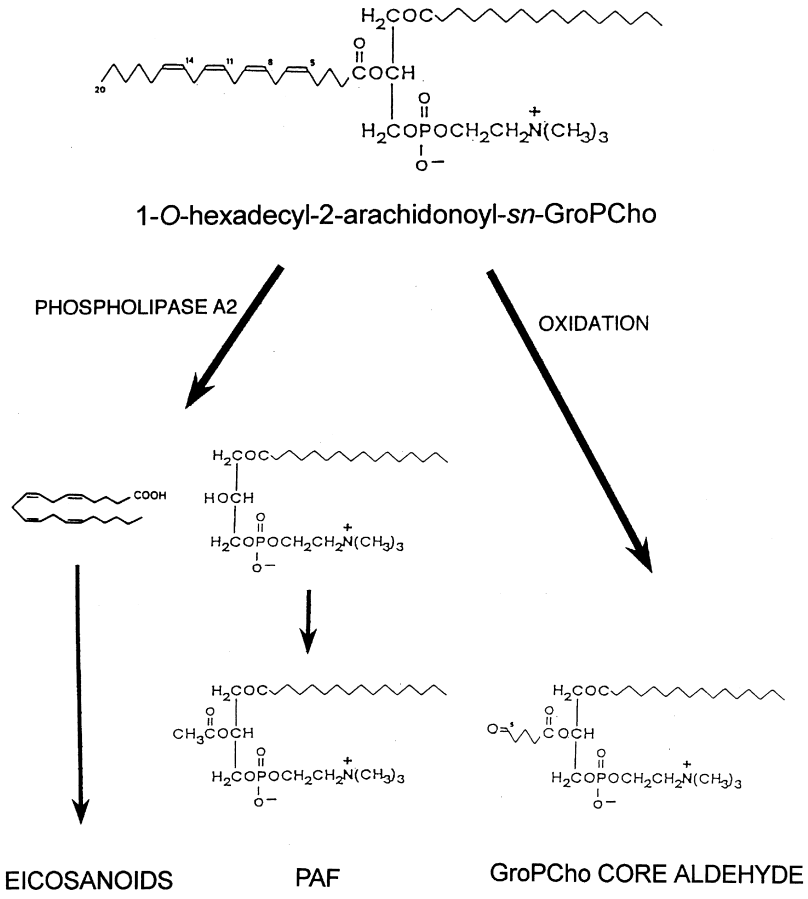

Fig. 11. 1-*O*-Hexadecyl-2-(5-oxovaleroyl)-*sn*-glycerophosphocholine (C_5 alkyl GroPCho core aldehyde) is generated by oxidation as the main product from the same precursor membrane lipid as eicosanoids and PAF.

from membrane lipid, have biological effects on both platelets and arteries. They are established physiological mediators of inflammation. C_5 alkyl GroPCho core aldehyde, which is generated by nonenzymatic oxidation from the same membrane lipid precursor as eicosanoids and PAF, also possesses biological activity toward both platelets and arteries. The C_5 alkyl GroPCho core aldehyde could therefore be a pathological lipid chemical mediator of inflammation and thrombosis.

In conclusion, we identified alkyl GroPCho core aldehydes from human atheromatous plaques and demonstrated that C_5 alkyl GroPCho core aldehyde can induce platelet aggregation through PAF receptor activation, and in addition can inhibit endothelium-dependent relaxation. Inflammation and thrombosis are now considered to be key features in the pathogenesis of acute coronary syndrome. The present results suggest that alkyl GroPCho core aldehydes present in atheroma lipid could be involved in precipitating acute coronary events, in which thrombus formation following lipid-rich plaque disruption is believed to play an important role.

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