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Membrane-Targeted Synergistic Activity of Docosahexaenoic Acid and Lysozyme against *P. aeruginosa*

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SYNOPSIS

Antimicrobial polypeptides including lysozymes (Ly) have membrane perturbing activity and are well documented effector molecules of innate immunity. In cystic fibrosis, a hereditary disease with frequent lung infection with *Pseudomonas aeruginosa*, the free fatty acid docosahexaenoic acid (DA), but not oleic acid (OA), is decreased and DA supplementation has been shown to improve the clinical condition in these patients. We hypothesized that DA may alone, or in conjunction with Ly, exert antibacterial action against *P. aeruginosa*. We found that DA and Ly synergistically inhibit the metabolic activity of *P. aeruginosa*, in contrast to OA. Electron microscopy and equilibrium dialysis suggest that DA accumulates in the bacterial membrane in the presence of Ly. Surface plasmon resonance with live bacteria and differential scanning calorimetry studies with bacterial model membranes reveal that, initially, DA facilitates lysozyme incorporation into the membrane, which in turn allows influx of more DA leading to bacterial cell death. Our study elucidates a molecular basis for the synergistic action of free fatty acids and antimicrobial polypeptides, which may be dysfunctional in cystic fibrosis.

Keywords

Innate host defence; antimicrobial peptides; free fatty acids; cystic fibrosis; bacterial cell wall; LUV (large unilamellar vesicles)

INTRODUCTION

The innate immune system is the first line of defence against invading micro-organisms. Among the key players are antimicrobial cationic peptides and proteins (AMPs), which are thought to kill micro-organisms by membrane perturbation [1,2] and possibly by disruption of membrane bound multienzyme complexes and intracellular events [3–5]. In addition, AMPs

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also have immune modulatory functions important for innate and adaptive host defence [6]. Human lysozyme (Ly, ~14.5 kDa) is one of the predominant antimicrobial polypeptides in all body fluids reaching up to mg/ml concentrations [7]. It is well known for its peptidoglycan hydrolyzing activity [8] but it also exerts antibacterial activity through membrane perturbation [9].

Free fatty acids such as docosahexaenoic acid (DA) and oleic acid (OA) are also found in body fluids including respiratory secretions at high nanomolar concentrations [10,11]. DA (C22:6) is a polyunsaturated fatty acid with documented activity against Gram-positive and Gram-negative bacteria [12]. OA (C18:1) is a monounsaturated fatty acid. Reduced levels of DA but increased levels of OA have been measured in patients with cystic fibrosis, a hereditary disease affecting the cystic fibrosis transmembrane regulator protein [13]. These patients produce an altered mucous leading to gravely delayed clearance of their airways and chronic lung infection with *Pseudomonas aeruginosa*, an opportunistic Gram-negative bacterium with characteristic phenotypic changes in cystic fibrosis patients [14]. The association between cystic fibrosis and diminished DA levels has been corroborated with an animal model [15] and dietary supplementation of DA has improved the clinical condition of cystic fibrosis patients in some studies [16].

Given that Ly is one of the essential antimicrobial polypeptides in the airways [7] and Ly levels are not reduced in cystic fibrosis patients [17] but DA levels are reduced, we hypothesized that DA may act synergistically with Ly, and we sought to define the contributions of DA to the effects of Ly on *P. aeruginosa* by employing metabolic assays and electron microscopy, and by performing biochemical and biophysical binding studies with live bacteria and bacterial model membranes.

EXPERIMENTAL

Lipids

Oleic and docosahexaenoic acid (OA and DA, respectively) were purchased from Sigma (Sigma-Aldrich) and kept at -20°C as 20 mM stock solutions in 95% ethanol under nitrogen. [^{14}C]-labeled DA and OA were purchased as sterile solutions in ethanol (American Radiolabeled Chemicals Inc., St. Louis, MO) and kept at -20°C .

Dipalmitoylphosphatidylglycerol (DPPG, Avanti Polar Lipids, Inc.) stock solutions were prepared in chloroform/methanol (2:1 v/v).

Human Lysozyme

Human lysozyme (Ly, pI 9.3) was purified from human milk (Mother's Milk Bank, Denver, CO) using a weak cationic exchange matrix (CM MacroPrep; BioRad; equilibrated in 25 mM NH_4Ac , pH 8.25) at a slurry to milk ratio of 1:20. After over night extraction at 4°C , the matrix was washed with equilibration buffer 3×5 min at room temperature. Ly was batch eluted with first 10% and then 5% glacial acetic acid, at a CM slurry to acetic acid ratio of 1:1 and 1:2, respectively, for 30 min each at 4°C , then dialyzed against 0.01% acetic acid (SpectraPor, Spectrum Laboratories; MWCO 12–14 kDa), concentrated, and further purified by reverse phase HPLC (10 mm $\times$ 250 mm Vydac C8 column, TP Silic, 300 Å, flow 2 ml/min) using 0.1% trifluoroacetic acid as pairing agent and an acetonitrile gradient in water: 5% for 6 min, to 35% in 10 min, to 38% in 3 min, to 39% in 16 min, to 43% in 2 min, to 45% in 5 min and to 60% in 5 min. Ly was adjusted to 1 mg/ml (68 μM) stocks in 0.01% acetic acid according to BCA Protein Assay (PIERCE Biotechnology Inc.) using hen egg white lysozyme (Sigma) as standard and stored at -20°C .

Bacteria

Mid-logarithmic growth phase *P. aeruginosa* (original strain from Dr. M.J. Welsh, University of Iowa) were prepared as previously described [18]. Briefly, one isolated colony was inoculated into 50 ml of 1-fold trypticase soy broth (TSB) and after incubation for 18–20 h at 37°C, 200 rpm, 500 µl of the culture were transferred into 50 ml of fresh, prewarmed TSB and incubation was continued for exactly 3 h. Thereafter, bacteria were washed and adjusted to McF 0.5 (equivalent to $\sim 1.0 - 2.0 \times 10^8$ CFU/ml) in assay buffer and kept on ice until further use (within 3 h). Assay buffer was 10 mM sodium phosphate (NaPi) pH 7.3, 100 mM NaCl, and 4% TSB.

Metabolic Assay

Reduction of the non-fluorescent dye resazurin to the fluorescent compound resorufin by bacterial metabolites like NADPH was used to assess bacterial viability [19] in the presence or absence of Ly and/or fatty acids over a prolonged period of time. Assay buffer was supplemented with 100 µM resazurin. Samples were prepared in 100 µl volumes and contained 10^6 CFU/ml, Ly at 0, 62.5, 125, 250 and 500 µg/ml (0, 4.25, 8.5, 17, and 34 µM, respectively), and free fatty acids at 0 and 10 µM. Incubation was in UV-sterilized microtiter plates with Non-Binding-Surface (Corning-Costar) in a SpectraMax GeminiEM fluorimeter (Molecular Devices) at 37 °C for 20 h with intermittent shaking. Readings of relative fluorescence were taken every 10 min (530 nm_{ex} and 590 nm_{em}). The area under the resulting curve representing the accumulated total reducing activity of bacteria, a function of the individual metabolic activity and the number of viable bacteria, was calculated by SoftmaxPro 4.1 software.

Quantification of Enzymatic Ly Activity

Enzymatic activity was quantified according to Jenzano & Lundblad [20]. Briefly, *Micrococcus lysodeikticus*, gram positive cocci with a thick peptidoglycan layer and high susceptibility to the enzymatic action of lysozyme, were added at 0.5 mg/ml to liquid 66 mM sodium phosphate buffer, pH 7.0 containing 1% agarose and square plates (10 cm × 10 cm area) were poured with 10 ml each. After solidification holes with 3 mm diameter were punched into the agar aseptically. Ly standards were prepared at 125, 250, and 500 µg/ml (8.5, 17, and 34 µM, respectively) in assay buffer. Test samples consisted of 1 part 250 µg/ml Ly (17 µM) or solvent plus 1 part 20 µM fatty acid or solvent in assay buffer. Duplicate samples of 5 µl/well were pipetted and plates were incubated for 18 h at room temperature. The resulting zones of clearing reflecting the enzymatic activity of lysozyme were measured in mm with a ruler and converted to arbitrary units: [(diameter – 3 (for the hole)) × 10].

Electron Microscopy

Reaction volumes were scaled up to obtain 10^8 CFU/ml per sample and a final sample volume of 2.38 ml. Ly was tested at 500 µg/ml (34 µM) and DA at 10 µM. After 20 h incubation bacteria were fixed in 2% EM grade glutaraldehyde (Ted Pella, Inc.) for 30 min at 4°C, washed, and resuspended in 1 ml of ice-cold PBS pH 7.4. For scanning electron microscopy (SEM), 100 µl of the sample were transferred into 950 µl of PBS and deposited onto 0.1 filter discs (Millipore). The discs were washed in PBS, dehydrated with 50%, 75%, 95% and 100% absolute alcohol, then gold coated with an EMS-76 Mini-coater (Ernest Fulham). Scanning was performed with a Cambridge 360 instrument. For transmission electron microscopy (TEM), the remaining bacteria were dehydrated as above and embedded in Epon. Ultrathin sections were stained with saturated uranyl acetate for 1 h at 60°C and examined at 80 keV with a Jeol CX-100 electron microscope [21].

Equilibrium dialysis

To measure fatty acid binding to *P. aeruginosa* in the presence or absence of Ly equilibrium dialysis was employed [22]. In this method ligand (*cis*, in our study free fatty acid) and target (*trans*, in our study bacteria) are separated by a dialysis membrane that allows only the free diffusion of the ligand. If the ligand binds to the target the free solute concentration of the ligand in the trans-compartment will be lowered thus forcing additional diffusion of ligand from the cis-compartment. Equilibrium dialysis units were constructed with 10 kDa cut-off Slide-A-Lyzer MINI Dialysis units (PIERCE, upper chamber) and sterile polypropylene transfer pipettes (bottom chamber). Bacteria (target) were placed in the lower (*trans*) chamber only. Fatty acids (ligands) were placed into the upper (*cis*) chamber only. Ly, when employed, was added to both chambers. The final test conditions were 10 μ M radiolabeled DA and OA (0.025 μ Ci/ μ l) or solvent only, 250 μ g/ml (17 μ M) Ly or solvent only, 5.0×10^5 CFU/ml bacteria or assay buffer only (10 mM NaPi, pH 7.3/100 mM NaCl/4% TSB/0.1% BSA) with a final volume of 300 μ l in each chamber. Incubation was at 37°C with constant agitation for 4h. Before and after incubation 50 μ l aliquots were taken from both chambers of each dialysis unit and subjected to liquid scintillation. In addition, 10 μ l aliquots from each lower chamber were spotted onto pre-dried trypticase soy agar plates (TSA) and incubated at 37°C over night to assess bacterial viability and purity.

Surface plasmon resonance (SPR)

SPR studies were performed on a BIAcore X analyzer (GE Healthcare Bio-Sciences Corp.) according to the manufacturer's recommended procedure (BIAcore X Control Software version 2.3, BIAevaluation Software version 4.1). CM5 chips (BIAcore) were activated with 60 μ l of a mixture of equal volumes of N-hydroxysuccinimide and N-ethyl-N'-[(3-dimethylamino)-propyl]-carbodiimide hydrochloride (BIAcore reagents) at 20 μ l/min. Then, Ly (10 μ M) was immobilized to the activate chip surface (60 μ l over 3 min). Unbound surfaces were blocked with 40 mM ethanolamine (50 μ l over 2.5 min). Lastly, 60 μ l of DA or OA (each 1 μ M) or a mixture of both were injected at 20 μ l/min. L1 chips (BIAcore) were pre-conditioned with 5 μ l of 20 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) at 5 μ l/min, washed with 100 μ l running buffer (10 mM NaPi pH 7.3/100 mM NaCl) at 25 μ l/min. Immobilization of live *P. aeruginosa* was achieved by injecting 80 μ l of a 1×10^9 CFU/ml suspension in running buffer at 2 μ l/min followed by a wash with 100 μ l of buffer at 25 μ l/min. Surface blocking was accomplished by injecting 5 μ l of 1 % BSA in running buffer at 2 μ l/min. Biomolecular interaction analysis of the immobilized bacteria with Ly and fatty acids was conducted by injecting 30 μ l of 25 μ g/ml (1.7 μ M) Ly, 1 μ M FA, or a mixture of both at 2 μ l/min in running buffer supplemented with 0.25% BSA. Alternatively, sequential injections of 30 μ l of 25 μ g/ml (1.7 μ M) Ly and 30 μ l of 1 μ M fatty acid or vice versa were made.

Preparation of large unilamellar DPPG vesicles (LUVs)

LUVs were prepared from dry lipid films dispersed in 20 mM NaPi buffer, pH 7.4, 130 mM NaCl, hydrated above the main transition, and extruded by applying 15 cycles and Millipore filters of 0.1 μ M VCTP. Aliquots of the respective fatty acid or Ly were added, mixed for 1 min and incubated again at 45°C for 30 min, then the other active reagent (Ly or fatty acid) was added and samples were incubated for another 30 min at 45°C. Samples were cooled to room temperature prior to differential scanning calorimetry.

Differential Scanning Calorimetry

Calorimetric experiments were performed with a MicroCal VP-DSC differential scanning calorimeter (MicroCal, Inc.) applying a scan rate of 30°C/h for DPPG and 60°C/h for lysozyme, respectively. Calorimetric enthalpies were calculated by integrating the peak areas after baseline adjustment and normalization with respect to solute concentration (phospholipid 1

mg/ml and lysozyme 2 mg/ml) using MicroCal's Origin software. Reversibility of transitions was verified by at least two heating and cooling cycles for samples with LUVs and reproducibility by measuring at least two samples. In the absence of LUVs samples were only heated once due to incomplete reversibility of the unfolding transition of lysozyme.

Data analysis

Data were calculated with Microsoft Excel, plotted with SigmaPlot version 9.0, and statistically analyzed with SPSS version 15.

RESULTS AND DISCUSSION

Ly inhibits the metabolic activity of *P. aeruginosa* in synergism with DA

Guided by the observed link between altered fatty acid profiles and chronic infectious diseases, we sought to investigate the potential of free fatty acids to act as an antimicrobial agent in conjunction with Ly, a predominant antimicrobial protein on mucosal surfaces. We first tested the prolonged effect of selected free fatty acids on the metabolic activity of *P. aeruginosa* in the presence or absence of Ly (Figure 1). The longer incubation time allowed observation of potential bacterial adaptation processes as they are likely to occur in the lungs of cystic fibrosis patients who have a severely impaired mucociliary clearance [23]. We found that Ly inhibited metabolic activity of *P. aeruginosa* and that this inhibition was significantly enhanced when DA was present and was decreased in the presence of OA ($p < 0.001$ for Ly concentration and $p < 0.05$ for the effects of fatty acids in an analysis of covariance). Synergism between free fatty acids and antimicrobial peptides has been reported [24] and *in vitro* acylation has been shown to increase the antibacterial activity of antimicrobial peptide derivatives of lactoferricin [25] and dermaseptin [26], as well as lipopeptides [27].

Ly is bimodal and the observed augmentation of the Ly effect could be the result of either enhanced peptidoglycan hydrolyzing activity or increased disruption of the bacterial membrane. Therefore, we tested the enzymatic activity of Ly in the presence of fatty acids and performed electron microscopy to examine alterations in the bacterial membrane structure.

Fatty acids do not affect the enzymatic activity of Ly

There was no significant change of the enzymatic activity of Ly in the presence of DA or OA or when tested at 250 µg/ml Ly (17 µM) and 10 µM fatty acid (data not shown).

Electron microscopy suggests accumulation of DA in the disrupted bacterial cell membrane in the presence of Ly

In scanning electron microscopy (Figure 2A–D), *P. aeruginosa* appeared after a 20-h incubation in assay buffer as short rods (Fig. 2A). After treatment with Ly the cells were aggregated and had formed spheroplasts (Figure 2B). Bacteria treated with DA alone were elongated (Figure 2C). After incubation with DA and Ly, spheroplasts and shortened rods were observed and bacterial surfaces appeared densely packed (Figure 2D). In transmission electron microscopy (Figure 2E–H), control bacteria (Figure 2E) showed an intact cell membrane (consisting of the outer membrane, peptidoglycan, and inner membrane) and intact cytoplasmic material. The three layers of the bacterial cell wall treated with Ly (Figure 2F) could not be differentiated and showed depositions on the outer surface and loss of cytoplasmic material. The ultrastructure of bacteria treated with DA alone was comparable to the structure of control bacteria (Figure 2G). In bacteria treated with both DA and Ly (Figure 2H) the cell wall was thickened and amorphous, and the cytoplasm appeared very condensed and displaced. The appearance of electron-dense material within the bacterial cell wall in the presence of Ly and DA is consistent with accumulation of DA in the bacterial cell wall in the presence of Ly,

possibly facilitated by Ly-initiated membrane lesions. Cell wall thickening after treatment with antimicrobial agents has been also reported for *Prototheca zopfii* in response to silver nitrate [28]. To further substantiate binding of DA to the bacterial surface in the presence of Ly we performed equilibrium dialysis and surface plasmon resonance.

Equilibrium dialysis is consistent with increased binding of DA to the bacterial cell membrane when Ly is present

Initial studies of the equilibrium dialysis kinetics established that in our experimental setup the optimal equilibrium dialysis time was 4 h (data not shown). We observed a significantly accelerated loss of DA from the *cis*-compartment when Ly was present (Figure 3A) consistent with binding of DA to bacteria in the presence of Ly. In average, the cpm were reduced in the *cis*-compartment by 837.0 ± 38.6 (mean $\pm$ S.D., $n = 3$, $p < 0.001$ in paired t-test), which translates to 1.83×10^7 more molecules of DA bound per bacterium in the presence of lysozyme. In contrast, the diffusion rate of OA, which acted antagonistically with Ly in the metabolic assay, was not significantly influenced by Ly. CFU assays conducted in parallel (Figure 3B) showed that the number of viable bacteria was reduced when Ly was added to DA compared to bacteria incubated with DA alone ruling out an accelerated diffusion of the fatty acid due to consumption by proliferating bacteria. The effect of Ly appears to be weakened for OA though there was no statistically significant difference between the means for DA + Ly and OA + Ly using a Tukey post-hoc comparison in a one-factor ANOVA. However, CFU/ml were determined after 4 h only whereas the metabolic activity of Ly +/- FA treated bacteria were assessed after 20 h. This implies that DA-mediated changes may require a longer time to fully develop. These binding studies validate that the ultrastructural changes observed in electron microscopy studies reflect accumulation of DA in the bacterial membrane in the presence of Ly.

DA does not interact with Ly or affect Ly thermal stability

To test whether the differences in the synergistic activity observed between DA and OA is due to differing effects on Ly structure we performed SPR and calorimetric studies (Figure 4). There was no binding of the fatty acids to Ly when applied individually or when applied together (Figure 4A). Calorimetric experiments on the thermal unfolding of Ly in the presence and absence of OA and DA, respectively, showed that both fatty acids do not affect the thermal stability of Ly, when added at concentrations at or below their critical micelle concentration (Figure 4B). This indicates that the fatty acids do not bind to Ly confirming the observation from SPR experiments. Hence, we conclude that the synergistic effects of DA and Ly result from direct action on the bacterial membrane.

SPR binding studies suggest that DA inserts into the bacterial membrane prior to Ly

We utilized SPR to analyze the interaction of Ly with the bacterial surface in the presence and absence of fatty acid. SPR is a highly sensitive and rapid technique for *in situ* surface interaction analysis. It has been used previously to study interaction of antimicrobial peptides with isolated bacterial lipids or bacterial model membranes [29,30]. Protocols for whole live bacteria in SPR have been previously described for carboxymethylated dextran chips which possess a hydrophilic surface [31]. However, *P. aeruginosa* could not be immobilized to this surface since the required buffers negatively impacted its viability (data not shown) and immobilization of Ly would likely adversely interfere with required molecule flexibility for embedding into the bacterial cell membrane [2]. Therefore, we developed a new protocol for whole live bacteria using L1 (lipid-covered gold) chips that offer a hydrophobic surface allowing non-covalent attachment [32]. Our immobilization protocol produced response units between 573–2944 (mean $\pm$ S.D. = 1026.71 ± 620.63 , $n = 14$), which was in the range of previously published data [33]. When DA and OA were applied simultaneously with Ly, we did not observe a statistically significant increase of response units for Ly + fatty acid compared to Ly and fatty

acid alone (Figure 5A). Therefore, we reasoned that Ly and DA should exert their synergistic activity by sequential damage. To discern whether Ly or DA inflicts the initial lesion in the bacterial membrane, we conducted SPR with Ly and DA injected consecutively (Figure 5B). We found that the SPR response was greater if DA was allowed to flow across the bacterial surface first, implying that DA facilitates Ly binding to the bacterial surface ($p < 0.05$ for [DA/Ly versus Ly/DA] and [DA/Ly versus buffer] for Tukey post-hoc comparisons in a one-factor ANOVA). The opposite trend was observed for OA injection that was followed by Ly injection ($p < 0.05$ for [OA/Ly versus Ly/OA] and for [OA/Ly versus buffer] for Tukey post-hoc comparisons in a one-factor ANOVA). However, similar SPR responses were observed for DA and OA when Ly was injected first (Figure 5C). *P. aeruginosa* has been shown to convert oleic acid to 7,10-dihydroxy-8(E)-octadecenoic acid (DOD), which can act as a surfactant to lower surface tension [34]. Such activity triggered by the additional presence of Ly may have possibly led to removal of bacteria from the surface, thus accounting for the observed decrease in the SPR response when OA injection preceded the Ly injection. Alternatively, oleic acid might have been rapidly internalized altering the bacterial and chip surface substantially thereby lowering the SPR response.

These data suggest that, in contrast to our original hypothesis, DA induces the initial lesion for the subsequent synergistic action of DA and Ly.

Calorimetric studies with large unilamellar DPPG vesicles confirm distinct membrane interaction of DA and OA

In order to gain further information on the membrane interaction of DA and OA in the presence or absence of Ly we conducted calorimetric studies with bacterial model membranes using unilamellar liposomes composed of DPPG. Figure 6 shows representative thermograms of liposomes treated with OA, DA and Ly in varying combinations using a molar ratio of DPPG to additive of 500:1. Table 1 summarizes the results from such thermodynamic measurements. Adding Ly to DPPG liposomes resulted in a shift of both pre- and main transition and a minor increase of enthalpy. This suggests that the protein binds to the negatively charged liposomal surface leading to charge compensation. While OA drastically lowered and markedly broadened the main transition, DA induced a highly cooperative main transition without significantly affecting the transition temperature. It is likely that the conformation of OA bent by the single *cis* double bond significantly perturbs the hydrocarbon chain packing of DPPG that contains saturated palmitoyl-residues. On the other hand, DA rather adopts a linear conformation because of its alternating 6 *cis* double bonds resulting in more homogeneous lipid packing as evident from the strong decrease of the transition half-width. Incubating DPPG liposomes with Ly first and then DA or vice versa resulted in thermograms very similar to pure DPPG. This observation is consistent with the observed increase of the SPR response under both conditions. Incubation of DPPG liposomes with Ly first and followed by OA resulted in thermodynamic characteristics similar to the DPPG/OA mixture. The broadening and shift to higher temperatures is indicative of additional surface-bound Ly in accordance with the increase of signal observed by SPR. Adding OA before Ly resulted in a strong destabilization of the DPPG gel phase (decrease of main transition by more than 6°C). The decrease of enthalpy for this sample can be indicative of the formation of small micellar particles, which would be in line with the decrease of SPR signal. Formation of membrane vesicles, which is increased by exposure to polycationic antibiotics, has been well described for *P. aeruginosa* [35]. OA may similarly increase membrane vesicle formation thus removing Ly and leading to its observed antagonistic activity. In contrast and as previously shown by SPR, the obvious differences between DA and OA treatments were lost when the fatty acids and Ly were added together to DPPG liposomes (Table 1). Overall, the DSC data demonstrate different effects on the bilayer organization of DA and OA in the presence and absence of Ly, and are in agreement with the results obtained by the techniques described in the previous paragraphs.

In conclusion, we have shown that docosahexaenoic acid and lysozyme synergistically inhibit the metabolic activity of *P. aeruginosa*. Our work provides novel insight into the mechanism of this activity. Electron microscopy revealed an ultrastructural damage of bacterial membranes that is consistent with the incorporation of DA in the presence of Ly and equilibrium dialysis confirmed increased binding of DA to *P. aeruginosa* in the presence of Ly. Unexpectedly, surface plasmon resonance and calorimetric studies both suggest that DA sets the initial lesion during early interaction with the bacterial membrane. Thus, the free fatty acid DA facilitates lysozyme mediated disruption of the bacterial membrane which in turn allows influx of more DA into the membrane. This study provides a molecular basis for the clinically observed association of DA deficiency and lung infection with *P. aeruginosa* in cystic fibrosis patients. The described mechanism may extend to other host-derived fatty acids and antimicrobial proteins and may lead to the development of novel antibiotics. This finding is highly significant considering that the increasing antibiotic resistance of microbes causing infectious diseases poses escalating threats to public health.

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Abbreviations used

AMP	Antimicrobial peptides
Ly	Lysozyme
OA and DA	Oleic and docosahexaenoic acid, respectively
NaPi	sodium phosphate
DPPG	dipalmitoylphosphatidylglycerol
SPR	surface plasmon resonance
RU	response unit
LUV	large unilamellar vesicles
CMC	critical micelle concentration

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Figure 1. DA augments Ly mediated metabolic inhibition of *P. aeruginosa*

Bacteria were incubated for 20 h with and without Ly (62.5, 125, 250, and 500 μg/ml or 4.125, 8.25, 17, and 34 μM, respectively) and/or 10 μM fatty acids (OA: oleic acid; DA: docosahexaenoic acid) in non-binding surface microtiter plates and the accumulated metabolic activity was quantified as relative fluorescence units (RFU) resulting from resazurin reduction to resofurin in the presence of bacterial metabolites. Shown are the combined data of 9 experiments, each conducted in duplicates. Data points represent means + S.E.M. (where applicable); n = 1 for DA + 62.5 and 125 μg/ml Ly; n = 4 or more for all other data points. An analysis of covariance revealed significant differences among treatment groups ($p < 0.05$).

Figure 2. DA increases ultrastructural damage induced by Ly in *P. aeruginosa*

Bacteria were incubated for 20 h with and without Ly and/or DA and subjected to scanning electron microscopy (A–D) and transmission microscopy (E–H). A, E: control bacteria; B, F: incubation with 500 μg/ml (34 μM) Ly only; C, G: incubation with 10 μM DA only; D, H: incubation with Ly and DA. Each bar represents 1 μm.

Figure 3. Equilibrium dialysis with FA and/or Ly and whole life bacteria suggest that DA but not OA accumulates in the bacterial cell wall in the presence of Ly

P. aeruginosa was placed in the *trans*-compartment of an equilibrium dialysis unit and radiolabeled fatty acid (DA and OA) were added to the *cis*-compartment separated by a 10kDa membrane. After 4 h incubation with (added to both compartments) and without Ly a 50 μ l aliquot was removed from the *cis*-compartment and subjected to liquid scintillation (A) and 10 μ l were removed from the *trans*-compartment for determination of CFU (B). A: The data points reflect averages of duplicate measurements for three different experiments. Lines connect the data points within one experiment. Accelerated diffusion with decreased residual

radioactivity is consistent with fatty acid binding to bacteria. * $p < 0.001$ in paired t-test. B: Shown are averages of three independent experiments + S.E.M.

Figure 4. Synergistic effects of Ly and DA do not arise from molecular interaction between Ly and DA

Molecular interaction analyses based on SPR and DSC. (A) Representative SPR sensogram showing Ly immobilization on a CM5 chip, blockage by ethanolamine (EA), and the injection of 1 μ M fatty acid. The arrows indicate injections of the species and the ratios of OA/DA are shown next to the respective injection peaks. B: Thermograms of Ly (2mg/ml) with and without DA or OA in buffer (10 mM NaPi, pH 7.4, 100 mM NaCl) at molar ratios of fatty acid to Ly of 1/50 (below CMC), 1/20 (at CMC), and 1/1 (above CMC with visible micelle formation). Experiments were performed at a scan-rate of 60°C/hr.

Figure 5. SPR binding studies suggest that DA inserts into the bacterial membrane prior to Ly
 A: L1 chips pre-immobilized with *P. aeruginosa* ($\sim 10^8$ bacteria) were treated with fatty acids and Ly alone or in combination. Shown are the combined data of 5 experiments. Where applicable, means are shown $\pm$ S.E.M. ($n = 3$ for Ly + DA and Ly + OA, for all others $n = 2$). Bu: buffer control. There is no statistically significant difference between Ly in combination with fatty acid and Ly or fatty acid alone. B: A representative SPR sensogram showing the immobilization of *P. aeruginosa* ($\sim 10^8$ bacteria), the blockage of active surface area by BSA, the treatment of 1.7 μ M Ly, and the final injection of 1 μ M DA. The arrows represent the injections of these species. C: Changes in SPR response upon exposure of DA followed by Ly (DA/Ly), Ly followed by DA (Ly/DA), oleic acid (OA) followed by Ly (OA/Ly), and Ly

followed by OA (Ly/OA); Bu: buffer control; RU: response units. The difference in response units at the end of each injection was calculated and shown are means $\pm$ S.E.M. of two experiments. * $p < 0.02$ for DA/Ly vs Ly/DA and $p < 0.01$ for OA/Ly vs Ly/OA for Tukey post-hoc comparisons in a one-factor ANOVA.

Figure 6. Calorimetric studies with bacterial model membranes support binding studies with live bacteria

Thermograms of large unilamellar vesicles of dipalmitoylphosphatidylglycerol (DPPG) with and without docosahexaenoic acid (DA) or oleic acid (OA) in the presence or absence of lysozyme (Ly) added in the order as indicated in the panels. DPPG was hydrated in buffer (10 mM NaPi, pH 7.3, 100 mM NaCl) above the main transition for one hour. Fatty acids and/or human lysozyme (Ly) were added to the lipid at a molar ratio of 500:1. The ratio of fatty acid to Ly was 1/50. Experiments were performed at a scan-rate of 30°C/hr. Shown are representative thermograms of two experiments measured in duplicates.

Table 1
Thermodynamic parameters of differential scanning calorimetric studies

Sample *	T _{pre} (°C)	ΔT _{1/2} (°C)	ΔH _{pre} (kcal/mole/°C)	T _m (°C)	ΔT _{1/2} (°C)	ΔH _m (kcal/mole/°C)
DPPG	33.8	3.4	1.05	40.7	1.3	12.8
DPPG + Ly	34.6	6.6	1.7	42.0	1.6	15.0
DPPG + DA	-	-	-	40.8	0.35	13.5
DPPG + DA + Ly ¹	34.0	5.0	1.2	40.7	1.4	14.9
DPPG + Ly + DA ¹	33.3	3.8	0.93	40.7	1.4	12.3
DPPG + Ly/DA ²	32.8	1.2	0.4	40.5	0.5	8.7
DPPG + OA	-	-	-	37.3	2.4	11.8
DPPG + OA + Ly ¹	-	-	-	34.2	4.3	10.0
DPPG + Ly + OA ¹	-	-	-	38.6	3.5	10.9
DPPG + Ly/OA, ²	34.1	1.4	0.5	40.1	0.9	9.6

DPPG: dipalmitoylphosphatidylglycerol; Ly: lysozyme; DA: docosahexaenoic acid; OA: oleic acid; T_{pre}: pre-transition; ΔT_{1/2}: transition width at half peak; ΔH_{pre}: enthalpy of pre- transition; T_m: melting transition; ΔT_{1/2}: ΔH_m: enthalpy of main transition.

* molar ratio of DPPG to additives 500:1;

¹ fatty and Ly applied in sequence;

² fatty acid and Ly applied mixed.