Cloning and expression of bovine p47-phox and p67-phox
by Peggy Lee Ohmstede Bunger

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Molecular Biology
Montana State University

Abstract:
Neutrophils play an essential role in bovine cellular host defense, and compromised leukocyte function has been linked to the development of respiratory and mucosal infections. During the host defense process, neutrophils migrate into infected tissues where they become activated, resulting in the assembly of neutrophil membrane and cytosolic proteins to form a superoxide anion-generating complex known as the NADPH oxidase. Two of the essential cytosolic components of the NADPH oxidase are p47-phox and p67-phox. Currently, only the human and murine homologues of these proteins have been sequenced. Because of the important role neutrophils play in bovine host defense, I carried out studies to clone, sequence, and express bovine p47-phox and p67-phox. Using PCR cloning techniques and a bovine bone marrow cDNA library, I cloned both of these bovine NADPH oxidase cytosolic components. Comparison of the bovine sequences with those of the human and murine homologues showed that they were highly conserved, but also revealed important information regarding key structural features of p47-phox and p67-phox, including location of putative phosphorylation sites. Functional expression of bovine p67-phox and p67-phox showed that these proteins could substitute for the human proteins in reconstituting NADPH oxidase activity in a cell-free assay system, again demonstrating the high degree of conservation between human and bovine homologues. This study greatly contributes to our understanding of the potential structural/functional regions of p47-phox and p67-phox as well as gives us information that can be used to study the role of neutrophils in bovine inflammatory diseases.

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Neutrophils play an essential role in bovine cellular host defense, and compromised leukocyte function has been linked to the development of respiratory and mucosal infections. During the host defense process, neutrophils migrate into infected tissues where they become activated, resulting in the assembly of neutrophil membrane and cytosolic proteins to form a superoxide anion-generating complex known as the NADPH oxidase. Two of the essential cytosolic components of the NADPH oxidase are p47-*phox* and p67-*phox*. Currently, only the human and murine homologues of these proteins have been sequenced. Because of the important role neutrophils play in bovine host defense, I carried out studies to clone, sequence, and express bovine p47-*phox* and p67-*phox*. Using PCR cloning techniques and a bovine bone marrow cDNA library, I cloned both of these bovine NADPH oxidase cytosolic components. Comparison of the bovine sequences with those of the human and murine homologues showed that they were highly conserved, but also revealed important information regarding key structural features of p47-*phox* and p67-*phox*, including location of putative phosphorylation sites. Functional expression of bovine p47-*phox* and p67-*phox* showed that these proteins could substitute for the human proteins in reconstituting NADPH oxidase activity in a cell-free assay system, again demonstrating the high degree of conservation between human and bovine homologues. This study greatly contributes to our understanding of the potential structural/functional regions of p47-*phox* and p67-*phox* as well as gives us information that can be used to study the role of neutrophils in bovine inflammatory diseases.

INTRODUCTION

Neutrophils play an essential role in host defense against bacterial and fungal pathogens (1). These inflammatory cells also play an important role (2) in serious inflammatory diseases, such as arthritis and adult respiratory distress syndrome (3,4). A key component in host defense is the NADPH oxidase system, which becomes activated when neutrophils contact and ingest foreign pathogens. The activated NADPH oxidase generates large quantities of superoxide anion (O_2^-) through reduction of molecular oxygen by NADPH-derived electrons (5). In addition, O_2^- is subsequently converted to other microbicidal products, such as H_2O_2 and HOCl. The NADPH oxidase has been most extensively studied in human neutrophils; however, the mammalian species investigated to date have also been shown to produce proteins corresponding to the membrane-bound proteins gp91-*phox* and p22-*phox* (6-10) and the cytosolic proteins p47-*phox* (10-16), p67-*phox* (10,14,16-18) and p40-*phox* (18,19). While conservation of these essential proteins would be expected, they have been characterized only in a few species (6-9,11-19).

Importance of Neutrophils in Host Defense

Across species, neutrophils play an important role in host defense, and a number of disease states have been associated with defective neutrophil function. In cattle, as in humans, these diseases are characterized by enhanced susceptibility to infections. For example, a genetic defect in CD18 (the common β-subunit of all β_2 integrins) is the cause of bovine leukocyte adhesion deficiency (BLAD) and its human counterpart, leukocyte

adhesion deficiency (LAD) (20). This defect severely impairs leukocyte adhesive functions, and neutrophils from animals with BLAD or humans with LAD fail to adhere to the vascular wall and therefore, cannot migrate into the tissue (20). An analog of human Chediak-Higashi syndrome, characterized by abnormal pigmentation, atypical azurophilic granules, and impaired bactericidal activity has also been described in cattle (21,22). Neutrophils also play an essential role in the cellular defense of the bovine mammary gland, where compromised leukocyte function has been linked to the development of bovine mastitis, the most common and costly disease afflicting dairy cattle around the world (23). The effectiveness of this defense system depends on the promptness and the magnitude of the neutrophil migratory response, as well as the phagocytic and bactericidal activities of these cells (24).

In cattle, neutrophil-derived products have been associated with adverse effects in acute pulmonary diseases, such as pulmonary thromboembolis, endotoxic shock, acute respiratory distress syndrome, and immune complex interstitial pneumonia. The pathogenesis of neutrophil-mediated injury has been postulated to be secretion and/or release of the toxic reactive oxygen species the neutrophil uses in microbial defense. As early as 1985, Slocombe *et al.* found neutrophils to be required for initiation of acute lung injury in neonatal calves with experimentally induced pneumonic pasteurellosis (25,26). The presence of high numbers of activated leukocytes in mammary tissues, which occurs in mastitis, also contributes to inflammatory tissue damage, including fibrosis, swelling and atrophy of the mammary tissue (24).

Hematopoiesis – Stem Cell to Mature Granulocyte

While neutrophils comprise about 60-75% of the blood leukocytes in most mammals, the blood of cattle contains only 20-30% neutrophils (27,28). Neutrophils originate in the bone marrow of all bones at birth, but only the flat bones continue hematopoiesis throughout life. Neutrophils begin as a blast-type cell, the myeloblast, and progress through maturation stages designated as progranulocyte, myelocyte, metamyelocyte, band and segmented neutrophil. In the band stage, the smooth nuclear membrane begins to indent at several points as the cells mature. At this stage the neutrophils are released into the peripheral blood where they age and the nucleus becomes segmented into several distinct lobes separated by filaments. Maturation of neutrophils from precursor cells requires approximately 5 to 6 days (29). The cells only remain in the blood for a few hours before entering the tissue. When neutrophils leave the blood to enter the tissues, a bone marrow reserve is stimulated to replenish those cells (27).

Electron microscopic analysis of bovine neutrophils shows them to be relatively small (diameter of about 7-10 μm), rounded or slightly elongated cells with few cytoplasmic projections (30,31). The nucleus of a neutrophil is distinct from other nucleated eukaryotic cells due to its size, shape, and a low number of nuclear pores (31). It is primarily composed of heterochromatin and seldom contains a nucleolus (31). The presence of a small drumstick-shaped lobe known as the female sex chromatin lobe is only an occasional occurrence in the bovine neutrophil (27). Several spherical, elongated, rod and dumb-bell shaped granules can been seen in the dense cytoplasm. A roughly circular, granule-free area

near the nuclear lobes contains stacks of four to nine Golgi cisternae arranged around one or two centrioles. The cytoplasm also contains particulate glycogen and a few vesicular profiles of rough-surfaced endoplasmic reticulum, but lacks appreciable quantities of most other organelles (e.g., mitochondria, ribosomes, and microtubules) (31). A narrow rim of cytoplasm around the periphery of the neutrophil is generally free of organelles and inclusions, but is rich in cytoskeletal proteins (31).

Unlike the nearly invisible granules of neutrophils from other domestic animals, the granules of bovine neutrophils are faintly visible in the cytoplasm of the cell (31). Three types of granules are present in bovine neutrophils; each type of granule arising at a different stage of cell maturation (32). The level at which the genes for granule components are sequentially activated is unknown. It is likely that their biosynthesis shutdown, which is coupled to the progress of myeloid differentiation, is carried out at the level of transcription (33). Peroxidase-positive granules, analogous to azurophil or primary granules from other species, are present in relatively small numbers. Primary granules are round or elongated and contain microbicidal enzymes (myeloperoxidase, acid hydrolases, and neutral proteases). These enzymes function in concert with membrane-bound and cytoplasmic enzymes to kill and digest phagocytized microbes, but can also be responsible for inducing tissue damage at sites of inflammation. The azurophilic granules also contain cationic proteins and other non-lysosomal enzymes (31). Compared with human neutrophils, bovine neutrophils lack lysozyme and show decreased levels of the azurophilic granule enzymes, such as β-glucuronidase, β-galactose, and myeloperoxidase. A survey of bovine granulocytes has indicated that myeloperoxidase is particularly concentrated in bovine eosinophils, suggesting

that the level of myeloperoxidase activity in some preparations of bovine neutrophils might reflect, in part, the presence of contaminating eosinophils (30,34,35).

The two remaining granule types, the large and specific granules, are round, peroxidase-negative, and distinguishable by size. The large granules are greater than 0.35 μm in diameter, have a pale, very homogenous matrix. These granules are found in the cytoplasm of bovine neutrophils, but not in neutrophils from other animals. Analysis of bone marrow specimens has shown that the formation of the large granules occurs in the myelocyte stage after the production of the peroxidase-containing azurophilic granules (32,33). A number of highly cationic proteins that are not found in other subcellular compartments are localized in the large granules. These proteins are thought to be responsible for the non-oxidative antimicrobial activity associated with this granule type. Unlike the azurophil or specific granules, the large granules contain no serine proteases or metalloproteases, acid hydrolases, or peroxidase (33,36).

The smaller, specific granules have a diameter of less than 0.3 μm and are formed after most of the large granules have matured. In other species, specific granules have been shown to contain lactoferrin and approximately 66% of the neutrophil's lysozyme (31). The concentrations of lactoferrin and vitamin B_{12}-binding protein, markers of specific granules in other species, appear to be higher in bovine cells than in the human neutrophils. However, as in the bovine azurophilic granules, lysozyme activity is undetectable in specific granules of this species (30). Bovine specific granules do have strong, nonspecific alkaline phosphatase activity, but show no acid phosphatase activity (31). Table 1.1 summarizes the major granule components that are known to exist in bovine neutrophils.

Table 1.1. Content and Function of Bovine Neutrophil Granules

Type of Granule	Factor	Function
Azurophilic (Primary)	Myeloperoxidase	Catalyzes the production of hypohalus acids for microbicidal activity
	Acid Hydrolases Arylsulfatase, β-glucuronidase β-galactosidase	Degrades some microbial macromolecules; degrades glucuronic acid and galactose in some bacterial capsules
	Neutral acid proteases Elastase Serine proteases Cathespin G	Breakdown of extra-cellular connetive tissue components; produce kinin-like mediators; alter vascular permeability; digest collagen, cartilage, and elastin; activate proforms of cationic proteins found in large granules. Kills Gram-positive and some Gram-negative microorganisms
	Acid phosphatase	Monophosphate esterase
Specific (Secondary)	Lactoferrin	Chelates iron. Binds to negatively charged cell surfaces and may deprive bacteria of iron. Catalyst causing O_2^- and H_2O_2 to generate $OH^•$; functions with antibody and possibly lysozyme for antimicrobial activity; contributes to neutrophil migration, increases adherence and aggregation.
	Collagenase	Digests microbial macromolecules
	Vitamin B_{12}-binding Protein	Binding and transport of vitamin-B_{12}
	Alkaline phosphatase	Monophosphatase esterase
Large	Cationic Proteins Bactenecins Prododecapeptide ProBMAP28	Antimicrobial peptides; phospholipase and chymotrypsin-like activity; Decrease cell surface charges; Facilitates neutrophil adherence and aggregation
	Lactoferrin	Chelates iron. Binds to negatively charged cell surfaces and may deprive bacteria of iron. Catalyst causing O_2^- and H_2O_2 to generate $OH^•$; functions with antibody and possibly lysozyme for antimicrobial activity; contributes to neutrophil migration, increases adherence and aggregation.

Table adapted from references 30, 34, 36, 90.

Neutrophil Response to Pathogenic Stimuli

Neutrophils capture and destroy dying cells, microorganisms, or foreign material by means of phagocytosis. Although a continuous process, phagocytosis can be divided into the distinct stages of adherence, chemotaxis, ingestion and digestion (28).

Adherence

Recruitment of neutrophils to the site of acute inflammation involves the combined action of multiple families of adhesion molecules, cytokines, and chemoattractants. The extravasation of neutrophils from the bloodstream initially depends on adhesive interactions. These adhesive interations are mediated through the selectins, which bind sialylated and fucosylated oligosaccharide ligands, such as sialyl Lewis X (sLe^x) (37-39). L-selectin, a protein expressed the neutrophil surface, binds to constitutively expressed ligands on high endothelial venules of peripheral lymph nodes [e.g., mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and peripheral lymph node addressin (PNAd)], to undefined ligands upregulated on endothelial cells at sites of inflammation, and to inducible ligands on other leukocytes [e.g., P-selectin glycoprotein ligand-1 (PSGL-1)] (38). On the endothelial cells, E-selectin and P-selectin are upregulated to bind to carbohydrate ligands on the surface of the neutrophil, including E-selectin ligand 1 (ESL-1) and P-selectin glycoprotein ligand-1 (PSGL-1) (37-39). The selectin-mediated interactions facilitate leukocyte rolling, slowing the flow of neutrophils through venules, and allow time for the neutrophils to sample the local environment as well as the endothelial cell surface. Binding of the selectins to their

ligands and the presence of chemoattractants can enhance the expression and adhesiveness of another receptor on the neutrophil surface, Mac-1 (CD11b/CD18). Mac-1, a member of the β_2-integrin family of leukocyte adhesion molecules, allows neutrophils to bind tightly to activated endothelium through its ligand, intercellular adhesion molecule-1 (ICAM-1) (40,41). In response to acute inflammatory stimuli, such as (42,43)interleukin-1 (IL-1), interleukin-8 (IL-8), platelet activating factor (PAF), or lipopolysaccharide (LPS), the neutrophil surface expression of Mac-1 is upregulated, while L-selectin is down-regulated due to cleavage by a specific protease (44,45). Neutrophils bound to ICAM-1 remain attached to the vessel wall and are, therefore, able to migrate along the endothelial surface and through the vessel wall into tissues along a concentration gradient of chemoattractant (43,46,47).

Chemotaxis

Bacterial invasion and the ensuing inflammatory response often results in the production of many different chemotactic molecules. As chemotactic molecules diffuse from the site of tissue damage, a concentration gradient is formed in the tissue and can be detected by adherent neutrophils. Bovine neutrophils have been shown to respond to opsonized particles or bacteria (48-50) as well as inflammatory mediators including prostaglandins (51), leukotriene B_4 (52,53), platelet activating factor (43,49,54), complement fragment C5a (55,56) and cytokines such as IL-1 (51), IL-8 (56,57), tumor necrosis factor (TNF-α) (58), and granulocyte-colony stimulating factor (59). An interesting characteristic of bovine neutrophils is the lack of a chemotactic response to the bacterial N-formylated peptides such

as fMLF (60,61). Formylated peptides have been shown to stimulate chemotaxis of primate, rat, rabbit, and guinea pig neutrophils, while like bovine cells, this response is not seen in porcine, feline, and canine neutrophils [reviewed in (53)].

Although the stimulus may vary, the mechanics of neutrophil movement appear to be conserved among species. Neutrophils migrate toward a chemotactic source with a broad leading edge (lamellipodium), which contains the actin network, and a knob-like posterior (uropod) (62,63). Actin, a major cytoskeletal protein, is responsible for the cytoskeletal changes that correspond with cell movement (49,64). In response to a chemotactic signal, monomeric actin assembles into filaments and microtubules. Microtubules extend to, but do not penetrate the lamellipodium and uropod. Microfilaments form a zone subjacent to the plasma membrane excluding cell organelles, where electron-dense polymerized areas serve as sites of cell attachment (65). The nucleus moves to the rear of the neutrophil and is separated from the lamellipodia by most of the granules and the Golgi apparatus (62,65). In polarized cells, the uropod membrane contains almost all of the coated pits and vesicles, as compared to the diffuse distribution found in unstimulated neutrophils. Before the neutrophil can change its direction of migration, reorientation of the nucleus, organelles and cytoskeletal elements must occur (62). Neutrophils are extremely sensitive to variations in chemoattractant concentration, and human neutrophils can detect as little as a 0.1% gradient difference between different regions of the cell surface (66). Changes in the diffusion time and the orientation of the stimulant may determine whether directional locomotion results in neutrophil aggregation (55), enhanced oxidative metabolism (51), or augmentation of complement receptor expression (67). These activities in turn modulate the chemotactic

response. Newly generated active oxygen radicals initiate the production of chemotactic and chemokinetic factors from cellular and extracellular lipids that may modify neutrophil function (51). When macrophages encounter invading organisms, they release IL-1 and TNF-α, which recruit neutrophils into inflamed tissues and prime these cells for a heightened oxidative burst to assist in microbial killing (58).

The response of neutrophils to a given chemoattractant is usually transient and results in a decreased responsiveness to subsequent stimulation by the same agonist (68). This type of homologous desensitization has been proposed to result from receptor phosphorylation, receptor association with the cytoskeleton, and receptor internalization (68,69). Heterologous desensitization can also occur and is characterized by the loss of responsiveness by a given receptor (e.g., C5a receptor or IL-8 receptor) following activation of a different receptor or process (e.g., fMLF receptor) (68,70). However, desensitization is highly dependent on the order in which the cell encounters the chemoattractant, and treatment of the neutrophils with IL-8 has no effect on the subsequent response to fMLF (70). The ability of chemoattractant receptors to "talk" with each other in this manner may help regulate leukocyte migration in the presence of complex chemoattractant arrays which are found at sites of inflammation (70).

Ingestion

When a neutrophil encounters a foreign particle, it extends pseudopodia around it, and binding occurs between opsonins on the organism and receptors on the neutrophil. Once bound firmly to the neutrophil surface, the particle is drawn into the cell and becomes

enclosed in a vacuole called a phagosome [Reviewed in ref. (28,31)].

Ingestion and degranulation are interrelated processes, which require intact neutrophil membranes and cytoskeletal elements (microbutules and microfilaments). When a phagocytizable particle binds to the cell membrane, cytoskeletal changes occur which are similar to those seen with chemotaxis. Neutrophil pseudopodia flow around the particle by the coordinated action of microfilament polymerization, changes in cell membrane fluidity, and receptor binding (31). The ease of this enclosure depends, in part, on the surface of the particle. Neutrophil cytoplasm readily flows over hydrophobic surfaces so that hydrophobic bacteria, such as *Mycobacterium tuberculosis,* are rapidly ingested. In contrast, *Streptococcus pneumoniae* has a hydrophilic carbohydrate capsule and is poorly phagocytosed unless made hydrophobic by a coating of antibodies or C3b (28). While opsoninization of a particle increases the rate of phagocytosis several fold, it is not required (34). In addition, the rate of phagocytosis is different between species of domestic animals. For example, bovine peripheral blood neutrophils ingest opsonized *Staphylococcus aureus* more rapidly than equine neutrophils (31).

The membranes near pseudopodia accumulate ligand-receptor complexes and are enriched in "coated" membranes. Endocytotic function is restricted to this region. The lamellipodial membrane excludes coated pits and lacks pinocytotic activity, but has preferential binding of immunoglobulin aggregates (Fc receptors). The uropodia of a neutrophil undergoing chemotaxis are characterized by receptor-mediated endocytosis, pinocytosis, and a high concentration of coated pits and vesicles. In neutrophils, the CD32 receptor, known as FcRγII, binds the Fc region of antibody molecules, triggers the respiratory

burst, and initiates ingestion. Another receptor, CD35 (or CR1) binds the complement component, C3b. Binding of C3b-coated particles leads to attachment, but may not necessarily trigger ingestion (31,51).

Destruction

Once a pathogen is phagocytosed, cytoplasmic granules fuse with the phagocytic vacuole and discharge their contents into the phagosome as a mixture of enzymes, including myeloperoxidase and highly reactive oxidizing agents associated with the respiratory burst (71). The antimicrobial systems of neutrophils can be classified into oxygen-dependent and oxygen-independent systems. The oxygen-dependent system involves consumption of molecular oxygen and production of O_2^-, hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hyroxide radical ($OH^\bullet$), and singlet oxygen ($^1O_2$). The oxygen-independent system is more diverse and is composed of various factors, including acid pH, lysozyme, cationic proteins, lactoferrin, vitamin B_{12}-binding protein, and acid hydrolases. The combination of various factors involved with killing a given type of microbe or in causing tissue damage is not well defined, and it is likely that all of these components participate to some in both of these processes (31).

The earliest events of degranulation are associated with changes in granule morphology. Degranulation, like phagocytosis, depends on an intact cytoskeletal system and is closely linked to chemotaxis, ingestion, and neutrophil metabolism. Secretion of granule proteins, such as elastase and collagenase at the lamellipodial edge, helps loosen the interstitial matrix promoting neutrophil migration (51). Granule enzymes may be autocrine-

like, controlling neutrophil function by destroying the hyaluronic acid, which can modulate neutrophil adhesion and directed migration. Granule exocytosis also releases mediators that influence the inflammatory response by inactivating as well as generating chemoattractants (31,51).

Microtubule and microfilament polymerization are required to move granules to the phagosomal membrane for intracellular release or to the plasma membrane for extracellular release. Release of granule enzymes initiates neutrophil oxidative metabolites, leading to microbial killing, tissue damage, and modulation of various inflammatory and immunologic processes.

Microbicidal Mechanisms of Bovine Neutrophils

Oxygen-independent killing

The oxygen-independent system is composed of numerous components, including cationic proteins, lactoferrin, vitamin B_{12}-binding protein, and acid hydrolases. Note, however, that oxygen-independent mechanisms are not strictly dependent on anaerobic conditions, as many of these mechanisms can function in the presence or absence of oxygen.

After phagocytosis, the hydrogen ion (H^+) concentration within the phagosome increases rapidly, likely as a result of the lactate produced by the glycolysis that occurs during phagocytosis. The low pH level in the phagosome may be microbicidal (attacking organic acids and lipophilic acids) or microbiostatic, depending on the sensitivity of the microbe to changes in pH. Acid pH also facilitates the function of some microbicidal enzyme systems (myeloperoxidase-H_2O_2-halide system, acid hydrolases, and lysozyme).

Enzymes considered to have a digestive role (hydrolases) function best at an acid pH; however, cationic proteins have a pH optimum at seven or above (72,73).

Lysozyme, a cationic protein that attacks β1,4-glycosidic bonds in bacterial cell walls, is found in both the azurophilic and secondary granules of some animal neutrophils, but requires other antimicrobial factors to induce microbial killing (72). Compared to human neutrophils (~30 μg), the amount of lysozyme in bovine neutrophils (~0.2 μg) is small, so the contribution of this enzyme to the overall killing and digestive functions is probably not significant (31,74).

Lactoferrin, from specific granules, inhibits bacterial growth by binding iron; however, a reduced ability to kill phagocytized *Escherichia coli* by lactoferrin-deficient neutrophils suggests a role in the killing of some microbes (31). One possibility is that lactoferrin, antibody, and lysozyme may function in concert to exert antimicrobial activity, although this has not been conclusively demonstrated. Lactoferrin also increases granulocyte adherence and aggregation and reduces the cell surface charge, contributing to the control of neutrophil migration and oxygen-radical production (31). Interestingly, bovine neutrophils have a higher concentration of lactoferrin than that of human granulocytes (74), suggesting lactoferrin may play a more important role in bovine neutrophil function.

Azurophil granules also contain cationic proteins, such as neutral and acid proteases that are associated with the breakdown of extracellular connective tissue components. These proteases are involved in the production of kinin-like mediators (leukokinins from serum proteins), the alteration of vascular permeability (possibly mediated by leukokinin), and the digestion of various types of collagen, cartilage, and elastin (75). Enzymatic activity is not

necessary for antimicrobial activity by these proteins. Cationic proteins bind to the microbe and impair replicative ability without altering the bacterial structure. These proteins likely act in concert with other antimicrobial mechanisms and may enhance the killing effects of the myeloperoxidase-H_2O_2-halide system (73).

Two peptide families have been isolated from neutrophil granules: the defensins and bactenicins. Defensins have been found in human, rabbit, rat and guinea pig neutrophils. Neutrophils from cattle and other ruminants also contain potent antimicrobial peptides; however, they are distinct from other species and are known as bactenecins. Bactenecins constitute an efficient cell-dependent defense system against bacteria, viruses, and parasites (33). These cyclic, defensin-like peptides are found in the large granules of bovine neutrophils. Bactenicins are highly cationic polypeptides with molecular weights ranging from 1,600 to 8,000 daltons (33). These peptides exert activity in a wide range of pH conditions (maximum pH 7-8) and remain active in physiological solutions (74). Two of these peptides, Bac5 and Bac7, have been shown to contain high levels of arginine and proline (> 60%), and have unique primary structures with repeated sequences. The Bac7 structure contains an "arginine-clustered region" (8 arginine residues within residues 1-17) and "three tandem repeats" of a tetradecapeptide (residues 18-31, 32-45, and 46-59). It has been suggested that Bac7 exhibits antimicrobial activity due to a bacteriostatic rather that a bacteriolytic effect (76). The peptide's mechanism of action involves binding to the outer membrane of Gram-negative bacteria, followed by a rapid translocation to the inner membrane, where the electron transport chain and some energy dependent membrane activities are impaired (33). Bactenicins appear to be stored in the large granules as harmless

propeptides, requiring cleavage by neutral serine protease(s) to generate antimicrobial molecules. In stimulated neutrophils, probactenecins are activated after their discharge into the phagocytic vacuoles by neutral serine proteases, such as elastase or cathepsin G, that are released into the phagosome by azurophil granules (33,74).

Oxygen-dependent killing by bovine neutrophils

Within seconds of binding to a foreign particle or stimulation of an antibody receptor, such as CD32, neutrophils increase their oxygen consumption nearly 100-fold. This increase in respiration results from activation of the respiratory burst oxidase or NADPH oxidase (27,77). The NADPH oxidase catalyzes the transfer of electrons from NADPH to molecular oxygen, converting it into superoxide anion (O_2^-), which is released into the phagosome or outside of the plasma membrane [Reviewed in ref. (78)] (See Reaction 1).

$$NADPH + 2O_2 \xrightarrow[\text{oxidase}]{\text{NADPH}} NADP^+ + H^+ + 2O_2^- \qquad \text{Reaction 1}$$

O_2^- is intrinsically unstable and spontaneously reacts to form secondary metabolites such as H_2O_2, HOCl, $OH^\bullet$, $^1O_2$, and $ONOO^-$ (79-84). O_2^- can also be scavenged by the enzyme superoxide dismutase (SOD) which is found in most tissues. SOD dismutates two molecules of O_2^- to generate one molecule of H_2O_2 (See Reaction 2).

$$2\ O_2^- + 2\ H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \qquad \text{Reaction 2}$$

As H_2O_2 accumulates in the cell, it can be scavenged by catalase which converts H_2O_2 intoH_2O (See Reaction 3). H_2O_2 can also be converted to other bactericidal compounds

through the action of myeloperoxidase from the primary granules (75). Myeloperoxidase (MPO) catalyzes a reaction between H_2O_2 intracellular halide ions (Cl^-, Br^-, I^-, or SCN^-) to produce hypohalides (See Reaction 3).

$$H_2O_2 + Cl^- + H^+ \xrightarrow{MPO} H_2O + HOCl \qquad \text{Reaction 3}$$

The chloride ion is probably used at most sites *in vivo* except in milk and saliva where SCN^- is employed (27). Hypochlorite ion (OCl^-) is a major product of neutrophil oxidative metabolism and kills bacteria by oxidizing bacterial proteins and enhancing the bactericidal activities of the lysosomal enzymes (27).

The reduction of H_2O_2 produces $OH^{\bullet}$, which is highly unstable and reacts with lipids and nucleic acids. It initiates lipid peroxidation by removing hydrogen atoms from unsaturated lipids to form hydroperoxides, which cause severe damage to cell membranes, organelles and associated enzymes (81).

Singlet oxygen is formed when oxygen absorbs sufficient energy to shift one of its unpaired electrons from its lowest energy level, or ground state, into an orbital of higher energy. The distorted electron shell of singlet oxygen has an affinity for compounds containing double bonds. Thus, polyunsaturated fatty acids can be attacked, resulting in the formation of hydroperoxides (71).

The reaction of O_2^- with nitric oxide, which is produced by neutrophils themselves as well as vascular endothelial cells, produces a toxic oxidant, peroxynitrite ($ONOO^-$) that is able to attack a wide variety of tissues (83). Recent research has implicated $ONOO^-$ as one of the damaging agents in a number of inflammatory diseases in humans (84). The formation

of $ONOO^-$ has also been shown to prime human neutrophils at sites of inflammation, increasing the efficiency by which neutrophils kill microorganisms (84).

Since all of these oxygen radical species are generated intracellularly, it is inevitable that some of these products leak into the cytoplasm. Thus, the cytoplasm is protected against the oxidative effects by the scavenging enzymes, SOD and catalase. H_2O_2 can also be removed by coupling to the glutathione reductase system, generating $NADP^+$ and subsequently increasing hexose monophosphate (HMP) shunt activity (71). Bovine neutrophils have low catalase activity, but high levels of two enzymes of the glutathione pathway, glutathione peroxidase and glutathione reductase, which may be the method used more often in this species (30). Defense mechanisms of bovine neutrophils are summarized in Figure 1.1.

NADPH Oxidase Components in Bovine Neutrophils

In the resting cell, the O_2^- generating machinery is dormant, and its protein components are segregated into cytoplasmic and plasma membrane compartments. Activation of the NADPH oxidase involves the interaction and assembly of several neutrophil proteins, and the transfer of electrons from NADPH to molecular oxygen occurs only after the cytosolic components of the oxidase are translocated to and assembled with the membrane-bound components. The membrane and cytosolic proteins involved in NADPH oxidase assembly are listed in Table 1.2.

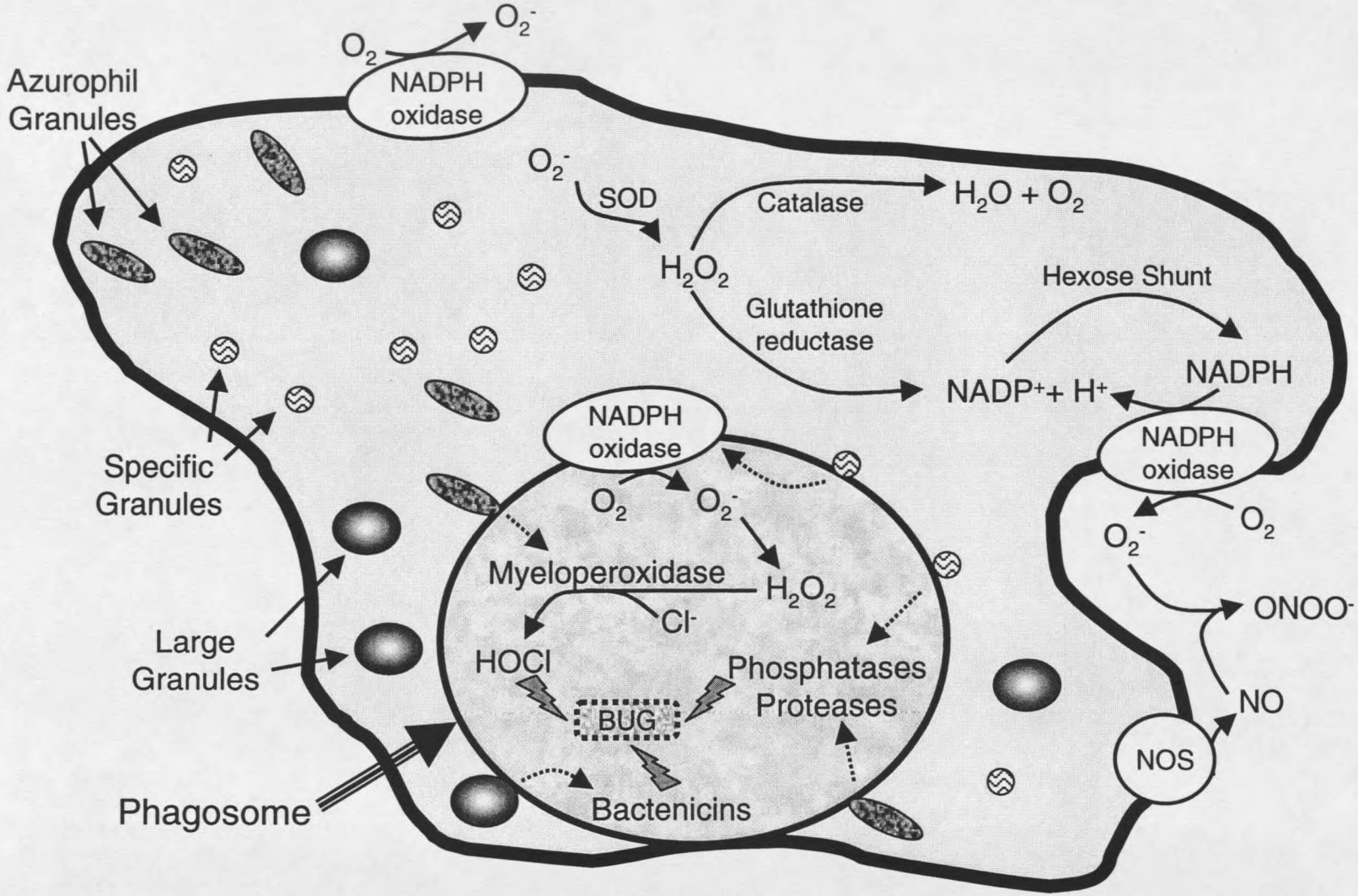

Figure 1.1. Schematic Representation of Bovine Neutrophil Microbicidal Mechanisms

Table 1.2. NADPH Oxidase Proteins

Membrane Components	Cytosolic Components
Flavocytochrome b	p40-*phox*
gp91-*phox*	p47-*phox*
p22-*phox*	p67-*phox*
Rap1A	Rac

Membrane Components

Flavocytochrome b is an integral membrane protein (5,6,85) composed of two subunits, gp91-*phox* and p22-*phox*, which are present at a 1:1 molar ratio (86). Studies have shown that both subunits are required for functional surface expression of the flavocytochrome b assembly, and a defect in one of the subunit proteins results in decreased expression of the other subunit (87). The heterodimer is responsible for coordinating at least two hemes, one which may be shared between gp91-*phox* and p22-*phox*. The larger subunit, gp91-*phox*, has 4-5 transmembrane helices (78), five potential glycosylation sites in the amino-terminal region, and has been shown to contain FAD and NADPH binding activities (88,89). The small subunit, p22-*phox*, has a molecular weight of 22 kDa and contains hydrophobic helices in the N-terminal portion of the protein that could also serve as membrane-spanning domains (87). Flavocytochrome b appears to act as the focal point of NADPH oxidase assembly, and the translocation of the cytosolic components does not occur in the absence of flavocytochrome b (90,91). In addition to the flavocytochrome b heterodimer, a low molecular weight GTP-binding protein, Rap1A, is also membrane bound

and associates with flavocytochrome b (92,93). In humans, Rap1A appears to play a regulatory role in the NADPH oxidase (78,94). A Rap homologue exists in cattle (95) and may function similar to human Rap1A, but this has yet to be determined.

Bovine gp91-*phox* has been sequenced by our lab and was shown to encode a protein of 570 amino acids. In addition to being identical in length to human and murine gp91-*phox*, the bovine protein is ~92% identical to the other species sequenced to date. Five potential glycosylation sites are located in regions similar to human gp91-*phox*, further suggesting that this protein is highly conserved across species. Bovine p22-*phox* has also been sequenced by our lab and was shown to be ~86% identical to homologues from other species (9).

Cytosolic components

Since the active enzyme complex forms at the membrane, the cytosolic proteins must translocate from the cytosol to associate with membrane-bound flavocytochrome b and possibly the cytoskeleton (Figure 1.2.). The human cytosolic NADPH oxidase proteins include p40-*phox*, p47-*phox*, p67-*phox* and a second low molecular weight GTP-binding protein, Rac2 [Reviewed in refs. (78,96,97)]. Extensive research of these proteins has been carried out in human and murine neutrophils. The isolation of bovine cytosolic proteins was not reported until 1990 (98). Since the functional role of the NADPH oxidase appears to be conserved across species, conservation of the cytosolic proteins would be expected. However, differences have been found to occur between species. For example, the importance of p47-*phox* and p67-*phox* has been demonstrated by the recurrent infections that