NAGging Hexokinase PEPs up NLRP3

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Recognition of peptidoglycan is integral to detection of gram-positive bacterial pathogens. In a recent issue of *Cell*, Wolf et al. (2016) report that detection of the N-acetylglucosamine component of peptidoglycan by the glycolytic enzyme hexokinase activates the NLRP3 inflammasome, revealing an intriguing interplay between pathogen detection and metabolism.

Metabolism drives diverse biological processes including differentiation, proliferation, and effector function in diverse cell types. In particular, the process of mounting an innate immune response to pathogens entails rapid sensing of a wide range of patterns displayed by invading microorganisms coupled to synthesis of cytokines, chemokines, and antimicrobial molecules within the time frame of a few minutes to hours after stimulation. This rapid shift from a quiescent to a highly active state incurs considerable, energetically expensive, metabolic burden on the cell. Reprogramming the cellular metabolic state to meet this energetic demand has thus emerged as a key feature of innate immune activation. For instance, in response to TLR ligands, macrophages and dendritic cells exhibit a rapid shift from oxidative phosphorylation to glycolysis accompanied by decreased oxygen consumption, which allows cells to produce ATP under hypoxic conditions encountered at sites of inflammation (Krawczyk et al., 2010). This increased glycolytic rate is essential for maturation; inhibition of glycolysis severely impairs ROS production and microbicidal activity of macrophages. In contrast to TLRs, activation of intracellular NLRs resulting in assembly of a macromolecular signaling complex called the inflammasome that activates caspase-1, is believed to inhibit glycolysis. Active caspase-1 triggered in response to *S. typhimurium* infection or septic shock cleaves and inactivates multiple enzymes (aldolase, triose-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, α-enolase, and pyruvate kinase) in the glycolysis pathway (Shao et al., 2007), and disruption of host cell glycolysis by *S. typhimurium* activates the NLRP3 inflammasome (Sanman et al., 2016). Wolf et al. (2016) now demonstrate that hexokinase (HK), an enzyme that catalyzes the first step in glycolysis, can itself act a sensor for bacterial peptidoglycan (PGN) that has been internalized and degraded in macrophage phagosomes resulting in activation of the NLRP3 inflammasome.

PGN, a component of the bacterial cell wall, is a polymer composed of N-acetylmuramic acid and N-acetylglucosamine linked by short amino acid side chains. Previous work by Underhill and colleagues showed that production of bioactive IL-1β in response to the gram-positive bacterium *Stapylococcus aureus* was dependent on phagocytosis and lysosome-dependent degradation of the bacterial cell wall, and independent of NOD2 which senses the muramyl dipeptide component of PGN, indicating that other cell wall degradation products are sensed to activate the inflammasome (Shimada et al., 2010).

In an initial set of experiments, Wolf et al. (2016) show that stimulation of LPS-primed bone marrow-derived macrophages (BMDMs) with PGN results in the secretion of IL-1β in an NLRP3-dependent manner, suggesting that the NLRP3 inflammasome is activated by a component of PGN. Activation of the NLRP3 inflammasome in response to many chemically distinct activators including ATP, the pore forming toxin nigericin and several crystalline substances has been classically associated with potassium efflux and an inflammatory form of cell death called pyroptosis (Muñoz-Planillo et al., 2013). Interestingly, Wolf et al. (2016) found that NLRP3-dependent production of IL-1β in response to PGN is potassium independent and does not induce pyroptosis, indicating that PGN activates NLRP3 by a mechanism distinct from that of classical NLRP3 activators. To determine the component of PGN that is responsible for activating NLRP3, Wolf et al. (2016) examined the inflammasome activating capacity of potential PGN degradation products by transfecting molecular subunits of PGN into BMDM and found that N-acetylglucosamine (NAG) was the minimal component of PGN that could induce NLRP3-dependent production of IL-1β (Figure 1). By stimulating cells with PGN from *Bacillus anthracis*, which is unique in that a majority of its NAG is deacetylated to glucosamine, Wolf et al. (2016) elegantly demonstrate that acetylation of NAG is necessary for its inflammasome activating potential. De-acetylated PGN from *Bacillus anthracis* induced substantially less IL-1β from BMDM and this defect could be rescued by its re-actylation. Consistent with this observation, de-acetylated anthrax PGN showed diminished neutrophil recruitment in an in vivo model of PGN-induced peritonitis, which could be rescued by its re-acetylation, demonstrating that acetylation of PGN is relevant to inflammatory responses in vivo.

Wolf et al. (2016) then proceed to interrogate the mechanism by which NAG activates the NLRP3 inflammasome. When examining potential triggers previously implicated in NLRP3 activation, they found that both PGN and NAG trigger appearance of mitochondrial DNA (mtDNA) in the cytosol, which led them to investigate the role of mitochondria in NAG induced NLRP3 activation. Early investigations into the glycolytic pathway showed that NAG could inhibit glycolysis (Spiro, 1958). Further studies demonstrated that by binding to its active

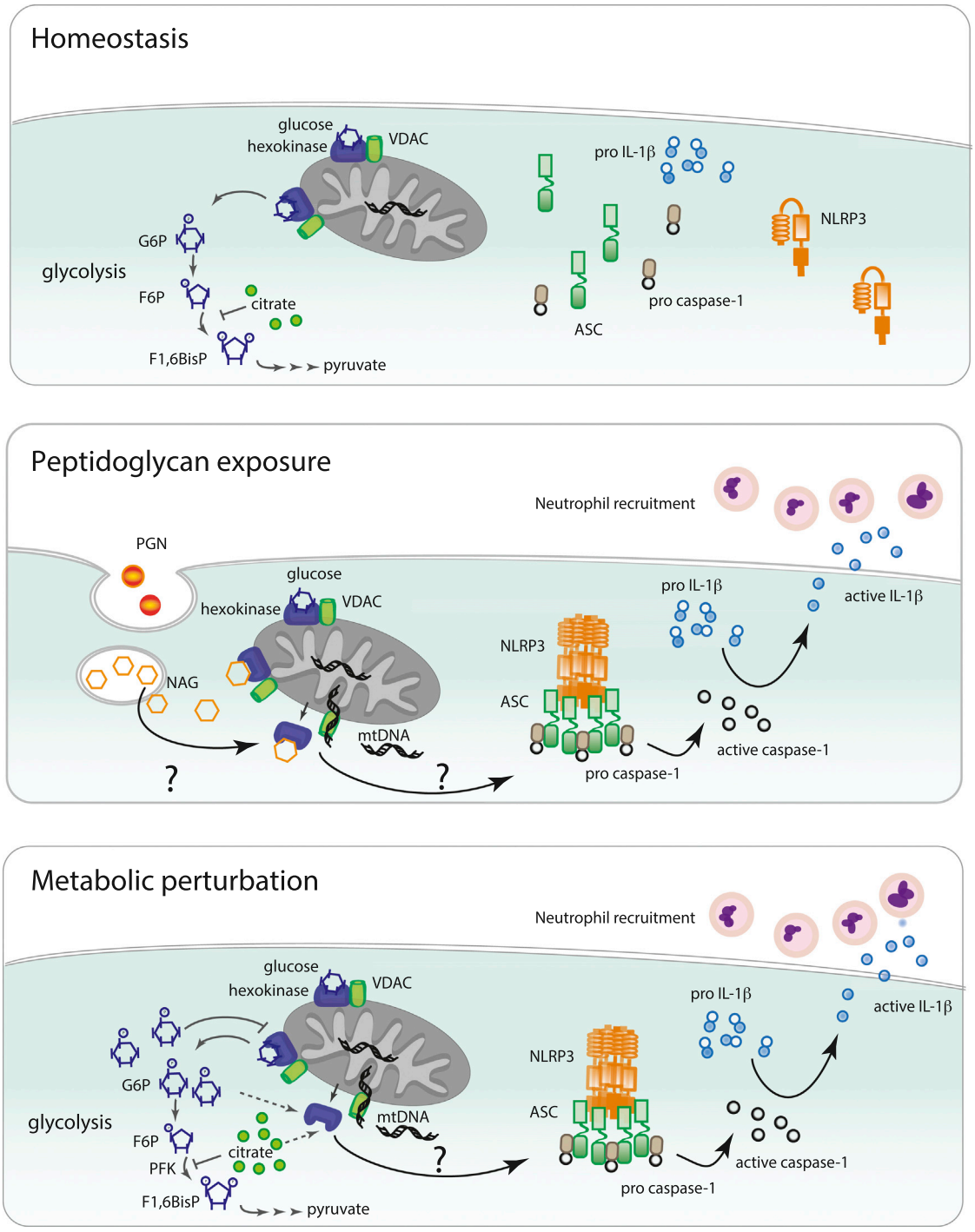

Figure 1. Inhibition of Hexokinase by NAG or Metabolic Intermediates Activates the NLRP3 Inflammasome
Under homeostatic conditions (top), hexokinase is tethered to the mitochondrial membrane. Phagocytosis of peptidoglycan (middle) is accompanied by its degradation in lysosomes resulting in release of N-acetylglucosamine (NAG) into the cytosol by an undefined mechanism. NAG binds and inhibits mitochondria-localized hexokinase resulting in its dissociation from the mitochondrial membrane. Detachment of hexokinase from the mitochondria is associated with release of mitochondrial DNA into the cytoplasm and eventual activation of the NLRP3 inflammasome resulting in production of active IL-1β. Glycolytic disruptions that inhibit hexokinase (bottom) such as high concentrations of glucose 6-phosphate and citrate, mimic the effect of NAG in inducing hexokinase dissociation from mitochondria resulting in NLRP3 inflammasome activation. G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F1,6BisP, fructose 1,6-bisphosphate; mtDNA, mitochondrial DNA; NAG, N-acetylglucosamine; VDAC, voltage-dependent anion channel; PFK, phosphofructokinase.

site, NAG competitively inhibits HK, which converts glucose to glucose 6-phosphate in the first step of glycolysis (Bertoni and Weintraub, 1984). Wolf et al. (2016) confirm the ability of NAG to inhibit HK by showing that glucose but not NAG is phosphorylated by HK. Because unacetylated NAG can be phosphorylated by HK, it lacks the inhibitory properties of acetylated NAG. By employing thermal shift technology that identifies sugar binding to a protein by modifying its thermal stability, they further show that NAG directly binds HK. Of the different isoforms of HK, hexokinase 2 (HK2) is normally tethered to the mitochondrial membrane through an interaction with voltage-dependent anion channels (VDACs), which are integral components of the mitochondrial permeability transition pore. HK has previously been identified as an inhibitor of VDAC channel opening, and dissociation of HK from VDAC leads to mitochondrial swelling, cytochrome *c* release, DNA laddering, and apoptotic cell death (Azoulay-Zohar et al., 2004). Previous work has also shown that ablating VDAC expression by shRNA inhibits NLRP3-dependent IL-1β production (Zhou et al., 2011). Thus, Wolf et al. (2016) hypothesized that HK-VDAC association may prevent release of mitochondrial danger signals into the cytosol through VDAC, and NAG may induce NLRP3 activation by disrupting HK association with mitochondria (Figure 1). To test this hypothesis, they examined the ability of NAG to induce HK2 dissociation from mitochondria and convincingly demonstrate that both PGN and NAG induce specific accumulation of HK protein as well as increased HK enzyme activity in the cytosol, which was not a result of a general loss of mitochondrial integrity. HK release into the cytosol was observed upon phagocytosis of a variety of gram-positive bacteria, and importantly, also in NLRP3-deficient macrophages indicating that it is a proximal event in inflammasome activation. Remarkably, by using a specific, cell-permeable peptide (HKVBD) previously known to induce dissociation of HK2 from mitochondrial VDAC, Wolf et al. (2016) demonstrate that forced dissociation of HK2 from mitochondria is sufficient to induce NLRP3-dependent production of active IL-1β, IL-18, and caspase-1 dependent neutrophil recruitment in vivo.

As the first enzyme in the glycolytic pathway, HK can be inhibited by intermediates of both the glycolysis and TCA cycle. Wolf et al. (2016) hypothesized that metabolic disruptions converging on HK inhibition may induce NLRP3 activation and offer insight into how metabolic perturbation impinges on inflammasome activation. Notably, transfection of LPS-primed BMDM with increasing amounts of glucose 6-phosphate (that inhibits HK by feedback inhibition), 2-deoxyglucose (a glycolysis inhibitor), or citrate (an intermediate in the TCA cycle that inhibits the rate-limiting glycolytic enzyme phosphofructokinase, resulting in elevated glucose 6-phosphate levels) mimics the effect of NAG and PGN in inducing NLRP3 inflammasome activation (Figure 1), suggesting an intriguing relationship between metabolism and inflammasome activation.

How HK release from mitochondria activates NLRP3 remains unknown. Wolf et al. (2016) speculate that release of mtDNA may constitute one such trigger. It is tempting to speculate that impairment of glycolysis creates a metabolic state permissible to NLRP3 activation, perhaps a pro-oxidant condition that promotes oxidative damage, another trigger previously linked to NLRP3 activation. Mitochondrial regulation of the NLRP3 inflammasome remains a complex puzzle. Nonetheless, the findings of Wolf et al. (2016) have major ramifications for metabolic diseases like type 2 diabetes, atherosclerosis, and obesity in which NLRP3 is implicated. Subject to future investigations into the role of HK and other glycolytic enzymes in the inflammasome response to different NLRP3 activators, their findings may raise the possibility of exploring the development of small molecules that restore glycolytic flux for therapeutic intervention in diverse autoinflammatory, autoimmune, infectious, and metabolic diseases linked to the NLRP3 inflammasome.

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