Mechanisms Involved in Lipopolysaccharide Derived ROS and RNS Oxidative Stress and Septic Shock

Gopala Kallapura¹, Neil R. Pumford¹, Xochitl Hernandez-Velasco ², Billy M. Hargis¹ and Guillermo Tellez¹

¹Department of Poultry Science, University of Arkansas, Fayetteville, AR, USA.
²Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autonoma de Mexico, 04510, Mexico.

E-mail for Correspondence: gtellez@uark.edu

Abstract

Host organisms detect the presence of infection by recognizing specific elements on pathogens that are not found in higher eukaryotic hosts. These elements are generally known as pathogen-associated molecular patterns (PAMPs). The endotoxin lipopolysaccharide (LPS) is a major component of the outer membranes of Gram-negative bacteria and a chief member of the PAMPs, which is largely responsible for most of the described toxic inflammatory reactions through reactive oxygen and nitrogen species. To understand the mechanistic nature of these redox molecules and their diverse immune functions, it is important to understand the type of chemical reactions that produces those outcomes and the relative time frame involved in this mode of defense. Here we review the major roles played by NADPH – Oxidases (NOX), CD14 and TLR4 in the initial recognition and initiation of inflammatory responses, followed by cascade of immune signals leading to generation of proinflammatory NF-kB. This LPS interaction with immune cells capable of producing reactive species is certainly beneficial. However, continued exposure of high doses of LPS that trigger prolonged production of inflammatory mediators, might lead to a deleterious condition termed oxidative stress. The excessive production of these radicals, associated with inflammation is a major contributing factor to the high mortality rates associated with several diseases and can sometimes potentially lead to a lethal systemic disorder called LPS toxicity induced septic shock. We discuss the importance of understanding the NF-kB system in detail, in particular the roles and interactions of individual effectors and modifiers of the activation cascade will be critical for the development of anti-endotoxin therapies.

Key words: Lipopolysaccharide, ROS, RN, septic shock, inflammation

INTRODUCTION

Host organisms detect the presence of infection by recognizing specific elements on pathogens that are not found in higher eukaryotic hosts. These elements are generally known as pathogen-associated molecular patterns (PAMPs) (Jabaut and Ckless, 2012). They have essential roles in the biology of the pathogen and therefore, are not subject to high mutation rates and hence preserved over generations. They include diverse bacterial cell wall components, such as lipopeptides, peptidoglycans and teichoic acids. The endotoxin lipopolysaccharide (LPS) is a major component of the outer membranes of Gram-negative bacteria and a chief member of the PAMPs. LPS is a relatively a large polysaccharide, in association with a core oligosaccharide and a highly conserved lipid-A region, which is largely responsible for most of the described toxic proinflammatory properties of LPS (Maripandi and Al-Salamah, 2010; Victor et al., 2004).

Primarily, LPS induces a receptor mediated signaling cascade resulting in nuclear factor-kB (NF-kB) activation and the transcription and subsequent release of cytokines and other proinflammatory mediators, by various polymorphonuclear
immune cells like monocytes and macrophages (Jabaut and Ckless, 2012; Li and Verma, 2002; Adams and Hamilton, 1984; Thannickal and Fanburg, 2000). The intracellular signaling events that proceed towards the activation of NF-kB are largely complex, both in terms of induction and the leading beneficial or detrimental consequences (Victor et al., 2004; Hensley et al., 2000).

Most of the polymorphonuclear cells, macrophages being at the forefront, combat pathogens containing variety of PAMPs, primarily by production of reactive redox species, via induction of the NADPH oxidase system (Robinson, 2008). These redox molecules are highly reactive low molecular weight lipophilic species, which can easily penetrate the microbial cell wall and inflict irreversible damage. The stimulating innate immune signal not only initiate the production of these redox molecules to intercept and kill pathogens, but lead to an extended expression of the immune responses, which in turn are modulated via complex downstream signaling pathways (Jabaut and Ckless, 2012; Pourova et al., 2010). Further, these molecules have the unique ability to serve a dual role for the host, both as immuno-modulators and, when overboard, unfortunately as immunotoxins. Overall, due to the diverse nature of this biological redox chemistry, in addition to the resulting complexity of immune functions, comprehension of the role of these redox molecules as key mediators of immunity has recently gathered renewed interest (Pourova et al., 2010).

To understand the mechanistic nature of these redox molecules and their diverse immune functions, it is important to understand the type of chemical reactions that produces those outcomes and the relative time frame involved in this mode of defense. In general, there are two categories of redox chemistry involved, ROS (Reactive Oxygen Species) being the starting molecule (Thannickal and Fanburg, 2000) and RNS (Reactive Nitrogen Species), made from the interaction of Nitric Oxide (NO), the prime reactive nitrogen molecule, with ROS (Wink et al., 2011; MacMicking et al., 1997). NO/RNS and ROS are either employed individually or in combination, to elicit immune responses and carry out immune regulation. If ROS is primarily used to target extracellular pathogens during phagocytosis or pathogens that are too large for phagocytose, RNS mainly target the intracellular/phagocytosed pathogens, and some extracellular pathogens and tumor cells upon appropriate stimulation (Jabaut and Ckless, 2012; Wink et al., 2011).

**Reactive Oxygen Species (ROS)**

The first category of biological redox system involved, in context to the above mentioned defense time line, is comprised of $O_2^-$, $H_2O_2$, and other reactive oxygen radicals, collectively known as ROS (Thannickal and Fanburg, 2000; Robinson, 2008). These species can function independently to carry out specific oxidation events that functionally activate intracellular signaling pathways resulting in regulation of migratory signals and cell proliferation with the ultimate objective of host defense. The primary cellular sources of ROS are the oxidases that generate $O_2^-$ by the transfer of a single electron to oxygen from NADPH (reduced form) (Robinson, 2008). Further single electron reduction to $H_2O_2$ or other ROS is catalyzed by a series of enzymes that includes super oxide dismutase (SOD) and myeloperoxidase (MPO) involving complex interactions with supporting transition metals (Pourova et al., 2010; Vanlaere and Libert, 2009; Schroder et al., 2004).

The reactive oxygen species (ROS) are generated during normal cellular metabolism with the respiratory chain in mitochondria being the major source. These ROS include superoxide anion ($O_2^-$), hydroxyl (OH-) peroxyl (ROO-) and alkoxyl (RO) radicals and certain non-radicals that are either oxidizing agents and easily converted into radicals, such as hydrogen peroxide ($H_2O_2$). They are very unstable, as they possess one or more unpaired electrons, which make these species highly reactive. ROS can easily react with and damage macromolecules including lipids, proteins and DNA (Robinson, 2008; Schroder et al., 2004; Nathan and Shiloh, 2000).

The NADPH – Oxidases (NOX) were initially recognized as an early participant in the initiation innate immune response and were later found to be part of phagocytic cells (hence belongs to Phox – Phagocytic Oxidase group of protein complexes) (Thannickal and Fanburg, 2000; Afanas’ev, 2011; Zhao et al., 2013; Sumimoto, 2008). It is now known that NOX involved mechanisms rapidly generate high level of superoxides when phagocytes are exposed to bacteria, which fits the previously coined terms called the “Respiratory Burst” or the “Oxidative Burst” (Robinson, 2008). In order to generate this burst of superoxides, there is an inherent requirement of the pathogen or its components to be presented to the phagocytes through a heteromeric assembly of multiple protein components. On the extracellular domain, the activation of the NOX is initiated by the direct interaction of NOX protein complex with immunogens or with complement or FcRs of the antibodies, which have marked the pathogens for destruction. This protein complex is preferentially membrane-bound and made of cytochrome b558 complex, which in turn is a conglomerate of gp91phox and p22phox catalytic subunits and four cytosolic proteins (p47phox, p67phox, p40phox, and GTPase Rac1) (Robinson, 2008; Sumimoto, 2008). The above mentioned interaction on the extracellular domain stimulates the cell resulting in the cytosolic proteins to assemble into a complex and translocate to the membrane, joining with the gp91phox-p22phox complex. This step results in full activation of the NOX enzyme complex and the production of $O_2^-$ (Thannickal and Fanburg, 2000; Afanas’ev, 2011; Sumimoto, 2008).
NOX is also demonstrated to be stimulated by treatment with agents that activate calcium entry or by treatment with cellular products such as arachidonic acid. Exposure of these cells to other agents, such as IFN-γ, TNF-α or IL-1β has shown to significantly increase the levels of O$_2^·$ produced during the immune response and are aptly called as the “priming” agents (Schroder et al., 2004).

**Reactive Nitrogen Species (RNS)**

Reactive nitrogen species (RNS) is a collective name that includes nitric oxide radical (NO$^·$), peroxynitrite (ONOO$^·$), nitrogen dioxide radical (NOO$^·$), other oxides of nitrogen and products arising when NO reacts with O$_2$, RO$^·$ and ROO$^·$ (MacMicking et al., 1997; Nathan and Shiloh 2000; Zhao et al., 2013). The primary source of NO is the NOS (nitric oxide synthases) enzyme, with three isoforms; two constitutively expressed Endothelial NOS – eNOS (NOS3) and Neuronal NOS - nNOS (NOS1), and one is inducible (iNOS) (Wink et al., 2011; MacMicking et al., 1997). eNOSs are constitutively expressed and in general, release short bursts of NO in a calcium-dependent manner upon stimulation. More recent studies have found that post-transcriptional modification of eNOS can convert these short, calcium-dependent NO bursts to sustained, calcium-independent production of NO. In the case of eNOS, these post-transcriptional regulations play a fundamental role in the health of endothelium during angiogenesis, as well as regulation of immune activation.

On the other hand, the iNOS (NOS2) is generally associated with the cells of innate immune system and produce NO for prolonged periods of time in a calcium independent manner, upon appropriate stimulation. Levels of NO produced by iNOS in their microenvironment can range from as low as 10 nM to high molar amounts and for extended periods of time. It is through producing high levels of NO, iNOS orchestrates various microenvironments that can be modulated within a tissue, with potentially different functions. However, the induction of iNOS is not solely defined by production of high, local amounts of NO, but the iNOS activity is shown to generate a wide range of levels of NO for variable periods of time and functions (Wink et al., 2011; MacMicking et al., 1997).

**LPS-induced signaling pathway**

LPS activates transcription, production and subsequent release of various proinflammatory cytokines, including TNF-α, through a receptor mediated signaling pathway/cascade. The first step in the LPS-associated signal transduction cascade leading to NF-κB activation and subsequent proinflammatory gene transcription is the recognition of the LPS molecule by specific cells of the immune system. LPS binds to a serum protein known as LPS binding protein (LBP). LBP is essential for the rapid induction of an inflammatory response by small amounts of LPS or Gram-negative bacteria. Once LPS is bound to LBP, the complex LPS-LBP activates different populations of cells by binding to its receptor (Thannickal and Fanburg, 2000).

Multiple cell bound receptors for endotoxins, in specific LPS, have been identified over the last decade, including β2-integrins, CD11/CD18, the macrophage scavenger receptor for acetylated LDL, L-selectin and CD14. Of these CD14 has consistently been studied and recognized as the major receptor involved, with two different forms: membrane CD14 (mCD14) and soluble CD14 (sCD14). The former mCD14, as the name indicates, is preferentially found on the surface of cells from myeloid lineage, functioning as glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein. In contrast, the latter lacks GPI properties, but can bind LPS to activate cells which lack mCD14 (e.g. endothelial cells). Evidence indicates that CD14 functions solely as a ligand-binding protein for LPS, or ore so to LPS-LBP complex and does not involve in directly transducing intracellular signaling. CD14 has also been implicated in cell activation processes involving other products of microbial pathogens, including lipoarabinomannans, peptidoglycans and outer membrane lipoproteins (Li and Verma, 2002; Schroder et al., 2004; Cadenas and Cadenas, 2002; Park et al., 2004; Möller et al., 2012).

Recent studies have elucidated major involvements of Toll Like Receptors (TLRs) involved in the activation of the proinflammatory signal transduction pathway which occur following the binding of the LPS-LBP complex to the GPI-anchored mCD14 with. TLRs are a family of receptors homolog to the Drosophila antifungal protein Toll that recognizes PAMPs (Schroder et al., 2004). 10 members have been identified and characterized so far and shown to be involved in innate immunity and inflammation responses. These TLR characterizations have reflected that different TLRs recognize different PAMPs, reflecting the fact that the extracellular domains of this Toll family of receptors are quite divergent. While the cytoplasmic domains have shown close similarities to the cytoplasmic portion of the interleukin 1 (IL-1) receptor and, therefore, are sometimes described as Toll/IL-1 receptor homologous region. This domain is largely responsible for cytosolic signal transduction (Li and Verma, 2002; Schroder et al., 2004; Möller et al., 2012).

Among 10 members of TLR, it has been demonstrated through studies that TLR4 is the major receptor involved (Park et al., 2004; Lee et al., 2012) in the activation of innate immune responses to Gram-negative bacteria, which is actually
the major source of LPS. There is enough evidence to show the direct involvement of TLR4 in recognizing LPS and the process by which LBP, CD14 and TLR4 interact concertedly to initiate and transduce immune signals, has been understood better than ever (Lee et al., 2012).

Studies have indicated that CD14 acts as an LPS-LBP binding fraction, on the surface of most monocytic cells. This complex LPS-CD14 physically reaches the adjacent TLR4 to transduce the LPS signal via the myeloid differentiation protein MyD88. If MyD88 serves as an intracellular adapter molecule, other proteins thought to be recruited during TLR ligation are MyD88-adapter-like (MAL), TNF-receptor-associated factor 6 (TRAF6) and the IL-1 receptor-associated (serine/threonine) kinase, known as IRAK. The transcription factor NF-kB mediates LPS-induced release of a number of inflammatory mediators (Jabaut and Ckless, 2012; Li and Verma, 2002; Adams and Hamilton, 1984; Park et al., 2004) and cytokines including TNF-α and IL-12. The activity of NF-kB is regulated by an inhibitor IκB. Activation of IκB kinases (IKK) leads to phosphorylation of IκB which, in turn, culminates in its proteolytic degradation and the translocation of NF-kB to the nucleus, where it associates with specific DNA binding sites initiating gene transcription (Jabaut and Ckless, 2012; Li and Verma, 2002; Thannickal and Fanburg, 2000;16, Lee et al., 2012).

The transcription factor NF-kB mediates LPS-induced release of a number of inflammatory mediators and cytokines including TNF-α and IL-12. The activity of NF-kB is regulated by an inhibitor IκB. Activation of IκB kinases (IKK) leads to phosphorylation of IκB which, in turn, culminates in its proteolytic degradation and the translocation of NF-kB to the nucleus, where it associates with specific DNA binding sites initiating gene transcription. The activated NF-kB is a heterodimer consisting of a 50 kDa (NF-kB1/p50) and a 65 kDa (RelA/p65) polypeptide. It induces the expression of mRNA of a variety of pro-inflammatory mediators including TNF-α, ILs (Interleukins), adhesion molecules and enzymes, such as cyclooxygenase 2 (COX-2) and iNOS, which are implicated in pathogenesis (Adams and Hamilton, 1984; Zhao et al., 2013; Lee et al., 2012). TNF-α stimulates leukocytes and vascular endothelial cells to release other cytokines (as well as additional TNF-α), to express cell-surface adhesion molecules and to increase arachidonic acid turnover. However, the unregulated release of TNF-α into the circulation results in circulatory dysfunction, increased endothelial permeability and inflammation of different organs (Hensley et al., 2000).

**Oxidative Stress and Septic Shock**

LPS interaction with myriad of immune cells capable of producing these reactive species, can be beneficial or detrimental to the host. Macrophages, the major producers of ROS and RNS, sense and get activated to eliminate bacterial infection through LPS recognition, a mechanism certainly required and advantageous to the host (Adams and Hamilton, 1984; Libby, 2007). However, continued exposure of high doses of LPS that trigger prolonged production of inflammatory mediators, might lead to a deleterious condition termed oxidative stress (Victor et al., 2004; Hensley et al., 2000), due to excessive production of these radicals, associated with inflammation. Oxidative stress is a major contributing factor to the high mortality rates associated with several diseases and can sometimes potentially lead to a lethal systemic disorder, LPS toxicity induced septic shock (Victor et al., 2004; Vanlaere and Libert, 2009; Cadenas and Cadenas, 2002; Aggarwal et al., 2012).

ROS are thought to be involved in the mechanism of LPS toxicity, in particular in NF-kB activation. There is a fine balance between ROS generation and antioxidant defenses in cells. When ROS generation overcomes the cellular antioxidants, oxidative stress is produced. Although ROS are generally considered to have beneficial functions for the individual, they are also known as be the toxic by-products of mitochondrial respiration, which can harm the host, even fatally (Vanlaere and Libert, 2009).

The transcriptional regulatory factor NF-kB is a central participant in modulating the expression of many of the immunoregulatory mediators involved in oxidative stress, and therefore in sepsis (Li and Verma, 2002). Numerous observations suggest that NF-kB activation is at least facilitated by some oxidation reactions and if continued uncontrollably can cause septic shock (Adams and Hamilton, 1984; Cadenas and Cadenas, 2002; Aggarwal et al., 2012).

Continuous oxidative damage may lead to cytotoxicity and the subsequent development of pathological conditions, such as degenerative diseases and cancer. The common mechanism by which tissues are damaged by the septic response is probably related to widespread vascular endothelial injury by hypotension and widespread vasodilation followed by microthrombosis. These, in turn, decrease oxygen and substrate supply to the tissues leading to anaerobic metabolism (Möller et al., 2012; Aggarwal et al., 2012). Glycolysis increases its rate and clearance of the resulting lactate and pyruvate by the liver and kidneys is impaired. As a consequence, blood lactate levels rise. Tissue hypoxia generates more lactic acid as hypoperfusion develops, contributing to metabolic acidosis. When regulatory control mechanisms are overwhelmed, homeostasis may fail and dysfunction of major organs may occur (Möller et al., 2012). Further regulatory imbalance leads to septic shock, which is characterized by hypotension and vascular collapse, with failure of the major organs within the body, including the heart, kidneys, lungs, liver, central nervous system and
coagulation system. The consequences are myocardial dysfunction (Afanas’ev, 2011; Cadenas and Cadenas, 2002; Libby, 2007; Aggarwal et al., 2012).

Aerobic organisms have developed an array of defense mechanisms against ROS damage. These consist of enzymatic and non-enzymatic defenses. The NF-κB pathway represents an attractive therapeutic target in strategies for controlling acute inflammation and septic shock (Li and Verma, 2002). However, it is important to recognize that although oxidation can lead to toxicity, the redox-based molecules are essential mediators of critical functions in physiological systems and are essential to immunity against disease. Hence, understanding the NF-κB system in detail, in particular the roles and interactions of individual effectors and modifiers of the activation cascade will be critical for the development of anti-endotoxin therapies for the medical treatment of septic shock (Victor et al., 2004; Hensley et al., 2000; Afanas’ev, 2011; Vane and Botting, 1987). In particular, vitamins are ideal antioxidants for increasing tissue protection from oxidative stress in humans due to their easy, effective and safe dietary administration in a large range of concentrations without harmful side effects. It is likely that the most efficient antioxidant supplementation for incidences of shock is that which includes combinations of antioxidants with known synergistic actions (Afanas’ev, 2011; Aggarwal et al., 2012). However, the nature, dose and time of intervention with antioxidants deserve further investigation.

REFERENCES


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