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REVIEW

How innate immunity proteins kill bacteria and why they are not prone to resistance

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Abstract Recent advances on antibacterial activity of peptidoglycan recognition proteins (PGRPs) offer some insight into how innate immunity has retained its antimicrobial effectiveness for millions of years with no frequent emergence of resistant strains. First, PGRP can bind to multiple components of bacterial envelope (peptidoglycan, lipoteichoic acid, and lipopolysaccharide). Second, PGRP simultaneously induces oxidative, thiol, and metal stress responses in bacteria, which individually are bacteriostatic, but in combination are bactericidal. Third, PGRP induces oxidative, thiol, and metal stress responses in bacteria through three independent pathways. Fourth, antibacterial effects of PGRP are enhanced by other innate immune responses. Thus, emergence of PGRP resistance is prevented by bacteriostatic effect and independence of each PGRP-induced stress response, as PGRP resistance would require simultaneous acquisition of three separate mechanisms disabling the induction of all three stress responses. By contrast, each antibiotic has one primary target and one primary antibacterial mechanism, and for this reason resistance to antibiotics can be generated by inhibition of this primary mechanism. Manipulating bacterial metabolic responses can enhance bacterial killing by antibiotics and elimination of antibiotic-tolerant bacteria, but such manipulations do not overcome genetically encoded antibiotic resistance. Pathogens cause infections by evading, inhibiting, or subverting host immune responses.

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Introduction

Modern medicine, despite its extensive armamentarium of powerful antibiotics, faces a crisis of ever-growing number of antibiotic-resistant bacteria. Introduction of every new antibiotic is followed by quick emergence of resistant strains. By contrast, innate immunity, which offers germline-encoded immediate protection for the host from infections, has retained its antimicrobial effectiveness for millions of years with no frequent emergence of resistant strains. In fact, innate immunity is the only protection from infections in all invertebrates and plants, and it is still an essential component of immunity in vertebrates. Why is innate immunity less prone to microbial resistance than antibiotics, since they both target conserved essential prokaryotic components not found in eukaryotes? We will address this question using human peptidoglycan recognition proteins (PGRPs) as a model of antibacterial innate immunity proteins.

PGRP targets multiple conserved structures in bacteria

Humans and other mammals have four PGRP proteins, coded by *PGLYRP1–4* genes. Mammalian PGRPs are all soluble secreted proteins with both recognition and effector functions (Dziarski et al. 2016b; Royet and Dziarski 2007). PGLYRP1, PGLYRP3, and PGLYRP4 are directly bactericidal for both Gram-positive and Gram-negative bacteria (Lu et al. 2006; Tydell et al. 2002, 2006; Wang et al. 2007),

and PGLYRP2 is an enzyme, peptidoglycan amidohydrolase (Gelius et al. 2003; Wang et al. 2003).

Each PGRP has one or two PGRP domains with a binding site specific for muramyl-peptide fragment of bacterial peptidoglycan (Dziarski et al. 2016b; Royet and Dziarski 2007). In Gram-positive bacteria, PGRP preferentially binds to muramyl peptides exposed by peptidoglycan-lytic endopeptidases at the separation sites of the newly formed daughter cells (Kashyap et al. 2011). But in addition, mammalian PGRPs have other binding sites specific for bacterial lipoteichoic acid and lipopolysaccharide located outside the peptidoglycan-binding groove (Sharma et al. 2011; Tydell et al. 2006). Thus, PGRPs also bind uniformly to the entire outer membrane of Gram-negative bacteria (Kashyap et al. 2011).

Because each PGRP is so versatile and can bind multiple bacterial components, bacteria cannot easily change these multiple structures to avoid PGRP binding. This binding of PGRPs to bacteria induces exaggerated stress responses in bacteria and initiates a cascade of events that eventually results in bacterial killing (Kashyap et al. 2011, 2014, 2017).

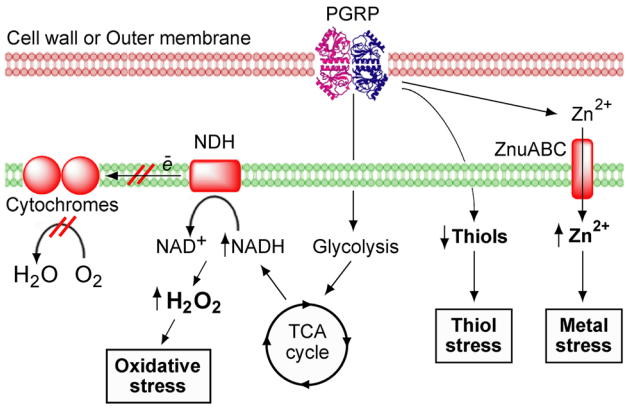

Fig. 1 PGRP induces oxidative, thiol, and metal stress through independent pathways, which individually are bacteriostatic and together become bactericidal. PGRP induces oxidative stress through a block in respiratory chain, which diverts electrons from respiratory chain NADH oxidoreductases to O_2 and generates H_2O_2. Production of H_2O_2 depends on increased supply of NADH from glycolysis and tricarboxylic acid (TCA) cycle. PGRP also induces thiol stress (depletion of thiols) and metal stress (increase in intracellular free Zn^{2+} through influx of extracellular Zn^{2+}), which are mostly independent of oxidative stress and of each other (Kashyap et al. 2014, 2017)

PGRP induces multiple synergistic antibacterial stress responses

PGRP kills bacteria by simultaneously inducing oxidative, thiol, and metal stress responses in bacteria (Fig. 1). PGRP-induced oxidative stress stems from increased production of hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\cdot}$) (Kashyap et al. 2011, 2014, 2017). PGRP-induced thiol (disulfide) stress stems from depletion of over 90% of intracellular thiols, and metal stress stems from increases in intracellular free Zn^{2+} and Cu^{+} (Kashyap et al. 2014, 2017). Induction of all three stress responses is required for PGRP-induced killing. Each stress response individually is only bacteriostatic, and only combined induction of all three stress responses is bactericidal (Kashyap et al. 2014).

PGRP induces oxidative, thiol, and metal stress through independent pathways

Resistance to PGRPs could easily arise if all three stress responses (i.e., oxidative, thiol, and metal stress) were induced by PGRP through a single pathway. However, PGRP induces oxidative, thiol, and metal stress through three mostly independent pathways (Kashyap et al. 2017). PGRP induces oxidative stress through an increase in central carbon catabolism and simultaneous block in electron transport through the respiratory chain, which results in premature diversion of electrons to oxygen and increased production of H_2O_2 (Fig. 1). PGRP-induced thiol stress is mostly independent of oxidative stress and metal stress, and may depend on the generation of endogenous electrophiles through a so far unidentified pathway. PGRP-induced metal stress is also independent of oxidative and thiol stress and depends on the increased influx of metals into bacterial cells (Fig. 1) (Kashyap et al. 2017).

Toxicity of oxidative stress is due to excessive production of superoxide anion (O_2^{-}) and H_2O_2, which oxidize and inactivate solvent-exposed [4Fe–4S] enzyme clusters and inactivate mononuclear iron enzymes by oxidizing Fe-coordinating cysteines or by replacing Fe^{2+} with Zn^{2+} (Anjem and Imlay 2012; Gu and Imlay 2013; Jang and Imlay 2007, 2010). H_2O_2 also reacts with Fe^{2+} and generates highly toxic $HO^{\cdot}$, which irreversibly damages DNA, proteins, and other organic molecules (Imlay 2013; Park et al. 2005). Toxicity of thiol stress is due to oxidation of thiols and loss of the redox balance and greater sensitivity to oxidative damage and metal toxicity (Chillappagari et al. 2010; Harrison et al. 2009; Leichert et al. 2003). Toxicity of metal stress depends on the metal involved. Zn and Cu toxicity is in part due to inactivation of solvent-exposed Fe–S clusters (Macomber and Imlay 2009; Xu and Imlay 2012). Zn also inactivates enzymes by replacing Fe^{2+} in their active sites (Chandrangsu and Helmann 2016; Gu and Imlay 2013), whereas Cu also increases thiol oxidation and sulfhydryl depletion, which magnify thiol stress and protein damage (Chillappagari et al. 2010; Harrison et al. 2009; Macomber and Imlay 2009).

Emergence of PGRP resistance is prevented by bacteriostatic effect and independence of each stress

Although disabling one of the PGRP-induced stress pathways greatly decreases or abolishes PGRP killing, PGRP still remains bacteriostatic. For this reason, full resistance to PGRP does not easily develop, because it would require simultaneous disabling of all three PGRP-induced independent stress responses, which is a very low probability event. Consistent with this notion, mutants fully resistant to antibacterial effects of PGRPs could not be found or generated, despite many efforts (Kashyap et al. 2017).

The initial events that follow PGRP binding to the cell wall in Gram-positive bacteria or to the outer membrane in Gram-negative bacteria and lead to the induction of oxidative, thiol, and metal stress are still not clear. Confocal microscopy indicates that PGRPs do not enter the cytoplasm and induce their lethal effects from their extracellular binding sites (Kashyap et al. 2011). It is not known whether PGRPs stay bound to the cell wall or to the outer membrane, or whether PGRPs also interact with the cytoplasmic membrane, because the resolution of these confocal experiments was not sufficient to make this distinction. However, PGRPs do not permeabilize the cytoplasmic membrane and do not induce osmotic lysis of bacteria (Kashyap et al. 2011; Lu et al. 2006; Wang et al. 2007). But independence of PGRP-induced oxidative, thiol, and metal stress of each other suggests that the initial PGRP-induced events that trigger these stress responses may also be different for each stress response and independent of each other.

Antibiotics and PGRPs have different mechanisms of action

The mechanisms of bacterial killing by PGRPs and antibiotics are different and PGRPs kill bacteria resistant to multiple antibiotics (Kashyap et al. 2014). Whereas PGRPs kill bacteria through simultaneous induction of multiple independent stress responses, each antibiotic has one primary target and one primary antibacterial mechanism. For this reason, resistance to antibiotics can be generated by inhibition of this primary mechanism. For example, antibiotics in each of the major groups, i.e., inhibitors of protein, RNA, DNA, or peptidoglycan synthesis, selectively and immediately inhibit the synthesis of only their respective target, whereas treatment with PGRP results in simultaneous inhibition of all biosynthetic reactions (Kashyap et al. 2011).

Much attention has been recently devoted to determining whether classical antibiotics kill bacteria through induction of oxidative stress or metabolic stress. However, the ability of antibiotics to induce oxidative stress in bacteria and the requirement for induction of oxidative stress for bacterial killing by antibiotics are still controversial (Brynildsen et al. 2013; Dwyer et al. 2014, 2015; Ezraty et al. 2013; Imlay 2015; Keren et al. 2013; Kohanski et al. 2007, 2008; Liu and Imlay 2013; Lobritz et al. 2015; Mahoney and Silhavy 2013). Manipulations of bacterial metabolism and enhancement of oxidative stress in bacteria can increase bacterial killing by antibiotics and help to eliminate antibiotic-tolerant bacteria (Belenky et al. 2015; Dwyer et al. 2014; Lobritz et al. 2015; Meylan et al. 2017). Consistent with this notion, bactericidal antibiotics increase the respiration rate in bacteria (Lobritz et al. 2015). By contrast, PGRPs induce a decrease in the respiration rate in bacteria due to a block in respiratory chain (Kashyap et al. 2017). Moreover, many effects of antibiotics on the metabolism and respiration most likely result from bacterial responses to the primary effects of antibiotics and happen late in antibiotic-induced killing (30–90 min) (Belenky et al. 2015; Kohanski et al. 2007). For this reason, metabolic manipulations do not overcome genetically encoded antibiotic resistance. By contrast, the kinetics of PGRP-induced changes in metabolism and respiration are much faster (5 min) and likely reflect the primary effects of PGRP on bacteria (Kashyap et al. 2017). There are also several additional differences between the mechanisms of bacterial killing by PGRPs and antibiotics (Kashyap et al. 2011, 2014, 2017).

PGRPs are bactericidal only under specific conditions

Natural antibiotics are produced by fungi or bacteria in the soil and have to be active in wide-ranging environmental conditions that the producing organisms cannot control. Therefore, antibacterial effects of antibiotics are generally independent of culture conditions, as long as bacteria can maintain vigorous growth, which is usually required for killing by bactericidal antibiotics. By contrast, PGRPs are produced in the host in very specific cells or tissues. PGLYRP1 is present in neutrophil, eosinophil, and macrophage granules (Dziarski et al. 2003; Liu et al. 2001; Tydell et al. 2002), PGLYRP2 is mostly present in the serum (Hoijer et al. 1996; Wang et al. 2003) and also in the skin, whereas PGLYRP3 and PGLYRP4 are produced on the skin and mucous membranes (especially in the moth and esophagus), and in sweat, sebum, and saliva (Liu et al. 2001; Lu et al. 2006).

Thus, PGRPs only need to be active under very specific conditions of these niches. These body fluids contain significant amounts of Zn and Cu, and PGRPs are only bactericidal in the media with very specific nutrient and ion composition that mimics body secretions or tissue fluids (Wang et al. 2007). The most important is the presence of Zn^{2+}, which is required for bactericidal activity of PGRPs

for both Gram-positive and Gram-negative bacteria (Wang et al. 2007), although for killing of Gram-positive bacteria (but not Gram-negative bacteria) Zn^{2+} can be substituted to some extent by other divalent cations, e.g., Ca^{2+} (Lu et al. 2006; Wang et al. 2007). PGRP killing also requires a precise type and amount of metabolic activity (Kashyap et al. 2017). For these reasons, PGRPs are usually not bactericidal, but can be bacteriostatic, in common laboratory media or buffers (Liu et al. 2000).

Antibacterial effects of PGRP are enhanced by other innate immune responses

The antibacterial effects of PGRPs are also enhanced by other innate immune responses of the host. For example, phagocytic cells, upon phagocytosis of bacteria, in addition to oxidative killing, pump Cu and Zn into phagolysosomes to enhance bacterial killing (Chandrangsu et al. 2017; German et al. 2013; Palmer and Skaar 2016). As already mentioned, PGRP-induced metal stress depends on the import of these extracellular cations (Kashyap et al. 2017). In response to PGRPs, bacteria up-regulate expression of Cu and Zn exporters (Kashyap et al. 2014). However, PGRPs defeat this bacterial defense by shutting down bacterial respiration and metabolism and depolarizing bacterial membranes (Kashyap et al. 2011, 2017), and thus depriving bacteria of energy and proton motive force needed to drive bacterial Cu and Zn efflux. PGRPs also kill bacteria synergistically with antimicrobial peptides, which are abundant in phagocytic granules and body secretions (Cho et al. 2005; Wang et al. 2007), where PGRPs are present. These synergistic effects with other host defenses further enhance antibacterial effectiveness of PGRPs and prevent development of resistance.

Pathogens evade innate immunity

If bacteria, including pathogens, do not easily develop resistance to PGRPs and other antibacterial innate immunity mechanisms, how do pathogens cause infections? Pathogens are successful in causing infections, because they developed an amazing variety of virulence factors that allow them to evade, inhibit, or otherwise subvert host immune responses (DiRita 2013; Olivos-García et al. 2016; Palmer and Skaar 2016; Reddick and Alto 2014). Greater sensitivity of *Pglyrp1*-deficient mice to infections with non-pathogenic, but not with pathogenic bacteria (which are both sensitive to PGRP killing in vitro) (Dziarski et al. 2003), suggests that in vivo pathogens can avoid antibacterial effects of PGRP. The ability of PGRPs to control the composition of microbiome also suggests differential antibacterial effects of PGRPs in vivo and differential in vivo sensitivity of various bacteria to PGRPs (Dziarski et al. 2016a, b; Royet et al. 2011). However, how pathogens evade PGRPs in vivo and how PGRPs differentially affect the composition of microbiome are not known and should be a fertile area for future studies.

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