Lipid Separation from *Urtica dioica*: Existence of Platelet-Activating Factor

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The common wild plant nettle, especially *Urtica dioica*, is one of the most potent plants in producing direct irritation to the skin (urticaria). In this study, total lipids of *Urtica dioica* were separated into neutral and polar lipids, which were further fractionated by high-performance liquid chromatography (HPLC). Triglycerides, sterol-esters, fatty acids, fatty acid methyl esters, glyceryl ethers, sterols, tocopherols, diglycerides, and galactosyldiglycerides were identified as the main neutral lipid classes by comparing their retention times on an HPLC column and their migration following spraying with specific reagents on thin-layer chromatography (TLC) with standards. Four main classes of phospholipids (i.e., phosphatidylinositol, phosphatidylethanolamine, phosphatidylcholine, and lysophosphatidylcholine) were also identified. A phospholipid that induced platelet aggregation was identified as platelet-activating factor on the basis of biological, chemical, and spectral methods.

Keywords: *Urtica dioica; Urticaceae; nettle; lipid analysis; platelet-activating factor*

INTRODUCTION

It is well known that lipids are major constituents of plants, and in most higher plants the polar lipid patterns are similar. The fatty acids in these lipids are usually unsaturated, although plants can biosynthesize both unsaturated and polyunsaturated fatty acids, and the distribution of fatty acids in polar lipids varies in different plant tissues. Platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a biologically active ether phospholipid, is biosynthesized by a variety of animal cell types, exhibits a wide spectrum of biological actions, and is the most potent platelet aggregating factor known today (Lee and Snyder, 1985). In many areas of the world, indigenous natural drugs are still widely used and plant products remain an integral part of modern therapeutics. Compounds with PAF-like activity have been isolated from various plant sources (Andrikopoulos et al., 1985; Koussissis et al., 1993), and PAF-analogs (Vakirtzi-Lemonias et al., 1992), which cause primary chemical irritation (Andrikopoulos et al., 1985), have also been found in plants. The common wild plant nettle, especially *Urtica dioica* with its well-known stinging properties, is one of the most potent plants in producing direct irritation to the skin (urticaria; Lampe and Fagerstrom, 1968). The whole plant is esteemed as an astringent and a diuretic (Lindley, 1985).

The properties of *Urtica dioica* prompted analysis of its components by several investigators. Considerable levels of vitamin K_1 and some trace elements (Sapronova et al, 1989); hydroxyl-fatty acids (Kraus et al, 1991), terpene diols, and terpene diol glycosides (Kraus and Spiteller, 1991); and hydrocarbons and di- and trigalactosyldiglycerides (Radunz, 1976) have been detected. It has also been reported that the extract of *Urtica dioica* manifested significant inhibitory activity towards adrenaline-induced aggregation of human platelets (Sajid et al., 1991). Other investigators reported that *Urtica dioica* contains high levels of leukotriene B_4 and C_4 (Czarnetzki et al., 1990) as well as histamine and serotonin (Oliver et al., 1991). However, as these investigators claim, part of the immediate reaction to *Urtica* stings is due to the immunoreactive substances just mentioned, and the persistence of the stinging sensation suggests the presence of substances in *Urtica* that are directly toxic or capable of secondary release of other mediators. The observed allergic response caused by *Urtica dioica* is not due to sensitization, but may be caused by compound(s) with PAF-like activity.

In this study we isolated and separated lipids from leaves and roots of *Urtica dioica* to clarify the mechanism of urtication by investigating the existence of lipids with PAF-like or anti-PAF-like activity, which may play a role in the irritating properties of this plant.

MATERIALS AND METHODS

Materials. *Urtica dioica* was collected in spring of 1989 from Marousi (Attica, Greece) and identified by Prof. G. Voliotis at the Agriculture University of Athens.

All reagents and chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany). High-performance liquid chromatography (HPLC) solvents were purchased from Ruthburn (Walkerburn, Peebleshire, UK). Lipid standards of HPLC grade were obtained from Supelco (Bellefonte, PA). Semisynthetic PAF (80% C-16PAF and 20% C-18PAF) was synthesized in our laboratory as previously described (Demopoulos et al., 1979). Chromatographic material used for thin-layer chromatography (TLC) was silica gel G-60 (Merck, Darmstadt, Germany).

Instrumentation. HPLC was performed on a dual pump (Jasko, Tokyo, Japan; model 880-PU HPLC), equipped with a 330-μL loop rheodyne (P/N 7125-047) injector. A Jasko model 875 UV spectrophotometer was used as detector (205−210 nm). The spectrophotometer was connected to a Hewlett-Packard (Avondale, PA) model HP-3396A integrator-plotter. The following columns were used: a cation-exchange column (SS, 10-μm Partisil, 25 cm × 4.6 mm i.d., PXS 10/25 SCX; Whatman, Clifton, NJ); and a reversed-phase column (7 μm, nucleosil-300, C18, 25 cm × 4 mm i.d., Analysentechnik, Mainz, Germany). The flow rate was 1 mL/min.

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The PAF-induced aggregation was measured in a Chrono-Log (Havertown, PA) aggregometer coupled to an Omniscribe recorder (Houston,TX).

Infrared (IR) spectra were recorded in air on a 1725X Perkin-Elmer FTIR (Fourier transform-IR) spectrophotometer. The sample, in a form of a thin film from $CDCl_3$ solution on KBr disks, was dried overnight at room temperature under reduced pressure before recording the spectra. Blanks of $CDCl_3$ residue on KBr disks were treated under identical conditions, and the recorded spectra are the difference between the blank and the sample spectra.

Ultraviolet (UV) spectra were recorded on a Hitachi U-2000 spectrophotometer. The sample was evaporated under a stream of nitrogen, dried in a dessicator over KOH under reduced pressure to remove traces of solvent, and finally dissolved in $CHCl_3$. The spectrum (180−400 nm) was recorded (1-cm path length) against a solvent blank with a double-beam recording spectrophotometer.

The samples were subjected to mass spectroscopy (MS) analysis on a Finnigan MAT 212/INCOS 2200. The ionization process was fast atom bombardment (FAB) argon atoms at 8 kV. The accelerating voltage in the mass spectrometer was 3 kV, and the ion source temperature was 70 °C. The matrix was glycerol, and the sample was applied in chloroform: methanol (1:1, v/v).

Extraction of Total Lipids. Roots (200 g) were separated from the rest of the plant (600 g) (leaves and stems) and each was cut into pieces and homogenized in a blender with ethanol. Ethanol extracts were separated by centrifugation and phased by adding appropriate volumes of chloroform and water to reach to a final ratio of chloroform:ethanol:water of 1:1:0.9 (v/v/v). Both residues from the previous centrifugation were reextracted according to the Bligh−Dyer method (Bligh and Dyer, 1959). The chloroform phases from ethanol extracts and from the Bligh-Dyer method, for roots as well as for leaves and stems, were combined separately.

Recovery of Pigment-Free Total Lipids. Pigment contamination from the two samples was removed by two successive preparative TLC procedures (Demopoulos et al., 1996). The chloroform phases from the extraction procedure that contains total lipids were evaporated to dryness, and the extracts were redissolved in a small volume of chloroform: methanol (1:1, v/v) and applied to TLC plates. The chromatograms were developed in petroleum ether (bp, 40−60 °C): benzene:acetic acid (30:70:2, v/v/v). Phospholipids and pigments remained in the origin, but neutral lipids migrated along the plates. The bands of neutral lipids and the band of phospholipids and pigments were scraped off separately, extracted according to the Bligh−Dyer method (Bligh and Dyer, 1959), and centrifuged. The organic solvents were phased by adding appropriate volumes of chloroform and water to arrive at a final chloroform:methanol:water ratio of 1:1:0.9 (v/v/v). The extracts that contained phospholipids and pigments were evaporated to dryness and redissolved in a small volume as just described. Phospholipids and pigments were rechromatographed on TLC plates, using acetone:methanol:water (40:20: 1, v/v/v) as the developing system. Pigments migrated near the solvent front, whereras phospholipids migrated along the plate. The fractions of pigments were discarded and the phospholipids were recovered. The fractions of phospholipids and neutral lipids from roots were pooled and those from leaves and stems were pooled, and two fractions of total lipids were obtained. By this procedure, total lipids that were free from pigments were recovered.

Separation of Polar Lipids from Neutral Lipids. The lipid fractions from roots and from leaves and stems were separated in neutral and polar lipids by current counter distribution (Galanos and Kapoulas, 1962).

Separation of Neutral Lipids into Classes and Species. Neutral lipids were further fractionated by HPLC (Antonopoulou et al., 1994). Briefly, neutral lipids were separated into classes and species with a Nucleosil-300 C_{18} column and a stepped gradient elution with the following solvents: A, methanol:water (4:1, v/v); B, acetonitrile:methanol (7:5, v/v); C, acetonitrile:tetrahydrofuran (99.5:0.5, v/v); D, isopropanol: acetonitrile (99:1, v/v), and E, cyclohexane. The stepped gradient elution was as follows: a linear gradient from solvent A to solvent B in 10 min; a hold for 5 min in B; a linear gradient to solvent C in 10 min; a second hold in C for 5 min; a linear gradient to solvent D in 10 min, a third hold in solvent D for 5 min; a linear gradient to solvent E in 10 min; and a hold in solvent E. A minor amount of the fraction eluted in the first 22 min was rechromatographed with the same HPLC column and a stepped gradient elution with the first three solvents (A, B, and C) of the first HPLC separation. The stepped gradient elution was as follows: a linear gradient from 100% A to A:B at 98:2 (v/v) in 5 min; a hold at A:B at 98:2 (v/v) for 5 min; a linear gradient to solvent B in 20 min; a second hold in B for 5 min; a linear gradient to solvent C in 10 min; and a hold in C.

Identification of Lipids. Lipids eluted from the HPLC systems were manually collected and concentrated by evaporating the solvents under a N_2 stream. These samples were developed on silica-gel TLC plates according to Mangold (1969) for neutral lipids, according to Demopoulos et al. (1979) for phospholipids, and according to Fischer et al. (1978) for glycolipids. Lipids were identified by comparison with commercial standards, and visualization was achieved by exposure to I_2 vapors and by spraying with specific reagents (Dittmer and Lester, 1964; Siakotos and Rouser, 1965).

Separation of Polar Lipids into Classes. Polar lipids were further fractionated by HPLC (Andrikopoulos et al., 1986). Briefly, polar lipids were separated on a cation-exchange HPLC column (10 μm) with an isocratic elution system consisting of 60% acetonitrile and 40% methanol:water (4:1, v/v).

Purification and Fractionation of PAF into Species. A small amount of PAF fraction, characterized as semipurified PAF, was further purified from overlapping phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) by fractionation on the same cation-exchange HPLC column by gradient elution with the following solvents: A, 70% acetonitrile and 30% methanol:water (4:1, v/v); and B, 50% acetonitrile and 50% methanol:water (4:1, v/v). The gradient system used was as follows: a hold in A for 10 min; a linear gradient from A to B in 10 min; and a hold in B. The amount remaining from the semipurified PAF fraction was fractionated into species with a Nucleosil-300, C_{18} column, and an isocratic elution system consisting of methanol:water:acetonitrile (85:10:2, v/v/v) was used. The flow rate was 1 mL/min, and detection was at 210 nm.

Biological Assay. PAF- and sample-induced aggregation were examined with washed rabbit platelets according to the method of Demopoulos et al. (1979). In some experiments, inhibitors [creatine phosphate (CP) 0.7 mM/creatine phosphate kinase (CPK), 13 units/ mL; indomethacin, 10 μM; and the ginkgolide BN 52021, 0.1 mM) were added to the washed rabbit platelets 1 min prior to the addition of the examined sample into the aggregometer cuvette.

Desensitization Experiment. This experiment was carried out according to the method of Lazanas et al. (1988). Briefly, in both desensitization and cross-desensitization experiments, platelets were desensitized by the addition of PAF or the examined phospholipid to the platelet suspension at a concentration that caused reversible aggregation. Next, stimulation was induced immediately after complete disaggregation by the addition of the examined phospholipid, PAF, or thrombin (PAF and thrombin were added at concentrations that induced a platelet aggregation pattern of the same height as that produced by the examined phospholipid).

Mild Alkaline Hydrolysis and Reacetylation. This procedure was carried out according to the method of Demopoulos et al. (1979). Briefly, the sample was dissolved in 1 mL of chloroform:methanol (1:4, v/v), and then 0.1 mL of 1.2 N NaOH in 50% methanol was added and allowed to stand for 20 min at 60 °C. The mixture was neutralized with 0.15 mL of 1 N acetic acid and 2 mL of chloroform:methanol (9:1, v/v), and then 1 mL of methanol and 2 mL of water were added. The two phases were separated, and the phased chloroform was washed with 1 mL of methanol:water (1:2, v/v). Both phases were subjected to reacetylation by the addition of 1 mL of acetic anhydrite and incubation at 60 °C for 45 min. Each phase was evaporated and extracted by the Bligh−Dyer method (Bligh and Dyer, 1959). All samples from this

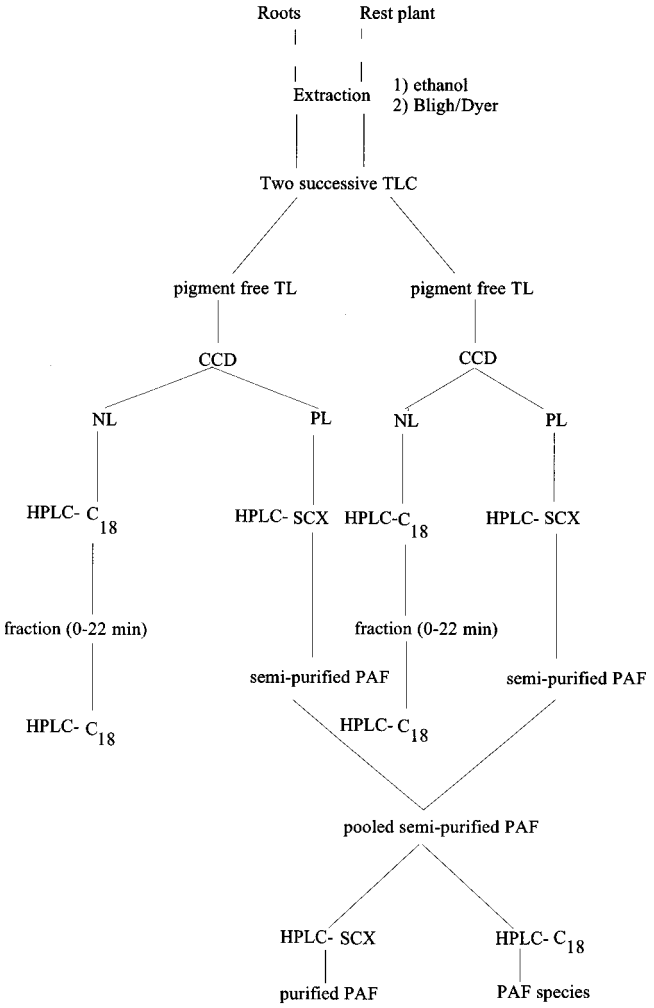

Figure 1. Schematic diagram of the extraction and the purification procedure: (CCD) current counter distribution; (NL) neutral lipids; (PL) phospholipids.

procedure were tested for their ability to induce washed rabbit platelet aggregation.

Treatment with Acetylhydrolase. Phosphate buffer (0.08 M, pH 7.5), human serum acetylhydrolase (purified by Dr. D. Stafforini; Stafforini et al., 1987), and the examined sample in bovine serum albumin (BSA) at 2.5 mg/mL saline were added to a prewarmed (37 °C) test tube. The enzymatic system was incubated at 37 °C, and, at different times, aliquots were taken and tested for their ability to induce washed rabbit platelet aggregation.

Phosphorus Determination. This determination was carried out according to the method of Bartlett (1959).

RESULTS AND DISCUSSION

The extraction scheme and the purification procedures are summarized in Figure 1.

Separation of Neutral Lipids into Classes and Species. A typical profile of neutral lipids separated from leaves and stems is shown in Figure 2. The fractions eluted in the first 22 min and were collected. A small amount (1/20 of the fraction) was rechromatographed with the same HPLC column and a stepped gradient elution with the first three solvents (A, B, and C) of the previous HPLC separation. Representative HPLC elution patterns of these fractions are shown in Figure 3 for leaves and stems.

For identification, some of the eluant fractions containing neutral lipids (from roots as well as from leaves and stems) were collected manually and cochromatographed on TLC plates with authentic standards as described in Materials and Methods.

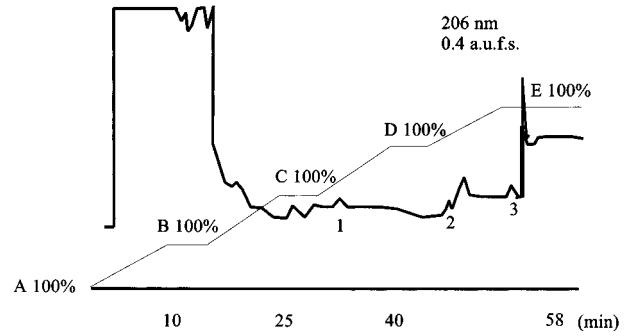

Figure 2. HPLC separation of neutral lipids of leaves and stems from *Urtica dioica* with a Nucleosil-300, C_{18} column: (peak 1) triglycerides; (peak 2) sterol esters; (peak 3) sterol esters; (A) methanol:water (4:1, v/v); (B) acetonitrile:methanol (7:5, v/v); (C) acetonitrile:tetrahydrofuran (99.5:0.5, v/v); (D) isopropanol:acetonitrile (99:1, v/v); (E) cyclohexane.

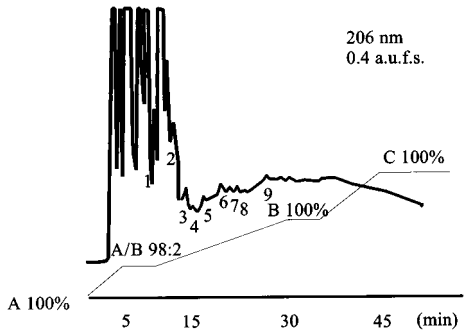

Figure 3. HPLC fractionation of the neutral lipids from *Urticas dioica* leaves and stems that eluted in the first 22 min of the HPLC separation shown in Figure 2: (peak 1) fatty acids; (peak 2) fatty acid methyl esters; (peak 3) galactosyldiglycerides; (peak 4) glycerylethers; (peaks 5, 6, and 7) sterols; (peak 8) tocopherols; (peak 9) diglycerides; (A) methanol:water (4:1, v/v); (B) acetonitrile:methanol (7:5, v/v); (C) acetonitrile: tetrahydrofuran (99.5:0.5, v/v).

The following order of elution of neutral lipid classes from the HPLC column was established: (1) Figure 2: triglycerides (TG) were eluted at 35 min and formed peak 1, and sterol-esters (St-Ests) were eluted from 50 until 59 min and formed peaks 2 and 3; (2) Figure 3: fatty acids (FA) were eluted at 9.5 min (peak 1), fatty acid methyl esters (FAME) were eluted at 14 min (peak 2), galactosyldiglycerides (GDG) were eluted at 15.5 min (peak 3), glycerylethers (GE) were eluted at 16.5 min (peak 4), sterols (St) were eluted from 17 to 23 min (peaks 5, 6, and 7), tocopherols (Toc) were eluted at 24.5 min (peak 8), and diglycerides (DG) were eluted at 28 min (peak 9). It is concluded that (a) TG and St-Ests are present in *Urtica dioica*, in small amounts compared with quantities of the other neutral lipids, and (b) neutral lipids are qualitatively the same in the leaves and stems samples as in the roots samples, but quantitatively there is almost twice the HPLC peak height in the case of leaves and stems.

Biological Activity of Neutral Lipids. All the HPLC fractions of neutral lipids (from both HPLC separations) derived from roots as well as from leaves and stems were tested for their ability to induce washed rabbit platelet aggregation and/or to inhibit PAF-induced and/or thrombin- induced washed rabbit platelet aggregation. We detected a significant number of biologically active lipid fractions that exhibited potent inhibitory effects against the aggregating agents and others that induced platelet aggregation. Their structures are under investigation.

Separation of Polar Lipids into Classes. Polar lipids from roots as well as from leaves and stems each

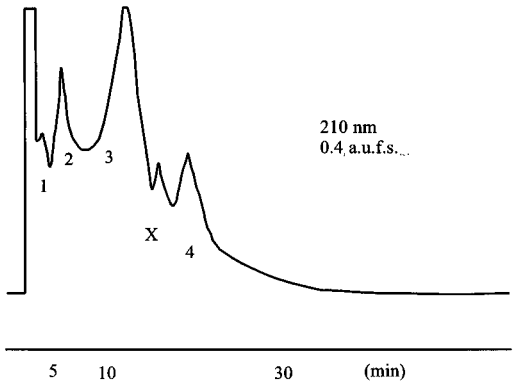

Figure 4. HPLC separation of polar lipids of leaves and stems from *Urtica dioica* by a cation-exchange column: (peak 1) phosphatidylinositol; (peak 2) phosphatidylethanolamine; (peak 3) phosphatidylcholine; (peak 4) lysophosphatidylcholine; (X) the fraction of semipurified PAF. The elution system was 60% acetonitrile and 40% methanol:water (4:1, v/v).

were further fractionated on HPLC (Andrikopoulos et al., 1986). A typical profile of the separation of polar lipids from leaves and stems is shown in Figure 4. Four main classes of phospholipids were tentatively identified by TLC analysis of the eluted HPLC peaks; namely, phosphatidylinositol (PI) was eluted at 4.5 min (peak 1), phosphatidylethanolamine (PE) was eluted at 6.5 min (peak 2), phosphatidylcholine (PC) was eluted at 14 min (peak 3), and lysophosphatidylcholine (LPC) was eluted at 23 min (peak 4). From the tentative identification of phospholipids as well as from HPLC chromatograms we conclude that roots share the same phospholipids as leaves and stems, but in a much smaller amount.

Biological Activity of Polar Lipids. All the HPLC fractions of polar lipids (from both HPLC separations) derived from roots as well as from leaves and stems were tested for their ability to induce washed rabbit platelet aggregation and/or to inhibit PAF-induced and/or thrombin-induced washed rabbit platelet aggregation. We detected a phospholipid fraction in roots as well as in leaves and stems extracts that induced rabbit platelet aggregation. As already mentioned, roots shared the same lipids with leaves and stems and because the aforementioned active fractions both eluted at the same time and also exhibited the same biological activity, they were pooled together to obtain the maximum amount of the active fraction. The combined fraction induced rabbit platelet aggregation with a dose-dependent aggregation pattern similar to that of PAF, but was much less potent than PAF (submaximal reversible aggregation on the order of 10^{-5} M, final concentration, based on phosphorus determination, compared with that for PAF on the order of 10^{-10} M, final concentration). It seems that this active phospholipid acts through PAF receptors because (1) platelets desensitized to PAF were not aggregated by this lipid and *vice versa*, (2) platelets desensitized to this lipid were aggregated by thrombin, (3) the enzymatic system CP/CPK and indomethacin, which specifically inhibit ADP and arachidonic acid -induced platelet aggregation, respectively, have no inhibitory effect on the aggregation induced by this active phospholipid, and (4) on the contrary, PAF-receptor-specific inhibitor BN 52021 does inhibit the aggregation induced by this active phospholipid (Figure 5).

This phospholipid comigrated with synthetic PAF on HPLC and on TLC. Alkaline hydrolysis rendered it inactive, and reacetylation of the chloroform soluble fraction, resulting from the hydrolysis just described,

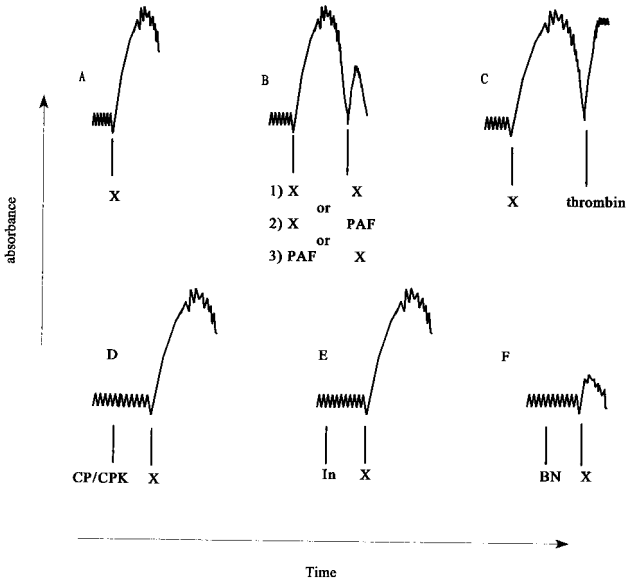

Figure 5. (A) Reversible platelet aggregation curve induced by the active phospholipid, where X corresponds to the fraction of active phospholipid. (B) Desensitization experiments with washed rabbit platelets: (1) X against X; (2) X against PAF; (3) PAF against X. (C) Desensitization experiments with washed rabbit platelets: X against thrombin. (D, E and F) Platelet aggregation curves induced by X after the addition of creatine phosphate/creatine phosphate kinase (CP/CPK), indomethacin (In), and BN 52021 (BN), respectively.

reinstated the lost activity. These data suggest the presence of a PAF backbone as well as the presence of acetyl group. Incubation of the phospholipid with serum acetylhydrolase resulted in its time-dependent inactivation, suggesting that the acetyl group is present at the *sn-2* position of the glyceryl backbone. The IR spectrum showed peaks at 2850 and 1450 cm^{-1} (CH_3 and CH_2), 1840 and 1730 cm^{-1} (C═O), 1300 cm^{-1} (P═O), 1080 cm^{-1} (P—O—C), 1110 cm^{-1} (C—O—C, ether bond), and 960 cm^{-1} (P—O—choline). Its UV pattern was similar to that of LPC, with peaks at 320 and 280 nm. The FAB-mass spectrum of the active phospholipid showed ions at m/z 184, which corresponds to the phosphocholine group, 524 $[M_1 + H]^+$, 466 $[M_1H - 58]^+$, 482 $[M_1H - 42]^+$, where M_1 corresponds to PAF 16:0, 552 $[M_2 + H]^+$, 494 $[M_2H - 58]^+$, 510 $[M_2H - 42]^+$, where M_2 corresponds to PAF 18:0, 522 $[M_3 + H]^+$, 464 $[M_2H - 58]^+$ and 480 $[M_3H - 42]^+$, where M_3 corresponds to PAF 16:1. The ions resulting from $[M + H]^+$ by subtracting 58 or 42 indicate the presence of an acetyl group at the *sn-2* position of the glyceryl backbone. The FAB-mass spectrum also gave ions indicating the presence of PC and LPC analogs. In conclusion, PAF should be present in the fraction of this biologically active phospholipid (characterized as semipurified fraction), but other lipids are also present because (1) the HPLC profile (Figure 4) shows insufficient separation of the active fraction from the fractions of PC and LPC, (2) its biological activity is lower than that of PAF, (3) its UV spectrum is characteristic of LPC, and (4) its FAB-mass spectrum indicates the presence of PC and LPC analogs.

Purification and Fractionation of PAF into Species. To purify PAF from PC and LPC, a small amount of the previous semipurified active fraction was further fractionated on HPLC with a cation-exchange column and a gradient eluting system consisting of acetonitrile, methanol, and water. The fraction that corresponded to the PAF retention time, determined with the aid of synthetic PAF, was collected and subjected to biological, chemical, and spectral methods. The amount remaining

was separated into molecular species with a C_{18} HPLC column, using a polar solvent mixture in an isocratic mode for elution.

Biological Activity of Purified PAF and PAF Species. As expected, the purified fraction from the cation-exchange column that eluted at the retention time of PAF exhibited biological activity had the same characteristics, in the biological and chemical tests, as the previously semipurified phospholipid fraction. This purified fraction induced submaximal reversible aggregation at the order of 10^{-10} M, final concentration, based on phosphorus determination. The results from the IR and from FAB-mass analyses confirmed the existence of PAF (18:0 and 16:0) without contamination by other lipids.

All the fractions derived from the C_{18} HPLC fractionation were tested for their ability to induce washed rabbit platelet aggregation and/or to inhibit PAF-induced and/or thrombin-induced washed rabbit platelet aggregation. A significant number of biologically active lipids was detected. Among them, we detected a phospholipid that eluted at the retention time of PAF 18:0 and shared the following characteristics: (1) induced rabbit platelet aggregation with a dose-dependent aggregation pattern similar to that of PAF; (2) acts through PAF receptors (results derived from desensitization experiments as well as from experiments with CP/CPK, indomethacin, and BN 52021); and (3) presence of PAF backbone and the presence of acetyl group at the *sn-2* position of the glyceryl backbone (results derived from chemical and enzymatic hydrolysis). In addition, the FAB-mass spectrum gave ions at *m/z* 184 (phosphocholine group), 550 $[M_1 + H]^+$, 492 $[M_1H - 58]^+$, and 508 $[M_1H - 42]^+$, where M_1 corresponds to PAF 18:1; and 494 $[M_2H - 58]^+$, where M_2 corresponds to PAF 18:0.

In conclusion, the presence of PAF in *Urtica dioica* is undoubtedly demonstrated. This is the first time that the existence of PAF in plants is documented with direct methods. Our findings offer new insight into the properties of *Urtica dioica* in that the urtication produced by this plant could be due to the presence of PAF.

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