Porphyromonas gingivalis INHIBITS MITOCHONDRION-INDUCED APOPTOSIS THROUGH AKT PATHWAY

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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To my parents
ACKNOWLEDGMENTS

I thank my mentor and dear friend Dr. Yilmaz for her endless support throughout my residency. I also thank Dr. Koutouzis for his contribution to my education and outstanding commitment to our profession.
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<td><em>P. gingivalis</em></td>
<td><em>Porphyromonas gingivalis</em></td>
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<td><em>B. forsythus</em></td>
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<td><em>T. denticola</em></td>
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<td>GECs</td>
<td>Gingival epithelial cells</td>
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<td>PI3 kinase</td>
<td>Phosphoinositide 3 kinase</td>
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<td>Bad</td>
<td>Mitochondrion-associated protein, a Bcl-2 family protein</td>
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<tr>
<td><em>F. nucleatum</em></td>
<td><em>Fusobacterium nucleatum</em></td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>siRNA</td>
<td>short interfering RNA</td>
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<td>Ser</td>
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<td>STS</td>
<td>Staurosporine</td>
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<td>Cas-9</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
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Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

*Porphyromonas gingivalis* INHIBITS MITOCHONDRION- INDUCED APOPTOSIS
THROUGH AKT PATHWAY

By
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Periodontal disease affects the supporting structures surrounding teeth in the oral cavity. Periodontal diseases are polymicrobial chronic infections of multifactorial etiology. Bacteria in the dental plaque colonizing human dentition are recognized as the primary etiologic agent in periodontal disease. Host susceptibility greatly modifies disease expression including the extent and severity of the disease. Chronic periodontitis is the most common type of periodontal disease treated today. Bacteria categorized in Socransky’s red complex; *P. gingivalis, B. forsythus, and T. denticola* are identified as the major pathogenic in chronic periodontal disease. Of the three pathogens, *P. gingivalis* has been widely associated with adult chronic forms of periodontal disease probably due to its ability to invade primary gingival epithelial cells (GECs) and modulate immune responses, local and systemic. Gingival epithelium is one of our first defense mechanisms against bacterial invasion. *P. gingivalis* can replicate and survive undetected inside GECs for extended periods, contributing to host tissue destruction. This capability is possibly due to the large number of virulence
factors *P. gingivalis* produces, which affect multiple intra-cellular pathways. In addition, *P. gingivalis*-infected GECs are protected from mitochondrion dependent apoptosis, partially through activation of the Phosphotidylinositol 3 kinase (PI3K)/Akt signaling pathway. Biochemical events associated with *P. gingivalis*-induced inhibition of apoptosis include blocking of mitochondrial membrane permeability and cytochrome-*C* release. The Akt signaling pathways were investigated during *P. gingivalis* infection along with other key mitochondrial molecules downstream from Akt, including pro-apoptotic Bad. We demonstrated that *P. gingivalis* infection caused significant phosphorylation of Bad, while mRNA levels for Bad slowly decreased. *P. gingivalis* infection resulted in the translocation of the mitochondria-associated protein (Bad) in the cytosol as seen with fluorescence microscopy. *P. gingivalis* lost the ability to induce phosphorylation and translocation of Bad in Akt-deficient GECs. Thus, *P. gingivalis* inactivates pro-apoptotic Bad by phosphorylation through Akt. In summary, our findings suggest that Akt is a key modulator of anti-apoptotic pathways activated by *P. gingivalis*. *P. gingivalis* utilizes multiple mitochondrial pathways to prevent gingival epithelial cells from cell death and secures its persistence in primary GECs.
CHAPTER 1
INTRODUCTION

Periodontal diseases are infections of multi-factorial etiology which affect the human dentition and ultimately lead to tooth loss (Machtei et al., 1999, Grossi et al., 1994; Grossi et al., 1995). Chronic periodontal disease is the most common of periodontal diseases treated today (Albandar et al., 1999). Many risk factors have been implemented in the etiology of chronic periodontitis including plaque control, smoking and age (Beck et al., 1992; Grossi et al., 1995, Grossi et al., 1994; Haffajje & Socransky, 1994; Haffajje & Socransky, 2000). It is established today that bacteria colonizing tooth surfaces in the dental biofilm are the causative agents (Beck et al., 1992, Socranksy et al., 1998) (Figure 1-1). However factors such as host susceptibility and host exposure to environmental and systemic factors such as smoking, socioeconomic status and diabetes can greatly modify expression of such infections (Seppala et al., 1993, Grossi et al., 1995, Axelsson et al., 1998, Haffajee & Socransky, 2000). Bacteria in the dental biofilm have been investigated over the past fifty years to identify key species involved in periodontal disease (Listgarten, 1976; Listgarten, 1988; Listgarten, 1999).

Several bacterial species in the subgingival microflora (Figure 1-2) are recognized today in the pathogenesis of chronic periodontitis, (Haffajee & Socrasnky, 1994; Socransky et al., 1998). Along with the complexity of such bacterial organization, an order of bacterial adhesion, colonization and maturation are found in the periodontal pocket. Microbiological analyses of subgingival plaque consistently show gram negative bacteria adjacent to the epithelial lining of the pocket (Liakoni et al., 1987). Subgingival bacterial complexes were first characterized by Socransky and coworkers in 1998.
(Figure 1-2). They used DNA probes from forty bacterial species frequently isolated from the oral cavity to identify species from periodontally diseased sites. Cluster and community ordination analyses were performed in attempt to characterize the complex relationships amongst bacteria in the subgingival biofilm. Therefore the so called “red complex” bacteria, *P. gingivalis, B. forsythus and T. denticola* were found to be associated with increased pocket depth probing and are recognized today as the causative agents in periodontal disease (Socransky et al., 1998). The intricate interactions of these species with others that colonize the periodontal pocket have been greatly investigated to shed light on the mechanism of action leading to periodontal tissue destruction and possible treatment modalities. Of Socransky’s red complex bacteria, *P. gingivalis* has been isolated from severely diseased but also healthy patients (Lamont & Yilmaz, 2002; Rudney et al., 2005).

*P. gingivalis* has also been recently implicated in cardiovascular disease and pre-term birth (Beck et al., 1996, Herzberg and Weyer, 1998, Offenbacher, 2004; Offenbacher et al., 2006, Stein et al., 2009).

Extensive research has been conducted in the pathogenesis of *P. gingivalis*. The structure of *P. gingivalis* is shown using scanning electron microscopy (Figure1-3). The mechanism of such pathogenesis is directly related to the multiple virulence factors this organism utilizes, including extracellular proteases such as gingipains (Sheets et al., 2008, Curtis *et al.*, 2005), endotoxins such as lipo-polysaccharide (LPS)(Darveau *et al.*, 1998), other adhesion proteins that modulate the host’s immunologic and inflammatory responses(Lamont Jenkinson, 2000; Tribble et al., 2006).
All of the above factors seem to allow for successful invasion and subsequent alterations of the host cells by *P. gingivalis* without the expected immune response. It has been shown that *P. gingivalis* can down-regulate the secretion of an important pro-inflammatory chemokine, interleukin-8 (IL-8) transcriptionally and post translation, even in the presence of another pro-inflammatory bacterium such as *F. nucleatum* (Darveau et al., 1998, Huang et al., 1998; Huang et al., 2001). IL-8 is responsible for attracting neutrophils to the site of an infection. The inhibition of production of IL-8 at infection sites would have a detrimental effect at the bacteria-epithelium interface (Lamont & Yilmaz, 2002). It has been shown that *P. gingivalis* can invade and replicate inside human gingival epithelial cells surviving for extended periods of time (Lamont et al., 1995; Belton et al., 1999; Yilmaz et al., 2006). In addition, *P. gingivalis* has been shown to affect multiple signaling pathways intra-cellularly such as the activation of an integrin-associated pathway leading to actin cytoskeletal rearrangements within epithelial cells (Darveau et al., 1998; Zhang et al., 2005; Yilmaz, 2008). These cytoskeletal arrangements appear to be the primary mediators for the bacterial internalization into GEC’s, after which *p. gingivalis* replicates rapidly in the cytoplasm of GEC’s, in the perinuclear region (Belton et al., 1999, Lamont et al., 1995). The adhesion of *P. gingivalis* to primary GEC’s and subsequent invasion is mainly mediated by the binding of major fimbriae to an integrin receptor and activation of a putative integrin signaling proteins FAK (focal adhesion kinase) and paxillin with simultaneous remodeling of the actin cytoskeleton (Yilmaz et al., 2002; Yilmaz et al., 2003). Several studies by using transformed non-oral-epithelial cell lines, such as HEp-2, also verified that the bacterial fimbriae – β1 integrin receptor’s involvement with the cell lipid membrane rafts could
lead to the activation of the actin cytoskeleton reorganization, thereby providing \textit{P. gingivalis}’ to internalize host cells (Tsuda et al., 2005). Subsequently, a number of studies have been published regarding \textit{P. gingivalis}’s survival intra-cellularly, its ability to maintain viability for extended periods of time in primary GECs, and spread from cell-to-cell through actin-based membrane projections later in the infection (Yilmaz et al., 2006; Yilmaz, 2008). Infected GECs harboring large numbers of intracellular \textit{P. gingivalis} do not undergo apoptotic or necrotic death. A large body of in vitro evidence indicated that \textit{P. gingivalis} infection induces an anti-apoptotic phenotype in primary GECs by rendering the host cells resistant to cell death from various potent pro-apoptotic agents including staurosporine, camptothecin, and extracellular ATP (Yilmaz et al., 2008a; Mao et al. 2007; Yilmaz et al., 2004; Nakhjiri et al., 2001).

In the same way, different studies examining the healthy human buccal epithelial cells for the identification of intra-cellular bacteria found high levels of \textit{P. gingivalis} in each sample (Rudney et al., 2005). Interestingly, a further study by Rudney et al. analyzed the oral epithelial samples collected from healthy subjects for viability using both markers of cell membrane integrity and metabolic activity, which indicated no significant level of apoptosis or necrosis in those heavily bacteria invaded epithelia (Rudney & Chen, 2006). These \textit{in vivo} studies provided logical results to the previously reported \textit{in-vitro} research on \textit{P. gingivalis} interaction with primary gingival epithelial cells. However, the contribution made by other intra-cellular bacteria in shaping the overall status of the oral epithelium needs to be considered.

It has also been shown that \textit{P. gingivalis} infected GECs undergo successful mitosis and that infection with \textit{P. gingivalis} accelerates the host-cell cycle progression
(Kuboniwa et al., 2008)(Figure 1-4). This organism impacts multiple anti-apoptotic and survival host pathways and can prevent host-cell death by partially blocking mitochondria-dependent apoptosis. Gingival epithelial cells have also been shown to express the functional purinergic receptor P2X7, which is involved in activating programmed cell death (apoptosis) by binding to extracellular ATP (Yilmaz et al., 2008). Infection with \textit{P. gingivalis} has demonstrated this organism's ability to consume the extra-cellular ATP that is released from the infected cells to induce apoptosis, and therefore inhibiting an apoptotic signal. The intrinsic anti-apoptotic mechanism include the inhibition of mitochondrial membrane depolarization and cytochrome-C release, up-regulation of the anti-apoptotic protein of the Bcl-2 family also named Bcl-2 and down-regulation of pro-apoptotic member Bax of the same family of proteins, in addition to the inhibition of caspase-3 activation through dual JAK/Stat and Akt signaling (Mao et al., 2007; Yilmaz et al., 2004; Nakhjiri et al., 2001). Furthermore, \textit{P. gingivalis} infection up-regulates an important cyclin; PI3 kinase/Akt pathway in primary gingival epithelial cells. A specific PI3 kinase inhibitor, LY294002, substantially diminishes the infected cells’ resistance to staurosporine-stimulated apoptosis. \textit{P. gingivalis’s} phosphorylation of Akt during the infection promotes a strong anti-apoptotic effect resulting in loss of mitochondrial membrane potential, cytochrome-C release, and DNA fragmentation (Yilmaz et al., 2004). Another important family of apoptosis-related family of proteins, the Bcl-2 family, has been shown to strictly control mitochondrial function in mammalian cells (Cosulich et al., 1999; Chao and Korsmeyer, 1998). One of the important members of the Bcl-2 family of proteins is Bad. Bad is a pro-apoptotic protein that mediates mitochondrial release of cytochrome-C inducing apoptotic death. These pro-
apoptotic activities of Bad have been shown down-regulated through phosphorylation by Akt kinase (Chen et al., 2005; Datta et al., 1999). Thus, Akt can partially modulate mitochondria-associated cell death along with key mitochondrial molecules. Bacteria-induced epithelial apoptosis can be regarded as a mechanism the host utilizes to inhibit bacterial infection. However, some bacteria may still be able to propagate to surrounding tissues through apoptotic bodies released from the dead epithelial cells. For example, *Pseudomonas aeruginosa* causes excessive apoptosis to disseminate infection (reviewed in Finlay et al., 1989). On the other hand, bacterial inhibition of apoptosis can provide a safe haven for bacterial proliferation and make intracellular pathogens invisible to the immune system. *Salmonella, Shigella, Mycobacterium tuberculosis*, some *Chlamydia*, and *Neisseria* species appear to delay or inhibit apoptosis in epithelial cells (Finlay et al., 1989; Zychlinsky et al., 1992; Monack et al., 1996; Knodler et al., 2001, Verbeke et al. 2006), preventing cell death. Similarly, successful persistent oral bacterium, *P. gingivalis* appears to use the latter strategy. These interactions between bacteria and epithelial cells are highly dynamic and result in complex responses. Thus, the modulation of epithelial apoptosis by bacterial pathogens is common theme and may have significant repercussions on ultimate status of epithelium and its functions including impaired immune response and imbalance in cellular homeostasis. Understanding the effects of *P. gingivalis*’ infection on inhibition of apoptosis in primary GECs and the molecular aspects associated with this process could add considerably to our knowledge of the fates of the infected GECs and the intracellular *P. gingivalis* besides mechanisms of host injury. Knowledge of these diverse mechanisms could aid
in the production of more targeted treatment modalities to control *P. gingivalis-*
associated periodontal disease. We propose anti-apoptotic Akt signaling pathway along
with other associated key molecules (Bad) can contribute the ability of *P. gingivalis* to
modulate GEC apoptosis to avoid host cell defense and intra-cellular killing, thereby
further disseminating the infection.

Thus, the overall hypothesis to be addressed is that Akt signaling is likely to
have functional significance on *P. gingivalis*-mediated survival of primary GECs and *P.
gingivalis’* association with some key mitochondrial molecules including Bad through Akt
may modulate apoptotic cell death.

The aims of this study are designed to determine the functional importance of Akt
during *P. gingivalis* infection by using RNA interference technology, and to characterize
the role of pro-apoptotic Bad in this interaction.

![Periodontitis -affected human dentition](image)

Figure 1-1. Periodontitis –affected human dentition- Property of the University of Florida.
Figure 1-2. Microbial complexes, Adapted from Socransky et al. 1998

Figure 1-3. *P. gingivalis* scanning electron microscopy by O. Yilmaz
Figure 1-4. Three-dimensional confocal scanning fluorescence microscopy showing a 24-hour-infected primary GEC (actin, red; nuclei, blue) with high numbers of intracellular *P. gingivalis* (green) undergoing successful mitosis, adapted from *Microbiology, Yilmaz.2008.*
CHAPTER 2
MATERIALS AND METHODS

Bacteria and Cell Culture

*P. gingivalis* ATCC 33277 was cultured anaerobically for 24 hours at 37°C in trypticase soy broth supplemented with yeast extract (1 ug/ml), haemin (5 ug/ml) and menadione (1 ug/ml). Bacteria were grown for 24 hrs, harvested by centrifugation at 6000 g and 4°C for 10 min, washed twice, and re-suspended in Dulbecco’s Phosphate-buffered saline (PBS), pH 7.3, before incubation with host cells. Bacteria were quantified using a Klett-Summerson photometer. Primary GECs were obtained after oral surgery in the clinics of University of Florida from healthy gingival tissue as previously described (Lamont *et al.*, 1995). Cells were cultured as mono-layers in serum-free keratinocyte growth medium (KGM) (Lonza, Walkersville, MD) at 37°C in 5% CO₂. GECs were used for experimentation at 80% confluence and cultured for 48 hours before infection with bacterial cells or exposure to other test reagents in KGM.

Infection of Cells with *P. gingivalis* and Treatment with Staurosporine, and PI3K Inhibitor

Gingival epithelial cells were infected at a multiplicity of infection of 100 with *P. gingivalis* 33277 for 30 minutes, 60 minutes, 2, 6, 12, and 24 hours at 37°C in a CO₂ incubator. All time points for the infections were carried out backwards; i.e., instead of beginning all infections at the same time, infections were initiated at the indicated times before time zero so that all incubations could be stopped at the same time. For induction of apoptosis studies, GECs were treated with a potent apoptotic inducer, staurosporine (STS), 2 uM or 4 uM (Sigma, St. Louise, MO), for 3 hours after 21 hours infections with or without the bacteria. Additionally, after 3 hours infection with *P.
*P. gingivalis* 33277, GECs were treated with the PI3 kinase inhibitor, LY294002 (LY), 20 μm (Sigma), for 18 hours (3 hours post infection) prior to the 3 hours STS treatment. All treatments were performed in GEC culture media at 37 °C in the CO2 incubator.

**Analysis of Apoptosis by Annexin-V and Propidium Iodide Staining**

Early apoptotic changes were identified by using fluorescein isothiocyanate (FITC)-conjugated Annexin-V-fluos (green fluorescence) (Roche Applied Science, Indianapolis, IN), which binds to phosphatidylserine (PS), a molecule exposed on the outer leaflet of apoptotic cell membranes. Propidium iodide (PI) (red fluorescence) (Sigma) was used for the discrimination of necrotic cells from the Annexin-V-positively stained cells. Briefly, GECs were grown on 4-well chambered slides (Nalge-Nunc International, Rochester, NY), infected with *P. gingivalis* for 24 hours and incubated with various agents as described above. The slides were washed with ice cold PBS and immediately treated with 100 ul Annexin-V-Fluos binding solution containing 10 ul Annexin-V-Fluos labeling reagent per 1000 ul HEPES buffer (10 mM HEPES/ NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl2) and 1 mg/ml PI. After 15 min incubation in the dark at room temperature, the slides were washed with ice cold PBS and fixed in 10 % neutral buffered formalin for 20 min. Slides were mounted in Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA) containing 4’, 6-diamidino-phenylindole (DAPI) for nuclear staining and examined using a fluorescence microscope (Zeiss Axio imager A1) equipped with band pass optical filter sets appropriate for imaging of dyes. The images were captured with a cooled CCD camera (Qimaging, Surrey, Canada) controlled by Q-capture software. Cells that were untreated and incubated in the binding buffer with Annexin-V and PI or only with Annexin-V or PI separately served as controls for determining the threshold of fluorescence intensity. Cells that were treated with only
STS or ethanol served as positive staining for Annexin-V and PI respectively. Approximately 1000 cells per condition from 3 separate experiments were analyzed to determine the percentage of cells positively stained for Annexin-V and PI. The same microscopy settings were employed throughout all experiments.

**Depletion of Akt by RNA Interference**

Primary cultures of GECs at 50% confluence were transfected in GECs growth media using 100 nM of siRNA duplexes in 5 ul siRNA Akt DharmaFECT1 agent (Dharmacon, Lafayette, CO). Briefly, 5 ul transfection agent was added drop-wise into 195 ul of GEC growth media and the incubation was performed for 10 min at room temperature. 100 nM siRNA Akt sequences (Dharmacon) were added to diluted transfection agent, mixed gently, and incubated for 10 min at room temperature. Finally, 50 ml of this mixture was added to each well, the plate was rocked gently, and further incubated for 48 hrs at 37 °C 5% CO2. Non-target pool siRNA (Dharmacon) and transfection agent alone were used as negative controls.

**Confirmation of Akt Knockdown by Western-Immunoblotting**

Western-blot analysis was performed up to 48 hours post-transfection using equal amount of protein in each sample determined by a bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL) and the samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis. Proteins were blotted onto nitrocellulose membrane, blocked in 5% dried milk solution diluted in Tris buffered saline containing 0.01% Tween 20 (TBS/T), incubated with Akt (1/2/3) antibody at a dilution of 1:2000 (Cell Signaling, Danvers, MA) and treated with horseradish peroxidase(HRP)- conjugated secondary antibody at 1:5000 (Cell Signaling). The blot was then stripped and probed with anti- B actin antibody 1:1000.
and HRP conjugated secondary antibody (Cell signaling) at 1:2000 used as control. Results were visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ). Densitometry scanning was quantified using NIH-Image J analysis (Bethesda, MD). The knockdown experiments were repeated at least 3 separate times.

**Assay of Bad Activation by Immunoprecipitation**

Gingival epithelial cells were infected with *P. gingivalis* 33277 for 6, 12, or 24 hours. Cells were washed twice with cold PBS and solubilized in lysis buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA with phosphatase inhibitor and protease cocktail inhibitor (Sigma). Lysates were clarified by centrifugation at 10000 g for 15 min at 4 °C and protein concentration was determined by the BCA protein assay (Pierce Company). Bad was precipitated from the cell lysates with anti Bad specific antibody (Cell Signaling) overnight at 4°C with 1:50 dilution. The protein-antibody complexes were collected with Protein A-Sepharose beads at 1:10 by volume and washed three times with lysis buffer. Samples were boiled in 12% SDS-PAGE sample buffer and transferred to nitrocellulose membranes. Ser$^{136}$ phosphorylation of Bad was assessed by reacting at 4°C overnight with a 1:500 dilution of specific anti-phospho Bad antibody to the Ser$^{136}$ (Cell Signaling) followed by a 1:2000 dilution of HRP conjugated secondary antibody (Cell Signaling). Results were visualized by the enhanced chemiluminescence detection system (Amersham Pharmacia), analyzed by scanning densitometry and quantified using NIH-Image J. Blots were then stripped and reprobed with a 1:1000 dilution of anti-Bad and 1:2000 dilution of HRP-conjugated secondary antibody (Cell Signaling) to determine total Bad in the samples.
Bad Localization Assay by Fluorescence Microscopy

Gingival epithelial cells were grown on 4-well chambered slides (Nalge-Nunc International), washed with ice cold PBS, and fixed in 10% neutral buffered formalin for 20 min. The cells were permeabilized for 10 min with 0.1% Triton X-100 at 4°C and the same slides incubated with anti-Bad monoclonal antibody 1:50 (Santa Cruz) and anti-\textit{P. gingivalis} 33277 rabbit polyclonal antibody at 1:1000 in PBS containing 0.1 % Tween and 3 % BSA for one hour. After washing with PBS twice, samples were stained with Oregon Green 488 goat anti-mouse secondary antibody and Alexa-Fluor 594 anti-rabbit secondary antibody respectively for one hour at room temperature (Invitrogen, Carlsbad, CA). Samples with no primary antibody incubation were included as control. Slides were mounted in Vectashield Mounting Medium (Vector Laboratories) containing 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining and examined using a fluorescence microscope (Zeiss Axio imager A1) as described previously (Yilmaz \textit{et al.}, 2006). The images were captured by multiple exposures using a cooled CCD camera controlled by Qcapture software. The images are representative of 100 cells studied per sample from at least two separate experiments performed.

Real-Time Quantitative PCR

Total RNA was isolated from triplicate independent control-and \textit{P. gingivalis} infected GECs using RNeasy Mini Kit (Qiagen, Germantown, MD). The genomic DNA was removed by DNase 1 treatment (Ambion, Austin, TX). Total RNA (1 \textmu g) from each sample was reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI). Real time quantitative PCR was conducted in triplicate for each cDNA sample with the iCycler iQ real-time PCR detection system using iQ\textsuperscript{TM} SYBR Green Supermix (Bio-Rad, Hercules, CA). Two microliters template cDNA was added to final
volume of 25 ul with 1X SYBR Green Supermix and 1ul (20 um) of the following primer pairs:
Bad Forward: 5’-GGGCACAGCAACGCAGATG-3’
Reverse: 5’-TGGGAACGGGTGGAGTTTCG-3’
18s rRNA Forward: 5’-CGCCGCTAGAGGTGAAATTC-3’
Reverse: 5’-TCTTTGCAAATGCTTTCGCT-3’

The sequence 18s rRNA was used as an endogenous control. Real-time results were analyzed using I Cycler™ iQ Optical System software (Bio-Rad). The melt curve profile was analyzed to verify a single peak for each sample, indicating primer specificity.

Preparation of standards: Specific DNA products for each gene under investigation were synthesized from chromosomal DNA using standard PCR methods and visualized by gel electrophoresis to verify that a single specific product had been generated. Each product was purified using the QIAquick PCR Purification Kit (Qiagen), and quantified using the GeneQuant spectrophotometer. DNA product copy number was calculated using the formula (Yin et al., 2001):

\[
\text{Copies/ul} = \frac{\text{Starting Quantity (SQ)}}{1000} = (\frac{6.023 \times 10^{23} \times [\text{DNA}] \text{ g/ml}}{\text{molecular weight of product (basepairs)}}) \times 1000
\]

A 10-fold dilution series of each DNA standard was prepared for starting quantities of 10^8 to 10^{10} copies/ ml. These were used at least in duplicate in each Real-time PCR assay to allow the Real-time PCR software to estimate SQ of that gene in cDNA samples.
CHAPTER 3
RESULTS

P. gingivalis-Induced Protection of GECs Against Cell Death is Reversed
with the Depletion of Akt by siRNA Technology

Akt signaling pathway is essential in the protection of cells from a variety of severe stresses that may develop during infection by intracellular pathogens (Datta et al., 1999; Verbeke et al., 2006). From our previous investigations (Yilmaz et al. 2004), the pharmacological inhibition and phosphorylation assays revealed a potentially important role for Akt pathway in P. gingivalis induced protection against cell death by exogenous apoptotic stimuli targeting mitochondria, such as STS). Therefore, we further examined the involvement of Akt in this process by RNA interference. Forty-eight hours after transfection with short interfering Akt RNA, about 85% of the protein levels of Akt were reduced in primary GECs as determined by Western blotting (Figure. 3-1).

We confirmed that Akt- siRNA by itself did not induce apoptosis of uninfected cells, and Akt-deficient cells had infection levels comparable to siRNA untreated cells demonstrating that Akt deficiency did not affect the internalization capability of P. gingivalis (not shown). We first measured 24 hours infected and uninfected GECs treated with the various concentrations (2mM and 4mM) of the apoptosis inducer, STS for 3 hours and analyzed cell death by immune-fluorescence microscopy using Annexin-V-PI double-staining (Figure. 3-2A and B). In addition, we utilized LY294002 (20 mM), a specific inhibitor of PI3 kinase, an immediate upstream mediator of Akt as we described previously (Yilmaz et al., 2004). Following treatment with LY294002, the infected and uninfected GECs were incubated with the varying concentrations of STS (Figure. 3-2A). Simultaneously, we measured the cell death in the Akt-deficient cells. The GECs were
first transfected with Akt siRNA, infected with *P. gingivalis*, and then treated with STS and/or LY294002 similar to the treatment of normal cells (Figure. 3-2 C and D).

Consistent with previous findings (Yilmaz et al. 2004), *P. gingivalis* did not promote cell death. Approximately 83% and 74% of normal cells that were infected with *P. gingivalis* and treated with 2 mM or 4 mM STS, respectively, were resistant to apoptosis, while 45% and 53% of normal cells without infection became severely apoptotic in a concentration-dependent manner following treatment with 2mM or 4 mM STS (Figure. 3- 2A). In the absence of Akt, the level of apoptosis in the infected cells and the cells infected and treated with STS displayed 3 to 4-fold increases. These results were statistically significant (*P* value < 0.05 and *P* value < 0.01 respectively) by student’s *t*-test (Figure. 3- 2C and D). Treatment of both normal GECs and the Akt deficient cells with LY294002 without infection and STS did not induce any significant level of apoptosis (not shown). In addition, the treatment of the infected deficient cells with PI3 kinase specific inhibitor followed by STS treatment did not cause further increase in the level of apoptosis proposing a specific role for Akt (Figure. 3- 2C and D) as LY294002 will also inhibit PI3 kinase and downstream pathways parallel to Akt. Hence, these results suggest that Akt plays a critical role in *P. gingivalis* mediated protection against mitochondrion-dependent cell death in primary GECs as knockdown of Akt by siRNA significantly inhibits the anti-apototic phenotype of primary GECs cells activated during infection with *P. gingivalis*.

**Pro-Apoptotic Bad Phosphorylation in *P. gingivalis*-Infected GECs is Mediated by Akt**

In a previous investigation (Yilmaz et al. 2004), it was shown *P. gingivalis* infection results in phosphorylation and subsequent activation of Akt. It has been
demonstrated that Akt promotes cell survival through its capability to phosphorylate Bad at the amino acid Serine, specifically and predominantly Ser\textsuperscript{136} (Seo et al., 2004; Yilmaz et al., 2004; Datta et al., 1997). Phosphorylated Bad dissociates from a heterodimeric complex formed with anti-apoptotic Bcl-2 and Bcl-xL proteins, thereby, increasing anti-apoptotic effects of the Bcl-2 family. In light of our new results, we investigated next whether activated Akt can phosphorylate pro-apoptotic Bad. We thus measured phosphorylation from the samples immunoprecipitated with specific Bad antibody over the course of infection in normal versus Akt-deficient GECs by immunoblots using antibodies against a phosphorylation site of Bad Ser\textsuperscript{136} residue. We found that phosphorylation of Bad noticeably increased (1.6-fold) at 6 hours infection, raised to more than 3-fold at 12 hrs and peaked to ~4.5-fold after a 24 hours infection in the ratio of phosphorylated: total Bad determined by densitometry analysis of Western-blotting products (Figure.3- 3A and B). There was no change detected at 2 hrs infection (not shown). In contrast, the level of phosphorylated Bad remained unchanged in Akt siRNA transfected cells over the course of infection (Figure.3-3A) as verified by the ratio of phosphorylated: total Bad (Figure. 3-3C). Thus, the protection of \textit{P. gingivalis}-infected GECs against cell death appears partially due to inactivation of pro-apoptotic Bad, which appears to be directly associated with activation of Akt signaling. These results were validated by the analysis of Akt phosphorylation on Ser\textsuperscript{473} residue by an quantitative immune-fluorescence assay, which revealed enhanced activation kinetics (maximal 2.5 fold increase) for Akt for the similar time points of \textit{P. gingivalis} infection in GECs (data not shown). This finding was consistent with previous
immunoblotting phosphorylation assays performed for Akt in P. gingivalis infected GECs (Yilmaz et al. 2004).

**Effect of *P. gingivalis* Infection on mRNA Levels of Pro-Apoptotic Bad**

In an investigation by Nakhjiri et al., it was shown that the *P. gingivalis* infection in primary GECs modulates the expression of Bcl-2 family members transcriptionally. This includes down-regulation of expression of pro-apoptotic molecule Bax and up-regulation in the expression of anti-apoptotic molecule Bcl-2. Similarly, we wanted to further characterize the effect of infection on Bad at the mRNA level both in normal and Akt deficient GECs. Although our protein phosphorylation assays (Figure. 3-4A) illustrated a major increase in the levels of Bad phosphorylation, quantitative (real time) RT-PCR was performed on mRNA extracted from primary GECs infected with *P. gingivalis* and showed gradual (approximately 60%) decrease in Bad mRNA levels following the 24 hrs incubation (Figure. 3-4A). Interestingly, there was no change in the levels of Bad mRNA from the uninfected samples deficient in Akt, but there was an approximately 20% increase in the samples treated with *P. gingivalis* for 24 hrs (Figure. 3-4B), indicating a potentially additional role for Akt pathway in down-regulating pro-apoptotic Bad at the transcriptional level.

**P. gingivalis** Infection Sequesters Bad in Cytosol of GECs Through Akt

Due to the noted significant increase in the levels of phosphorylated Bad during the 24 hr infection of *P. gingivalis* in GECs and phosphorylated Bad is normally maintained in cytosol in an inactive form, we wanted to next determine whether 24 hour infection alters the intracellular distribution of Bad in GECs using immuno-fluorescent microscopy. Akt deficient GECs were also analyzed in order to further verify the importance of Akt in this interaction. Uninfected control cells demonstrated that large
proportion of Bad is localized in the cytosol in the absence of apoptotic stimuli (Figure. 3-5A). Addition of STS to the uninfected cells resulted in strong staining of Bad around the nuclei where typically mitochondria are clustered in the cell indicating a large amount of Bad translocated to mitochondria (Figure.3- 5A). However, the cells infected with *P. gingivalis* showed abundant amount of Bad accumulated in cytosol.(Collins & Bootman, 2003) On the other hand, GECs that are lacking Akt displayed similarly strong staining of Bad around the nucleus both in infected and STS treated uninfected cells (Figure. 3-5B). Overall results further confirmed the role of Akt associated signaling in sequestration of pro-apoptotic Bad in cytosol, during *P. gingivalis* infection, thereby, increasing anti-apoptotic effects of the organism in primary GECs.

![Figure 3-1](image)

**Figure 3-1.** Knockdown of Akt by siRNA in primary GECs. A target-specific Akt antibody was used to confirm the inhibition of Akt expression by western blotting. A non-target antibody (Beta-actin) was used to control proper loading and specificity of Akt siRNA. Column 1 is non-target siRNA (control), column 2 is transfection agent alone (control), and column 3 is target siRNA with transfection agent. Densitometric scanning of the products from the 48 hrs post-transfected samples (column 3, Akt lane) displayed a ~85% decrease in the level of Akt demonstrating a successful inhibition of the protein in the primary cultures of GECs.
Figure 3-2. Quantitative analysis of cell death by Annexin-V and Propidium iodide (PI).

The percentage of dying cells was determined by dual staining with FITC-conjugated Annexin-V and PI using fluorescence microscopy. The threshold of fluorescence intensity was determined with samples that were uninfected and untreated, respectively, were used to evaluate the positively Annexin-V and/or PI stained cells. At least 10 separate fields containing an average of 50 cells were studied quantitatively from 3 independent experiments performed in duplicate A). Primary GEC monolayers grown on microscopic chambers were incubated in the binding buffer containing Annexin-V (green fluorescence) and PI (red fluorescence). PI was used for the discrimination of necrotic or late apoptotic cells from the Annexin-V-positively stained cells. The nuclear stain DAPI (blue fluorescence) was used to visualize the number of cells in the field. B) The simultaneous quantitative analysis and microscopic examinations were employed in the Akt-deficient GECs under the similar conditions employed with normal GECs (C and D). (*), (**), and (*** denote). Statistical significance (P = 0.006, P = 0.001, and P = 0.01 t-test) for 24 hrs uninfected + STS (2mM-treated GECs versus 24 hrs infected + STS (2 uM-treated GECs, for 24 hrs uninfected + STS (4 uM-treated GECs versus 24 hrs infected + STS (4 uM-treated GECs versus 24 hrs infected + STS treated (4 uM-GECs, and for 24 hrs infected GECs versus 24 hrs infected + STS (2 uM+LY treated GECs, respectively (A). (*), and (** denote statistical significance (P = 0.03 and P= 0.005 t-test) for 24 hrs uninfected +siRNA treated GECs versus 24 hrs infected + siRNA treated GECs and 24 hrs infected + siRNA treated GECs versus 24 hrs infected + STS+ siRNA treated GECs, respectively (C).
Figure 3-2. Continued.
Figure 3-2. Continued

Figure 3-3. *P. gingivalis* infection induces a large increase in Bad Phosphorylation. Primary GECs and the Akt-deficient GECs were infected with *P. gingivalis* for 0 min (control), 6, 12, and 24 hrs. Cell lysates immune-precipitated with anti-Bad specific antibody were analyzed by immunoblotting with antibodies against phosphorylated Bad (Ser\(^{136}\)) (A). Blots were analyzed by scanning densitometry and ratios of phosphorylated: total Bad determined relative to ratios in control cells. The values show relative fold change calculated for a representative experiment and represent results obtained from at least three experiments (B and C).
Figure 3-3. Continued
Figure 3-4. Bad mRNA levels slowly decreased by *P. gingivalis* infection. Gene expression was measured by quantitative RT-PCR on mRNA from primary GECs A) and Akt-deficient GECs B) infected with *P. gingivalis* for 0 min (control), 6, 12, and 24 hrs. Relative fold change was calculated by dividing the copy number of the gene transcript in *P. gingivalis* infected cells by the copy number in control cells. Data are representative of three independent experiments performed in triplicate. (*) denotes statistical significance (*P* = 0.05 *t*-test) for 24 hrs uninfected GECs versus 24hrs infected GECs.
Figure 3-5. *P. gingivalis* infection redistributes Bad localization in primary GECs through Akt. Intracellular Bad localization was detected by immune-fluorescence using antibodies against Bad (green). The samples were also stained with *P. gingivalis* antibody (red) and DAPI (blue) to visualize the nuclei. A) Uninfected cells displayed large proportion of Bad localized in cytosol. However, incubation with the apoptosis inducer STS caused strong staining in the perinuclear area indicating translocation of Bad to mitochondria. Infection with *P. gingivalis* showed sequestration of Bad in cytosol. B) Akt knockdown cells were prepared as in Figure 3-5A. The localization of Bad showed intense staining around the nuclei, where mitochondria are. This was similar in the uninfected STS treated cells (control). The images were captured with a fluorescence microscope equipped with a cooled CCD.
CHAPTER 4
DISCUSSION

Chronic adult periodontitis is primarily a mixed infection of multi-factorial etiology (Machtei et al., 1999, Grossi et al., 1994, Grossi et al., 1995). Host factors play a critical role in extent and expression of the disease in the oral cavity. Host factors including immunologic, and environmental factors such as smoking, age, race, and socioeconomic status greatly influence the phenotypic expression of periodontitis, mainly the extent and severity of periodontitis (Grossi et al., 1994, Grossi et al., 1995, Haffajee & Socransky, 1994). Other systemic factors also influence and are influenced by these periodontal infections including diabetes mellitus, cardiovascular disease, and pre-term births (Grossi et al. 1994; Herzberg & Weyer 1998; Offenbacher et al., 2004; Kibumitsu et al. 2002). As previously discussed multiple organisms have been implicated in the pathogenesis of periodontal disease (Haffajje & Socransky 1994; Socransky et al. 1998). Recent focus has shifted from a single organism infection to include the dental biofilm as a whole in the initiation and progression of periodontitis (Haffajee & Socransky, 2000). Synergistic relationships exist in the biofilm leading to accumulation of key periodontal pathogens, along with the presence of the necessary host factors ultimately resulting in destruction of the periodontium and eventual tooth loss (Haffajee and Socransky, 1994). As a successful colonizer of the dental biofilm and key pathogenic organism in chronic periodontitis, *P. gingivalis* has established itself as a powerful and opportunistic organism of the oral cavity (Lamont & Yilmaz, 2002). *P. gingivalis*’s multiple virulence factors contribute to the ability of this bacterium to utilize multiple pathways to invade, and replicate successfully in oral epithelial cells (Lamont et al., 2002; Yilmaz et al., 2006). As it invades gingival epithelial cells, *P. gingivalis*
modulates multiple signaling pathways to insure its survival and possible escape from immune responses. Namely, *P. gingivalis* infection intra-cellularly down-regulates the secretion of IL-8, an important chemokine attracting neutrophils to sites of infection. Other mechanisms include the modulation of intra-cellular calcium concentration and the activation of integrin-receptor-associated pathway (paxillin), leading to internalization of *P. gingivalis* and cytoskeletal arrangements (Darveau et al., 1998, Yilmaz et al., 2002; Zhang et al., 2005). This activation results in cell actin cytoskeletal rearrangements facilitating *P. gingivalis*’s ability to propagate within the cell and possibly outside. In addition, intracellular spreading capabilities of *P. gingivalis* contribute to this organism’s endurance by modulating local and systemic immune responses such as host-cell apoptotic pathways (Yilmaz et al., 2006). Moreover, infection by the intracellular pathogens illustrates that there is a great level of control on these host-cell apoptotic pathways (Byrne & Ojcius, 2004; Danelishvili et al., 2003; Collins, 1995). Apoptosis is a significant process in host-pathogen dynamics that can be advantageous to the host by contributing to pathogen removal. Induction of apoptosis can also be used by the pathogen as a virulence strategy facilitating dissemination of infection. For microorganisms that require a eukaryotic cell structure for survival, a viable host is essential for replication and colonization. Consequently, inhibition of apoptosis can provide a safe haven for microbes, and make intracellular organisms invisible to the immune system (Hacker *et al.*, 2006). The sites of entry for the potential pathogens frequently include mucosal regions lined by epithelial tissues, which function as an important part of innate immunity. As a result, the epithelial cells can serve as effectual colonizing niches for these opportunistic species. A common finding for an increasing
number of successful host-adapted bacteria is that they delay or inhibit apoptotic pathways in epithelial cells while the same bacteria act as pro-apoptotic stimuli in other tissue types (Hacker et al., 2006; Byrne & Ojcius, 2004). For example, *Salmonella typhimurium* infection is shown to cause apoptosis in macrophages but not epithelial cells (Monack et al., 1996). Similar to *Salmonella*, *Shigella flexneri*, which kills macrophages rapidly through apoptosis, also does not induce cell death in epithelial cells (Knodler & Finlay, 2001; Monack et al., 1996; Zychlinsky et al., 1992). *P. gingivalis*, one of the major constituents of oral subgingival microflora, is predominantly identified in severe forms of periodontal disease, possesses the ability to successfully colonize, adapt, and persist in its target gingival epithelial cells without being destructive until the environment becomes favorable for disease initiation and progression (Yilmaz et al., 2008). The suppression of cell death and promotion of cell survival in GECs by *P. gingivalis* appears to trigger the bacterium’s capacity to disseminate inter-cellularly. GECs harboring high level of intracellular *P. gingivalis* also demonstrate increased host-cell cycle progression and successful mitosis following one day infection (Kuboniwa et al., 2008; Yilmaz et al., 2006). In conjunction with the above findings, a recent study showed *P. gingivalis* infection can inhibit the P2X7 receptor-dependent apoptosis of primary GECs by consuming extra-cellular ATP via its secreted putative nucleoside diphosphate kinase (NDk) and preventing activation of P2X7 receptors by ATP binding (Yilmaz et al., 2008). This ATP signal is recognized as a universal danger signal released by stressed and/or apoptotic cells and is being consumed by a *P. gingivalis* enzyme during the infection, thus blocking this apoptotic signaling pathway (Yilmaz et al. 2008). Previous studies showed that *P. gingivalis* promotes the gingival epithelial
host-cell survival partially by activating PI3 kinase/Akt pathway, blocking caspase-3 activation via JAK/Stat signaling, and targeting the members of the Bcl-2 family proteins (Mao et al., 2007; Yilmaz et al., 2004; Nakhjiri et al., 2001). Our study verified the predicted functional role of PI3 kinase/Akt signaling in promoting epithelial cell survival stimulated by *P. gingivalis* infection since PI3kinase is an immediate regulator of Akt.

Knock down of Akt by siRNA confirmed that Akt is likely central to the ability of the organism in limiting mitochondrion-dependent host cell death and in colonizing gingival epithelial tissues as a thriving persistent opportunistic pathogen. The biochemical events that are associated with inhibition of apoptosis during *P. gingivalis* infection include blocking of mitochondrial permeability transition and inhibition of cytochrome-c release from mitochondria (Yilmaz et al., 2004). Anti-apoptotic Bcl-2 and Bcl-xLproteins of Bcl-2 family are located at the outer membrane of mitochondria and can inhibit release of cytochrome C. In the presence of apoptotic inducer, pro-apoptotic Bad translocates to mitochondria to associate with Bcl-2 and Bcl-XL. This induces membrane depolarization of mitochondria and subsequent release of cytochrome-C (Chao & Korsmeyer, 1998). On the other hand, the apoptotic activity of Bad can be inhibited through its phosphorylation by Akt (Datta et al., 1997). The phosphorylated form of Bad dissociates from a heterodimeric complex formed with anti-apoptotic Bcl-2 and/or Bcl-xL proteins and it stays in the cytosol in an inactive form, thereby, increasing anti-apoptotic effects of the Bcl-2 family. Hence, the phosphorylation status and intracellular localization of Bad are critically important biochemical events in cell-death pathways. Accordingly, our results showed *P. gingivalis* infection causes a significant level of Bad phosphorylation in GECs through Akt. This results in sequestering of Bad
away from mitochondria and blocking its pro-apoptotic action. The results also suggested that the inactivation of Bad mediated by Akt during the infection occurred gradually and it was a time-dependent process. The inactivation of Bad appears to be maximized at 24 hour infection. Interestingly, the large decrease in Bad mRNA levels following the 24 hours infection proposed a potentially additional mechanism for the Bad inactivation. Recent studies investigating the quantitative proteomics of intracellular \textit{P. gingivalis} in gingival epithelial cells showed the secretion of a number of distinctive \textit{P. gingivalis} proteins that could be important for adaptation and survival (Yilmaz et al., 2008; Xia et al., 2007). It is certainly valid to hypothesize that secretion of effector molecules by the intracellular bacterium may provide additional strategy for the inhibition of pro-apoptotic Bcl-2 family proteins. On the other end, Caspase-3 activation, which significantly increases the mitochondrial permeability, is shown to be inhibited by \textit{P. gingivalis} infection through dual Akt and JAK/Stat signaling (Mao et al., 2007). Similarly, Yao et al. recently demonstrated that caspase-9 activation is significantly impaired by \textit{P. gingivalis} in a time-dependent manner. Yet, the inhibition of caspase-9 was independent of Akt and JAK as indicated by the joint siRNA and the pharmacological inhibition assays (Yao et al., 2010). The early and short-lived detection of caspase-9 activation was not unexpected, since our previous study showed \textit{P. gingivalis} triggers activation of caspases at early time points of infection in GECs determined by the inhibition of rapid PS exposure by the broad-spectrum caspase inhibitor zVAD-fmk. However, neither caspase activation nor PS externalization lead to host-cell apoptosis (Yilmaz et al., 2004). In addition, caspase-9 activation is also required for transcription factor p53 dependent cell death. Infection of GECs with \textit{P. gingivalis}
*gingivalis* causes a large reduction in p53 levels determined by a recent study (Kuboniwa et al., 2008). Nonetheless, Yao et al. study showed Akt is not responsible for the caspase-9 inhibition stimulated by *P. gingivalis* infection.

To date, an extensive body of research indicates that *P. gingivalis* utilizes multiple virulence factors and manages to regulate many host-cell molecules, which are involved in cell to cell signaling in order to survive and successfully replicate intracellularly and simultaneously modulating the immunologic response to bacterial invasion. Furthermore *P. gingivalis*’ ability to prolong host cell survival and enhance the proliferation of gingival cells seems to allow the organisms to contribute to periodontal disease progression provided that necessary host and microbial factors are present. Association of *P. gingivalis* with apoptosis has been examined in a variety of cell types. Previous studies have indicated *P. gingivalis* induces apoptosis in Jurkat T-cells, KB cells, B cells, human gingival fibroblasts, and human trophoblasts, yet inhibits apoptosis in human monocytes, macrophages, neutrophils and GECs (Geatch et al. 1999, Chen et al. 2001, Belibasakis et al. 2010, Bostanci et al. 2009, Pollreisz et al, 2009)

Thus, this investigation outlined significant events occurring between the intracellular molecules Akt and Bad, as a result of *P. gingivalis* infection, which may be central for the organism’s survival and colonization in oral epithelial tissues. Collectively, these findings illustrate the complexity of the modulation of mitochondrion-dependent cell death during the infection of *P. gingivalis* in GECs and may be used as therapeutic targets to treat periodontal disease in the future. These interactions are summarized in Figure 4-1.
In conclusion, appreciating these complex microbiological and immunological interactions would help understand and possibly provide novel therapeutic modalities for the treatment of periodontitis especially in patients where conventional methods have failed.

Figure 4-1. The mechanisms for primary GECs protection against cell death induced by *P. gingivalis* infection, adapted from Dr. Yilmaz’s work.
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BIOGRAPHICAL SKETCH

Dr. Caroline Jermanus studied Biochemistry at the University of Florida, where she graduated in the spring of 2003. After which, she attended dental school at Nova Southeastern University where she received his Doctor of Dental Medicine degree in the summer of 2007. In July of 2007, she started her post-doctoral residency in periodontics and her Master of Science degree at the University of Florida. Dr. Jermanus received prestigious AADR travel bloc grant and won second place at the University of Florida Annual Research day. Upon graduation in the spring of 2010, Caroline will return to Jacksonville, Florida to practice clinical periodontics.