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Short communication

Formyl peptide receptor-mediated proinflammatory consequences of peptide deformylase inhibition in *Staphylococcus aureus*

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Abstract

The biosynthesis of proteins with N-terminal formylated methionine residues and subsequent protein deformylation are unique and invariant bacterial processes. They are exploited by the capacity of the human innate immune system to sense formylated peptides (FPs) and targeted by the deformylation-blocking antibiotic actinonin. We show that human polymorphonuclear leukocytes respond via the formyl peptide receptor (FPR) with increased calcium ion fluxes, chemotactic migration, IL-8 release, and CD11b upregulation to the human pathogen *Staphylococcus aureus* upon actinonin treatment. These data underscore the crucial role of bacterial FPs in innate immunity and indicate that deformylase inhibition may have considerable proinflammatory consequences.

Keywords: *Staphylococcus aureus*; Actinonin; Methionyl-tRNA formyltransferase; Peptide deformylase; Formyl peptide receptor; Inflammation; Chemotaxis; IL-8

Staphylococcus aureus is a leading cause of severe community and hospital-associated infections [1] and causes alarming outbreaks by new, highly antibiotic-resistant and virulent clonal lineages such as the emerging community-associated methicillin-resistant *S. aureus* (CA-MRSA) [2]. Abscesses and other local *S. aureus* infections, characterized by a massive influx of polymorphonuclear leukocytes (PMN), are often followed by life-threatening complications such as sepsis or endocarditis [3]. Consequently, *S. aureus* virulence depends on several factors with the capacity to limit neutrophil influx [4,5]. Leukocytes migrate to sites of infection and inflammation along gradients of stimuli, which are recognized by G protein-coupled receptors (GPCRs) [6]. These chemotaxis receptors respond to chemokines, lipid-derived stimuli such as leukotriens, or bacterial ligands the best studied of which are the formylated peptides (FPs) (e.g. fMLF) [7,8]. Bacterial FPs represent a hallmark of bacterial infections since only bacterial cells start protein biosynthesis with a formylated methionine, while the cytoplasmic ribosomes of human cells use an unmodified methionine residue. Accordingly, FPs have been shown to represent major chemotactic pathogen-associated molecular patterns (PAMPs), which are recognized by the GPCR formyl peptide receptor (FPR) [9,10]. However, it has also become clear that bacteria produce further chemotactic molecules, whose nature has remained elusive [9]. The phenol-soluble modulins (PSMs), a new class of peptide toxins produced by highly pathogenic *S. aureus* such as the CA-MRSA represent a new group of chemotactic PAMPs [11,12]. Moreover, there is evidence that *S. aureus* releases further, non-proteinaceous molecules that can attract leukocytes [9].

FPR plays a crucial role in mammalian innate immunity against bacterial infections [13] and many *S. aureus* strains produce a specific inhibitor of FPR, the chemotaxis-inhibitory

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protein of *S. aureus* (CHIPS) [14], which underscores the importance of FPR in staphylococcal infections and indicates that it may be advantageous for bacterial pathogens to suppress FPR activation [4].

The methionyl-tRNA formyltransferase gene *fmt* required for generation of formyl-Met tRNA can be inactivated in *S. aureus*. The resulting *fmt* mutants have been shown to be viable, albeit with severe growth and fitness defects [15]. While the correct function of some bacterial proteins appears to depend on the presence of an N-terminal formyl group, many others need to be deformylated to be active. Accordingly, the enzyme peptide deformylase (PDF) is essential for bacterial viability and is used as the major target for a group of antibiotics (e.g. actinonin), some of which are currently in clinical trials to combat infections caused by otherwise antibiotic-resistant bacteria such as CA-MRSA [16]. It has been tempting to assume that PDF inhibition may lead to increased release of FPs by bacteria and, consequently, to increased PMN influx and activation. Accordingly, actinonin-treated *Escherichia coli* have previously been shown to have an increased capacity to stimulate PMN [17].

In this study, we investigated the impact of actinonin treatment on *S. aureus*-mediated PMN responses. We used an *S. aureus fmt* mutant, which is incapable of producing FPs and an FPR-transfected cell line to demonstrate that actinonin treatment leads to a considerable increase in PMN-stimulating activity via FP-mediated FPR activation.

A recently described *S. aureus fmt* mutant (Δ*fmt*) [15], the corresponding wild-type strain RN4220 (WT) and Δ*fmt* complemented with a plasmid-encoded intact copy of *fmt* [9] were incubated with increasing amounts of actinonin to determine at which concentrations actinonin starts to inhibit the growth of *S. aureus*. Bacterial overnight cultures grown in tryptic soy broth (Fluka) were used to inoculate 25 ml of lipopolysaccharide-free Iscove's modified Dulbecco's medium (IMDM) without phenol red (Gibco) at an OD_{578} of 0.1, which were grown at 37 °C on a shaker for 8 h. Actinonin inhibited growth of WT and complemented mutant by about 20 and 50% at 1 μg/ml and 10 μg/ml, respectively (Fig. 1). Δ*fmt* was actinonin-resistant because PDF inhibition has no consequence in the absence of protein formylation as described before [15].

In order to investigate a potential impact of PDF inhibition on the leukocyte-attracting capacity of *S. aureus*, PMN were incubated with *S. aureus* culture supernatants and intracellular calcium ion fluxes usually occurring concomitantly with leukocyte migration were measured. Of note, *S. aureus* RN4220 does not contain the CHIPS gene and produces only trace amounts of PSM peptides [9]. Supernatants were obtained from bacteria grown overnight in 15 ml IMDM as described above in the presence or absence of 1 μg/ml actinonin. Then, the OD_{578} was adjusted to 1.0, bacteria were removed by centrifugation and supernatants were passed through a 0.22 μm-pore size sterile filter. PMN from healthy human volunteers were isolated by Ficoll/Histopaque (GE Healthcare/Sigma–Aldrich) centrifugation as described recently [9]. 5×10^6 PMN per ml were loaded with the

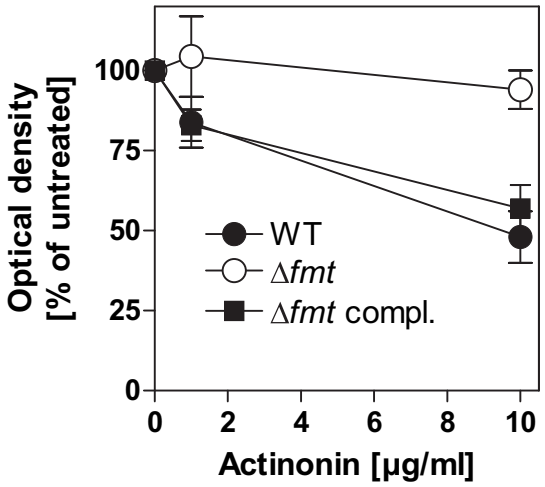

Fig. 1. Inhibition of growth of *S. aureus* strains by actinonin. The indicated strains were grown for 8 h in IMDM in the presence of increasing concentrations of actinonin and OD_{578} was measured. Data represent means ± SEM of three independent experiments.

calcium-sensitive dye Fluo-3-AM (Molecular Probes) at a final concentration of 2 μM for 20 min at room temperature in RPMI 1640 medium (Biochrom) supplemented with 0.05% human serum albumin (RPMI–HSA). Subsequently, PMN (10^6 cells/ml) were mixed with diluted staphylococcal culture supernatants (12.5% final concentration) or with the synthetic FP fMLF (10 nM final concentration; Sigma) in RPMI–HSA and calcium fluxes were monitored after 15 s of incubation in a FACScalibur (Beckton Dickinson) as described recently [9]. Actinonin itself did not stimulate PMN and it had no impact on the stimulating capacity of fMLF at the concentrations used for the preparation of the culture supernatants (1 μg/ml). However, the calcium flux-inducing activities of WT and complemented Δ*fmt* were significantly (1.8-fold) increased in actinonin-treated compared to non-treated cultures (Fig. 2A) indicating that PDF inhibition has in fact a profound impact on the ability of PMN to sense *S. aureus* molecules. In accord with this finding, Δ*fmt* supernatants induced only very week calcium fluxes, which were not increased in actinonin-treated cultures.

The consequences of PDF inhibition could also be observed when the chemotactic migration of PMN in response to supernatants was measured. Here, the supernatants from actinonin-treated WT led to a 1.7-fold and supernatants from the complemented Δ*fmt* to a 1.4-fold increase in PMN migration compared to supernatants from cultures without actinonin while the residual activities in Δ*fmt* supernatants remained unaffected by actinonin (Fig. 2B). In agreement with previous data PMN exhibited a substantial degree of unspecific migration in the absence of any stimulus [9]. Neither the unspecific nor the PAMP-induced migration was directly affected by actinonin. PMN chemotaxis was measured using trans-well inserts (Costar) as described recently [9]. In brief 0.5×10^6 PMN loaded with the fluorescent dye BCECF-AM (Molecular Probes) in 100 μl Hank's balanced salt solution containing 0.05% HSA (HBSS–HSA) were added to the upper compartments. 600 μl bacterial supernatants (6.25%),