Identification of a new endogenous platelet-activating factor-like molecule in gingival crevicular fluid

Smaragdi ANTONOPOULOU*, Constantinos A. DEMOPOULOS*¹, Dimitris ARGYROPOULOS*, George BALTAS†, Helen KOTSIFAKI‡ and Anthoula DIAMANTI-KIPIOTI†

*Department of Chemistry, University of Athens, Athens, GR-15771, Greece, †Department of Periodontology, University of Athens, Athens, Greece, and ‡Department of Physiology, University of Athens, Athens, Greece

Periodontal disease is an inflammatory disease and the major cause of tooth loss in adults. Bacteria and their products are the causative agents of this disease. Endogenous molecules mediate the inflammatory process and play a major role in its amplification and perpetuation as well as in the ensuing tissue destruction. The relationship between platelet-activating factor (PAF) and periodontal disease has not so far been examined thoroughly. We have isolated a phospholipid molecule with PAF-like activity from gingival crevicular fluid. This molecule, purified on HPLC, causes washed platelet aggregation with EC₅₀

value 0.1 μ M, based on phosphorus determination. It acts through PAF-receptors and is inactivated by PAF-acetylhydrolase. In addition, this phospholipid presents biological activity towards human platelets. The combination of the results obtained from the chemical and enzymic treatments, the biological assays as well as results from the electrospray analysis, leads to the conclusion that this phospholipid is a hydroxyl-PAF analogue with relative molecular mass 703. This PAF-like molecule may be implicated in periodontal disease.

INTRODUCTION

In periodontal disease, bacteria and their products trigger inflammatory host responses which, in addition to the direct destructive action of the bacteria, cause most of the tissue destruction [1]. Initiation of the disease by bacteria is well documented, as well as the resulting destruction of the alveolar bone and the other connective tissue. However, the molecular events that mediate this procedure are only now being understood. These mediators are produced and released from a variety of activated inflammatory cells (e.g. polymorphonuclears, macrophages and lymphocytes) [2]. Molecules such as cytokines and prostaglandins have been studied more than some others, e.g. platelet-activating factor (PAF), in mediating destruction of the tissue in periodontitis.

The most bioactive mediator PAF is produced by activated inflammatory cells such as polymorphonuclear leucocytes, macrophages, platelets and endothelial cells [3]. It has also been reported that PAF is produced and released from bacteria [4]. Previous research showed that PAF *in vivo* increases vascular permeability and infiltration of polymorphonuclears [5]. Recent studies, however, have implicated PAF with virtually all inflammatory and immune responses [6].

The inactivation of PAF into the biologically inactive lyso-PAF is catalysed by PAF-acetylhydrolase (PAF-AH). This enzyme was detected in many tissues and cells as well as in biological fluids and apparently plays a major role in the regulation of PAF levels [7].

The relationship between PAF and periodontal disease has not been thoroughly examined so far. The presence of substance(s) in certain samples of saliva which inhibited platelet aggregation induced by PAF as well as by arachidonic acid, has been noted [8]. Positive correlation between PAF levels in saliva and the severity of periodontal disease has been demonstrated [9]. Also

the levels of PAF produced in inflamed gingivae were higher than in normal ones but the activity of PAF-AH did not show any significant difference [10]. In our previous study, we estimated PAF and PAF-AH levels in gingival crevicular fluid (GCF) (an exudation, mainly inflammatory) in comparison to clinical parameters of periodontal disease, during experimental gingivitis as well as before and after conventional periodontal treatment [11] and we isolated a phospholipid molecule with PAF-like activity which is implicated in periodontal disease.

In this study, we elucidate the structure of the above inflammatory molecule with PAF-like activity.

EXPERIMENTAL PROCEDURES

Patient selection and gingival crevicular fluid sampling

In this study 20 patients with chronic adult periodontitis participated. The group consisted of 14 males and six females, aged 35–67 years, none of them had received any periodontal treatment or antibiotic therapy during the last 6 months. Standardized filter papers were used for GCF collection from the six maxilla's anterior teeth. Collection sites were isolated with cotton wool rolls, air dried and obvious supra-gingival plaque carefully removed with a curette. The paper strips were inserted into the crevice until mild resistance was felt and left for 60 s. Care was taken in order to avoid mechanical injury of the tissues. Strips contaminated by blood or visible plaque were discarded. The PAF-like molecule was extracted according to the Bligh–Dyer method [12].

Solvents and reagents

All solvents used were of analytical grade purchased from Merck (Darmstadt, Germany). HPLC solvents were purchased from

Abbreviations used: PAF, platelet-activating factor; PAF-AH, PAF-acetylhydrolase; GCF, gingival crevicular fluid; CP, creatine phosphate; CPK, creatine phosphate kinase; PRP, platelet-rich plasma; ESMS, electrospray mass spectrometry.

¹ To whom correspondence should be addressed.

Ruthburn (Walkerburn, Peeblesshire, U.K.). Semisynthetic PAF (80% C₁₆PAF and 20% C₁₈PAF) was synthesized in our laboratory as previously described [13]. Standard lipids were purchased from Supelco (Bellefonte, PA, U.S.A.). BSA and thrombin were purchased from Sigma (St. Louis, MO, U.S.A.). Human plasma acetylhydrolase was a gift from Dr. D. Stafforini.

HPLC purification

Bioactive phospholipid was further purified on a cation exchange column SS 10 μ m Partisil 25 cm $\times$ 4.6 mm I.D., PXS 10/25 SCX from Whatman (Clifton, NJ, U.S.A.). Acetonitrile/methanol/water (60:32:8, v/v/v) were used as eluting system. The flow rate was 1 ml/min. HPLC was performed on a dual pump Jasco (Tokyo, Japan) model 880-PU HPLC, supplied with a 330 μ l loop Rheodyne (P/N 7125-047) injector. A Jasco model 875 UV spectrophotometer was used as detector (210 nm). The spectrophotometer was connected to a Hewlett-Packard (Avondale, PA, U.S.A.) model HP-3396A integrator-plotter.

Biological assays

The PAF-like activity of the phospholipid was estimated by bioassay studies based on washed rabbit platelets. PAF and the examined sample were dissolved in 2.5 mg BSA per ml saline. Thrombin was dissolved in saline. Various concentrations of the examined phospholipid were added into the aggregometer cuvette and the aggregation induced by the sample was studied in a Chronolog aggregometer (Havertown, PA, U.S.A.) [10]. Experiments with specific inhibitors, creatine phosphate (CP) 0.7 mM/creatine phosphate kinase (CPK) 13 units per ml saline, indomethacin 10 μ M (10% ethanol in water) and BN52021 0.1 mM (0.3% DMSO in water), were also performed. These inhibitors were added to washed rabbit platelets 1 min prior to the addition of the examined sample into the aggregometer cuvette. This experiment was carried out according to Lazanas et al. [14]. In desensitization and cross-desensitization experiments platelets were desensitized by the addition of the phospholipid to the platelets suspension at a concentration which caused reversible aggregation. Second stimulation was performed immediately after complete desegregation with the addition of the phospholipid or PAF or thrombin, all added in concentrations which induced a platelet aggregation pattern of the same height as the first one.

The PAF-like activity of the phospholipid towards human platelet-rich plasma (PRP) was estimated according to Demopoulos et al. [15]. The PRP was obtained from a healthy volunteer.

Treatment with acetylhydrolase

In a test tube prewarmed at 37 °C, phosphate buffer (0.08 M, pH 7.5) was added along with human serum acetylhydrolase and the examined sample in 2.5 mg BSA per ml saline. 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.

Mild alkaline hydrolysis and reacylation

An amount of the phospholipid was subjected to mild alkaline hydrolysis and reacylation while another amount of this lipid was only subjected to acetylation. The biological activities of the lipids derived from the above chemical reactions were then tested on washed rabbit platelets. These procedures were carried out according to Demopoulos et al. [13].

Phosphorus determination

Phosphorus was determined using the method of Bartlett [16].

Mass spectrometry

The purified phospholipid as well as the acetylated phospholipid were subjected to mass spectrometry analysis. The Electrospray Mass Spectrometry (ESMS) was recorded on a Fisons VG Quattro instrument with a VG Biotech Electrospray source, having a hexapole lens. Nitrogen 99.99% pure was used as the nebulizing and bath gas at flows of 20 and 150 dm³ min⁻¹ respectively. The samples were injected in the flow of solvent (10 μ l/min) of a Varian 9012 solvent delivery system, via a Fisons interface with a Rheodyne 7125 injector. The capillary voltage was optimum at about 3.30 kV for positive ions. The high voltage lens potential was kept at 0.56 kV. The focus and skimmer lenses voltages were 40 and 45 V, respectively, in the majority of the measurements, as these values produced the highest peak intensities and minimum fragmentation. HPLC grade methanol/water (70:30, v/v) 0.01 M in ammonium acetate was used as solvent.

RESULTS AND DISCUSSION

The biologically active phospholipid extracted from GCF was purified onto a cation exchange HPLC column. This active molecule was eluted after the standard lysophosphatidylcholine at 32 min, indicating that it is not a typical PAF molecule and it should be more polar than PAF. The purified phospholipid induced washed rabbit platelet aggregation in a dose-dependent manner, with PAF-like aggregation tracing. In lower concentrations, it induced platelet shape change or reversible aggregation while in higher concentrations, induced irreversible aggregation. The EC₅₀ value based on phosphorus determination was in the order of 0.1 μ M, final concentration, being less active than PAF which induces aggregation with EC₅₀ value in the order of 0.1 nM, final concentration. Furthermore, this molecule desensitized platelets against itself and PAF but not against thrombin. The aggregation induced by this PAF-like molecule was inhibited by BN52021, a specific PAF-receptor antagonist. No inhibition was observed by indomethacin and CP/CPK which are specific inhibitors of the aggregation induced by arachidonic acid and ADP, respectively. These results suggest that this phospholipid acts through receptors which are identical to the ones of PAF. This PAF-like molecule was inactivated in a time-dependent manner by human plasma PAF-AH at the same rate as the one of PAF, indicating the presence of an acetyl group in the *sn*-2 position of the glycerol backbone.

Mild alkaline hydrolysis rendered this active phospholipid completely inactive while reacylation of the chloroform soluble fraction, resulting from the above hydrolysis, increased its biological activity. Acetylation of the initial molecule resulted in an increase of its biological activity, identical to the one observed after the reacylation of the above chloroform soluble fraction (Figure 1). The complete inactivation of this phospholipid after alkaline hydrolysis, demonstrated the existence of an ester group(s) indispensable for displaying its biological activity. The recovery of the biological activity after the reacylation of the chloroform soluble fraction, demonstrated that the phospholipid has a glyceryl ether backbone and also indicated the presence of an acetyl group at the *sn*-2 position of the glycerol backbone. The increased biological activity observed after the reacylation of the chloroform soluble fraction, resulting from the alkaline hydrolysis which is identical to the one observed after acetylation

Figure 1 Diagram illustrating the biological data and the structural alterations of the biologically active compound after mild alkaline hydrolysis followed by re-acetylation as well as acetylation of the initial compound

of the initial molecule established on one hand the presence of at least one free hydroxyl group in the molecule and on the other hand confirmed the presence of the acetyl group at the *sn*-2 position of the glycerol backbone.

The purified phospholipid also activated human platelets. It induced platelet shape change or reversible aggregation with the same aggregation profiles compared to regular PAF.

The ESMS analysis of the active compound (Figure 2) showed that an ion, presumably M^+ , was present at m/z 704 accompanied by an MNa^+ ion at m/z 726. After fragmentation of this compound, the mass spectrum (Figure 3) showed ions at m/z 184 and 125 demonstrating the presence of the phosphocholine

Figure 2 Positive ion electrospray mass spectrum of the active compound

Figure 3 Positive ion electrospray mass spectrum of the active compound after fragmentation

Figure 4 The proposed structure of the active compound

group in the molecule. The ESMS analysis of the acetylated molecule, showed an ion at m/z 746, M_1^+ , corresponding to the previous molecular mass plus 42 Da which demonstrated the existence of one acetyl group.

The combination of the results obtained from the chemical and enzymatic treatments, the biological assays along with the ones from the electrospray analysis, leads to the proposed structure presented in Figure 4.

It should be noticed that this is the first time that an endogenous hydroxyl-PAF analogue is reported which is also implicated in

the pathogenesis of the periodontal disease. Evidence for the existence of a PAF-analogue, sharing the same chromatographic and biological characteristics as the hydroxyl-PAF analogue isolated from GCF samples, has been reported in human blood [17]. The above data reinforce the assumption that this inflammatory mediator could be released from the inflammatory cells, such as polymorphonuclears, lymphocytes and monocytes, after their infiltration and activation, induced by the bacteria or their products being present in dental plaque.

REFERENCES

- Genco, R. J. (1992) *J. Periodontol.* **63**, 338–355
- Page, R. (1991) *J. Periodont. Res.* **26**, 230–242
- Lee, T. C. and Snyder, F. (1985) *Phospholipids and Cellular Regulation* (Kuo, J. F. ed.), vol. 2, pp. 1–39, CRC Press Inc. Boca Raton, FL
- Andriotis, M., Demopoulos, C. A., Tournis, S., Siafaka, A. and Mavris, M. (1986) Second International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, TN, U.S.A., p. 120 (Abstr)
- Humphrey, D. M., Hanahan, D. J. and Pinckard, R. N. (1982) *Lab. Invest.* **47**, 227–234
- Braquet, P. and Rola-Pleszczynski, M. (1987) *Immunol. Today* **8**, 345–352
- Blank, M. L., Lee, T. C., Fitzgerald, V. and Snyder, F. (1981) *J. Biol. Chem.* **256**, 175–178
- Smal, M. A. and Baldo, B. A. (1991) *Lipids* **26**, 1144–1147
- McDonnell, H. T. (1993) *J. Periodontol.* **64**, 1109
- Noguchi, K., Morita, I. and Murota, S. (1989) *Arch. Oral. Biol.* **34**, 37–41
- Baltas, G., Kotsifaki, H., Antonopoulou, S., Kipioti, A. and Demopoulos, C. A. (1996) in *Platelet-Activating Factor and Related Lipid Mediators in Health and Disease* (Nigam, S., Kunkel, G. and Prescott, S. M., eds.), pp. 135–141, Plenum Publishing Corporation, NY
- Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Demopoulos, C. A., Pinckard, R. N. and Hanahan, D. J. (1979) *J. Biol. Chem.* **254**, 9355–9358
- Lazanas, M., Demopoulos, C. A., Tournis, S., Koussissis, S., Labrakis-Lazanas, K. and Tsarouhas, X. (1988) *Arch. Dermatol. Res.* **280**, 124–126
- Demopoulos, C. A., Andrikopoulos, N. K. and Stathopoulou-Sparou, R. (1988) *Int. J. Biochem.* **20**, 599–604
- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
- Antonopoulou, S. (1994) Doctoral dissertation, University of Athens, Athens, Greece

Received 11 June 1997/16 October 1997; accepted 11 November 1997