Characterization of the Beta-2 Adrenergic Receptor Mechanism in Bovine Neutrophils, and Some Effects of Inflammatory Stimuli on its Function

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i. ABSTRACT

The bovine polymorphonuclear leukocyte (neutrophil) is a central component of the acute inflammatory response, and is capable of reacting to a myriad of pro-inflammatory chemical signals that have been characterized in the context of bovine respiratory disease (BRD). Human neutrophils and bovine macrophages are known to react to pro-inflammatory signals as well; however, they are also capable of responding to anti-inflammatory signals from the autonomic nervous system. In particular, activation of the β_2-adrenergic receptor on these cells decreases several aspects of inflammatory activity, including reactive oxygen species production, chemotaxis, degranulation, and inflammatory mediator production. Dysfunction of β-adrenergic receptors is known to contribute to the pathophysiology of numerous diseases in both people and animals. For example, congestive heart failure, asthma, cystic fibrosis, atopic dermatitis, pheochromocytoma, myasthenia gravis, hypertension, and sepsis have all been linked to decreased β_1- / β_2-adrenergic receptor density (depending on the cell type) and / or uncoupling of the respective receptor from its effector enzyme, adenylyl cyclase. Dysfunction of the β_2-adrenergic receptor mechanism has also been described in pulmonary airway and vascular smooth muscle tissue from cattle, sheep, and rats exposed to *Manheimia haemolytica*, which provides insight into the pathophysiology of BRD. Despite the prominent role of the bovine neutrophil in the acute inflammatory stage of BRD, and despite the potential for dysfunction following excessive exposure to inflammatory stimuli, there are no reports that describe the presence of the β_2-adrenergic receptor on bovine neutrophils, nor function of the components responsible for its signal transduction cascade. Without complimentary work with bovine neutrophils, using data from human neutrophils to examine treatment options for the acute inflammatory stage of

BRD is unrealistic. For this reason, the present dissertation proposed that 1) bovine neutrophils possess the β_2-adrenergic receptor mechanism, 2) components of the β_2-adrenergic receptor mechanism work in concert to increase bovine neutrophil adenosine 3,5-cyclic monophosphate (cAMP) levels and suppress superoxide anion production, and 3) the β_2-adrenergic receptor mechanism is dysfunctional following exposure to inflammatory stimuli. Using the nonselective β_1- / β_2-adrenergic receptor antagonist $^{[3H]}$CGP-12177 we observed a maximum specific binding density (B_{max}) value of 0.19 fmol per 10^6 bovine neutrophils. Although this value is approximately equal to what we observed with dairy cow neutrophils, human neutrophil B_{max} values with this radioligand are anywhere from five to ten-fold greater, which suggests a significant species difference. We further defined the adrenergic receptor population on bovine neutrophils to be dominated by the β_2-subtype. Next, we characterized the function of β_2-adrenergic receptors by stimulating cAMP production with the β_2-adrenergic receptor agonist, terbutaline. The role of the β_2-subtype was confirmed when the terbutaline-mediated effect was negated by ICI-118,551, a β_2-adrenergic receptor antagonist. Also, the role of the phosphodiesterase enzyme in cAMP recycling in bovine neutrophils was illustrated, as the terbutaline-mediated rise in cAMP concentration was dependent upon phosphodiesterase inhibition by 3-isobutyl-1-methylxanthine (IBMX). This study confirms the anti-inflammatory nature of the β_2-adrenergic receptor on bovine neutrophils by demonstrating the ability of terbutaline and IBMX to decrease superoxide anion production in a dose-dependent manner. The synthetic cAMP analog, 8-bromo-cAMP also decreased superoxide anion production, but the effect was time-dependent because of its need to diffuse across the cell membrane. Moreover, IBMX exaggerated the terbutaline-mediated effect on superoxide anion production, while cAMP exaggerated the IBMX-mediated effect on superoxide anion, demonstrating that the β_2-adrenergic receptor acts in concert with adenylyl cyclase, while the phosphodiesterase enzyme functions to decrease their signal. By increasing the dose of the inflammatory stimulant opsonized zymosan eight-fold, we were able to eliminate the ability of various concentrations of terbutaline and IBMX to reduce superoxide anion production. We sought to provide a more specific demonstration of this phenomenon by activating protein kinase C (PKC) via phorbol 12-myristate 13-acetate (PMA) administration.

However, preincubation with PMA actually increased terbutaline-mediated cAMP production, in a dose and time-dependent manner. At this time, we cannot explain why increasing the dose of opsonized zymosan and PMA had opposite effects on β_2-adrenergic receptor mechanism function. The answer may reside in the many reported functions of PKC isoforms. Additional studies that identify the PKC isoform repertoire in bovine neutrophils may illustrate the potential for selective inhibition, and may lead to more specific identification and treatment of β_2-adrenergic receptor mechanism dysfunction. Also, it remains to be seen how the various components of the bovine neutrophil β_2-adrenergic receptor mechanism function *in-vivo* during the acute inflammatory stage of BRD.

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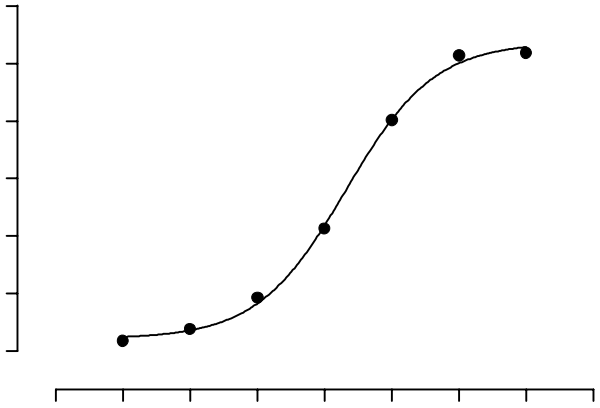

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iii. DECLARATION OF WORK PERFORMED

I declare that I, Timothy Paul LaBranche, performed all the work necessary to collect, interpret, and discuss the data reported in this dissertation except that which is described below.

All collections of whole blood from the steers were performed by Kevin Weaver and Chris Wakley at the Virginia-Maryland Regional College of Veterinary Medicine's Non-Client Animal Facility.

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vi. GLOSSARY OF TERMS AND ABBREVIATIONS

α_1: Alpha subtype-1 (adrenergic receptor)
AC: Adenylyl (adenylate) cyclase
ACD: Acid citrate dextrose
ACTH: Adrenocorticotropic hormone
ADCC: Antibody-dependent cell-mediated cytotoxicity
AICC: Antibody-independent cell-mediated cytotoxicity
ATP: Adenosine triphosphate
β_1: Beta subtype-1 (adrenergic receptor)
β_2: Beta subtype-2 (adrenergic receptor)
B_{max}: Maximum (radioligand) binding density
BHV-1: Bovine herpes virus type-1
BLAD: Bovine leukocyte adhesion deficiency
BRD: Bovine respiratory disease
BRSV: Bovine respiratory syncytial virus
BVDV: Bovine viral diarrhea virus
C3b: Complement fragment 3b
C5a: Complement fragment 5a
cAMP: Adenosine 3,5-cyclic monophosphate
CD11: Cluster of differentiation molecule 11 (β_2-integrin protein complex component)
CD14: Cluster of differentiation molecule 14 (FcγRII receptor)
CD18: Cluster of differentiation molecule 18 (β_2-integrin protein complex component)
CD54: Cluster of differentiation molecule 54 (ICAM-1)
CD62L: Cluster of differentiation molecule 62 (L-selectin)
cGMP: Guanosine 3,5-cyclic monophosphate
CPM: Counts per minute
CRH: Corticotropin releasing hormone
DAG: Diacylglycerol
DNA: Deoxyribonucleic acid
fMLP: N-formylated methionine-leucine-phenylalanine
GPCR: G-protein-coupled receptor
G-protein: Guanyl nucleotide-binding-regulatory protein
GRK-2: G-protein receptor kinase type-2
G_s: Stimulatory G-protein
HBSS: Hanks balanced salt solution
HOCl: Hypochlorous acid
IBMX: 3-isobutyl-1-methylxanthine
IBR: Infectious bovine rhinotracheitis
IC_{50}: 50% inhibitory concentration
ICAM-1: Intercellular adhesion molecule type-1
IL-1β: Interleukin-1 beta
IL-6: Interleukin-6
IL-8: Interleukin-8
IP3: Inositol 1,4,5-triphosphate

K_D: Dissociation constant
LAD: Leukocyte adhesion deficiency
LBP: LPS-binding protein
LFA-1: Leukocyte function associated antigen-1
LPS: Lipopolysaccharide, endotoxin
LTB_4: Leukotriene B4
M_3: Muscarinic subtype-3
MAPK: p38 Mitogen-activated protein kinase
MPO: Myeloperoxidase
NADPH: Nicotinamide adenine dinucleotide phosphate
NF-kB: Nuclear transcription factor- kappa B
NO: Nitric oxide
O.D.: Optical density
PAF: Platelet-activating factor
PI-3: Parainfluenza type-3
PIP2: Phosphatidyl inositol 4,5-bisphosphate
PKA: Protein kinase A
PKC: Protein kinase C
PLA_2: Phospholipase A2
PLCγ: Phospholipase C gamma
PMA: Phorbol 12-myristate 13-acetate
PMN: Polymorphonuclear leukocyte / neutrophil
RNA: Ribonucleic acid
ROS: Reactive oxygen species
RPMI: Roswell Park Memorial Institute-1640 medium
RTX: Repeats in structure family of toxins
SEM: Standard error of the mean
SOD: Superoxide dismutase
TLR-4: Toll-like receptor type-4
TNF-α: Tumor necrosis factor-alpha
TXA_2: Thromboxane A2

PART I: INTRODUCTION

A. Statement of hypotheses:

1) The β_2-Adrenergic Receptor Mechanism Exists in Bovine Neutrophils and its Activation Results in Decreased Superoxide Anion Production.

2) The Bovine Neutrophil β_2-Adrenergic Receptor Mechanism is Dysfunctional Upon Exposure to Increasing Concentrations of Inflammatory Stimuli.

B. Justification for the study:

Bovine respiratory disease (BRD), also known as shipping fever, is an infectious, acute fibrino-necrotizing bronchopneumonia that affects young beef and dairy calves. BRD accounts for 75% of all beef cattle illnesses, significantly affects feedlot feed efficiency, and is considered to be the number one disease problem of beef cattle in the United States and Canada.[1, 2] Affected animals demonstrate increased respiratory rate, decreased tidal volume and lung compliance, ventilation-perfusion mismatch and pulmonary hypertension.[3] These insults to the animal are the manifestation of an acute inflammatory response within the lung, involving large numbers of neutrophils and severe amounts of edema, fibrin, and necrotic cellular debris in the lumens of bronchioles and alveoli. Neutrophils are directed into the lung via a multistep process, which is initiated when airway and vascular macrophages stimulate endothelial cells to present both P-and E-selectins. The presentation of selectins stimulates neutrophils to express the β2-integrin protein complex and adhere to the vascular wall.[4, 5] The process continues when neutrophils undergo a protein kinase C (PKC) and calcium-driven rearrangement of their cytoskeleton and diapedese across the blood vessel wall in an organized, deliberate manner.[6-8] When neutrophils reach exposed basement membrane, they are capable of then traversing the extracellular matrix of the pulmonary interstitium, sensing along the way an inflammatory mediator (chemoattractant) concentration gradient pathway. As neutrophils exocytose into the alveolar space, high chemoattractant concentrations prepare the cellular machinery for phagocytosis, degranulation, and reactive oxygen species (free radical) production.[9-11]

This migration in the lung is orchestrated by three basic classes of receptors found on the neutrophil plasma membrane: ion channel-coupled receptors, enzyme-coupled receptors, and G-protein-coupled receptors. These receptors enable the neutrophil to recognize and integrate a myriad of chemical signals. For instance, it is widely reported that chemoattractants initiate migration and other pro-inflammatory actions by activating neutrophil G-protein-coupled receptors (i.e. platelet activating factor, interleukin-8, and leukotriene B_4 receptors).[10, 12-15] However, comparatively little is known about the cell surface receptors that damper the neutrophil inflammatory response.

This question has been partially answered by work with human and bovine leukocytes. For instance, it is now generally accepted that during the sympathetic response, epinephrine and norepinephrine are released and bind to beta subtype-2 (β_2) adrenergic receptors on eosinophils,[16] natural killer cells,[17] monocytes,[18] lymphocytes,[19-21] macrophages,[22, 23] mast cells,[24] and neutrophils.[25] In particular, activation of human neutrophil β_2-adrenergic receptors decreases chemotaxis,[26] decreases degranulation,[27, 28] decreases adhesion to endothelium,[29-31] decreases adhesion to bronchial epithelium,[32] decreases release of inflammatory mediators,[33-35] and decreases apoptosis.[36, 37] Indeed, stimulation of the β_2-adrenergic mechanism (collective term for the β_2-adrenergic receptor and the cellular machinery for its signal transduction cascade - the stimulatory G_s-protein and the effector enzyme, adenylyl cyclase) seems to regulate several pro-inflammatory aspects of human neutrophil activity. However, to the author's knowledge, there are no reports describing the presence of the β_2-adrenergic receptor mechanism in bovine neutrophils. Therefore, investigation into the presence and function of the β_2-adrenergic receptor on bovine neutrophils is prudent. Neutrophils play a central role in the acute inflammatory stage of BRD, and demonstrating the presence and putative anti-inflammatory effects of the β_2-adrenergic receptor mechanism will advance our understanding of bovine neutrophil physiology.[22, 23]

The bovine β_2-adrenergic receptor mechanism may also prove to be dysfunctional under certain circumstances, as several other disease states (congestive heart failure,[38, 39]

asthma,[40] cystic fibrosis,[41] atopic dermatitis,[42] pheochromocytoma,[43] myasthenia gravis,[44] hypertension,[45] and sepsis[46]) have been successfully linked to decreased β-adrenergic receptor mechanism function. In these examples, the β-adrenergic receptors were considered to be "desensitized". The process of desensitization, where a G-protein-coupled receptor (such as the β_1- or β_2-adrenergic receptor) becomes functionally uncoupled from its effector enzyme, is believed to be the product of excessive tissue exposure to catecholamines and / or inflammatory mediators. When this occurs, receptors are rendered non-responsive to further stimulation.

Desensitization is classified as either homologous or heterologous in cause. Homologous desensitization of a G-protein-coupled receptor is the manifestation of excessive exposure to a ligand which is specific to that same receptor (i.e., a β_1-adrenergic agonist causing homologous desensitization of the β_1-adrenergic receptor), whereas heterologous desensitization of one G-protein-coupled receptor is caused by excessive exposure to a ligand specific to another G-protein-coupled receptor (i.e., platelet-activating factor causing heterologous desensitization of the interleukin-8 receptor).[47, 48] Perhaps the most widely-known example of homologous desensitization is that which occurs during heart failure. When an ischemic event occurs, the heart suffers from a net loss of functional mass. To compensate for the sudden decrease in workload capacity, blood catecholamine levels may increase as high as 3-fold.[49] Yet, the resultant increase in cardiac output may be temporary, as the same patient may soon experience a continued decrease in cardiac output.[50] This is due to a progressive decrease in cardiac myocyte β_1-adrenergic receptor density, and is associated with high blood norepinephrine levels in monitored heart failure patients.[51] In addition to loss of receptor density, the remaining β_1-adrenergic receptors may be functionally uncoupled from their effector enzyme, adenylyl cyclase. As a result, cardiac tissue may not respond as vigorously to additional catecholamine exposure and / or to β_1-adrenergic agonist drugs.[51] While the role of homologous desensitization during heart failure has provided insight into cardiac β-adrenergic receptor dysfunction, investigators have also found that heterologous desensitization of pulmonary β-adrenergic receptors affects the pathophysiology of

asthma. Indeed, dysfunction of airway smooth muscle cell β_2-adrenergic receptors is thought to be the result of excessive parasympathetic activity and exposure to inflammatory mediators.[52, 53] Parasympathetic (specifically, muscarinic) receptors and most inflammatory mediators are known to raise smooth muscle PKC concentrations, and PKC is suspected of causing heterologous desensitization of both pulmonary smooth muscle and human leukocyte β2-adrenergic receptors.[54, 55]

Similar to the asthma model, studies have examined the effect of BRD on the functionality of pulmonary airway and vascular smooth muscle β_2-adrenergic receptor signaling mechanism. For example, intra-tracheal inoculation of bull calves with $1x10^6$ live *Manheimia haemolytica* organisms resulted in the impairment of β_2-adrenergic receptor-dependent pulmonary venous, but not arterial, dilation.[56] This is the first published work which describes impaired autonomic control of the lung following infection with *M. haemolytica*. Also, sheep pulmonary artery and vein samples exhibit a reduced ability to relax in response to isoproterenol administration *ex-vivo* following *M. haemolytica* infection.[57] Finally, inoculation with a commercially-available *M. haemolytica* vaccine causes aortic hyperresponsiveness to the vasoconstricting agent methoxamine (a selective α_1-adrenergic receptor agonist), and decreased responsiveness to the vasodilating agents carbachol and isoproterenol.[58, 59]

Measuring blood cell β-adrenergic receptor number and functionality as a means of monitoring and / or explaining disease states in major organ systems has been performed for over 20 years. Drawing from insight provided by studies on heart failure, asthma and BRD, it is reasonable to ask whether the β_2-adrenergic receptor mechanism in bovine neutrophils is dysfunctional following excessive exposure to inflammatory stimuli. A compromise in the ability to "shut down" neutrophils may lead to an exaggerated acute inflammatory response, and ultimately to lesions observed both grossly and microscopically. The hypotheses proposed by the author ask if autonomic control of neutrophils is present in juvenile cattle, and whether dysfunction of such a regulatory mechanism occurs following exposure to inflammatory stimuli that are believed to be relevant to the acute inflammatory stage of BRD.

PART II: LITERATURE REVIEW

CHAPTER 1: Literature Review

1.1 Bovine Respiratory Disease and the Beef Industry

Bovine respiratory disease (BRD), also known as shipping fever, is an acute fibrino-necrotizing bronchopneumonia that affects young beef and dairy calves. BRD is characterized by an acute inflammatory response to a bacterial infection in the lung, involving large numbers of neutrophils and severe amounts of edema, fibrin, and necrotic cellular debris in the lumen of bronchioles and alveoli.[60, 61] The clinical manifestations of this disease may include fever, depression, labored breathing, tissue hypoxia, and eventual multiple organ failure and / or death. Most cases of BRD are initiated by viral or stress-mediated immunosuppression, followed by bacterial colonization of the lower respiratory tract. Management practices play a significant role in whether or not an animal is deemed "at risk" for the disease. Considering the collective influence of individual management practices, pathogen exposure, and immune status, BRD provides an excellent platform for describing the basic mechanisms of the acute inflammatory response and its regulation.

1.1.1 Epidemiology

BRD, which accounts for 75% of all beef cattle illnesses, is considered to be the number one disease problem of beef cattle in the United States and Canada. BRD affects 30% (total production morbidity rate) of all beef cattle. Although the total production mortality rate is 1-2%, BRD is the most common cause of mortality (24% of all beef calf mortalities).[1, 2] BRD remains a disease that affects more calves than adult cattle. For instance, in 1995 more adult cattle died from calving problems than BRD. In 1995 an estimated 374,000 beef calves and another 256,000 dairy calves died of BRD. However, the percent of total lost was greater for dairy calves (35% vs. 24%, respectively).[62]

1.1.2 Economic Impact

BRD has a large impact on profits, costing the beef industry an estimated $640 million to $1 billion per year.[63, 64] Although BRD affects many facets of the beef industry, from cow-calf operator to retailer, it is the feedlot operators who are most affected. In 1995, the estimated total value of all beef cattle and calves that died of BRD was $478 million.[3] Although cattle death is a concern among feedlot operators and veterinarians alike, mortality is not the primary reason for monetary loss. BRD significantly affects feedlot feed efficiency and human labor costs, as attempts to regain the efficiency of diseased animals are made. The average cost for chemotherapy (antimicrobial agent) and / or an anti-inflammatory drug is $10 per animal, not including costs incurred from a higher plane of nutrition, labor, and the indirect cost of decreased weight gain. The overall decrease in feedlot efficiency affects the profitability of the operation, and ultimately the costs are passed on to the consumer.

1.1.3 Clinical Manifestation of Disease

Depending on how long an animal is sick, the clinical signs associated with BRD may progress from nasal discharge, coughing and fever to labored breathing, decreased food and water intake, and depression.[64] Major mechanical pulmonary disturbances are often noted with moderate to extreme cases. For instance, increased respiratory rate, decreased tidal volume and lung compliance, ventilation-perfusion mismatch and pulmonary hypertension have all been observed in experimentally-induced BRD.[3] These disturbances are primarily the result of inflammatory responses within the pulmonary interstitium; however, inherent anatomic features of the bovine lung are believed to put cattle at higher risk for infection.[65] Functional lung mass (gaseous exchange capacity) in bovine species is smaller than that in other mammalian species, including horses, dogs, and humans, and cattle have been found to use a greater proportion of their lung mass for

basal breathing. Also, the bovine lung lacks Pores of Kohn, thus increasing pulmonary compartmentalization and the potential for airway (individual bronchiole) collapse / loss of function. Post mortem examinations of lung tissue from animals with BRD typically reveal large numbers of neutrophils and macrophages, lesser numbers of lymphocytes, and severe amounts of edema, fibrin, and necrotic cellular debris in the lumen of bronchioles and alveoli, affecting several lobules at a time to entire lobes of lung.

The typical case of BRD invariably originates at a cow-calf operation, where 4-6 month old calves are weaned, castrated, vaccinated, dehorned, and loaded on to an 18-wheel diesel truck for transportation to auction. This is usually performed over a period of 1-3 days, most often in Autumn. Approximately 85% of all beef cattle operations sell their calves by auction.[66] Calves may be sold at up to 5-10 different auctions, possibly changing ownership each time. Each auction may last from 1-3 days, and the calves are usually off feed and water while transported. Hydration is believed to be an important factor for adequate mucociliary apparatus function and therefore pulmonary mechanical defense function.[65] Upon arrival at an auction, the calves are unloaded from the trucks, mixed and sorted according to breed, body type, or size. Here, exposure to potentially novel viral and bacterial pathogens from other herds is a major challenge to the immune system. If not vaccinated already, calves are often given primary vaccinations for a number of viral and bacterial pathogens at the auction site. Although the bovine immune system is not fully developed until at least one year of age, calves are exposed to these stressors within days of weaning. Not surprisingly, the BRD rate is higher in calves than yearlings in these situations.

Upon their final arrival at a feedlot, approximately 30% of calves exhibit subtle clinical signs of upper airway infection. Despite this fact, 84% of calves are processed within 24 hours of arrival at feedlots. Processing may include castration, dehorning, administration of vaccines and / or growth implants, treatment for parasites, or other procedures; 98% of feedlots vaccinate calves for BRD when they arrive.[67] The validity of these processing procedures comes into question when one considers the data from the Bruce County (Ontario, Canada) Beef Cattle Project, which states that mortality rates are

highest when calves are vaccinated from 0-14 days post arrival at a feedlot.[1] Indeed, placing further demands upon an immune system that is already compromised and immature may bring about new cases of BRD in calves that were not displaying clinical signs on arrival.

The infection may progress to pneumonia within 3-7 days if bacteria are successful at entering the lower lung. As airways become partially to completely occluded by an inflammatory cell infiltrate (consolidation), pulmonary function decreases and animals decrease their feed efficiency and growth rate. These effects are first noted upon at least 15% total lung mass consolidation; a more significant portion (up to 50%) of the lung may be consolidated upon diagnosis of clinical pneumonia. This process may become more severe; however, greater than 65% consolidation generally results in death of the animal. Calves may then take up to 2 weeks to clear the infection and repair damaged lung tissue, and 1-2% of the afflicted will die. In addition to possibly processing and / or vaccinating calves upon arrival, the typical routine for feedlot employees is to carefully observe the calves as they come off the truck and continue to monitor them during their first few days in the feedlot. Approximately 80% of all feedlot operations check calves more than once a day.[68] Calves that appear sick will be separated from the group and placed in a sick pen, where the therapeutic regime may consist of antimicrobials, anti-inflammatory agents, and / or a higher plane of nutrition.

1.1.4 Management Practices

Vaccine prophylaxis and chemotherapy comprise by far the largest efforts to reduce the impact of BRD on the beef industry. Yet, the argument has been raised that the most effective approach to consistently reduce BRD may not be “better medicine”, but changing the ways in which beef cattle are raised and brought to market. Indeed, numerous individual and industry-wide management-based approaches that agree with this argument have attempted to provide stable, cost-effective means for controlling BRD. Overall, they have experienced sporadic success, for a number of reasons.

Possibly the most promising approach is the "preconditioning program", where certain weaning and vaccination protocols are certified prior to shipping. Most states have their own programs, where a producer may select any one of several vaccination and / or weaning protocols. While in principle a majority of producers feel that this is effective in reducing calf sickness and death loss, most do not consistently see satisfactory returns for the added expense.[69] Moreover, a lack of flexibility in management of when to wean may play a role. This is evidenced by a recent United States Department of Agriculture survey of beef cattle producers. While the leading factor for determining when to wean is calf age or weight (50%), a significant percentage (25%) of those surveyed base their decision upon either tradition, grazing permits, or market contracts.[70]

Other options for a producer include having direct agreements with buyers, where calves are raised on a cow-calf farm, and are then directly transported to a stocker or feedlot operation. An estimated 10% of all beef cattle operations used this method in 1997.[71] Also, direct sales, where auctions are held over the phone and the cattle are transported directly from the producer to the feedlot, illustrate how the process can be streamlined even further. From the standpoint of reducing the incidence of BRD, there are a number of critical control points in bringing a calf to market. Because of these different control points, the communication between buyers, sellers, and transporters becomes very important to assuring that an animal will remain healthy and gain weight. For instance, decreasing the interaction between cattle from different cow / calf operations and / or feedlots, with the intention of decreasing potential exposure to pulmonary pathogens the animals have yet to be exposed, to may decrease the rate of BRD before they reach the slaughter house. This may take the form of direct to slaughter, all-in-one operations (following the model of the poultry and more recently the hog industry).

1.2 Pathogenesis of Bovine Respiratory Disease

1.2.1 Predisposing Factors

There are a number of predisposing factors associated with the development of BRD. Management and environmental factors, including co-mingling and transporting calves, extreme temperatures, and weaning are known to contribute. Stress experienced by the animal in these situations, as well as subsequent exposure to viral and / or bacterial organisms, is also considered to play an important role in the pathogenesis of BRD. More specifically, viral and bacterial insult, in addition to corticosteroid-mediated immunosuppression, provides an environment favorable for colonization of the lower airways by opportunistic bacteria. Interrelationships among the predisposing factors for BRD have intrigued producers, veterinary practitioners, and researchers alike for over 40 years, and much work has been performed to describe the link between stress, pathogen invasion, and impairment of the upper airway physical and immune defenses.

A correlation between the incidence rate of BRD, the time of year, and pattern of auction market sales is plausible. Autumn is known to be associated with the highest number of calves at auction and the highest number of BRD cases, it is traditionally the time of year when cattle are castrated, weaned, vaccinated, and shipped to auction markets and feedlots. The mixing of cattle, particularly calves, from different sources at these auction markets and feedlots seems to be a strong factor for the onset of BRD.[67] Also, regions that experience cold weather see a disproportionately higher number of BRD cases.[70] Road transportation increases plasma cortisol levels, resulting in increased neutrophil numbers, decreased T-lymphocyte numbers and decreased lymphocyte blastogenesis[68]. Yet, the risk of mortality due to BRD does not increase as the distance traveled from auction to feedlot increases.[69, 70]

1.2.2 Normal Flora of the Upper Respiratory Tract

For this review of the literature, the regions of the nasal cavity, nasopharynx, oropharynx, laryngopharynx, and trachea will be collectively termed the upper

respiratory tract, while the regions of the bronchi, bronchioles and alveoli will be collectively termed the lower respiratory tract. *Manheimia haemolytica*, *Pasteurella multocida* and *Haemophilus somnus* are the bacteria known to primarily contribute to the clinical presentation of BRD. These organisms are small, non-motile, encapsulated gram-negative cocco-bacilli with no flagellae. Their isolation from calf nasal mucus may vary from week to week, during which clinical signs of BRD may not be present.[71] Moreover, these organisms are rarely cultured successfully from tonsilar tissue of the oropharynx and nasopharynx unless the cattle have been previously inoculated.[72] Yet, these bacteria are considered normal flora of the upper respiratory tract with the potential to colonize the lower respiratory tract, and are carried between calves by nasal mucus, which is aerosolized following bellowing, coughing or sneezing, or is physically shared by contact. Rapid and complete horizontal transmission of *M. haemolytica* from calf to calf has been documented within days of co-mingling.[73, 74]

Until recently, *Pasteurella haemolytica* was thought to have two biotypes, type A and type B. However, after analyzing ribonucleic acid (RNA) sequences and performing deoxyribonucleic acid (DNA) hybridizations, *P. haemolytica* biotype A is now called *Manheimia haemolytica*, while *P. haemolytica* biotype B is now considered to be a completely different bacterial organism, *Pasteurella trelahosi*.[75] *M. haemolytica* retains the two serotypes (1 and 2) originally assigned to *P. haemolytica*. Although both *M. haemolytica* serotypes may be present in the nasal passages, serotype-2 typically predominates in healthy individuals. However, a stressful event may result in a shift in population towards serotype-1 (the more pathogenic of the two) for several days.[71, 73] *M. haemolytica* is considered by many as the leading causative organism in the establishment and progression of BRD. The role of *P. trelahosi* at this moment is unclear. Because of the long-standing belief that *P. haemolytica* was the primary bacterium responsible, BRD was commonly called pneumonic pasteurellosis. Moreover, the traditional association of BRD with the stressors of transportation has lead to the common name, "shipping fever".

1.2.3 Viral-Bacterial Synergy

Despite their significant contribution to the development of lower airway infection and pneumonia, bacteria are not the only type of pathogen involved in BRD. A number of viruses, too, have been identified in the upper respiratory tract from cattle infected with BRD. Among these viruses are Infectious Bovine Rhinotracheitis, also known as Bovine Herpes Virus Type-1 (IBR / BHV-1), Parainfluenza Type-3 (PI-3), Bovine Respiratory Syncytial Virus (BRSV), and Bovine Viral Diarrhea Virus (BVDV).[76-78] The relative populations of viruses in the airways of commercially raised beef cattle vary significantly and, although these pathogens are not believed to be normal flora of the bovine upper respiratory tract, they may be isolated during times of stress and infection.

The significance of respiratory viral infection in the pathogenesis of BRD is apparent when one carefully studies the lack of a consistent method for experimentally inducing BRD over the past 80 years. For instance, inoculation with isolates of *M. haemolytica*, *P. multocida*, or *H. somnus* from sick animals or stock cultures was a technique used from the 1920's through the 1950's to experimentally induce BRD, with little success.[79] This approach became popular again in the late 1970's, and continued through the 1980's, with only moderate success.[60, 61, 80, 81] In contrast, reports from the late 1950's through today that describe an initial viral exposure followed by an intra-tracheal deposition of a single species or combinations of species of bacteria illustrate a high success rate for inducing BRD. The most notable studies involved infecting calves with IBR, followed by inoculation with *M. haemolytica* four days later.[82, 83] Another study reported success when BRSV infection was followed by *H. somnus* intra-tracheal inoculation 8 days later.[84] Experimental infection studies have also illustrated the importance of using either calves that have not been vaccinated for *M. haemolytica*, or those considered negative for *M. haemolytica* and *P. multocida* infection following repeated nasal swabbing.[80, 85] Additionally, dose of the organism, as well as inclusion of *M. haemolytica* serotype-1 in the inoculate, are other factors that have been proven necessary for successful experimental induction of BRD in calves.[61]

The inflammatory events that constitute the body's response to viral infection of the upper respiratory tract may result in inflammatory cell influx, decreased airway epithelial cilia density, airway epithelium cell death, decreased goblet cell content, and interstitial edema.[86] The net effect of this mass movement of fluid, cells, and soluble components is edema, pain, and compromised function. Viral infection may also directly harm lymphoid tissue (tonsils) in the bovine oropharynx. Several investigations on the effects of BVDV on neutrophil and lymphocyte function, *in-vitro*, report that lymphocytes from infected calves demonstrate reduced blastogenesis in response to mitogen. Also, neutrophil *Staphylococcus aureus* ingestion, random migration under agarose, and production of reactive oxygen species (ROS, also known as free radicals) may decrease following BVDV infection.[87-89] Similar effects on bovine neutrophils by IBR have also been noted.[90] Viral infections are also known to result in increased levels of cortisol and epinephrine in the blood, which presumably inhibits neutrophil and lymphocyte activity.

1.2.4 The Role of Stress

Cold weather, dust, truck exhaust particulate matter, and viral infection all have the potential for causing direct, physical damage to nasal epithelium and the acute inflammatory response which follows. Upon adequate suppression of the upper respiratory tract's physical and immunological defense systems, colonization of the lower respiratory tract by *M. haemolytica*, *P. multocida*, and / or *H. somnus* is possible. Stress, defined by increased blood levels of cortisol as well as the catecholamines norepinephrine and epinephrine, also contributes to the pathogenesis of BRD.[91, 92] Whereas both acute and chronic stress result in increased blood neutrophil levels, chronic stress is associated with a decrease in circulating lymphocytes.[93]

A number of studies have examined the importance of stress as a variable in experimentally-inducing BRD. Cold stress, followed by intratracheal inoculation with *M.*

haemolytica, has been successful at inducing BRD in calves.[94] Weaning and transportation have a negative impact on vaccine-stimulated antibody production[91], and data from goats corroborate the idea that transportation stress is a significant predisposing factor for respiratory infection. For instance, goats inoculated intransally with *M. haemolytica* after first being transported and given the anti-inflammatory drug dexamethasone, displayed gross lung lesions consistent with pneumonic pasteurellosis, and positive cultures were obtained from the upper and lower respiratory tract. However, lungs from goats exposed to dexamethasone and *M. haemolytica* alone did not exhibit any signs of pneumonic pasteurellosis, nor did they yield positive cultures.[95] Inoculation of beef cattle with *M. haemolytica*, followed by repeated treadmill exercise yielded similar results.[96] Intrabronchial inoculation of *H. somnus* (which is not traditionally viewed as the primary pathogen responsible for BRD) was able to induce BRD in another study where the calves were treated with dexamethasone 24 hours prior to challenge.[97] However not all studies with beef cattle have proven as consistent. For instance, when calves were inoculated with IBR or *M. haemolytica* and transported to a feedlot, the calves proved more susceptible to IBR infection than *M. haemolytica* infection.[98]

Knowledge of how cattle handle stressful situations, both psychologically and physiologically, may be used to better understand the pathogenesis of BRD. Cattle are capable of sensing small changes in their environment. Indeed, they are creatures of habit, and readily respond to disturbances in their routine or environment by displaying signs of stress, such as excitement and increased adrenal gland secretions. These effects may persist for days or weeks. For instance, stressed cattle arriving at feedlots may require as long as 3 weeks for their normal feed intake to resume.[99]

Psychogenic stress results in hypothalamic production of corticotropin releasing hormone (CRH) by the hypothalamus.[100] CRH is released to the adenohypophysis of the pituitary gland via the adenohypophyseal portal system. Corticotropes in the *pars distalis* of the adenohypophysis respond to the CRH by producing adrenocorticotropic hormone (ACTH) and releasing it into circulation. ACTH arrives at the adrenal gland and stimulates the release of corticosteroids into the caudal vena cava. Corticosteroids such

as cortisol and corticosterone display both mineralocorticoid and glucocorticoid properties. Glucocorticoids are known to significantly affect the immune system, while mineralocorticoids modulate body water and electrolyte balance. The cells of the adrenal medulla, on the other hand, are directly innervated by pre-ganglionic neurons belonging to the sympathetic division of the autonomic nervous system (sympathetic nervous system). Acetylcholine, secreted by these neurons, stimulates the immediate release of norepinephrine and epinephrine by the adrenal medulla into the caudal vena cava.

The sympathetic nervous system-initiated production of epinephrine and the adenohypophysis-initiated production of glucocorticoids are physiologically dependent upon each other. More specifically, epinephrine released into the circulation initiates CRH release by activating alpha- (α) and beta- (β) adrenergic receptors in the central nervous system[101, 102] and adrenal cortex.[103] Conversely, glucocorticoids have been shown to enhance the effectiveness of epinephrine and norepinephrine in the body by increasing catecholamine output (blocking reuptake) and increasing tissue responsiveness to catecholamines. In summary, when a calf encounters a stressful situation, both epinephrine and glucocorticoid levels in the circulation increase.

Infection-mediated (physiologic) stress, such as acute inflammation, can also result in elevated blood glucocorticoid levels. For example, the inflammatory cytokines interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-six (IL-6) have all been demonstrated to increase glucocorticoid levels.[104] IL-1β-mediated glucocorticoid production is thought to be due to activation of both central nervous system and adrenal gland receptors.[103, 105, 106] Bacterial lipopolysaccharide (LPS), also known as endo-'toxin' because of its propensity to induce an acute inflammatory response, has also been recognized as a stimulant of CRH production and release.[100, 107] In summary, the onset of an acute inflammatory response in the bovine lung (involving the production of inflammatory cytokines), as well as the onset of bacterial replication in the lung (involving the production of LPS), contributes to an increase in blood glucocorticoid levels.

Whether the stress response by cattle is psychogenic or infection-mediated, glucocorticoids are known to alter pulmonary tissue and immune cell function. For instance, in one study, cold stress induced rapid changes in pulmonary function, such as increased carbon dioxide production, increased oxygen consumption, and increased alveolar ventilation.[94] Glucocorticoids have also been reported to inhibit secretion of mucus[108]. Stress is known to result in reduced lymphocyte counts in the peripheral blood, both in an absolute (sequestering lymphocytes in lymph nodes)[93] and relative manner (reducing the binding of marginal pool neutrophils to endothelium, effectively increasing the sampled population).[109] In addition to altering white blood cell kinetics, corticosteroids alter a number of neutrophil functions. For example, following administration of ACTH to live cattle, harvested neutrophils were found to demonstrate increased random migration, decreased myeloperoxidase-induced ROS production, and decreased lymphocyte blastogenesis.[110] Similar effects were seen when lymphocytes were incubated *in-vitro* with cortisol.[111] Infection with BVDV, as noted earlier, may result in decreased lymphocyte blastogenesis and neutrophil activation.[87] Moreover, when infection with BVDV is coupled with ACTH administration, the immunosuppressive effect on lymphocytes and neutrophils is enhanced.[112] Similarly, work with sheep that received ACTH showed an enhanced killing of neutrophils by *M. haemolytica* leukotoxin.[113] Glucocorticoids have also been demonstrated to reduce macrophage-mediated killing of bacteria and fungi.[114]

Although the significance of glucocorticoid-mediated immunosuppression and altered pulmonary tissue function has been appreciated for some time, only recently has attention been paid to the mechanism(s) of action. Glucocorticoids are able to pass through cell membranes and, once in the cell, they bind to cytoplasmic steroid receptors. The glucocorticoid-receptor complex translocates to the nucleus and may increase or decrease the transcription rate of various genes. This is done by binding to the regulatory region (response element) of certain genes, modulating transcription in response to external stimuli.[115] Glucocorticoids are known to increase the transcription rate of the gene(s) responsible for annexins (previously known as lipocortins), a class of cytosolic proteins that decreases the production rate of phospholipase A2 (PLA_2).[116, 117] PLA_2 is an

enzyme which acts on membrane phospholipids, generating the production of arachidonic acid. In the presence of either cyclooxygenase or lipoxygenase, arachidonic acid is then cleaved to form a family of prostaglandins and thromboxane A2 (TXA_2), or a family of leukotrienes, respectively. Modulation of PLA_2 activity, and thus reduction in the production of the ensuing infamous inflammatory mediators, is generally believed to be the primary mechanism of action of glucocorticoids.[118]

There has been significant debate over the advantage of glucocorticoid production during times of stress. For instance, glucocorticoids are known to provide energy by stimulating gluconeogenesis, decreasing glucose uptake by adipocytes, skin, fibroblasts and thymocytes by inducing translocation of the glucose transporter from the membrane to the cytosol, and by enhancing catecholamine-induced lipolysis. Glucocorticoids are also known to support the sympathetically-mediated fight-or-flight response by increasing vascular and myocardial responsiveness to catecholamines, which allows an animal to maintain blood pressure and, coupled with increased gluconeogenesis, assures glucose delivery to important "core" tissues (brain, kidney, liver, lungs and skeletal muscle). Also, glucocorticoids are thought to increase resistance to bacterial infection by redistributing neutrophils from the marginal pool to a circulating pool. However, redistribution of energy comes with a cost, resulting in reduced immune system activity due to heavy use of protein. Indeed, if glucocorticoid production is sustained long enough, and / or at a high enough level, the animal may be placed at a higher level of risk for infection. Nonetheless, by assuring adequate core tissue perfusion and nutrition, and by increasing tolerance to pain, authors argue that glucocorticoids allow an animal to successfully elude a wide variety of threats.[119]

1.3 Pathophysiology of Bovine Respiratory Disease

Viral infection of the upper respiratory tract, reinforced by stress, causes increased mucus production, decreased cilia activity, increased fluid extravasation, pain, and a loss of function.[76] This effectively sets the stage for bacterial multiplication along

the damaged airway epithelium of the pharynx and eventual deposition in the trachea, leading to a compromised mucociliary apparatus. As colonization of the primary and then secondary bronchi is attained, bacteria invade the bronchioles, alveoli, and vasculature. Clinical signs are not usually present until successful colonization of the terminal bronchioles and alveoli is achieved. A typical calf with BRD presents itself as a depressed animal that is coughing, and has nasal discharge and airway consolidation.[60, 61] If severe enough, hypoxemia may ensue, eventually resulting in multiple organ failure.

1.3.1 Activation of Airway Macrophages

Pulmonary bronchiolar and alveolar macrophages are the first immune cells to interact with replicating *M. haemolytica* in the microairways of the lower lung. Just beneath the alveolar macrophages are capillaries and venules that bathe the airways and facilitate an inflammatory response. Such facilitation involves an increase in intravascular hydrostatic pressure, endothelial cell contraction, and basement membrane exposure. This affords neutrophils, erythrocytes, plasma and soluble proteins access to the alveolar space.[120] A significant amount of fibrin may then be formed, functioning to trap bacteria and facilitate their removal. However, the accumulation of cells and protein-rich fluid may cause a ventilation / perfusion mismatch, as airway diameter is decreased and terminal bronchioles and alveoli are occluded.[121] Poiseuille's Law states that a smaller diameter results in greater velocity. Indeed, it has been proposed that narrowing of the upper airways may accelerate the deposition of bacteria in the lower respiratory tract, as higher velocity air may pass deep into the lung and carry microcolonies of bacteria.[64]

M. haemolytica replicating in the airways passively shed LPS and actively secrete leukotoxin. LPS is a component of the bacterial cell wall, and is shed by many gram-negative bacteria as they replicate. The cell wall of gram negative bacteria is composed of inner and outer plasma membranes. The outer membrane contains numerous proteins and carbohydrates. One of the carbohydrates is LPS and consists of what is called the

lipid-A moiety which embeds in the outer bacterial membrane, as well as a core oligosaccharide and an O-chain (repeating units chain of sugars). The lipid component and the polysaccharide O-chain justify this molecule's name, lipo-polysaccharide. The production of LPS reaches a maximal rate during log-phase bacterial growth in the lower airways, and macrophages possess a receptor to identify the presence of LPS.[122-124] Bovine macrophages are capable of also recognizing leukotoxin, a polypeptide and member of the repeats in structure family of toxins (RTX).[125] Other toxins in this family include the *Bordetella pertussis* adenylate cyclase toxin and the *Escherichia coli* 0157:H7 hemolysin toxin. Leukotoxin is actively produced and secreted by *M. haemolytica* in the airways, and binds to bovine neutrophils and platelets.[126]

Alveolar macrophages react to both *M. haemolytica* LPS and leukotoxin by releasing IL-1β, TNF-α, interleukin eight (IL-8) and nitric oxide (NO) into the pulmonary microenvironment.[125-133] Such an inflammatory response is initiated intracellularly as an increase in calcium concentration and nuclear transcription factor-kappa B (NF-κB) activity. The response continues as nearby macrophages, acting in a paracrine fashion, respond to TNF-α by releasing IL-8 for a prolonged duration. The NO produced by macrophages decreases vascular tone, adding to the increased hydrostatic pressure and fluid exudation.[134]

1.3.2 Neutrophil Migration in the Lung

A unique and elegant cooperation exists between pulmonary macrophages and endothelium to attract neutrophils to the lung. As the initial macrophages inflammatory response accelerates itself, an inflammatory mediator concentration gradient radiates from sentinel macrophages. Endogenous inflammatory mediators and bacterial products diffuse through the pulmonary interstitium into the vasculature. Once in blood, LPS is quickly bound to the endogenous blood proteins, LPS-binding protein (LBP) and soluble CD14. LBP and soluble CD14 are considered to be amplifiers, such that they allow for small amounts of LPS in the blood to induce a significant bodily response. The response

is initiated when vascular endothelial cells recognize soluble CD14 - LPS complexes, among other inflammatory mediators, and allow leukocytes to emigrate into the interstitium.

Two populations of neutrophils exist in blood circulation, those in the circulating pool and those in the marginal pool. The marginal pool consists of cells found along the blood vessel wall, outside the central laminar flow. There are no major phenotypic differences between the two neutrophil populations,[135] and all mature neutrophils are known to constitutively express L-selectin (CD62L) molecules on their plasma membrane.[136] As neutrophils enter the marginal pool, L-selectin interacts and forms a weak bond with endothelium. Many of these bonds break (as neutrophils shed L-selectin) and form again as neutrophils "roll" along wall of blood vessels. Indeed, the marginal pool is comprised of neutrophils temporarily attached to endothelium during the non-inflammatory state.

However, during the acute stage of BRD, neutrophils are directed outside the vasculature and into the lung. This multistep process is initiated when soluble CD14 - LPS complexes, TNF-α, and IL-1β stimulate capillary and post-capillary endothelial cells to express both P- and E-selectins on their surface, effectively "capturing" neutrophils.[4, 5] P-selectins are pre-formed and stored in intracellular granules for quick recruitment to the cell surface, whereas E-selectin expression on the endothelial surface first requires gene transcription. In the presence of other chemokines released from the site of injury, the stationary leukocytes are then stimulated to express the β_2-integrin protein complex components CD18 and CD11, also known as leukocyte function-associated antigen-1 (LFA-1) and MAC-1.[136] Human neutrophils are not known to have receptors for IL-1β and TNF-α; however, bovine neutrophils may in fact recognize TNF-α, which was noted in one study on initiating neutrophil deformation following exposure to TNF-α *in-vitro*.[9] This process of forming a stable adhesion to the vascular wall constitutes the second step in neutrophil emigration into the lung. When the neutrophil β2-integrin complex binds to the endothelial cell surface immunoglobulin intercellular adhesion molecule type-1 (ICAM-1, also known as CD54), bovine neutrophils adhere to

endothelium.[136, 137] ICAM-1 is only presented by endothelial cells stimulated by IL-1β and TNF-α. Adhesion with neutrophils stimulates endothelial cells to secrete IL-8 and platelet-activating factor (PAF),[4, 138] two inflammatory mediators that then recruit additional neutrophils to the blood vessel wall, thus enhancing the adhesion process. The significance of the leukocyte adhesion process to animal health is best demonstrated by the types of leukocyte adhesion deficiency (LAD) that occur in Holstein cattle, Irish Setter dogs, and humans. Specifically, with bovine leukocyte adhesion deficiency (BLAD), neutrophils do not express the β_2-integrin (CD18) and the affected individuals experience recurrent pneumonia, enteritis, periodontitis, and delayed wound healing, among other clinical signs.[139, 140]

The expression of one of the β_2-integrin complex proteins, LFA-1, on both bovine neutrophils and mononuclear leukocytes is accelerated by simultaneous exposure to either LKT or the LPS-LBP complex,[141, 142] evidence that these bacterial products are necessary for and may enhance bovine neutrophil recruitment to the lung. Regardless of the significance, the upregulation is due to membrane-bound CD14 glycoprotein molecules (also known as FcγRII receptors) and Toll-like receptor type-4 (TLR4) receptors on the surface of neutrophils functioning as binding sites for LPS-LBP complexes.[143-145] When TLR4 receptors are activated by the lipid-A portion of LPS, neutrophils experience an intracellular calcium flux and increase in activated PKC, allowing for β_2-integrin presentation on the cell surface, IL-8 release, and superoxide anion production.

Adhesion of the neutrophil to activated endothelium results in increased neutrophil intracellular tyrosine phosphorylation and calcium levels, causing a rearrangement of the cytoskeleton. As neutrophils spread across the endothelium, they appear almost flat and pseudopods become evident. Meanwhile, the endothelial cells may become activated by the vasoactive amines histamine and serotonin from nearby mast cells, contract, and expose underlying basement membrane. Leukotoxin has been demonstrated *in-vitro* to induce bovine mast cell histamine release, and may also facilitate neutrophil extravasation.[146] Although the vasodilatory effect of bradykinin, a

basic peptide generated as a result of activation of the Hagemen factor, on bovine pulmonary vasculature has been described *in-vitro*,[147] there are no reports describing its facilitation to neutrophil extravasation into the lung during BRD. As the neutrophils begin the last step in migration into the lung, they diapedese across the blood vessel wall in an organized, deliberate manner, reach exposed basement membrane and traverse the extracellular matrix of the pulmonary interstitium. Along the way, neutrophils themselves will secrete PAF, leukotriene B4 (LTB_4) and IL-8, upregulating their own adhesion and diapedesis processes.[6-8] These processes may be further supported by the neutrophil interaction with the extracellular matrix proteins collagen IV, laminin, fibronectin, thrombospondin, and heparin sulfate.[148]

Neutrophils emigrate toward the site of injury along an inflammatory mediator (chemoattractant) concentration gradient "highway". Indeed, once in the extracellular matrix, neutrophils are exposed to distinct concentration gradients of several inflammatory mediators. The mediators may be produced by a number of cell types in close proximity to the infection, such as macrophages in the airways, airway epithelium, mast cells in the interstitium, monocytes, lymphocytes, platelets, other neutrophils in the interstitium, and the bacteria themselves. Neutrophils have distinct machinery for sensing the concentration gradients, including receptors for the inflammatory mediators and intracellular signaling pathways. The signaling pathways may then result in the production of more inflammatory mediators, or may dictate cell movement and / or activation.

Three families of receptors dictate neutrophil activity: ion channel-coupled receptors, enzyme-coupled receptors, and G-protein-coupled receptors. Such a variety of receptor types allow the neutrophil to respond to different types of stimuli. For example, most inflammatory mediators produced by other host cells, such as PAF, IL-8, histamine, LTB_4, and complement fragment 5a (C5a) are known to activate G-protein-coupled receptors. However, bacterial-derived mediators, such as LPS in addition to host immunoglobulin ligands (Fc portion of antibodies) are recognized by enzyme-linked tyrosine kinase receptors.[12-14] The role of ion channel-coupled receptors is not described

in the literature as extensively, with the exception of PAF .[15]

Chemoattractants direct neutrophil migration through the fibrous structural proteins, proteoglycans, and glycosaminoglycans (including fibronectin and laminin), placing specific neutrophil actions under exquisite control. Low concentrations (10^{-10}M to 10^{-7}M) of chemoattractants such as PAF cause actin polymerization and an increase in intracellular calcium concentration.[10] These actin responses are rapid and transient, and are independent of extracellular calcium concentrations, suggesting that they are dependent upon release of calcium from the smooth endoplasmic reticulum. Low concentrations of several chemoattractants are also known to initiate calcium-mediated gene transcription, resulting in increased neutrophil pro-inflammatory receptor populations. For example, molecules of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (a multiprotein enzyme complex recruited to the cell surface by calcium and PKC) increase in number following neutrophil exposure to various chemoattractants. The same is true for CD14, the endothelial cell, macrophage, and neutrophil receptor responsible for recognizing LPS, as well as for the receptor for the bacterial-derived N-formylated methionine-leucine-phenylalanine polypeptide (fMLP) chemoattractant human neutrophil receptor.[149-152] Thus, chemoattractants effectively up-regulate the neutrophil's ability to respond to further stimulation, and are why neutrophils are considered "primed" following initial exposure to inflammatory mediators. Primed neutrophils elicit much greater effects on bacteria and surrounding tissue than non-primed neutrophils.[153] As neutrophils come in closer proximity to the site of infection, they are bathed in micromolar (or higher) concentrations of chemoattractants. This sets the stage for activation. For example, 10^{-6}M and 10^{-5}M concentrations of IL-8 and PAF initiate the neutrophil respiratory burst, as well as induce phagocytosis and degranulation.[9-11]

Leukotoxin is a secretory product of *M. haemolytica*, and diffuses to surrounding macrophages and blood vessels during an infection in the lower lung. Leukotoxin is a unique member of the RTX family, as it specifically binds to bovine neutrophils and platelets, recognizing transferrin-binding proteins on these cells.[154] At sublytic

concentrations, leukotoxin initiates the release of neutrophil granular constituents and inflammatory mediators, such as LTB_4, arachidonic acid metabolite, and ROS. Both intracellular and extracellular-source calcium are utilized, involving adenosine 5-triphosphate (ATP)-consuming calcium channels.[155-159] Thus, it is reasonable to conclude that in addition to host-derived chemoattractant inflammatory mediators, leukotoxin also forms a concentration gradient from the infection site. In addition to its chemotactic properties, leukotoxin has been known for some time to be cytotoxic to neutrophils.[160] At high concentration *in-vitro*, leukotoxin selectively destroys bovine neutrophils by forming hydrophilic pores in the cell membrane. This ultimately results in cell necrosis, specifically, apoptosis.[161-163] At this moment, the effect of neutrophil apoptosis on the acute inflammatory response is unknown.

The increase in chemoattractant concentration closer to the site of infection prepares the neutrophil for the killing and engulfment of bacteria. For instance, PAF elicits two different effects on neutrophils as the concentration of PAF increases. Low concentrations of PAF operating via the Gq-protein-coupled receptor pathway cause calcium release from the neutrophil smooth endoplasmic reticulum. This calcium flux allows for actin polymerization-based chemotaxis and secretory granule degranulation. However, at higher concentrations (micromolar and above), PAF initiates the oxidative (respiratory) burst, phagocytosis and degranulation.[10] This high concentration effect is dependent on cell membrane calcium channels to bring in extracellular calcium[15, 164] It is possible that PAF receptors are coupled to both G-proteins and calcium channels,[165-167] and thus a single chemoattractant may have two distinct effects on neutrophils. In summary, the process of neutrophils being primed and activated while they migrate along a chemoattractant concentration gradient involves many specific, sequential intracellular events.

1.3.3 Killing and Removal of *Manheimia haemolytica*

Bovine neutrophils possess a variety of tools used to destroy *M. haemolytica*. Whether the process involves phagocytosis or the production of destructive enzymes and reactive oxygen species, the bovine neutrophil response is dependent upon an orchestration of intracellular events. Activation of γ-immunoglobulin (antibody) receptors on bovine neutrophils by the Fc portion of antibodies results in a cellular "respiratory burst", and drives chemotaxis, phagocytosis, degranulation, and inflammatory mediator production. This process, called antibody-dependent cell-mediated cytotoxicity (ADCC), is how bovine neutrophils ultimately ingest and kill *M. haemolytica*.[168, 169] Ingestion of other materials by bovine neutrophils may experimentally occur in the absence of antibodies, such as chicken, turkey and human erythrocytes.[170, 171] This phenomenon is called antibody-independent cell-mediated cytotoxicity (AICC), and despite some evidence of lymphokine secretion by activated mononuclear cells, is poorly understood.

The activation of the complement system represents an additional mechanism for neutrophil activation during a response to infection. The formation of complement fragments support ADCC, such that bacterial organisms may be coated with both antibodies and complement and go on to stimulate neutrophils (a process known as the classical pathway for complement activation). However, complement may coat microbial surface molecules (LPS) or complex polysaccharides (i.e., yeast cell wall) in experimental settings with little or no additional exposure to antibodies and initiate the same neutrophil response (alternative pathway).[172] For example, the incubation of bovine plasma with *Saccharomyces cerevisiae* cell wall particles (zymosan) initiates the formation of complement fragment 3b (C3b) and its deposition on the cell wall particles. The coated cell wall particles are then capable of inducing superoxide anion production by bovine neutrophils via activation of its respective (C3) G-protein-coupled receptor.[172] However, antibodies may also coat the zymosan particles if the donor was previously sensitized to *S. cerevisiae*. Regardless, the process of C3b and antibody deposition on the surface of zymosan is called opsonization, and opsonized zymosan alone may initiate neutrophil phagocytosis, degranulation, and ROS production.

When describing the phenomenon of neutrophil activation and resultant capacity to kill bacteria, the term "respiratory burst" is used because of the neutrophil's shift from anaerobic (basal) metabolism to an aerobic glycolysis. This change occurs to fuel the biochemical reactions necessary to evoke significant cellular action. For instance, as a bovine neutrophil comes into contact with (opsonized) particles coated in antibody and C3b, the Fc-portion of individual antibodies binds to the neutrophil cell-surface tyrosine kinase receptor CD32 (also called FcγRII) and C3b receptor, respectively.[172] Activation of the CD32 signal transduction pathway in turn activates cytoplasmic phospholipase Cγ (PLCγ), which then cleaves the phospholipid cell membrane component, phosphatidyl inositol 4,5-bisphosphate (PIP_2), into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is thus freed from the membrane, opens smooth endoplasmic reticulum calcium channels (but not extracellular calcium channels),[173, 174] and activates the enzyme responsible for enhanced arachidonic acid metabolite (and thus prostaglandin) formation, namely PLA_2. The rise in intracellular calcium which follows IP_3 activation enables numerous actin polymerization-dependent neutrophil activities to then take place. For example, the pseudopods that allow neutrophils to "crawl" through the extracellular matrix and engulf particles are dependent upon protein filament synthesis and destruction, a calcium-dependent event.[175] Also, degranulation requires deliberate movement of vesicles from the cytoplasm to the cell membrane along microtubules, also a calcium-dependent event.[176] DAG, which remains attached to the cell membrane, recruits and activates PKC, the molecule responsible for recruiting the NADPH oxidase components along microtubules (also dependent upon the rise in intracellular calcium following IP_3 activation) to the cell membrane, thus allowing ROS to be formed. Activation of bovine neutrophil p38 mitogen-activated protein kinase (MAPK) also occurs following exposure to opsonized zymosan, and is known to be dependent upon IP_3 liberation.[177] MAPK is responsible for regulating the transcription rate of genes that encode cytokines (TNF-α, IL-1β, IL-8), among other proteins secreted by neutrophils.[178] In summary, ligation of neutrophil CD32 by the Fc portion of antibodies, and ligation of the C3b receptor by its ligand initiates a fundamental shift in neutrophil metabolism, a shift which drives chemotaxis, phagocytosis, degranulation, ROS formation, and inflammatory mediator production.

The production of ROS by neutrophils has intrigued clinicians and basic scientists investigating the inflammatory component of various diseases for over 30 years. In the case of BRD, bovine neutrophils are assumed to produce ROS in a similar manner as human neutrophils. In the presence of oxygen, NADPH oxidase transports electrons from NADPH to oxygen. This creates superoxide anion, which is then converted into hydrogen peroxide by superoxide dismutase (SOD). Myeloperoxidase (MPO), a neutrophil membrane-associated (hemoprotein peroxidase) enzyme found in azurophilic primary granules, completes the bactericidal process as the granules fuse with the phagosome, forming the phago-lysosome. Once degranulation has taken place, MPO gains access to hydrogen peroxide. In the presence of the halide ion chloride, MPO converts hydrogen peroxide into hypochlorous acid (HOCl). HOCl, the active ingredient in house bleach, is the most reactive of the ROS (100 times more than superoxide anion or hydrogen peroxide), as it binds to the cell wall and initiates destruction of *M. haemolytica*. Alternatively, if lysed erythrocytes are present during the acute inflammatory response and ROS production, the iron contained within free hemoglobin provides the substrate for the conversion of hydrogen peroxide into the hydroxyl radical, giving rise to the reaction's name - the fenton reaction. Also, NO produced by macrophages may combine with superoxide anion to form a much more potent reactive oxygen species molecule, perioxynitrite.[129]

1.3.4 Host Tissue Damage

The role of neutrophils in BRD is infection control, yet the deleterious effects of this inflammatory activity on the host may be greater than the lesions induced by the bacteria themselves. Thus, BRD is potentially a cascading disease. The second law of thermodynamics states that entropy, the degradation of matter to inert uniformity, is unavoidable and constant in the universe. In a word, entropy is disorder, and the living organism is thought to be at constant battle with entropy. The concept of homeostasis is based upon this classic struggle. Although disorder is never eliminated, it is kept at a

level where life can occur. Homeostatic mechanisms, such as fever, the immune system, hormones, etc., maintain that level. All types of cells in the animal body (different, but interdependent elements) have the capability, and responsibility, to maintain some aspect of homeostasis. In the case of BRD, disorder is created as bacteria replicate and disrupt the integrity of pulmonary tissue. The body responds to this altered state by inducing a fever and calling on phagocytic and other inflammatory cells to not only remove the source of injury, but to promote reconstruction of damaged tissue. Thus, the body attempts to bring itself back to a sustainable level of disorder. However, the result of the inflammatory response is often not resolution, but rather a progression of cell injury.

As neutrophils engulf a bacterium, a number of enzymes and other tissue-degrading molecules are produced and / or released in the phagosome, including ROS and proteases. Ultimately, a complete phagosome is achieved, and digestion is completed. However, until the phagosome is complete, the digestive molecules may be spilled into the extracellular space. This phenomenon of phagocytosis is referred to as "messy-eating". ROS are of particular concern during phagocytosis, because of their damaging effects on host tissue. Specifically, ROS damage tissue by radical addition (covalent attachment to lipids), electron transfer (thiol crosslinking of proteins), or atom abstraction (single strand breaks in DNA).[179] As a result, various structural and functional cell membrane components are damaged by the ROS, as the normally protective protease-antiprotease balance is altered. If ROS are spilled into the extracellular environment, host cells may be damaged or die. As host cells die, bacteria may gain access to vital nutrients released into the extracellular space. Thus, host tissue damage by ROS may elevate the severity of an infection.

The relationship between the neutrophil and endothelial cells during BRD is a particularly important one because of the role endothelium plays in initiating and amplifying inflammation in the lung. Therefore, damage to the endothelium by ROS is critical to the progression of BRD. Activated neutrophils are known to alter vascular responsiveness, as one study demonstrated the capacity of bovine neutrophils to reduce endothelium-dependent relaxation of bovine mesenteric artery.[180] This effect was

abolished with the addition of SOD, suggesting that the ROS superoxide anion is responsible. Also, experiments in this laboratory describe the presence of endothelial "blebs" following inoculation of sheep and rats with a modified-live *M. haemolytica* vaccine[59, 181] These vascular effects may either be caused by ROS or by the *M. haemolytica* organisms.[182]

In addition to endothelium, alveolar interstitium and larger airway epithelium are also sensitive to the acute inflammatory response. For example, as neutrophils leave the pulmonary vasculature and migrate towards the airways, matrix metalloproteinases and ROS are known to degrade glycosaminoglycans, the structural component of the lung's extracellular matrix [183]. However, some argue that neutrophils are not activated during migration through the parenchyma, at least to the extent of producing ROS.[184, 185]

The oxygen-dependent bactericidal effect of ROS is of paramount importance to the neutrophil and its ability to kill invading bacteria, as well as to the potential for host tissue damage. Equally as important are the oxygen-independent bactericidal mechanisms. Proteases, including elastase, may initiate a significant inflammatory response by degrading extracellular matrix tissue and increasing epithelial permeability.[186]

1.4 The Autonomic Nervous System and Lung Homeostasis

The autonomic nervous system is composed of neurons located in both the central and peripheral nervous systems. The central efferent (leaving the brain) motor part of the autonomic nervous system includes neurons in the spinal cord and brain stem. Spinal cord neurons mediate reflex responses, such as sweating or blood pressure alterations, while brain stem neurons receive input from afferent fibers and are responsible for several bodily functions, such as medullary control of respiration and hypothalamic control of body temperature. In contrast, the peripheral efferent motor part of the

autonomic nervous system includes neurons primarily in the abdomen and thorax, and provides involuntary control over all innervated structures of the body, excluding skeletal muscle cells. Autonomic nervous system neurons can be further categorized into either sympathetic or parasympathetic divisions. The two divisions differ in their anatomy, pharmacology, and physiologic role in the body, yet they typically innervate the same tissues.[187]

The peripheral efferent sympathetic neurons exit the spinal cord from the first thoracic vertebra to the first or second lumbar vertebrae. These efferent neurons leave the ventral horn of the spinal cord, synapse in sympathetic ganglia (most are paravertebral, along the rami communicans) with multiple (as many as 20) post-ganglionic neurons, and continue on to innervate their target tissues.[188] The sympathetic nervous system primarily affects the cardiovascular and pulmonary systems, along with visceral organs and other smooth muscle tissues. The effects of the sympathetic nervous system are most often characterized as those relating to the "fight or flight" response. Such effects include elevated heart rate and blood pressure, increased blood glucose, increased airway diameter, pupillary dilation, decreased digestive activities and increased leukocytes in circulation.

The peripheral efferent parasympathetic neurons exit the brain and spinal cord via cranial nerves III (oculomotor), VII (facial), IX (glossopharyngeal) and X (vagus), and between sacral vertebrae two to four, respectively. In contrast with the sympathetic division, parasympathetic neuron synapses occur in close proximity to the target tissue, often with just one post-ganglionic neuron. [188] The effects of the parasympathetic division are much more localized because of this. Such effects include decreased heart rate, increased digestive and absorptive capabilities, decreased airway diameter and pupillary constriction. Predominantly, these actions conserve energy and maintain organ function during rest. It is this reciprocity or functional antagonism of the two divisions of the autonomic nervous system which provides for exquisite autonomic control over the tissues of the body. Yet, these two divisions may complement each other, such as with male sexual function.

1.4.1 Autonomic Control of Airway Diameter

Regulation of airway diameter is under control of the autonomic nervous system. The parasympathetic division controls the primary (basal resting) tone, and is enforced by acetylcholine release from the vagus (10^{th} cranial) nerve at terminal junction sites in the lung. Acetylcholine binds to and activates muscarinic subtype-3 (M_3) receptors on airway smooth muscle cells, and causes an increase in intracellular calcium.[189] As a result, the cells contract and airway diameter decreases. The sympathetic division can reverse the primary tone and cause bronchodilation. This is achieved principally by pre-ganglionic sympathetic neurons stimulating the adrenal medulla to release norepinephrine and epinephrine into circulation. Norepinephrine and epinephrine then find their way to the lung and activate beta subtype-2 (β_2) adrenergic receptors on airway smooth muscle cells. Sympathetic effects on the lung are also achieved by post-ganglionic neurons that release norepinephrine at terminal junction sites on airway smooth muscle cells. Norepinephrine released by these neurons activates smooth muscle cell β_2-adrenergic receptors and thus induces bronchodilation.

β_2-adrenergic receptors are responsible for airway smooth muscle cell relaxation. This relaxation is accomplished by the receptor activating the membrane-bound, allosteric enzyme adenylyl cyclase (AC).[189] AC accelerates the conversion of the nucleotide ATP into a cyclic species, adenosine 3,5-cyclic monophosphate (cAMP). Once produced, cAMP activates protein kinase A (PKA). PKA phosphorylates smooth muscle myosin light chain kinase, causing smooth muscle cell relaxation and a resultant bronchodilation. Stimulation of the sympathetic nervous system may also cause a paradoxical bronchoconstriction. While epinephrine has a greater affinity for the β_2-adrenergic receptor than norepinephrine, both epinephrine and norepinephrine share the same affinity for the pulmonary smooth muscle cell alpha subtype-1 (α_1)-adrenergic receptor. Sympathetic neurons release norepinephrine at the terminal junction site near smooth muscle cells, and therefore may activate α_1-adrenergic receptors. These receptors

elevate intracellular calcium levels and cause smooth muscle contraction. It is not known precisely how well bovine airways are innervated by post-ganglion sympathetic neurons.

1.4.2 Autonomic Control of Vascular Tone

Primary vascular tone is under the control of the vasomotor center and the sympathetic division of the autonomic nervous system. This is accomplished by post-ganglion neurons directly innervating blood vessels, as well as by the adrenal medulla releasing norepinephrine and epinephrine into circulation. At the sympathetic terminal junctions on blood vessels, α_1-receptors predominate.[190] Because of this, and because sympathetic neurons innervate more resistance vessels (arteries and arterioles) than capacitance vessels (capillaries, venules and veins), the net effect of activating the sympathetic division of the autonomic nervous system is an increase in blood pressure. However, there exists one exception. Skeletal muscle arterioles and coronary arterioles have greater populations of β_2-adrenergic receptors. Therefore, activation of the sympathetic division also results in dilation of the vessels that feed skeletal and cardiac muscle.

Activation of the parasympathetic division causes vasodilation. To be more specific, post-ganglionic parasympathetic neurons release acetylcholine at the terminal junction site, endothelium. Acetylcholine binds to and activates the endothelial M3 receptor, stimulating the production of NO by the endothelial cell. This process is mediated by the endothelial cyclic nucleotide, guanosine 3,5-cyclic monophosphate (cGMP). When NO is released from the endothelial cell following parasympathetic stimulation, it reaches the underlying vascular smooth muscle by diffusion. There, NO causes smooth muscle relaxation (vasodilation), although the precise mechanism of action is not known.[191] Also, it is not clear whether NO and β_2-adrenergic receptors share similar signal transduction pathways within vascular smooth muscle. Alternatively, acetylcholine released at the terminal junction site of parasympathetic neurons may cause vasoconstriction. This is achieved when acetylcholine released by the neurons directly

reaches vascular smooth muscle cells, where it causes contraction. It is unclear how the anatomy of the parasympathetic terminal junction on vasculature lends itself to either relaxation or contraction at any particular time. However, during severe vascular damage or when endothelial cells have been denuded, acetylcholine no longer induces vasodilation and may cause vasoconstriction. This phenomenon may be significant during the acute inflammatory response.

1.4.3 Autonomic Control of Neutrophil Activation

The physiological domain of the autonomic nervous system is not limited to tissues directly innervated by sympathetic and / or parasympathetic division neurons. By secreting epinephrine and norepinephrine into circulation, the adrenal gland is the essential organ for autonomic control over circulating inflammatory and other immune cells. The idea that the nervous system is able to regulate inflammation is gaining support by researchers and clinicians, as they consider the effects of brain function and xenobiotics on the immune system.

In addition to smooth muscle tissue, β_2-adrenergic receptors have been found on eosinophils,[16] natural killer cells,[17] monocytes,[18] lymphocytes,[19-21] macrophages,[22, 23] mast cells,[24] and neutrophils[25] from numerous species of animals, including humans. However, to the author's knowledge, there are no reports describing the presence, or absence, of β_2-adrenergic receptors on bovine neutrophils.

The β_2-adrenergic receptor is a cell surface protein receptor that has seven transmembrane-spanning domains. Because of its reliance upon a heterotrimeric guanyl nucleotide-binding-regulatory protein (G-protein) for activation of its effector enzyme, AC, the β_2-adrenergic receptor is categorized as a G-protein-coupled receptor (GPCR). Activation of the neutrophil β_2-adrenergic receptor results in a signal transduction pathway that is quite similar to that found in smooth muscle cells. Following β_2-adrenergic receptor ligation, a specific species of the heterotrimeric G-protein,

stimulatory G-protein (G_s-protein), is activated and the α-subunit shuttles towards the membrane-associated allosteric enzyme, AC. AC accelerates the conversion of cellular stores of ATP into cAMP, which is responsible for activating cytosolic PKA. This results in the inhibition of myosin light chain kinase activity, causing inhibition of microfilament activity.[192]

The number of β_2-adrenergic receptors per human neutrophil (approximately 1,000) is quite small, compared to the amount of the chemotactic fMLP receptors (50,000). Despite this, only half of a human neutrophil's β_2-adrenergic receptors need to be occupied for complete inhibition of fMLP-mediated neutrophil superoxide anion production.[193] This feat is largely attributed to the amplifying capabilities of the β_2-adrenergic receptor signal transduction mechanism. For instance, for every human neutrophil β_2-adrenergic receptor, there exist 700 molecules of G_s-protein, resulting in the formation of 10,000 molecules of cAMP Therefore, a relatively low level of hormone in the blood is capable of having a significant effect on the inflammatory response. In contrast, the rat cardiac cell contains 17 molecules of G_s-protein and 3 molecules of AC for every beta subtype-1 (β_1)-adrenergic receptor.[194]

A functional role for neutrophil β_2-adrenergic receptors during the acute inflammatory response has been questioned. When studying the inhibition of fMLP-induced human neutrophil superoxide anion production *in-vitro*, the 50% inhibitory concentration (IC_{50}) of isoproterenol and epinephrine was found to be between $1x10^{-8}$M and $1x10^{-7}$M, a concentration commonly seen *in-vivo*.[25, 195-198] Other effects of activating human neutrophil β_2-adrenergic receptors and / or increasing intracellular cAMP concentration include: decreased chemotaxis,[26] decreased degranulation,[27, 28] decreased adhesion to endothelium,[29-31] decreased adhesion to bronchial epithelium,[32] decreased release of inflammatory mediators,[33-35] and decreased apoptosis.[36, 37] Thus, stimulation of the β_2-adrenergic mechanism affects all aspects of the neutrophil's role in acute inflammation. A paradoxical response to β-adrenergic receptor activation occurs in the bovine basophils. β_1-adrenergic receptors (a receptor subtype predominantly found in

cardiac tissue), enhance antigen-induced histamine release from protein-sensitized calf granulocytes.[199]

1.5 β-Adrenergic Receptor Mechanism Dysfunction

Dysfunction of the β-adrenergic receptor mechanism contributes to the pathophysiology of numerous diseases in both people and animals. Congestive heart failure,[38, 39] asthma,[40] cystic fibrosis,[41] atopic dermatitis,[42] pheochromocytoma,[43] myasthenia gravis,[44] hypertension,[45] and sepsis[46] have all been linked to decreased β-adrenergic receptor mechanism function. The etiologic factors involved are increased levels of catecholamines and / or inflammatory mediators. Provided with the appropriate concentration and duration of exposure, these endogenous molecules are capable of causing homologous (catecholamines) or heterologous desensitization (inflammatory mediators) of the β-adrenergic receptor mechanism on a variety of tissues.

1.5.1 Homologous Desensitization

Homologous desensitization is an eloquent and effective process, and its elucidation has opened the doors to a new paradigm in cell biology: receptor regulation. Homologous desensitization of G-protein-coupled receptors occurs when excessive exposure to ligand results in functional uncoupling of the receptors from their effector enzyme. This has been illustrated *in-vitro*, where β_2-adrenergic receptors on human neutrophils were uncoupled from AC, following exposure to a high concentration of isoproterenol ($1x10^{-4}$M).[200] As described earlier, activation of the β_2-adrenergic receptor mechanism results in the production of cAMP and an ensuing activation of PKA. However, upon reaching a threshold concentration within the cell, PKA may induce the production of the cytosolic enzyme G-protein receptor kinase type-2 (GRK-2). GRK-2 then phosphorylates the intracellular portions of agonist-occupied β_2-adrenergic

receptors,[201] which allows a second type of molecule, β-arrestin, to bind to the internal domain of β_2-adrenergic receptors.[202] In doing so, β-arrestin causes a stearic hindrance and keeps the β_2-adrenergic receptors from successfully transferring its signal to AC. When this occurs, a β_2-adrenergic receptor is considered functionally uncoupled from its effector enzyme. Homologous desensitization may progress to endocytosis, where the receptor is recycled back to the cell surface within 10-30 minutes.[203, 204] Once recycled, the receptor may be functionally coupled again to AC within 2 hours.[205] Worthy of mention are the findings that isoproterenol-induced homologous desensitization of human lymphocyte β-adrenergic receptors may be reversed by the addition of propranolol, a β-adrenergic receptor antagonist which competes with isoproterenol at the receptor site.[206] Thus, homologous desensitization is concentration, as well as time-dependent.

Although homologous desensitization is an adaptive response to β_2-adrenergic receptor stimulation, it may result in a clinically significant loss of receptor function. Most of the pioneering work on homologous desensitization of β-adrenergic receptors focused on the cellular and molecular mechanisms of heart failure in humans, in addition to animal models of heart failure. Homologous desensitization of cardiac myocyte β_1-adrenergic receptors, due to either elevated plasma catecholamines and / or exogenous β_1-adrenergic receptor agonists, is a common, yet seemingly paradoxical phenomenon.[49] When β_1-adrenergic receptors are functionally uncoupled from their effector enzyme (AC), cardiac myocytes do not respond as vigorously to additional catecholamines and / or β_1 agonist drugs.[51] Indeed, high plasma norepinephrine levels in human patients have been linked to a decrease in cardiac β_1-adrenergic receptor density and coupling to AC, and the body's attempt to increase cardiac output actually causes homologous desensitization of cardiac β_1-adrenergic receptors and a resultant decreased cardiac output.[50] Therefore, β_1-adrenergic receptor antagonists (popularly termed beta-blockers) have proven to be a rational approach to treating heart failure.[207] Beta-blockers decrease the amount of norepinephrine and epinephrine able to bind to β-adrenergic receptors, thereby decreasing the amount of PKA produced and the likelihood of GRK-2 activation.[208, 209]

The relevance of the pathophysiology of heart failure to that of BRD resides within the fact that homologous desensitization of β-adrenergic receptors may occur during episodes of elevated catecholamine blood levels in otherwise healthy subjects. Such acute stress may be experienced by beef cattle during the auction and transportation processes. Moreover, homologous desensitization of human neutrophil β_2-adrenergic receptors *in-vitro* has specifically been described.[200]

1.5.2 Heterologous Desensitization

Heterologous desensitization describes a process where activation of one type of GPCR results in the functional uncoupling of another type of GPCR. Heterologous desensitization is similar to homologous desensitization in that the intracellular domain of a GPCR is phosphorylated by a GRK (β_2-adrenergic receptors are phosphorylated by GRK-2). However, GRK-2 is one of many types of kinases present in cells, and these kinases may become activated by a number of signal transduction cascades and phosphorylate other GPCRs.[201, 210] Because of this, heterologous desensitization is often referred to as "receptor cross-talk". [Note that earlier another type of 'receptor cross-talk' was discussed, neutrophil priming. Neutrophil priming is also the result of one chemoattractant altering a cell's response to a second. However, with priming, the two signaling pathways *enhance* each other. Heterologous desensitization defines two signaling pathways that *antagonize* each other. For instance, fMLP, C5a, PAF and IL-8 are capable of desensitizing each other's primary effect on human neutrophil calcium mobilization.[47, 48] Also, human neutrophil exposure to PMA, LTB_4, or fMLP results in the decreased ability of isoproterenol to ligate β_2-adrenergic receptors.[211]]

The pathophysiology of asthma is suspected to revolve around dysfunction, but not depletion, of airway smooth muscle cell β_2-adrenergic receptors.[52, 53] A putative cause of dysfunction is continued parasympathetic nerve activity, in addition to airway smooth muscle exposure to inflammatory mediators. Pulmonary parasympathetic

neurons secrete acetylcholine at the airway smooth muscle-neuron junction, serving to decrease airway diameter and maintain a resting tone. Acetylcholine activates the airway smooth muscle M_3 receptor, causing a rise in intracellular PKC and calcium concentrations. Most inflammatory mediators also raise intracellular PKC and calcium concentrations. PKC and calcium work together to drive gene expression and smooth muscle cell contraction. However, at sufficient concentration, PKC is capable of causing heterologous desensitization of G-protein-coupled receptors, including the β_2-adrenergic receptor. For instance, high concentrations of PMA (a synthetic analogue of DAG, the membrane-bound molecule which activates PKC), and the muscarinic / nicotinic agonist, carbachol, (10^{-6}M & 10^{-4}M, respectively) demonstrated the ability to reduce the number of β_2-adrenergic receptors on bovine tracheal smooth muscle. The tissue's capacity to produce cAMP after exposure to isoproterenol was also diminished in the presence of the PKC-raising compounds.[54]

Heterologous desensitization has also been described in leukocytes. Human neutrophil exposure to PMA, LTB_4, or fMLP has been demonstrated to result in the decreased ability of isoproterenol to ligate β_2-adrenergic receptors.[211] It is believed that activation of PKC is the critical step in heterologous desensitization of β_2-adrenergic receptors.[212] To be more specific, one study illustrated how PMA may cause heterologous desensitization of human mononuclear leukocyte β_2-adrenergic receptors, in a dose-dependent manner. cAMP production (following exposure to 10^{-4}M isoproterenol) was reduced 55% when the leukocytes were pre-incubated with 10^{-6}M PMA for 30 minutes. Basal cAMP levels were also reduced (54%) when the leukocytes were incubated with PMA.[55] Similar to the work with bovine pulmonary smooth muscle just discussed, the activation of GRK-2 by PKC is believed to be the critical step in heterologous desensitization of human leukocyte β_2-adrenergic receptors.[212]

Taken together, these data illustrate the relevance of catecholamines to homologous desensitization of β-adrenergic receptors, and PKC to heterologous desensitization of β-adrenergic receptors. Measuring blood cell β-adrenergic receptor numbers and functionality as a means of monitoring and / or explaining disease states has

been performed for over 20 years. With most situations, cAMP production, but not β-adrenergic receptor number, is reduced, suggesting that the receptor is uncoupled from its effector enzyme, but is not internalized. This is in contrast to 'complete' desensitization, which requires both uncoupling and internalization of the receptor. Thus, it appears that a dysfunctional receptor signaling pathway is not dependent upon internalization of the receptor.

1.5.3 Pulmonary Dysfunction During Bovine Respiratory Disease

The opinion that viral infection and / or stress may lead to bacterial colonization of the lower lung is one shared by veterinarians and beef cattle producers alike. Yet, the mechanism of pulmonary immune and physical defense system impairment have not been explained adequately. It is possible that autonomic control of the lung is impaired or altered during infection, which would invariably alter pulmonary homeostasis. Indeed, impairment of adrenergic receptor function in pulmonary airway and vascular tissue from animals exposed to *M. haemolytica* is a unique, yet relevant approach to understanding the pathophysiology of BRD.

The effects of live *M. haemolytica* organisms on ruminant lung tissue function have been studied. Intra-tracheal inoculation of bull calves with 10^6 live *M. haemolytica* organisms impairs β-adrenergic receptor-dependent pulmonary venous, but not arterial, dilation.[56] As a result, inability to reduce pulmonary venous resistance may lead to increased hydrostatic pressure. This increases the potential for interstitial and alveolar edema and congestion. The effect of *M. haemolytica* on ovine pulmonary vasculature has also been studied. Both sheep pulmonary artery and vein samples exhibit reduced ability to relax in response to isoproterenol administration *ex-vivo* following *M. haemolytica* infection. This effect was only demonstrated when the endothelium was removed, suggesting a protective role for endothelium.[57] Also, a reduced ability of ovine pulmonary arteries to relax to the muscarinic agonist, carbachol, in addition to an exaggerated contractile response by pulmonary veins to carbachol,[213] may add to our

understanding of how *M. haemolytica* infection leads to pulmonary edema and congestion, as well as an increased influx of inflammatory cells into the lung.

Heat-inactivated *M. haemolytica* vaccines have long been known to cause immunologically-mediated hypersensitivity pneumonitis and fibrin exudation in the intact calf.[80, 214] This has sparked interest in characterizing the insult. The vascular effect of *M. haemolytica* vaccine inoculation in rats was observed. Inoculation with a commercially-available *M. haemolytica* vaccine caused aortic hyperresponsiveness to the vasoconstricting agent methoxamine (an α_1-adrenergic receptor agonist), and a decreased responsiveness to the vasodilating agents carbachol and isoproterenol.[58, 59] In addition to gross dysfunction of vascular responsiveness, microvascular insults may occur during *M. haemolytica* infection. This is evidenced by disturbances of the rat aorta endothelial surface following inoculation [181]. Numerous blebs were observed with scanning electron microscopy 3 days post vaccination. Such a disturbance may provide evidence of neutrophil influx and activation, and may either be caused by neutrophil ROS or by the *M. haemolytica* organisms.[182]

Viral infection may adversely affect autonomic control of upper respiratory tract epithelium and smooth muscle in a manner similar to *M. haemolytica*. Trachea and tertiary bronchial tissue sections taken from calves infected with IBR, PI-3, or both, exhibit reduced relaxation when exposed to isoproterenol.[215] This suggests a dysfunctional β-adrenergic receptor mechanism. Other researchers, who infected guinea pigs with *H. influenzae* or PI-3, observed reduced bronchial relaxation and enhanced constriction.[86, 216] In one study, such hyperreactivity was partly reversed by atropine administration, suggesting a role for exaggerated cholinergic innervation causing and / or adding to heterologous desensitization.[217]

Dysfunction of the β_2-adrenergic receptor mechanism during infection may be caused by the invading organism (putative production of a toxin that specifically interferes with the β_2-adrenergic receptor signaling pathway). Dysfunction following infection may also be the result of continued exposure to inflammatory mediators[218] and /

or catecholamines. Interestingly, *M. haemolytica* LPS defies categorization as either a bacterial toxin or inflammatory mediator. LPS is a component of the gram-negative bacterial cell wall that is shed during replication. LPS is recognized by neutrophil and endothelial cell LPS receptors and initiates an inflammatory response.[219] As such, LPS is not a specific toxin for the β_2-adrenergic receptor signaling mechanism. However, LPS is not an inflammatory mediator produced by the animal body. Rather, LPS is an inflammatory mediator produced by bacteria, and activates a tyrosine-kinase-coupled receptor signaling pathway. This pathway may interfere with β_2-adrenergic receptor signaling, likely by heterologous desensitization.[220] Thus, it is reasonable to consider the possibility that the events observed may be the result of continuous LPS exposure to vascular smooth muscle, causing β_2-adrenergic receptor desensitization.[56-59, 181, 213] Additionally, cattle are stressed during BRD. Continued catecholamine presence in the blood may increase the chances of β_2-adrenergic receptor homologous desensitization, as it typically increases blood catecholamine levels.

β_2-adrenergic receptor dysfunction following infection may also be the result of free radical damage to cell membranes. A common sequela to infection and inflammation is the production of free radicals, or reactive oxygen species (ROS). ROS provide a direct, generalized and physical insult to tissue and their receptors, and have been implicated in β-adrenergic receptor dysfunction. For instance, activated bovine neutrophils can reduce endothelium-dependent relaxation of bovine mesenteric artery.[180] This effect is abolished with the addition of SOD, suggesting that superoxide anion is responsible for the pathology. This study is important because it demonstrates the role endothelium plays in initiating and amplifying inflammation in the lung; damage to the endothelium by ROS is critical to progression of BRD. As a side note, neutrophil-produced ROS have also been implicated in cardiac β_1-adrenergic receptor dysfunction following ischemia-reperfusion.[221]

Migration of the bovine neutrophil into the lung is an important component to the acute inflammatory stage of BRD, and is orchestrated following interaction of chemical signals with cell-surface receptors. Although there currently is a significant body of

knowledge which illustrates how pro-inflammatory signals regulate neutrophil migration and activation in response to *M. haemolytica* infection, comparatively little is known about a bovine neutrophil receptor that may respond to anti-inflammatory signals and dampen the acute inflammatory response. Work with human leukocytes suggests that such a receptor may exist. For instance, the β-adrenergic receptor has been illustrated to decrease human neutrophil chemotaxis, degranulation, and activation.

In the context of various human disease states, the β-adrenergic receptor is found to be functionally uncoupled from its effector enzyme and, more rarely, decreased in number. For instance, work with human heart failure and asthma has characterized the process responsible for such decreased β-adrenergic receptor function, desensitization. Desensitization may be homologous or heterologous in nature, yet ultimately results in the phosphorylation of the intracellular component of β-adrenergic receptors. More recently, studies have also demonstrated dysfunction of β-adrenergic receptors in bovine and rat tissues following exposure to *M. haemolytica*, a major etiologic agent with BRD. However, it remains to be seen if any similarities and / or differences exist between β-adrenergic receptor dysfunction in human and bovine tissues in the context of clinically-relevant disease states.

PART III: MATERIALS AND METHODS

CHAPTER 2: Materials and Methods

2.1 Experimental Design

2.1.1 β-adrenergic receptor quantification

A comparative study of β-adrenergic receptor populations on various cell types was executed using the tritiated version of the hydrophilic β_1- / β_2-adrenergic receptor antagonist CGP-12177 ($^{[3H]}$CGP-12177, PerkinElmer, Boston, MA),[222] and employed the radioligand binding technique described elsewhere.[21] The total, specific and non-specific binding of $^{[3H]}$CGP-12177 to steer and dairy cow neutrophils, steer and dairy cow lymphocytes, and rat lymphocytes was quantified using a scintillation cocktail and a liquid scintillation counter (LS 6500, Beckman Coulter, Fullerton CA). Specificity of binding (receptor versus non-receptor sites) was determined by subtracting nonspecific binding values (binding of $^{[3H]}$CGP-12177 to neutrophils in the presence of $1x10^{-6}$M (-) propranolol, a general β_1- and β_2-adrenergic receptor antagonist) from total binding values (binding of $^{[3H]}$CGP-12177 to neutrophils in the presence of Hank's balanced salt solution).

The predominant β-adrenergic receptor subtype (β_1 or β_2) was investigated by comparing competitive inhibition curves for (-) isoproterenol, (-) epinephrine and (-) norepinephrine. Although isoproterenol, epinephrine, and norepinephrine each bind to β-adrenergic receptors; the known rank order of potency for the β_2-adrenergic receptor is as follows: isoproterenol > epinephrine > norepinephrine.[187] The presence of β_1-adrenergic receptors was investigated by comparing competitive inhibition curves for (-) propranolol, (+/-) ICI-118,551 (a selective β_2-adrenergic receptor competitive antagonist), and (-) atenolol (a selective β_1-adrenergic receptor competitive antagonist).

Preliminary experiments revealed that equilibrium binding of $^{[3H]}$CGP-12177 to bovine neutrophils was reached within 60 minutes of incubation at 39 ºC. Significant non-specific (non-receptor) $^{[3H]}$CGP-12177 binding was observed when neutrophil density exceeded $2x10^6$ cells per microwell. This finding was corroborated by the Combi-12 Cell Harvester operating manual, which states that the diameter of the impression made by the O-rings (9 mm) will hold a maximum of $2x10^6$ cells (Molecular Devices, Sunnyvale, CA). A counting cutoff parameter (7.5% counting precision) was selected for the liquid scintillation counter, which is the counting of radioactive disintegrations performed until 95 out of 100 counts per minute were within 7.5% of the mean. Such a parameter was chosen as the counting cutoff parameter rather than counts per given period of time because of the relatively low amount of radioactivity in the samples (and therefore high degree of variability). Indeed, the counting precision protocol allowed for standardization of the precision for all samples. The counting precision could not be set for a value lower than 7.5% due to time constraints; to improve the counting precision from 7.5% to 5.0%, counting time per sample would at least double, from 30-40 minutes to 60-80 minutes, for example.

The concentration of (-) propranolol used for $^{[3H]}$CGP-12177 binding specificity ($1x10^{-6}$M) was chosen from a competitive inhibition binding study (Figure 8), and was corroborated by numerous reports that describe the ability of (-) propranolol to express non-specific, membrane-stabilizing activity at concentrations equal to and greater than $1x10^{-6}$M.[223-225]

Hypothesis 1:

Saturation Binding of $^{[3H]}$CGP-12177 for the determination of maximum binding site density (B_{max}) and dissociation constant (K_D):

Treatment groups

a. Total Binding
    i. Dose range of $^{[3H]}$CGP-12177 + cells

b. Nonspecific Binding
   i. Dose range of $^{[3H]}$CGP-12177 + cells + $1x10^{-6}$M (-)propranolol
c. Specific Binding = [Total Binding] – [Nonspecific Binding].

Competitive Inhibition Binding of $^{[3H]}$CGP-12177 for the determination of dominant β-adrenergic receptor subtype.

Treatment groups

a. $^{[3H]}$CGP-12177 concentration @ 90% B_{max} ($3x10^{-10}$M) + dose range of either (-) isoproterenol, (-) epinephrine, (-) norepinephrine, (-) propranolol, (+/-) ICI-118,551, or (-) atenolol.

2.1.2 cAMP production

Activation of the β_2-adrenergic receptor on various cell types is known to result in G_s-protein-mediated activation of the membrane-bound enzyme adenylyl cyclase (AC) and rise in intracellular cAMP concentration. Therefore, quantification of intracellular cAMP is a useful endpoint for the characterization of a functional β_2-adrenergic receptor signaling mechanism in intact cells.

The Direct cAMP Enzyme Immunoassay Kit was employed to quantify bovine neutrophil intracellular cAMP concentration (Sigma Chemical Co., St. Louis, MO). The kit includes rabbit-source polyclonal antibodies to cAMP, which bind to either sample (bovine neutrophil) cAMP or kit cAMP, which is covalently attached to alkaline phosphatase. As sample cAMP concentrations increase, fewer molecules of alkaline phosphatase are allowed to bind to the goat-source, anti-rabbit antibody-coated well. A coloring reagent allows for a spectrophotometer to measure the degree of alkaline phosphatase binding in the well. The concentration of sample cAMP is calculated from a daily standard curve.

Terbutaline is a selective β_2-adrenergic receptor agonist, while isoproterenol will activate both β_1- and β_2-adrenergic receptors. 3-isobutyl-1-methylxanthine (IBMX) is a nonselective phosphodiesterase inhibitor, and has been employed in various studies on leukocyte cAMP production.[27, 28] Adenosine 3',5'-cyclic monophosphate, 8-bromo-sodium salt (8-bromo-cAMP) is a cell-permeable cAMP analog, which preferentially activates PKA and is constructed to be more resistant to phosphodiesterases than endogenous cAMP.[36] ICI-118,551 hydrochloride is a selective β_2-adrenergic receptor competitive antagonist, and has been used in various published studies on leukocyte β-adrenergic receptor density and function at $1x10^{-6}$M.[43, 211] PMA is a diacylglycerol (DAG) analog which imbeds itself within the cell membrane. PMA constitutively activates PKC, a second messenger for Gq-protein and tyrosine-kinase-coupled inflammatory mediator receptors.

Various concentrations of these compounds were used to investigate cAMP production by bovine neutrophils, and were identified following review of the literature and trial and error in the laboratory. No significant increase in cAMP production was observed when bovine neutrophils were pre-incubated with $5x10^{-4}$M IBMX for 15, 30, 60 or 120 minutes before exposure to various concentrations of terbutaline, compared to simultaneous exposure to terbutaline and $5x10^{-4}$M IBMX. Therefore, IBMX and terbutaline were added simultaneously to the neutrophils. For the competitive inhibition experiments, where the effect of terbutaline on cAMP production was antagonized by ICI-118,551, the EC_{80} concentration of terbutaline ($5x10^{-6}$M) was used. For the experiments that illustrated the effect of PMA on terbutaline-mediated cAMP production, the EC_{100} concentration of terbutaline ($5x10^{-6}$M) was used. The decision to use $1x10^{-6}$M PMA was made following consideration of results from preliminary studies, where (a) a dose-response study demonstrated $1x10^{-6}$M PMA to have a maximal effect on bovine neutrophil cAMP production, and (b) $1x10^{-6}$M PMA was demonstrated to increase bovine neutrophil extracellular acidification rate six-fold (data not shown).

Hypothesis 1:

Prior to using the kit, neutrophils were exposed to cAMP-elevating agents (isoproterenol or terbutaline and / or IBMX) for 5 minutes at room temperature. The reaction was stopped by placing the tubes on ice for 5 minutes and centrifuging at 100 x g for 5 minutes at 4 °C:

Treatment Groups:

a. Isoproterenol Dose-Response
b. Terbutaline Dose-Response
c. Terbutaline Dose-Response + $5x10^{-4}$M IBMX
d. $5x10^{-6}$M Terbutaline + $5x10^{-4}$M IBMX + $1x10^{-12}$M to $1x10^{-5}$M ICI-118,551

Hypothesis 2:

Prior to using the kit, neutrophils were incubated with PMA for 30 minutes at 39º C, and then cAMP-elevating agents for an additional 5 minutes at 39º C. The reaction was stopped by placing all tubes on ice for 5 minutes and centrifuging at 100 x g for 5 minutes at 4 ºC:

Treatment Groups:

a. $1x10^{-6}$M PMA
b. $5x10^{-4}$M IBMX
c. $1x10^{-6}$M PMA + $5x10^{-4}$M IBMX
d. $1x10^{-5}$M Terbutaline + $5x10^{-4}$M IBMX
e. $1x10^{-5}$M Terbutaline + $5x10^{-4}$M IBMX + $1x10^{-6}$M PMA

2.1.3 Superoxide anion production

Superoxide anion is a member of the reactive oxygen species, along with the hydroxyl radical and hydrogen peroxide. Both human and bovine neutrophils readily

produce superoxide anion upon exposure to various concentrations of certain inflammatory mediators, including opsonized zymosan.[47, 137] For the following experiments, superoxide anion molecules were identified and quantified using the cytochrome C reduction assay. Upon exposure to superoxide anion, iron within cytochrome C reduces, thus changing the optical properties of cytochrome C. The resulting change in optical density was measured by a 96-well plate spectrophotometer at 550 nanometer (nm), with a subtraction of background at 650nm.

Opsonized zymosan is made by incubating serum with zymosan, which is a powder of yeast cell wall particles (Zymosan A, Sigma Chemical Co.). The mode of action of opsonized zymosan is thought to be due to the leukocyte response to the presence of complement (activated via the alternate pathway) and immunoglobulins on the zymosan (thus "opsonized" zymosan). The concentration of 0.50 mg / well was in accordance with the protocol for opsonized zymosan provided by Dr. Dagmar Frank (College of Veterinary Medicine, Iowa State University, Ames, IA). This has been employed in previous veterinary studies.[87, 226, 227] However, preliminary studies revealed that β_2-adrenergic receptor activation has no effect on superoxide anion production when at least 0.50 mg / well of opsonized zymosan was used. Therefore, a study was designed to compare the effect of β_2-adrenergic receptor activation on bovine neutrophil superoxide anion production when 0.50 mg or 0.063 mg / well opsonized zymosan were used as the stimulant.

In testing the second hypothesis, 0.063 mg / well of opsonized zymosan was employed in addition to 0.50 mg / well because 0.063 mg / well was identified in a preliminary experiment as the threshold concentration for β-adrenergic receptor-mediated decrease in opsonized zymosan-dependent superoxide anion production (data not shown).

Hypothesis 1:

A total of $2x10^6$ neutrophils were added directly to wells of a 96-well plate, already containing Hank's balanced salt solution (HBSS), cytochrome C and

0.063 mg opsonized zymosan with or without terbutaline, IBMX and / or 8-bromo cAMP. A baseline optical density reading was then obtained and the plate vortexed at 52 x g for 30 minutes at 37 °C. After incubation, a second optical density reading was obtained, with the initial values subtracted.

Treatment Groups:

a. Terbutaline Dose-Response
b. IBMX Dose-Response
c. 8-bromo cAMP Time-Response
d. $1x10^{-4}$M IBMX + Terbutaline Dose-Response
e. $1x10^{-3}$M 8-bromo-cAMP at 15 Minutes Exposure + IBMX Dose-Response

Hypothesis 2:

A total of $2x10^{6}$ neutrophils were added directly to wells of a 96-well plate already containing HBSS, cytochrome C and opsonized zymosan. Terbutaline and / or IBMX and were then added. A baseline optical density reading was then obtained and the plate vortexed at 52 x g for 30 minutes, 39 °C. After incubation, a second optical density reading was obtained, with the initial values subtracted. Concentrations of opsonized zymosan were chosen based on the justification provided above.

Treatment Groups:

a. 0.063 mg opsonized zymosan / well + terbutaline dose-response
b. 0.50 mg opsonized zymosan / well + terbutaline dose-response

2.2 Materials

2.2.1 Animals

Two groups of 5 Angus-cross, castrated, male beef cattle (steers) 6-9 months of age were purchased by the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) from a local farmer and maintained on pasture while fed a supplementary concentrate diet (16% crude protein, Stocker / Heifer Grain Mix, Southern States, Richmond, VA) at 10 lbs / head / day. The first group was used for the radioligand binding studies, while the second group was used for the cAMP and superoxide anion production assays. All aspects of animal husbandry and procedures performed (blood collection) were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech, and implemented by staff at the VMRCVM Non-Client Animal Facility.

Whole blood was also collected from 5 adult lactating Holstein cows. At the time of blood collection, the animals were owned and housed by the College of Agriculture, Virginia Tech at the dairy center.

Male Sprague-Dawley rats (n=20), weighing between 350 and 375 grams, were purchased from a commercial supplier (Harlan Sprague Dawley, Indianapolis, IN). During and after the one-week quarantine period, the rats were maintained on a commercial diet (Purina Laboratory Rodent Chow, Purina Mills, St. Louis MO) and had access to water *ad-libitum*. Housing at the Virginia Tech Laboratory Animal Resources (LAR) Facility was under controlled conditions of temperature and humidity, with a 12-hour light-dark cycle. All such aspects of animal husbandry, as well as procedures performed (blood collection) were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech.

2.2.2 Reagents

All chemicals and buffers, unless otherwise noted, were purchased from Sigma Chemical Co. (St. Louis, Mo.).

2.3 Methods

2.3.1 Bovine neutrophil isolation

Neutrophils were harvested from steer and dairy cow whole blood following the method of Roth and Kaeberle[227], with modifications. For all experiments, neutrophils were harvested and analyzed within the same day. Flash hypotonic lysis was employed for the removal of erythrocytes in these experiments, and has been proven not to cause changes in human neutrophil morphology or chemotaxis, nor affect $^{[3H]}$CGP-12177 binding to human mononuclear leukocyte β_2-adrenergic receptors.[204, 228]

1) For each animal, 45 ml of whole blood were obtained by jugular venipuncture, using a 60 ml regular-tip polypropylene syringe and a 16-gauge 1.5" stainless steel needle.
2) Once collected, the needle was removed from the syringe and the blood was immediately emptied into a single 50 ml polypropylene centrifuge tube containing 5 ml of cold, sterile-filtered acid citrate dextrose (ACD) solution. The blood was mixed well by manual rotation of the tubes (end-over-end 3 times).
3) The tubes were transported to the laboratory (transit time of 15 minutes) and centrifuged at 1000 x g for 20 minutes at 25 ºC, utilizing no brake.
4) The plasma, buffy coat and 5ml of red blood cell pellet were aspirated from each tube using a 25 ml capacity glass pipetter and a hand-held electric pump, leaving behind 20 ml of red blood cell pellet in each tube.
5) 30 ml sterile-filtered Hank's balanced salt solution without calcium or magnesium (HBSS, Sigma Chemical Co.) were added to each tube at room temperature. The tubes were then mixed by manual rotation (end-over-end 10 times) and centrifuged at 1000 x g for 20 minutes at 25 ºC, utilizing no brake.
6) The diluted plasma and any remaining buffy coat material were aspirated. The red blood cell pellet was then aspirated until approximately 12 ml remained. 10 ml of the red blood cell pellet was then transported to a new 50 ml polypropylene centrifuge tube.
7) 20 ml of cold, sterile-filtered hypotonic phosphate-buffered water were added to each tube, 2 tubes at a time. The tubes were mixed well by manual rotation (end-over-end) for 1 minute, causing lysis of the red blood cells. Tonicity was then restored by adding 10 ml of cold, sterile filtered hypertonic phosphate-buffered saline to each tube and mixing by manual rotation (end-over-end 5 times).
8) Tubes were centrifuged at 480 x g for 10 minutes at 25 ºC.

9) The lysed red blood cell solution was decanted and the remaining neutrophil pellet was resuspended with 10 ml of HBSS, using a glass Pasteur pipette.
10) Steps 7 and 8 were repeated.
11) The lysed red blood cell solution was decanted and the remaining neutrophil pellet was resuspended with 10 ml of HBSS, using a glass Pasteur pipette. Tubes were centrifuged at 300 x g for 10 minutes at 25 ºC.
12) Step 11 was repeated two more times, for a total of 3 washes.
13) The neutrophil suspensions from all 5 tubes were pooled into one 50 ml polypropylene tube.
14) 200 µl of the cell suspension was removed and purity of the neutrophil population was determined using a flow cytometer. Usual result was 95-98 % neutrophils.
15) 10 µl of the cell suspension was removed and density of the neutrophil population was determined using a hemocytometer. The usual result was $4x10^6$ neutrophils per ml, total yield of 50 ml.
16) Neutrophil concentration was adjusted accordingly for each experiment ($2\text{-}4x10^7$ cells / ml).

2.3.2 Bovine Lymphocyte Isolation

Lymphocytes were harvested from steer and dairy cow whole blood following the method described in the Histopaque®-1077 technical bulletin (Sigma Chemical Co.), with modifications. For all experiments, lymphocytes were harvested and analyzed within the same day.

1) For each animal, 45 ml of whole blood were obtained by jugular venipuncture, using a 60 ml regular-tip polypropylene syringe and a 16-gauge 1.5" stainless steel needle.
2) Once collected, the needle was removed from the syringe and the blood was immediately emptied into a single 50 ml polypropylene centrifuge tube containing 5 ml of ACD solution. The blood was mixed well by manual rotation of the tubes (end-over-end 3 times).
3) The tubes were transported to the laboratory (transit time of 15 minutes) and centrifuged at 1000 x g for 20 minutes at 25 ºC, utilizing no brake.
4) All but 5 ml of plasma (closest to the buffy coat) were aspirated and discarded, using a 25 ml capacity glass pipette and an electric pump. Without altering the underlying red blood cell pellet, a 2ml capacity glass Pasteur pipette was used to remove 50% of the buffy coat material and transfer it to an empty 15 ml polystyrene centrifuge tube.
5) HBSS was then added to the 15 ml tube such that the final volume reached 9 ml. The contents were mixed well using a glass Pasteur pipette.

6) Using the same pipette, the 9 ml of buffy coat solution were removed and carefully placed over 6 ml of room temperature Histopaque®-1077 (Sigma Chemical Co.) in an empty 15 ml polystyrene centrifuge tube.
7) Tubes were centrifuged at 1000 x g for 30 minutes at 25 ºC, utilizing no brake.
8) Without removing any supernatant, 90% of the buffy coat material (from the top down) was removed using a glass Pasteur pipette. Care was taken not to aspirate any of the underlying Histopaque®-1077 and / or red blood cell pellet. The buffy coat was transferred to an empty 15 ml polystyrene centrifuge tube and 10 ml of HBSS was added, or until each tube was full.
9) Tubes were centrifuged at 300 x g for 10 minutes at 25 ºC.
10) Tube contents were emptied by decanting and 10 ml of HBSS was then added. The buffy coat pellet was gently resuspended using a glass Pasteur pipette.
11) Tubes were centrifuged at 100 x g for 10 minutes at 25 ºC.
12) Steps 10 and 11 were repeated two more times, for a total of 3 washes.
13) The pellets were resuspended in 10 ml HBSS gently using a glass Pasteur pipette.
14) The lymphocyte suspensions were pooled from all 5 tubes into one 50 ml polypropylene tube.
15) 200 µl of cells were removed for flow cytometry analysis. Usual result was 90 % lymphocytes.
16) 10 µl of cells were removed for hemocytometer-based cell counting. Usual result was $2x10^6$ cells per ml, with a total yield of 20 ml.
17) Cell concentration was adjusted for radioligand binding ($2x10^7$ cells / ml).

2.3.3 Rat Lymphocyte Isolation

Lymphocytes were harvested from rat whole blood following the method described in the Histopaque®-1077 lymphocyte isolation kit (Sigma Chemical Co.), with modifications. For all experiments, lymphocytes were harvested and analyzed the same day.

1) Rats were anesthetized individually via inhalation of 5% isoflurane for 2 minutes, using an induction chamber. Oxygen was provided at 2 liters / minute, and the combined gases were scavenged using an F-air canister. Once induced in the chamber, each rat was then placed on its back and general anesthesia was maintained via placement of a mask over the nose.
2) The abdomen of each rat was opened and 8-10 ml of whole blood was collected via abdominal vena cava venipuncture, using a 13 ml regular-tip polypropylene syringe which contained 1.29 ml of heparin (diluted with sterile saline, concentration of 100 units / ml), and a 21-gauge 1.5" stainless steel needle.

3) The needle was removed from each syringe and the whole blood was immediately emptied into a single 15 ml polystyrene centrifuge tube. Tubes were mixed well by manual rotation of each tube (end-over-end 3 times).
4) Tubes were transported to the laboratory (transit time of 15 minutes) and centrifuged at 1000 x g for 20 minutes at 25 ºC, utilizing no brake.
5) Using a 2 ml capacity glass Pasteur pipette, 50% of the buffy coat material was carefully removed, making sure not to remove any underlying red blood cell pellet. The buffy coat material was transferred to a new 15 ml polystyrene centrifuge tube.
6) HBSS was added to the tube such that the final volume reached 3 ml. The tube was mixed well using a glass Pasteur pipette.
7) Using the same pipette, the 3 ml of buffy coat solution were removed and carefully placed over 3 ml of room temperature Histopaque®-1077 (Sigma Chemical Co.) in a new 15 ml polystyrene centrifuge tube.
8) Tubes were centrifuged at 400 x g for 30 minutes at 25 ºC, utilizing no brake.
9) Without removing any supernatant, 90% of the buffy coat material (from the top down) was removed using a glass Pasteur pipette. Care was taken not to aspirate any of the underlying Histopaque®-1077 and / or red blood cell pellet. The buffy coat was transferred to a new 15 ml polystyrene centrifuge tube and up to 10 ml of HBSS was added or until each tube was full.
10) Tubes were centrifuged at 300 x g for 10 minutes at 25 ºC.
11) The contents of the tube were decanted and 10 ml of HBSS was added. The buffy coat pellet was gently resuspended using a glass Pasteur pipette.
12) Tubes were centrifuged at 100 x g for 10 minutes at 25 ºC.
13) Steps 10 and 11 were repeated two more times, for a total of 3 washes.
14) The pellet was resuspended in 10 ml of HBSS gently using a glass Pasteur pipette.
15) The lymphocyte suspensions from all tubes (1-2 per day) were pooled into one 15 ml polypropylene tube.
16) 200 µl of the suspension was removed for flow cytometry analysis. Usual result was 90 % lymphocytes.
17) 10 µl of the suspension was removed for hemocytometer-based cell counting. Usual result was $2x10^6$ cells per ml, with a total yield of 20 ml.
18) Cell concentration was adjusted for radioligand binding ($2x10^7$ cells / ml).

2.3.4 Total and Specific $^{[3H]}$CGP-12177 Binding for Receptor Quantification

Saturation Binding of $^{[3H]}$CGP-12177

1. 125 µl of RPMI and 25 µl of various concentrations of [5,7 – ^{3}H] (-) CGP-12177 (33 Ci / mmol, lot # 3499-043, PerkinElmer Life and Analytical Sciences, Inc., Boston, MA) were added to the first row of 300 µl wells in round bottomed 96-well microplates.
2. 100 µl of cells at $2x10^{7}$ cells / ml ($2x10^{6}$ cells) were then added to the second and third row of microwells.
3. For total binding, 25 µl of various concentrations of $^{[3H]}$CGP-12177 and 25 µl of HBSS were then added to the second row of microwells.
4. For nonspecific binding, 25 µl of various concentrations of $^{[3H]}$CGP-12177 and 25 µl of $6x10^{-6}$M (-) propranolol were added to the third row of microwells.
5. The plate was placed in a water bath at 39 ºC for 60 minutes.
6. The plate was removed from the bath and placed on ice for 5 minutes.
7. Harvesting of intact cells onto 1.0 µm glass fiber FilterMat paper (Molecular Devices, Sunnyvale, CA) was performed using the Combi Cell Harvester model # 11025 (Molecular Devices, Sunnyvale, CA). Specifically, contents of the microplate wells (12 at a time) were simultaneously washed with room-temperature HBSS and aspirated, for 9 seconds. To reduce non-specific binding, the FilterMat paper was pre-moistened with HBSS for 2 seconds before harvesting.
8. The FilterMat paper was then placed in a drying oven, for 60 minutes at 50 ºC.
9. The FilterMat paper was removed from the drying oven and placed on the 96-well FilterPunch apparatus (Molecular Devices, Sunnyvale, CA). The round impressions made by the O-rings (diameter 9 mm) following harvesting were punched into 5 ml- capacity VialKit plastic tubes (Molecular Devices, Sunnyvale, CA).
10. 3 ml of Ultima Gold F non – aqueous scintillation cocktail (PerkinElmer) were added to each of the VialKit tubes.
11. The VialKit tubes were capped, and disintegrations were counted using the LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Fullerton, CA), until a counting precision of 7.5% was achieved (typically 30 – 45 minutes per sample).
12. Disintegrations per minute were converted to counts per minute by the scintillation counter. Counts per minute (CPM) were then converted to fmol $^{[3H]}$CGP-12177 bound / 10^{6}cells, which comprised the Y-axis (assuming $2x10^{6}$ cells / well and 50% counting efficiency).
13. For each concentration of $^{[3H]}$CGP-12177 examined, nonspecific binding values were subtracted from total binding values to obtain specific binding values. Methods for calculation of maximal receptor binding (Bmax) and the dissociation constant (KD) are found in section 2.3.7 (Data Analysis).

<u>Competitive Inhibition Binding of $^{[3H]}$CGP-12177</u>

1) 125 µl of RPMI and 25 µl of various concentrations of $^{[3H]}$CGP-12177 were added to the first row of 300 µl wells of round bottomed 96-well microplates.
2) 100 µl of cells at $2x10^7$ cells / ml ($2x10^6$ cells) were then added to the second, third, fourth and fifth rows of microwells.
3) For total binding, 25 µl of $2x10^{-9}$M $^{[3H]}$CGP-12177 and 25 µl of HBSS were then added to the second row of microwells.
4) For nonspecific binding, 25 µl of $2x10^{-9}$M $^{[3H]}$CGP-12177 and 25 µl of various concentrations of either (-) isoproterenol, (-) epinephrine, (-) norepinephrine, (-) propranolol, ICI-118,551, or (-) atenolol were added to the third, fourth and fifth row of microwells, respectively. The experiment continued as described in steps 5 – 11 above.
5) Disintegrations per minute were converted to counts per minute by the scintillation counter. CPM was then converted to fmol $^{[3H]}$CGP-12177 bound / 10^6cells, which comprised the Y-axis (assuming $2x10^6$ cells / well and 50% counting efficiency).
6) The Y-axis percent of control values were obtained by dividing the specific binding value (fmol $^{[3H]}$CGP-12177 bound / 10^6cells) of $3x10^{-10}$M $^{[3H]}$CGP-12177 (90% of B_{max})in the presence of various concentrations of other (competing) ligands by the specific binding value of $3x10^{-10}$M $^{[3H]}$CGP-12177 alone.

2.3.5 cAMP Production

Neutrophil Preparation

1) Steer neutrophils were harvested as noted above and brought to a final concentration of $2x10^7$ cells / ml.
2) 500µl of the cells were added to 12 x 75 mm borosilicate glass tubes already containing 50 µl of HBSS or varying concentrations of cAMP-elevating agents (isoproterenol or terbutaline with or without IBMX) or PMA.
3) The tubes were gently agitated (vortexed at 500 rpm for 2 seconds) and allowed to sit either at room temperature for 5 minutes or in a water bath at 39 ºC for 30 minutes.
4) The reaction was stopped by placing all tubes on ice for 5 minutes and then centrifuging at 300 x g for 5 minutes at 4 ºC.
5) Tubes were then decanted and blotted on tissue paper, and the cell pellets were resuspended with 500 µl of 0.1M HCl (containing 0.1% Triton X-100 detergent) using a 100 µl pipetter.
6) Tubes then remained at room temperature for 20 minutes, followed by centrifugation at 600 x g for 5 minutes at 25 ºC.
7) Without disturbing the pellets, 100 µl of supernatant was removed from each tube and added to a single well of the cAMP Immunoassay Kit, in accordance with the experimental design.

For instructions for use of kit, please see: Technical Bulletin: Direct cAMP Enzyme Immunoassay Kit, 96 Well Kit, Product Number CA-200, Sigma Chemical Co, St. Louis, MO.

2.3.6 Superoxide Anion Production

Preparation of opsonized zymosan reagent

Preparation of bovine serum

1) 50 ml of whole blood were collected via jugular venipuncture (60 ml syringe with 16 gauge 1.5"needle) from 5 adult dairy cows. The blood was added to five 10ml glass "red top" Vacutainer® blood collection tubes (Becton Dickenson, Franklin Lakes, NJ).
2) The tubes were allowed to rest at room temperature for 2 hours, and then at 4 ºC overnight, to complete the clot.
3) Tubes were centrifuged at 1000 x g for 20 minutes at 25 ºC.
4) The serum from each tube was aspirated and pooled in 50 ml polypropylene centrifuge tubes.
5) Tubes were centrifuged at 480 x g for 10 minutes at 25 ºC, to pellet any remaining red blood cells.
6) The pooled serum was warmed to 39 ºC, using a magnetic stirrer and hot plate.
7) Warmed serum was used in step 5 of the opsonized zymosan procedure (see below).

Procedure

1) 1 g of zymosan A (Sigma Chemical Co.) was suspended in 100 ml filter-sterilized Hanks Balanced Salt Solution containing calcium and magnesium (HBSS, Sigma Chemical Co.).
2) The suspension was then homogenized using a glass, hand-held tissue grinder.
3) The homogenized suspension was then centrifuged at 450 x g for 10 minutes at 25 ºC.
4) The supernatant was decanted and the pellets were resuspended with 66.7 ml of HBSS (warmed to 39 ºC). The suspension was mixed with a magnetic stirrer for 5 minutes.
5) The 100 ml of warmed bovine serum (see previous protocol) was added, and the entire suspension was mixed with a magnetic stirrer for 60 minutes.
6) 16.7 ml of EDTA (pH 7.20) was added, and the entire suspension was mixed with a magnetic stirrer for 5 minutes.
7) The suspension was placed into 50 ml polypropylene tubes, and centrifuged at 450 x g for 10 minutes at 25 ºC.
8) The supernatant was decanted and the pellets were resuspended with 100 ml HBSS. The suspension was mixed with a magnetic stirrer for 5 minutes.

9) The suspension was then divided into twenty aliquots of 5 ml each, using glass vials. Aliquots were stored at -80 °C for up to 2 years.
10) Aliquots were stored at the concentration of 10 mg / ml. When 50 µl of this suspension was added to a 300 µl well, the concentration was 0.50 mg / well (1.67 mg / ml).

Preparation of Cytochrome C

1) 500 mg of cytochrome C from horse heart (Sigma Chemical Co., St. Louis, MO) was put into solution with 13.3 ml of filter-sterilized HBSS with calcium and magnesium (Sigma Chemical Co.).
2) The solution was then divided into 24 aliquots of 500 µl (18.8 mg) each, using micro-centrifuge tubes. Aliquots were stored at -20 ºC for up to 3 months.

Preparation of Superoxide Dismutase (SOD)

1) 15,000 units of SOD (Sigma Chemical Co.) were put into solution with 3 ml of filter-sterilized HBSS with calcium and magnesium.
2) The solution was then divided into twenty-four aliquots of 150 µl (750 units) each, using micro-centrifuge tubes. Aliquots were stored at -20 ºC for up to 3 months.

Preparation of Sodium Hydrosulfite (Dithionide)

1) 100 mg sodium hydrosulfite (Sigma Chemical Co.) was put into solution with 2.87 ml of filter-sterilized HBSS with calcium and magnesium (Sigma Chemical Co.).
2) A 1:10 dilution was performed to achieve $2x10^{-2}$M. This reagent was prepared and used daily.

Procedure

1) Two aliquots of cytochrome C, one aliquot of SOD, and one aliquot of opsonized zymosan were placed on the counter and allowed to warm to room temperature.
2) 500 µl from one aliquot of cytochrome C was transferred to Tube #1, which contained 7 ml of filter-sterilized HBSS w/ calcium and magnesium. 500 µl from the second aliquot of cytochrome C was transferred to Tube #2, which contained 6.85 ml of HBSS w/ calcium and magnesium.
3) 150 µl of SOD was then added to Tube #2.
4) Depending on the experiment, the opsonized zymosan suspension was either kept at the concentration of 0.50 mg / well or diluted 1:1 three times with HBSS to obtain a concentration of 0.063 mg / well.
5) 150 µl of cytochrome C with or without SOD was added to the appropriate wells.
6) 50 µl of opsonized zymosan was added to the appropriate wells.
7) 50 µl of neutrophils at $4x10^{7}$cells / ml ($2x10^{6}$ neutrophils) were added to the appropriate wells.

8) Either 50 µl HBSS or 50 µl treatments (cAMP-elevating agents, such as IBMX, terbutaline, or 8-bromo cAMP) were added to the appropriate wells.
9) 150 µl of $2x10^{-2}$M sodium hydrosulfite was added to a control well, and was brought to a final concentration of $1x10^{-2}$M by adding 150 µl of cytochrome C without SOD.
10) A baseline optical density (O.D.) reading was obtained using a spectrophotometer (550 nm, narrow band filter required), with 650 nm subtracted for specificity.
11) The 96-well plate was placed on a vortexer, using a platform head adapter. The plate was vortexed at 52 x g for 30 minutes at 37 ºC.
12) Values from the second reading on the spectrophotometer were subtracted from the baseline reading, and a net O.D. value was obtained for all wells.

2.3.7 Data Analysis

Radioligand Binding

For saturation and competitive inhibition binding experiments, data points (fmol $^{[3H]}$CGP-12177 bound / 10^6 cells) represent observations of pooled cells obtained from 5 animals per day over 3 days for n=3. The exception is rats, where pooled cells were obtained from 1-2 animals per day, over 8 days for n=8, where each day is represented by the average of two wells and each well contained $2x10^6$ pooled cells. The data points are graphed as either the mean or mean +/- standard error of the mean (SEM). Nonlinear regression curves were fit to the competitive inhibition (percent of control) binding data, using a sigmoidal dose-response (variable slope) approach.

Saturation of specific binding, as all available receptors are occupied, is expressed in this study as the maximum binding density (B_{max}). The concentration at which ½ the B_{max} is achieved is considered the equilibrium binding dissociation constant (K_D). The B_{max} and K_D values were obtained by performing nonlinear regression analyses on specific binding values for each cell type. These best-fit values for the binding parameters were then used to create Scatchard plots for each specific binding curve. To be exact, the specific binding data were transformed, to which X- and Y-axis intercepts were added using each curve's B_{max} and K_D (the B_{max} is the X-axis intercept, while the K_D is the negative reciprocal of the slope on the Scatchard Plot). The intercepts were

connected by a line, accurately reflecting the B_{max} and K_D values found by nonlinear regression. When completed, the X-axis represents specific binding (bound) with the units of fmol $^{[3H]}$CGP-12177 bound / 10^6 cells, while the Y-axis represents the ratio of specific binding to concentration of free radioligand (bound / free). Where relevant, the specific binding value at K_D was divided by the total binding value at K_D to provide the percent of total binding. The percent of total binding (at K_D) is a useful endpoint for comparing the amount of non-specific $^{[3H]}$CGP-12177 binding for each cell type. For competitive inhibition binding experiments, the percent $^{[3H]}$CGP-12177 bound was obtained by dividing $^{[3H]}$CGP-12177 with antagonist values by the $3x10^{-10}$M $^{[3H]}$CGP-12177 alone value. All statistical methods were performed, and all graphs were created using Prism 4.0 (GraphPad Software, San Diego, CA).

cAMP Production

Data points represent cAMP concentration in supernatant of $1x10^7$ lysed neutrophils (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, number of days sampled ranging between experiments). Statistically significant differences ($p<0.05$) between data points were identified by performing paired, two-tailed T-tests. All statistical methods were performed, and all graphs were created using Prism 4.0 (GraphPad Software, San Diego, CA).

Superoxide Anion Production

Data points represent superoxide anion production by $2x10^6$ neutrophils (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, number of days sampled ranging between experiments). Statistically significant differences ($p<0.05$) between data points were identified by performing paired, two-tailed T-tests. All statistical methods were performed, and all graphs were created using Prism 4.0 (GraphPad Software, San Diego, CA).

PART IV: RESULTS

CHAPTER 3: Presence and Function of the β_2-Adrenergic Receptor Mechanism in Bovine Neutrophils.

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3.1 Abstract

The objective of this study was to determine whether the bovine neutrophil possesses the β_2-adrenergic receptor, and whether activating various components of the β_2-adrenergic receptor signaling mechanism results in both an increase in intracellular cAMP production and a decrease superoxide anion production. A comparative study of β-adrenergic receptor populations on bovine neutrophils and lymphocytes and rat lymphocytes was executed using the hydrophilic β_1- / β_2-adrenergic antagonist $^{[3H]}$CGP-12177. Neutrophils and lymphocytes were obtained from five healthy 6 month-old Angus-cross steers and five adult Holstein cows, while lymphocytes were obtained from twenty 250g male Sprague-Dawley rats. Rat lymphocytes were found to possess the highest number of specific binding sites, followed by steer and dairy lymphocytes, and then by steer and dairy neutrophils. The β_2-adrenergic receptor was then identified as the dominant adrenergic receptor subtype on bovine neutrophils. The selective β_2-adrenergic receptor agonist terbutaline was able to stimulate bovine neutrophil cAMP production and decreased superoxide anion production in a dose-dependent manner, and was

dependent upon phosphodiesterase inhibition by IBMX. Superoxide anion production was also decreased by both 8-bromo-cAMP and IBMX, with the IBMX-mediated effect exaggerated by concurrent incubation with 8-bromo-cAMP. In summary, bovine neutrophils possess the β_2-adrenergic receptor, as well as the adenylyl cyclase and phosphodiesterase enzymes. The β_2-adrenergic receptor appears to act in concert with adenylyl cyclase to raise cAMP levels and decrease bovine neutrophil superoxide anion production, while the phosphodiesterase enzyme functions to antagonize these actions.

3.2 Introduction

The bovine respiratory disease complex (BRD), also known as shipping fever, is an infectious, acute fibrino-necrotizing bronchopneumonia that affects young beef and dairy calves. BRD accounts for 75% of all beef cattle illnesses, significantly affects feedlot feed efficiency, and is considered to be the number one disease problem of beef cattle in the United States and Canada.[2, 62] Affected animals demonstrate increased respiratory rate, decreased tidal volume and lung compliance, ventilation-perfusion mismatch and pulmonary hypertension.[3] These insults to the animal are the manifestation of an acute inflammatory response within the lung, involving large numbers of polymorphonuclear leukocytes (neutrophils) and severe amounts of edema, fibrin, and necrotic cellular debris in the lumen of bronchioles and alveoli. Neutrophils are directed into the lung via a multistep process, which is initiated when airway macrophages stimulate endothelial cells to present both P-and E-selectins. These proteins induce neutrophils to express the β_2-integrin protein complex and resultantly adhere to the vascular wall.[136, 137] The process continues when neutrophils undergo a protein-kinase-C and calcium-driven rearrangement of their cytoskeleton and diapedese across the blood vessel wall in an organized, deliberate manner. Neutrophils are capable of traversing the extracellular matrix of the pulmonary interstitium sensing, along the way, an inflammatory mediator (chemoattractant) concentration gradient.[6-8, 148] As neutrophils exocytose into the alveolar space, high endogenous and bacterial-source chemoattractant

concentrations prepare the cellular machinery for phagocytosis, degranulation, and reactive oxygen species (free radical) production.[15]

The migration of the neutrophil in the lung is orchestrated by three basic classes of cell-surface receptors that enable the recognition and integration of a myriad of chemical signals: ion channel-coupled receptors, enzyme-coupled receptors, and G-protein-coupled receptors.[12, 13] Chemoattractants such as platelet activating factor, interleukin-8, and leukotriene B_4 initiate pro-inflammatory actions by activating their respective G-protein-coupled receptors.[7, 8, 10, 11] However, comparatively little is known about cell surface receptors that may dampen the neutrophil inflammatory response.

Some characterization of leukocytes regulation has been done with human neutrophils and bovine macrophages, as it is now generally believed that the physiologic domain of the autonomic nervous system includes not only those tissues directly innervated by sympathetic and / or parasympathetic neurons, but also circulating leukocytes. For instance, β_2-adrenergic receptors have been characterized on eosinophils,[16] natural killer cells,[17] monocytes,[18] lymphocytes,[19-21] macrophages,[22, 23] mast cells,[24] and neutrophils.[25] Activation of the β_2-adrenergic receptor on these cells has been reported to result in the regulation of several aspects of leukocyte inflammatory activity including reactive oxygen species production,[25, 195-198] chemotaxis,[26] degranulation,[27, 28] and inflammatory mediator production.[33-35] However, despite such work describing the presence and function of the β_2-adrenergic receptor on leukocytes from various species, there are no reports that describe the presence of the β_2-adrenergic receptor on bovine neutrophils, nor the cellular machinery responsible for its signal transduction cascade. Therefore, because of the central role neutrophils play in the acute inflammatory stage of BRD, we investigated the presence and function of the β_2-adrenergic receptor mechanism in bovine neutrophils.

Several direct and indirect approaches are available for investigating the β_2-adrenergic receptor mechanism in bovine neutrophils. The radiolabeled (tritiated) version of the hydrophilic, β_1 / β_2-adrenergic receptor antagonist CGP-12177 has been

used successfully with quantifying β-adrenergic receptor density on human neutrophils, bovine lymphocytes, and rat lymphocytes.[21, 222, 229, 230] Activation of the β_2-adrenergic receptor is known to result in a rise in intracellular cAMP production in numerous cell types. Therefore, quantification of intracellular cAMP concentration was chosen as a tool for characterizing a functional β_2-adrenergic receptor signaling mechanism in intact bovine neutrophils.[26, 28, 32, 192] Superoxide anion, a member of the reactive oxygen species, is reported to be produced by both human and bovine neutrophils upon exposure to various concentrations of inflammatory mediators, including opsonized zymosan, and is easily identified via cytochrome C reduction.[226, 227, 231, 232] In particular, human neutrophil production of superoxide anion production reportedly decreases, in a dose-dependent manner, when β_2-adrenergic receptors are activated.[25, 198] Therefore, the use of a β_2-adrenergic agonist and other agents that modify the β_2-adrenergic receptor mechanism may prove useful for modulating bovine neutrophil superoxide anion production.

The present study compares β-adrenergic receptor density on steer neutrophils to that of dairy cow neutrophils, steer lymphocytes, dairy cow lymphocytes, and rat lymphocytes. This study also identifies the predominant β-adrenergic receptor subtype on steer neutrophils, and investigates the individual components of a functional bovine neutrophil β_2-adrenergic receptor signaling mechanism. In summary, this study demonstrates for the first time the presence and anti-inflammatory effects of the β_2-adrenergic receptor mechanism in bovine neutrophils.

3.3 Methods

3.3.1 Animals

A group of 5 Angus-cross, beef cattle (steers) 6-9 months of age were purchased by the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) from a local farmer and maintained on pasture while fed a supplemental concentrate diet (16%

crude protein, Stocker / Heifer Grain Mix, Southern States, Richmond, VA) at 10 lbs / head / day. All aspects of animal husbandry and procedures performed (blood collection) were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech, and implemented by staff at the VMRCVM Non-Client Animal Facility.

Whole blood was also collected from 5 adult lactating Holstein cows. The animals were owned and housed at the Virginia Tech, College of Agriculture, Dairy Center at the time of blood collection.

Male Sprague-Dawley rats (n=20), weighing between 350 and 375 grams, were purchased from a commercial supplier (Harlan Sprague-Dawley, Indianapolis, IN). During and after the one-week quarantine period, the animals were maintained on a commercial diet (Purina Laboratory Rodent Chow, Purina Mills, St. Louis MO) and had access to water *ad-libitum*. Housing at the Virginia Tech Laboratory Animal Resources Facility was under controlled conditions of temperature and humidity, with a 12-hour light-dark cycle. All such aspects of animal husbandry, as well as procedures performed (blood collection) were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech.

3.3.2 Bovine neutrophil isolation

Neutrophils were harvested from steer and dairy cow whole blood following the method of Roth and Kaeberle[227], with modifications. Briefly, whole blood was collected via jugular venipuncture and centrifuged at 1000 x g for 20 minutes at 25 ºC, utilizing no brake. Acid-citrate-dextrose was used as the anticoagulant. Following aspiration and removal of the plasma, buffy coat material, and approximately ½ of the remaining red blood cell pellet, 2 volumes of cold phosphate-buffered water were added for 1 minute to initiate red blood cell lysis. Tonicity was restored by adding 1 volume of hypertonic phosphate - buffered saline. The neutrophils were then washed 3 times with sterile-filtered Hanks Balanced Salt Solution without calcium and magnesium (HBSS, Sigma Chemical Co., MO), following centrifugation at 300 x g for 10 minutes at room

temperature. For all experiments, neutrophils were harvested and analyzed within the same 8 hour day. Cells were counted using a hemocytometer and a microscope, while purity was identified using forward and side scatter flow cytometry.

3.3.3 Bovine and rat lymphocyte isolation

Lymphocytes were harvested from steer, dairy cow and rat whole blood following the method described in the Histopaque®-1077 technical bulletin (Sigma Chemical Co., St. Louis MO), with modifications. Briefly, whole blood was collected via either jugular venipuncture (steers and dairy cows) or caudal vena cava venipuncture during general anesthesia (rats). Anticoagulated blood (acid-citrate-dextrose for steer and dairy cow, heparin for rat) was centrifuged at 1000 x g for 20 minutes at 25 ºC, utilizing no brake. The buffy coat material was then removed and added to HBSS. The resultant buffy coat suspension was then carefully layered over Histopaque®-1077, such that the final ratio of either 1.5:1 (steer and dairy cow) or 1:1 (rat) was achieved. Tubes were then centrifuged for either 1000 x g (steer and dairy cow) or 400 x g (rat) for 30 minutes at room temperature, utilizing no brake. The purified buffy coat material was removed just above the interface with Histopaque®-1077 and washed 3 times with HBSS following centrifugation at 300 x g for 10 minutes at room temperature. For all experiments, lymphocytes were harvested and analyzed within the same 8 hour day. Cells were counted using a hemocytometer and a microscope, while neutrophil purity was determined using forward and side scatter flow cytometry.

3.3.4 β-adrenergic receptor quantification

A comparative study of β-adrenergic receptor populations on various cell types was executed using the tritiated version of the β_1- / β_2-adrenergic receptor antagonist CGP-12177 ($^{[3H]}$CGP-12177, PerkinElmer, Boston, MA), and employed the radioligand binding technique described elsewhere.[21] Briefly, the total, specific and non-specific binding of $^{[3H]}$CGP-12177 to steer and dairy cow neutrophils, steer and dairy cow lymphocytes, and rat lymphocytes was quantified using a liquid scintillation counter and

scintillation cocktail. All binding studies were conducted on 100 µl aliquots of cell suspension ($2x10^6$ cells) in 300 µl wells of round bottomed 96-well microplates. Following 60 minutes of incubating the cells with various concentrations of $^{[3H]}$CGP-12177 at 39 °C, the reaction was stopped by placing the cells on ice for 5 minutes, followed by harvesting on to 1.0 µm glass fiber filter paper and washing for 9 seconds with room-temperature HBSS (Sigma Chemical Co., St. Louis, MO) using the Combi-12 Cell Harvester (Molecular Devices, Sunnyvale, CA). To reduce non-specific binding, the glass fiber filter paper was pre-moistened with HBSS for 2 seconds before harvesting. Once dried (60 minutes at 50 °C in a drying oven), the 9 mm diameter sections of filter paper were punched into individual 5 ml plastic tubes using the 96-well capacity FilterPunch (Molecular Devices, Sunnyvale, CA). To these tubes, 3 ml of non-aqueous scintillation cocktail (Ultima Gold F, PerkinElmer, Boston, MA) was added. The tubes were then capped and disintegrations were counted using the LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA). Counting was automatically stopped once a counting precision of 7.5% was achieved.

Specificity of binding (receptor versus non-receptor sites) was determined by incubating neutrophils with $^{[3H]}$CGP-12177 and / or $1x10^{-6}$M (-) propranolol, a general β_1- and β_2-adrenergic receptor antagonist. The predominant beta-adrenergic receptor subtype (β_1 or β_2) was investigated by comparing competitive inhibition curves for the β-adrenergic receptor agonists (-) isoproterenol, (-) epinephrine and (-) norepinephrine. The known rank order of potency of these drugs for the β_2-adrenergic receptor is as follows: isoproterenol > epinephrine > norepinephrine. The presence of β_1-adrenergic receptors was also investigated, and was done so by comparing competitive inhibition curves for the β-adrenergic receptor antagonists (-) propranolol, (+/-) ICI-118,551 and (-) atenolol. The known rank order selectivity of these drugs for the β_2-adrenergic receptor is (+/-) ICI-118,551 > (-) propranolol > (-) atenolol.[187]

3.3.5 cAMP production

cAMP production by bovine neutrophils was quantified using the Direct cAMP Enzyme Immunoassay 96 Well Kit (Sigma Chemical Co.). Neutrophils were harvested from steer whole blood and brought to a final concentration of $2x10^7$ cells / ml in HBSS containing calcium and magnesium. 500 µl aliquots of the cell suspension were added to 12 x 75 mm borosilicate glass tubes, to which 50 µl of HBSS or any of the cAMP-elevating agents isoproterenol, terbutaline, 3-isobutyl-1-methylxanthine (IBMX) were added. When necessary, co-incubation with ICC-118,551 was included. The neutrophils then remained at room temperature for 5 minutes. The reaction was stopped by placing all tubes on ice for 5 minutes and centrifuging at 300 x g for 5 minutes at 4 ºC. Tubes were then decanted and the neutrophils were resuspended with 500 µl of 0.1M HCl + 0.1% Triton X-100 for cell lysis. Following a 20 minute incubation period at room temperature, tubes were centrifuged at 600 x g for 5 minutes at room temperature. From each tube, 100 µl of supernatant were removed and placed into the corresponding wells of the enzyme immunoassay kit. Analysis of cAMP concentration in each supernatant was performed in accordance with the kit's technical bulletin.

3.3.6 Superoxide anion production

Superoxide anion production was measured using the cytochrome C reduction assay, as described by Roth and Kaeberle[227]. Reduction of cytochrome C was measured using a spectrophotometer 96-well microplate reader (UVmax, Molecular Devices, Sunnyvale, CA), set to read at 550 nm with correction (subtraction) at 650 nm, and was communicated as optical density (O.D.) values with a blank subtracted. First, neutrophils were harvested from steer whole blood and brought to a concentration of $4x10^7$ cells / ml in HBSS containing calcium and magnesium. Next, 50 µl of neutrophils ($2x10^6$ cells), 50 µl of HBSS, 150 µl (0.41 mg) of cytochrome C with or without 15 units of superoxide dismutase (SOD), and 50 µl (0.50, 0.25, 0.125, 0.063, 0.031, or 0.016 mg) of opsonized zymosan were added to microplate wells. When necessary, $5x10^{-10}$M to $1x10^{-3}$M terbutaline, and $1x10^{-9}$M to $1x10^{-3}$M IBMX substituted for HBSS in the microwells, either alone or in combination. Experiments with 8-bromo cAMP required preincubation of neutrophils with the drug at room temperature before their addition to the microwells

for the period of either, 0, 10, 20 or 30 minutes. For all experiments, regardless of treatment group, the final volume in each microwell was 300 µl.

Immediately after all the reagents were added to the wells, a baseline optical density measurement was obtained. The plate was then vortexed at 52 x g for 5, 10, 15, 20, 25, 30, 35, 40, or 45 minutes in an incubator set to 39 °C. After incubation, a second optical density measurement was obtained, with the initial values subtracted. Data points are either expressed as net increase in optical density (O.D.), or percent of the control, where the optical density following bovine neutrophil exposure to the respective concentration of opsonized zymosan alone was established as the control and all data points are expressed as a percentage of that control.

Each experimental day, maximum assay optical density values were obtained by incubating cytochrome C alone with 10 mM sodium hydrosulfide (a reducing agent, also known as sodium dithionide). To be more specific, O.D. values from wells containing neutrophils and opsonized zymosan were compared to the maximum assay optical density value to determine percent available cytochrome C molecules reduced per well.

3.3.7 Statistical Analysis

For saturation and competitive inhibition $^{[3H]}$CGP-12177 binding experiments, data points (fmol bound / 10^6 cells) represent observations of pooled cells, obtained from 5 animals per day over 3 days for n=3. The exception is rats, where pooled cells were obtained from 1-2 animals per day over 8 days for n=8. Each day is represented by the average of two wells, and each well contained $2x10^6$ pooled cells. The data points are graphed as either the mean or mean +/- standard error of the mean (SEM). Nonlinear regression curves were fit to the competitive inhibition (percent of control) binding data using a sigmoidal dose-response (variable slope) approach. Maximum binding density (B_{max}) and dissociation constant (K_D) values were obtained by performing nonlinear regression analyses on specific binding values for each cell type. These best-fit values for the binding parameters were then used to create Scatchard plots for each specific

binding curve. Where relevant, the specific binding value at K_D was divided by the total binding value at K_D to provide the percent of total binding. All statistical methods were performed, and all graphs were created using Prism 4.0 (GraphPad Software, San Diego, CA).

cAMP production data points represent cAMP concentration in the supernatant of $1x10^7$ lysed neutrophils (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, number of days sampled ranging between experiments). Statistically significant differences ($p<0.05$) between data points were identified by performing paired, two-tailed T-tests. All statistical methods were performed, and all graphs were created using Prism 4.0 (GraphPad Software, San Diego, CA).

Superoxide anion production data points represent superoxide anion production by $2x10^6$ intact neutrophils (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, number of days sampled ranging between experiments). Statistically significant differences ($p<0.05$) between data points were identified by performing paired, two-tailed T-tests. All statistical methods were performed, and all graphs were created using Prism 4.0 (GraphPad Software, San Diego, CA).

3.4 Results

3.4.1 β-adrenergic receptor quantification

The total, non-specific and specific binding of $^{[3H]}$CGP-12177 to intact steer neutrophils and lymphocytes (Figures 1 and 2, respectively), intact dairy cow neutrophils and lymphocytes (Figures 3 and 4, respectively), and intact rat lymphocytes (Figure 5) were measured. The specific binding of $^{[3H]}$CGP-12177 to these cell types ranged from 87 to 99% of total binding at concentrations equal to the respective K_D, while for all

specific binding data Scatchard plots were linear. Figure 6 provides a comparison of the specific binding of $^{[3H]}$CGP-12177 to various cell types in addition to the respective maximum $^{[3H]}$CGP-12177 binding density (B_{max}) values. The cell type with the highest specific binding B_{max} value was the rat lymphocyte, with 1.63 fmol $^{[3H]}$CGP-12177 binding per 10^6 cells. Steer and dairy cow lymphocyte specific binding B_{max} values were significantly lower than the rat lymphocyte value, but were not different from each other, with 0.62 (steer lymphocyte) vs. 0.65 (dairy cow lymphocyte) fmol $^{[3H]}$CGP-12177 binding per 10^6 cells. Steer and dairy cow neutrophil specific binding B_{max} values were significantly lower than rat, steer, and dairy lymphocyte values, but were also not different from each other, with 0.19 (steer neutrophil) vs. 0.16 (dairy cow neutrophil) fmol $^{[3H]}$CGP-12177 binding per 10^6 cells. In this study, specific binding at K_D (as a percentage of total binding) is greatest for dairy cow lymphocytes (99%), followed by dairy cow neutrophils (98%), steer lymphocytes (97%), steer neutrophils (92%), and rat lymphocytes (87%).

The predominant β-adrenergic receptor subtype on steer neutrophils is the β_2-adrenergic receptor as indicated by the rank order IC_{50} of the various ligands used. Figure 7 illustrates the competition for specific binding of $^{[3H]}$CGP-12177 to intact steer neutrophils by various concentrations of (-) isoproterenol, (-) epinephrine, and (-) norepinephrine (rank order of potency: isoproterenol > epinephrine > norepinephrine). The β_1-adrenergic receptor was also confirmed to be present on bovine neutrophils. Figure 8 illustrates the competition for specific binding of $^{[3H]}$CGP-12177 to intact steer neutrophils by various concentrations of the non-selective β_1- / β_2-adrenergic receptor antagonist (-) propranolol, the selective β_2-adrenergic receptor antagonist (+/-) ICI-118,551, and the selective β_1-adrenergic receptor antagonist (-) atenolol (rank order of potency: propranolol > ICI-118,551 > atenolol).

3.4.2 cAMP production

The effect of activating components of the bovine neutrophil β_2-adrenergic receptor mechanism on cAMP production was evaluated. Bovine neutrophil exposure to

various concentrations of the β_1- / β_2-adrenergic receptor agonist isoproterenol resulted in a dose-dependent increase in cAMP production; for instance, $1x10^{-3}$M isoproterenol increased cAMP production 3-fold higher than baseline (Figure 9). However, various concentrations of the selective β_2-adrenergic receptor agonist terbutaline ($1x10^{-8}$M to $1x10^{-3}$M) did not initiate a statistically significant increase in cAMP production over baseline levels without co-incubation with $5x10^{-4}$M IBMX, a phosphodiesterase inhibitor (Figures 10 and 11). The ability of the selective β_2-adrenergic receptor antagonist ICI-118,551 to decrease the terbutaline and IBMX effect was illustrated, as exposure to various concentrations of ICI-118,551 resulted in as much as a 90% decrease in cAMP production (Figure 12). The cAMP level representing exposure to the lowest concentration of terbutaline with $1x10^{-4}$M IBMX equals the cAMP level representing exposure to $1x10^{-4}$M IBMX alone, which suggests a threshold (data not shown).

3.4.3 Superoxide anion production

A time- and dose-response for opsonized zymosan-dependent bovine neutrophil superoxide anion production was evaluated (Figures 13, 14 and 15). Maximum superoxide anion production was realized when $2x10^6$ neutrophils / well were incubated for 30 minutes at 39 °C with 0.063 mg per well of opsonized zymosan. Using these parameters, approximately 50% of the molecules of cytochrome C in each well were reduced, which assured adequate assay sensitivity (Figure 16). Also, superoxide anion production was quenched by 15 units / well of superoxide dismutase (SOD) (Figure 17).

The effect of activating components of the β_2-adrenergic receptor mechanism on opsonized zymosan-dependent bovine neutrophil superoxide anion production was evaluated. First, superoxide anion production was decreased in a dose-dependent manner by various concentrations of terbutaline (Figure 18) and IBMX (Figure 19). Maximum efficacies for terbutaline and IBMX were both realized at $1x10^{-3}$M, with superoxide anion production decreased by 40% and 74%, respectively. Second, addition of $1x10^{-4}$M, but not $5x10^{-6}$M IBMX, resulted in an exaggeration of the suppressive effect of various concentrations of terbutaline on superoxide anion production (Figure 20). Third, 8-

bromo-cAMP decreased opsonized zymosan-dependent bovine neutrophil superoxide anion production. This effect was not dose-dependent, regardless of the time of preincubation with cells (Figure 21); however, the effect was dependent upon preincubation time (Figure 22). The maximum effect of 8-bromo cAMP on neutrophil superoxide anion production (a 50% decrease) was realized when $1x10^{-3}$M 8-bromo-cAMP was incubated with the cells for 30 minutes at room temperature. Finally, the IBMX dose-dependent effect on superoxide anion production was enhanced when the cells were preincubated with $1x10^{-3}$M 8-bromo cAMP 15 minutes at room temperature (Figure 23).

3.5 Discussion

As a central component to the acute inflammatory response, neutrophils are capable of reacting to a myriad of chemical signals. Pro-inflammatory signals have been carefully studied in the context of BRD and more specifically, neutrophil migration in the lung,[137, 141] production of reactive oxygen species,[15, 149] degranulation,[10, 138] and phagocytosis.[11, 172] Results from work with human neutrophils and bovine macrophages suggest that leukocytes may also react to anti-inflammatory signals from the autonomic nervous system via adrenergic receptors; for instance, activation of the β_2-adrenergic receptor mechanism decreases human neutrophil adhesion to the vascular wall,[29, 30, 136] reactive oxygen species production,[25, 195-198] chemotaxis,[26] degranulation,[27, 28] and inflammatory mediator production.[33-35] Similarly, activation of the bovine macrophage β_2-adrenergic receptor is reported to decrease reactive oxygen species production.[22] Without complimentary work with bovine neutrophils, using these data to examine treatment options for the acute inflammatory stage of BRD is unrealistic. For this reason, we tested the hypothesis that bovine neutrophils possess components of the β_2-adrenergic receptor mechanism, and the components work in a cooperative manner to raise intracellular cAMP and decrease superoxide anion production.

With high specific binding by the β_1- / β_2-adrenergic receptor antagonist $^{[3H]}$CGP-12177 at concentrations equal to the respective K_D, and with linear orientation of Scatchard plots, non-specific (non-receptor) binding was kept to a negligible level and a single population of binding sites was identified on intact steer neutrophils and lymphocytes, intact dairy cow neutrophils and lymphocytes, and intact rat lymphocytes, respectively. We report here the maximum number of $^{[3H]}$CGP-12177 binding sites for rat lymphocytes is approximately two and a half times that of both dairy cow lymphocytes and steer lymphocytes. These values support other published rat lymphocyte values,[230] and roughly match published human lymphocyte values.[204, 233] The maximum number of $^{[3H]}$CGP-12177 binding sites for both dairy cow and steer lymphocytes in this study are one-third of that previously reported for dairy cow lymphocytes.[21] We cannot explain this discrepancy; however, the lack of a difference between the values for dairy cow and steer lymphocytes suggests that neither a breed nor age difference in receptor population exists in this study. Moreover, there is no discernable difference in the technique / handling of the cells to suggest a reason for the difference, and although lymphocytes were collected from primiparous lactating East Flemish Red Pied cows for the previously reported study, the lack of a difference between Angus-cross steer and the Holstein dairy cow values in this study suggests that genetics are most likely not a major contributing factor for the difference in results.

This study demonstrates that β-adrenergic receptors exist on bovine neutrophils, with the β_2-adrenergic receptor subtype predominating. The maximum $^{[3H]}$CGP-12177 specific binding density (B_{max}) values for steer and dairy cow neutrophils are not different from each other; however, they are one-third the density of dairy cow and steer lymphocytes and one-tenth the density of rat lymphocytes. Because these experiments were performed by the same person, using the same techniques and equipment during the same time period, we feel that such discrepancies between cell types and species represent true differences in the comparative anatomy of these cells. It is prudent to compare bovine neutrophil β-adrenergic receptor density to that of human neutrophils. Despite ample discussion in the literature on the function of β-adrenergic receptors on intact human neutrophils, there are only two reports that describe the specific binding

density of [3H]CGP-12177 on these cells, where the authors report B_{max} values 5- and 10-fold greater than bovine neutrophils.[211, 234] Indeed, without investigating β-adrenergic receptor density on both human and bovine neutrophils in the same experiment, any claim that there is a real difference in receptor number between these two cell types would remain inconclusive. In this study, we illustrate the competition for specific binding of [3H]CGP-12177 to intact steer neutrophils by various concentrations of (-) isoproterenol, (-) epinephrine, and (-) norepinephrine, with a rank order of potency as follows: isoproterenol > epinephrine > norepinephrine. This rank order suggests that the β_2-adrenergic receptor is the predominant subtype present. This study also confirms the presence of β_1-adrenergic receptors on bovine neutrophils.

In reference to the lack of reports that describe β-adrenergic receptor numbers on human neutrophils, there is adequate description of human neutrophil β-adrenergic receptor labeling with the β_1- / β_2-adrenergic receptor antagonists [^{3}H]dihydroalprenolol (DHA) and ^{125}I-iodocyanopindolol (^{125}I-ICYP); however, these studies either used purified receptor protein,[41, 42, 200] or the radioligands demonstrated unacceptable levels of non-specific binding, which was speculated to be due to their hydrophilic nature.[20, 233] Although use of [3H]CGP-12177 was first reported in 1983,[222] it was not until recently that it became the radioligand of choice for β-adrenergic receptor studies.[229] We feel that using [3H]CGP-12177 to quantify β-adrenergic receptors is a relevant approach because the compound is a hydrophilic ligand with minimal non-specific binding. Moreover, [3H]CGP-12177's hydrophilic property lends well to using intact cells, which allows the researcher to more readily extrapolate their radioligand binding data for clinical use.

This is the first report of cAMP production in the bovine neutrophil. Measuring intracellular cAMP concentration is a relevant technique for screening putative anti-inflammatory compounds because the immunoassay used does not require the use of radioactive agents and because it is accepted that cAMP, via activation of PKA, modulates cytoskeletal rearrangements and therefore regulates NADPH oxidase formation and resultant superoxide anion formation.[192, 196] In this study, various concentrations of the β_1- / β_2-adrenergic receptor agonist isoproterenol increased bovine

neutrophil cAMP production in a dose-dependent manner, up to 300% above baseline. However, even high concentrations (up to $1x10^{-3}$M) of the specific β_2-adrenergic receptor agonist terbutaline did not stimulate cAMP production, unless neutrophils were also incubated with $5x10^{-4}$M IBMX, a phosphodiesterase inhibitor. One possible explanation for this is isoproterenol alone may have increased cAMP production due to concurrent activation of β_1-adrenergic receptors, whereas terbutaline is a specific β_2-adrenergic receptor agonist. However, this hypothesis could not be confirmed because an experiment using isoproterenol with the β_1-adrenergic receptor antagonist atenolol was not performed. Regardless, the dependency upon phosphodiesterase inhibition (and the resultant increased lifespan of molecules of cAMP) for measurable cAMP production following β-adrenergic receptor activation is readily found in the literature,[26, 28, 32, 192] suggesting that bovine neutrophil cAMP may experience a short half-life and / or inherent bovine neutrophil phosphodiesterase activity is vigorous. Therefore, to ensure proper assay sensitivity, neutrophils were co-incubated with $5x10^{-4}$M IBMX in all experiments where terbutaline was used to stimulate cAMP production, with the $5x10^{-4}$M IBMX alone values subtracted from all relevant data points to account for the contribution of IBMX alone on cAMP production. This study characterized the role of the β_2-adrenergic receptor in bovine neutrophil cAMP production. Increasing concentrations ($1x10^{-11}$M to $1x10^{-5}$M) of the selective β_2-adrenergic receptor antagonist ICI-118,551 decreased terbutaline-dependent cAMP production by as much as 90%. Although high concentrations ($1x10^{-6}$M to $1x10^{-4}$M) of ICI-118,551 have been reported by others to cause membrane stabilization,[235] $1x10^{-6}$M and $1x10^{-5}$M ICI-118,551 were not found to alter basal bovine neutrophil cAMP production when used alone. Similar to this study, reports on human neutrophils have demonstrated the ability of β-adrenergic receptors to stimulate cAMP production; however, the nonselective β_1- / β_2-adrenergic receptor agonist isoproterenol was used,[26, 28, 32, 195] and therefore the specific role of the β_2-subtype in cAMP production was not addressed. Also, the reports describe significant cAMP production using isoproterenol without concurrent phosphodiesterase inhibition, a feat we were not able to accomplish in the present study. One possible explanation for this may be a difference in receptor number between human and bovine neutrophils, as sufficient β_2-adrenergic receptor activation may saturate phosphodiesterase molecules,

leading to an overflow of cAMP. As discussed earlier, bovine neutrophils were found in the present study to possess 5- to 10-fold fewer β-adrenergic receptors than that reported for human neutrophils.

The present study is the first to demonstrate the ability of various components of the β_2-adrenergic receptor mechanism to decrease bovine neutrophil superoxide anion production using the cytochrome C reduction assay. The cytochrome C reduction assay is a relevant technique for monitoring superoxide anion production and for identifying a functional β_2-adrenergic receptor mechanism in intact bovine neutrophils. It has been employed extensively with mononuclear and polymorphonuclear leukocytes from various species of animals, including humans.[28, 33, 198, 226, 231, 232, 234] In this study, terbutaline and IBMX were demonstrated to activate the β_2-adrenergic receptor and increase cAMP levels, respectively. Here, we also demonstrate that these two compounds decrease superoxide anion production by 40% and 74%, respectively, in a dose-dependent manner. These results support published work with human neutrophils that also claim activation of various components of the β_2-adrenergic receptor mechanism (for instance, the β_2-adrenergic receptor, adenylyl cyclase, or phosphodiesterase inhibition) will decrease superoxide anion production.[25, 195-198, 236, 237] In this study, the synthetic cAMP analog 8-bromo-cAMP decreased bovine neutrophil superoxide anion production by up to 50% in a time-dependent, but not dose-dependent, manner. This time-dependent behavior by 8-bromo-cAMP supports other reports of its effects on leukocytes,[32, 238, 239] as it passively diffuses across the cell membrane before reaching the enzyme PKA, its site of action. When $1x10^{-4}$M, but not $5x10^{-6}$M IBMX was added to each of the terbutaline concentrations, the inhibitory effect of terbutaline on superoxide anion production in this study was exaggerated. This finding, which demonstrates the dose-dependent ability of phosphodiesterase inhibition to enhance β_2-adrenergic receptor-mediated modulation of superoxide anion production, supports similar work with isoproterenol and IBMX and illustrates an additive effect on the human neutrophils.[28] Also, the present study describes for the first time the exaggerated inhibitory effect of IBMX on superoxide anion production when neutrophils are first preincubated with $1x10^{-3}$M 8-bromo cAMP

for 15 minutes. By preincubating with 8-bromo-cAMP, there was presumably additional activation of PKA.

In summary, this study demonstrates for the first time that bovine neutrophils possess a functional β_2-adrenergic receptor mechanism, and illustrates how components of the mechanism work to stimulate cAMP production and decrease superoxide anion production. We also report here that rat lymphocytes possess more β-adrenergic receptors than dairy cow or steer lymphocytes, and that diary cow lymphocytes contain no more β-adrenergic receptors than steer lymphocytes. It is also worthy to note that dairy cow and steer lymphocytes possess more β-adrenergic receptors than dairy cow and steer neutrophils, with diary cow neutrophils containing no more β-adrenergic receptors than steer neutrophils. This may have physiologic consequence, as lymphocytes may be more heavily regulated by the autonomic nervous system than neutrophils.[188] By allowing for molecules of cAMP that are either produced or given in an exogenous manner to remain intact, phosphodiesterase inhibition by IBMX works in synergy with both terbutaline-mediated β_2-adrenergic receptor activation and 8-bromo-cAMP administration to reduce bovine neutrophil superoxide anion production and expose possible targets for pharmacologic manipulation of the acute inflammatory stage of BRD.

3.6 Acknowledgements

The authors gratefully acknowledge the technical assistance of Kevin Weaver and Chris Wakley for animal care / bleeding, Delbert Jones, Kristen Knight, Kristel Fuhrman, and Dr.'s Dagmar Frank, Matthew Landry and Rafael Burgos for assistance with technique development, Joan Kalnitsky for assistance with Flow Cytometry, and Jan Murphy for glassware. The Virginia-Maryland Regional College of Veterinary Medicine supported this work.

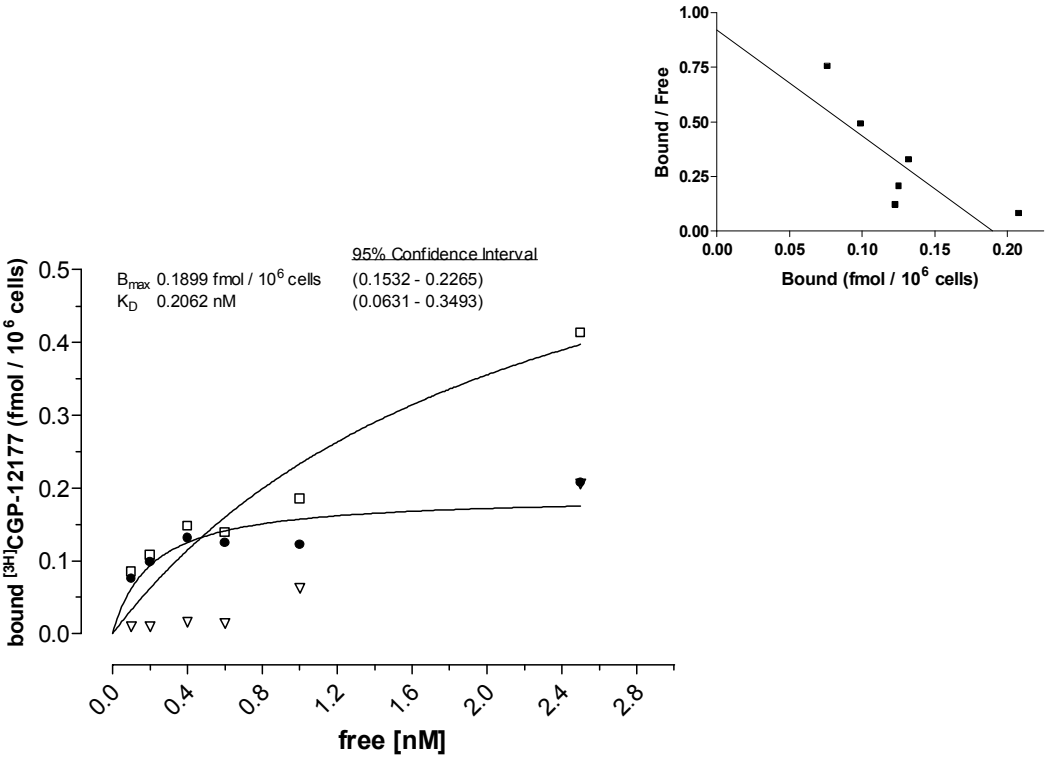

Figure 1. Total (open square), specific (closed circle) and nonspecific (open triangle) binding of $^{[3H]}$CGP-12177 to steer neutrophils. Specific binding at dissociation constant (K_D) was 92% of total binding. Data points represent mean of observations (1 observation per day, pooled sample of neutrophils from 5 steers per observation, 3 days total for n=3). Inset: Scatchard analysis of data for maximum $^{[3H]}$CGP-12177 binding density (B_{max}).

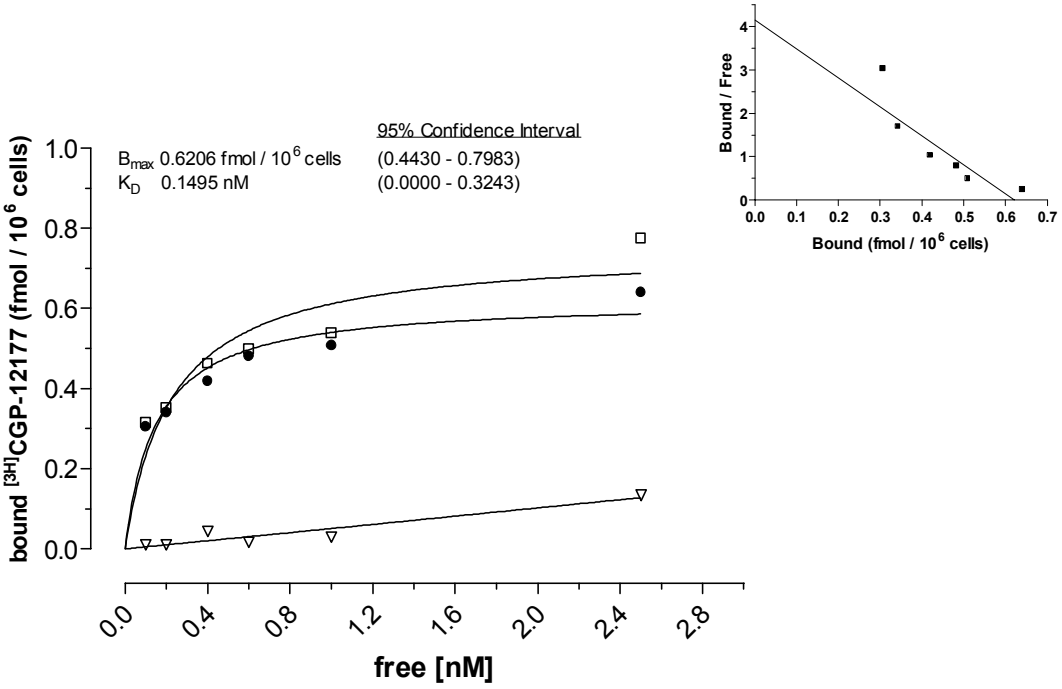

Figure 2. Total (open square), specific (closed circle) and nonspecific (open triangle) binding of $^{[3H]}$CGP-12177 to steer lymphocytes. Specific binding at dissociation constant (K_D) was 97% of total binding. Data points represent mean of observations (1 observation per day, pooled sample of lymphocytes from 5 steers per observation, 3 days total for n=3). Inset: Scatchard analysis of data for maximum $^{[3H]}$CGP-12177 binding density (B_{max}).

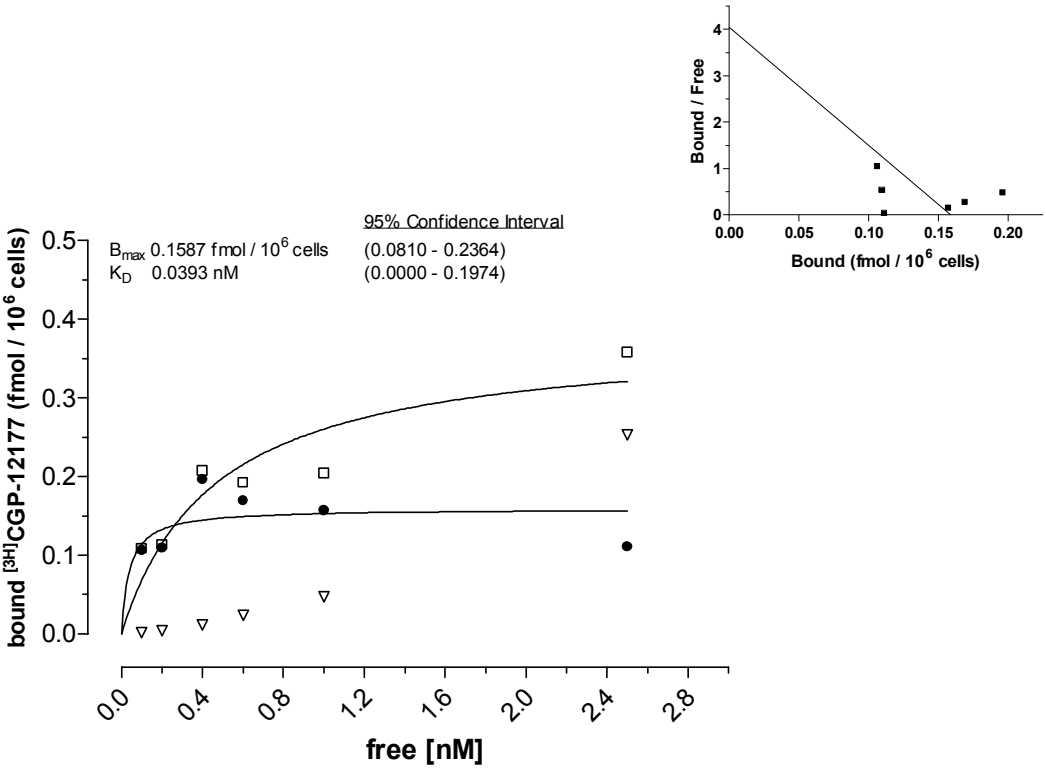

Figure 3. Total (open square), specific (closed circle) and nonspecific (open triangle) binding of $^{[3H]}$CGP-12177 to dairy cow neutrophils. Specific binding at dissociation constant (K_D) was 98% of total binding. Data points represent mean of observations (1 observation per day, pooled sample of neutrophils from 5 steers per observation, 3 days total for n=3). Inset: Scatchard analysis of data for maximum $^{[3H]}$CGP-12177 binding density (B_{max}).

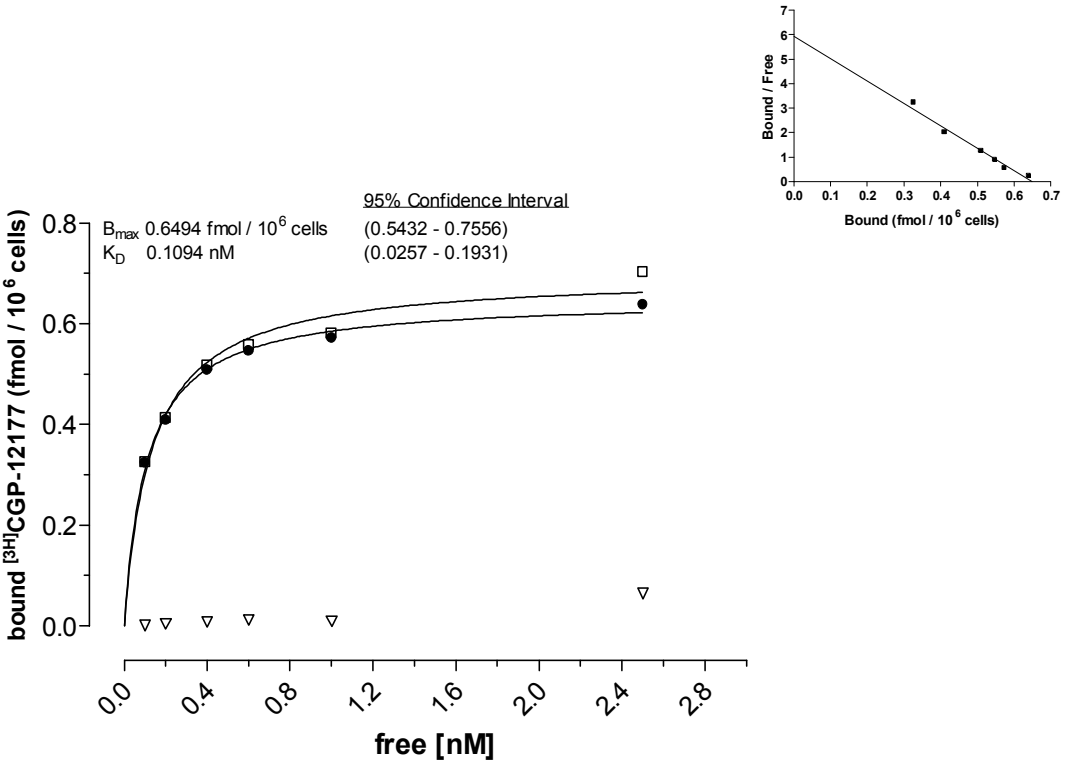

Figure 4. Total (open square), specific (closed circle) and nonspecific (open triangle) binding of $^{[3H]}$CGP-12177 to dairy cow lymphocytes. Specific binding at dissociation constant (K_D) was 99% of total binding. Data points represent mean of observations (1 observation per day, pooled sample of lymphocytes from 5 steers per observation, 3 days total for n=3). Inset: Scatchard analysis of data for maximum $^{[3H]}$CGP-12177 binding density (B_{max}).

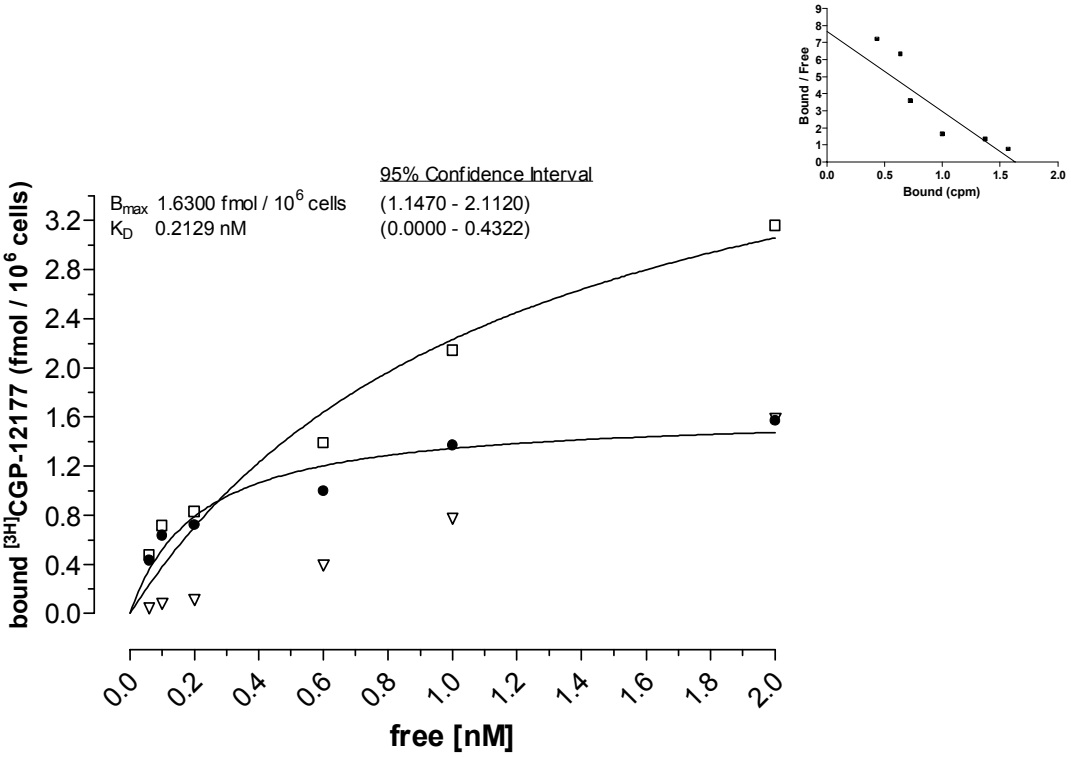

Figure 5. Total (open square), specific (closed circle) and nonspecific (open triangle) binding of [3H]CGP-12177 to rat lymphocytes. Specific binding at dissociation constant (K_D) was 87% of total binding. Data points represent mean of observations [1 observation per day, pooled sample of lymphocytes from 1-2 rats per observation, 8 days (11 rats) total for n=8]. Inset: Scatchard analysis of data for maximum [3H]CGP-12177 binding density (B_{max}).

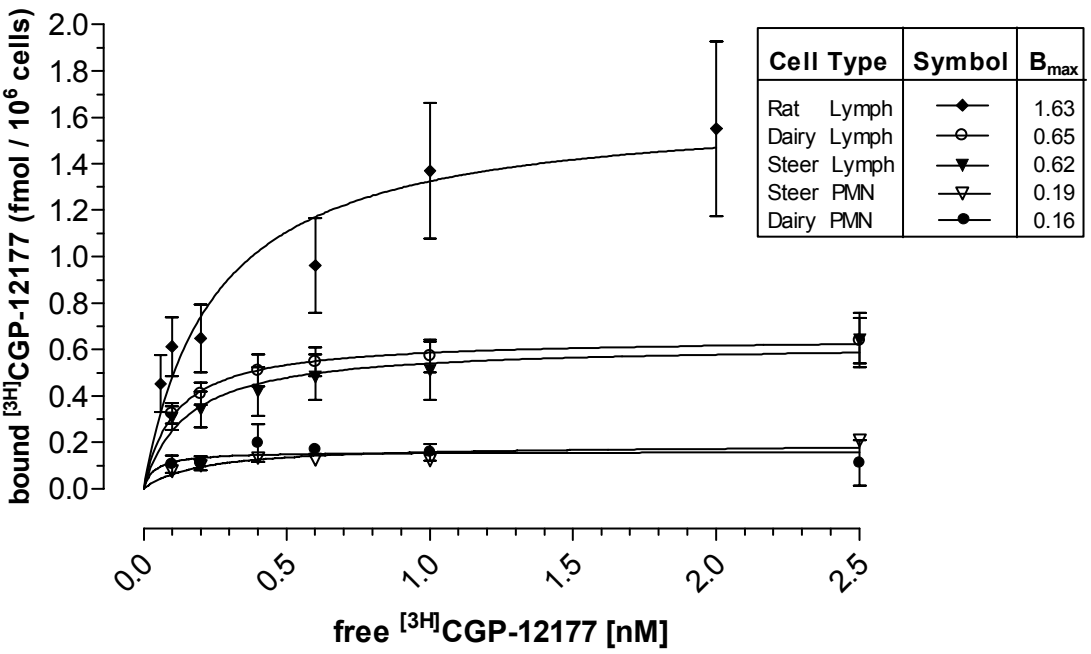

Figure 6. Specific binding of $^{[3H]}$CGP-12177 to rat lymphocytes (closed diamond; B_{max} = 1.63 fmol per 10^6 cells; K_D = 0.21 nM), dairy cow lymphocytes (open circle; B_{max} = 0.65 fmol per 10^6 cells; K_D = 0.11 nM), steer lymphocytes (closed triangle; maximum $^{[3H]}$CGP-12177 binding density (B_{max}) = 0.62 fmol per 10^6 cells; dissociation constant (K_D) = 0.15 nM), steer neutrophils (PMN) (open triangle; B_{max} = 0.19 fmol per 10^6 cells; K_D = 0.21 nM), and dairy cow PMN (closed circle; B_{max} = 0.16 fmol per 10^6 cells; K_D = 0.04 nM). Data points represent mean +/- SEM. Inset: Comparison of B_{max} values (fmol $^{[3H]}$CGP-12177 per 10^6 cells) for specific binding to the various cell types.

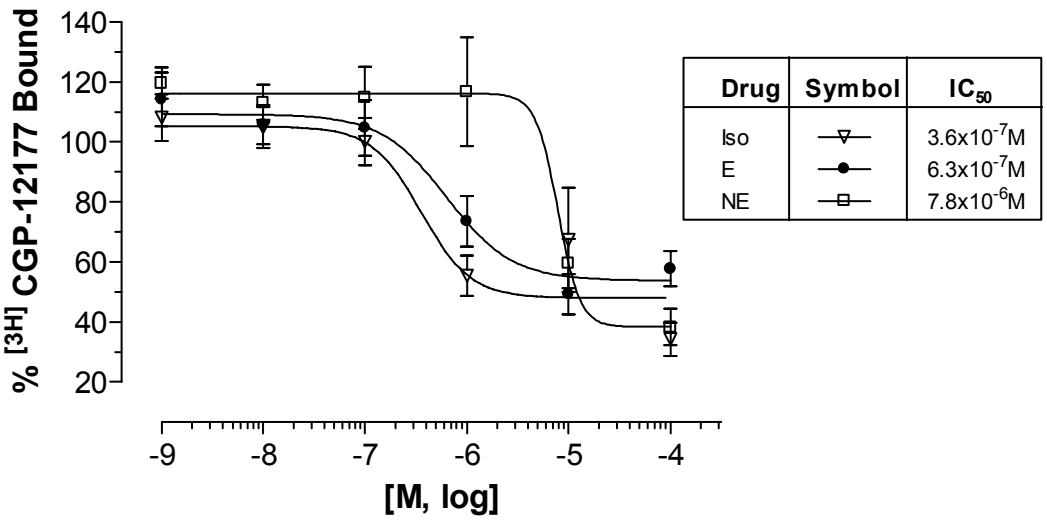

Figure 7. Competition of specific $^{[3H]}$CGP-12177 binding to steer neutrophils in the presence of various concentrations of the β-adrenergic receptor agonists isoproterenol (Iso, open triangle), epinephrine (E, closed circle), or norepinephrine (NE, open square). Inset: Comparative IC_{50} values for Iso, E, and NE – mediated displacement of $^{[3H]}$CGP-12177 binding. Data points represent mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 10 days total for n=10.

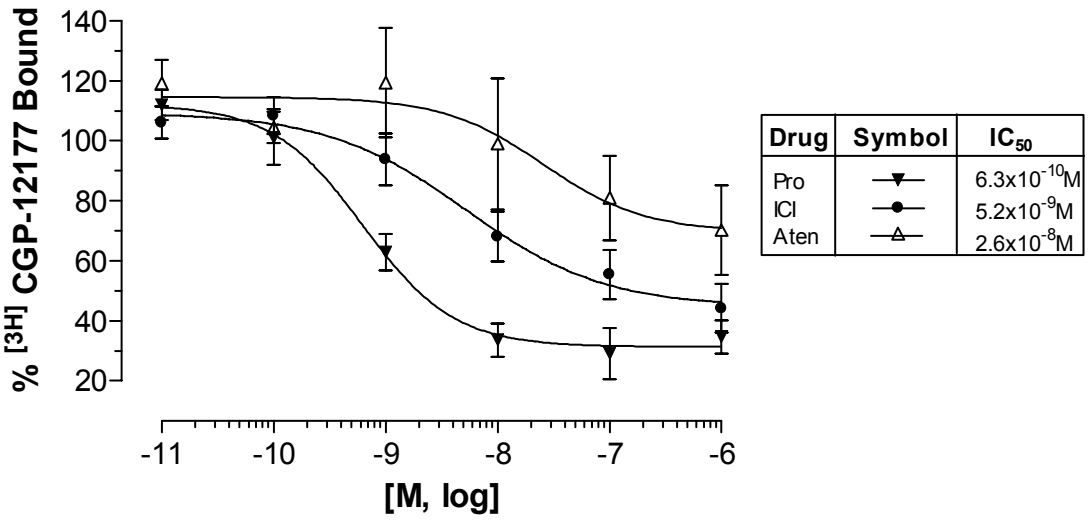

Figure 8. Competition of specific [3H]CGP-12177 binding to steer neutrophils in the presence of various concentrations of the β-adrenergic receptor antagonists propranolol (Pro, closed triangle), ICI-118,551 (ICI, closed circle), or atenolol (Aten, open triangle). Inset: Comparative IC_{50} values for propranolol, ICI-118,551 and atenolol – mediated displacement of [3H]CGP-12177 binding. Data points represent mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 10 days (propranolol & ICI-118,551, n=10) or 4 days (atenolol, n=4) total.

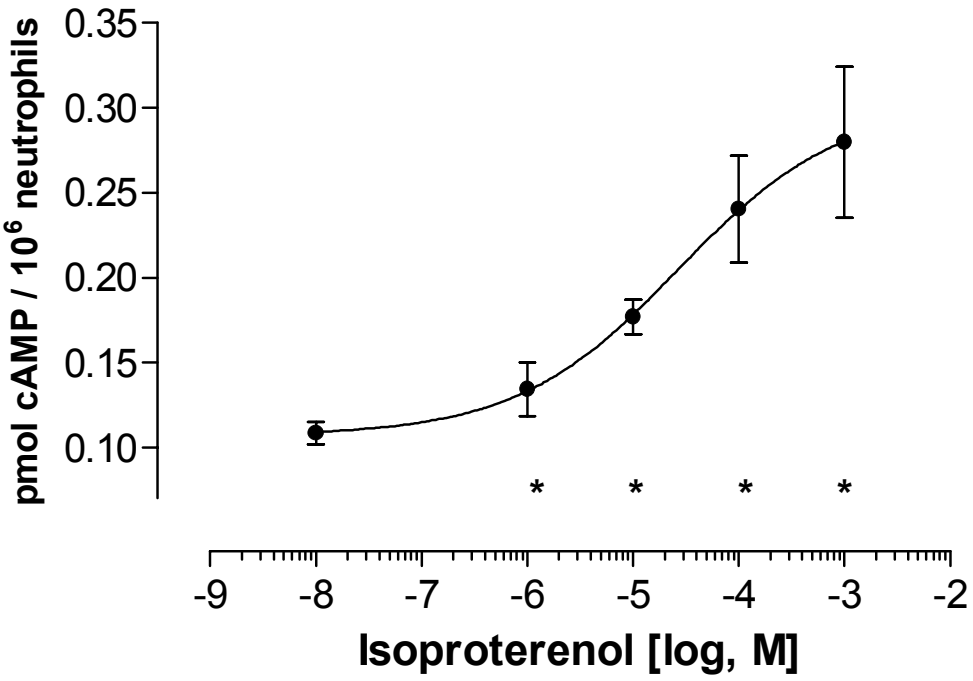

Figure 9. Cyclic AMP (cAMP) production by bovine neutrophils exposed to various concentrations of the β_1- / β_2-adrenergic receptor agonist, isoproterenol. Data points represent cAMP concentration in supernatant from 10^7 lysed cells (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 6 days total for n=6). *Difference in cAMP concentration between neutrophils exposed to isoproterenol and those exposed to Hank's balanced salt solution (HBSS) is statistically significant at concentrations of isoproterenol $1x10^{-6}$M and higher (paired, two-tailed T-test, $p<0.05$).

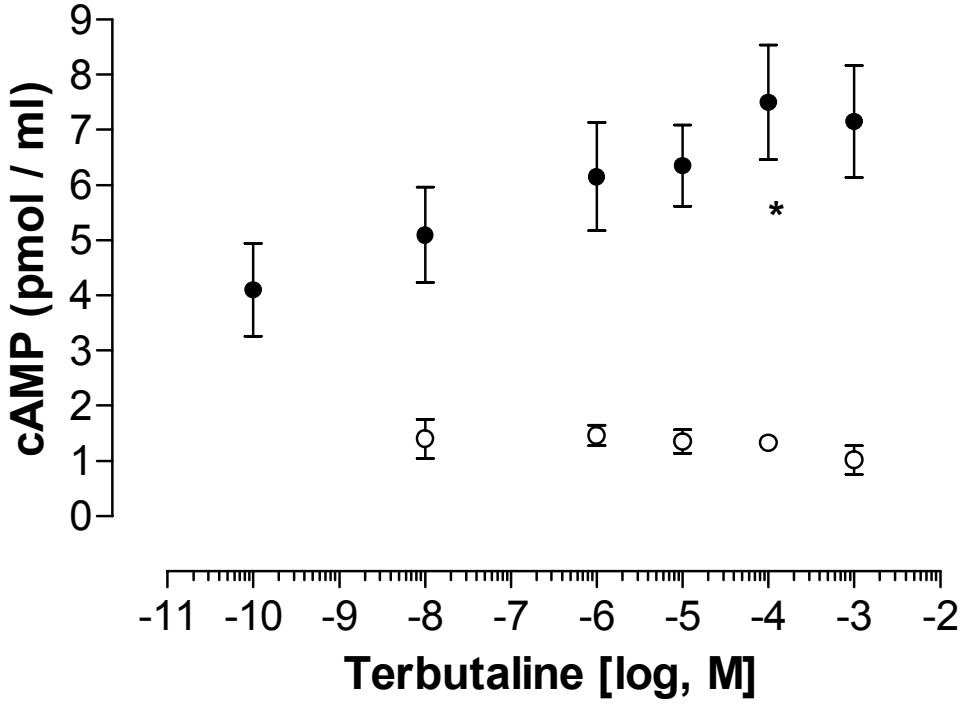

Figure 10. Cyclic AMP (cAMP) production by bovine neutrophils exposed to various concentrations of the selective β_2-adrenergic receptor agonist, terbutaline, with (solid circle) and without (open circle) $5x10^{-4}$M 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor. Data points represent cAMP concentration in supernatant from 10^7 lysed cells (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 8 days total for n=8). *Difference in cAMP concentration between $1x10^{-4}$M terbutaline with $5x10^{-4}$M IBMX (solid circle, mean = 7.503) and $5x10^{-4}$M IBMX alone (data point not shown, mean = 5.123) is statistically significant (paired, two-tailed T-test, $p<0.05$).

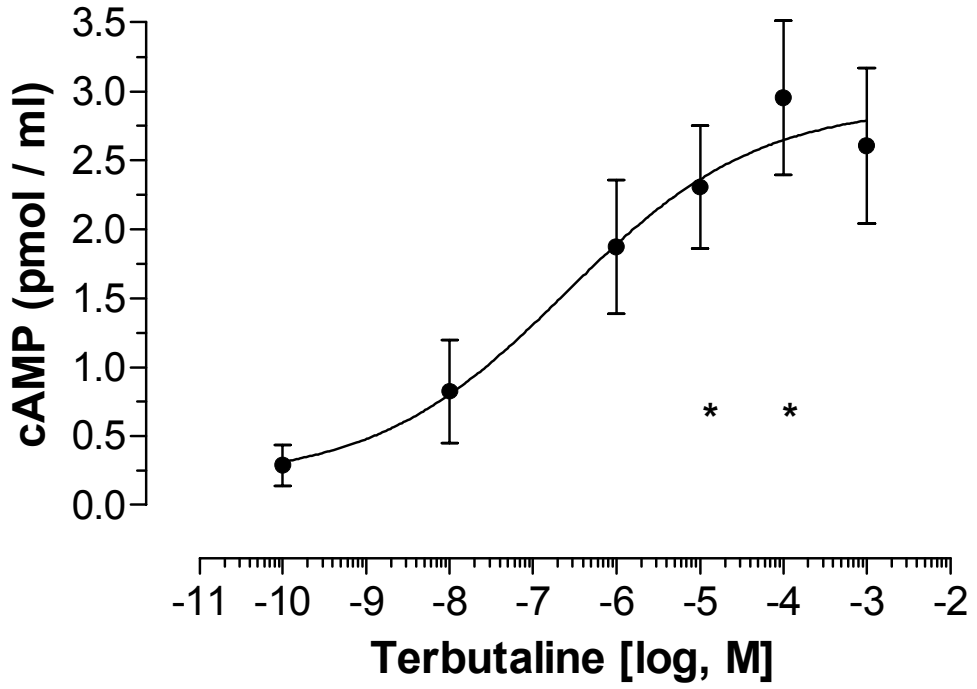

Figure 11. Cyclic AMP (cAMP) production by bovine neutrophils exposed to various concentrations of the selective β_2-adrenergic receptor agonist, terbutaline, with $5x10^{-4}$M 3-isobutyl-1-methylxanthine (IBMX). Data points represent the concentration of cAMP in supernatant from 10^7 lysed cells, with $5x10^{-4}$M IBMX alone values subtracted (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 8 days total for n=8). *Difference in cAMP concentration between terbutaline ($1x10^{-5}$M and $1x10^{-4}$M; mean = 2.306 and 2.954, respectively) and Hank's balanced salt solution (HBSS, mean = 0.837) is statistically significant (paired, two-tailed T-test, $p<0.05$).

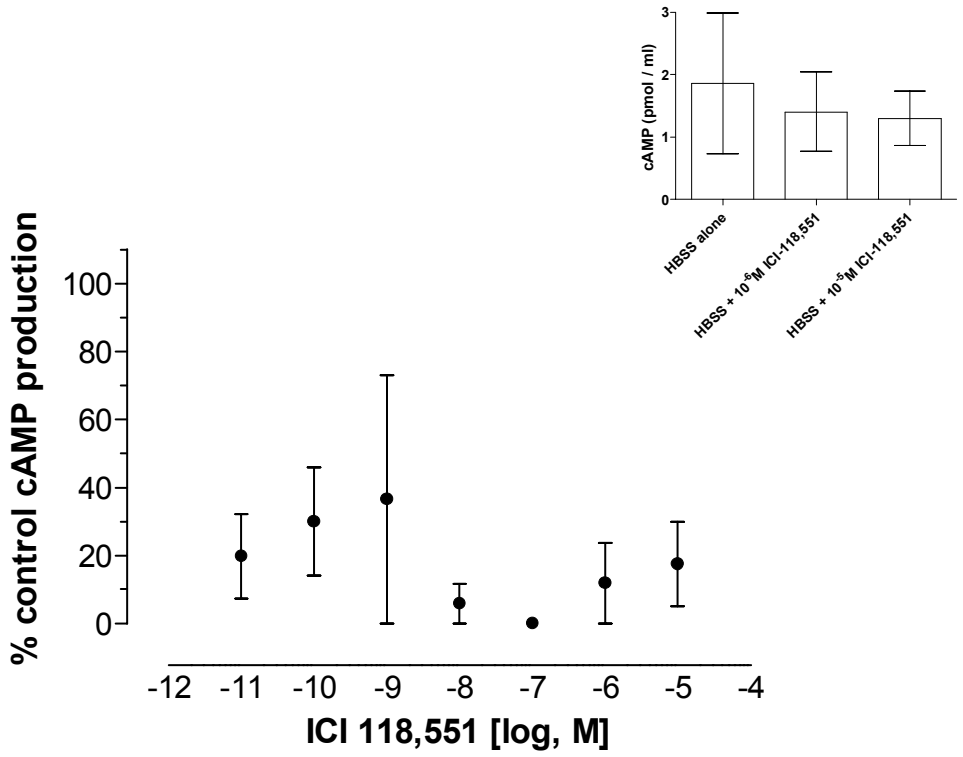

Figure 12. Cyclic AMP (cAMP) production by bovine neutrophils exposed to the selective β_2-adrenergic receptor agonist terbutaline ($5x10^{-6}$M) with various concentrations of the selective β_2-adrenergic receptor antagonist, ICI-118,551. Values are expressed as a percentage of control cAMP concentration (12.07 pmol cAMP / ml; neutrophils exposed to $5x10^{-6}$M terbutaline + $5x10^{-4}$M IBMX, with $5x10^{-4}$M IBMX alone value subtracted), and represent the concentration of cAMP in supernatant from 10^7 lysed cells (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 3 days total for n=3). Inset: Effect of ICI-118,551 on basal cAMP production by bovine neutrophils, compared to Hank's balanced salt solution (HBSS).

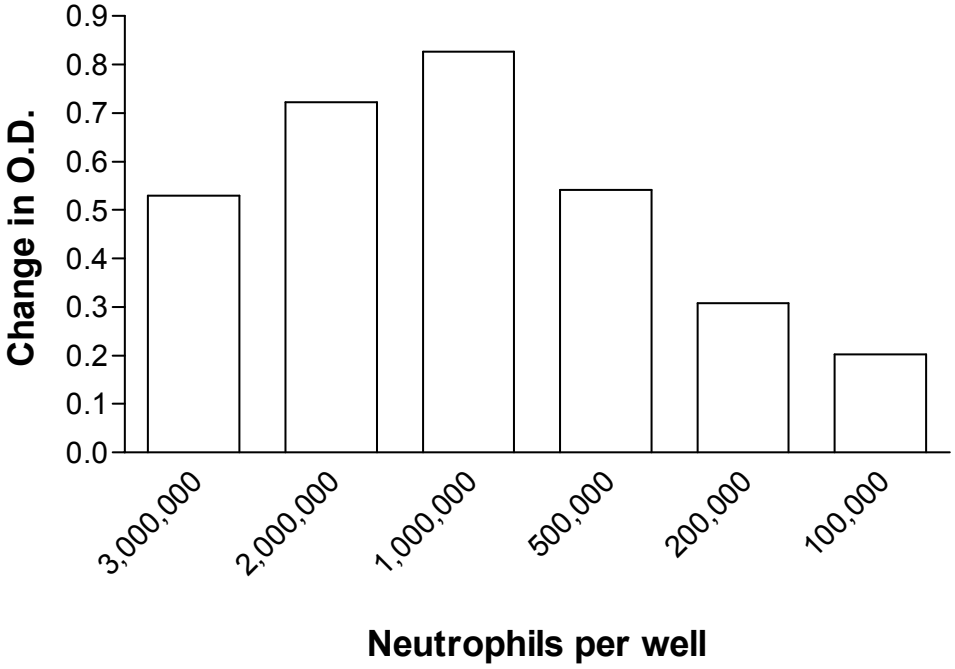

Figure 13. Superoxide anion production by varying amounts of bovine neutrophils exposed to 0.50 mg opsonized zymosan. Change in optical density (O.D.) at 550 nm indicates cytochrome C reduction. Data points represent values from 1 observation, pooled sample of neutrophils from 5 steers.

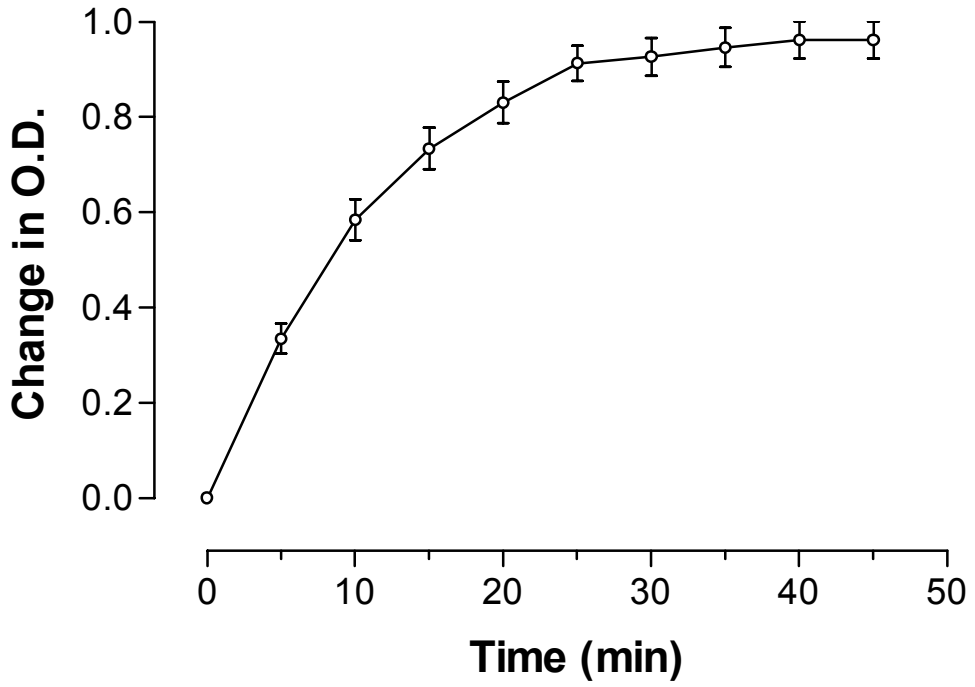

Figure 14. Superoxide anion production by $2x10^6$ bovine neutrophils following incubation with 0.50 mg of the inflammatory stimulant, opsonized zymosan, for various lengths of time at 39 ºC. Change in optical density (O.D.) at 550 nm indicates cytochrome C reduction. Data points represent mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 11 days total for n=11.

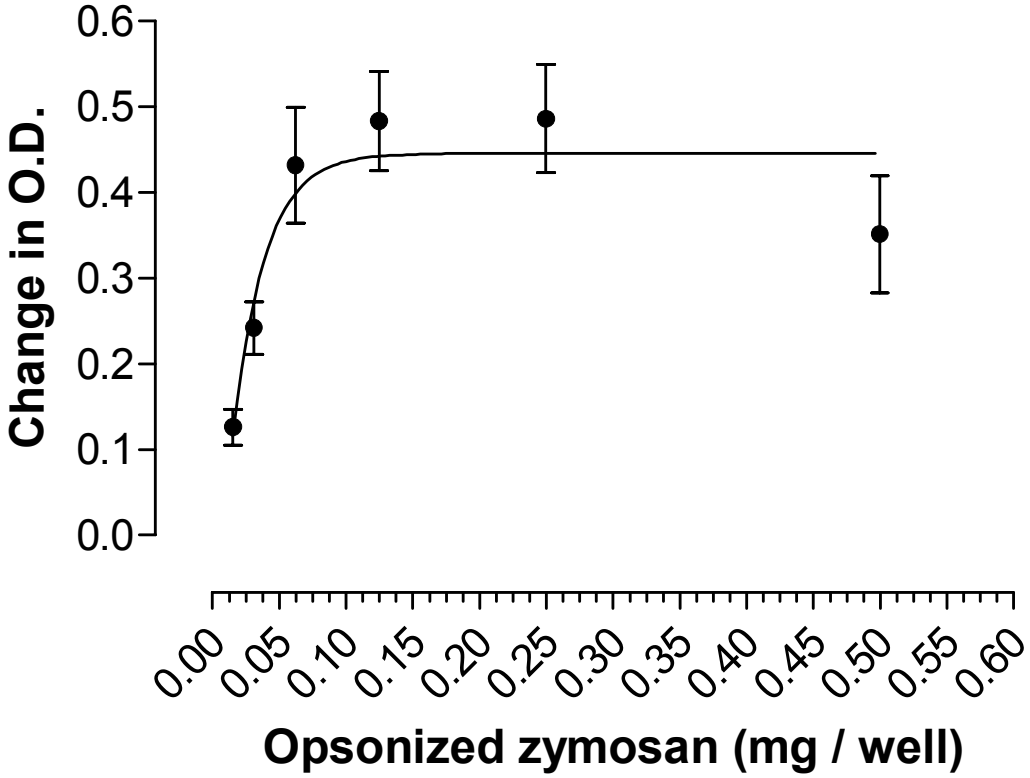

Figure 15. Superoxide anion production by $2x10^6$ bovine neutrophils following incubation with 0.016, 0.031, 0.063, 0.125, 0.25, or 0.50 mg opsonized zymosan for 30 minutes at 39 ºC. Change in optical density (O.D.) at 550 nm indicates cytochrome C reduction. Data points represent mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 3 days for n=3.

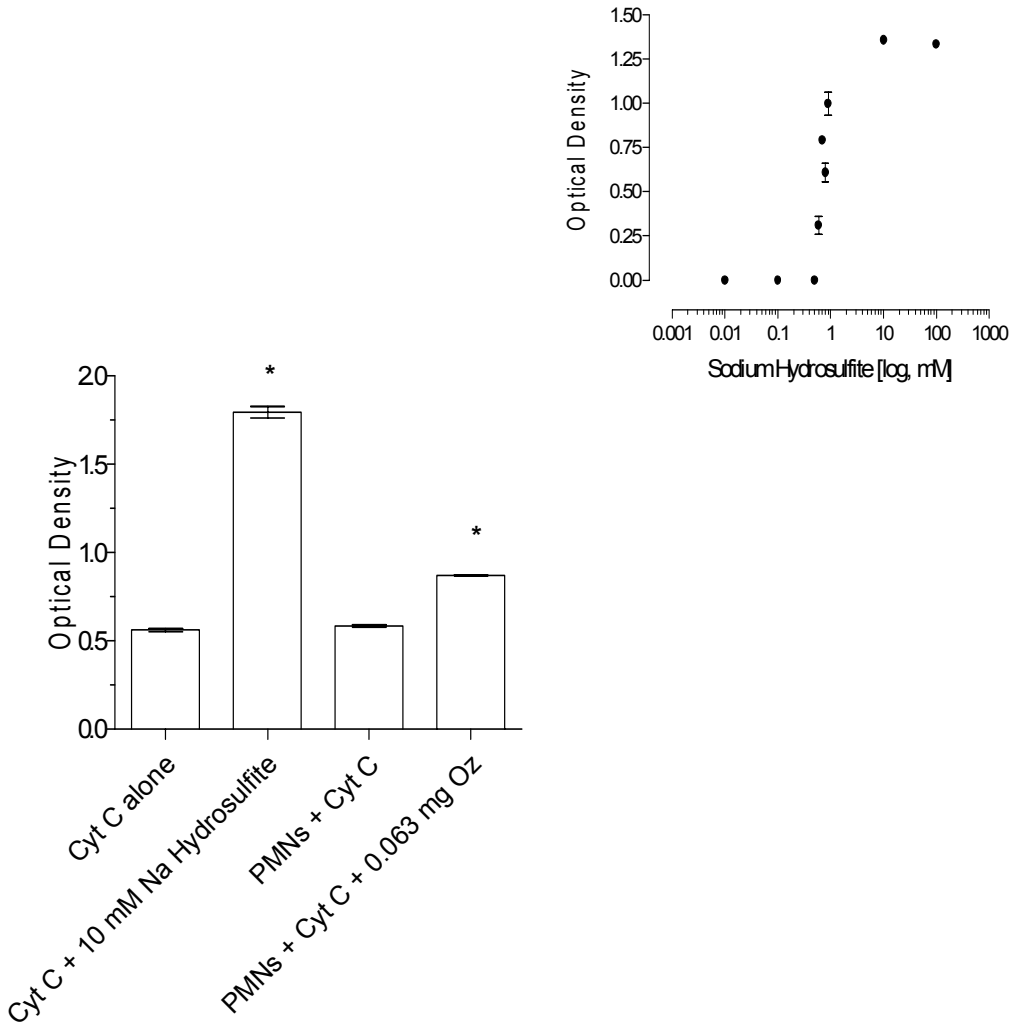

Figure 16. Superoxide anion production by $2x10^6$ bovine neutrophils (PMNs) following incubation with 0.063 mg opsonized zymosan (Oz) [PMNs + Cyt C + 0.063 mg Oz], compared to maximum optical density achievable with the (Cytochrome C) reducing agent, sodium hydrosulfite [Cyt C + 10 mM Na Hydrosulfite]. Raw optical density at 550 nm after 30 minutes of incubation at 39 ^oC indicates cytochrome C (Cyt C) reduction and is illustrated by the Y axis values. Data points represent mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 3 days total for n=3. *Statistically significant differences between [Cyt C alone] and [Cyt C + 10mM Na hydrosulfite], as well as between [PMNs + Cyt C] and [PMNs + Cyt C + Oz] were observed (paired, two-tailed T-test, $p<0.05$). Inset: Cyt C in the presence of various concentrations of Na hydrosulfite. No bovine neutrophils were present. Data points represent mean of 2 wells, +/- SEM.

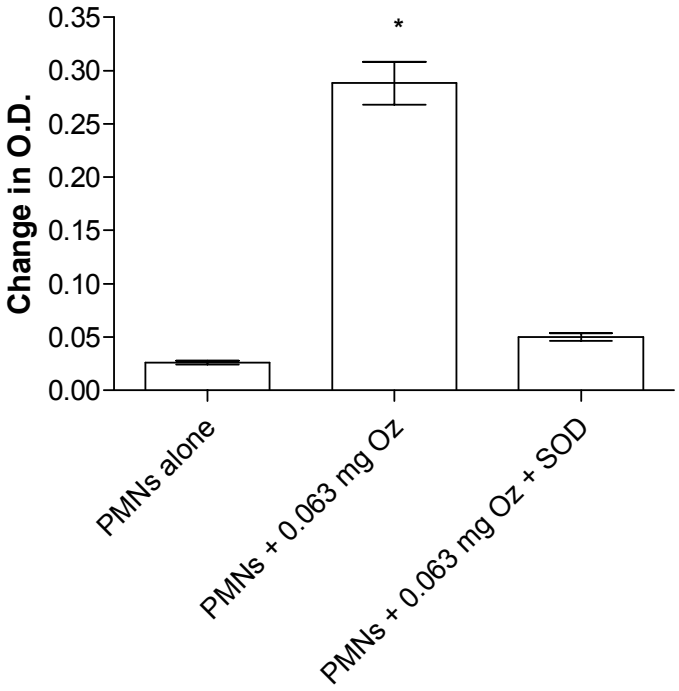

Figure 17. Superoxide anion production by $2x10^6$ bovine neutrophils (PMNs) following incubation with 0.063 mg opsonized zymosan (Oz) with and without 15 units of the free radical scavenger, superoxide dismutase (SOD). Change in optical density (O.D.) at 550 nm indicates cytochrome C reduction. Data points represent mean +/- SEM of pooled sample of neutrophils from 5 steers per day, 3 days for n=3. *Statistically significant differences between [PMNs + 0.063 mg Oz] and both [PMNs alone] and [PMNs + 0.063 mg Oz + SOD] were observed (paired, two-tail T-test, $p<0.05$).

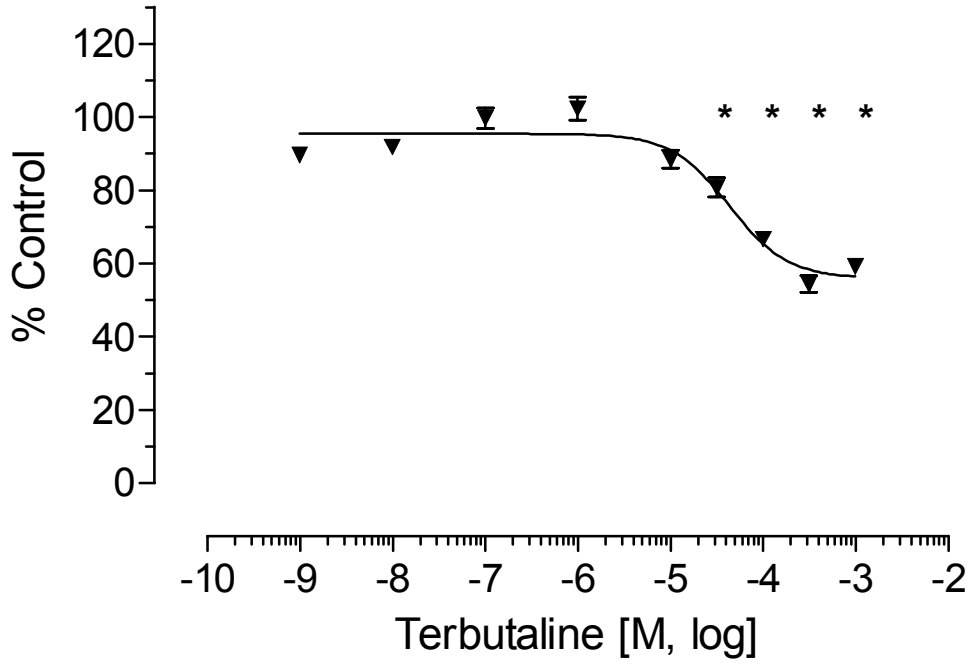

Figure 18. Superoxide anion production by $2x10^6$ bovine neutrophils exposed to 0.063 mg opsonized zymosan with various concentrations of the selective β_2-adrenergic receptor agonist, terbutaline, for 30 minutes at 39 ºC. Values represent the percent of control superoxide anion production (Optical Density = 0.294 +/- 0.097; 95 % confidence interval), identified as neutrophil exposure to 0.063 mg opsonized zymosan alone (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 3 days total for n=3). Maximum decrease in superoxide anion production (40%) occurred with $5x10^{-4}$M terbutaline. *A statistically significant difference from control superoxide anion production was achieved with terbutaline at concentrations equal to and greater than $5x10^{-5}$M (Paired, two-tail T-test, $p<0.05$).

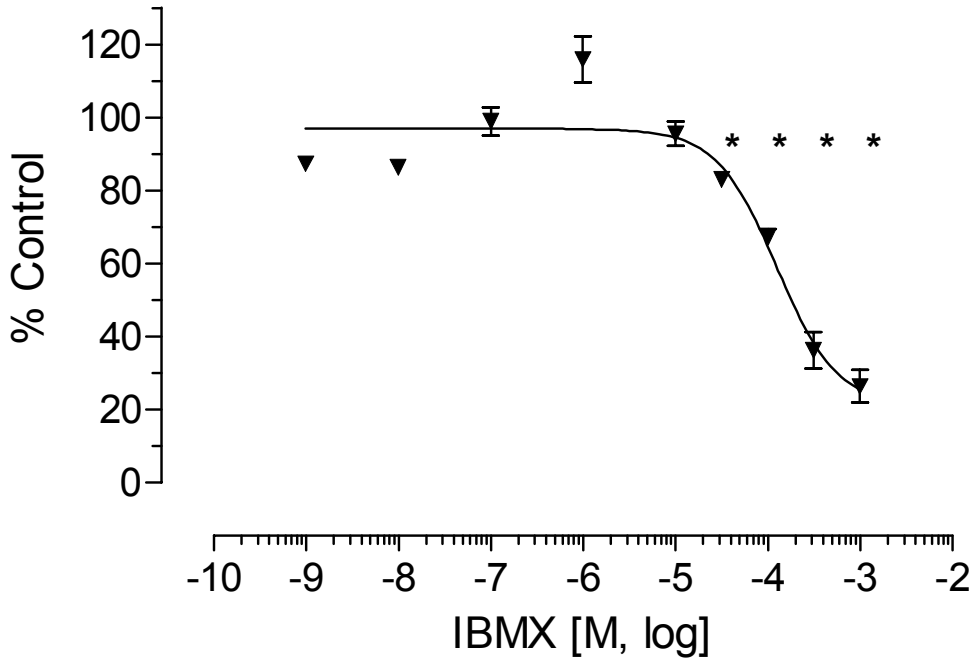

Figure 19. Superoxide anion production by $2x10^6$ bovine neutrophils exposed to 0.063 mg opsonized zymosan with various concentrations of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), for 30 minutes at 39 °C. Values represent the percent of control superoxide anion production (Optical Density = 0.294 +/- 0.097; 95% confidence interval), identified as neutrophil exposure to 0.063 mg opsonized zymosan alone (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 3 days total for n=3). Maximum decrease in superoxide anion production (74%) occurred with IBMX at $1x10^{-3}$M. *A statistically significant difference from control superoxide anion production was achieved with IBMX at concentrations equal to and greater than $5x10^{-5}$M (Paired, two-tail T-test, $p<0.05$).

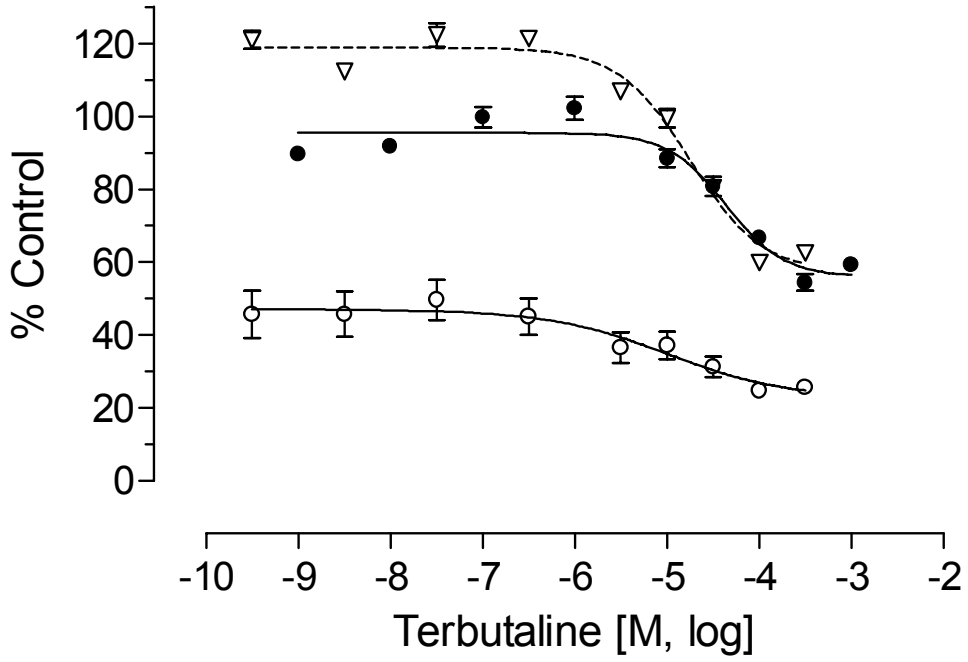

Figure 20. Superoxide anion production by $2x10^6$ bovine neutrophils exposed to 0.063 mg opsonized zymosan with various concentrations of terbutaline alone (closed circle) or with either $5x10^{-6}$M 3-isobutyl-1-methylxanthine (IBMX) (open triangle) or $1x10^{-4}$M IBMX (open circle), for 30 minutes at 39 °C. Values represent the percent of control superoxide anion production (Optical Density = 0.294 +/- 0.097; 95% confidence interval), identified as neutrophil exposure to 0.063 mg opsonized zymosan alone (mean +/- SEM, IBMX, 3 days total for n=3). *Statistically significant differences were observed between data points representing all concentrations of terbutaline alone and terbutaline + $1x10^{-4}$M IBMX (Paired, two-tail T-test, $p<0.05$).

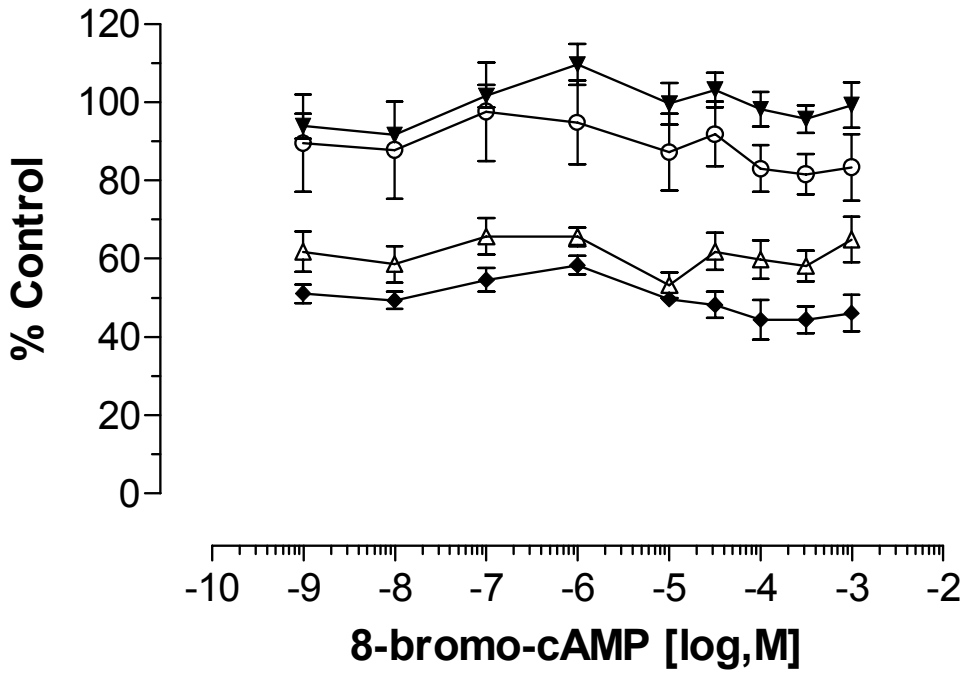

Figure 21. Superoxide anion production by $2x10^6$ bovine neutrophils exposed to 0.063 mg opsonized zymosan, following preincubation with various concentrations of the synthetic version of cyclic AMP (cAMP), 8-bromo-cAMP, at 25 °C for either 0 minutes (closed triangle), 10 minutes (open circle), 20 minutes (open triangle), or 30 minutes (closed diamond). Values represent the percent of control superoxide anion production (Optical Density = 0.369 +/- 0.098; 95% confidence interval), identified as neutrophils pre-incubated with Hanks balanced salt solution (HBSS) for the respective length of time before exposure to 0.063 mg opsonized zymosan (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 3 days total for n=3). There were no consistent statistically significant differences between data points reflecting various doses to support the presence of a dose-response for each 8-bromo-cAMP pre-incubation time period.

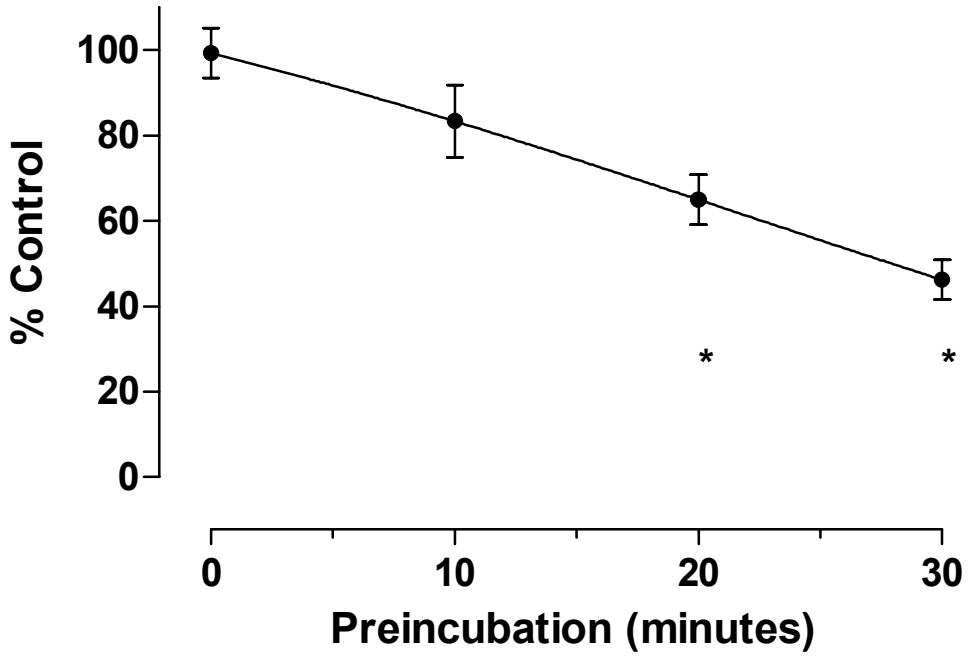

Figure 22. Superoxide anion production by $2x10^6$ bovine neutrophils exposed to 0.063 mg opsonized zymosan, following pre-incubation with $1x10^{-3}$M 8-bromo-cAMP at 25 °C for either 0 minutes, 10 minutes, 20 minutes, or 30 minutes. Values represent the percent of control superoxide anion production (Optical Density = 0.369 +/- 0.098; 95% confidence interval), identified as neutrophils pre-incubated with Hanks balanced salt solution (HBSS) for the respective length of time before exposure to 0.063 mg opsonized zymosan (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 3 days total for n=3). *A statistically significant difference from control superoxide anion production was achieved by pre-incubating the neutrophils with 8-bromo-cAMP ($1x10^{-3}$M) for 20 minutes or 30 minutes at 25 °C (paired, two-tail T-test, $p<0.05$).

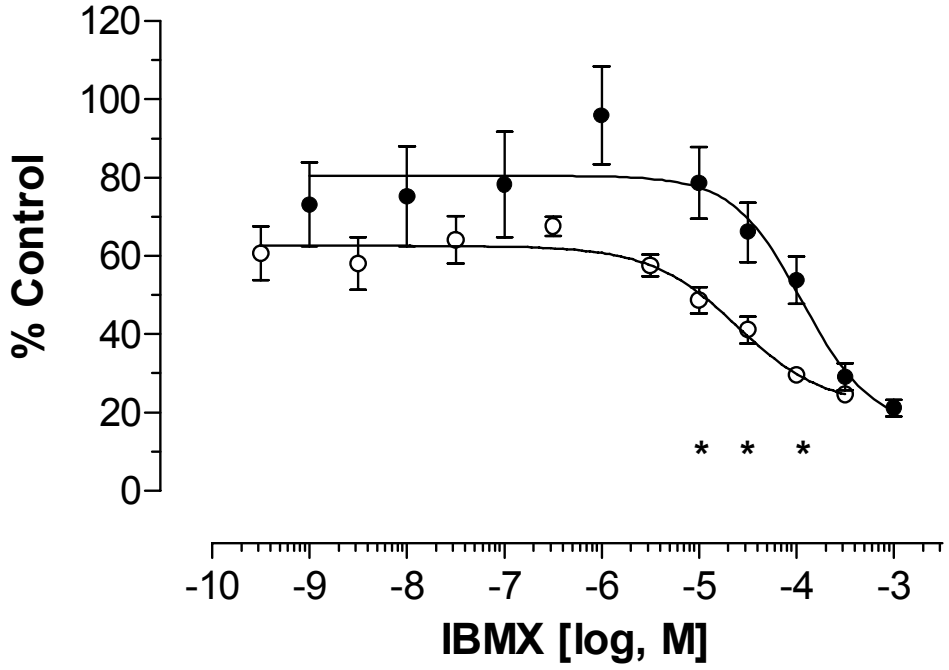

Figure 23. Superoxide anion production by $2x10^6$ bovine neutrophils exposed to 0.063 mg opsonized zymosan with various concentrations of 3-isobutyl-1-methylxanthine (IBMX), following pre-incubation with Hanks balanced salt solution (HBSS) alone (closed circle) or with $1x10^{-3}$M 8-bromo-cAMP (open circle) for 15 minutes at 25 °C. Values represent the percent of control superoxide anion production (Optical Density = 0.432 +/- 0.291; 95% confidence interval), identified as neutrophils pre-incubated with HBSS before exposure to 0.063 mg per well of opsonized zymosan (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 3 days total for n=3). *Statistically significant differences in superoxide anion production are present between IBMX alone ($1x10^{-5}$M, $5x10^{-5}$M and $1x10^{-4}$M) and respective concentrations of IBMX + $1x10^{-3}$M 8-bromo cAMP (paired, two-tail T-test, $p<0.05$).

CHAPTER 4: Modulation of the Bovine Neutrophil β_2-Adrenergic Receptor Mechanism by Inflammatory Stimuli.

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4.1 Abstract

The objective of this study was to determine whether the bovine neutrophil β_2-adrenergic receptor mechanism is dysfunctional following exposure to inflammatory stimuli. Neutrophils were obtained from five healthy 6 month-old Angus-cross steers. The ability of the selective β_2-adrenergic receptor agonist terbutaline and the non-selective phosphodiesterase inhibitor IBMX to raise intracellular cAMP levels and decrease superoxide anion production was assessed following exposure to increasing concentrations of an inflammatory stimulant (opsonized zymosan) and a PKC activator (PMA). By increasing the concentration of opsonized zymosan eight-fold, the ability of various concentrations of terbutaline and IBMX to reduce superoxide anion production was reduced. However, exposure to PMA resulted in increased terbutaline-mediated cAMP production, with the effect being dependent upon both PMA dose and time of incubation with the cells. Opsonized zymosan and PMA are both known to activate PKC; however, based on these data there appear to be multiple, discrete effects of PKC activation on the bovine neutrophil β_2-adrenergic receptor mechanism function.

4.2 Introduction

The bovine neutrophil is a central component of the acute inflammatory response to pulmonary pathogens and plays a particularly important role in the pathophysiology of bovine respiratory disease (BRD), also known as shipping fever.[137] There are three basic classes of receptors on the neutrophil plasma membrane (ion channel-coupled receptors, enzyme-coupled receptors, and G-protein-coupled receptors) that enable the cell to respond to distinct pro-inflammatory[10, 12, 13] and anti-inflammatory[25, 26, 28, 30, 196] chemical signals. In particular, the β_2-adrenergic receptor (a G-protein-coupled receptor) has been identified on bovine neutrophils, its anti-inflammatory effects have been characterized *in-vitro*, and the importance of the autonomic nervous system in regulation of bovine neutrophil function has been discussed (Chapter 3).

However, it remains unknown how the bovine neutrophil β_2-adrenergic receptor signaling mechanism is affected by experimental and / or spontaneous disease, such as BRD. To be more specific, although dysfunction of the β_1-adrenergic receptor mechanism in human cardiac tissue and dysfunction of the β_2-adrenergic receptor mechanism in both human neutrophils and smooth muscle tissue has been described in numerous disease states, including congestive heart failure,[38, 39, 49-51] asthma,[40] cystic fibrosis,[41] atopic dermatitis,[42] pheochromocytoma,[43] myasthenia gravis,[44] hypertension,[45] and sepsis,[46] and although dysfunction of the β_2-adrenergic receptor mechanism in bovine and ovine pulmonary airway and vascular smooth muscle following exposure to *M. haemolytica* has been described,[56-59] there are no reports that describe dysfunction of the bovine neutrophil β_2-adrenergic receptor mechanism. Therefore, it is important to study the effect of inflammatory stimuli on bovine neutrophil β_2-adrenergic receptor mechanism function.

Investigative efforts to describe the pathogenesis of various diseases affecting the β_1- or β_2-adrenergic receptor mechanism have yielded a multifactor mechanism of disease entitled, "desensitization". Desensitization is a process where a G-protein-coupled receptor (such as the β_1- or β_2-adrenergic receptor) becomes functionally

uncoupled from its effector enzyme, such as adenylyl cyclase.[200] When desensitization occurs, receptors are rendered non-responsive to further stimulation. This leads to cellular and / or systemic malfunction, with the type and degree of malfunction dictated by the receptor type and tissue. Desensitization is further categorized into either homologous desensitization or heterologous desensitization. Homologous desensitization of a receptor is the manifestation of excessive exposure to a ligand which is specific to that same receptor (i.e., catecholamines and other β_1-adrenergic receptor agonists causing homologous desensitization of the cardiac β_1-adrenergic receptor during heart failure),[49, 200, 207, 208] while heterologous desensitization of a receptor is the manifestation of excessive exposure to a ligand which is specific to another type of receptor (i.e., platelet-activating factor causing heterologous desensitization of the human neutrophil interleukin-8 receptor, or excessive activation of airway muscarinic receptors causing heterologous desensitization of pulmonary smooth muscle β_2-adrenergic receptors).[47,48,54]

The direct cAMP detection immunoassay and the cytochrome C reduction assay are valuable tools for testing bovine neutrophil β_2-adrenergic receptor mechanism function. To be more specific, because activation of the β_2-adrenergic receptor on bovine neutrophils results in a rise in intracellular cAMP production (Chapter 3), measuring intracellular cAMP concentration following β_2-adrenergic receptor activation is a useful approach to determining if the bovine neutrophil β_2-adrenergic receptor signaling mechanism is functional following exposure to inflammatory stimuli.[26, 28, 32, 192] Superoxide anion, a member of the reactive oxygen species, is produced by both human and bovine neutrophils upon exposure to various concentrations of certain inflammatory mediators, including opsonized zymosan, and is readily measured using the cytochrome C reduction assay.[28, 33, 198, 226, 227, 231, 232, 234] The production of superoxide anion has been demonstrated to decrease, in a dose-dependent manner, following activation of the bovine neutrophil β_2-adrenergic receptor mechanism (Chapter 3). Therefore, identification of a decrease in superoxide anion production following β_2-adrenergic receptor activation is a second useful approach to determining if the bovine neutrophil β_2-adrenergic receptor signaling mechanism is functional following exposure to inflammatory stimuli.[25, 198]

Identifying blood cell β-adrenergic receptor functionality as a means of monitoring and / or explaining disease states in major organ systems has been reported for over 20 years. Drawing from insight provided by studies on heart failure, asthma and BRD, it is reasonable to ask whether inflammatory stimuli can modulate β_2-adrenergic receptor mechanism function. In this study opsonized zymosan and PMA were used as the inflammatory stimulus to investigate modulation of bovine neutrophil β_2-adrenergic receptor mechanism function.

4.3 Methods

4.3.1 Animals

A group of 5 Angus-cross, 6-9 months of age beef cattle (steers) were purchased by the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) from a local farmer and maintained on pasture while fed a supplemental concentrate diet (16% crude protein, Stocker / Heifer Grain Mix, Southern States, Richmond, VA) at 10 lbs / head / day. All aspects of animal husbandry and procedures performed (blood collection) were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech, and implemented by staff at the VMRCVM Non-Client Animal Facility.

4.3.2 Bovine Neutrophil Isolation

Neutrophils were harvested from steer and dairy cow whole blood following the method of Roth and Kaeberle[227], with modifications. Briefly, whole blood was collected via jugular venipuncture and centrifuged at 1000 x g for 20 minutes at 25ºC, utilizing no brake. Acid-citrate-dextrose was used as the anticoagulant. Following aspiration and removal of the plasma, buffy coat material, and approximately ½ of the remaining red blood cell pellet, 2 volumes of cold phosphate-buffered water was added for 1 minute to initiate red blood cell lysis. Tonicity was restored by adding 1 volume of hypertonic phosphate - buffered saline. The neutrophils were then washed 3 times with sterile-

filtered Hanks balanced salt solution without calcium and magnesium (HBSS, Sigma Chemical Co., MO), following centrifugation at 300 x g for 10 minutes at room temperature. For all experiments, neutrophils were harvested and analyzed within the same 8 hour day. Cells were counted using a hemocytometer and a microscope, while purity was identified using forward and side scatter flow cytometry.

4.3.3 cAMP Production

cAMP production by bovine neutrophils was quantified using the Direct cAMP Enzyme Immunoassay 96 Well Kit (Sigma Chemical Co.). Neutrophils were harvested from steer whole blood and brought to a final concentration of $2x10^7$ cells / ml in HBSS containing calcium and magnesium. 500 µl aliquots of the cell suspension were added to 12 x 75 mm borosilicate glass tubes, to which 50 µl of HBSS or 50 µl of the PKC activator, PMA were added. The neutrophils were then placed in a 39 ^oC water bath for 10 or 30 minutes. Following this incubation period, 100 µl of either HBSS or cAMP-elevating agents (terbutaline and / or IBMX) were added. The tubes then remained in the water bath for an additional 5 minutes. The reaction was stopped by placing all tubes on ice for 5 minutes and centrifuging at 300 x g for 5 minutes at 4 ºC. Tubes were then decanted and the neutrophils were resuspended with 500 µl of 0.1M HCl + 0.1% Triton X-100 for cell lysis. Following a 20 minute incubation period at room temperature, tubes were centrifuged at 600 x g for 5 minutes at room temperature. From each tube, 100 µl of supernatant were removed and placed into the corresponding wells of the enzyme immunoassay kit. Analysis of cAMP concentration in each supernatant was performed in accordance with the kit's technical bulletin.

4.3.4 Superoxide Anion Production

Superoxide anion production was measured using the cytochrome C reduction assay, as described by Roth and Kaeberle.[227] Reduction of cytochrome C was measured using a spectrophotometer 96 – well microplate reader (UVmax, Molecular Devices, Sunnyvale, CA), set to read at 550 nm with correction (subtraction) at 650 nm, and was

communicated as optical density (O.D.) values with a blank subtracted. First, neutrophils were harvested from steer whole blood and brought to a concentration of $4x10^7$ cells / ml in HBSS containing calcium and magnesium. Next, 50 µl of neutrophils ($2x10^6$ cells), 50 µl of HBSS, 150 µl (0.41 mg) of cytochrome C with or without 15 units superoxide dismutase (SOD), and 50 µl (0.063 mg or 0.50 mg) of opsonized zymosan were added to microplate wells. When necessary, $5x10^{-6}$M terbutaline and / or $5x10^{-4}$M IBMX substituted for HBSS in the microwells. For all experiments, regardless of treatment group, the final volume in each microwell was 300 µl.

Immediately after all the reagents were added to the wells, a baseline optical density measurement was obtained. The plate was then vortexed at 52 x g for 30 minutes in an incubator set to 39 °C. After incubation, a second optical density measurement was obtained, with the initial values subtracted. Data points are either expressed as net increase in optical density (O.D.), or percent of the control, where the optical density following bovine neutrophil exposure to the respective concentration of opsonized zymosan alone was established as the control and all data points are expressed as a percentage of that control.

Each experimental day, maximum assay optical density values were obtained by incubating cytochrome C alone with 10 mM sodium hydrosulfide (a reducing agent, also known as sodium dithionide). To be more specific, O.D. values from wells containing neutrophils and opsonized zymosan were compared to the maximum assay optical density value to determine percent available cytochrome C molecules reduced per well.

4.3.5 Statistical Analysis

Data points represent cAMP concentration in the supernatant of $1x10^7$ lysed neutrophils (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation). The number of days sampled ranged between experiments. Statistically significant differences ($p<0.05$) between data points were identified by

performing paired, two-tail T-test ($p<0.05$). All statistical methods were performed, and all graphs were created using Prism 4.0 (GraphPad Software, San Diego, CA).

Data points represent percent of control superoxide anion production by $2x10^6$ intact neutrophils (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation. The number of days sampled ranged between experiments. Statistically significant differences ($p<0.05$) between data points were identified by performing paired, two-tailed T-tests. All statistical methods were performed, and all graphs were created using Prism 4.0 (GraphPad Software, San Diego, CA).

4.4 Results

4.4.1 cAMP Production

The effect of activating PKC on cAMP production by bovine neutrophils was measured. Exposure to various concentrations of the PKC activator, PMA, for 30 minutes at 39 ºC did not result in altered bovine neutrophil basal intracellular cAMP levels (Figure 24). However, the combination of $5x10^{-4}$M IBMX (a non-selective phosphodiesterase inhibitor) and $1x10^{-6}$M PMA resulted in cAMP production at a level greater than that following exposure to $5x10^{-4}$M IBMX alone (Figure 25). Also, PMA was found to exaggerate, in a dose-dependent manner, the rise in cAMP following exposure to the combination of $5x10^{-6}$M of the selective β_2-adrenergic receptor agonist terbutaline and $5x10^{-4}$M IBMX (Figure 26). In addition to the dose-dependency, this effect was time-dependent. Incubation with PMA for only 10 minutes at 39 ºC did not result in a significant exaggeration of the rise in cAMP following exposure to the combination of $5x10^{-6}$M terbutaline and $5x10^{-4}$M IBMX (Figure 27).

4.4.2 Superoxide Anion Production

Time and dose-responses for opsonized zymosan-dependent bovine neutrophil superoxide anion production were previously characterized (Chapter 3). These studies found that maximum superoxide anion production was realized when $2x10^6$ neutrophils / well were incubated for 30 minutes at 39 °C with 0.063 mg per well of opsonized zymosan. Using these parameters, approximately 50% of the molecules of cytochrome C in each well were reduced, which assured adequate assay sensitivity. Also, superoxide anion production was quenched by 15 units / well of superoxide dismutase (SOD).

The effect of opsonized zymosan dose on the terbutaline-dependent decrease in bovine neutrophil superoxide anion production was evaluated. Neutrophils were exposed to increasing concentrations of terbutaline and either 0.063 mg or 0.50 mg per well of opsonized zymosan (Figure 28). The use of 0.50 mg opsonized zymosan per well has been reported in previous studies.[87, 226, 227] However, preliminary experiments (dose-response curve - Chapter 3, Figure 15) revealed that β_2-adrenergic receptor activation had no inhibitory effect on superoxide anion production when at least 0.013 mg was used. In this study, superoxide anion production was decreased in a dose-dependent manner by various concentrations of terbutaline when 0.063 mg, but not 0.50 mg per well opsonized zymosan was used as the superoxide anion stimulant. For example, $5x10^{-4}$M terbutaline reduced 0.063 mg per well opsonized zymosan-dependent superoxide anion production by 55%. However, no such effect of terbutaline was observed at any concentration when 0.50 mg / well opsonized zymosan was used as the stimulant.

The effect of opsonized zymosan dose on IBMX-dependent decrease in bovine neutrophil superoxide anion production was also evaluated. Neutrophils were exposed to $1x10^{-3}$M IBMX and either 0.063 mg or 0.50 mg per well of opsonized zymosan (Figure 29). Superoxide anion production was decreased by $1x10^{-3}$M IBMX when 0.063 mg, but not when 0.50 mg opsonized zymosan per well was used as the superoxide anion stimulant. For example, $1x10^{-3}$M IBMX decreased 0.063 mg / well opsonized zymosan-dependent superoxide anion production by 74%, in contrast to reducing 0.50 mg per well opsonized zymosan-dependent superoxide anion production by 35%.

4.5 Discussion

Dysfunction of the β-adrenergic receptor mechanism is known to contribute to the pathophysiology of diseases in both people and animals. For example, congestive heart failure,[38, 39] asthma,[40] cystic fibrosis,[41] atopic dermatitis,[42] pheochromocytoma,[43] myasthenia gravis,[44] hypertension,[45] and sepsis[46] have all been linked to decreased β_1- / β_2-adrenergic receptor density (depending on the cell type) and / or uncoupling of the respective receptor from adenylyl cyclase. Decreased function of the β_2-adrenergic receptor mechanism has also been described in pulmonary airway and vascular smooth muscle tissue from cattle, sheep, and rats exposed to *M. haemolytica*,[56-59] and provides insight into the pathophysiology of BRD. However, despite their prominent role in the acute inflammatory stage of BRD, there are no reports that describe the dysfunction of the bovine neutrophil β_2-adrenergic receptor mechanism.

This is the first study to investigate the effect of inflammatory stimuli on components of the β_2-adrenergic receptor mechanism in bovine neutrophils. The β_2-adrenergic receptor mechanism's ability to function in the presence of (and indeed decrease the response to) the inflammatory stimulant opsonized zymosan has been described (Chapter 3); however, here we report that this function is not retained when the dose of opsonized zymosan is increased eight-fold to 0.50 mg per $2x10^6$ neutrophils, a dose used by others for neutrophil inflammatory function assays.[88, 226, 227] Indeed, increasing concentrations of terbutaline ($1x10^{-9}$M to $1x10^{-3}$M) were unable to decrease bovine neutrophil superoxide anion production when the opsonized zymosan dose was at 0.50 mg. The results suggest that the site of dysfunction could be the β_2-adrenergic receptor, the G_s-protein, adenylyl cyclase, or any site "down-stream", such as PKA activation or microtubule assembly because the treatment affected β_2-adrenergic receptor mechanism function following exposure to the selective β_2-adrenergic receptor agonist. The data support other studies that describe heterologous desensitization of the β_2-adrenergic receptor mechanism in human mononuclear leukocytes and neutrophils during

an inflammatory state.[46, 211] Also, we demonstrate here for the first time that an increased dose of opsonized zymosan disables the IBMX-mediated decrease in bovine neutrophil superoxide anion production, reducing the maximal percent inhibition from 74% to 30%. These data more specifically suggest that the β_2-adrenergic receptor mechanism dysfunction is centered upon downstream actions, such as enhanced phosphodiesterase activity, decreased PKA activation, or enhanced microtubule assembly. Essentially any site "down-stream" to cAMP production is open for further investigation into the mechanism for this receptor cross-talk.

In this study, we also asked the more specific question of how activation of PKC, a component to various inflammatory mediator signal transduction pathways, may affect bovine neutrophil β_2-adrenergic receptor mechanism function. The opsonized zymosan product used in this study is known to activate PKC, and is presumed to be composed of zymosan particles coated by activated complement fragment C3b and gamma-immunoglobulin (IgG).[172] When approached by a neutrophil, these opsonins are believed to bind to G_q-protein-coupled and tyrosine kinase-coupled receptors, respectively. The signal transduction pathway for both of these types of receptors converges at the level of PKC recruitment and activation.[172, 240, 241] Therefore, we believe that raising intracellular PKC levels in bovine neutrophils via PMA administration is a relevant *in-vitro* technique for modeling the acute inflammatory stage of BRD. Indeed, several inflammatory mediators (IL_1-β, LTB_4, and fMLP) have been linked to PKC-dependent β_2-adrenergic receptor mechanism dysfunction in airway epithelial cells and human neutrophils.[211, 242] Moreover, the intracellular domain of the β_2-adrenergic receptor is known to be phosphorylated by both PKC and GRK-2,[201, 202, 243, 244] with additional upregulation of GRK-2 provided by PKC.[212] PMA is a compound known to imbed itself into plasma membranes and function as a synthetic analogue of DAG by constitutively activating PKC. Therefore it is of little surprise that PMA exposure is reported to result in decreased β-adrenergic receptor mechanism function in human lymphocytes, human airway smooth muscle, duck erythrocytes, turkey erythrocytes, rat reticulocytes, Chinese hamster fibroblasts expressing β_2-adrenergic receptors, and 1321N1 human neuroblastoma cells.[54, 245-250]

The present study is the first time bovine neutrophil β_2-adrenergic receptor mechanism function was examined following PMA administration. Surprisingly, various concentrations of PMA were not able to decrease cAMP production; PMA exaggerated terbutaline and IBMX-mediated cAMP production, in a time and dose-dependent manner. This suggests that PMA increases the number of cAMP molecules presented to phosphodiesterase, but not enough to saturate the enzyme and have a "spill-over" effect. It is likely that this is accomplished by either activating the β_2-adrenergic receptor, enhancing G_s-protein activity, decreasing G_i-protein activity and / or by activating adenylyl cyclase. Indeed, we suggest that PKC may promote any component of the β_2-adrenergic receptor mechanism upstream to the phosphodiesterase enzyme. The time-dependency of the PMA effect is intriguing (efficacy at 30 minutes but not 10 minutes), but alone does not confirm the site of action (receptor vs. enzyme).

We are uncertain why an elevated dose of opsonized zymosan decreased β_2-adrenergic receptor mechanism function yet PMA enhanced cAMP production in a time and dose-dependent manner. As already discussed, some published data support the phenomenon of PMA-induced β-adrenergic receptor mechanism dysfunction.[212, 243] However, several reports also illustrate the ability of PMA to increase β_2-adrenergic receptor mechanism function. For example, exposure to PMA exaggerated (in a time and dose-dependent manner) isoproterenol-dependent cAMP production in human neutrophils and eosinophils, as well as in RAW 264.7 macrophages and bovine aortic endothelial cells.[251-255] The authors of these reports commented on the conflicting nature of their data compared to others, and suggested that the conflicting effects may be due to either preincubation conditions (time, concentration) or cell type used.[250] Indeed, the reports that demonstrate β-adrenergic receptor mechanism dysfunction describe the incubation of cells with approximately $1x10^{-6}$M PMA for 30 minutes at 37 ºC, while those that demonstrate hyperfunction describe the incubation of cells with a lower concentration of PMA (approximately $1x10^{-8}$M) for shorter periods of time (5 to 30 minutes). However, the current study demonstrates β_2-adrenergic receptor mechanism

hyperfunction after incubation with various concentrations of PMA ($1x10^{-12}$M up to $1x10^{-6}$M) for 10 or 30 minutes.

The existence of various PKC isoforms is also reported as an explanation of PMA's contrasting effects on β-adrenergic receptor mechanism function.[256, 257] There are currently 12 members in the PKC isoform family, which have been primarily characterized in T and B lymphocytes and are classified into three groups based on their various functions: conventional (α, βI, βII, and γ), novel (δ, ε, η, ϕ, and μ), and atypical (ι, λ, and ζ) isoforms. Indeed, it is conceivable that the production of particular individual isozymes by cells may be coordinated. For example, PKC-μ is reported to increase β-adrenergic receptor density in human airway epithelial cells exposed to IL-1β,[172] while PKC-μ and PKC-ε increase cAMP production in RAW 264.7 macrophages[254] and PKC-δ increases cAMP production in human eosinophils.[253] In contrast, bovine PKC-α and PKC-βII are reported to reduce human β_2-adrenergic receptor-mediated cAMP production in human embryonic kidney (HEK-293) cells.[258] This last study also describes direct phosphorylation of the human β_2-adrenergic receptor by bovine PKC-α, in addition to decreased cAMP production following exposure to $1x10^{-8}$M PMA, suggesting that PMA causes heterologous desensitization of the β_2-adrenergic receptor via PKC-α activation. Also, PKC-β and PKC-ε but not PKC-α are reported to translocate to the plasma membrane of human monocytes following cross-linking and activation of either FcγRI or FcγRII, with PMA stimulating the translocation of all three isozymes.[259] Yet another report describes the translocation of PKC-α, βI, βII, δ, ε, and μ isoforms to the RAW 264.7 macrophage plasma membrane following PMA administration.[254] Finally, one report suggests a dual-role for PKC in human neutrophils, but does not comment on the individual roles by PKC isoforms, by demonstrating cAMP-dependent F-actin reorganization during receptor-mediated phagocytosis,[260] suggesting that inflammatory stimuli and phagocytosis may be dependent upon cAMP production. Clearly, extrapolation of PKC isoform data between species and cell types requires standardization of isozyme profiles, cell type and stimuli. Because of this, and because there are no reports of PKC isoform distribution or function in neutrophils from any

species, extrapolation of these data for use with bovine neutrophils should be approached with caution.

In summary, this study illustrates a compromised terbutaline and IBMX-dependent decrease in bovine neutrophil superoxide anion production when opsonized zymosan is increased from 0.063 to 0.50 mg per $1x10^6$ cells. This study also demonstrates the ability of PMA to exaggerate terbutaline and IBMX-dependent cAMP production, in a time and dose-dependent manner. Additional studies that identify the PKC isoform repertoire expressed in bovine neutrophils will undoubtedly shed light on the potential for selective PKC isoform manipulation, and may therefore lead to more specific identification and treatment of β_2-adrenergic receptor mechanism dysfunction. Also, it remains to be seen how the various components of the bovine neutrophil β_2-adrenergic receptor mechanism function *in-vivo* during the acute inflammatory stage of BRD.

4.5 Acknowledgements

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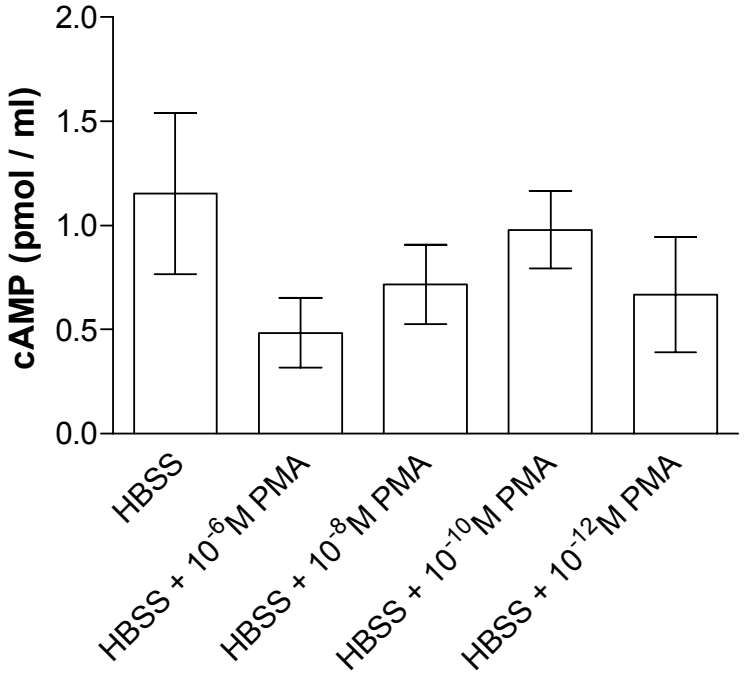

Figure 24. Cyclic AMP (cAMP) production by bovine neutrophils exposed to Hank's balanced salt solution (HBSS) alone or with various concentrations of the protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA), for 30 minutes at 39 °C. Values represent the concentration of cAMP in supernatant of $1x10^7$ lysed neutrophils (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 3 days total for n=3). A statistically significant difference from control cAMP production (HBSS) was not achieved by pre-incubating the neutrophils with various concentrations of PMA (Paired, two-tail T-test).

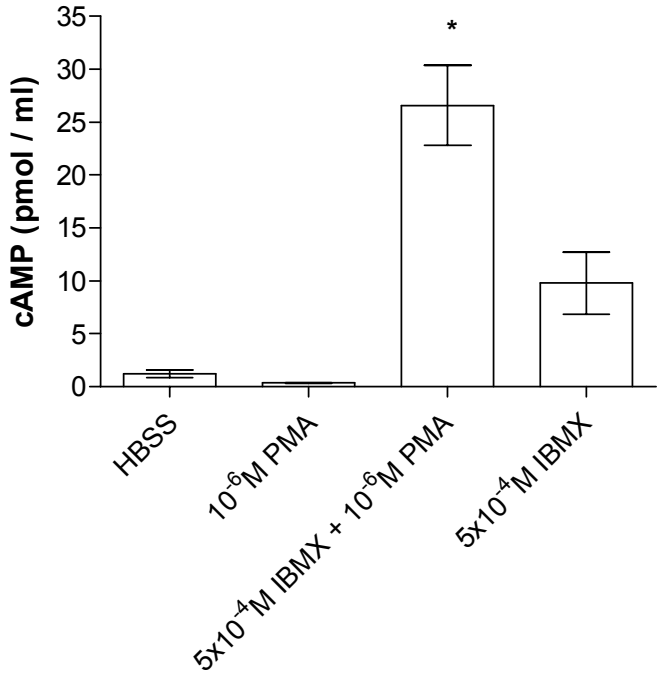

Figure 25. Cyclic AMP (cAMP) production by bovine neutrophils exposed to Hank's balanced salt solution (HBSS) alone or with $1x10^{-6}$M phorbol 12-myristate 13-acetate (PMA), $5x10^{-4}$M of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), or the combination thereof for 30 minutes at 39 °C. Values represent the concentration of cAMP in supernatant of $1x10^{7}$ lysed cells (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 3 days total for n=3). *The differences between the value representing $5x10^{-4}$M IBMX + $1x10^{-6}$M PMA and both PMA alone and IBMX alone were statistically significant (Paired, two-tail T-test, $p<0.05$).

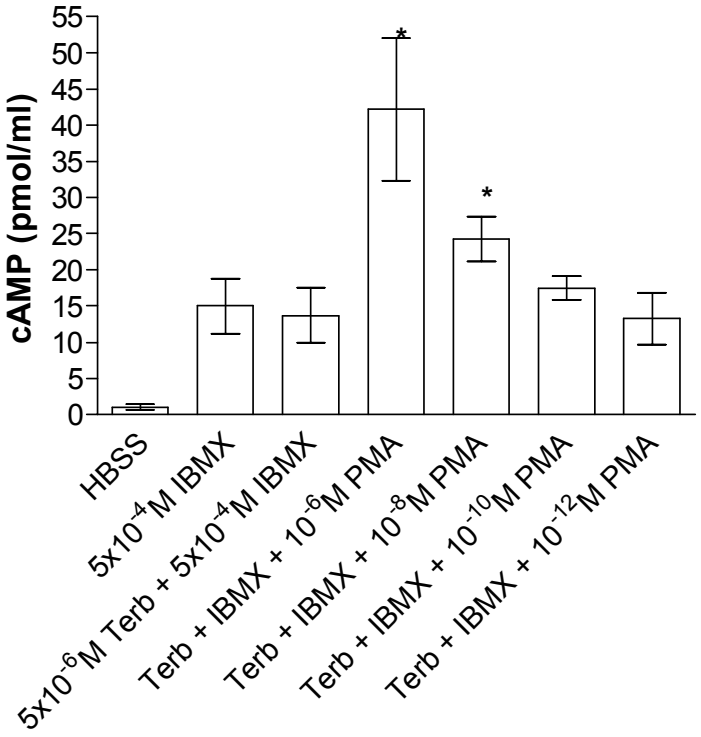

Figure 26. Cyclic AMP (cAMP) production by bovine neutrophils exposed to Hank's balanced salt solution (HBSS), $5x10^{-4}$M 3-isobutyl-1-methylxanthine (IBMX), or the combination of various concentrations of phorbol 12-myristate 13-acetate (PMA) with $5x10^{-6}$M of the selective β_2-adrenergic receptor agonist terbutaline (Terb), with or without $5x10^{-4}$M IBMX, for 30 minutes at 39 ºC . Values represent the concentration of cAMP in the supernatant of $1x10^{7}$ lysed cells (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 4 days total for n=4). *The differences between $5x10^{-6}$M Terb + $5x10^{-4}$M IBMX and Terb + IBMX + 10^{-6}M PMA, and between $5x10^{-6}$M Terb + $5x10^{-4}$M IBMX and Terb + IBMX + 10^{-8}M PMA were both statistically significant (Paired, two-tail T-test, $p<0.05$). These data demonstrate a dose-dependent, PMA-mediated rise in bovine neutrophil cAMP concentration.

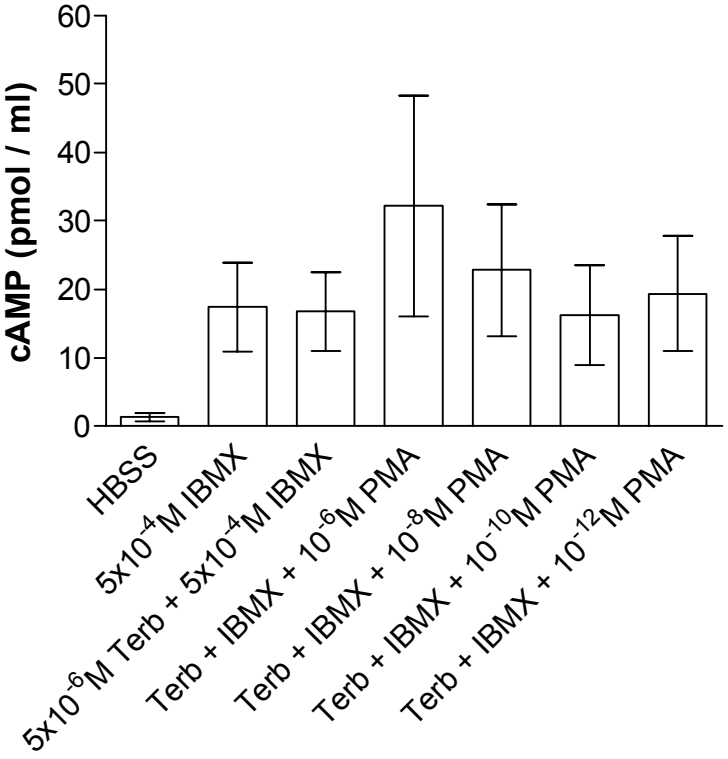

Figure 27. Cyclic AMP (cAMP) production by bovine neutrophils exposed to Hank's balanced salt solution (HBSS), $5x10^{-4}$M 3-isobutyl-1-methylxanthine (IBMX), or the combination of various concentrations of phorbol 12-myristate 13-acetate (PMA) with $5x10^{-6}$M of the selective β_2-adrenergic receptor agonist terbutaline (Terb), with or without $5x10^{-4}$M IBMX, for 10 minutes at 39 ºC . Values represent the concentration of cAMP in the supernatant of $1x10^7$ lysed cells (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 4 days total for n=4). The differences between $5x10^{-6}$M Terb + $5x10^{-4}$M IBMX and Terb + IBMX + 10^{-6}M PMA, and between $5x10^{-6}$M Terb + $5x10^{-4}$M IBMX and Terb + IBMX + 10^{-8}M PMA were not statistically significant.

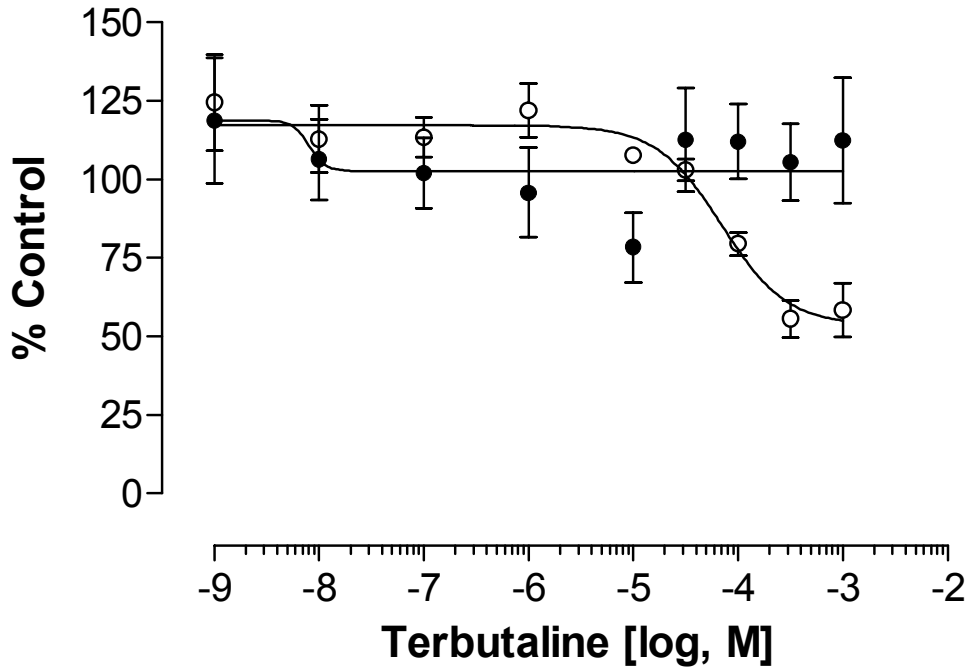

Figure 28. Superoxide anion production by $2x10^6$ bovine neutrophils exposed to either 0.063 mg (open circle) or 0.50 mg (closed circle) opsonized zymosan with various concentrations of terbutaline for 30 minutes at 39 ºC. Values represent the percent of control superoxide anion production, defined as Optical Density at 550 nm = 0.279 +/- 0.099 for neutrophil exposure to 0.063 mg opsonized zymosan, and 0.229 +/- .225 for neutrophil exposure to 0.50 mg opsonized zymosan; 95% confidence interval (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 4 days total for n=4). Statistically significant differences in superoxide anion production values at each terbutaline concentration were not noted between 0.50 mg and 0.063 mg opsonized zymosan doses [paired, two-tail T-test; p values = 0.0823 (at $1x10^{-4}$M terbutaline), 0.0616 (at $5x10^{-4}$M terbutaline), and 0.1303 (at $1x10^{-3}$M terbutaline), respectively].

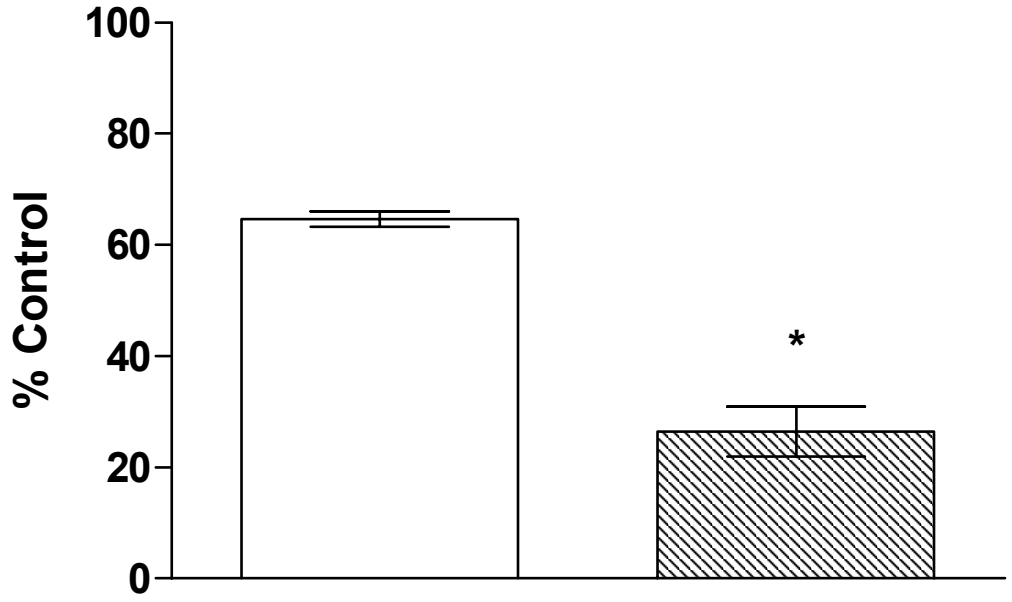

Figure 29. Superoxide anion production by $2x10^6$ bovine neutrophils exposed to either 0.063 mg (shaded bar) or 0.50 mg (open bar) opsonized zymosan, with $1x10^{-3}$M 3-isobutyl-1-methylxanthine (IBMX) for 30 minutes at 39 ºC. Values represent the percent of control superoxide anion production (Optical Density at 550 nm = 0.294 +/- 0.097; 95% confidence interval), identified as neutrophil exposure to either 0.063 mg or 0.50 mg opsonized zymosan alone (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 3 days total for n=3). *A statistically significant difference was noted between $1x10^{-3}$M IBMX with 0.063 mg opsonized zymosan and $1x10^{-3}$M IBMX with 0.50 mg opsonized zymosan (paired, two-tail T-test; $p<0.05$).

Part V: DISCUSSION

CHAPTER 5: General Discussion and Conclusions

Hypothesis 1

The β_2-Adrenergic Receptor Mechanism Exists in Bovine Neutrophils and its Activation Results in Decreased Superoxide Anion Production.

The components of the β_2-adrenergic receptor mechanism previously identified in various tissues from several animal species include the β_2-adrenergic receptor, the intermediary G_s-protein, the effector enzyme adenylyl cyclase, the second messenger cAMP, and the phosphodiesterase enzyme. Positive identification of these components may involve various techniques and logical approaches. The data in this study support the hypothesis that bovine neutrophils possess the components of the β_2-adrenergic receptor signaling mechanism.

Support for the hypothesis is provided here in more detail. First, we identified β-adrenergic receptors on bovine (steer) neutrophils and concluded that the β_2-adrenergic receptor is the predominant receptor subtype. We also examined β-adrenergic receptor densities on steer lymphocytes, dairy cow neutrophils and lymphocytes, and rat lymphocytes. There is no statistically significant difference between the β-adrenergic receptor density on steer neutrophils and dairy cow neutrophils, suggesting that breed or sex-related differences in neutrophil β_2-adrenergic receptor density do not exist between beef calves and dairy cows. However, a difference does exist between bovine (both steer and dairy cow) neutrophil and bovine lymphocyte β-adrenergic receptor density, with lymphocytes possessing a three-fold greater density. Moreover, bovine lymphocytes have a β-adrenergic receptor density two and a half-fold less than rat lymphocytes.

Second, we investigated whether activation of the β_2-adrenergic receptor results in cAMP production in steer neutrophils. The specific β_2-adrenergic receptor agonist terbutaline did not stimulate cAMP production unless neutrophils were also incubated with $5x10^{-4}$M IBMX, a phosphodiesterase inhibitor (Chapter 3, Figures 10 and 11). The dependency upon phosphodiesterase inhibition (and the resultant increased lifespan of molecules of cAMP) for measurable cAMP production following β-adrenergic receptor activation is readily found in the literature,[26, 28, 32, 192] suggesting that bovine neutrophil cAMP may experience a short half-life and / or inherent bovine neutrophil phosphodiesterase activity is vigorous. The β_2-adrenergic receptor was confirmed as the mechanism of action for terbutaline-mediated initiation of cAMP production when the selective β_2-adrenergic receptor antagonist ICI-118,551 negated terbutaline's effect (Chapter 3, Figure 12). Measuring intracellular cAMP concentration in the bovine neutrophil is a good model for characterizing anti-inflammatory drugs because bovine neutrophils can be harvested in great number and remain functionally intact for at least 8 hours at room temperature after isolation from whole blood, and the immunoassay approach to measuring cAMP concentration does not require the use of radioactive agents. Also, because cAMP modulates cytoskeletal rearrangements via activation of PKA and therefore regulates NADPH oxidase formation and resultant superoxide anion formation, it is reasonable to follow-up cAMP production data with the cytochrome C reduction assay.

The cytochrome C reduction assay, the third technique used in this study to demonstrate the presence of the β_2-adrenergic receptor mechanism in bovine neutrophils, measures superoxide anion production, and has been employed extensively with mononuclear and polymorphonuclear leukocytes from various species of animals, including humans.[28, 33, 198, 226, 231, 232, 234] In this study, terbutaline and IBMX decreased bovine neutrophil superoxide anion production by 40% and 74%, respectively, in a dose-dependent manner (Chapter 3, Figure 18 and 19). Also, the synthetic cAMP analog 8-bromo-cAMP decreased bovine neutrophil superoxide anion production by up to 50% in a time-dependent, but not dose-dependent, manner (Chapter 3, Figure 21 and 22). This time-dependent behavior by 8-bromo-cAMP supports other reports of its effects on

leukocytes,[32, 238, 239] as it passively diffuses across the cell membrane before reaching its site of action, PKA. The interdependency between the β_2-adrenergic receptor and adenylyl cyclase on putative PKA activation was illustrated when the inhibitory effect of terbutaline on superoxide anion production in this study was exaggerated following co-incubation with the nonselective phosphodiesterase inhibitor, IBMX (Chapter 3, Figure 20). This finding demonstrates the dose-dependent ability of phosphodiesterase inhibition to bolster β_2-adrenergic receptor-mediated modulation of superoxide anion production. Finally, the present study describes for the first time the exaggerated inhibitory effect of IBMX on superoxide anion production when neutrophils are first preincubated with $1x10^{-3}$M 8-bromo cAMP for 15 minutes (Chapter 3, Figure 23). By preincubating with 8-bromo-cAMP, there were presumably more molecules of cAMP present to activate PKA, in addition to those basally produced and saved from metabolism by IBMX.

In summary, this study suggests that several approaches may be employed to activate the components of the β_2-adrenergic receptor mechanism in bovine neutrophils and decrease pro-inflammatory free radical production. For example, the data discussed in the this study indicate that by allowing for molecules of cAMP that are either produced (following activation of β_2-adrenergic receptors by terbutaline) or given directly in an exogenous manner (8-bromo cAMP administration) to avoid hydrolysis, phosphodiesterase inhibition (by IBMX) works in synergy with both the β_2-adrenergic receptor and adenylyl cyclase to decrease bovine neutrophil superoxide anion production. This gives rise to various clinical uses of therapeutics already on the marketplace for treating the acute inflammatory stage of BRD. There are several β_2-adrenergic receptor agonists currently purchased by veterinarians for use in treating various respiratory disorders in horses, such as clenbuterol and albuterol. Perhaps these products may also be relevant to treating BRD. Also, various phosphodiesterase inhibitors, including aminophylline and theophylline are used by equine and small animal practitioners for treating chronic cough and bronchoconstriction. Perhaps the time has come to coordinate clinical trials to examine their benefit to calves suffering from BRD. The species differences in β-adrenergic receptor density are certainly intriguing. Perhaps diseases of

humans and rodents that center around β-adrenergic receptor mechanism dysfunction would benefit from using bovine neutrophils as a positive control, instead of trying to experimentally reproduce decreased receptor population in human and rodent tissue.

Hypothesis 2

The Bovine Neutrophil β_2-Adrenergic Receptor Mechanism is Dysfunctional Upon Exposure to Increasing Concentrations of Inflammatory Stimuli.

The components of the β_2-adrenergic receptor mechanism work in concert to decrease superoxide anion production in bovine neutrophils. However, during certain disease states the β_2-adrenergic receptor mechanism in human and other animal tissues is reported to be dysfunctional. Observing dysfunction of an individual component allows for positive mechanism of disease identification, and offers the promise of future targeted therapy. The data in this study support the hypothesis that inflammatory stimuli modulate components of the bovine neutrophil β_2-adrenergic receptor signaling mechanism.

Support for the hypothesis is provided here in more detail. First, we investigated how well terbutaline-dependent β_2-adrenergic receptor activation and IBMX-dependent phosphodiesterase inhibition decrease bovine neutrophil superoxide anion production when the dose of the inflammatory stimulant, opsonized zymosan, is increased eight-fold. Terbutaline's ability to decrease superoxide anion production was eliminated, and IBMX's efficacy was significantly compromised (reduced from a 74% decrease in superoxide anion production to 30%). The use of PAF, another inflammatory stimulus relevant to BRD,[10, 124, 261-263] could not be used as an inflammatory stimulant to study β_2-adrenergic receptor dysfunction because PAF itself induces bovine neutrophils to produce superoxide anion production and would confound the superoxide anion production data. For instance, PAF exaggerates (up to 167 %) opsonized zymosan-dependent bovine neutrophil superoxide anion production in a dose-dependent manner (Appendix A), and the effect is independent of the vehicle (Appendix C). Based on the data from the

opsonized zymosan experiments, the mechanism of bovine neutrophil β_2-adrenergic receptor mechanism dysfunction is either enhanced phosphodiesterase activity, decreased PKA activation, or enhanced microtubule assembly; essentially any site "down-stream" to cAMP production is open for further investigation.

Second, we measured the ability of terbutaline and IBMX to reduce bovine neutrophil superoxide anion production when the cells were also incubated with various concentrations of the PKC activator, PMA. In contrast to the general effect of an increase in opsonized zymosan dose rate, PMA exaggerated terbutaline and IBMX-dependent cAMP production, and did so in a time- and dose-dependent manner. These data, in addition to the observation in this study that PMA alone does not alter basal bovine neutrophil cAMP production, suggest that PMA enhances any one or several of the β_2-adrenergic receptor mechanism components upstream to the phosphodiesterase enzyme. The time-dependency of the PMA effect is intriguing (efficacy at 30 minutes but not 10 minutes), but alone does not confirm the site of action (receptor vs. enzyme).

The results from the opsonized zymosan and PMA experiments appear to contradict expectations. Indeed, several reports describe the phenomenon of PMA-induced β-adrenergic receptor mechanism dysfunction, and the bovine neutrophil signal transduction pathways activated by opsonized zymosan and PMA are reported to converge at the level of PKC recruitment and activation.[172, 211, 240-242] Moreover, the intracellular domain of the β_2-adrenergic receptor is known to be phosphorylated by both PKC and GRK-2,[201, 202, 243, 244] with additional upregulation of GRK-2 provided by PKC.[212] PMA is a compound that is known to imbed itself into plasma membranes, functioning as a synthetic analogue of DAG by constitutively activating PKC. Therefore, it is to be expected that PMA exposure is reported to result in decreased β-adrenergic receptor mechanism function in human lymphocytes, human airway smooth muscle, duck erythrocytes, turkey erythrocytes, rat reticulocytes, 1321N1 human neuroblastoma cells, and Chinese hamster fibroblasts expressing β_2-adrenergic receptors.[54, 245-250] However, several reports also illustrate PMA's ability to increase β_2-adrenergic receptor mechanism function. For example, exposure to PMA exaggerated (in a time and dose-dependent

manner) isoproterenol-dependent cAMP production in human neutrophils and eosinophils, in addition to RAW 264.7 macrophages and bovine aortic endothelial cells.[251-255] The authors of these studies commented on the conflicting nature of their data compared to the literature, and suggest that effects may be influenced by preincubation conditions (time, concentration) or cell type used, and may be independent of each other with contradicting results.[250] However, this hypothesis does not explain our results, as we demonstrate β_2-adrenergic receptor mechanism hyperfunction in both a time- and dose-dependent manner.

We propose that the dual effects of different inflammatory stimuli on bovine neutrophil β_2-adrenergic receptor mechanism function may be partially or completely explained by the existence of various PKC isoforms in these cells. For instance, bovine PKC-α and PKC-βII reportedly reduce human β_2-adrenergic receptor-mediated cAMP production in human embryonic kidney (HEK-293) cells.[258] This last study also describes direct phosphorylation of the human β_2-adrenergic receptor by bovine PKC-α, in addition to decreased cAMP production following exposure to $1x10^{-8}$M PMA, suggesting that PMA causes heterologous desensitization of the β_2-adrenergic receptor via PKC-α activation. In contrast, PKC-μ is reported to increase β-adrenergic receptor density in human airway epithelial cells exposed to IL-1β,[172] and PKC-μ and PKC-ε are reported to increase cAMP production in RAW 264.7 macrophages,[254] with PKC-δ increasing cAMP production in human eosinophils.[253] Finally, one report suggests a dual-role for PKC. In human neutrophils, inflammatory stimuli and phagocytosis may both be dependent upon cAMP production, as cAMP-dependent F-actin reorganization was observed during receptor-mediated phagocytosis.[260]

Although it is tempting to explain the results in this study using the language of PKC isoform activity reported elsewhere, extrapolation of data between species and cell types requires standardization of PKC isoform profiles, cell type and stimuli. Moreover, because there are no reports of PKC isoform distribution or function in neutrophils from any species, extrapolation of these data for use with bovine neutrophils should be approached with caution. Future work that characterizes the PKC isoform profile in

bovine neutrophils may then provide the necessary platform from which to design isoform knock-out experiments. Such experiments may illustrate which PKC isoforms contribute to β_2-adrenergic receptor mechanism modulation. Indeed, it should be a goal of future work to bring to the marketplace PKC isoform inhibitors that selectively reduce β_2-adrenergic receptor mechanism dysfunction, because such drugs may prove useful to the veterinarian treating the acute inflammatory stage of BRD.

During preliminary studies, platelet-activating factor (PAF) was also evaluated for potential modulatory effect on the β_2-adrenergic receptor mechanism. Because the PAF receptor is a G_q – protein coupled receptor, and is therefore thought to be coupled to PKC activation, it is considered to be a relevant inflammatory mediator for bovine neutrophils. We have demonstrated in this study that PAF enhances opsonized zymosan-mediated bovine neutrophil superoxide anion production (Appendix A), and have previously demonstrated that PAF increases bovine neutrophil extracellular acidification rate in a dose-dependent manner.[264] However, the effect of various concentrations of PAF on bovine neutrophil cAMP concentration was not reported in the results of this study because PAF did not change basal cAMP production, nor did it change $1x10^{-4}$M IBMX or $5x10^{-6}$M terbutaline-stimulated cAMP production (Appendix B). We are uncertain why this property is inconsistent with the inflammatory stimulant opsonized zymosan and PMA; however, we speculate that PAF may activate a PKC isoform that is not involved with β_2-adrenergic receptor mechanism function.

In conclusion, this study illustrates the presence and function of the β_2-adrenergic receptor mechanism in bovine neutrophils. This study also demonstrates that modulation of the β_2-adrenergic receptor mechanism function occurs when bovine neutrophils are exposed to PMA or differing concentrations of opsonized zymosan. This information may be used to further characterize the effect of the acute inflammatory stage of bovine respiratory disease on pulmonary function. Also, additional studies that identify the PKC isoform repertoire expressed in bovine neutrophils may shed light on the potential for selective inhibition, and may lead to more specific identification and treatment of bovine neutrophil β_2-adrenergic receptor mechanism dysfunction. Finally, a considerable

amount of research is still needed to illustrate how the various components of the bovine neutrophil β_2-adrenergic receptor mechanism function *in-vivo* during the acute inflammatory stage of BRD.

PART VI: GENERAL REFERENCES

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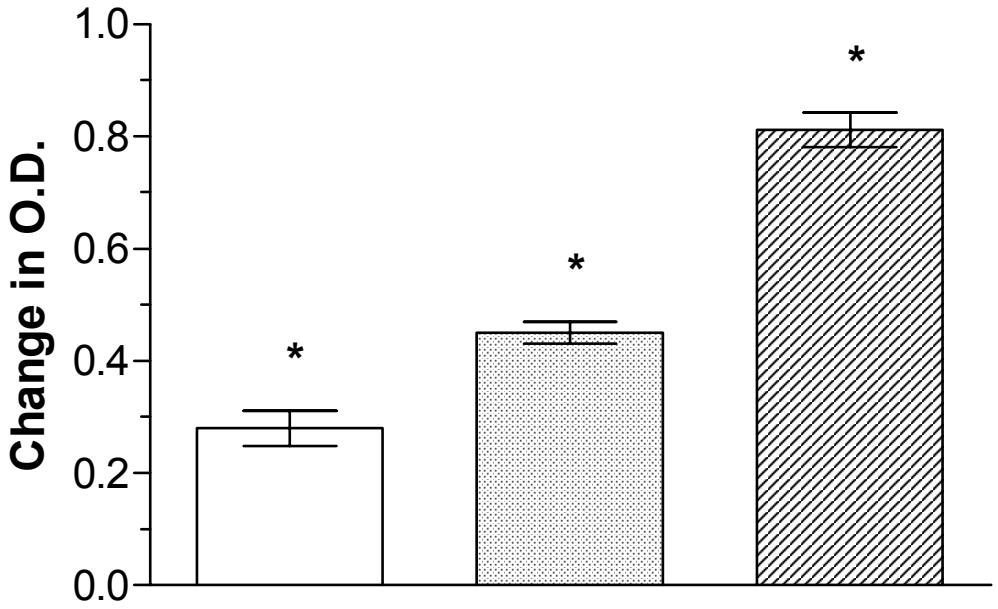
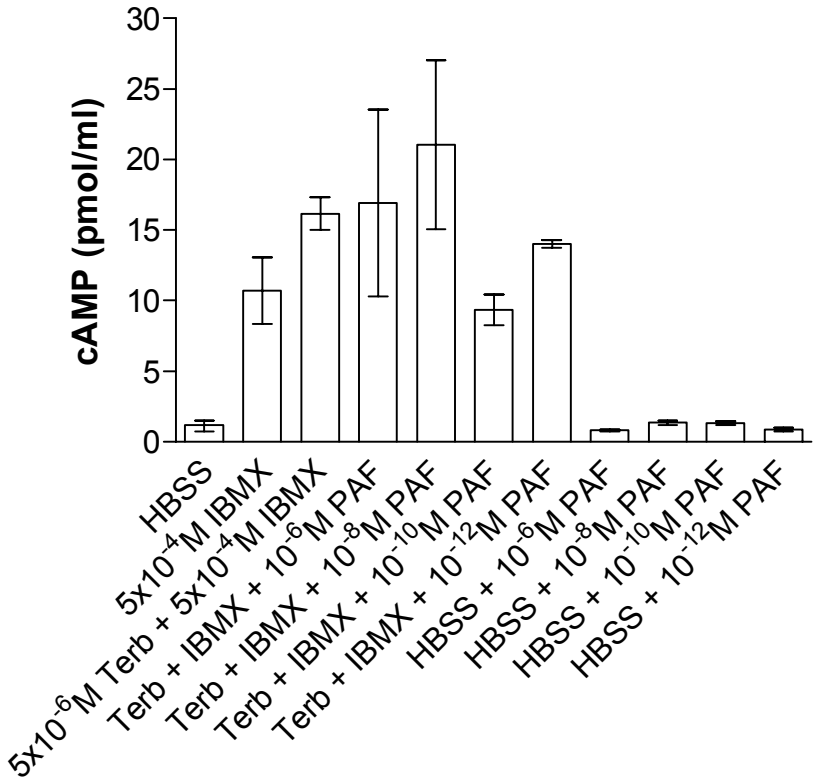
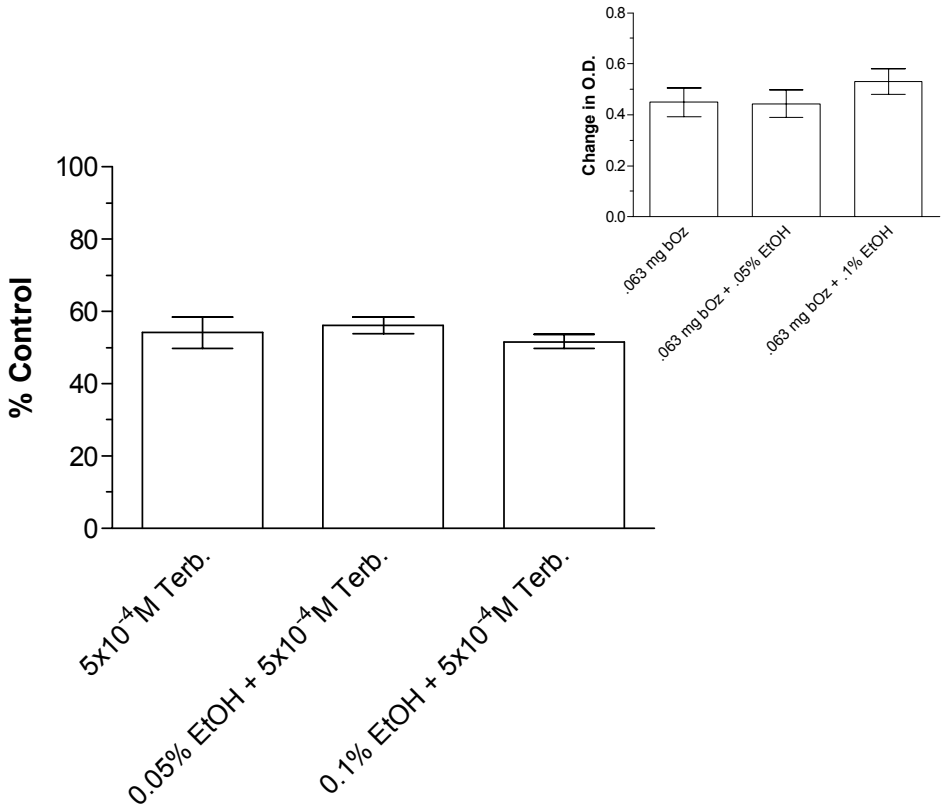
PART VII: APPENDIX



Appendix A. Superoxide anion production by $2x10^6$ bovine neutrophils exposed to 0.063 mg opsonized zymosan alone (open bar) and with the inflammatory mediator, platelet-activating factor at concentrations of either $5x10^{-7}$M (shaded bar) or $5x10^{-6}$M (striped bar) for 30 minutes at 39 ºC. Change in optical density (O.D.) at 550 nm indicates cytochrome C reduction (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 4 days total for n=4). *Statistically significant differences are observed between opsonized zymosan alone and both opsonized zymosan + $5x10^{-7}$M platelet-activating factor and opsonized zymosan + $5x10^{-6}$M platelet-activating factor. A statistically significant difference was also noted between opsonized zymosan + $5x10^{-7}$M platelet-activating factor and opsonized zymosan + $5x10^{-6}$M platelet-activating factor (Paired, two-tail T-test, $p<0.05$), suggesting a dose-dependent, platelet-activating factor-mediated enhancement of opsonized zymosan-stimulated superoxide anion production.

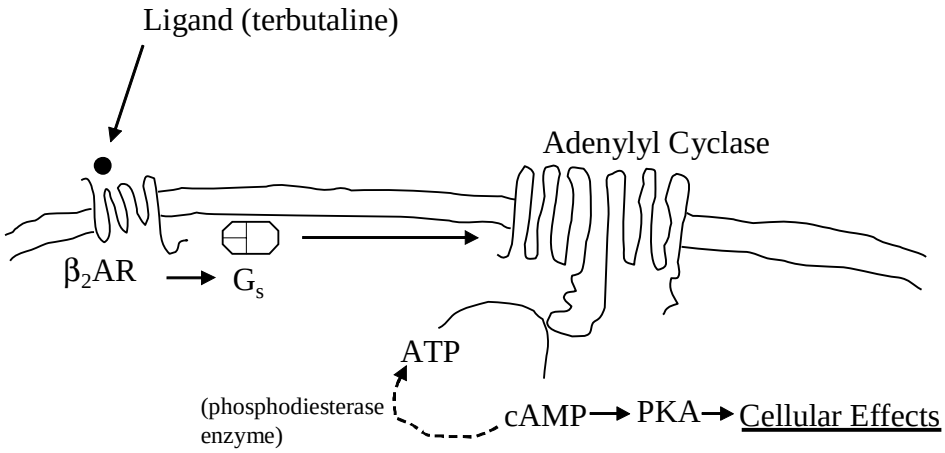


Appendix B. Cyclic AMP (cAMP) production by bovine neutrophils exposed to Hank's balanced salt solution (HBSS), $5x10^{-4}$M 3-isobutyl-1-methylxanthine (IBMX), or the combination of various concentrations of platelet-activating factor (PAF) with $5x10^{-6}$M terbutaline (Terb), with or without $5x10^{-4}$M IBMX, for 30 minutes at 39 ºC. Values represent the concentration of cAMP in supernatant of $1x10^{7}$ lysed cells (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 3 days total for n=3). Statistically significant differences were not noted between $5x10^{-6}$M Terb + $5x10^{-4}$M IBMX and Terb + IBMX + 10^{-6}M through 10^{-12}M PAF.



Appendix C. Superoxide anion production by $2x10^6$ bovine neutrophils exposed to 0.063 mg opsonized zymosan (bOz) with $5x10^{-4}$M terbutaline (Terb.), with and without increasing concentrations of ethanol (EtOH) for 30 minutes at 39 ^oC. Values represent percent of control superoxide anion production, optical density = 0.444 +/- 0.686 for neutrophils incubated with opsonized zymosan and 0.05% EtOH, and 0.531 +/- 0.635 for neutrophils incubated with opsonized zymosan and 0.1% EtOH; 95% confidence interval (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 2 days total for n=2). Inset: Superoxide anion production by $2x10^7$ bovine neutrophils exposed to 0.063 mg Oz alone and with increasing concentrations of ethanol (EtOH) for 30 minutes at 39 ºC. Change in optical density (O.D.) at 550 nm indicates cytochrome C reduction (mean +/- SEM, 1 observation per day, pooled sample of neutrophils from 5 steers per observation, 2 days total for n=2).

Appendix D. The β_2-Adrenergic Receptor Mechanism



PART VIII: VITA

Timothy Paul LaBranche was born on November 24, 1974 in Providence, Rhode Island to Paul and Janet LaBranche. Tim has one brother, Matthew, and two sisters, Adrienne and Danielle. After enduring 18 New England winters, and not knowing a heifer from a Holstein, Tim came to Blacksburg to study Animal Science. In 1996 Tim graduated from Virginia Tech with a B.S. and entered the graduate program at the Virginia-Maryland Regional College of Veterinary Medicine. After three years of studying pharmacology under the tutelage of Dr.'s Peter Eyre and Marion Ehrich, Tim entered the Doctor of Veterinary Medicine program, enrolling in the Government / Corporate Track. Upon graduation from veterinary school in 2003, he stayed in Blacksburg to complete his research. In the summer of 2004, Tim entered the anatomic pathology residency program at the University of Georgia. Tim currently lives outside Atlanta, in a real house, with his wife, Lora, and his three kids, Mallard (a Yellow Lab), Cedar (a Jack Russell) and Meadow (a Gray Tabby). During his spare time, Tim enjoys eating good food and working on his dissertation, but can also be spotted traveling, mountain biking, or listening to jazz.