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Protease-Activated Receptors in the Prostate

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Protease-Activated Receptors in the Prostate" submitted by Aaron F. Hirschfeld in partial fulfilment of the requirements of the degree of Master of Science.

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Abstract

Prostatitis is a poorly understood disease characterized by inflammation of the prostate gland. Proteinase-activated receptors (PARs) are novel G protein-coupled receptors that are cleaved by extracellular proteinases. They are involved in inflammatory pathways and could play a role in prostatitis. In the present investigation, PAR-1 and PAR-2 were detected in human prostate cells (RWPE-1) and in rat prostate tissue. PAR-2 was found to be highly expressed in the glandular epithelial cells of the rat prostate. PAR agonists induced calcium fluxes *in vitro* and modulated cytokine levels in human cells. PAR-1 and PAR-2 specific peptide agonists did not induce inflammatory responses in the rat prostate as judged by gross and histopathology, MPO levels or cytokine expression. Bacterial proteinases derived from uropathogenic *E. coli* did not activate PARs. PARs appear to modulate cytokine expression in human epithelial cells, however their effect on prostatitis *in vivo* has yet to be elucidated.

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List of Symbols, Abbreviations and Nomenclature

AMC	7-amido-4-methylcoumarin
ANOVA	analysis of variance
ATP	adenosine triphosphate
AY-NH_2	PAR-4 activating peptide AYPGKF-NH_2
bp	base pair
BPH	benign prostatic hyperplasia
cAMP	cyclic adenosine monophosphate
CDB	cell dissociation buffer
CFU	colony forming units
CGRP	calcitonin gene-related peptide
DAG	diacylglycerol
DMEM	Dulbecco modified Eagle medium
DMSO	dimethylsulfoxide
EGF	epidermal growth factor
EPS	expressed prostatic secretions
ELISA	enzyme-linked immunosorbant assay
ERK	extracellular regulated kinase
Fluo-3 AM	fluo-3 acetoxymethyl ester
GEF	guanine-nucleotide exchange factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
Gro/CINC-1	growth related oncogene/cytokine-induced neutrophil chemoattractant-1
h	hour
H&E	haematoxylin and eosin
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HMW-SV	high molecular weight seminal vesicle
HTAB	hexadecyl-trimethylammonium bromide
IL	interleukin
IP_3	inositol trisphosphate

kDa	kilodalton
KSFM	keratinocyte serum-free medium
LR-NH_2	PAR-2 reverse peptide LRGILS-NH_2
LT	leukotriene
LUTS	lower urinary tract symptoms
MAPK	mitogen-activated protein kinase
min	minute
MOI	multiplicity of infection
MPO	myeloperoxidase
OD	optical density
PAR	proteinase-activated receptor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
PI(3)K	phosphoinositide 3 kinase
PKC	protein kinase C
PLC	phospholipase C
PSA	prostate-specific antigen
RL-NH_2	PAR-1 reverse peptide RLLFT-NH_2
RT-PCR	reverse transcriptase polymerase chain reaction
RWPE	non-transformed human prostate epithelial cell line
SL-NH_2	PAR-2 activating peptide SLIGRL-NH_2
TF-NH_2	PAR-1 activating peptide TFLLR-NH_2
TMP-SMX	trimethoprim-sulfamethoxazole
TNBS	trinitrobenzenesulfonic acid
TNF-α	tumour necrosis factor alpha
U	unit of enzymatic activity (mmol substrate catalyzed per minute)
UPEC	uropathogenic *Escherichia coli*
UTI	urinary tract infection

CHAPTER ONE: INTRODUCTION

The prostate, a gland found in the male urogenital system, has the principal function to produce secretions that are critical for fertility and to sequester sperm from the immune system. With increasing age, the prostate becomes gradually more susceptible to disease in many individuals. Benign prostatic hyperplasia, a non-malignant disease, is found in prostate tissue biopsies from men in their twenties. As men approach middle age, their risk of developing prostate cancer increases. One of the most common prostatic diseases is prostatitis, inflammation of the prostate gland. The symptoms are often painful, irritating and majority of patients report a general decrease in their quality of life. A bacterial aetiology can only be established in a small fraction of patients, in which treatment usually resolves the infection in the acute form of the disease. However, in most cases the aetiology is of unknown origin. Current research is focused on elucidating novel factors which may play a role in this inflammatory disease of the prostate.

Proteinase-activated receptors (PARs) are G protein-coupled receptors that are cleaved by proteinases to unmask an endogenous "tethered" ligand that binds intramolecularly to the receptor, initiating signalling. These unique receptors are stimulated by various proteinases in the extracellular milieu. The effects of PARs are still being catalogued but it has been shown they play important roles in haemostasis, inflammation and hyperalgesia. By using proteinases and selective peptide agonists, the functions of these receptors can be probed in experimental systems. It is the goal of this study to analyze the effects of PAR activation in the prostate gland using a human

prostate cell line and an animal model of prostatitis. PARs and their possible link to prostatic inflammation have not been investigated and it would be important to evaluate the role of PARs in the prostate.

1.1 The Prostate

1.1.1 Anatomy and Function

The prostate is a large exocrine gland found in males that's primary function is to produce secretions that are a major component of semen. The human prostate lies between the urinary bladder and the pelvic floor (Blacklock and Bouskill, 1977). It is a fibromuscular organ that surrounds the prostatic urethra with ejaculatory ducts opening into the urethra before exiting the prostate. A thin layer of connective tissue at the periphery of the prostate forms a fibro-elastic capsule around the organ. The capsule forms incomplete septa that extend into the stroma, dividing the prostate into poorly defined lobes. Randomly arranged smooth muscle bundles can also be found in the stroma. The smooth muscle fibres, contained in supporting tissue, are innervated by the sympathetic nervous system which stimulates contraction of the prostate during ejaculation.

Histology of the glandular prostatic regions reveals the prostate is mainly composed of tubular acinar glands, and the prostatic ducts arranged concentrically around the urethra (Blacklock and Bouskill, 1977). The acini are lined by columnar secretory epithelial cells with basal nuclei, which form branching folds resulting in a columnar appearance. The epithelial layer of cells is separated from the basement membrane by a

layer of flattened basal cells that give rise to the secretary cells. Small diameter striated muscle fibres, separated by connective tissue, surround the distal prostatic urethra.

The prostate has been described as an immunologically privileged organ, that is an organ in which an allograft could be placed without a primary immunological rejection (Head and Billingham, 1985). The lack of afferent lymphatic vessels, high incidence of carcinoma and immunosuppressive properties of seminal plasma are other factors that have contributed to this status (Quayle *et al.*, 1987). In contradiction, other studies have shown there is extensive immunosurveillance in the prostate (Bostwick *et al.*, 2003). Lymphocytes consisting of T cells are normally found in the prostate, whose function and CD4/CD8 distribution are strictly regulated. Inflammatory infiltrate may be found in the prostate which could be a result of bacterial infection, nodular hyperplasia, carcinoma and the transient presence of spermatozoa (McClinton *et al.*, 1990).

1.1.2 Components of Seminal Fluid

The secreted seminal fluid contains a diverse mixture of carbohydrates, lipids and high molecular weight seminal vesicle (HMW-SV) proteins (Wilson, 1995). The secretions are also rich in citric acid, fucose and zinc (Wilson, 1995). Zinc is an important molecular building block and component of over fifty human enzymes (Vallee and Galdes, 1984) and the highest concentration of zinc in body is found in the prostate gland (Cho *et al.*, 2002a). Zinc may also act as an antibacterial factor and changes in prostatic zinc concentration may affect disease susceptibility (Cho *et al.*, 2002b; Fair and Parrish, 1981).

Immediately following ejaculation, HMW-SV proteins are the major proteins which constitute the structural protein of the seminal clot (Lilja and Weiber, 1984). The enzymes in seminal fluid are diverse and play a crucial role in male fertility. Semen contains proteinases and inhibitors of the haemostatic system, most notably clotting and fibrinolytic factors. Fibrinolysin is very important in seminal clotting during fertilization and acts to liquefy semen once it has been deposited in the female genital tract (Lwaleed *et al.*, 2004). The role of clotting and fibrinolytic activities in humans still remains poorly understood. Other proteinases that can be found in semen include tissue factor, which originates from the prostate, prothrombin fragments 1 and 2 (a thrombin-like enzyme), pepsinogen II, metalloproteinases, tissue and urinary plasminogen activator and proteinases with caseinolytic and gelatinolytic activity. There are traces of plasminogen, plasminogen activator inhibitor-1 (PAI-1), factor VIII coagulant activity and fibrinogen (Lwaleed *et al.*, 2004). Several genes of the serine proteinase encoding kallikrein family are also expressed at high levels in the prostate (Takayama *et al.*, 2001).

1.1.3 Prostate-Specific Antigen

Kallikrein-like serine proteinases, such as prostate specific antigen (PSA) are known to be present in high concentrations in prostatic fluids (Lwaleed *et al.*, 2004). The term kallikrein was originally introduced to describe an enzyme that reacts with kininogen to release the bioactive peptide kinin (Bhoola *et al.*, 1992). PSA is released from the glandular lumen form epithelial cells in the prostate where it is active in the 10 to 50 μM range (Takayama *et al.*, 2001). Inactive forms of PSA (pro-enzyme form) are also present in the extracellular milieu at 10^6 times lower concentrations. Although there

is no evidence that PSA acts as a mitogen, elevated levels of PSA have been used as a marker for prostate cancer and benign prostatic hyperplasia, however much remains unknown about its physiological function (Lilja, 2003).

1.1.4 Diseases of the Prostate

1.1.4.1 Prostatitis

Prostatitis is defined as inflammation of the prostate gland. Acute prostatitis is characterized by inflammation of the prostatic ducts and glands as a consequence of neutrophil infiltration of the stroma and epithelia followed by subsequent destruction of secretory cell layers. The prostate glands often become atrophic and irregular after the initial inflammatory reaction (Kohnen and Drach, 1979).

Clinical studies have revealed that up to half of all men will develop prostatitis at some point within their lifetime (Roberts *et al.*, 1997). Recent studies suggest the prevalence of the disease is 5% to 9% among men in the United States (Roberts and Jacobsen, 2000). Prostatitis is characterized by three key characteristics: lower urinary tract.symptoms, evidence of inflammation and pain in the prostate gland (Nickel, 2003). These three characteristics may show varying degrees of overlap that are unique to each patient. The disease is further subdivided into defined categories of acute or chronic bacterial prostatitis, non-bacterial prostatitis or non-inflammatory pelvic pain syndrome (Batstone *et al.*, 2003; Nickel, 2003; Theodorou and Becopoulos, 1999). Acute and chronic bacterial prostatitis are the best understood, but the least common of prostatic diseases.

1.1.4.1.1 Acute Bacterial Prostatitis

Acute prostatitis is a serious condition often associated with sudden onset of fever, chills and malaise. Patients often present symptoms of increased urinary frequency, incomplete emptying, bacterial infection, discomfort in the pelvis and occasionally back pain (Gurunadha Rao Tunuguntla and Evans, 2002). Patients with prostatitis also exhibit negative psychological effects which may include sexual dysfunction, depression and a general decrease in the quality of life (Krieger, 2003). A diagnosis of acute prostatitis is made based on patient history, clinical examination and laboratory studies.

Acute prostatitis typically develops as a secondary infection from acute urethritis and is accompanied by symptoms and signs of systemic toxicity (Theodorou and Becopoulos, 1999). Upon rectal examination, the prostate has obvious signs of severe inflammation and is usually hot, enlarged and bearing abscesses which may require drainage (Gurunadha Rao Tunuguntla and Evans, 2002). Examination of urine and expressed prostatic secretion (EPS) cultures reveals possible microbiological aetiologies of the disease. Causative organisms include pathogenic aerobic Gram-negative rods including *Enterobacteriaceae*, *Pseudomonas* and occasionally *Enterococcus* species (Krieger, 2003). Possible routes of infection include ascending urethral infection, urine reflux into the prostatic ducts and invasion by rectal bacteria (Meares, 1991).

Antimicrobials such as fluoroquinolones and antibiotics are routinely used to cure acute bacterial prostatitis (Nickel, 1999). Patients who require hospitalization are usually put on a regimen of intravenous antibiotics such as gentamycin, cephalosporin, vancomycin and trimethoprim-sulfamethoxazole (TMP-SMX) alone or in combination

(Nickel, 1999). Appropriate administration of antimicrobial and supportive therapy usually results in a dramatic improvement with occasional complications.

1.1.4.1.2 Chronic Bacterial Prostatitis

Chronic prostatitis is a disorder that is characterized by persistent and recurrent inflammation which is associated with bacterial infection and the presence of inflammatory cells in EPS (Roberts *et al.*, 1997) and may or may not be preceded by a known episode of acute prostatitis. Recurrent urinary tract infections are caused by the same uropathogens implicated in cases of acute prostatitis (Krieger, 1984). Patients report varying degrees of pelvic discomfort associated with irritative and obstructive voiding symptoms. The symptoms are less severe than reported in acute bacterial prostatitis and therefore diagnosis of chronic bacterial prostatitis is more difficult. Between episodes of detectable bacterial infection, patients are often asymptomatic (Krieger, 1984). Bacteria that have been implicated in chronic bacterial prostatitis are uropathogenic *E. coli*, *Enterococcus faecalis* and *Staphylococcus epidermidis*, while *Chlamydia trachomatis*, mycoplasma, mycobacteria and fungi are suspected to be involved (Weidner *et al.*, 1991). However, in only 7% of cases of chronic prostatitis are defined as having a bacterial aetiology which is an enigma that plagues clinicians and researchers (Nickel, 2003).

Treatment of chronic bacterial prostatitis is either curative or suppressive. Patients who have a positive bacterial infection, as diagnosed by the Meares and Stamey test (Meares and Stamey, 1968), are treated for 12 weeks with TMP-SMX or fluoroquinolones due to their favourable pharmacodynamics and excellent penetration into the prostate gland (Gurunadha Rao Tunuguntla and Evans, 2002). Use of these antimicrobials usually results in eradication of bacteria in 60-75% of the cases (Weidner

et al., 1998). Eradication of bacteria does not necessarily lead to the ablation of symptoms associated with prostatitis, which is the goal of therapy. Many patients are still not cured after prolonged (3 month) therapy (Krieger, 2003). Surgery, local injection of antibiotics and α_1-blockers may also be administered to help alleviate persisting symptoms (Weidner *et al.*, 1991). During suppressive therapy, the asymptomatic patients with positive cultures are given low-dose suppressive antibiotics such as TMP-SMX, tetracycline and cephalothin for 6-10 weeks (Nickel, 1999). These antibiotic treatments suppress bacteriuria and limit symptomatic infections to a point which is effective for many patients (Krieger, 2003).

Levels of zinc in the prostate may also influence the ability for bacteria to colonize the prostate. It has been shown that exogenous zinc inhibits *E. coli* in the rat prostate and that zinc levels in clinical cases may be decreased in chronic prostatitis, however there are variations reported in different studies (Cho *et al.*, 2002b; Evliyaoglu and Kumbur, 1995). Clinical studies have shown a decrease in reported severity of symptoms when zinc treatment is administered (Deng *et al.*, 2004). Further research is warranted to determine all of the underlying factors, of an endogenous or exogenous origin, that may contribute to the disease.

1.1.4.1.3 Nonbacterial Prostatitis and Pelvic Pain Syndrome

A majority of patients with the symptoms of chronic prostatitis when tested for bacterial prostatitis have negative cultures, yet an inflammatory condition is present. Causative pathogens are detected in only 5-10% of patients with chronic prostatitis (Weidner *et al.*, 1991), while in the remaining 90% of cases, a bacterial aetiology cannot be readily established (Nickel, 2003). Patients with lower urinary tract symptoms (LUTS)

and no evidence of urinary tract infection are classified as having nonbacterial prostatitis if they have evidence of leukocytes in their EPS (Krieger, 2003). If a patient exhibits all of the symptoms associated with nonbacterial prostatitis with the exception of leukocytes in their EPS, they are classified as having chronic pelvic pain syndrome (Mehik *et al.*, 2003; Nickel, 2003; Theodorou and Becopoulos, 1999). The aetiologies of these diseases are unclear, which results in difficulties in diagnosis and speculative treatment regimens.

Possible aetiologies could be cryptic infection, neuromuscular dysfunction of the bladder neck or urethral sphincter, autoimmune disorders or reflux of urinary constituents into the prostatic ducts (Krieger, 2003). Imbalances of pro-inflammatory and anti-inflammatory interleukins may also play a role in the progression of these diseases. Nerve growth factor (NGF) has been found to be up-regulated in pelvic pain syndrome which in turn enhances transcription of IL-6, a pro-inflammatory cytokine (Miller *et al.*, 2002b). On the contrary, IL-10, an anti-inflammatory cytokine, is also found at higher concentrations in similar patients (Miller *et al.*, 2002a). Elevated IL-10 also induces NGF which directly induces pain in the patient population (Miller *et al.*, 2002b). It is clear that the dynamics of neurotrophic, pro-inflammatory and anti-inflammatory cytokines and regulatory systems operating during chronic prostatitis need to be better understood.

Antimicrobial agents are routinely administered to patients, with mixed results, based on an uncertain aetiology of cryptic microrganism involvement (Nickel, 1999). Combinations of α_1-blockers, muscle relaxants and fluoroquinolones coupled with supportive therapy yield a relief in pain. Some antibiotics may have anti-inflammatory activity and it is possible that these treatments reduce inflammation, rather than

eradicating bacteria (Batstone *et al.*, 2003; Nickel, 2003). It is not clear as to whether the treatment for nonbacterial prostatitis and pelvic pain syndrome should differ based on the poorly understood role of inflammation in each of the diseases (Nickel, 1999). Benefits from this course of treatment are questionable and in order to effectively treat the disease, idiopathic aetiologies must be fully explored. A rat model of abacterial prostatitis, based on the loss of mucosal integrity, has been established (Lang *et al.*, 2000) to further characterize this disease.

1.1.4.2 Benign Prostatic Hyperplasia and Prostate Cancer

Benign prostatic hyperplasia (BPH) is one of the most common non-malignant prostate diseases characterized by abnormal increases in glandular and stromal tissue when compared to epithelial tissue (Skolarikos *et al.*, 2004). It is associated with LUTS, classified as obstructive and irritative voiding and storage difficulties (Nickel, 1994; Skolarikos *et al.*, 2004). These symptoms are primarily due to urinary obstruction caused by prostatic enlargement. Patients with mild symptoms do not undergo treatment, while patients that exhibit more severe symptoms will be administered α_1-blockers to reduce smooth muscle tone. In extreme cases, surgical treatment is one of the only options available which includes transurethral resection of the prostate and possible prostatectomy (Steers and Zorn, 1995).

The prevalence of the prostate cancer in men greater than 60 years of age is approximately 60% (Jemal *et al.*, 2004). Prostate adenocarcinoma is histologically similar to BPH and hyperplasia of prostate tissue is often misdiagnosed as prostate cancer (Cheville and Bostwick, 1995). In contrast to BPH, premalignant lesions known as high

grade intraepithelial neoplasias, occur when the basal cells are more proliferative than in BPH and extend to the border of the lumen. There are increases in collagen and non-collagenous proteins in the extracellular matrix as well as higher levels of elastase activity which contribute to malignancy (Nakada and Kubota, 1994). PSA levels in the serum are routinely used as a marker for prostate cancer, although not as a diagnostic, because studies have found they are significantly elevated relative to BPH and other prostatic diseases (Richie and Swanson, 2004). Treatment is in the form of radiotherapy, androgen ablation therapy and surgery, although patients may relapse and develop androgen independent tumours (Long *et al.*, 2004).

There has been an increasing amount of evidence suggesting that there is an increased risk of prostate cancer with a prior history of sexually transmitted disease or prostatitis (Dennis *et al.*, 2002). The contributions of the cellular components of inflammation are non-specific reactive oxygen and nitrogen species that may cause somatic gene alterations. Free-radicals can alter proteins involved in DNA repair, apoptosis and cell signalling pathways which could relate to prostate carcinogenesis (Jaiswal *et al.*, 2001; Melino *et al.*, 1997). There are still many questions left to be answered as to whether chronic prostatitis may contribute to the initiation and progression of prostate cancer.

1.2 Animal Model of Prostatitis

Laboratory animal models are essential for studying the impact of inflammation and infection in an *in vivo* setting. The rat prostate is divided into dorsal, ventral and lateral lobes, with the ventral lobe displaying the most similar morphology and histology

to the human prostate gland (Ceri *et al.*, 1999a; Nickel *et al.*, 1990). One notable difference is that upon fertilization, the seminal fluid rapidly coagulates and a copulatory plug is formed, unlike humans where the seminal clot is dissolved (Williams-Ashman, 1984). The rat has been successfully used as an experimental model for induced nonbacterial and bacterial prostatitis by several groups. Nickel *et al.* have developed a model of chronic bacterial prostatitis by anaesthetizing, catheterizing and instilling uropathogenic *E. coli* into the ventral prostate of a rat (Nickel *et al.*, 1990). This model displayed similar pathology to chronic prostatitis in humans, with the presence of bacterial micro-colonies and neutrophil infiltration of the prostatic ducts.

Other researchers have successfully reproduced this work, testing other organisms to determine if cytotoxic necrotizing factor type 1 increases prostatic neutrophil infiltrates (Rippere-Lampe *et al.*, 2001a). The model has also been adapted to model nonbacterial prostatitis by instilling ethanol, to disrupt mucosal integrity, and dinitrobezenesulfonic acid as an irritant (Lang *et al.*, 2000). The prostate tissues harvested from these animals showed signs of inflammation upon histological examination and increased levels of IL-1β over a 48 hour time period. Infection based models must employ controlled delivery of the stimulus to prevent infection of the surrounding tissue, create a pathology similar to human disease and avoid producing a systemic infection. If these conditions are met, the rat model of prostatitis should be considered a reasonable model to simulate prostatitis as observed in humans.

1.3 Proteinase-Activated Receptors

1.3.1 Discovery

Proteinases are enzymes that participate in a broad range of biological processes from the degradation of proteins in the gastrointestinal tract to various regulatory mechanisms at the cellular level. Thrombin is a major proteinase involved in the coagulation cascade, a series of zymogen conversions that is triggered by a disruption in vascular integrity (Coughlin, 1999). Thrombin is the enzyme responsible for converting fibrinogen to fibrin, which polymerizes to form blood clots, the aggregation of platelets, cytokine release and many other effects in a multitude of cell types (Coughlin *et al.*, 1992; Hung *et al.*, 1992b). The proteinase-activated receptor family was recently discovered in the 1990s following a search for cell surface receptors through which thrombin could exert its potent cellular effects (Rasmussen *et al.*, 1991; Vu *et al.*, 1991a).

1.3.1.1 The Thrombin Receptor

A functional thrombin receptor was expression cloned by injecting RNA from human thrombin-responsive cells into oocytes from *Xenopus laevis* (Rasmussen *et al.*, 1991; Vu *et al.*, 1991a). The oocytes were then screened for thrombin-responsiveness by measuring increases in intracellular calcium concentration ($[Ca^{2+}]_i$). Clones that were positively identified encoded a 425 residue protein which showed homology to G-protein coupled receptors (GPCRs) with the exception of a unique amino-terminal signal sequence that appeared to be potential cleavage site for thrombin. The thrombin receptor, termed proteinase-activated receptor-1 (PAR-1), is activated when thrombin cleaves the amino-terminal extracellular domain at a specific site, which exposes a new amino-

terminus that serves as a tethered ligand (Vu *et al.*, 1991a). The search for other PAR-like genes that could act as receptors for proteinases resulted in the discovery of three additional receptor family members termed PAR-2, PAR-3 and PAR-4.

1.3.1.2 Other Proteinase-Activated Receptors

PAR-2 was discovered when a mouse genomic library was screened using degenerate primers to the second and sixth exoloops of the bovine neurokinin-2 receptor (Nystedt *et al.*, 1994). One of the clones showed homology to GPCRs and had a ~30% identity to PAR-1. The most interesting feature was a putative cleavage site for trypsin at the amino-terminus (Nystedt *et al.*, 1995). The unexpected discovery of PAR-2 suggested there may be other unknown receptors of the PAR family.

Degenerate primers to conserved sequences in PAR-1 were used to screen RNA from rat platelets and PAR-3 was cloned from murine and human cells (Ishihara *et al.*, 1997). PAR-3 was found to share ~28% homology to PAR-1 and exhibited a putative cleavage site for thrombin. Further PAR-like sequences were found after searching expressed sequence tag libraries and a new GPCR was cloned and termed PAR-4 (Kahn *et al.*, 1998; Xu *et al.*, 1998). Sequence analysis of PAR-4 revealed a potential cleavage site for thrombin and trypsin. To date, four PARs have been identified and efforts have been ongoing to characterize their physiological functions.

1.3.2 The PAR Family

PARs share many characteristics of typical GPCRs, an extracellular N-terminal domain, seven trans-membrane spanning domains and an intracellular C-terminal signalling domain (Ji *et al.*, 1998; Nystedt *et al.*, 1994; Vu *et al.*, 1991a). The trans-

membrane spanning domain forms the core embedded in the cell membrane, with three exoloops and three cytoloops. Substantial degrees of amino acid homology are found between members of the PAR subfamily of GPCRs. Differences in structure and functionality are found in the amino-terminus, the second exoloops and the carboxy-terminus (O'Brien *et al.*, 2001). PARs have also been found in other mammalian species such as the rat and mouse (Vergnolle *et al.*, 2001b). Although the amino acid structure does not share 100% identity to human PARs, they have similar physiological functions and can be activated by the same proteinase and selective peptide agonists used for human PARs.

Cleavage of the N-terminal domain by serine-like proteinases results in the unmasking of a tethered ligand which forms the new amino-terminus. The freed ligand binds to the second exoloop of the receptor, activating the receptor and initiating signalling through a series of conformational changes (Coughlin *et al.*, 1992; Ji *et al.*, 1998). There is no know function for the short peptide that is released following receptor cleavage (Ossovskaya and Bunnett, 2004). The conformational changes lead to guanine nucleotide exchange on associated G-proteins followed by a multitude of downstream effects. The amino acid sequence in the domain containing the cleavage site is crucial for receptor activation, yielding different proteinase specificities for different PARs (Vu *et al.*, 1991b). There are also distinct differences in the carboxy-terminal domains which affect the signalling of the receptor. The main role of PARs appears to be the ability to sense, react and initiate signalling cascades in response to different extracellular proteinases.

1.3.2.1 PAR-1

The human amino acid sequence of PAR-1 has a 75 residue extra-cellular amino-terminal domain that contains a cleavage site for thrombin at the 42nd serine residue ($LDPR^{41}\downarrow S^{42}FLLRN$) (Vu *et al.*, 1991b). The carboxy sequence that flanks the cleavage site resembles the tail of the thrombin inhibitor hirudin and binds to thrombin in a similar matter (Hung *et al.*, 1992b). Thrombin uses this sequence to bind to PAR-1 and then cleaves the receptor resulting in unmasking of the tethered ligand SFLLRN. The SFLLRN sequence binds to the second exoloop which initiates signal transduction. Mutation of the cleavage site inhibits thrombin signalling, representing the significance of the amino-terminal domain (Vu *et al.*, 1991a). Replacement of the thrombin cleavage site with an enterokinase cleavage site renders the receptor responsive to enterokinase, underlining the importance of proteolytic activation (Vu *et al.*, 1991b). PAR-1 is also responsive to factor Xa, granzyme A, trypsin and other serine proteinases, but to a lesser extent than thrombin, presumably due to the lack of recognition of domains surrounding the cleavage site (Molino *et al.*, 1997b; Suidan *et al.*, 1996; Vouret-Craviari *et al.*, 1995).

Selective PAR-1 agonists have been developed that mimic the tethered ligand sequence and are able to bind to and activate PAR-1 in the absence of thrombin (Hollenberg *et al.*, 1997; Vassallo *et al.*, 1992). Synthetic peptide agonists such as TFLLR-NH_2 are useful tools in determining the effects of PAR activation independent of other effects proteinases may have on cells, as well as the effects they may have on other PARs. PAR-1 is expressed in human platelets, epithelium, endothelium, fibroblasts, myocytes, neurons and astrocytes (Ossovskaya and Bunnett, 2004).

1.3.2.2 PAR-2

The human PAR-2 gene encodes a protein of 397 residues with an amino-terminus of 46 residues and a trypsin cleavage site at the 35th residue (SKGR^{34}↓S^{35}LIGKV) (Nystedt *et al.*, 1995). PAR-2 is cleaved in a similar manner as PAR-1 and mutation of the trypsin cleavage site also results in the inability of the receptor to respond to trypsin (Nystedt *et al.*, 1994). The tethered ligand SLIGKV is required for receptor activation and functionality. Exposure of trypsin to cells expressing PAR-2 results in a loss of immunoreactivity to an antibody against a PAR-2 amino-terminal epitope, confirming that trypsin cleaves PAR-2 at the surface of the cell (Bohm *et al.*, 1996b). Like PAR-1, there are other proteinases that are able to cleave PAR-2 *in vitro*. Other significant PAR-2 activating proteinases include tryptase and factors VIIa and Xa which cleave PAR-2 with less potency than trypsin (Camerer *et al.*, 2000; Molino *et al.*, 1997a; Nystedt *et al.*, 1994). Synthetic peptides such as SLIGRL-NH_2, trans-cinnamoyl-LIGRLO-NH_2 and 2-furoyl-LIGRLO-NH_2 have been used to activate PAR-2 independently of other PARs (al-Ani *et al.*, 1995; McGuire *et al.*, 2004; Roy *et al.*, 1998). PAR-2 has been found in epithelium, endothelium, fibroblasts, myocytes, neurons and astrocytes (Ossovskaya and Bunnett, 2004).

1.3.2.3 PAR-3

The human PAR-3 gene encodes a protein of 374 amino acid residues with an amino-terminus thrombin cleavage site at the 38th residue (LPIK^{38}↓T^{39}FRGAP) (Ishihara *et al.*, 1997). Cleavage by thrombin unveils the TFRGAP tethered ligand which interacts with the receptor. Mutation of the cleavage site renders the receptor insensitive to

thrombin (Ishihara *et al.*, 1997). There are no other known proteinase activators of PAR-3 and oddly, synthetic ligands that correspond to the tethered ligand sequence TFRGAP-NH_2 do not activate PAR-3 (Hansen *et al.*, 2004; Hollenberg, 1999). PAR-3 is expressed on platelets, the endothelium, myocytes and astrocytes (Ossovskaya and Bunnett, 2004).

1.3.2.4 PAR-4

Human PAR-4 is a 385 amino acid protein with a potential cleavage site for thrombin and trypsin ($PAPR^{47}\downarrow G^{48}YPGQV$) in the amino-terminal domain (Xu *et al.*, 1998). The neutrophil granule proteinase, cathepsin G has also been shown to activate PAR-4 (Sambrano *et al.*, 2000). In addition to proteinases, peptides corresponding to the tethered ligand, GYPGQV-NH_2 or AYPGKF-NH_2 can also activate PAR-4 (Xu *et al.*, 1998). As with PAR-1, PAR-2 and PAR-3, mutation of the cleavage site renders PAR-4 inactive when exposed to thrombin or trypsin (Kahn *et al.*, 1998). PAR-4 is localized on human platelets, endothelium, myocytes and astrocytes (Ossovskaya and Bunnett, 2004).

1.3.2.5 Receptor Interactions

Thrombin can not only activate PAR-1, but PAR-3 and PAR-4 as well with reduced effectiveness, while PAR-2 may be activated by many different proteinases. Since PARs are frequently co-expressed on the cell surface, this degree of complexity indicates that there may be interactions between PARs which may increase the diversity of PAR responses in a particular cell type.

Platelets express PAR-1 and PAR-4 which are both activated by thrombin, yet the kinetics of each receptor is very different. PAR-1 activation occurs in response to very

low thrombin concentrations and results in large increases in $[Ca^{2+}]_i$, while PAR-4 activation requires significantly higher thrombin concentrations and produces a delayed and prolonged signal (Covic *et al.*, 2000). This difference may be important for the later phase of platelet aggregation. PAR-3 has a hirudin-like domain, similar to PAR-1, and there is evidence to suggest that PAR-3 acts as a cofactor for PAR-4 by binding thrombin which cleaves PAR-4 molecules in its vicinity (Nakanishi-Matsui *et al.*, 2000).

There is also evidence that PARs may form homodimers and heterodimers where a cleaved receptor may activate an uncleaved receptor. When the amino-terminus of PAR-1 was attached to a CD8 membrane anchor and expressed in *Xenopus*, an increase in $[Ca^{2+}]_i$ was not observed after thrombin stimulation (Chen *et al.*, 1994). However, when the PAR-1 amino-terminus was co-expressed with a truncated PAR-1 receptor which lacked the amino-terminus, intermolecular PAR-1 signalling was observed (Chen *et al.*, 1994). There is additional evidence that indicates that intermolecular signalling may occur between different PARs. PAR-2 does not respond thrombin, but when a PAR-1 signalling defective mutant was co-expressed with PAR-2 and stimulated with thrombin, a significant $[Ca^{2+}]_i$ increase was measured (O'Brien *et al.*, 2000). PARs appear to not only require their own respective proteinase activators for function, but other PAR receptors subtypes for a broader range of functionality in different tissues and cells.

1.3.3 PAR Signal Transduction

The importance of the tethered ligand sequence binding to the second exoloop of the receptor, following cleavage, has been demonstrated by studying chimeras of PAR-1 from different species as well as chimeras of PAR-1 and PAR-2 (Gerszten *et al.*, 1994).

Replacement of the second exoloop in *Xenopus* PAR-1 with the human PAR-1 sequence renders *Xenopus* PAR-1 selective for human PAR-1 activating peptides (AP). This study reveals the importance of the second exoloop for PAR agonist and tethered ligand selectivity. The mechanism of PAR signal transduction uses multiple pathways, as with other GPCRs, and is best understood for PAR-1 and PAR-2.

1.3.3.1 Proteinase Concentration and PAR Signalling

GPCRs not activated in a proteolytic manner produce graded responses to different concentrations of their respective agonists (Ji *et al.*, 1998). PAR activation by proteinases, by their inherent enzymatic nature could effectively cleave the entire PAR complement of a cell at very low concentrations given time, may make regulation less concentration specific. However, this is not the case, and it has been shown that graded responses in PARs depend on the rate of receptor cleavage (Ishii *et al.*, 1993). Each cleavage of a PAR receptor by an agonist proteinase generates a discreet phosphatidylinositol signal that is rapidly terminated by receptor desensitization mechanisms. This allows the cell to detect the rate of receptor cleavage rather than the cumulative amount of receptor cleavage over longer time periods.

1.3.3.2 PAR-1 Signalling

PAR-1 signalling has been studied extensively using knock-out mice. PAR activation results in the coupling to heterotrimeric G proteins (α, β and γ), guanine nucleotide exchange and the initiation of multiple downstream signalling pathways (Hung *et al.*, 1992a). PAR-1 interacts with several different α-subunits, most importantly

$G_{q11}\alpha$, $G_{12/13}\alpha$ and $G_i\alpha$, which is believed to account for the pleiotropic effects of its ligands.

1.3.3.2.1 Gα Protein Function

PAR-1 couples to $G_{q11}\alpha$ to play an important signalling role in platelets and fibroblasts. Knock-out mice that are deficient in $G_q\alpha$ possess platelets that exhibit reduced thrombin-induced aggregation and degranulation (Offermanns *et al.*, 1997). $G_{q11}\alpha$ activates phospholipase C-β_1 (PLC), generating inositol trisphosphate (IP_3) and diacylglycerol (DAG) from phosphoinositide hydrolysis (Taylor *et al.*, 1991). IP_3 functions to promote a release of sequestered calcium from stores in the smooth endoplasmic reticulum, leading to increases in $[Ca^{2+}]_i$. DAG activates protein kinase C (PKC) which provides a pathway to calcium-regulated kinases and phosphatases, guanine-nucleotide exchange factors (GEFs), mitogen-activated protein kinase (MAPK) cassettes and numerous other proteins. Collectively these mechanisms influence many cell properties including cell mobility, shape, metabolic responses, integrin activation and transcription and are similar to other G-protein signalling systems (Coughlin, 2000; Offermanns *et al.*, 1997).

PAR-1 also couples to the α-subunits G_{12} and G_{13} which bind RhoGEFs providing a pathway to Rho-dependent cytoskeletal responses involving shape changes in platelets, permeability and migration in endothelial cells (Kozasa *et al.*, 1998). Platelets from $G_q\alpha$-deficient animals show impaired degranulation and aggregation, yet they undergo normal shape changes in response to thrombin (Offermanns *et al.*, 1997). Thrombin-induced DNA synthesis is inhibited when antibodies to $G_{12}\alpha$ were microinjected into astrocytoma cells (Aragay *et al.*, 1995) and fibroblasts from $G_{13}\alpha$-deficient mice show decreased

migration in response to thrombin and the inability to develop an organized vascular system (Klages *et al.*, 1999). Collectively these findings indicate $G_{12/13}\alpha$ are involved in PAR-1 signalling and are crucial for the activation of Rho-mediated control of cell shape through the activation of Rho-kinase and myosin light-chain kinase.

PAR-1 couples to $G_i\alpha$ proteins which primarily function to inhibit adenylyl cyclase which catalyzes the formation of cAMP from ATP (Coughlin, 1999). Activation of PAR-1 in fibroblasts was shown to inhibit cAMP which suggests a role for $G_i\alpha$ protein involvement (Hung *et al.*, 1992c).

1.3.3.2.2 Gβγ Protein Function

The Gβγ subunits promote downstream signalling by activating phosphoinositide 3 kinase (PI(3)K), other protein kinases and ion channels (Dery *et al.*, 1998). Collectively, these effects change cell mobility, shape, metabolic responses, integrin activation and most importantly, transcription (Leevers *et al.*, 1999). Wortmannin, which blocks PI(3)K, hinders the activation of extracellular response kinases (ERKs) 1/2 following PAR-1 activation in astrocytes (Wang *et al.*, 2002). Calcium regulated phosphatases and kinases mediate most of the transcriptional changes.

1.3.3.3 PAR-2 Signalling

PAR-2 activation results in many of the same effects involving G proteins as PAR-1 with a few exceptions. PAR-2 agonists stimulate the production of IP_3 and increases in $[Ca^{2+}]_i$ in numerous cell lines (Ossovskaya and Bunnett, 2004). As with PAR-1, PAR-2 most likely couples to $G_q\alpha$, however PAR-2 signalling is unaffected by pertussis toxin, which inhibits cAMP, indicating that PAR-2 does not signal through G_i

proteins (DeFea *et al.*, 2000). PAR-2 activation has been shown to stimulate the activation of phospholipase A_2 and cyclooxygenase-1 to facilitate the generation of PGE_2 and F_1 (Kong *et al.*, 1997). The MAP kinase ERK 1/2 pathway is also activated as well as the NFκB pathway in keratinocytes and myocytes (Kanke *et al.*, 2001). Activation of PAR-1 and PAR-2 results in a wide array of cellular effects including changes in cell shape, migration, proliferation, secretion and transcription.

1.3.3.4 Termination of PAR Mediated Signals

Once PARs are activated the ligand is not able to dissociate from the receptor, which would result in continuous stimulation if it were not for a variety of desensitization mechanisms. Proteinases at the cell surface may inactivate PARs by cleaving the second exoloop or by cleaving the amino-terminus to remove the activation site. These disabling proteinases would dampen signalling and provide one mechanism to disable PAR signalling. Cathepsin G activates PAR-4 but cleaves PAR-1, 2, and 3, at the amino-terminus to abolish tethered ligand signalling regardless if it has been unmasked or not (Molino *et al.*, 1995). Elastase and proteinase 3 can also inactivate PAR-1 prior to stimulation with proteinases but not with agonist peptides, suggesting the binding domain is conserved (Renesto *et al.*, 1997). PAR-mediated responses to various proteinases in the extracellular environment depend on the complement of PAR subtypes expressed on the cell surface which may inhibited or activated.

The principal mechanism that terminates and prevents constitutive signalling is similar to desensitization of other GPCRs (Grady *et al.*, 1997). Activation induces the membrane translocation of G protein receptor kinases (GRKs) from the cytosol to the

receptor. GRKs interact with β-arrestins and phosphorylate the carboxy-terminus of the receptor which results in the dissociation of G proteins (Ishii *et al.*, 1994). PKC plays an important role stimulating phosphorylation of PAR-2 and a partial role for stimulating phosphorylation of PAR-1 (Bohm *et al.*, 1996a). PARs undergo clatherin-dependent endocytosis where they are trafficked to early endosomes and sorted to lysosomes for degradation. Not much is known about PAR-3 and PAR-4 desensitization except that PAR-4 slowly desensitizes which may allow it to signal for longer periods of time (Shapiro *et al.*, 2000).

1.3.3.5 PAR Regeneration

To generate a sustained signal through PAR activation, there must be constant regeneration of the receptor since PARs are activated by an irreversible mechanism. Prolonged signalling depends on the cell's internal store of new receptors. PAR-1 mobilization is rapid in endothelial cells and fibroblast where there are large internal stores in the Golgi apparatus (Hein *et al.*, 1994). On the other hand, there is a slow recovery observed in megakaryoblasts, indicating PAR-1 regeneration depends on the synthesis of new receptors (Hoxie *et al.*, 1993). There are intracellular pools of PAR-2 in many cell types that result in resensitization to PAR-2 agonists (Bohm *et al.*, 1996a). Platelets are unable to synthesize new receptors and do not recover responsiveness to thrombin, which does not impair their function since they are only required to respond once to thrombin in order to undergo aggregation. It appears that the resensitization phase of PARs are important in determining the nature of a response to a persisting stimulus.

1.3.4 Physiological Roles of PARs

PARs are involved in a number of physiological processes throughout various mammalian tissues and cell types. As selective peptide agonists became available, it was possible to study the effects of selective PAR activation *in vitro* and *in vivo* (al-Ani *et al.*, 1995; Coughlin, 1999). These tools have allowed researchers to focus on the effects of PAR activation, isolated from the broad effects that proteinases may have on other systems, not only on PARs. A problem with this approach is that peptides are administered at high concentrations which may have biological effects that are unrelated to PAR receptor activation.

1.3.4.1 Circulatory System

PAR-1 plays a key physiological role in haemostasis due to the action of thrombin on platelet aggregation and endothelial cell regulation (Vu *et al.*, 1991a). PAR-1 is involved in mediating coordinated responses to tissue injury by promoting tissue repair and pathogen clearance (Coughlin, 2000). Thrombin causes platelet aggregation, granular secretion and coagulation by binding to PAR-1 and PAR-3, signalling through PAR-1 and PAR-4. PAR-1 APs yield similar responses while PAR-4 APs are less efficacious which implicates PAR-1 as an early response receptor on platelets.

PARs are also found on endothelial cells and vascular smooth muscle cells functioning as a regulator of blood flow and extravasation of plasma proteins and granulocytes (Coughlin, 2000). These effects are typically studied by examining organ bath physiology. Thrombin and PAR-1 APs cause relaxation of contracted blood vessels via the release of nitric oxide from the vascular endothelium (Hamilton and Cocks,

2000). Inhibition of NO synthase suppresses relaxation and interestingly in pulmonary arteries, inhibiting cyclooxygenase yields similar results, implying lipid mediators may be released as well. PAR-2 also causes relaxation of a wide range of vessels from a number of species (Hamilton *et al.*, 2002; Roy *et al.*, 1998). It has been shown in murine animal models that PAR-1 and PAR-2 causes dilation of vessels and hypotension *in vivo* (Cicala *et al.*, 1999).

PARs may play an important physiological role in the circulatory system during inflammation and sepsis. The discovery of increased levels of PAR-2 mRNA in endothelial cells after the addition of IL-1α, TNF-α or LPS supports a role for PAR-2 during inflammation (Corvera *et al.*, 1997). PAR-2, but not PAR-1, is up-regulated during exposure to TNF-α and lipopolysaccharide which results in a hypotensive response to PAR-2 APs (Nystedt *et al.*, 1996). Oedema, plasma extravasation and granulocyte migration into tissues are caused by alterations in vessel permeability triggered by proteinases from the coagulation cascade and inflammatory cells. It has been shown that thrombin stimulates leukocyte rolling and adhesion to post-capillary venules of the rat mesentery and PAR-2 APs cause oedema formation in the rat paw (Vergnolle, 1999; Vergnolle *et al.*, 1999). The widespread distribution of PAR-2 compared to the limited distribution of pancreatic trypsin suggests other enzymes may function in PAR-2 activation. The induction of PAR-2 responses by mast cell tryptase suggests a role for this enzyme during inflammatory states that involve mast cell infiltration and degranulation (Akers *et al.*, 2000; Molino *et al.*, 1997a). Since trypsin is not normally found in the blood stream, PAR-1 may initiate an acute immune response, leading to mast

cell recruitment, degranulation and release of tryptase, thereby activating PAR-2. These observations are supported by the fact there is a decrease in rolling of neutrophils in PAR-2 deficient mice (Lindner *et al.*, 2000). PARs are important for a coordinated response to vascular injury, but prolonged activation by proteinases released from granulocytes may actually be detrimental.

1.3.4.2 Gastrointestinal System

The gastrointestinal system is exposed to a vast number of proteinases during normal biological processes and disease. All four PAR subtypes are expressed in the small intestine (Vergnolle, 2000). Physiological concentrations of trypsin can signal enterocytes by cleaving PAR-2 at the apical surface of the lumen. PAR-1 and PAR-2 stimulates chloride ion secretion from the intestinal mucosa which serves to enhance fluid secretion (Buresi *et al.*, 2001). Activation of PAR-1 in SCBN cells, a human small intestinal epithelial cell line, results in an increase of short-circuit current that is due to Cl^- secretion via ERK 1/2, phospholipase A_2 and cyclooxygenase-1 and -2.

PARs have been shown to have many other effects in the gastrointestinal tract including contraction of the gastric longitudinal muscle, pancreatic amylase and salivary and gastric mucus secretions (Bohm *et al.*, 1996b). PAR-2 activation stimulates pepsin secretion in rats while suppressing gastric acid secretion (Kawao *et al.*, 2002; Nishikawa *et al.*, 2002). The secretion of mucus in the gastrointestinal tract may have a protective effect by promoting the clearance of bacterial toxins and debris. In contrast to protective role, PARs may have a detrimental effect on intestinal epithelial cells. It has also been shown that PAR-1 agonists induce a loss of mucosal barrier function by the disruption of

tight junctions and promoting intestinal apoptosis (Chin *et al.*, 2003). This may be a pathophysiological mechanism for enteric microbes to invade and colonize the gastrointestinal tract. PARs are also widely expressed in the enteric nervous system that controls motility, secretion and fluid transport. Selective APs for PAR-1, -2 and -4 excite neurons in the gut which may lead to alterations in fluid secretion and pain sensation during inflammation (Bertog *et al.*, 1999; Coelho *et al.*, 2002).

Intestinal inflammation is associated with the release of proteinases which makes PARs a potential mediator of inflammatory bowel diseases. PAR-2 APs and proteinases produce an inflammatory reaction in the mouse colon but not in PAR-2 deficient mice (Cenac *et al.*, 2002). Inhibition of NO synthase, calcitonin gene-related peptide (CGRP) type 1 receptor and ablation of the sensory nerves suppresses PAR-2-induced inflammation, suggesting there may be a neurogenic component to the disease (Cenac *et al.*, 2003). In contrast, PAR-2 protects against inflammation in animal models of colitis produced by instillation trinitrobenzene sulfonic acid (Fiorucci *et al.*, 2001). In certain tissues, especially mucosal surfaces, PAR-2 activation may lead to a suppression of Th1-mediated inflammatory responses and protection from any pathological inflammatory effects. Through the stimulation of prostaglandin production and gastric mucus secretion, there may be a dual role for PAR-2 in the gastrointestinal tract.

1.3.4.3 Nervous System

PARs are expressed throughout the brain in rats, in neurons, glial cells astrocytes and the dorsal root ganglia (Vergnolle *et al.*, 2001a). Oscillations of $[Ca^{2+}]_i$ in neuronal cells are important for the control of excitability and the release of neurotransmitters.

Low concentrations of thrombin protect neurons from oxidative stress during ischemia *in vivo* while this protective effect is attenuated when Rho is inhibited, suggesting the involvement of the actin cytoskeleton (Donovan and Cunningham, 1998). PAR-1 may play a protective role in ischemic brain injury, however high concentrations of thrombin leads to neurodegeneration, causing apoptosis through caspase-3 in neurons and astrocytes (Donovan *et al.*, 1997). PAR-4 activation may contribute to inflammation in the brain by triggering the release of TNF-α through the activation of the NFκB pathway (Suo *et al.*, 2003).

There is also evidence that PAR-2 activation leads to the release of neuropeptides, substance P and calcitonin gene-related protein (CGRP) from primary sensory neurons contributing to hyperalgesia (Vergnolle *et al.*, 2001a). CGRP induces arteriolar dilation and SP induces gap formation in endothelial cells causing oedema and granulocyte infiltration (de Garavilla *et al.*, 2001; Steinhoff *et al.*, 2000). PAR-1 also promotes the release of substance P and the extravasation of plasma proteins, but causing analgesia instead of hyperalgesia by unknown mechanisms (Asfaha *et al.*, 2002). There are clearly different roles for PAR receptors for their involvement in neurogenic inflammation that still needs to be explored.

1.3.4.4 Airway

Proteinases activating PARs in the airway most likely arise from inflammatory cells and products of the coagulation cascade. There is new evidence (see section 1.4) that microbial pathogens may release proteinases that activate PARs in the lungs. PAR-1 and PAR-2 are expressed in the epithelial cells, endothelial cells and the airway smooth

muscle. Coagulation proteinases and tryptase are found at high levels in patients that suffer from airway inflammation (Idell *et al.*, 1987). PARs may be important mediators of allergic inflammation and contraction of the airway. PAR-1 and -2 APs cause bronchodilation in murine airways through the release of prostaglandin E_2 indicating a protective role in the lung (Chow *et al.*, 2000). In other models, PAR-2 stimulation leads to the contraction of segmental bronchial rings (Schmidlin *et al.*, 2001). There is strong evidence that activations of PARs contributes to pathophysiology in the lungs, however there are also observations that clearly give PARs a protective role against inflammation in some cases. Thrombin stimulates the release of GM-CSF from airway smooth muscle cells which may contribute to fibrosis in the lung (Tran and Stewart, 2003). Activation of PAR-1, -2 and -4 triggers the release of pro-inflammatory cytokines IL-6 and IL-8 which are potent inflammatory mediators (Asokananthan *et al.*, 2002a). PAR-2 in the airway may function to mediate allergic inflammation through mast cell tryptase and has shown to be up-regulated in asthmatics. Thus selective inhibitors of PARs could prove useful in attenuating the severity of allergic inflammation.

1.4 Microbial and Insect Proteinases

Proteinases have long been recognized as important virulence factors for a variety of pathogenic organisms. Proteinases that may possess the ability to activate PARs not only originate from endogenous sources, but also from exogenous sources such as bacteria, fungi, and insects (Hong *et al.*, 2004; Lourbakos *et al.*, 2001). It has been shown that various proteinases produced by these organisms signal through PARs via proteolytic cleavage, contributing to the virulence of the pathogen.

Porphyromonas gingivallis is a bacterial pathogen implicated in causing periodontitis in humans. The bacteria produce arginine-specific proteinases, gingipains-R (RgpB and HRgpA) that have been implicated in the disease process (Nakayama *et al.*, 1995). Upon stimulation with these proteinases, PAR-1 and PAR-2 transfected cells mobilize $[Ca^{2+}]_i$ and oral epithelial cells release IL-6, a pro-inflammatory cytokine, which functions to trigger immune responses (Lourbakos *et al.*, 1998; Lourbakos *et al.*, 2001). Desensitization studies also show that these proteinases cleave PAR-1 and PAR-4 on platelets causing aggregation. These data suggest that bacteria could trigger an immune response in-part by activating PARs.

It has been recently shown that dust mites may signal mammalian cells by cleaving PAR-2 in airway epithelia. *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* produce proteinase allergens (trypsins, chymotrypsins, cysteine proteinases) that cause allergic airway inflammation and asthma. Desensitization studies show that serine proteinases Der P3 and Der P9 induce pro-inflammatory cytokine release from lung epithelial cells through a PAR-2 mechanism (Sun *et al.*, 2001). Cysteine proteinase Der P1 has also been shown to activate PAR-2 and inactivate PAR-1 by proteolytic cleavage, resulting in $[Ca^{2+}]_i$ mobilization and the release of IL-6 and IL-8 (Asokananthan *et al.*, 2002b). The potential for non-mammalian proteinases to interact with PARs and contribute to an overall inflammatory reaction is a strong possibility. Whether the *in vitro* data are equivalent to the disease *in vivo* is still unknown. Organisms involved in other infectious diseases that secrete proteinases may also function through PARs modulate the host reaction.

1.5 PARs and Prostatitis

Prostatitis is a disease caused by many different factors, most of which are believed to be of a non-bacterial origin and are presently unknown. Bacterial infection is one aetiological factor that contributes to the pathology and symptoms of the disease. A growing body of knowledge has demonstrated the importance PARs play as inflammatory mediators throughout many tissues of the body. It has been shown that PAR-1 and PAR-2 are expressed in prostate cancer cell lines and that activation produces increased levels of matrix metalloproteinases (MMP) MMP-2 and MMP-9 which contributes to metastasis (Liu *et al.*, 2003; Wilson *et al.*, 2004; Zain *et al.*, 2000). Reflux of uric acid into the prostate, high K^+ levels or bacterial infection could provoke an inflammatory response by which the proteinases released during inflammation could trigger PARs. The effects of PARs in the prostate during inflammation have not yet been studied and it would be essential to determine the significance of PARs in the prostate and their influence on prostatic inflammation.

Proteinases secreted from uropathogenic bacteria could also activate PARs thereby contributing to the inflammatory reaction. A uropathogenic strain of *E. coli* CFT073 was isolated from a patient with urinary tract infection, which secretes a 107 kD secreted autotransporter (Sat) protein (Guyer *et al.*, 2000). This protein has been characterized and shows homology to the Pet and EspC, serine proteinases of the *Enterobacteriaceae* and has cytopathic activity in kidney and bladder cell lines (Guyer *et al.*, 2002). It is possible this proteinase has the potential to cleave PARs and to activate PAR signalling cascades which could contribute to the pathogenesis of this organism.

1.6 Hypothesis and Research Objectives

Hypothesis: PARs are found on prostatic epithelial cells where activation by endogenous or bacterial proteinases leads to the expression of pro-inflammatory cytokines thereby contributing to the inflammatory response seen in prostatitis.

1. The first objective will be to determine whether PAR-1, -2, -3, and -4 are expressed in the normal human prostate epithelial cell line RWPE-1 and in rat prostate tissues.
2. By using PAR activating proteinases and selective peptide agonists, it will be determined if these agonists induce intracellular calcium fluxes in the RWPE-1 cells. The production of inflammatory cytokines from these cells following stimulation will also be assessed to determine if PAR activation may have a pro-inflammatory role *in vitro*.
3. The rat model of bacterial prostatitis will be adapted to study the effects of PAR activation *in vivo* by instilling PAR agonists in the ventral prostate gland of rats. The nature of PAR-mediated responses will be evaluated by comparing pro-inflammatory markers to a bacterial control group of animals.
4. To analyze the effects of the bacterial proteinase (Sat) secreted from *E. coli* CFT073, the proteinase will be semi-purified and examined for proteolytic activity. By desensitizing the cells used in the Ca^{2+} bioassay that express PAR receptors, it will be possible to verify that any calcium fluxes observed are due to PAR activation and not other mechanisms.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Cell Lines and Propagation

Cells from the non-tumourigenic SV40 transformed human prostate epithelial cell line (RWPE-1) were purchased from American Type Culture Collection (CRL-11609) and propagated at 37°C in an atmosphere of 5% (v/v) CO_2. Cells were cultured in sterile 75 cm^2 T-flasks (BD Clontech) using keratinocyte serum-free medium (KSFM) supplemented with 5 ng/ml EGF, 0.05 mg/ml bovine pituitary extract (Gibco) and 1% penicillin/streptinomycin (Sigma). The growth medium was changed every 2 days and cells were passaged using 0.05% trypsin (Gibco) to detach cells from culture flasks using standard cell culturing protocols. Cells used for experiments were passaged a minimum of six times before use. Cell viability was quantified by the trypan blue exclusion method using a haemocytometer.

2.2 Peptides and Proteinases

Proteinases used in the study included thrombin (specific activity = 2.80×10^3 U/mg protein, Calbiochem #605195), a PAR-1 proteinase agonist, or trypsin (specific activity = 3.00×10^3 U/mg protein, Calbiochem #650200), a PAR-2 proteinase agonist, diluted in PBS to a stock concentration of 1000 U/ml. All peptides used were kindly provided by Dr. Morley Hollenberg and were obtained from and synthesized by the Peptide Synthesis Facility at the University of Calgary. The composition and purity of all peptides was established by high performance liquid chromatography, mass spectral and amino acid analysis. Stock peptide solutions were prepared in 25 mM HEPES buffer at pH 7.4 with either PAR-1 AP TFLLR-NH_2 (TF-NH_2), PAR-2 AP SLIGRL-NH_2 (SL-

NH_2) or PAR-4 AP AYPGKF-NH_2 (AY-NH_2). Negative control peptides, RLLFT-NH_2 (RL-NH_2) and LRGILS-NH_2 (LR-NH_2) corresponding to the reverse sequences of PAR-1 and PAR-2 were also used.

2.3 PAR Expression

2.3.1 RT-PCR

Human and rat specific cDNA primers and random cDNA hexameric primers were synthesized and gel purified by University of Calgary DNA Services.

2.3.1.1 Animal Tissues

A reverse transcription PCR (RT-PCR) reaction was performed to assess expression of PAR-1, PAR-2, PAR-3 and PAR-4 at the messenger RNA level. The ventral prostate glands of three CO_2 asphyxiated male Sprague-Dawley rats were immediately harvested, washed with sterile PBS and flash frozen by immersion in liquid nitrogen. The prostate tissues were either used immediately or stored at -70°C until the RNA extraction procedure was performed. The tissues were disrupted in liquid nitrogen by mortar and pestle and subsequently homogenized by passage through a 30 gauge needle. The total RNA was extracted from the homogenized sample using an RNeasy kit (QIAGEN). The concentration of the purified RNA was determined by measuring the A_{260}. From the RNA purification, 2 μg of RNA was transcribed and amplified at 37°C for 60 min using an Omniscript Reverse Transcriptase kit (QIAGEN) and random hexameric cDNA primers.

Following the reverse transcription, 2 μl of the product was combined with primer pairs for PCR analysis designed to amplify rat PAR-1, PAR-2, PAR-3, PAR-4. Rat PAR-

1 primer pair (711 bp product): forward primer 5′-GTGCTCCCCTTCAAGATCAG-3′ and reverse primer 5′-CGTAAAACACGACGTCTCTT-3′. Rat PAR-2 primer pair (598 bp product): forward primer 5′-CAACAGTAAAGGGAGAAGTCT-3′ and reverse primer 5′-GCAGCACGTCGTGACAGGT-3′. Rat PAR-3 primer pair (580 bp product): forward primer 5′-GTGTCTCTGCACACTTAGTG-3′ and reverse primer 5′-ATAGC ACAATACATGTTGCC-3′. Rat PAR-4 primer pair (557 bp product): forward primer 5′-GAATGCCAGACGCCCAGCATC-3′ and reverse primer 5′-GGTGAGGCGTTG ACCACGCA-3′. A commercial β-actin primer pair (R&D Systems) was used as a positive control and negative controls consisted of cDNA or primer pairs exclusively.

Taq PCR (QIAGEN) was used for amplification, running 30 cycles beginning with a 1 min denaturing step at 94°C, followed by a 1 min reannealing step at T_m-5°C and a 1 min primer extension step at 72°C. PCR products were separated by 2% (w/v) agarose gel electrophoresis and visualized with ethidium bromide under UV illumination. Bands were excised from the gel and the DNA was purified using a QIAquick gel extraction kit (QIAGEN) and sequenced by the University of Calgary DNA Services using an ABI Prism 3730XL DNA Analyzer (Applied Biosystems).

2.3.1.2 Cultured Cells

RWPE-1 cells were harvested from a 75 cm^2 T-flask, homogenized with a 30 gauge needle and syringe and total RNA was extracted using an RNeasy kit (QIAGEN). The quantity of RNA was determined by measuring the A_{260} and the RT-PCR amplification procedures were performed as described in the previous section for animal tissues.

Primers were designed to amplify human PAR-1, PAR-2, PAR-3 and PAR-4. Human PAR-1 primer pair (300 bp product): forward primer 5′-CGGCAGTGA TTGGCAGTTTG-3′ and reverse primer 5′-TCGAGCAGGGTTTCATTGAGC-3′. Human PAR-2 primer pair (399 bp product): forward primer 5′-TTGATGGCA CATCCGACGTC-3′ and reverse 5′-AATACCTCTGCACACTGAGGCAG-3′. Human PAR-3 primer pair (372 bp product): forward primer 5′-ATGAAAGCCCTCA TCTTTGCAG-3′ and reverse primer 5′-TCTGGTCCTGAAGAAAAGCATCC-3′. Human PAR-4 primer pair (392 bp product): forward primer 5′-ATTACTCG GACCCGAGCC-3′ and reverse primer 5′-TGTAAGGCCCACCCTTCTC-3′. β-actin primers (R&D Systems) were used as a positive control and negative controls consisted of solely cDNA or primer pairs in the reaction mixture.

PCR products were separated by 2% (w/v) agarose gel electrophoresis and visualized with ethidium bromide under UV illumination. Bands were excised from the gel and the DNA was purified using a QIAquick gel extraction kit (QIAGEN) and sequenced by the University of Calgary DNA Services using an ABI Prism 3730XL DNA Analyzer (Applied Biosystems).

2.3.2 Immunohistochemistry

2.3.2.1 Animal Tissues

The prostate glands of three male Sprague-Dawley rats were immediately harvested, cut in laterally and placed in 4% paraformaldehyde to fix the tissue. To visualize the localization of PAR-2, the sections (4 μm) were embedded in paraffin, sectioned, mounted on silane coated glass slides and immunostained with a rabbit

polyclonal anti-PAR-2 antibody (B5) diluted 1:500, kindly provided by Dr. Morley Hollenberg, which is antigen specific for the (G^{30}PNSKGR...SLIGRLDTP^{45}) amino acid sequence of PAR-2 (Kong *et al.*, 1997). A second anti-PAR-2 antibody (SLAW-A), which binds to the (^{5}SLAWLLG^{11}-G^{30}PNSKGR^{36}) epitope was used as well. The antibody binding was visualized by a streptavidin-conjugated HRP enzyme bound to a biotinylated goat anti-rabbit antibody (Sigma). Negative controls consist of sections incubated with only secondary antibody, primary antibody incubated with immunizing peptide prior to application to the section and pre-immune rabbit serum. Slides were observed on a Leica DMR fluorescence microscope at 40x magnification and photomicrographs were taken using a Photometrics CoolSNAP digital camera and Openlab 3.0.3 imaging software.

2.4 Experiments with Cultured Cells

2.4.1 Calcium Signalling Bioassay

Growth medium was aspirated from confluent RWPE-1 cell monolayers cultured in 75 cm^2 T-flasks. Flasks were rinsed with sterile calcium-free PBS (Gibco) and aspirated. Cells were lifted from T-flasks and disaggregated using an enzyme-free cell dissociation buffer (CDB) (Gibco) followed by a rinse with calcium-free PBS. Cells were collected, pelleted by centrifugation and resuspended in KSFM to a concentration of approximately 8-10 $\times$ 10^6 cells/ml in preparation for loading of the calcium indicator, fluo-3 acetoxymethyl ester (Fluo-3 AM) (Molecular Probes Inc.). Fluo-3 AM was added to a final concentration of 22 μM to the cell suspension in the presence of 0.4 mM sulfinpyrazone and 1 μl of Pluronic F-127 (Biotium) to enhance dye loading. Fluo-3 AM

uptake was facilitated by gentle agitation for 25 min at room temperature. The calcium assay buffer used in this experiment was of the following composition: NaCl (150 mM), KCl (3 mM), $CaCl_2$ (1.5 mM), HEPES (20 mM), dextrose (10 mM) and sulfinpyrazone (0.25 mM). Free Fluo-3 AM was removed from the cell suspension by repeated washing in Ca^{2+} assay buffer. Cells were resuspended in Ca^{2+} assay buffer for stimulation.

Changes in intracellular Ca^{2+} concentration was analyzed by measuring Fluo-3 AM fluorescence at an excitation wavelength of 480 nm and an emission wavelength of 530 nm using an Aminco Bowman Series 2 luminescence spectrophotometer. Cell suspensions were made to yield baseline fluorescence values between 1.8 and 2.2. Suspensions were stirred in disposable plastic cuvettes, to which stock solutions of PAR agonists were added directly, while measuring fluorescence in real-time. After establishing a stable baseline, Ca^{2+} ionophore calcimycin A-23187 (Sigma #16569), thrombin (Calbiochem), trypsin (Calbiochem), TF-NH_2, SL-NH_2, AY-NH_2, PAR-1 sham peptide NR-NH_2, PAR-2 sham peptide LR-NH_2 or Ca^{2+} assay buffer was added to the cuvettes.

Proteinase concentrations ranged from 0.025-250 U/ml while peptide concentrations ranged from 6.25-100 μM to observe concentration-responses. For desensitization experiments, agonists were applied consecutively at 5-10 min intervals. Fluorescence changes induced by an agonist were measured as a percentage of the change in fluorescence induced by 0.5 mM of the Ca^{2+} ionophore. Results consisted of two independent trials, each performed in quadruplicate.

2.4.2 Stimulation of Cultured Cells

RWPE-1 cells from 80-90% confluent 75 cm^2 T-flasks were lifted with CDB and seeded into 24-well cell culture plates (BD Clontech) at a concentration of approximately 5×10^4 cells/well in KSFM. Cells were grown to 80% confluency and incubated with fresh KSFM for 24 h, after which time they were exposed to varying concentrations of thrombin (0.025-250 U/ml), trypsin (0.025-250 U/ml), TF-NH_2 (6.25-100 μM) and SL-NH_2 (6.25-100 μM). As a positive control, cells were incubated with 50 ng/ml TNF-α (BioSource #PHC3015). The negative controls consisted of HEPES buffer and the PAR-1 and PAR-2 reverse peptides (RL-NH_2) and (LR-NH_2). Following a 24 h incubation period, the cell culture medium was removed, centrifuged at 12,000 × *g* at 4°C for 5 min, and the supernatant was removed and stored at -70°C. Cytokine expression was measured using human IL-6, IL-8 and IL-10 ELISA kits (R&D Systems) following the manufacturer's instructions. Cytokine concentration was normalized to the number of cells in each well quantified by the trypan blue exclusion method using a haemocytometer. Results consisted of two independent trials, each performed in quadruplicate.

2.5 Animal Model

2.5.1 Care and Handling

All animal studies were carried out under protocols approved by the Life Science Animal Care Committee and met standards set by the Canadian Council on Animal Care. The animal model adapted to this study has been previously described as a model for bacterial prostatitis (Ceri *et al.*, 1999a; Nickel *et al.*, 1990). All animals were obtained

from the University of Calgary Life & Environmental Sciences Animal Resource Centre. Adult male Sprague-Dawley rats weighing between 300 and 350 g (18 to 20 weeks old) were caged in groups of four in polycarbonate box cages with corn cob bedding. Animals were housed at a temperature of 20 ± 2°C and relative humidity of 30 ± 10%, with a 12 h illumination period. They were provided with standard rat chow and tap water ad libitum.

2.5.2 Agonist Instillation

2.5.2.1 PAR Agonists

Rats were instilled with the following solutions: 200 U thrombin (Calbiochem), 200 U trypsin (Calbiochem), 100 μM TF-NH_2, 100 μM SL-NH_2, 100 μM RL-NH_2 and 100 μM LR-NH_2. PBS (vehicle solution) alone was used as negative control along with the PAR reverse peptides. A positive control for inflammation consisted of a uropathogenic strain of *Escherichia coli* CP9 *cnf*$^+$, an O4:H5:K54, haemolysin-positive, CNF1-positive bacterial isolate from a patient with pyelonephritis (Rippere-Lampe *et al.*, 2001b). This strain was grown on Luria agar plates at 37°C and suspended in PBS at a concentration of 1.0×10^6 CFU/ml. To disrupt the mucin barrier in the prostate and test the reactions to PAR agonists, a solution of 50% ethanol was used as a vehicle solution and a barrier breaker (Lang *et al.*, 2000). Rats were instilled with 50% ethanol or TF-NH_2 and SL-NH_2 made up in a 50% ethanol solution.

2.5.2.2 Instillation

Rats were randomly divided into groups (n=6) and anaesthetized by exposure to 4% halothane. While anaesthetized, the rats were swabbed with 70% alcohol in the penile

region and catheterized with an ethylene oxide sterilized and lubricated polyethylene (PE 10) tube attached to a 30 gauge needle. A volume of 0.2 ml of the appropriate test solution was instilled into the ventral prostate gland while the catheter was held in place. The accuracy of the catheterization and instillation procedure was verified by observing blue dye in the ventral prostate gland following the instillation of a methylene blue solution into a non-test animal. The time after instillation was designated as time 0 h. Rats were sacrificed by CO_2 asphyxiation at either 6 h or 24 h following instillation of material.

2.5.3 Tissue Analysis

2.5.3.1 Morphology

The ventral prostate gland of the rat was removed and scored on the basis of size, congestion and hyperemia relative to an untreated control prostate gland. A score ranging from 0 to 3 (normal tissue to severe inflammation) was assigned to each category in a blinded fashion. The scores were added to obtain a total score from 0 to 9 for each animal. Following scoring, the prostate was divided into sections to be used for the following assays.

2.5.3.2 Histology

Prostatic tissue sections were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin wax using standard protocols. Tissue blocks were cut into 5 μm sections using a microtome, and sections were stained by standard haematoxylin and eosin (H&E) staining reagents and procedures. Sections were scored in a blinded manner from 0 to 3 (normal tissue to severe inflammation) in categories of oedema, haemorrhage

and leukocyte infiltration into the tissue. The scores for each category were totalled to obtain a score from 0 to 9 for each animal.

2.5.3.3 Myeloperoxidase Activity

The myeloperoxidase (MPO) activity assay was performed as previously described by (Bradley *et al.*, 1982). The hexadecyl-trimethylammonium bromide (HTAB) buffer used in the MPO assay consisted of HTAB (13.7 mM) in potassium phosphate buffer at pH 6.0 of the following composition: KH_2PO_4 (50 mM), K_2HPO_4 (50 mM). The O-dianisidine substrate consisted of: O-dianisidine (526 μM), 10% (v/v) potassium phosphate buffer and 5×10^{-4}% (v/v) H_2O_2.

A section of tissue was homogenized and sonicated in HTAB buffer for analysis of MPO activity. Samples were centrifuged at 1,800 × *g* for 10 min and the supernatants were removed and stored at -70°C. Assays were performed in 96-well microtitre plates containing 7 μl HTAB buffer, 7 μl of sample and 200 μl of O-dianisidine substrate. All samples were run in triplicate along with blank controls. Immediately following the addition of the O-dianisidine, the A_{450} was read at 30 sec intervals for 5 min in a kinetic assay by a plate reader (Molecular Devices Thermomax) using SoftMax software. The mean MPO activity from the assay negative control was subtracted from the mean activity for each sample. 1 unit of MPO activity is equal to 1 μmol H_2O_2 converted to O^- / min (Δ11.3 mOD / min). The mean MPO activities were normalized to the protein concentration of each respective sample, measured by the Bradford assay (Bio-Rad). Slides were observed on a Leica DMR fluorescence microscope at 20x magnification and

photomicrographs were taken using a Photometrics CoolSNAP digital camera and Openlab 3.0.3 imaging software.

2.5.3.4 Cytokine Assays

Sections of prostate tissue were homogenized in 1 ml of PBS containing 2 µl of a proteinase inhibitor cocktail (Sigma) and centrifuged at 1,800 × *g* at 4°C for 10 min. The supernatant was collected and stored at -70°C until analyzed. The concentration of the following cytokines was measured by using commercially available murine ELISA kits: IL-1β, TNF-α, IL-10 (R&D Systems) and Gro/CINC-1 (Amersham). The ELISAs were performed following the manufacturer's instructions, using diluted tissue supernatant samples run in duplicate. The absorbance was read by a Molecular Devices Thermomax plate reader using SoftMax software. The mean cytokine concentrations were normalized to the protein concentration of each respective sample, measured by the Bradford assay (Bio-Rad).

2.5.4 *In situ* Fluorescence Zymography

2.5.4.1 Tissue Preparation

Prostate tissue was excised from adult Sprague-Dawley rats (n=6) that had been instilled with either PBS or 1.0×10^6 CFU/ml *cnf*$^+$ UPEC 24 h earlier, as described in section 2.3.2. Fresh tissue was snap frozen in Optimal Cutting Temperature compound (VWR Scientific) by immersion in liquid nitrogen. Frozen tissue blocks were stored at -70°C until sectioned. Cryosections were prepared from the frozen blocks at -20°C using a Leica Jung CM 3000 Cryostat and cut to a thickness of 10 µm. The sections were

mounted on pre-cleaned Superfrost Plus slides (VWR Scientific) and were used immediately to assay proteinase activity.

2.5.4.2 Fluorescence Zymography

In situ proteinase activity was analyzed using the substrate Boc-QAR-7-amido-4-methylcoumarin (AMC) (Sigma #B4153) which is cleaved by trypsin and thrombin-like proteinases. When bound to a peptide, AMC does not emit significant fluorescence; however when cleaved from the peptide the AMC group is liberated and can be detected under the DAPI filter of a fluorescence microscope or by using a spectrophotometer with excitation and emission wavelengths of 380 nm and 460 nm, respectively.

The peptide substrate Boc-QAR-AMC (10 mg/ml) and AMC (2.63 mg/ml) were dissolved in DMSO. Unbound AMC was used as a positive control solution. Aliquots of each stock solution were serially diluted to concentrations of 0.5 mg/ml (0.37 mM; substrate) and 0.13 mg/ml (0.37 mM; AMC) using reaction buffer consisting of Tris-HCl (50 mM), $CaCl_2$ (10 mM), Brij 35 (0.05% v/v) (Sigma). A sample of DMSO was diluted 20-fold for use as a negative control. Samples of the dilute substrate, AMC and DMSO solutions (100 µl) were combined 1:1 with 1% molten agarose prepared in reaction buffer at 65°C. A 10 µl sample of the agarose solution was applied to the mounted sections as previously described by (Yi *et al.*, 2001) . Coverslips were immediately applied and the slides were allowed to cool to 0°C. Slides were incubated at 37°C for 30-35 min and viewed under a DAPI filter for fluorescence as marker for thrombin and trypsin-like proteinase activity, using a Leica DMRXA2 microscope. Image-J software was used to evaluate the fluorescence for each image taken of each slide.

2.6 Bacterial Proteinase Analysis

2.6.1 Bacterial Strains

Escherichia coli CFT073 was originally obtained from the blood and urine of a female patient suffering from acute pyelonephritis (Mobley *et al.*, 1990). This *hly*$^+$ *pap*$^+$ *sfa*$^+$ *pil*$^+$ strain of uropathogenic *E. coli* (UPEC) is phenotypically positive for P fimbriae, haemolysin and type 1 fimbriae, and is virulent in the CBA mouse model of ascending UTI (Mobley *et al.*, 1993) This particular strain is also cytotoxic for human renal epithelial cells and has found to produce a secreted auto-transporter toxin (Sat) which shows homology to SPATE (serine proteinase autotransporters of *Enterobacteriaceae*) proteins. An *E. coli* CFT073 *sat*::pGP704 Sat-deficient mutant (Guyer *et al.*, 2000) was used as a negative control strain which contains ampicillin and nalidixic acid resistant genes as selective markers.

2.6.2 Proteinase Purification

2.6.2.1 Filtration and Concentration

E. coli CFT073 was cultured at 37°C overnight on Luria Bertrani (LB) agar plates while the isogenic *sat* mutant was cultured on Luria agar plates containing 200 μg/ml ampicillin and 50 μg/ml nalidixic acid. Negative controls consisted of sterile media. The bacteria were inoculated into Luria broth cultures or Vincent's Minimal medium (VMM) (100 ml), with the isogenic *sat* mutant culture receiving appropriate antibiotics. The flasks were placed on a shaker and were left to grow for two days at 37°C. The bacteria were removed from the broth culture by centrifugation at 12,000 × *g* for 10 min at 4°C. The culture supernatants were collected and passed through 0.22 μm pore-size sterile

filters. Supernatants were concentrated using 50,000 kDa molecular weight cut-off Centricon Plus-80 filters (Millipore) to a volume of about 1 ml. Culture supernatants that had not been concentrated were also collected. Protein concentration was measured in all samples using the Bradford assay (Bio-Rad).

2.6.2.2 Dialysis and Lyophilization

Bacterial cultures were grown in LB flasks or minimal salts media for two days as described in the previous section. Negative controls consisted of sterile media. The bacteria were removed from the broth culture by centrifugation at 12,000 × *g* for 10 min at 4°C and filtration using 0.22 μm pore-size sterile filters. Spectra/Por Biotech membranes with a 50,000 kDa molecular weight cut-off were soaked and rinsed in deionized water for 30 min at room temperature to remove glycerine. The sterile supernatants were dialyzed for 18 h and decanted into in 50 ml Falcon tubes. The samples were frozen at -20°C for 2 h and subsequently at -70°C for 4 h. Samples were transferred to a lyophilization chamber for three days or until white, dry residues formed. Lyophilized samples were resuspended in PBS and the protein concentrations were measured using the Bradford assay (Bio-Rad).

2.6.2.3 Bacteria Grown in Co-Culture with Prostate Cells

RWPE-1 cells were grown in 6-well plates until 95% confluent in KSFM and were counted by microscopy using a grid. *E. coli* CFT073 and CFT073 *sat*::pGP704 cells were grown as previously described on Luria agar plates containing appropriate antibiotics and suspended in PBS and added to the cell culture media to attain an MOI of approximately 100:1. The cells were incubated at 37°C for 24 h after which time the

media was removed and the cells were lifted from the wells with 0.05% trypsin and viability was quantified using the trypan blue exclusion method. The culture supernatants were centrifuged at 12,000 × *g* for 10 min at 4°C and subsequently passed through a 0.22 μm pore-size sterile filter to remove bacteria. The filtered cell culture supernatants were stored at -20°C until used in the calcium signalling bioassay.

2.6.3 Proteinase Activity

2.6.3.1 Fluorometric Activity Assay

The proteinase activity of *E. coli* CFT073 and *E. coli* CFT073 *sat*::pGP704, which were grown in LB, filtered and concentrated as described in section 2.5.2.1, was analyzed by cleavage of a fluorescent substrate. The trypsin substrate Boc-QAR-AMC (7.5 mM in DMSO) was conjugated to AMC which fluoresces when cleaved by trypsin-like proteinases. Standard solutions of AMC ranging from 0 μM to 7.5 μM were made in DMSO. An assay buffer consisting of 50 mM Tris and 20 mM $CaCl_2$ (pH 7.4) was used to dilute the standard AMC solutions 100X to final volumes of 2 ml which were placed in disposable plastic cuvettes. An Aminco Bowman Series 2 luminescence spectrophotometer was used with an excitation wavelength of 380 nm and an emission wavelength of 460 nm to measure the fluorescence of each standard to calibrate the machine. Bacterial culture supernatants diluted 100X in the assay buffer solution were measured over a period of 10 min for changes in fluorescence with the substrate. As a positive control, the activity from 10U of trypsin (CalBioChem) was also measured.

2.6.3.2 Colourimetric Activity Assay

The proteinase activity of each sample: LB + *E. coli* CFT073, LB + *E. coli* CFT073 *sat*::pGP704, VMM + *E. coli* CFT073, VMM + *E. coli* CFT073 *sat*::pGP704, was measured using a QuantiCleave™ Proteinase Assay Kit (Pierce Biotechnology) which uses succinylated casein in conjugation with TNBS and has a sensitivity of 2 ng/ml. The assay was performed following the manufacturers instructions. Cleavage of the succinylated casein substrate exposes primary amines which TNBS reacts with to yield a coloured product. The activity of the test solutions was calculated by measuring the ΔA_{450} by subtracting the A_{450} of the blank negative controls using a plate reader (Molecular Devices Thermomax) using SoftMax software, which consisted of LB and VMM without bacteria, from the A_{450} of the test solutions.

2.6.3.3 Calcium Signalling Bioassay

The calcium signalling bioassay as previously described in Section 2.4.1 was used to determine PAR-activating proteinase activity in the filtered and concentrated bacterial supernatants. Supernatants from the filtered and lyophilized bacterial culture supernatants were tested at various concentrations for the ability to induce an increase in fluorescence at an emission wavelength of 530 nm from RWPE-1 cells incubated with Fluo-3 AM. Supernatants from bacteria grown in co-culture with RWPE-1 cells were also tested in the same manner.

2.7 Statistical Analysis

Group data are expressed as mean values ± SEM unless otherwise stated. Figures and statistical analyses were compiled using GraphPad Prism v4.00 software for

Windows (GraphPad Software). Morphological and histological scores were analysed by the non-parametric Kruskal-Wallis ANOVA on ranks test and Dunn's multiple comparison test of groups. The results of ELISA and MPO assays from *in vivo* and *in vitro* experiments were transformed and analysed by one-way ANOVA and Tukey's multiple comparison test. The unpaired t-test was used for analysing the results from the calcium mobilization bioassays. P values < 0.05 were considered statistically significant.

CHAPTER THREE: RESULTS

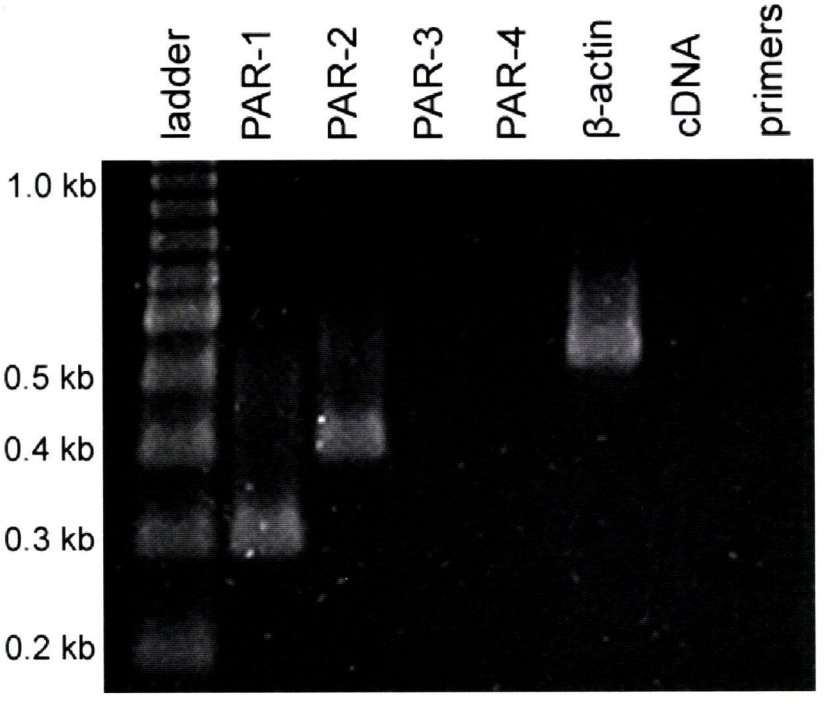
3.1 PAR Expression in Human Prostate Cells

At the initiation of the study there was only a single reference to PARs being present in the human prostate and no indication as to PARs being present in prostate of non-human mammals. The initial studies were aimed at determining the presence of members of the PAR family in the human prostate epithelial cell line RWPE-1 and in the rat prostate, which has been used by Ceri *et al.* as a model for prostate disease. In this study, the expression of the PAR family was determined by RT-PCR on extracted mRNA from the human prostate epithelial cell line RWPE-1 and from rat prostate tissues, as well as by immunohistochemistry of rat prostate tissues.

To demonstrate the presence of PARs in RWPE-1 cells, RT-PCR was performed on mRNA extracted from these cells. Primers specific for human PAR-1, -2, -3 and -4 were used along with β-actin primers as a positive control. It was found that PAR-1 and PAR-2 were expressed in this cell line, while PAR-3 and PAR-4 were not expressed (Figure 3.1). The experiment was repeated three times with separate cell cultures for each and the results were identical in each trial. The PAR-1 and PAR-2 fragments were excised, sequenced and found to be approximately 300 bp and 399 bp in length, respectively, with DNA sequences that corresponded to human PAR-1 and PAR-2.

Figure 3.1: PAR mRNA expression in human prostate epithelial cells. Primers specific for human PAR-1, -2, -3 and -4 were used to amplify cDNA that was reverse transcribed from homogenized RWPE-1 cell pellets. RT-PCR products were separated by 2% (w/v) agarose gel electrophoresis, stained with ethidium bromide and visualized under UV illumination. Lanes from left to right: 1 kb+ DNA ladder, PAR-1 primers, PAR-2 primers, PAR-3 primers, PAR-4 primers, β-actin primers (positive control), cDNA without primers (negative control), primers alone (negative control).

Figure 3.1: PAR-1, -2, -3 and -4 mRNA expression in RWPE-1 cells.



3.2 PAR-1 and PAR-2 Activation in RWPE-1 Cells

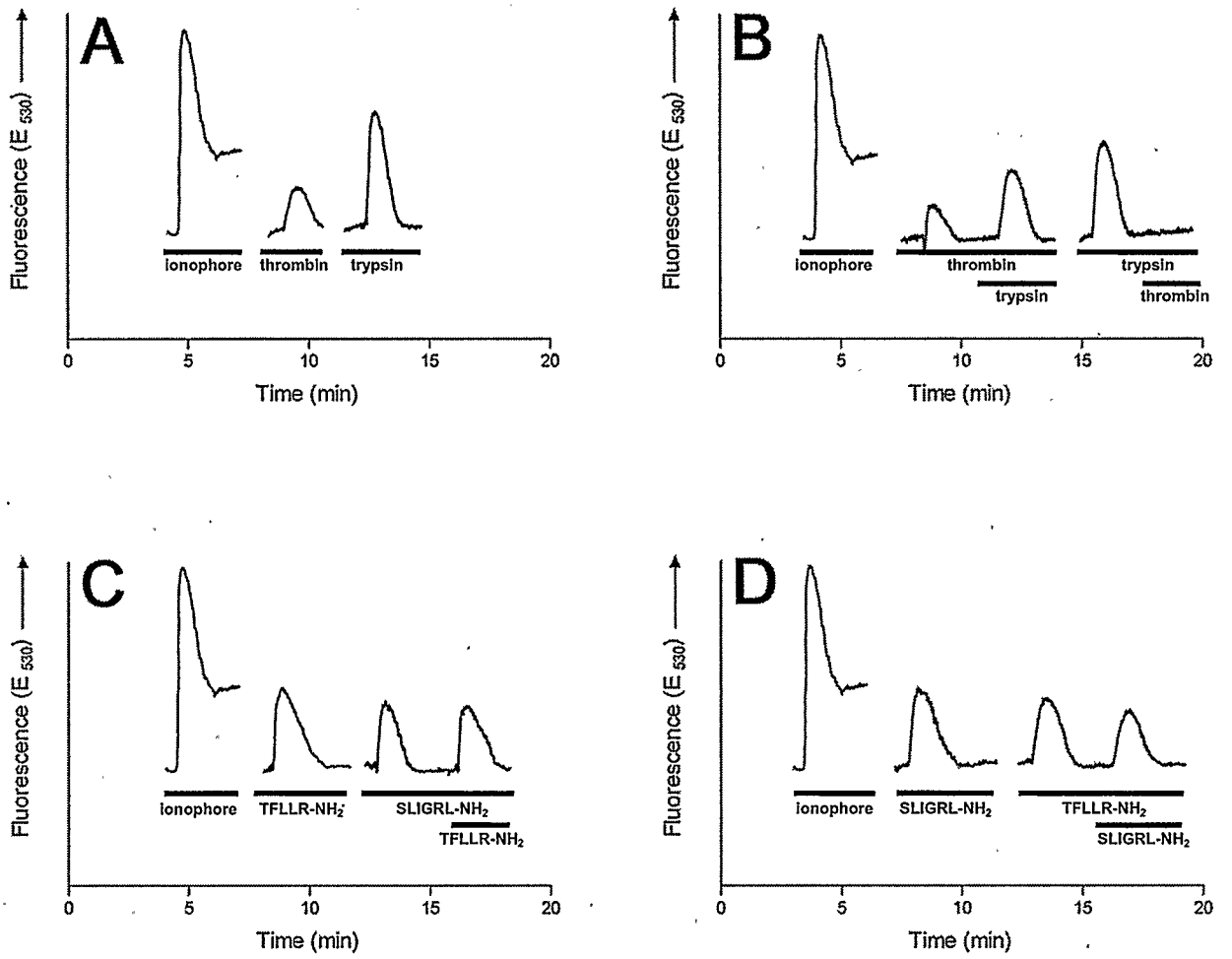
3.2.1 Intracellular Calcium Mobilization

With the knowledge that PAR-1 and PAR-2 were expressed in RWPE-1 cells, the second objective of this study was to evaluate the effects that PARs may have in the human prostate, specifically during inflammation. To model PAR activation *in vitro* using RWPE-1 cells, it had to be determined if proteinase and selective peptide agonists signal through the PAR-1 and PAR-2 receptors expressed in these cells. This was accomplished by loading the cells with a calcium indicator Fluo-3 AM and measuring temporal changes in intracellular calcium after the addition of agonist or control solutions to the cells (Figure 3.2). A calcium ionophore was added to the cells as a positive control for increases in $[Ca^{2+}]_i$. Fluxes in $[Ca^{2+}]_i$ were quantified by measuring the height of the peak from the initial level of baseline fluorescence. Calcium fluxes induced by PAR APs or proteinases were measured and normalized to the ionophore and expressed as a percentage of the calcimycin-induced change in $[Ca^{2+}]_i$.

Thrombin (250 U/ml) and trypsin (250 U/ml) induced increases in intracellular calcium concentration relative to the increases induced by a calcium ionophore in RWPE-1 cells (Figure 3.2A). After Ca^{2+} mobilization due to proteinase exposure, the level of fluorescence returned to the baseline, unlike the calcium ionophore which returned to a higher baseline level. It was also shown that after the cells were desensitized with thrombin they could still respond to trypsin (Figure 3.2B). However, when cells were desensitized with trypsin they no longer remained responsive to thrombin. To test

Figure 3.2: PAR agonist-induced change in $[Ca^{2+}]_i$ in human prostate epithelial cells. RWPE-1 cells were loaded with Fluo-3 AM calcium indicator and exposed to PAR-1 and PAR-2 agonists. $\Delta[Ca^{2+}]_i$ was measured over time by exciting with a wavelength of 480 nm and measuring the emission at 530 nm. Each unbroken line indicates a fresh sample of cells that had not been previously exposed to a stimulus. Bars underneath the traces indicate when solutions were added to the cells. From left to right: **(A)** calcium ionophore A23187, thrombin (250 U/ml), trypsin (250 U/ml), **(B)** calcium ionophore A23187, thrombin (250 U/ml) followed by trypsin (250 U/ml), trypsin (250 U/ml) followed by thrombin (250 U/ml), **(C)** calcium ionophore A23187, TFLLR-NH_2 (100 μM), SLIGRL-NH_2 (100 μM) followed by TFLLR-NH_2 (100 μM), **(D)** calcium ionophore A23187, SLIGRL-NH_2 (100 μM), TFLLR-NH_2 (100 μM) followed by SLIGRL-NH_2 (100 μM). Results consisted of two independent trials, each performed in quadruplicate.

Figure 3.2: Intracellular calcium release in RWPE-1 cells following addition of PAR agonists or controls.



selective PAR-1 and PAR-2 peptide agonists, cells were exposed to TF-NH_2 which yielded an increase in $[Ca^{2+}]_i$ (Figure 3.2C). When cells were desensitized with SL-NH_2 they still remained responsive to TF-NH_2. SL-NH_2 also induced a similar increase in fluorescence as TF-NH_2 (Figure 3.2D). When TF-NH_2 was used to desensitize the cells, they remained responsive to subsequent challenge with SL-NH_2. HEPES buffer (vehicle solution) and the PAR-1 and PAR-2 reverse peptides did not induce a change in fluorescence (data not shown).

Concentration-response curves were obtained for thrombin and trypsin by adding 0.0250, 0.250, 2.50, 25.0 or 250 U/ml of proteinase to RWPE-1 cells loaded with Fluo-3 AM (Figure 3.3). TF-NH_2, SL-NH_2, AY-NH_2 (PAR-4 AP), RL-NH_2 (PAR-1 reverse peptide) and LR-NH_2 (PAR-2 reverse peptide) were added to RWPE-1 cells at concentrations of 1.50, 3.10, 6.25, 12.5, 25.0, 50.0 and 100 μM to obtain concentration-response curves for PAR APs (Figure 3.4). HEPES buffer and the PAR-1 and PAR-2 reverse peptides were used as negative controls. Unexposed cells were used to test each concentration of proteinase or peptide agonist. At concentrations of 2.50, 25.0 and 250 U/ml of trypsin, increases in $[Ca^{2+}]_i$ were significantly higher than increases in $[Ca^{2+}]_i$ due to thrombin at identical concentrations (Figure 3.3). At all concentrations except for 0.0250 U/ml, thrombin and trypsin induced increases in $[Ca^{2+}]_i$ that were significantly higher than HEPES buffer, which did not result in any increase in $[Ca^{2+}]_i$. Thrombin induced a maximal response at 2.50 U/ml that was 27% of the response from the calcium ionophore whereas trypsin induced a maximal response at 25.0 U/ml that was 71% of the calcium ionophore. At the highest concentration of trypsin, the resulting calcium

mobilization slightly decreased from the maximum deviation observed. At higher concentrations of thrombin, there were no additional increases in calcium mobilization in the cells as were observed with trypsin and the response appeared to plateau.

At all concentrations except 1.50 μM, both the PAR-1 and PAR-2 AP induced changes in $[Ca^{2+}]_i$ that were significantly higher than HEPES buffer (Figure 3.4). TF-NH_2 and SL-NH_2 both exhibited maximal increases in $[Ca^{2+}]_i$ at 100 μM which were 62% and 64% of the calcium ionophore-induced response. At a concentration of 12.5 μM, the SL-NH_2 PAR-2 AP induced a significantly higher calcium response than TF-NH_2, however at all other concentrations there were no significant differences between the two peptides. At all concentrations with the exception for 1.50 μM, the calcium responses were significantly higher than the reverse peptides, RL-NH_2 and LR-NH_2 (data not shown). The PAR-4 AP was used as a negative control to verify there would be no effect from a PAR-4 selective agonist. AY-NH_2 did not induce changes in $[Ca^{2+}]_i$ at any concentration and the measured values were not different from those obtained from adding buffer.

3.2.2 Production of Inflammatory Mediators in RWPE-1 Cells

To analyze if activation of PAR-1 and PAR-2 in RWPE-1 cells involved the initiation of inflammatory pathways, IL-6 and IL-8 expression were analyzed in response to incubation with PAR APs and proteinases. A concentration-response curve was generated by incubating the cells with 0.00, 0.0250, 0.250, 2.50, 25.0 or 250 U/ml of thrombin or trypsin and measuring the concentration of IL-6 and IL-8 in cell culture supernatants after 24 h. The cells were also incubated with 0.00, 6.25, 12.5, 25.0, 50.0 or 100 μM of TF-NH_2, SL-NH_2 or the PAR-1 and PAR-2 reverse peptides. To verify that

Figure 3.3: Change in $[Ca^{2+}]_i$ after exposure to PAR-1 and -2 activating proteinases in human prostate epithelial cells. RWPE-1 cells were loaded with Fluo-3 AM calcium indicator and were exposed to 0, 0.025, 0.25, 2.5, 25 or 250 U/ml of thrombin or trypsin. Changes in $[Ca^{2+}]_i$ were measured over time by using an excitation wavelength of 480 nm and measuring emission at 530 nm. Increases in fluorescence, indicating calcium mobilization within the cell, were normalized to increases in fluorescence resulting from the addition of calcium ionophore A23187. Results consisted of two independent trials, each performed in quadruplicate. The unpaired t-test was used to compare the proteinase responses to each other and to the negative control. * ($p<0.05$) and *** ($p<0.001$) denotes concentrations of proteinases that produce calcium responses that were significantly different from each other.

Figure 3.3: Concentration-response curves measuring $[Ca^{2+}]_i$ in RWPE-1 cells after exposure to PAR-1 and -2 activating proteinases.

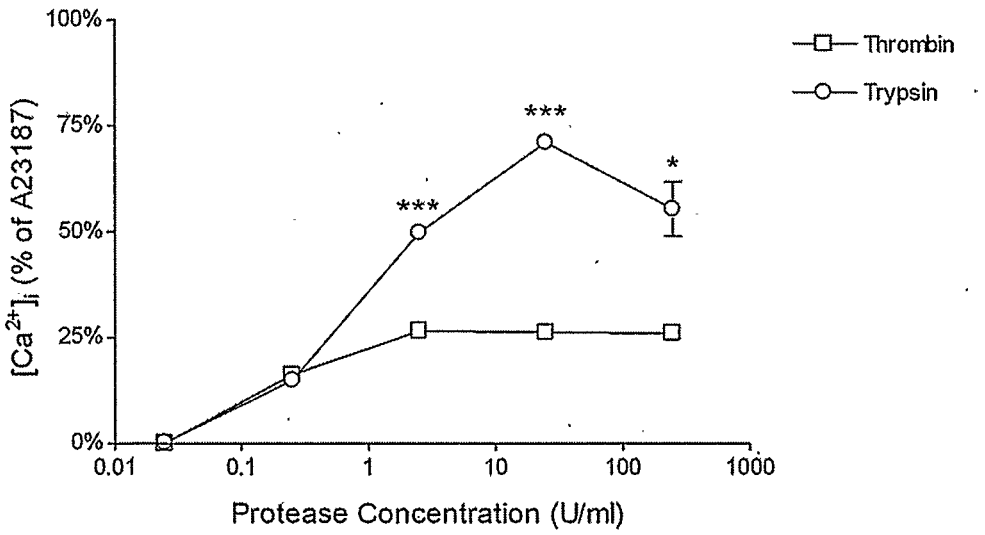
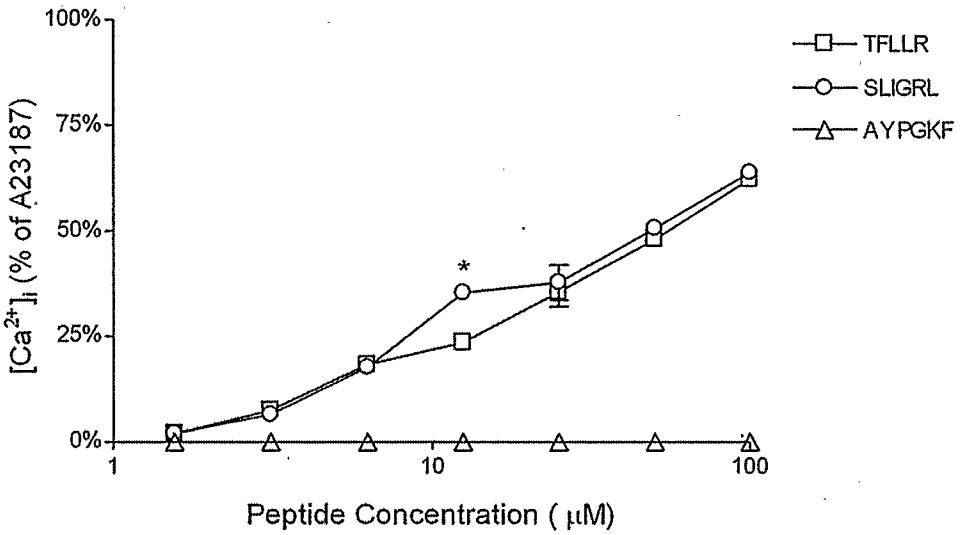


Figure 3.4: $\Delta[Ca^{2+}]_i$ after exposure to PAR-1 and -2 activating peptides in human prostate epithelial cells. RWPE-1 cells were loaded with Fluo-3 AM calcium indicator and were exposed to 0, 6.25, 12.5, 25, 50 or 100 μM of TF-NH_2, SL-NH_2 or AY-NH_2. Changes in $[Ca^{2+}]_i$ were measured over time by using an excitation wavelength of 480 nm and measuring emission at 530 nm. Increases in fluorescence, indicating calcium mobilization within the cell, were normalized to increases in fluorescence resulting from the addition of calcium ionophore A23187. Results consisted of two independent trials, each performed in quadruplicate. The unpaired t-test was used to compare the peptide responses to each other and to the negative control. * ($p<0.05$) denotes concentrations of peptides that produce calcium responses that were significantly different from each other.

Figure 3.4: Concentration-response curves measuring $[Ca^{2+}]_i$ in RWPE-1 cells after exposure to PAR-1 and -2 activating peptides.

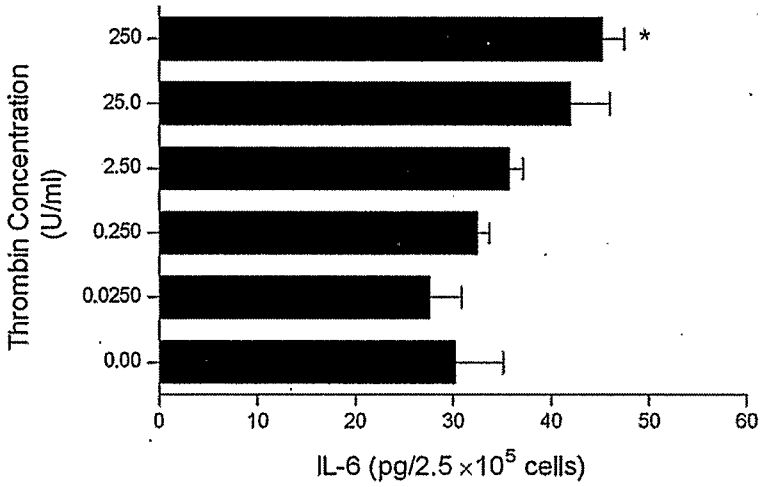


these cells were able to express IL-6 and IL-8 they were incubated with TNF-α which stimulates the production of these cytokines in other epithelial cell types. The cells from each well were counted and the cytokine concentrations were normalized to the cell numbers from each well.

It was found that 50.0 ng/ml TNF-α resulted in increases in IL-6 (2.28-fold, $p<0.05$) and IL-8 (1.73-fold, $p<0.001$) over the buffer control which would correspond to the baseline level of expression (data not shown). Similar increases were observed for the PAR-1 agonists. IL-6 expression increased with higher thrombin concentrations and at 250 U/ml the IL-6 expression was significantly higher (45.2 pg/2.5×10^5 cells) than the HEPES buffer treated cells (30.2 pg/2.5×10^5 cells) and cells incubated with 0.0250 U/ml thrombin (27.6 pg/2.5×10^5 cells) (Figure 3.5). There was no significant difference in IL-6 expression when the cells were incubated with 6.25, 12.5, 25.0 or 50.0 μM of TF-NH_2 but at 100 μM, IL-6 expression increased (50.0 pg/2.5×10^5 cells) and was significantly higher than the buffer control (27.7 pg/2.5×10^5 cells). When IL-8 was measured, a similar concentration-response for thrombin was observed, however these increases were not statistically significant (Figure 3.6). The baseline level of IL-8 expression was 3.00×10^3 pg/2.5×10^5 cells and thrombin (250 U/ml) resulted in 3.57×10^3 pg/2.5×10^5 cells. When IL-8 was analyzed after TF-NH_2 exposure, there was also an increase in expression with higher concentrations of the peptide as observed for thrombin which was significant. TF-NH_2 concentrations of 25.0, 50.0 and 100 μM resulted in IL-8 levels (4.50×10^3, 5.03×10^3, 5.71×10^3 pg/2.5×10^5 cells respectively) that were significantly higher than the buffer control (3.14×10^3 pg/2.5×10^5 cells). The expression of IL-6 and IL-8 after incubation

Figure 3.5: PAR-1 agonist-induced IL-6 expression in human prostate epithelial cells. RWPE-1 cells were grown in 24-well plates, incubated with PAR-1 agonists and the IL-6 concentration in the cell culture supernatant was measured after 24 h. **(A)** IL-6 concentration after incubation with 0.00, 0.0250, 0.250, 2.50, 25.0 or 250 U/ml of thrombin. **(B)** IL-6 concentration after incubation with 0.00, 6.25, 12.5, 25.0, 50.0 or 100 μM TFLLR-NH_2. Results consisted of two independent trials, each performed in quadruplicate. Data was transformed and analysed by one-way ANOVA and Tukey's multiple comparison test. * ($p<0.05$) denotes concentrations of proteinases or peptides that resulted in IL-6 concentrations that were significantly different from the negative control (HEPES buffer).

Figure 3.5: IL-6 expression in RWPE-1 cells following exposure to PAR-1 agonists.



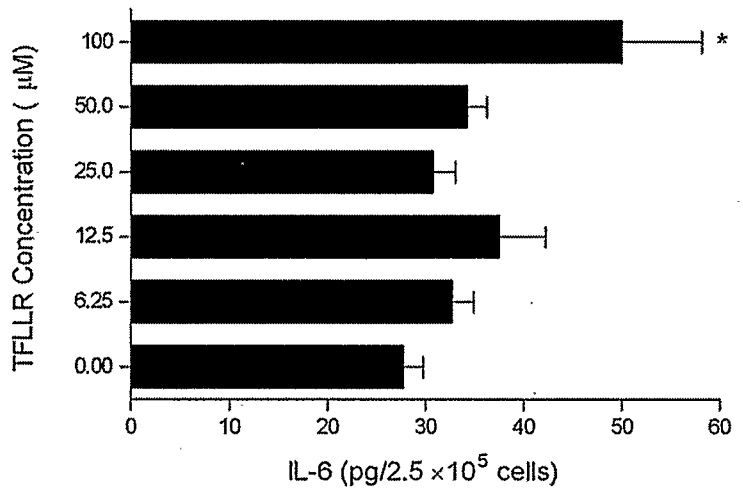
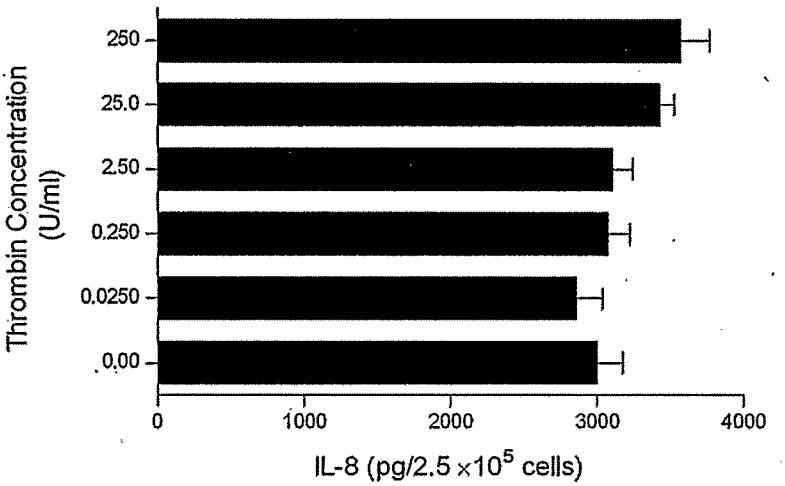
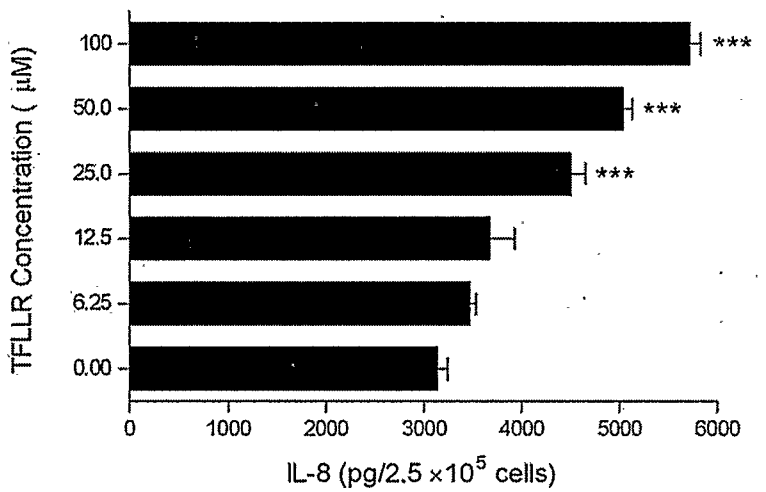


Figure 3.6: PAR-1 agonist-induced IL-8 expression in human prostate epithelial cells. RWPE-1 cells were grown in 24-well plates, incubated with PAR-1 agonists and IL-8 concentration in the cell culture supernatant was measured after 24 h. **(A)** IL-8 concentration after incubation with 0.00, 0.0250, 0.250, 2.50, 25.0 or 250 U/ml of thrombin. **(B)** IL-8 concentration after incubation with 0.00, 6.25, 12.5, 25.0, 50.0 or 100 μM TFLLR-NH_2. Results consisted of two independent trials, each performed in quadruplicate. Data was transformed and analysed by one-way ANOVA and Tukey's multiple comparison test. *** ($p<0.001$) denotes concentrations of proteinases or peptides that resulted in IL-8 concentrations that were significantly different from the negative control (HEPES buffer).

Figure 3.6: IL-8 expression in RWPE-1 cells following exposure to PAR-1 agonists.





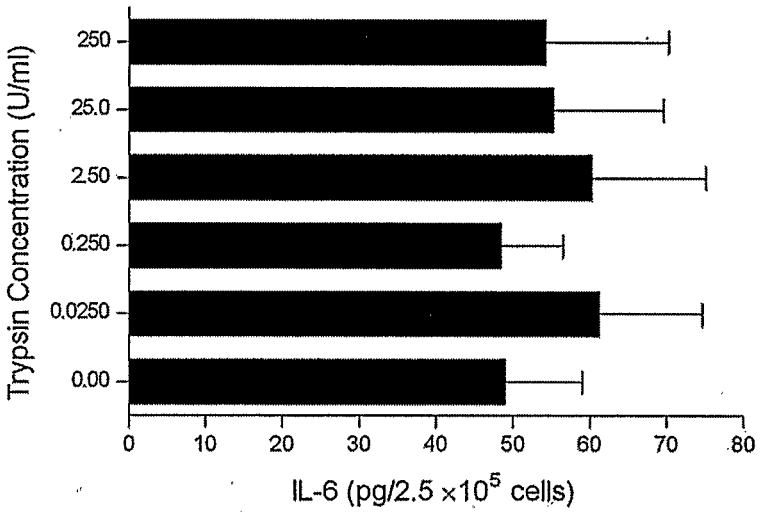
with the reverse peptides was insignificant when compared to HEPES buffer (data not shown).

When RWPE-1 cells were exposed to PAR-2 APs and proteinases, a dissimilar concentration-response was observed. At all concentrations of trypsin, the level of IL-6 expression was highly variable and was not statistically different from the buffer control (27.7 pg/2.5×10^5 cells) (Figure 3.7). Similarly, when cells were incubated with concentrations of 6.25, 12.5, 25.0, 50.0 and 100 μM SL-NH_2, significant increases in IL-6 when compared to the buffer control (26.7 pg/2.5×10^5 cells) were not observed. It appeared that concentrations of 12.5 and 25.0 μM led to a reduction baseline IL-6 expression but this observation was not statistically significant. PAR-2 negative control peptides resulted in negligible changes in IL-6 concentration when compared to the HEPES buffer control (data not shown). IL-8 expression also did not appear to change with increasing trypsin concentration and at all trypsin concentrations, IL-8 expression did not differ from the control (4.85×10^3 pg/2.5×10^5 cells) (Figure 3.8). The mean IL-8 expression increased relative to the control (3.21×10^3 pg/2.5×10^5 cells) when 12.5 and 25.0 μM SL-NH_2 (5.75×10^3 and 5.68×10^3 pg/2.5×10^5 cells respectively) were added to the cells but these variations were not statistically significant.

Epithelial cells may also secrete anti-inflammatory cytokines and PAR-1 and PAR-2 may play a protective role in this cell line. IL-10 expression was measured after stimulation with PAR-1 and PAR-2 proteinase and selective peptide agonists and it was found that the IL-10 concentration in the cell culture medium did not change in comparison to the baseline under any of the test conditions (data not shown).

Figure 3.7: PAR-2 agonist-induced IL-6 expression in human prostate epithelial cells. RWPE-1 cells were grown in 24-well plates, incubated with PAR-2 agonists and IL-6 concentration in the cell culture supernatant was measured after 24 h. **(A)** IL-6 concentration after incubation with 0.00, 0.0250, 0.250, 2.50, 25.0 or 250 U/ml of trypsin. **(B)** IL-6 concentration after incubation with 0.00, 6.25, 12.5, 25.0, 50.0 or 100 μM SLIGRL-NH_2. Results consisted of two independent trials, each performed in quadruplicate. Data was transformed and analysed by one-way ANOVA and Tukey's multiple comparison test.

Figure 3.7: IL-6 expression in RWPE-1 cells following exposure to PAR-2 agonists.



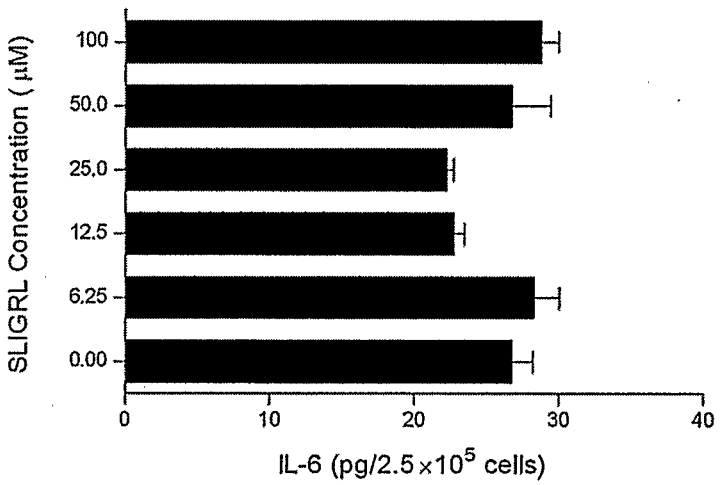
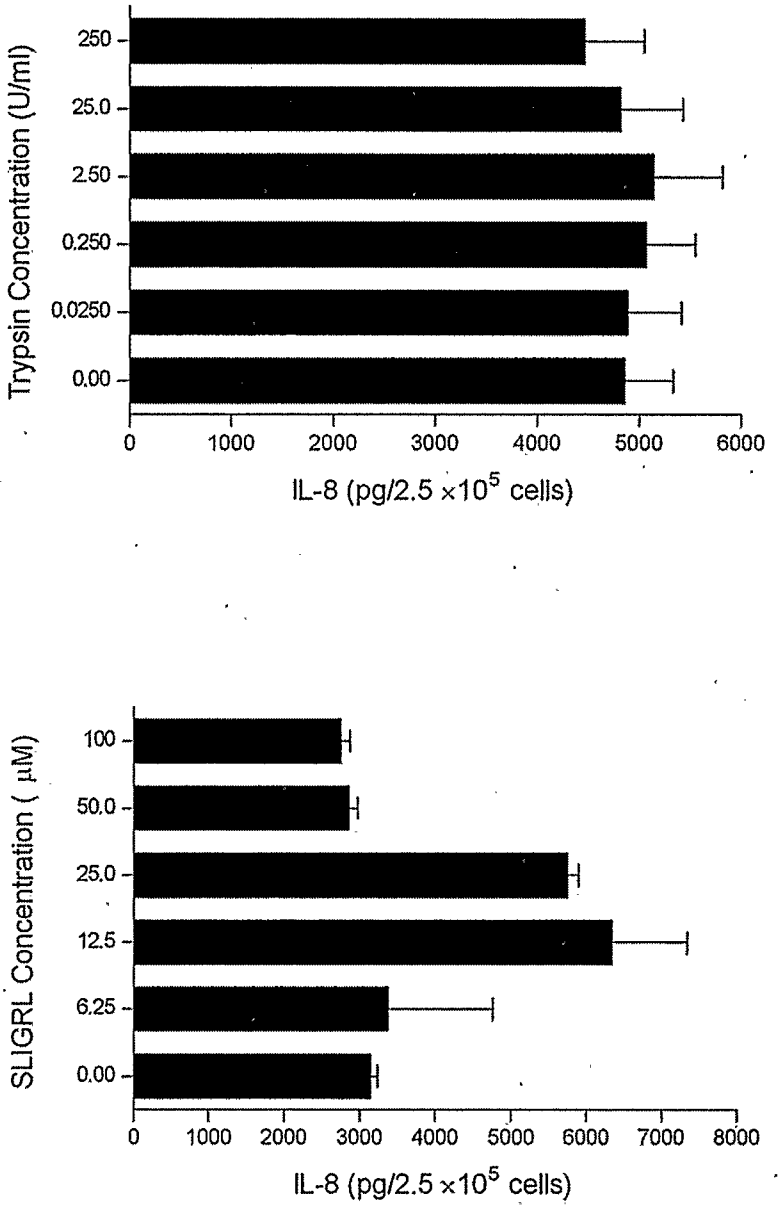


Figure 3.8: PAR-2 agonist-induced IL-8 expression in human prostate epithelial cells. RWPE-1 cells were grown in 24-well plates, incubated with PAR-2 agonists and IL-8 concentration in the cell culture supernatant was measured after 24 h. **(A)** IL-8 concentration after incubation with 0.00, 0.0250, 0.250, 2.50, 25.0 or 250 U/ml of trypsin. **(B)** IL-8 concentration after incubation with 0.00, 6.25, 12.5, 25.0, 50.0 or 100 μM SLIGRL-NH_2. Results consisted of two independent trials, each performed in quadruplicate. Data was transformed and analysed by one-way ANOVA and Tukey's multiple comparison test.

Figure 3.8: IL-8 expression in RWPE-1 cells following exposure to PAR-2 agonists.



3.3 PAR Expression in the Rat Prostate

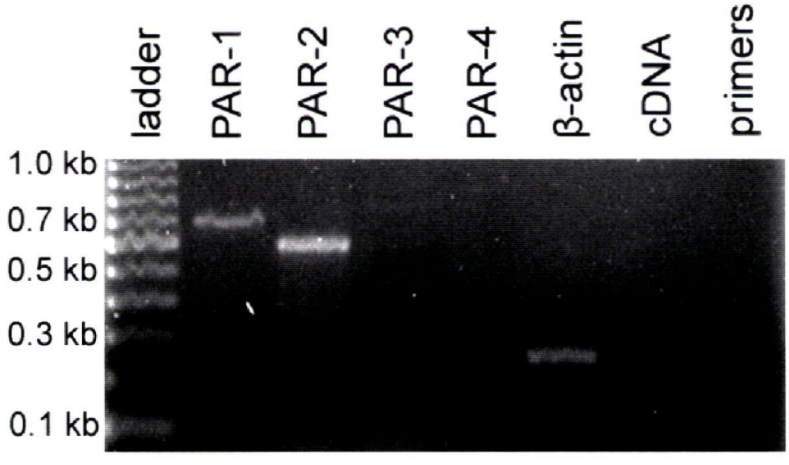
The objective of this research was to determine if activation of PARs in the prostate contributes to inflammation in the tissue. Since pro-inflammatory responses were observed in an *in vitro* system, it was logical to extend this study to observe PAR-mediated effects in a complex *in vivo* system. To accomplish this, an animal model of prostatitis was adapted so that PAR agonists could be instilled into the prostatic ducts. Any pathological responses could be graded by analyzing the tissue morphology, histology and by molecular assay of inflammatory mediators. Taken together, these data should provide a picture as to the overall inflammatory state of the tissue following agonist instillation. The expression of PARs in the rat prostate had not been previously described in the literature so RT-PCR was performed on extracted mRNA from the ventral rat prostate.

Primers specific for rat PAR-1, -2, -3 and -4 were used to amplify PARs from the ventral prostate gland of Sprague-Dawley rats. PAR-1 and PAR-2 were detected along with β-actin (positive control) in rat prostate tissue from three separate animals in independent experiments (Figure 3.9). PAR-3 and PAR-4 were not detected in the tissues in any animals. The bands were excised, sequenced and found to correspond to the 711 bp and 703 bp fragments of PAR-1 and PAR-2, respectively.

As anti-PAR-2 polyclonal antibodies (B5 and SLAW-A) were available, the tissue distribution of PAR-2 was examined in the rat ventral prostate. Paraffin sections (4 μm) of the ventral rat prostate from Spraque-Dawley rats were incubated with the B5 antibody and visualized by peroxidase staining (Figure 3.10A). The B5 antibody, which

Figure 3.9: PAR mRNA expression in the ventral prostate of Sprague-Dawley rats. Primers specific for rat PAR-1, -2, -3 and -4 were used to amplify cDNA that was reverse transcribed from homogenized rat tissues. RT-PCR products were separated by electrophoresis in a 2% (w/v) agarose gel, stained with ethidium bromide and visualized under UV illumination. Lanes from left to right: 1 kb+ DNA ladder, PAR-1 primers, PAR-2 primers, PAR-3 primers, PAR-4 primers, β-actin primers (positive control), cDNA without primers (negative control), primers alone (negative control).

Figure 3.9: PAR-1, -2, -3 and -4 mRNA expression in rat prostate tissue.



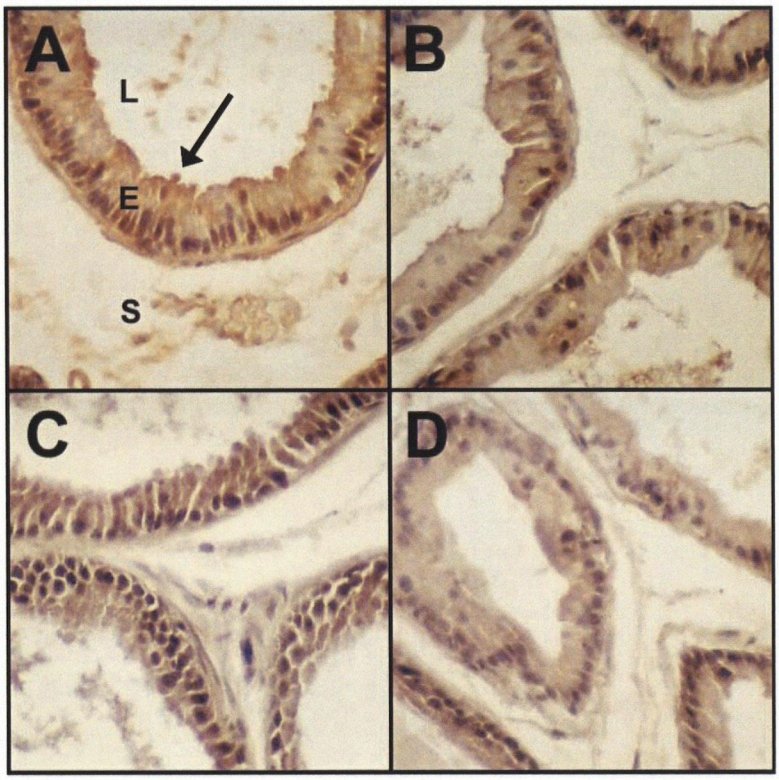
is specific for the PAR-2 epitope G^{30}PNSKGR...SLIGRLDTP^{45} which bridges the cleavage site, was mostly found on epithelia lining the ducts of the prostate. Control sections consisted of sections incubated with primary antibody incubated with immunizing peptide prior to application to the section (Figure 3.10B), pre-immune rabbit serum (Figure 3.10C) or only secondary antibody (Figure 3.10D). SLAW-A, an alternative antibody to PAR-2 which binds the epitope ^{5}SLAWLLG^{11}-G^{30}PNSKGR^{36}, resulted in inconsistent immunostaining (data not shown) and the negative competitive controls also showed staining, indicating that this antibody was not as antigen specific as B5 is in this tissue.

3.4 PAR-1 and PAR-2 Activation in an Animal Model

To study the possible inflammatory effects of PAR activation in this model, proteinase and peptide agonists of PAR-1 and PAR-2 were used in this study. Sprague-Dawley rats (n=6) were instilled with saline (the vehicle solution as a negative control), 200 U thrombin, 200 U trypsin, 100 μM TF-NH_2, 100 μM SL-NH_2, 100 μM RL-NH_2, 100 μM LR-NH_2 or 1.0×10^6 CFU/ml *E. coli* CP9 *cnf*$^+$ (positive control for a moderate to severe inflammatory response in the tissue). These concentrations were determined by preliminary dose-response studies performed to determine the minimal concentrations that would result in deviations from the negative control (data not shown). They were also based on concentrations used in similar studies involving mucosal tissues. Colonization of the prostate with *cnf*$^+$ UPEC was confirmed by plate counting tissue homogenates and it was found that infected animals generally had 10^5 to 10^6 CFU per mg

Figure 3.10: PAR-2 immunohistochemistry in the ventral prostate of Spraque-Dawley rats. Prostate tissue sections were fixed in 4% paraformaldehyde, sectioned and incubated with the following: **(A)** 1:500 PAR-2 antibody (B5), **(B)** B5 pre-incubated with immunizing peptide, **(C)** only secondary antibody, **(D)** only blocking serum. Following peroxidase staining the sections were photographed. The (L) lumen of the acinus, (E) epithelial cell layer and the (S) stroma is indicated in (A). The arrow specifies peroxidase staining of PAR-1 with the B5 antibody. 40x magnification.

Figure 3.10: PAR-2 expression in the ventral prostate tissue of Sprague-Dawley rats.



of tissue. PAR-1 and PAR-2 reverse peptides were also used as negative controls to confirm that any effects due to administration of the peptides were due to PAR activation and not other non-PAR related signal transduction pathways. Following similar studies, the animals were sacrificed 6 h or 24 h after the instillation of the various solutions.

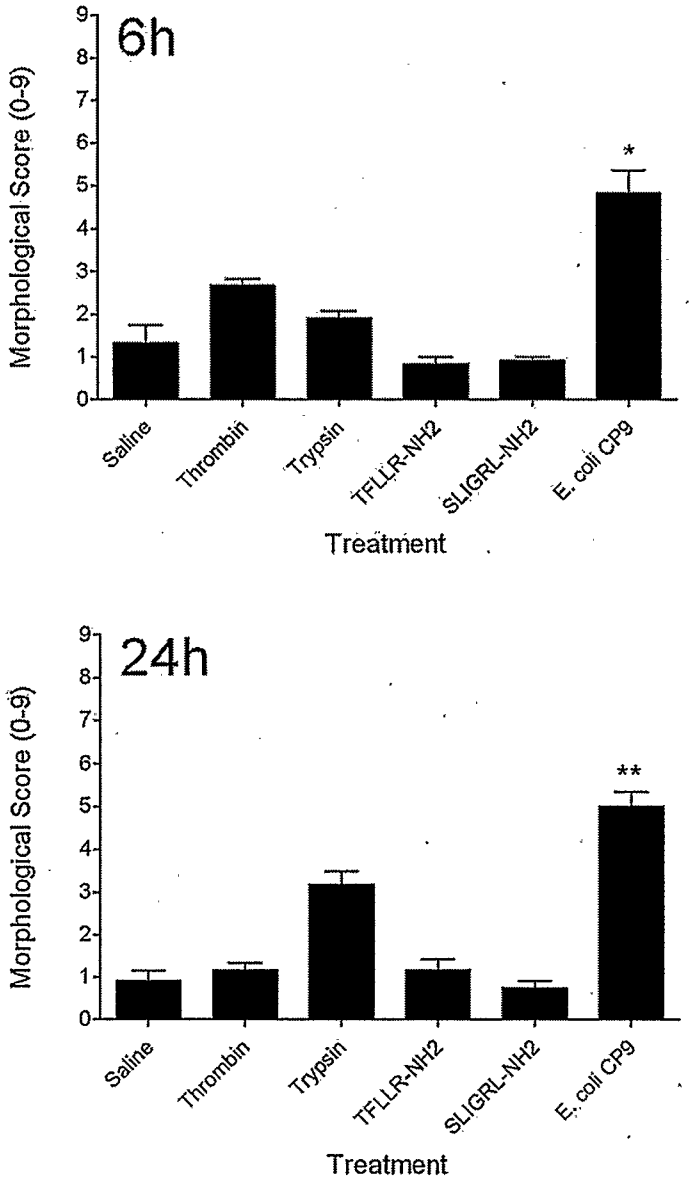
3.4.1 Morphological and Histological Analysis

The tissues were scored for gross morphology after 6 h and it was found that *cnf*$^+$ UPEC infected rats had significantly more damage with a mean score of 4.8 when compared to the damage (size, congestion and hyperaemia) scores from saline (1.3), TF-NH_2 (0.8) and SL-NH_2 (0.9) (Figure 3.11). Thrombin (2.6) also induced significant amount of damage when compared to TF-NH_2 (0.8) but none of the PAR-1 or PAR-2 activating proteinase or peptides resulted in significant damage scores when compared to the saline control. The reverse peptides were comparable to the saline control for both 6 hand 24 h (data not shown). After 24 h, similar results were observed as in the 6 h groups with one exception, the trypsin damage score (3.1) was significantly higher when compared to the corresponding PAR-2 AP (0.7). None of the PAR-1 or PAR-2 agonists at both 6 h and 24 h were significantly different from the saline control.

Samples of tissue were taken from each animal to be used for the preparation of H&E stained histological sections (5 μm). The saline control sections displayed normal tissue architecture, closely associated acini, minimal stromal tissue and a lack of oedema and neutrophils (Figure 3.12A). The *cnf*$^+$ UPEC infected animals showed neutrophil infiltrate in acini, a loss of tissue integrity in some cases and oedematous stromal tissue (Figure 3.12F). The histological sections from animals instilled with PAR-1 and PAR-2

Figure 3.11: Gross tissue morphology scores of the ventral prostate of Sprague-Dawley rats instilled with control or PAR agonist solutions. Male rats were anesthetised and instilled with 0.2 ml of one of the following solutions in the ventral prostate via catheter: saline (vehicle solution), 200 U thrombin, 200 U trypsin, 100 μM TFLLR-NH_2, 100 μM SLIGRL-NH_2 or 1.0×10^6 CFU/ml *E.coli* CP9 *cnf*+ (n=6). Rats were sacrificed at 6 h or 24 h, as indicated, and the prostate glands were harvested and scored for size (0-3), hyperemia (0-3) and congestion (0-3). The scores were totalled to obtain a cumulative score (0-9). Scores were analysed by the Kruskal-Wallis ANOVA on ranks test and Dunn's multiple comparison test of groups. * ($p<0.05$) and ** ($p<0.01$) denotes groups that were significantly different from the negative control (saline).

Figure 3.11: Gross tissue morphology scores of rat prostates instilled with control or PAR agonist solutions.



agonists were similar when compared to the saline control. Generally, these sections lacked the neutrophil infiltration into the tissues and oedema as seen in the *cnf*$^+$ UPEC sections (Figures 3.12A-E). One of the rats that instilled with trypsin resulted in small amounts of neutrophil infiltration in some of the prostatic acini.

The sections were scored and it was found that *cnf*$^+$ UPEC infected rats had significantly higher histological damage scores (3.0) after 6 h than saline (0.3) and TF-NH_2 (0.4) (Figure 3.13). None of the PAR-1 or PAR-2 agonists were significantly different from the saline control. After 24 h the UPEC damage score (5.2) was significantly higher than saline (0.3), trypsin (0.8), TF-NH_2 (0.7) and SL-NH_2 (0.7). As with the 6 h groups, none of the PAR-1 or PAR-2 agonists had scores that were significantly different from saline. Also reverse peptides did not have scores that differed from saline at 6 h or 24 h (data not shown). The deviations from the saline control observed in the gross morphological scores for thrombin and trypsin were not reflected in the histological score.

3.4.2 Analysis of Inflammatory Mediators

3.4.2.1 MPO Activity

MPO activity is representative of an inflammatory response typically associated with polymorphonuclear leukocyte infiltration into tissues and was measured to determine a quantitative measure of relative tissue infiltration by activated neutrophils. Fractions of tissue homogenates were sonicated and centrifuged to quantify the MPO activity of the samples. *cnf*$^+$ UPEC colonization of the rat prostate after 6 h and 24 h resulted in MPO levels that were significantly higher (57.4 U/mg protein and 1.08×10^3

Figure 3.12: Histological sections of the ventral prostate from Sprague-Dawley rats. H&E stained sections from rats administered **(A)** saline, **(B)** 200 U thrombin, **(C)** 200 U trypsin, **(D)** 100 μM TFLLR-NH_2, **(E)** 100 μM SLIGRL-NH_2 or **(F)** 1.0×10^6 CFU/ml *E.coli* CP9 *cnf*+ via catheter. Rats were sacrificed after 24 h and the ventral prostates were removed, fixed in 10% formalin, sectioned and stained. Arrow indicated neutrophil infiltration into the prostatic ducts. 20x magnification.

Figure 3.12: Histological sections of ventral prostate sections from Sprague-Dawley rats.

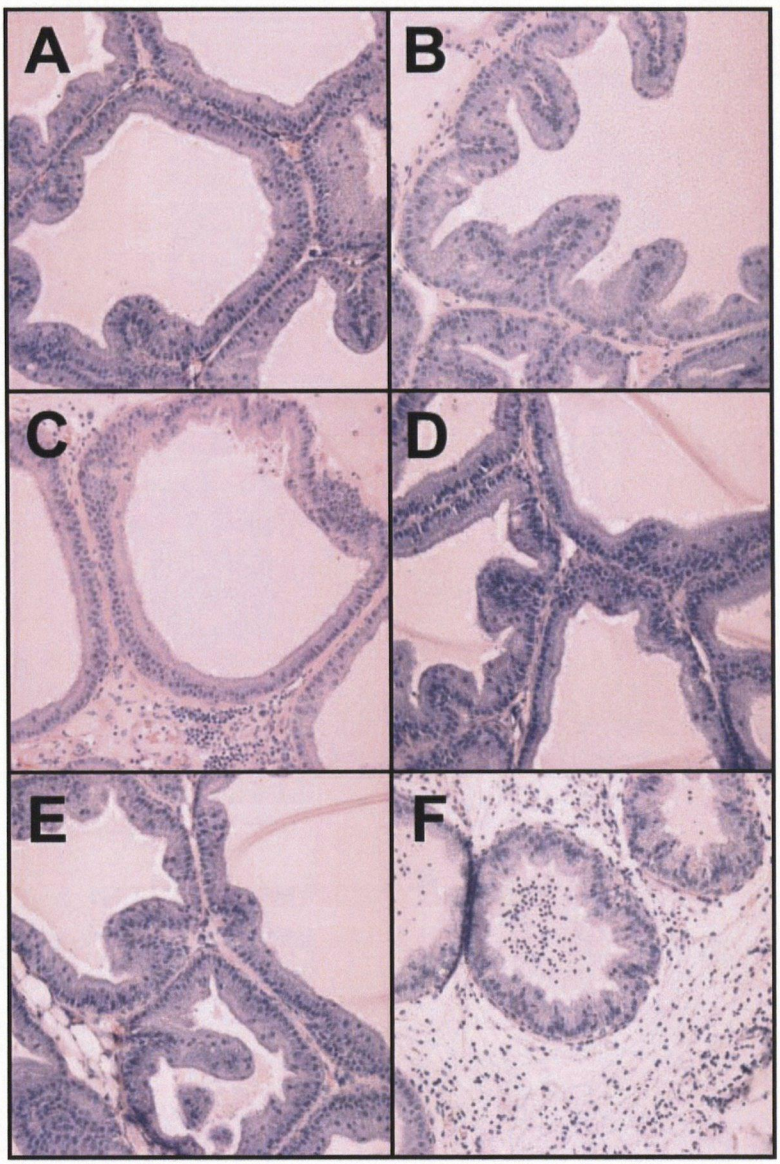
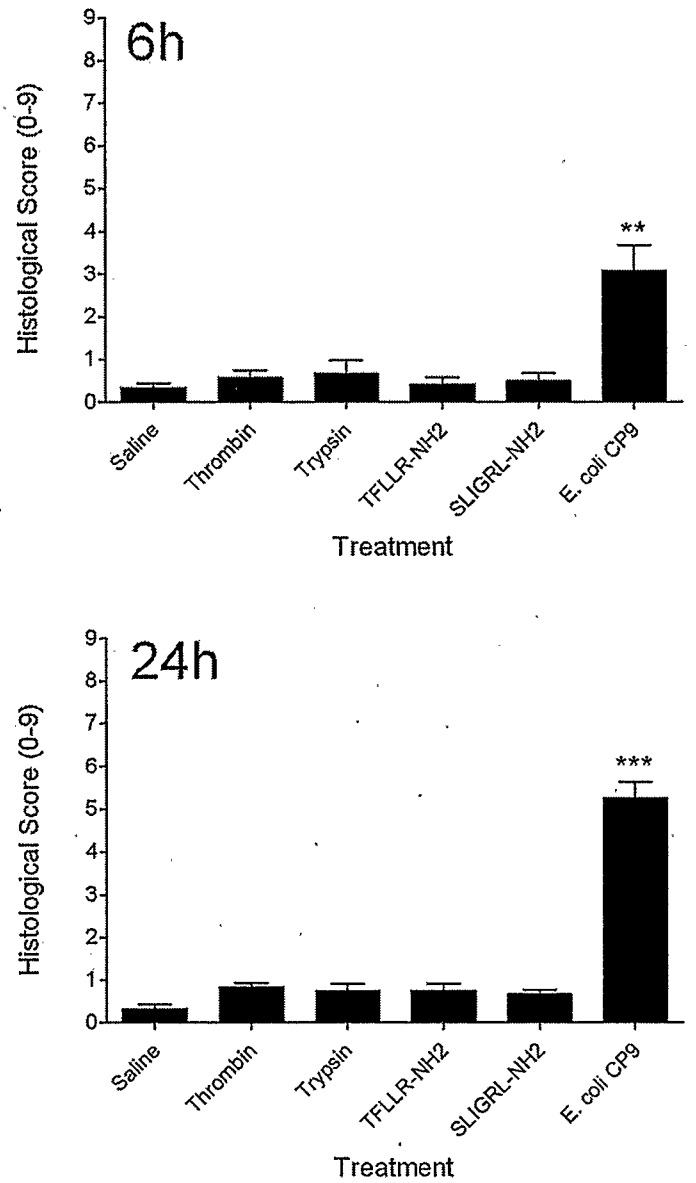


Figure 3.13: Histological scores of the ventral prostate of Sprague-Dawley rats instilled with control or PAR agonist solutions. Male rats were anesthetised and instilled with 0.2 ml of one of the following solutions in the ventral prostate via catheter: saline (vehicle solution), 200 U thrombin, 200 U trypsin, 100 μM TFLLR-NH_2, 100 μM SLIGRL-NH_2 or 1.0×10^6 CFU/ml *E.coli* CP9 *cnf*+ (n=6). Rats were sacrificed at 6 h or 24 h, as indicated, and the prostate glands were harvested, H&E stained and histologically scored for oedema (0-3), haemorrhage (0-3) and leukocyte infiltration (0-3). The scores were totalled to obtain a cumulative score (0-9). Scores were analysed by the Kruskal-Wallis ANOVA on ranks test and Dunn's multiple comparison test of groups. ** ($p<0.01$) and *** ($p<0.001$) denotes groups that were significantly different from the negative control (saline).

Figure 3.13: Histological scores of rat prostates instilled with control or PAR agonist solutions.



U/mg protein, respectively) than rats in the other test groups, including the saline control (7.0 U/mg protein) (Figure 3.14). The animals in the 24 h group exhibited a significant increase in MPO activity. None of the PAR-1 or PAR-2 activating proteinases or peptides induced a similar response and these groups were not statistically different from rats instilled with saline.

3.4.2.2 Pro-inflammatory Cytokine Expression

IL-1β and TNF-α are important cytokines secreted by activated macrophages and endothelial cells that function to regulate inflammatory responses through NO production, leukocyte recruitment and the acute phase response. Concentrations of these cytokines were measured by commercially available ELISA kits and were normalized to the protein concentration of each sample. IL-1β levels from rats infected with *cnf*$^+$ UPEC were significantly higher than saline at 6 h and 24 hours and were higher than the levels of all other groups (Figure 3.15). The 6 h *cnf*$^+$ UPEC treated animals had a mean IL-1β concentration of 18.3 pg/mg protein and the 6 h saline treated animals was 0.6 pg/mg protein. Thrombin (0.8 pg/mg protein), trypsin (5.3 pg/mg protein), TF-NH_2 (2.7 pg/mg protein) and SL-NH_2 (0.8 pg/mg protein) did not have IL-1β levels that were significantly different from the saline control animals. After 24 h the IL-1β concentration in the *cnf*$^+$ UPEC treated animals was 61.9 pg/mg protein and was significantly higher than animals in the 6 h group. Thrombin (8.2 pg/mg protein) and TF-NH_2 (10.1 pg/mg protein) 24 h groups had higher IL-1β concentrations than saline (0.1 pg/mg protein) but this was also not significant.

Figure 3.14: MPO activity in the ventral prostate of Sprague-Dawley rats instilled with control or PAR agonist solutions. Male rats were anesthetised and instilled with 0.2 ml of one of the following solutions in the ventral prostate via catheter: saline (vehicle solution), 200 U thrombin, 200 U trypsin, 100 μM TFLLR-NH_2, 100 μM SLIGRL-NH_2 or 1.0×10^6 CFU/ml *E.coli* CP9 *cnf*+ (n=6). Rats were sacrificed at 6 h or 24 h, as indicated, and the prostate glands were harvested, homogenized and analyzed for MPO activity. MPO activity was normalized to the amount of protein in each sample measured by the Bradford assay. Data was transformed and analysed by one-way ANOVA and Tukey's multiple comparison test. ** ($p<0.01$) and *** ($p<0.001$) denotes groups that were significantly different from the negative control (saline).

Figure 3.14: Myeloperoxidase activity in rat prostate tissue instilled with control or PAR agonist solutions.

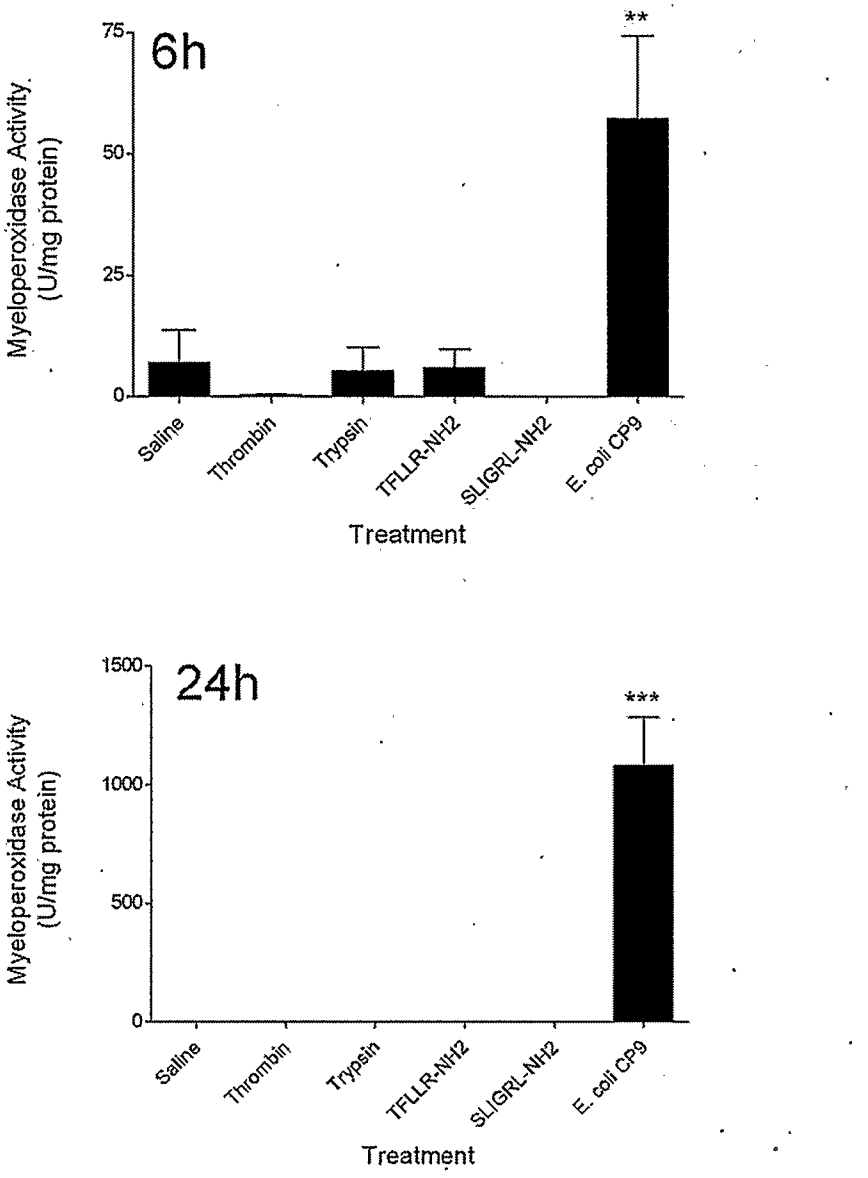
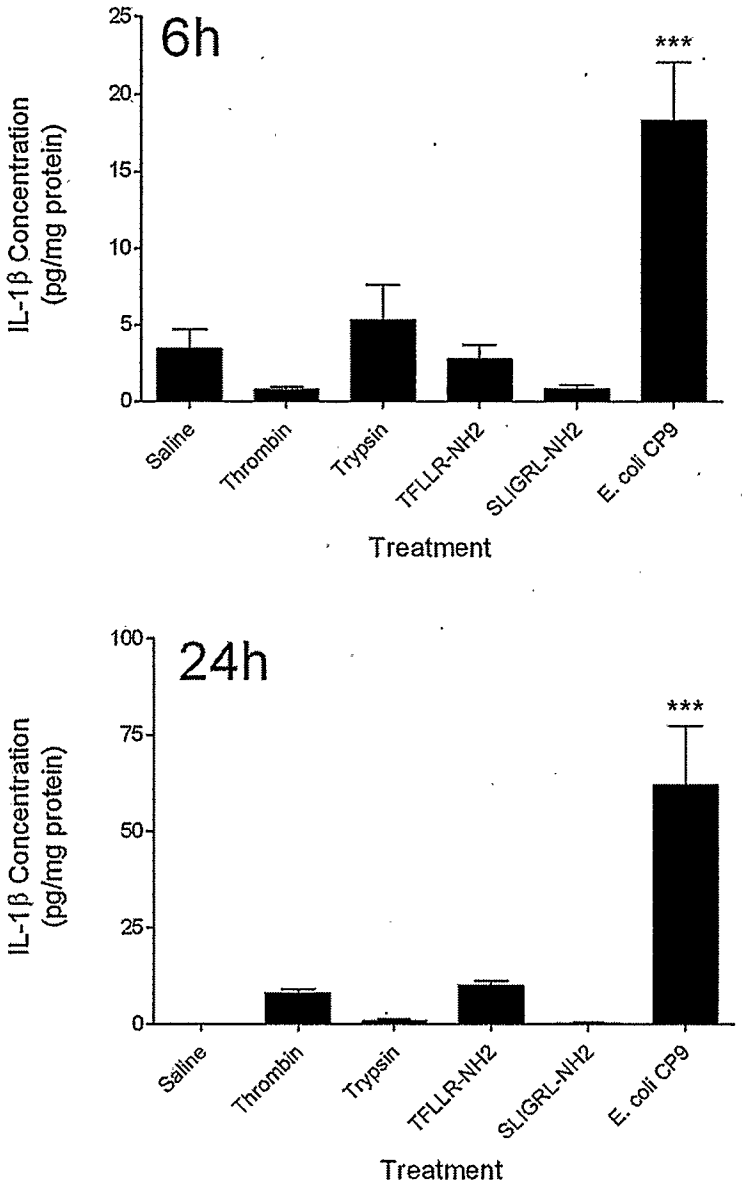


Figure 3.15: IL-1β concentration in the ventral prostate of Sprague-Dawley rats instilled with control or PAR agonist solutions. Male rats were anesthetised and instilled with 0.2 ml of one of the following solutions in the ventral prostate via catheter: saline (vehicle solution), 200 U thrombin, 200 U trypsin, 100 μM TFLLR-NH_2, 100 μM SLIGRL-NH_2 or 1.0×10^6 CFU/ml *E.coli* CP9 *cnf*+ (n=6). Rats were sacrificed at 6 h or 24 h, as indicated, and the prostate glands were harvested, homogenized and analyzed for IL-1β by ELISA. Cytokine concentration was normalized to the amount of protein in each sample measured by the Bradford assay. Data was transformed and analysed by one-way ANOVA and Tukey's multiple comparison test. *** ($p<0.001$) denotes groups that were significantly different from the negative control (saline).

Figure 3.15: IL-1β concentration in rat prostate tissues instilled with control or PAR agonist solutions.



The TNF-α concentration from the positive control was similar at 6 h and 24 h at 4.8 pg/mg protein and 5.0 pg/mg protein, respectively (Figure 3.16). Thrombin-induced TNF-α levels (2.6 pg/mg protein) were significantly higher than saline (1.3 pg/mg protein), TF-NH_2 (0.8 pg/mg protein) and SL-NH_2 (0.9 pg/mg protein). Thrombin (2.6 pg/mg protein) and trypsin (1.9 pg/mg protein) treated groups did not have significant increases in TNF-α levels. The concentration of TNF-α in UPEC treated animals was significantly higher than any other group after 6 h and 24 h UPEC. After 24 h it was found that TNF-α levels in the trypsin group (3.1 pg/mg protein) was significantly higher than the saline control (0.9 pg/mg protein), thrombin (1.1 pg/mg protein), TF-NH_2 (1.1 pg/mg protein) and SL-NH_2 (0.7 pg/mg protein). Thrombin and the PAR-1 and PAR-2 APs were not statistically different from the saline control.

Levels of Gro/CINC-1 (rat analogue of human IL-8) in the tissue homogenates were also measured in order to obtain a profile of pro-inflammatory cytokines. For the 6 h and 24 h UPEC infected animals; Gro/CINC-1 concentrations were 4.50×10^3 pg/mg protein and 8.39×10^3 pg/mg tissue, respectively (Figure 3.17). These values were significantly higher than the saline control (48.0 pg/mg tissue (6 h) and 58.0 pg/mg tissue (24 h)) and the PAR-1 and PAR-2 agonist treatment groups, which had very low concentrations of Gro/CINC-1. The Gro/CINC-1 cytokine concentrations from the saline control, the test groups and reverse peptides were negligible.

IL-10 was also measured to analyze a key anti-inflammatory cytokine which may be expressed under certain conditions. PAR-1 and PAR-2 activation in this model could be anti-inflammatory in nature, so it was important to look at the levels of IL-10

Figure 3.16: TNF- α concentration in the ventral prostate of Sprague-Dawley rats instilled with control or PAR agonist solutions. Male rats were anesthetised and instilled with 0.2 ml of one of the following solutions in the ventral prostate via catheter: saline (vehicle solution), 200 U thrombin, 200 U trypsin, 100 μM TFLLR-NH_2, 100 μM SLIGRL-NH_2 or 1.0×10^6 CFU/ml *E.coli* CP9 *cnf*+ (n=6). Rats were sacrificed at 6 h or 24 h, as indicated, and the prostate glands were harvested, homogenized and analyzed for TNF-α by ELISA. Cytokine concentration was normalized to the amount of protein in each sample measured by the Bradford assay. Data was transformed and analysed by one-way ANOVA and Tukey's multiple comparison test. * ($p<0.05$) and *** ($p<0.001$) denotes groups that were significantly different from the negative control (saline).

Figure 3.16: TNF-α concentration in rat prostate tissues instilled with control or PAR agonist solutions.

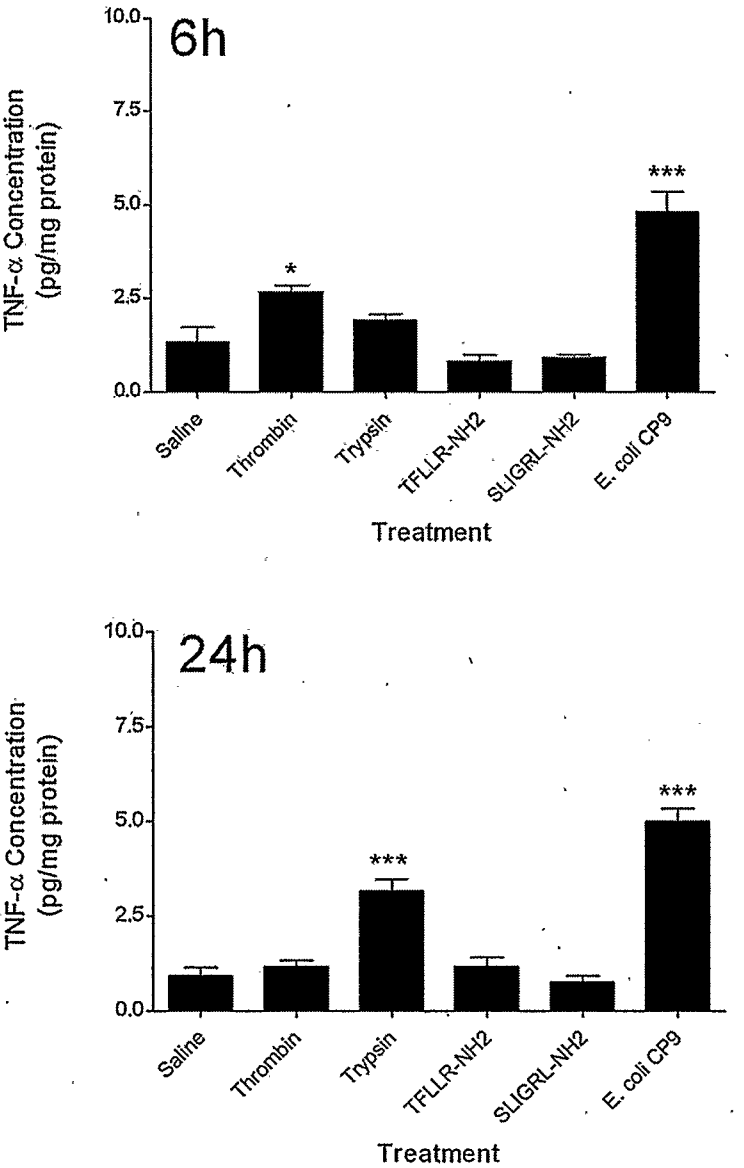


Figure 3.17: Gro/CINC-1 concentration in the ventral prostate of Sprague-Dawley rats instilled with control or PAR agonist solutions. Male rats were anesthetised and instilled with 0.2 ml of one of the following solutions in the ventral prostate via catheter: saline (vehicle solution), 200 U thrombin, 200 U trypsin, 100 μM TFLLR-NH_2, 100 μM SLIGRL-NH_2 or 1.0×10^6 CFU/ml *E.coli* CP9 *cnf*+ (n=6). Rats were sacrificed at 6 h or 24 h, as indicated, and the prostate glands were harvested, homogenized and analyzed for Gro/CINC-1 by ELISA. Cytokine concentration was normalized to the amount of protein in each sample measured by the Bradford assay. Data was transformed and analysed by one-way ANOVA and Tukey's multiple comparison test. *** ($p<0.001$) denotes groups that were significantly different from the negative control (saline).

Figure 3.17: Gro/CINC-1 concentration in rat prostate tissues instilled with control or PAR agonist solutions.

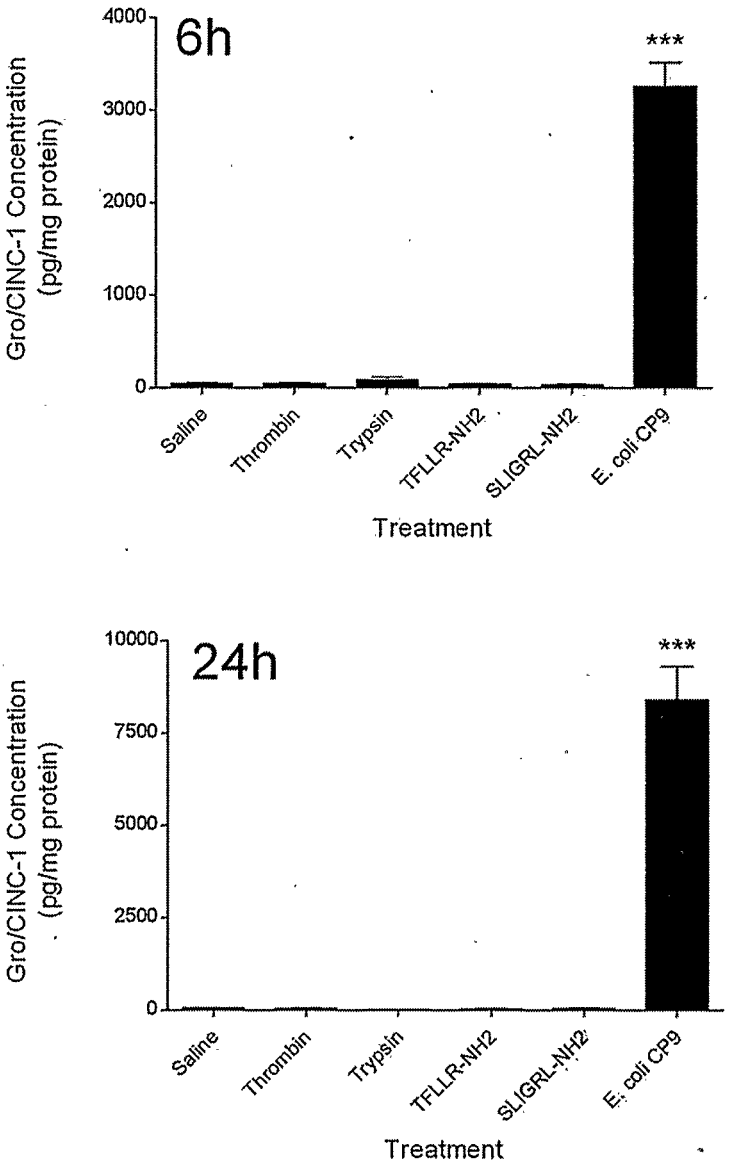
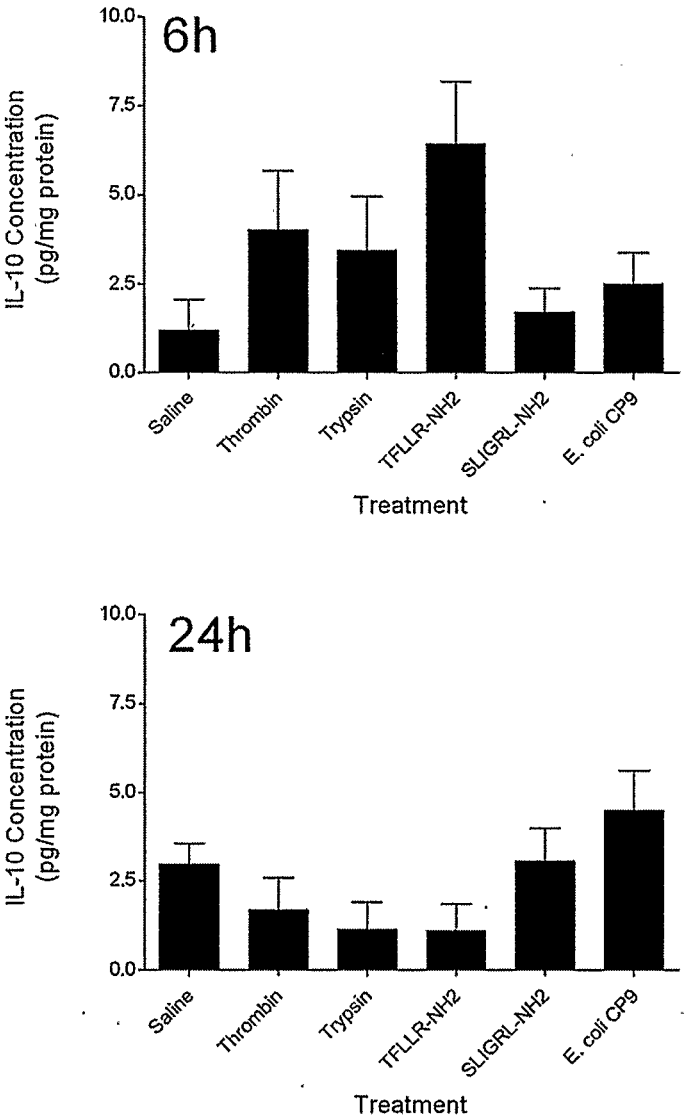


Figure 3.18: IL-10 concentration in the ventral prostate of Sprague-Dawley rats instilled with control or PAR agonist solutions. Male rats were anesthetised and instilled with 0.2 ml of one of the following solutions in the ventral prostate via catheter: saline (vehicle solution), 200 U thrombin, 200 U trypsin, 100 μM TFLLR-NH_2, 100 μM SLIGRL-NH_2 or 1.0×10^6 CFU/ml *E.coli* CP9 *cnf*+ (n=6). Rats were sacrificed at 6 h or 24 h, as indicated, and the prostate glands were harvested, homogenized and analyzed for IL-10 by ELISA. Cytokine concentration was normalized to the amount of protein in each sample measured by the Bradford assay. Data was transformed and analysed by one-way ANOVA and Tukey's multiple comparison test.

Figure 3.18: IL-10 concentration in rat prostate tissues instilled with control or PAR agonist solutions.



expression in the tissues. All treatment groups showed similar concentrations of IL-10 at both 6 h and 24 h (Figure 3.18). Variability in expression between the groups of animals sacrificed was insignificant and the IL-10 levels from animals sacrificed at 6 h or 24 h were not statistically significant.

3.4.3 Disruption of the Mucosal Barrier

In some models of colitis and prostatitis the normal mucosal barrier is disrupted with ethanol to remove the mucosal lining in the tissue (Lang *et al.*, 2000). Then the effects of chemical irritants were observed in the normal and disrupted tissues and it was found that mucosal integrity was important during inflammatory processes. In this model of PAR activation in the rat prostate, the mucosal lining of the prostatic ducts could be a barrier to PAR-1 and PAR-2 agonists so 50% ethanol was used to disrupt the mucosal surfaces in the prostate.

Animals were instilled with a vehicle solution of 50% ethanol to reduce the mucosal integrity or PAR-1 and PAR-2 APs in a 50% ethanol solution. The test groups were compared to saline and *cnf*$^+$ UPEC treated control groups. Upon examination of the gross morphology of the test groups, only the SL-NH_2 + 50% ethanol group was significantly higher than both saline and ethanol (Figure 3.19). The histology revealed no significant differences in any of the PAR AP instilled groups from the saline or ethanol groups. Ethanol as a vehicle solution did not lead to abnormal histopathology when compared to saline. MPO activity was also evaluated and it was determined that there was an absence of significant activity in any of the peptide treated groups (Figure 3.20A). All test groups were not statistically different from saline and UPEC treated rats had

Figure 3.19: Morphological and histological scores of the ventral prostate of Sprague-Dawley rats instilled with control or PAR agonist solutions in 50% ethanol solutions. Male rats were anesthetised and instilled with 0.2 ml of one of the following solutions in the ventral prostate via catheter: saline, 50% ethanol, 50% ethanol + TF-NH_2, 50% ethanol + SL-NH_2 or 1.0×10^6 CFU/ml *E.coli* CP9 *cnf*+ (n=6). Rats were sacrificed after 6 h and the prostate glands were harvested and **(A)** scored for size (0-3), hyperemia (0-3) and congestion (0-3). The scores were totalled to obtain a cumulative gross morphology score (0-9). **(B)** The prostate glands were harvested, H&E stained and histologically scored for oedema (0-3), haemorrhage (0-3) and leukocyte infiltration (0-3). The scores were totalled to obtain a cumulative score (0-9). Scores were analysed by the Kruskal-Wallis ANOVA on ranks test and Dunn's multiple comparison test of groups. * ($p<0.05$) and ** ($p<0.01$) denotes groups that were significantly different from the 50% ethanol group.

Figure 3.19: Gross morphological and histological scores of rat prostates instilled with control or PAR agonist solutions in 50% ethanol.

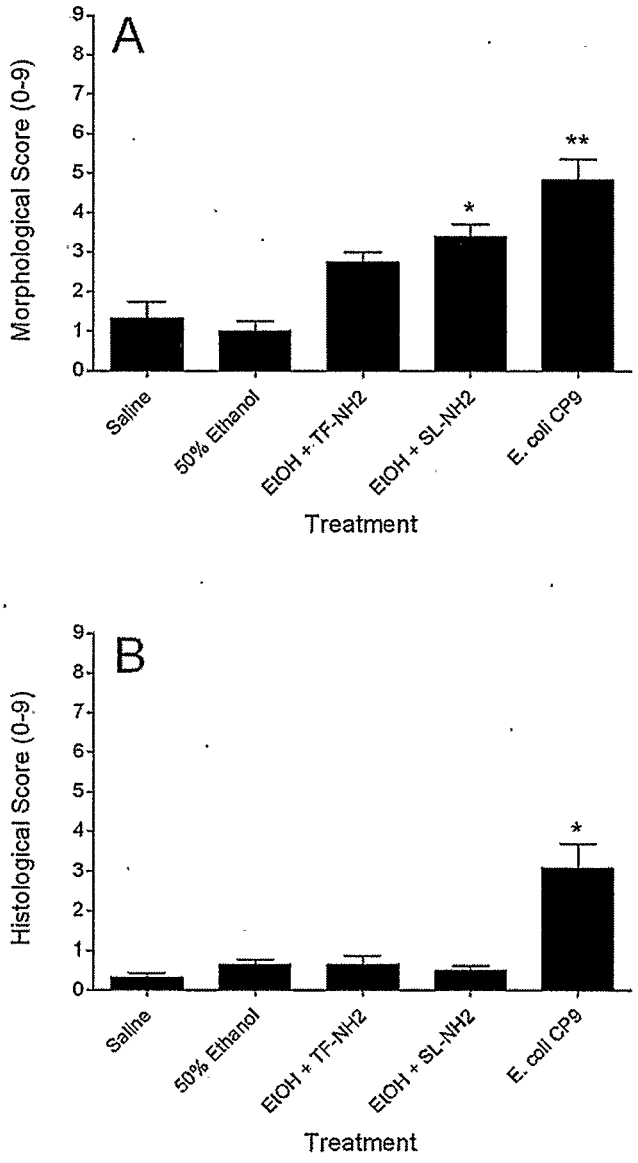
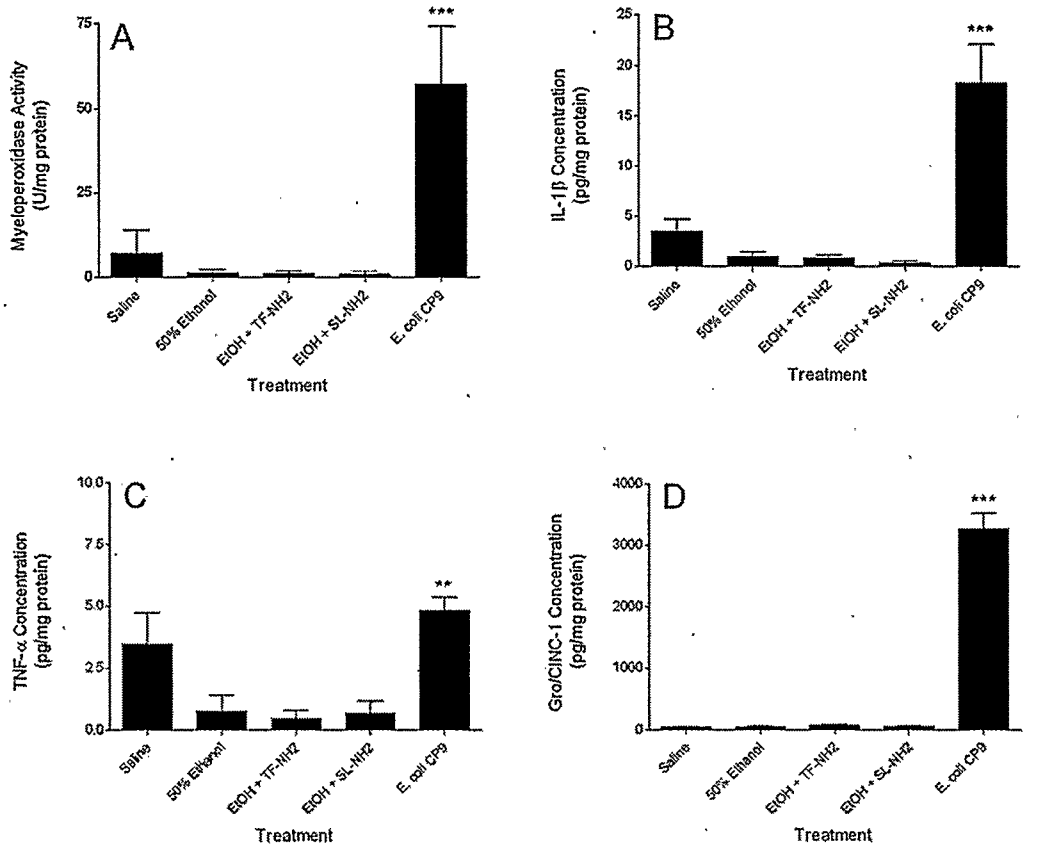


Figure 3.20: Concentrations of MPO activity, IL-1β, TNF-α and Gro/CINC-1 in the ventral prostate of Sprague-Dawley rats instilled with control or PAR agonist solutions in 50% ethanol solutions. Male rats were anesthetised and instilled with 0.2 ml of one of the following solutions in the ventral prostate via catheter: saline, 50% ethanol, 50% ethanol + TF-NH_2, 50% ethanol + SL-NH_2 or 1.0×10^6 CFU/ml *E.coli* CP9 *cnf*+ (n=6). Rats were sacrificed after 6 h and the prostate glands were harvested, homogenized and analyzed for **(A)** MPO activity, **(B)** IL-1β, **(C)** TNF-α and **(D)** Gro/CINC-1 concentration. The activity or concentrations of inflammatory mediators were normalized to the amount of protein in each sample measured by the Bradford assay. ** denotes groups that are significantly different ($p<0.01$) from the saline group. Data was transformed and analysed by one-way ANOVA and Tukey's multiple comparison test. ** ($p<0.01$) and *** ($p<0.001$) denotes groups that were significantly different from the 50% ethanol group.

Figure 3.20: Concentrations of MPO activity and inflammatory cytokines in rat prostate tissues instilled with control or PAR agonist solutions in 50% ethanol.



dramatically higher levels of MPO activity. Cytokine concentrations of IL-1β (Figure 3.20B), TNF-α (Figure 3.20C) and Gro/CINC-1 (Figure 3.20D) were measured in the tissues and were compared to *cnf*$^+$ UPEC treated animals and like MPO, the peptide treated animals were not statistically different from saline or ethanol treated animals. IL-10 was also evaluated for the presence of an anti-inflammatory cytokine in the prostate under these test conditions (Figure 3.21). Levels of IL-10 did not increase when compared to saline and ethanol. When the ethanol + PAR AP groups were compared to groups administered the peptide agonists without ethanol, they were not higher, but in some cases lower.

3.4.4 *In situ* Fluorescence Zymography

One theory put forth in this study was that PARs may be activated, leading to prolonged or exacerbated inflammatory responses, by increases in endogenous proteinases in response to a bacterial stimulus. To test this hypothesis, tissue sections were compared for thrombin- or trypsin-like proteinase activity from rats (n=6) infected with 1.0×10^6 CFU/ml *E. coli* CP9 *cnf*$^+$ or a negative control group instilled with saline. The rats were sacrificed 24 h after instillation of the solutions and the mean fluorescence in the tissue sections was representative of the amount of cleaved thrombin or trypsin substrate (Boc-QAR-7-AMC). The tissue sections were incubated with the substrate and intensity of the fluorescent AMC product was photographed and evaluated with Image-J software (Figure 3.22). The mean fluorescence of the positive control sections (incubated with AMC) and the assay negative control sections (lacking substrate) were significantly different from each other. The tissue sections from both the rats infected with *cnf*$^+$ UPEC

Figure 3.21: IL-10 concentration in the ventral prostate of Sprague-Dawley rats instilled with control or PAR agonist solutions in 50% ethanol solutions. Male rats were anesthetised and instilled with 0.2 ml of one of the following solutions in the ventral prostate via catheter: saline, 50% ethanol, 50% ethanol + TF-NH_2, 50% ethanol + SL-NH_2 or 1.0×10^6 CFU/ml *E.coli* CP9 *cnf*+ (n=6). Rats were sacrificed after 6 h and the prostate glands were harvested, homogenized and analyzed for IL-10 by ELISA. Cytokine concentration was normalized to the amount of protein in each sample measured by the Bradford assay. Data was transformed and analysed by one-way ANOVA and Tukey's multiple comparison test.

Figure 3.21: IL-10 concentration in rat prostate tissues instilled with control or PAR agonist solutions in 50% ethanol.

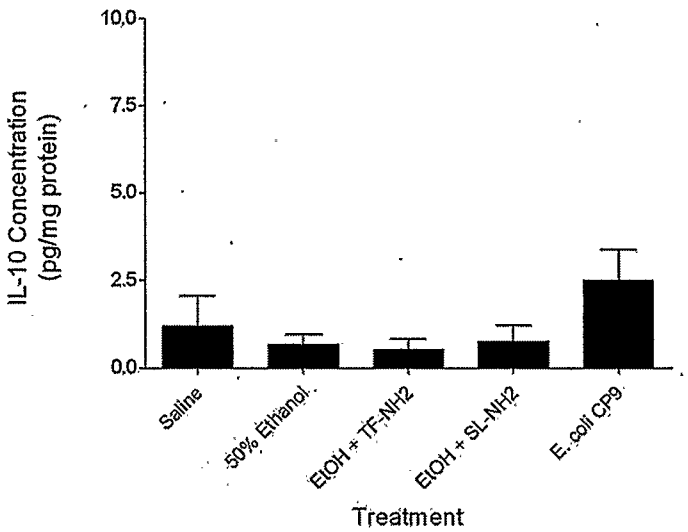
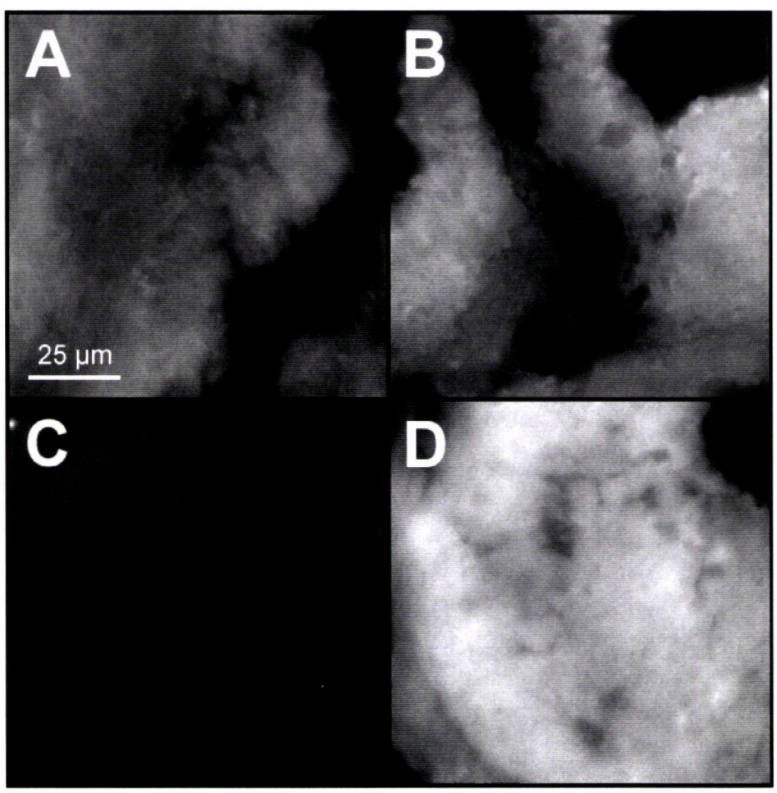


Figure 3.22: *In situ* fluorescence zymography of rat ventral prostate sections from Sprague-Dawley rats instilled with *E. coli* CP9 *cnf*$^+$. Prostate tissue sections were embedded in OCT, sectioned and incubated with the substrate Boc-QAR-7-amido-4-methylcoumarin. The slides were observed using a fluorescence microscope exciting at a wavelength of 380 nm and measuring the emission with a wavelength of 460 nm. Fluorescence, represented by lighter colour, was indicative of trypsin- or thrombin-like activity in the tissue preparations: **(A)** 24 hour PBS treated control, **(B)** 24 hour *E. coli* CP9 *cnf*$^+$ treated animal, **(C)** tissue without substrate (negative control) and **(D)** tissue incubated with unbound AMC (positive control).

Figure 3.22: *In situ* fluorescence zymography of rat prostate sections from Sprague-Dawley rats instilled with *E. coli* CP9 *cnf*$^+$.



and instilled with saline had mean fluorescence values that were not statistically different from each other and they were both higher than the assay negative control indicating that there is endogenous thrombin- and trypsin like proteinase activity in the rat prostate.

3.5 Bacterial Proteinases

A uropathogenic strain of *E. coli* CFT073 was studied for the ability to activate or inactivate PAR receptors expressed on the surface of human prostate epithelial cells RWPE-1. Bacterial culture supernatants of *E. coli* CFT073 which express the Sat protein have been previously shown to induce cytopathic activity in the human bladder cells and prostatitis in a rat model of inflammation (Guyer *et al.*, 2002). Since Sat has sequence homology to serine-proteinases of the *Enterobacteriaceae*, it was proposed that virulence may in part be mediated by PAR activation. To test this theory, two strains were obtained; one was phenotypically wild type and expressed Sat and the other was an isogenic mutant which had been shown not to express the Sat protein. The bacteria were cultured and the supernatants were concentrated following previously described methods (Guyer *et al.*, 2002).

The concentrated supernatants from LB, lyophilized LB and lyophilized VMM were collected for each strain and for sterile media that had not been inoculated. The supernatants were diluted 100-fold and used in a fluorometric trypsin-like activity assay which measures the rate of substrate cleavage. When all supernatants were tested for the ability to cleave the Boc-QAR-AMC substrate, there was no change in fluorescence indicating a lack of trypsin-like proteinase activity (data not shown).

The supernatants (100 ng/ml) were assayed for the ability to cleave a succinylated casein substrate which would correlate to general proteinase activity when compared to the negative and positive controls. None of the bacterial supernatants were able to yield proteinase activity that differed from the assay negative control or the sterile media control (data not shown). Collectively from these two different proteinase assays it appeared that the supernatants from *E. coli* CFT073 *sat*$^{+}$ did not possess proteinase activity under these conditions. Since the RWPE-1 calcium bioassay had proved to be a reliable system to measure PAR-1 and PAR-2 agonist responses, it was worthwhile to determine if these concentrated supernatants had measurable effects on these cells.

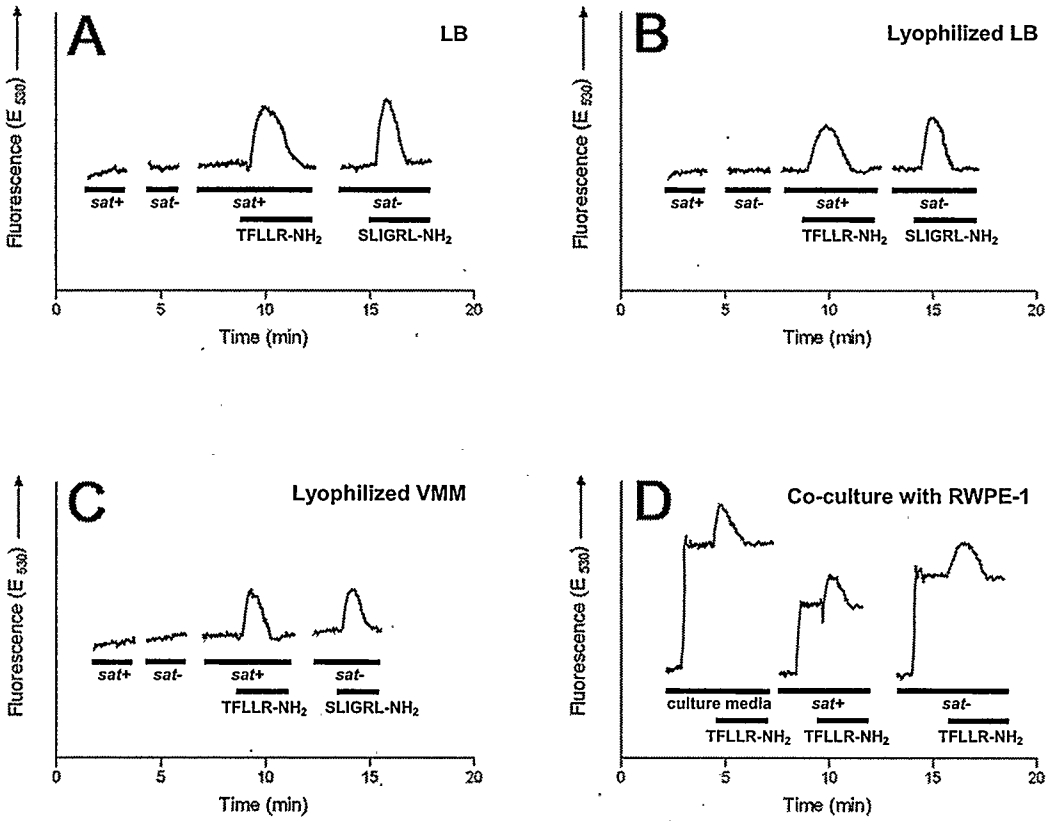
The supernatants were added to RWPE-1 cells in a calcium signalling bioassay (described in section 3.2.1) to observe direct effects on the ability to mobilize $[Ca^{2+}]_i$ within the cells (Figure 3.23). LB and lyophilized LB supernatants (100 ng/ml) were added to the cells and there were no observed increases in $[Ca^{2+}]_i$ (Figures 3.23A and 3.23B). To determine if the supernatants inactivated PAR-1 or PAR-2, the supernatant fractions from the wild type stain and the isogenic mutant were added to the cells and then the cells were challenged with TF-NH_2 and SL-NH_2 (50 μM). The RWPE-1 cells remained responsive to the PAR agonists and increases in fluorescence were similar to previously established levels. The bacteria were also grown in VMM to reduce the amount of protein that was not of bacterial origin. RWPE-1 cells were exposed to approximately 10.0 ng/ml of this solution; however the cells remained unresponsive to this challenge (Figure 2.23C). As with the LB supernatants, the minimal salts supernatant did not appear to desensitize PAR-1 and PAR-2 when subsequently challenged with PAR

APs and proteinases. The supernatants from media that had not been inoculated (negative controls) also did not yield a calcium signal in this assay system.

The bacteria may not secrete large amounts of the Sat protein under normal laboratory growth conditions, so it was decided to try to grow them in co-culture with RWPE-1 cells and then assay the centrifuged supernatants for the ability to mobilize calcium in RWPE-1. Cells were incubated with the wild type strain, the isogenic mutant and no bacteria at all. Once the co-culture media was collected after 24 h it was tested in the calcium bioassay. Different volumes (2.0 µl to 20.0 µl) of culture media were added to the cells in the calcium assay and a large spike in fluorescence was immediately observed for every test sample for both of the negative controls (Figure 3.23D). The increase resulted in higher baseline fluorescence and was most likely due to calcium or some other substance that influenced the fluorescence of the sample. The cells were still responsive to subsequent PAR AP challenge (data for PAR-1 shown) indicating that PAR-1 and PAR-2 were not desensitized.

Figure 3.23: The effect of *E. coli* CFT073 *sat*$^{+}$ and CFT073 *sat*$^{-}$ culture supernatants on $\Delta[Ca^{2+}]_i$ in human prostate epithelial cells. RWPE-1 cells were loaded with Fluo-3 AM calcium indicator and exposed to bacterial culture supernatants. Changes in $[Ca^{2+}]_i$ were measured over time by an excitation wavelength of 480 nm and measuring emission at 530 nm. Each unbroken line indicates a sample of cells that had not been previously exposed to a stimulus. RWPE-1 cells were exposed to the following culture supernatants from *E. coli* CFT073 *(sat*$^{+}$*)*, the isogenic mutant *E. coli* CFT073 (*sat*$^{-}$) and untreated culture media. PAR-1 and PAR-2 peptide agonists were added to following exposure to supernatants as indicated **(A)** filtered supernatant from bacteria grown in LB, **(B)** filtered and lyophilized supernatant from bacteria grown in LB, **(C)** filtered and lyophilized supernatant from bacteria grown in VMM, **(D)** culture media from bacteria grown in co-culture with RWPE-1 cells.

Figure 3.23: Changes in $[Ca^{2+}]_i$ in RWPE-1 cells following exposure to *E. coli* CFT073 *sat*$^+$ and CFT073 *saf* culture supernatants.



CHAPTER FOUR: DISCUSSION

The aetiology of non-bacterial prostatitis is an enigma that has yet to be fully comprehended. Efforts have been made to understand the mechanisms involved in prostatic inflammation and to determine the factors which may trigger an initial inflammatory response. Still, a majority of the current prostatitis research focuses on clinical studies that concentrate on alleviating the symptoms associated with prostatitis without exploring the underlying causes of the disease. To fully understand what courses of action may be taken to treat or to cure the disease, it is critical to answer questions regarding the triggers of the inflammatory reaction. In the past decade, it has become clear that PARs have a function in many biological systems whether it may be in a homeostatic, pathological or protective role (Ossovskaya and Bunnett, 2004). It has been demonstrated that PARs are expressed in many cell types and tissues, including exposed surfaces such as in the airway and intestinal mucosa. The prostate is a gland that contains a large mucosal surface area in the form of prostatic ducts that are lined by mucus-secreting epithelium. Given that PARs are expressed on other mucosal surfaces and are involved in inflammatory pathways, these unique receptors may also play a similar role in the prostate gland.

When this research was initiated, PARs had only been detected in a few prostate cancer cell lines (Chay *et al.*, 2002; Huang *et al.*, 2000). The body of literature on PAR expression and cellular effects following PAR activation in prostate cells or tissues was minimal, and what studies that had been done focused on PAR-mediated effects on prostate tumour cell growth and proliferation (Wilson *et al.*, 2004). Studying PARs and

prostatic inflammation was of great interest because no research had been previously undertaken that looked at PAR expression and function in normal prostate cells and tissues, in either murine or human systems. With the prostate tissue exhibiting some of the greatest diversity and quantity in terms of proteinase and peptidase activity (Lwaleed *et al.*, 2004), it seemed logical that PARs could potentially play a role in the normal or pathological physiology of the prostate. High proteinase and zinc concentrations in the prostate are necessary components for the innate immunity of the prostate since the prostate is an immune privileged tissue (Quayle *et al.*, 1987). Considering the aetiologies of non-bacterial prostatitis are largely unknown, it was hypothesized that PARs may be involved in mediating and triggering inflammatory responses in the prostate gland. Further, secreted virulence factors from uropathogenic bacteria include proteinases which could also activate PARs to trigger an inflammatory response. To answer these questions, it was important to examine PAR expression in normal human prostate cells and to use an animal model to study the effects of PAR activation in a complex *in vivo* system.

4.1 PAR Expression and Activation in Prostate-Derived Cells

The objective of this research was to analyze PAR expression and activation in normal prostate tissue which is why the RWPE-1 cell line was chosen for the *in vitro* experiments. The RWPE-1 cell line is an immortalized human papilloma virus 18 transfected epithelial line derived from the peripheral zone of a histologically normal adult human prostate, the only cell line available that was not tumourigenic or hyperplasic (Bello *et al.*, 1997). RT-PCR of RWPE-1 mRNA showed that PAR-1 and PAR-2 genes were expressed by these cells under normal growth conditions. This finding

was based on the presence of appropriate product appearing on gels primed with human specific PAR-1 and PAR-2 primers and from sequencing the derived bands to demonstrate they did in fact represent the expected products. These findings are similar to PAR expression in prostate-derived tumour cell lines. For example, PAR-1 and PAR-2 have been previously detected in prostatic tumour cell lines PC-3, DU-145 and androgen-dependent LNCaP cells (Cottrell *et al.*, 2004; Greenberg *et al.*, 2003; Huang *et al.*, 2000). LNCaP cells were also shown to express PAR-4 but not PAR-3 while none of the other cell lines expressed PAR-3 or PAR-4 (Greenberg *et al.*, 2003). The expression of only PAR-1 and PAR-2 in RWPE-1 cells was consistent with PAR expression found in the PC-3 and DU-145 cell lines.

It has been well established in many cell lines (such as KNRK and HEK cells) (Bohm *et al.*, 1996b; Kawabata *et al.*, 1999) that PAR activation causes calcium to be released from intracellular stores to modulate the functions of phosphatases and kinases. The next objective was to determine if PAR-1 and PAR-2 specific proteinases and selective peptides agonists induced measurable changes in intracellular calcium concentration in RWPE-1 cells. The addition of thrombin resulted in concentration-dependent increases in $[Ca^{2+}]_i$ which was substantially higher than increases observed for trypsin at higher concentrations. Since these proteinases may have other cellular effects not related to PAR activation that could influence calcium levels, it was prudent to use selective PAR-1 or PAR-2 agonists peptides to see if similar results could be obtained. The peptides mimic the tethered ligand sequences of PARs and are able to bind to the receptor without proteolysis of the receptor's amino-terminus. Similar concentrations of

the PAR-1 or PAR-2 APs produced nearly identical increases in $[Ca^{2+}]_i$ for both PAR-1 and PAR-2 receptors in a concentration-dependent manner. Calcium fluxes observed in response to the peptide agonists were strictly due to PAR-1 or PAR-2 activation. This was supported by the observations that none of the reverse peptides induced any change from the baseline and when the cells were desensitized by one peptide agonist the other still produced a signal. It appeared that calcium flux due to thrombin plateaus at a lower level than TF-NH_2, the corresponding PAR-1 AP. This difference suggests that there may be different receptor kinetics involved in proteinase or peptide activation of PAR-1.

Desensitization with trypsin rendered the cells unresponsive to a subsequent thrombin challenge and when thrombin was added to fresh cells, they remained responsive to trypsin. These data are in agreement with evidence that suggests trypsin activates PAR-1 and PAR-2 while thrombin only signals through PAR-1 and not PAR-2. The peptides were not able to induce calcium fluxes following incubation of the cells with their corresponding PAR proteinase agonists, however signalling through PAR-1 and PAR-2 was still achieved regardless if the cells were incubated with one peptide prior to the other. This demonstrates the calcium fluxes observed in the peptide concentration-response experiments are solely due to the independent effects of either PAR-1 or PAR-2 activation. It is clear PAR-1 and PAR-2 are expressed on the surface of RWPE-1 cells and that specific agonists were able to provoke a response in a receptor specific fashion that could be quantitatively measured as an increase in intracellular calcium.

The measurable increases in $[Ca^{2+}]_i$ in RWPE-1 cells after PAR-1 and PAR-2 stimulation suggested IP_3 has been generated, which releases Ca^{2+} from the endoplasmic reticulum. The release of calcium from cytoplasmic stores mediates a wide variety of cellular functions, one being the regulation of kinases and phosphatases involved in the transcription of inflammatory mediators. While it is assumed that other signalling cascades involved in G protein activation had been initiated, the transcriptional end products of inflammatory pathways were examined. Other studies have shown that stimulation of PAR-1 and PAR-2 in airway and retinal epithelial cells results in the expression of pro-inflammatory cytokines IL-6 and IL-8 (Asokananthan *et al.*, 2002a; Scholz *et al.*, 2004). High levels of IL-6 and IL-8 have been found in the EPS of patients with chronic prostatitis, which indicates that abnormal expression of these cytokines may be important for the progression and severity of the disease (Paulis *et al.*, 2003). The question was asked as to whether PAR-1 or PAR-2 induces the release IL-6 and IL-8 in prostatic epithelium, which could promote inflammation in the prostate *in vivo*.

By incubating RWPE-1 cells with different concentrations of PAR-1 or PAR-2 proteinases and selective peptide agonists, the production of IL-6 and IL-8 was measured in the cell culture medium. High concentrations of thrombin and TF-NH_2 caused IL-6 to be expressed at higher concentrations in the culture media than in untreated cells. This suggests that PAR-1 activation under these conditions results in IL-6 production. Since the PAR-1 agonist TF-NH_2 induced IL-6, similar to the effect of thrombin, it could be assumed thrombin stimulated IL-6 release is mediated by PAR-1. Interestingly, thrombin did not stimulate a significantly higher increase in IL-8 but TF-NH_2 did in a

concentration-dependent fashion. Knowing that the reverse peptide, RL-NH_2 did not initiate IL-8 production, the effects of TF-NH_2 must be mediated by PAR-1. Thrombin could have other effects on the cells, not mediated by PAR-1, which may suppress increased IL-8 expression even if PAR-1 was activated. Activation of PAR-2 using trypsin and SL-NH_2 did not result in significant IL-6 or IL-8 expression from RWPE-1 cells which could indicate PAR-2 plays a separate role from PAR-1 in the prostate. Interestingly, PAR-2 peptides were the most potent causing the release of IL-6 and IL-8 in lung epithelial cells (Asokananthan *et al.*, 2002a) which emphasizes how PARs could have variable effects in different tissues. In studies of TNBS colonic inflammation, IL-10 expression following TNBS exposure with SL-NH_2 was anti-inflammatory but did not alter baseline IL-10 expression (Fiorucci *et al.*, 2001).

Collectively these data indicate that PAR-1 activation leads to the expression and release of IL-6 from RWPE-1 cells and IL-8 after exposure to proteinases and selective PAR agonists. Thrombin may have non-PAR-1 mediated effects in RWPE-1 cells that inhibit increased expression of IL-8. PAR-2 activation does not appear to have a pro-inflammatory effect in terms of IL-6 and IL-8 release from RWPE-1 cells. Epithelial cells are sources for many different chemokines and cytokines, and therefore IL-10 was screened to test for anti-inflammatory activity. IL-10 levels in the medium were found to be negligible under all test conditions and similar to the saline control.

Working with an *in vitro* model system has the distinct advantage in comparison to *in vivo* models because the variables and conditions of an experiment may be tightly controlled. The experimental conditions may be easily repeated and manipulated to test a

variety of scenarios. The disadvantage is that it does not accurately represent a complex system as found in humans and animal models of disease. There are many other endogenous factors that come into play in a complex system that may affect the outcome of the experiment. This is why it was necessary to study PAR activation in an animal model so that the global effects of PARs in the prostate could be assessed.

4.2 PAR Expression and Activation in an Animal Model

An animal model of prostatitis using Sprague-Dawley rats has been previously used to successfully study acute bacterial infection and the disruption of the mucosal barrier in the prostate (Ceri *et al.*, 1999b; Lang *et al.*, 2000; Rippere-Lampe *et al.*, 2001a). It has been demonstrated that the mucosal lining of the prostate is important for protection against chemical irritants (Lang *et al.*, 2000). Using ethanol as a mucosal barrier breaker was required for DNBS to act as an irritant, inducing inflammation. Inflammation was characterized by morphological and histological scoring of prostate tissues in studies using animal models. Quantitative measures of inflammation include measuring MPO activity, which is representative of activation of a number of cells including neutrophils, and their infiltration into tissues, and by measuring pro-inflammatory cytokines. These measures have been used to accurately gauge the relative levels of inflammation in the prostate. To study PAR activation *in vivo*, the model of prostatitis was adapted such that various control and PAR agonist solutions could be instilled into the ventral prostate of rats. Following instillation of these solutions, the tissues could be harvested and examined for changes in the inflammatory condition of the

prostate. An advantage to using this animal model is that the ventral rat prostate is histologically and morphologically similar to the human prostate.

PARs had not yet been examined in the rat prostate and PAR expression had not been established. By RT-PCR using rat specific primers, it was found that like in RWPE-1 cells only PAR-1 and PAR-2 were expressed based on sequencing of the PCR products. PAR-3 and PAR-4 primers did not amplify a PCR product in these tissues. This result was an indication the rat could be a good model system since it expressed the same complement of receptors as found in human prostate cells. PAR-1 and PAR-2 have also been found to be highly expressed in other mucosal surfaces such as the gastrointestinal tract and the airways which would suggest that PAR-1 and -2 may play a critical role in mucosal tissues (Chow *et al.*, 2000; Vergnolle, 2000). PAR-2 immunostaining was localized to the glandular epithelium which suggested that PARs are highly expressed in the lumen of prostatic acini. Proteinases that are secreted by the epithelium into the lumen at high physiological concentrations and PARs may function to sense and respond to proteinase imbalances within the prostate.

To study the effects of PAR activation on inflammation in the rat prostate, PAR proteinases and selective peptide agonists were instilled into the ventral prostate gland. The concentrations of PAR agonists used were similar to concentrations used in studies in the rat colon (Cenac *et al.*, 2002). A positive control group, consisting of *cnf*$^+$ UPEC treated animals, was used to compare inflammatory reactions observed in the test groups of PAR agonist treated animals. This measure was taken so that the relative level of inflammation could be compared to a bacterial strain (*E. coli* CP9 *cnf*$^+$) that had been

previously shown to cause a moderate to severe inflammatory response (Rippere-Lampe *et al.*, 2001a). Having a positive control in the study also ensured that the instillation technique was effective during an experiment.

Rats infected with *cnf*$^+$ UPEC showed significantly higher morphological and histological scores than the saline control group. Blinded scoring of the prostate gland morphology and histology was a non-biased, subjective measure that was used to judge the level of damage in the test animals. Although it could be expected that the scores may vary between studies, it was found that the range of scores observed in this study were comparable to past studies using this particular stain of *cnf*$^+$ UPEC (Rippere-Lampe *et al.*, 2001a). The gross morphological scores indicated that the PAR-1 and PAR-2 agonist treated rats were not significantly different from the saline treated animals, except for at 6 h when thrombin and at 24 h when trypsin resulted in higher scores when compared to their corresponding agonist peptides. At both time points, the corresponding PAR-1 (6 h) and PAR-2 (24 h) agonist peptides did not induce a similar morphological score which indicates that the observed pathology could be due to non-PAR mediated mechanisms. Histologically, the tissue sections from all proteinase and peptide treated animals did not appear to have abnormal pathology when compared to the controls. Multiple sections were taken from different areas of the whole tissue and stained to account for differential localization of inflammation in the tissue, however no differences were observed. The conclusions drawn from the prostate histology and prostate morphology scores were similar, that PAR agonists did not induce an overt inflammatory reaction in the tissue. The lack of oedema in the histological sections correlates with the normal size the

prostate tissue reflected in the morphological score. Yet thrombin (6 h) and trypsin (24 h) instillation resulted in increased tissue size and congestion which was not reflected in the histological scores.

Pro-inflammatory cytokines IL-1β and TNF-α have a synergistic effect during inflammation and are often both up-regulated and released from activated macrophages during bacterial infection. High levels of IL-1β were seen in the positive controls instilled with *cnf*$^+$ UPEC and TNF-α levels were also higher than the saline treated animals. The cytokine profile for IL-1β and TNF-α in animals treated with proteinases or peptides was comparable to the saline controls for both time points with the exception of TNF-α levels for thrombin (6 h) and trypsin (24 h). These observed increases in TNF-α correspond to the results observed in the morphological pathology of the tissue. TF-NH_2 and SL-NH_2 did not result in significant increases parallel to the proteinase agonists, suggesting that this observation could be due to PAR-independent mechanisms. Gro/CINC-1 is a CXC chemokine which belongs to the IL-8 family and acts as a neutrophil chemoattractant (Watanabe *et al.*, 1989). The presence of this chemokine was tested for in the rat tissues since neutrophil infiltration of the prostatic ducts is a hallmark of acute bacterial prostatitis in the animal model (Rippere-Lampe *et al.*, 2001a). Gro/CINC-1 levels were extremely high in the positive control and were insignificant in the saline control and the test groups. The low levels of Gro/CINC-1 correlate with the lack of neutrophils found in these sections when the histology was analyzed.

These data collectively suggest that the instillation of PAR-1 and PAR-2 proteinase and selective peptide agonists into the rat prostate does not initiate an

inflammatory response as indicated by the lack of oedema or granulocyte migration into the prostate gland. In contrast, other studies have shown that mucosal surfaces in the gastrointestinal tract become inflamed when exposed to PAR-1 agonists and exacerbated inflammation in TNBS treated mice (Vergnolle *et al.*, 2004). PAR-2 has also been shown to have a pro-inflammatory role in the gastrointestinal tract by increasing permeability through the activation of myosin light chain kinase and the disruption of barrier permeability (Cenac *et al.*, 2002; Cenac *et al.*, 2003; Chin *et al.*, 2003). PAR-2 activation in C57Bl/6 mice following intracolonic instillation of SL-NH_2 and trypsin resulted in increased macroscopic damage scores, granulocyte infiltration and MPO activity from 4 h to 24 h (Cenac *et al.*, 2002). There is strong evidence that PAR-1 and PAR-2 may contribute to inflammatory bowel diseases by the previously described mechanisms. In this model of PAR agonist instillation into the prostate, the effects of PAR activation did not mirror those witnessed in colonic models of PAR activation and inflammation. It should be considered that PARs may have a physiological role in the prostate that is non-inflammatory as indicated by leukocyte infiltration and inflammatory mediators.

Other studies have shown that PAR-2 activation is anti-inflammatory in the colon and protects against TNBS-induced colitis by the down-regulation of Th1 cytokines (Fiorucci *et al.*, 2001). IL-10 expression was examined during TNBS-induced colitis and did not change upon exposure to SL-NH_2. In this model, levels of IL-10 in the tissue homogenates were measured to ascertain if PAR activation could affect expression of an anti-inflammatory cytokine. There were no increases in IL-10 following PAR-1 or PAR-2 agonist instillation into the prostate. Anti-inflammatory effects may not be mediated

solely by IL-10 so other factors that would protect the tissues may also confer mucosal protection in this system.

PAR activation in the prostate was also scrutinized after disruption of the protective mucosal barrier lining the prostatic epithelia. Ethanol disruption may allow for increased exposure for PAR agonists to the epithelia and a reduction in mucin may yield the prostate more susceptible to the effects of PARs. To test this theory, rats were instilled with TF-NH_2 and SL-NH_2 in a 50% ethanol solution and the same parameters were evaluated as in the previous experiment. Neither the morphological or histological scores changed from the saline control after administration of the ethanol and PAR APs. Signaling molecules (MPO, IL-1β, TNF-α, Gro/CINC-1 and IL-10) involved in inflammation also did not appear to be up-regulated under these conditions. This suggests that disruption of the prostatic mucosa does not render the tissue more prone to inflammation after PAR-1 or PAR-2 activation.

It seems highly unlikely that PAR-1 and PAR-2 do not have a physiological role in the proteinase-rich environment of the prostate. It was speculated that during bacterial infection in acute prostatitis, there may be host proteinases that are up-regulated during a microbial insult. Proteinases such a mast cell tryptase, proteinases of the coagulation cascade or trypsin expressed by epithelial cells (Cottrell *et al.*, 2004) may be increased during infection, perpetuating the inflammatory response by activating PARs. By *in situ* zymography of *cnf*$^+$ UPEC infected and normal tissues is was observed that there were not measurable increases in thrombin- or trypsin-like activity.

4.3 PAR Agonists of Bacterial Origin

Current studies have shown that bacterial and insect proteinases may activate PARs on mucosal surfaces. It has recently been shown that *P. gingivallis*, *Dermatophagoides sp.* and *Blattella germanica* (German cockroach) produce proteinases that are able to initiate PAR-mediated signalling which may contribute to the pathogenesis of these organisms (Hong *et al.*, 2004; Lourbakos *et al.*, 2001; Sun *et al.*, 2001). There is a growing body of evidence that implies PARs may not only be cleaved by host proteinases, but by invading pathogens as well. A UPEC strain (*E. coli* CFT073 sat^+) that was isolated from a patient with urinary tract infection has shown to produce a Type V secreted autotransporter (Sat) protein. This strain (CFT073 sat^+) was of interest in this study for the following reasons: pathogenic organisms cultured from patients with bacterial prostatitis likely colonize the prostate due to urine reflux and secondly, the Sat protein secreted by this organism has sequence homology to serine proteinases of the *Enterobacteriaceae* and could potentially activate PARs.

It has been demonstrated that *E. coli* CFT073 sat^+ instillation into the ventral prostate gland of rats induces acute bacterial prostatitis which was significantly higher than what was produced by a sat^- isogenic mutant strain (Ceri *et al.*, unpublished data). Both supernatants from wild type and isogenic mutant liquid cultures were concentrated following the procedure used by Guyer *et al.*, 2000. These samples were found not to have significant activity in a quantitative broad spectrum colourimetric proteinase assay. The ability for the supernatant fractions to cleave the trypsin substrate Boc-QAR-AMC was also negligible when compared to positive trypsin controls. Considering these results, a Western blot analysis was performed using a polyclonal antibody against Sat

obtained from Mobley *et al.* to determine if this protein was being expressed. The immunoreactivity of the supernatants from the wild type and mutant strains showed non-specific banding patterns (Phan *et al.*, unpublished data). Using PCR primers to amplify the Sat gene, it was confirmed the wild type strain did in fact possess the *sat* gene and in the isogenic mutant it was disrupted (Phan *et al.*, unpublished data). These data confirmed *sat* was present in the genome of the wild type, but it couldn't be confirmed that it was actually expressed by the bacteria.

Even though the results from the previous proteinase activity tests were negative, the ability of the sat^+ supernatant to activate PARs in the calcium bioassay was tested using the RWPE-1 bioassay system. If a response was observed, PAR-1 or PAR-2 could be desensitized with peptide agonists and then the same cells could be exposed to the supernatant to determine if signalling was via PARs. In this assay system, the filtered supernatants from both the wildtype and isogenic mutant strains did not result in deviations from baseline fluorescence levels which indicated there was not calcium mobilization within the cells.

It was also possible that the bacteria couldn't produce high levels of secreted protein unless they needed to produce it for survival. It would be logical for the bacteria to conserve energy and reduce the level of Sat synthesis when it was not required and to turn on these pathways during infection and evasion of the immune system. Perhaps the bacterial cells required a signal from eukaryotic cells in order to produce a significant amount of Sat? To test this hypothesis, the two strains were grown in co-culture with RWPE-1 cells and the supernatants were tested for PAR agonist activity using the

calcium bioassay. When the supernatants from both strains were added to RWPE-1 cells there was not a change calcium signalling within the cells and subsequent addition of TF-NH_2 or SL-NH_2 still induced calcium mobilization. This indicates the cells still retained function and PARs on the cell surface had not been desensitized and affected by the bacterial supernatants.

These data collectively suggest that either Sat is not a proteinase with high activity, though it bears homology to known bacterial proteinases, or not enough Sat protein is produced by the bacteria to obtain a measurable response since the expression of Sat could not be confirmed by Western blot analysis. Under these test conditions is does not appear that *E. coli* CFT073 *sat*$^+$ secretes a proteinase that signals via PAR activation although it may use other unknown mechanisms to trigger an inflammatory response.

4.4 Conclusions

It could be assumed that PAR-1 and PAR-2 have physiological functions in the prostate since they were found to be expressed in both human prostate epithelia and in the rat prostate tissue. In these experiments, the production of inflammatory mediators following PAR activation was focused on as one of the possible roles PARs may have in the prostate. It was found that activation of PAR-1 with thrombin and TF-NH_2 led to increased expression of pro-inflammatory cytokines IL-6 and IL-8, while the PAR-2 agonists did not increase the expression of IL-6 or IL-8. PAR-2 may have physiological functions in the prostate besides involvement in inflammatory pathways.

PAR-1 and PAR-2 were also found to be expressed in the rat prostate and activation in an *in vivo* system with agonists peptides did not result in an inflammatory reaction as measured by macro and microscopic damage and cytokine expression. However thrombin and trypsin administration resulted significantly increased morphological scores and tissue levels TNF-α in some cases. PAR-1 and PAR-2 could have multiple roles in many pathways, not only inflammatory, under differing conditions. The hallmarks of inflammation are swelling, redness, heat, pain and loss of function. The variables measured in this study assessed swelling and redness reflected by the morphological and histological scores as well as the cytokine concentrations which be a sign of relative oedema, inflammation and leukocyte recruitment. Heat and pain were variables that could not be quantified under the parameters of the experiments defined in this study. It is possible that through non-PAR mediated pathways that thrombin and trypsin may cause inflammation, or the pain associated with inflammation, that is not defined by mass granulocyte infiltration into the prostate.

The prostate is rich in endogenous proteinases that are present to facilitate liquefaction of the seminal clot in humans, not in rats. Knowing that PARs are expressed in the lumen of the rat prostate, there must be a physiological reason for this observation, one of which could be innate immunity. PAR-1 and PAR-2 may be present to sense alterations in normal proteinase levels in the prostate which may change during disease states. Since there are always proteinases expressed in the prostatic ducts, it seems reasonable that there would be a baseline level of PAR activation. Changes in the rate of receptor cleavage due to change in proteinase concentration be enough to trigger a PAR-

mediated response. Proteinases such as trypsinogen and enteropeptidase have been found to be expressed in PC-3 cells, a tumour cell line, localized to vesicles within the cells (Cottrell *et al.*, 2004). The production of trypsin in the lumen of the prostate may in part, serve to signal PAR-1 and PAR-2. PAR activation may not only be inflammatory, but actually protective in the prostate gland since it has been found that PAR-2 mediates gastric mucus secretion by stimulating the release of CGRP from sensory neurons (Kawabata *et al.*, 2001).

To prevent antigen processing by immune cells in the prostate and seminal vesicles, anti-phagocytic activity is found in prostatic fluid (Binks and Pockley, 1999). During bacterial infection there would be an increase in the phagocytic activity in the lumen of the prostate to remove the insult and increases in proteinase concentration during these states may activate PARs triggering IL-6 production and an acute phase response. PARs may not only play a role in non-bacterial prostatitis, but bacterial prostatitis as well through signalling via bacterial proteinases. The supernatants of *E. coli* CFT073 *sat*$^{+}$ were not found to have proteinase activity which would activate or desensitize PAR-1 or PAR-2. Certainly, proteinase virulence factors of other uropathogenic bacteria may function though PAR activation. Also inactivation of PARs may be beneficial for the organism by reducing the activation of macrophages and neutrophils.

In this model of PAR activation in the rat prostate, the collective results suggest they are not inflammatory in nature. However, *in vitro* data suggest that PAR-1 has a role in mediating prostatic inflammation by the release in pro-inflammatory cytokines. PAR

activation in human cells and the animal model is conflicting and needs to be resolved. Since the biology and function of the semen, notable the seminal clot, is different in humans and in rats, the role of PARs may also be quite dissimilar in these species. To ascertain how PARs may play a role in the normal function of the prostate, PAR knock-out animals would be a powerful tool. Adapting this model to the mouse to take advantage of PAR-1 and PAR-2 knock-outs would allow for testing of bacterial and non-bacterial prostatitis to see how the diseases are modulated by the lack of PARs. An alternative method may be to use newly developed PAR-1 antagonists which would be powerful tools to inhibit endogenous PAR-1 activation (Selnick *et al.*, 2003). In this study it seems that PAR-1 in RWPE-1 cells play an important role in inflammation and by using new molecular tools to study PARs in the rat and mouse prostate as models of human disease, much would be learned about their function.

For the first time it has been shown that PAR-1 and PAR-2 are expressed in a non-tumourigenic human prostate cell line and in the prostate tissue of Sprague-Dawley rats. It was demonstrated that PAR-1 activation induces IL-6 and IL-8 expression from human prostate epithelium which may serve to modulate human prostatitis. The expression of PAR-1 and -2 are accompanied by changes in intracellular calcium ion concentration after stimulation. PAR-2 activation did not generate cytokine responses as tested for in this study which suggests that PAR-2 may have other roles, possibly anti-inflammatory. PAR-1 activation did not stimulate the production of inflammatory mediators and inflammation *in vivo* and the effects of PARs in this model are unclear. An animal model using PAR-1 and PAR-2 agonists did not result in the expected effects of

IL-6 and IL-8 generation which would be inflammatory infiltrate in the prostate tissue and higher levels of inflammatory mediators. Signalling through PARs by bacterial proteinases from *E. coli* CFT073 *sat*$^{+}$ did not occur which indicates this strain may not use PAR activation as a virulence mechanism. Further study of PARs in the prostate is warranted to fully examine all of the possible mechanisms involved after activation which could modulate prostatic disease.

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APPENDICES

Appendix A

Luria Bertrani Broth (LB)

Yeast extract	5.0 g
Tryptone	10.0 g
NaCl	10.0 g
dH_2O	1.0 L

For solid media add 15 g agar/L broth

Vincent's Minimal Medium (VMM)

Solution A

KH_2PO_4	1.0 g
K_2HPO_4	1.0 g
KNO_3	0.6 g
dH_2O	1.0 L

Solution B

$FeCl_3$	0.1 g
Mg SO_4	2.5 g
$CaCl_2$	1.0 g
dH_2O	1.0 L

Solution C

Biotin	0.01 g
Thiamine	0.01 g
Calcium pantothenate	0.01 g
dH_2O	1.0 L

After autoclaving, add 1/10 solution B and 1/100 solution C to solution A. Add 10 g mannitol.