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Synthesis of paf-acether from exogenous precursors by the prokaryote *Escherichia coli*

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Paf-acether (paf) is a potent mediator of inflammatory diseases and septic shock. Using normal-phase HPLC, a paf-like activity was found in culture supernatants from *E. coli*. Prokaryotic paf exhibited the same biological and physico-chemical properties as eukaryotic cells and synthetic paf. Further, reverse-phase HPLC indicates that paf generated by bacteria is predominantly of the hexadecyl and octadecyl species. When cultures were supplemented with lyso-paf, a dramatic increase in paf production was observed. The purity and molecular structure of bacterial paf were further characterized by mass spectral analysis. These results could be of importance considering the pathogenetic role of enterobacteria. Further, it appears that the competence to form and release paf is an early phylogenetic development.

Platelet activating factor-acether; Mass spectral analysis; (*Escherichia coli*)

1. INTRODUCTION

Paf-acether (paf: 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine [1,2], formerly platelet-activating factor) is a phospholipid mediator initially described as a product of IgE-sensitized rabbit basophils [3]. It is now known to be rapidly synthesized by a variety of cells after appropriate stimulation [4,5]. The growing importance of this mediator in inflammatory processes [6], along with the striking similarities of its properties in many eukaryotic species, suggested to us that paf may be a molecule that has been conserved through evolution. We show here that *E. coli* contains paf and can acetylate exogenous lyso-paf to generate and release large amounts of the bioactive compound. This work reports for the first time the release of paf by *E. coli*, a result that could be of some importance since (i) the potent proinflammatory mediator paf could contribute to the pathology produced by enterobacteria; and (ii) the existence of paf in a prokaryote suggests that it is an ancient and functionally conserved molecule, possibly with a wider role than that of a proinflammatory autacoid.

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2. MATERIALS AND METHODS

2.1. *Bacterial culture conditions*

Four different strains of bacteria were grown aerobically at 37°C in a rich complex medium (ML) [7], supplemented with 0.25% lipid-free bovine serum albumin (Sigma, St. Louis, MO, USA). After 16 h, bacteria from a standard culture (10 ml, 1×10^9 cells) were harvested by centrifugation ($600 \times g$, 15 min). In most of the experiments bacteria were supplemented at the beginning of the culture with lyso-paf (1-*O*-alkyl-glycero-3-phosphocholine; Bachem, Bubendorf, Switzerland).

2.2. *HPLC on lipid fractions*

Lipids were extracted from both supernatants and cell pellets according to Bligh and Dyer methods [8]. The dried residue was dissolved in 500 μl of HPLC solvent (dichloromethane/

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methanol/water, 60:50:5). Samples were applied to a Microporasil column 3.9 mm ID × 300 mm length (Waters Associates, Milford, MA), which was then eluted at a flow rate of 1 ml/min. 1 ml fractions were dried and resuspended in 50 μl of 60% ethanol and then assayed for platelet-aggregating activity.

2.3. *Paf assay*

Washed rabbit platelets were prepared as in [9]. Aspirinated platelets (1.6×10^8) in Tyrode's (300 μl) containing 2.5% gelatin and the ADP scavenger mixture, creatine phosphate (1 mM)/creatine phosphokinase (10 U/ml) were stirred in an aggregometer (Icare, Marseille, France). Aggregating activity of the samples was measured over the linear portion of the calibration curve obtained with 5–50 pg synthetic paf (Bachem).

2.4. *Mass spectral analysis*

The HPLC fractions were introduced into an Extrel 400-2 quadrupole mass spectrometer via a Vestec thermospray interface (Vestec, Houston, TX, USA) as described previously [10]. Vaporisation and ionisation were achieved by heating the capillary vaporiser and also by applying an electron-emitting filament current (0.2 A). Without this auxiliary ionising source, sufficient ionisation did not occur since a mobile phase extremely high in organic content was required to dissolve and elute the phospholipids. Excess solvent was pumped away through a vacuum line via a dry ice/isopropyl alcohol cold trap. The vaporiser tip temperature was maintained at 145–148°C and the source temperature at 300°C. All data were acquired with an Extrel EL-1000 data system.

3. RESULTS

As shown in table 1, when lipids extracted from bacteria cultured for 16 h were submitted to normal phase HPLC, some effluent fractions contained an activity that induced aggregation of washed rabbit platelets unresponsive to ADP and arachidonic acid. The active fractions were eluted from the column with a retention time identical to that of authentic paf whereas no paf activity was detected elsewhere in the chromatogram. In the majority of individual experiments ($n = 28$), more than 90% of this activity was recovered in the culture media, the rest remaining cell-associated. In the absence of bacteria, no paf was detected in either culture media.

Apart from its chromatographic behaviour, the platelet-aggregating substance appeared undistinguishable from authentic paf: (i) the aggregating activity found in the extract was totally inactivated by treatment with phospholipase A_2 but not with lipase A_1 [11]. (ii) The three antagonists of the paf putative receptor L 652,731 (generously provided by Dr J. Chabala, Merck Sharp and Dohme, Rahway, NJ, USA), CV-3988 (a gift from Dr Nagawa, Takeda Chemical Indust. Ltd, Osaka, Japan) and BN 52021 (obtained from IHB, Le Plessis-Robinson, France), inhibited platelet aggregation induced by submaximal concentration of both *E. coli* paf and synthetic paf [12]. (iii) The activity was eluted from reverse-phase HPLC at the same retention time as synthetic C16 and C18 paf (for about 50% of the activity each) (not shown).

Table 1

Formation of paf-acether in *E. coli* strains

Strains	Genotype	paf[a]
POP 1021	*F⁻, metA, trp*	132 ± 48
PAP 271	*F⁻, metA, bio, thyA, endA*	138 ± 90
PAP 274	*F⁻, bio, thyA, pldA*	60 ± 40
0111:B4	NA[b]	200 ± 120

[a] In pg paf for 1×10^9 bacteria (mean ± SE of 7 experiments)
[b] NA, not available

We next investigated whether paf release could be limited by the amounts of its precursor, lyso-paf. The latter compound, totally devoid of ag-

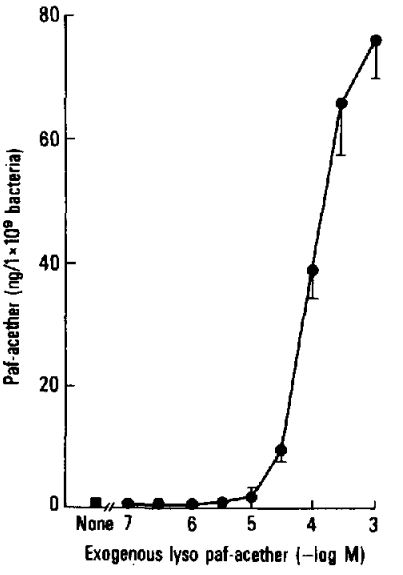

Fig.1. Effects of exogenous lyso paf on paf formation. *E. coli* POP 1021 was grown in the absence or presence of varying amounts of synthetic C16 lyso-paf. After 16 h, lipids were extracted and paf content assayed as indicated in section 2. Results are expressed in ng paf/1 × 10^9 bacteria (mean ± SE of 4 experiments).

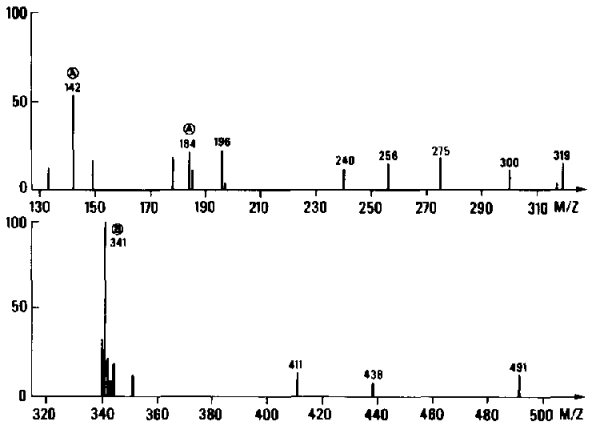

Fig.2. Separation and detection of molecular species of bacterial paf using coupled HPLC and thermospray mass spectrometry. A pool of bacterial paf, obtained following supplementation with 0.5 mM C16 lyso-paf, and eluted from direct phase HPLC was analysed by HPLC coupled thermospray mass spectrometry. Peaks denoted A and B in the spectra represent fragments derived from phosphocholine and diglyceride ions, respectively.

gregating property, is the immediate paf precursor as it is a substrate for an acetyltransferase; it is also a paf catabolite after acetylhydrolase attack [13]. As shown in fig.1, exogenous C16 lyso-paf markedly enhanced in a dose-dependent manner the synthesis and release of paf by *E. coli* K12. As compared to medium alone a 10- to 420-fold increase in paf production was observed when bacteria were cultured in the presence of lyso-paf from 10 μM to 1 mM, whereas lyso-paf from 0.1 μM to 10 μM was not effective. Of note, lyso-paf (up to 1 mM) had no effect on cell growth or viability. In a further attempt to characterize bacterial paf, the active fraction obtained following supplementation with 0.5 mM C16 lyso-paf and eluted from the normal-phase HPLC was analyzed using thermospray mass spectrometry. The mass fragmentation pattern of the extract is depicted in fig.2. The major fragment peak resulting from the head group loss (*m/z* 341) represents the diglyceride ion containing the 16:0 fatty alkyl moiety. Ions from the choline head group (*m/z* 142 and 184) were also present.

4. DISCUSSION

In the present work, large amounts of paf (up to 80 ng) were produced by a relatively low number of bacteria (1×10^9), indicating that biological activity of high physiological and pathological significance can be expressed in the microorganism environment. Identity of the prokaryotic molecule with authentic paf is based on the stringent functional and biochemical criteria detailed above including two types of HPLC and mass spectrometric analysis. It should be pointed out, however, that the amount of paf formed by bacteria in the absence of exogenous lyso-paf was insufficient for structural analysis using the latter method.

Since ether-linked and choline-substituted phospholipids have not yet been reported in *E. coli*, what is the mechanism by which *E. coli* can synthesize de novo the alkyl-ether, choline-containing mediator? The simplest interpretation of these findings is that paf results from the bioconversion of paf precursors present in culture media, indeed 1 nM lyso-paf was detected in culture media. An alternative view is that *E. coli* itself contains small amounts of 1-*O*-alkyl-glycero-3-phosphocholine undetectable using conventional means yet sufficient to support paf synthesis. In this regard, our bioassay is able to detect sub-picogram amounts of active ether-linked phospholipids, thus several orders of magnitude more sensitive than conventional physico-chemical methods. Clearly, further studies will be required to allow definitive conclusions on the endogenous vs exogenous origin of the alkyl-ether phosphatidylcholine backbone of paf found in unsupplemented bacteria. Nonetheless, supplementation with the lyso precursor of paf led to a major increment in paf production, indicating most likely the existence in *E. coli* of an acyltransferase activity as previously reported for long chain fatty acids [14]. There is also evidence to suggest that the lyso-paf resulting from the degradation of most eukaryotic cell membranes is abundant in the natural environment of the bacteria, thus providing the 'raw material' to be acetylated into paf.

At the present time, the function of paf in *E. coli* is not known. For example, it could be implicated in bacteria-related pathogenic events. Indeed, the role of paf has been suspected in necrotizing enterocolitis [15], gastric ulceration [16] and Gram-negative septic shock [17].

Therefore, the existence of bacterial paf either alone or associated with bacterial endotoxin could

support the concept of the local and/or general release of this mediator from bacterial and/or host sources initiating or amplifying gastro-intestinal damages or septic shock. The release of lyso-paf following phospholipase A_2 activation and/or cell damage during the initial local injury could trigger an increment of paf synthesis by saprophyte or pathogenetic bacteria, thus producing an auto-amplifying injury.

Finally, the strict similarity of prokaryotic and eukaryotic paf, considered along with the phylogenic position of the enterobacteria, suggests a more fundamental role for paf than the now classical ones in allergy and inflammation and opens a new area for speculation. For instance, is paf involved in the physiology of the bacteria itself? Was this mediator, a major effector of cell-cell communication, also regulating interactions between bacteria and, later, between bacteria and more complex organisms. Bacterial mutants-lacking paf could be helpful probes in approaching these issues.

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