The antioxidant resveratrol down-regulates inflammation in an *in vitro* model of *Pseudomonas aeruginosa* infection of lung epithelial cells

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Abstract

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen capable of infecting the lungs and causing severe pulmonary disease in immunocompromised individuals. During the infectious process, *P. aeruginosa* provokes a potent inflammatory response and induces the release of reactive oxygen species (ROS). Cells undergo oxidative stress when cellular antioxidants are unable to effectively scavenge and detoxify ROS resulting in lung damage. Resveratrol (3,5,4‟-trihydroxystilbene) is a natural polyphenolic compound with recognized antioxidant effects. In this study we have tested the hypothesis that the antioxidant activities of resveratrol can attenuate an inflammatory response in *P. aeruginosa*-infected cells. Human lung epithelial (A549) cells were pre-treated with resveratrol for 5 hours followed by infection with *P. aeruginosa in vitro*. Intracellular ROS generation measured with CM-H_2DCFDA was used as an indicator of *P. aeruginosa*-induced oxidative stress. Surface expression of Fas/CD95 and activation of caspases-3 and -7 were used as indicators of cellular apoptosis. To further study the effects of resveratrol we also measured protein expression of intercellular adhesion molecule (ICAM)-1 and gene expression of pattern recognition receptors and enzymes related to inflammation and redox signaling. Resveratrol significantly reduced ROS generation, ICAM-1 expression, human beta-defensin-2 expression, and markers of apoptosis in A549 cells infected with *P. aeruginosa*, and up-regulated levels of glutathione peroxidase, suggesting that this compound may play an important therapeutic role in protecting the lungs against the deleterious effects of *P. aeruginosa* infection.

Lay Summary

The Lakehead University Department of Biology lists its mission statement as: "Faculty and students in the Department of Biology are bound together by a common interest in explaining the diversity of life, the fit between form and function, and the distribution and abundance of organisms." This research project, centered in the realm of the human sciences, aims to understand the mechanisms behind *Pseudomonas aeruginosa* infection of immunocompromised lungs using an *in vitro* model, and suggests that resveratrol, an antioxidant component of several fruits, nuts, and red wines, is a natural therapeutic agent capable of protecting the lungs against damage during infection.

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As a first year Biology student walking onto campus for the first time I could not have imagined how important becoming involved with research would be to both my personal and professional development. My experiences in the Lakehead Biology Department, and most recently at the Northern Ontario School of Medicine (NOSM), have taught me many life lessons and awarded me many friends among my colleagues.

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Abbreviations

·OH	Hydroxyl radical
AP-1	Activator protein-1
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CF	Cystic Fibrosis
CM-H_2DCFDA	5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester
COPD	Chronic obstructive pulmonary disease
ddH_2O	Double-distilled water
EPO	Eosinophil peroxidase
FBS	Fetal bovine serum
FLICA	Fluorochrome inhibitors of caspases
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSR	Glutathione reductase
GSSG	Glutathione disulphide (oxidized glutathione)
H_2O_2	Hydrogen peroxide
HSLs	Homoserine lactones
IL	Interleukin
LPS	Lipopolysaccharide

MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappaB
NLR	Nucleotide binding and oligomerization domain-like receptor
$O_2^{\cdot}$-	Superoxide anion
PAK	*Pseudomonas aeruginosa* strain K
PBS	Phosphate buffered saline
qPCR	Quantitative real-time polymerase chain reaction
QS	Quorum sensing
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SEM	Standard error of the mean
SOD	Superoxide dismutase
TNF	Tumour necrosis factor
TNF-α	Tumour necrosis factor-alpha
TNFR	Tumour necrosis factor receptor
γ-GCS	Gamma-glutamylcysteine synthetase
γ-GT	Gamma-glutamyl transpeptidase

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1 –Introduction

1.1 –*Pseudomonas aeruginosa* & Disease

Pseudomonas aeruginosa is a Gram negative, aerobic, rod-shaped bacterium responsible for a variety of acute and chronic infections. Infection by *P. aeruginosa* is predominantly seen in immunocompromised individuals, including patients with Cystic Fibrosis, HIV/AIDS, burn wound victims, transplant recipients, and patients undergoing chemotherapy (Kipnis et al. 2006; Sadikot et al. 2005). Its presence in nature is ubiquitous, and *P. aeruginosa* is capable of inhabiting almost any aqueous environment including soil, surface water, sewage, plants, and various foods including fruit and vegetables (Bonten et al. 1999). Found also within the sinks and humidifiers of health care settings, *P. aeruginosa* can be easily transferred to patients by hospital staff (Nseir et al. 2002) and as a result has become a common cause of nosocomial, or hospital-acquired, pneumonia, second only to *Staphylococcus aureus* (Santucci et al. 2003). Indeed, *P. aeruginosa* infection accounts for 10% of all hospital-acquired infections (Diekema et al. 1999) and approximately 25% of all ventilator-associated pneumonia (VAP), leading to a 69% mortality rate primarily due to septic shock following lung infection (Chastre et al. 2002; Crouch Brewer et al. 1996). Lung injury due to *P. aeruginosa* infection results from the direct destructive effects of the organism on the lung tissue and the potent host immune response (Sadikot et al. 2005).

1.2 – The Role of Bacterial Virulence Factors

To aid in its pathogenicity, *P. aeruginosa* is equipped with multiple virulence factors that allow it to adhere, invade, infect, and destroy human cells. *P. aeruginosa* can also form anaerobic bacterial communities called biofilms, which restrict the entry of

antimicrobial compounds and provide a platform for the bacteria to produce virulence factors (Lau et al. 2004). This resourcefulness coupled with an easily mutable nature make *P. aeruginosa* an important pathogen capable of developing resistance against therapeutic agents targeting it. Indeed, *P. aeruginosa* is particularly difficult to eradicate (Kipnis et al. 2006; Lau et al. 2004).

P. aeruginosa utilize surface appendages, which play an important role in adhesion to cellular surfaces. A single monotrichous flagella serves to propel bacteria via corkscrew motions (Kipnis et al. 2006). These thick, hair-like protein structures also mediate adhesion by binding to glycoprotein asialo-GM1 (Kipnis et al. 2006). Flagella also interact with toll-like receptors (TLRs) 5 and 2 to stimulate pro-inflammatory cytokine production critical in initiating the inflammatory response. In the lung environment, *P. aeruginosa* adapts to its surroundings and loses some virulence factors (i.e. flagella), resulting in aflagellar mutants that are more adept at avoiding host innate immune response during chronic infection (Kipnis et al. 2006; Sadikot et al. 2005). Pili are smaller finger-like surface appendages that facilitate movement using a "twitching" movement to sweep over cellular surfaces. Pili also use the same asialo-GM1-dependant mechanism to adhere to cells, and both flagella and pili are under study as potential targets for specific therapy (Kipnis et al. 2006).

Within the outer membrane of *P. aeruginosa* lies lipopolysaccharide (LPS). Found in all Gram-negative bacteria, LPS is composed of a hydrophobic domain, Lipid A, located in the phospholipid bilayer and a complex consisting of a core polysaccharide and an O-specific polysaccharide (O-antigen), which together form the hydrophilic tail. LPS maintains structural integrity within the bacterial cell wall and protects against the

effects of chemical attack (Kipnis et al. 2006). In particular, the O-antigen can be used to both distinguish between *P. aeruginosa* serotypes and to make vaccines. These vaccines, however, are of limited value because of the rate of genomic recombination and the high adaptability of the pathogen (Holloway 1955; Kipnis et al. 2006). It has also been observed that Lipid A composition changes in the process of airway colonization in CF patients. LPS is crucial to bacterial survival, and its removal leads to death of the pathogen (Ernst et al. 2003; Kipnis et al. 2006).

P. aeruginosa also utilizes a Type III secretion system (TTSS) to inject colonized cells with a variety of exotoxins. A syringe-like structure on the bacterial surface serves to inject effector proteins through the host cell membrane into the cytoplasm. Exotoxins of *P. aeruginosa* include ExoY, ExoS, ExoT, and ExoU, and while differing in their specific effects all are implicated in inflammation and cell death (Kipnis et al. 2006). ExoY compromises host membrane integrity by increasing cytoplasmic cAMP and host membrane permeability. ExoU possesses phospholipase A2 activity to destroy the host membrane and cause rapid necrotic death (Engel et al. 2009). ExoS and ExoT disrupt the structure of cytoplasm using N-terminal GTPase activating protein (GAP) domains that inhibit actin polymerization. ExoS also prevents normal cytoskeletal protein function by inhibiting interaction of Erzin-Radixin-Moesin, and ExoT prevents wound healing by inhibiting the Rac/integrin healing pathway (Kipnis et al. 2006).

Persistent inflammation is common in chronic *P. aeruginosa* infection of CF patients, and is partially caused by the Type II Secretion System (T2SS), which releases toxins and extracellular enzymes into the extracellular milieu. Including elastase and

pyocyanin, these secretins are commonly implicated in pathogen invasion and tissue damage (Durand et al. 2003; Kipnis et al. 2006).

Pyocyanin is a blue/green-pigmented secondary metabolite secreted via T2SS, which plays a distinct role in the pathogenicity of *P. aeruginosa*. Effects of pyocyanin include the recruitment of neutrophils to the airway epithelia, prevention of apoptotic cell engulfment by macrophages, depression of host immune responses, and it also acts to increase production of interleukin (IL)-8 by host cells (Bianchi et al. 2008; Lau et al. 2004). Pyocyanin is also an oxireducer and inflicts oxidative-stress-related damage on host epithelial cells by oxidizing host antioxidants like glutathione (Kipnis et al. 2006).

Large volumes of pyocyanin are commonly seen in sputum samples from infected CF patients, however the impact of pyocyanin alone is difficult to determine as numerous virulence factors play different roles in *P. aeruginosa* pathogenicity (Kipnis et al. 2006). *In vitro* studies using purified pyocyanin demonstrated an extensive amount of cellular damage resulting from an inhibition of normal ciliary function, epidermal cell growth, and cell respiration. While antioxidant therapy has been shown to improve lung function in CF patients, inhibiting pyocyanin production is also a potential therapeutic strategy (Kipnis et al. 2006). In addition, increased levels of IL-8 are also seen when human airway epithelial cells undergo oxidative stress (Lau et al. 2004). This increase in IL-8 recruits pro-inflammatory cells through a chemical gradient, perpetuating inflammation (Lau et al. 2004; Vlahopoulos et al. 1999).

Quorum sensing (QS) is a sophisticated form of communication between bacterial cells and is found in both acute and chronic *P. aeruginosa* infections. QS recognizes low-weight signaling molecules called homoserine lactones (HSLs) (Winstanley et al. 2009).

HSLs are secreted by *P. aeruginosa* and accumulate in the external environment as the pathogen persists and multiplies. Depending on the extracellular concentrations, *P. aeruginosa* can effectively coordinate the expression of other virulence factors like pyocyanin. Biofilm production is also cued via QS (Winstanley et al. 2009).

1.3 – The Lung, Respiratory Disease & Inflammation

Composed of over 40 different cell types, the human lung is an organ responsible for respiration and is an active player in innate immunity and front-line defense against pathogens (Hippenstiel et al. 2006; Shek et al. 1994).The terminal ends of the respiratory tree are composed to alveolar epithelial cells, which are divided further into type I and type II cells(Evans et al. 2010; Hippenstiel et al. 2006).Type I cells are responsible for gas exchange in the lung between the air space and the capillaries, and while making up only 33% of the total number of alveolar epithelial cells, cover 93% of the alveolar surface area. Alveolar type II cells, on the other hand, are the precursors for type I cells and account for 67% of the total number of epithelial cells present in the lung, but cover only 7% of the alveolar surface area (Dinis-Oliveira et al. 2008; Evans et al. 2010). These cells facilitate water and ion transport, and the surfactant proteins secreted by type II cells act as a chemical barrier against foreign environmental particulates and help prevent lung collapse (Dinis-Oliveira et al. 2008; Shek et al. 1994).

The respiratory epithelium represents an important mucosal barrier and provides mucociliary clearance via tiny cilia that brush bacteria and debris upwards via wave-like motions where material can be diverted to the gastrointestinal tract and digested (Evans et al. 2010; Knowles et al. 2002). During lung infection, however, inflammatory responses can lead to severe impairment and dysfunction of the barrier properties within the

endothelium and epithelium, leading to a disseminated, or systemic, response (Chow et al. 2003).

1.3.1 – The Innate Immune & Inflammatory Response

An effective host response against *P. aeruginosa* infection requires a variety of cell types and mediators in order to recognize, respond, and clear the bacteria from the airways (Sadikot et al. 2005). Almost immediately upon infectious insult, activation of the pulmonary endothelium and epithelium leads to the secretion of low-molecular weight signaling molecules, called cytokines (i.e. TNF-α, IL-1β, IL-6), which facilitate the activation of alveolar and interstitial macrophages; while a special class of cytokines with chemoattractant properties, called chemokines (i.e. IL-8), direct the transmigration of the activated macrophages into the alveolar space and sequester large amounts of neutrophils within the pulmonary microvasculature (Chow et al. 2003; Sadikot et al. 2005). Once recruited, these cells release a battery of cytotoxic and pro-inflammatory agents including proteolytic enzymes, reactive oxygen species, cationic proteins, lipid mediators, and more inflammatory cytokines (Chow et al. 2003).

The up-regulation of adhesion molecules is a step imperative to the inflammatory response, and allows activated macrophages and neutrophils to adhere tightly to the endothelial wall and move throughout the pulmonary epithelium (Chow et al. 2003; Sadikot et al. 2005). Currently, there are three recognized classes of adhesion molecules: integrins (α- and β- integrins), selectins (L-, P- and E-selectin), and the immunoglobulin superfamily of cell surface proteins (ICAM-1, VCAM-1) (Cavallaro et al. 2003). More specifically, intercellular adhesion molecule-1 (ICAM-1; CD54) is a transmembrane glycoprotein expressed constitutively on epithelial, endothelial and immune cells (Hogg et al. 1991); however, its expression increases during inflammation in response to

cytokines such as TNF-α, INF-γ, and IL-1(Dustin et al. 1988; Look et al. 1992). ICAM-1 is a ligand for the integrin LFA-1 (CD11a,b/CD18), that both facilitates leukocyte transmigration into tissues during inflammation and intercellular interactions (Krunkosky et al. 2000).

Responses to *P. aeruginosa* also involve innate chemical epithelial defense via the induction of low molecular weight antimicrobial peptides (Schroder et al. 1999), which demonstrate antimicrobial activity with a preference towards Gram-negative bacteria and yeasts (Schroder et al. 1999). These peptides are expressed almost immediately following contact with human epithelial cells, and function independently from leukocyte-mediated immune defense mechanisms (Schroder et al. 1999). In particular, human beta-defensin-2 (HBD-2) is a 5 kDa, highly cationic antimicrobial peptide produced on the surface of airway epithelial cells and within phagolysosomes in phagocytes, and functions to kill bacteria and fungi (Schroder et al. 1999).

1.3.2 – The Role of Nod-like Receptors

In addition to TLRs, the innate immune system also includes a class of pathogen recognition receptors called nucleotide binding and oligomerization domain-like receptors (NLRs) (Geddes et al. 2009). The human NLR family contains 23 known members to date, and is a large family of intracellular receptors that regulate both inflammation and apoptosis (Travassos et al. 2005). However, studies have only recently highlighted the importance of Nod1 and Nod2 in innate immune response and as a result they have become the best characterized members in the NLR family (Inohara et al. 2005). Where Nod1 recognizes diaminopimelic acid found in the peptidoglycan of many Gram-negative bacteria, Nod2 is more of a general sensor of bacteria and detects muramyl dipeptide present in the peptidoglycan of both Gram-positive and -negative

bacteria (Girardin et al. 2003). As well, recognition of *P. aeruginosa* during infection has been traditionally associated with TLRs, however, following internalization *P. aeruginosa* still elicits an inflammatory response supporting the idea that the cell would be equipped with an internal sensor (Plotkowski et al. 1999). This is believed to be due to activation of Nod1, which has been shown to play a critical role in the TLR-independent activation of NF-κB during infection with Gram-negative bacteria like *P. aeruginosa* (Travassos et al. 2005), however, the role of Nod2 during infection remains unknown.

1.3.3 – Oxidant generation in the Airspace

Large evidence supports the role of oxidants and oxidative injury in the pathogenesis of lung infections (Chow et al. 2003). Following migration into the lungs, macrophages and neutrophils generate reactive oxygen species (ROS) in response to inflammatory mediators (Chow et al. 2003; Rahman et al. 2006). In particular, leukocyte activation generates the $O_2^{\cdot}$- radical via the (NADPH) oxidase system; $O_2^{\cdot}$- is quickly converted into O_2 and H_2O_2 by superoxide dismutase (SOD) enzymes (Chow et al. 2003; Rahman et al. 2006). Phagocytic cells may also make use of other enzymes to produce ROS including myeloperoxidases (MPO) and eosinophil peroxidase (EPO) (Rahman et al. 2006). Eosinophils contribute to the oxidant burden significantly because these cells possess a greater capacity for $O_2^{\cdot}$- and H_2O_2 production compared to neutrophils, and the amount of EPO in eosinophils is 3 to 10 times higher than the amount of MPO present in neutrophils. Another potent oxidant of considerable importance, ·OH, is formed non-enzymatically in the presence of free iron (Fe^{2+}) via the Fenton reaction and immediately reacts with surrounding target molecules (Rahman et al. 2006). A diverse array of stimuli including LPS, cytokines, chemokines, complement fragments and lipid mediators are elevated during airway stress and stimulate neutrophil-produced ROS.

During the infectious process, *P. aeruginosa* induces ROS production within epithelial cells in a few ways. Following its secretion into the microenvironment, pyocyanin permeates the epithelial cell membrane and directly oxidizes intracellular pools of NADPH and glutathione, producing superoxide and downstream ROS (Rada et al. 2011). Recognition of *P. aeruginosa* LPS by the epithelial cells leads to ROS production through protein kinase C (PKC)-NADPH oxidase signaling pathway in human epithelial cells (Yan et al. 2008). Other potential sources of ROS are derived from the activated epithelium itself via induction of the mitochondrial electron transport chain, cytochrome P450, and xanthine oxidase. In case of mechanical ventilation, the introduction of excess oxygen can also fuel the production of ROS (Chow et al. 2003). In acute lung injury, however, stimulated phagocytes produce the majority of ROS (Ward 2010). Overwhelming oxidant injury may lead to alveolar collapse and extensive fibrotic scarring, impairing gas exchange between the affected airways and the capillary system (Ward 2010).

1.4 – Cellular Redox Balance

1.4.1 – Reactive Oxygen Species

Reactive oxygen and nitrogen species (ROS, RNS) refer to a large group of free radicals derived from superoxide ($O_2^{\cdot -}$) or nitric oxide (NO), respectively, and include O2·- and NO, as well as hydrogen peroxide (H_2O_2), the hydroxyl radical (·OH), hypochlorous acid (HOCl), peroxynitrite (ONOO), and ozone (O_3) (Park et al. 2009). Within the airway epithelium, ROS are formed endogenously through the reduction of molecular oxygen to water following mitochondrial electron transport as part of cellular

respiration (Ciencewicki et al. 2008; Gram 1997; Mak 2008; Rahman et al. 2006). As well, cellular enzymes including cyclooxygenases, lipoxygenases, peroxidases, cytochrome P450 oxidase and xanthine oxidase can be released by activated inflammatory cells (Ciencewicki et al. 2008; Gram 1997; Mak 2008; Rahman et al. 2006). At moderate levels ROS, however, are essential for maintaining cellular homeostasis and play an important role in regulating many signal transduction pathways, often acting as second messengers (Bilska et al. 2005; Droge et al. 1994; Winrow et al. 1993).

1.4.2 – Oxidative Stress

Oxidative stress refers to an imbalance in the redox status of the cell favouring an oxidizing environment. Extensive ROS production leads to the depletion of antioxidants and results in cellular damage. In particular, ROS can damage DNA strands by reacting with base pairs and the deoxyribose phosphate backbone of DNA, a main target of radical damage (Gram 1997). Without the protection of antioxidants, ROS can also initiate lipid peroxidation of polyunsaturated fatty acid components of cell membrane phospholipids, affecting cellular integrity (Rahman et al. 2006). Amino acids can also be damaged by ROS, leading to protein denaturation and enzyme deactivation (Gram 1997; Rahman et al. 2006). When left unmanaged, oxidative stress can eventually lead to cell death.

1.4.3 – Apoptosis

Apoptosis refers to programmed cell death essential for the natural process of clearing unwanted or excess cells and can occur via one of two pathways, i.e. the Type I (extrinsic) and Type II (intrinsic) pathways (Kuribayashi et al. 2006) (**Figure 1**). The

intrinsic pathway becomes activated following the loss of mitochondrial membrane potential causing the release of pro-apoptotic factors, like cytochrome *c*, from the inner membrane space into the cytoplasm. Upon the release of cytochrome *c*, intracellular cysteine proteases called caspases are activated which lead to the activation of downstream effector caspases-3 and -7 (Kuribayashi et al. 2006).

Similarly, activation of the death receptor Fas (FasR, CD95) also leads to apoptosis. Following activation, Fas/CD95 aggregates with the FADD adaptor protein, where procaspase-8 is recruited and cleaved in a signaling cascade ultimately leading to the activation of caspases-3 and -7 (Barnhart et al. 2003).

1.4.4 – Antioxidants

In order to cope with oxidant attack, intricately equipped antioxidant systems provide a mechanism to detoxify and maintain lung redox balance *in vivo*. Currently, there are two recognized classes of antioxidants: enzymatic and non-enzymatic. Enzymatic antioxidants degrade ROS to less toxic molecules, and include catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, and the thioredoxins (Mak 2008; Rahman et al. 2006). Non-enzymatic antioxidants can interact directly with ROS to regulate their levels, and are regulated by feedback mechanisms such that balanced levels of both antioxidants and ROS are maintained in the cell (Mak 2008; Rahman et al. 2006). Examples include ascorbic acid, α-tocopherol, β-carotene, melatonin, and low molecular-weight thiol-containing compounds (i.e. the reduced form of glutathione) (Mak 2008; Rahman et al. 2006).

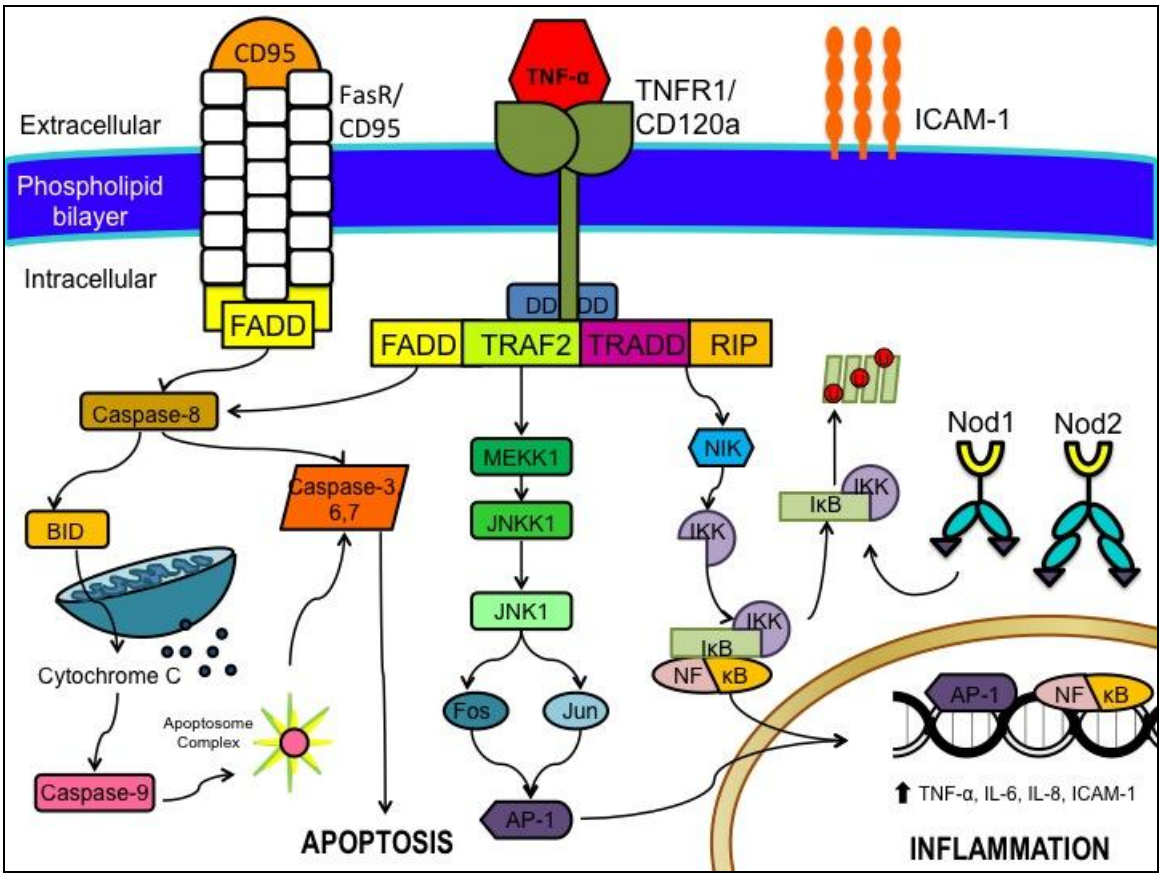

Figure 1. The Intrinsic and Extrinsic Pathways of Apoptosis. The extrinsic apoptosis pathway is triggered by the activation of death receptors which initiate caspase-8 activation. Caspase-8, in turn, activates caspases-3 and -7leading to apoptosis. In the intrinsic apoptosis pathway, apoptotic stimuli trigger the release of cytochrome *c* from the mitochondria independent of caspase-8, leading to the formation of the apoptosome complex and activate caspases-3 and -7.

1.4.5 – Glutathione

Glutathione (GSH), a ubiquitous thiol-containing tripeptide (L-γ-glutamyl-L-cysteinyl-glycine), is the most important antioxidant in terms of ROS detoxification and plays a vital role in protecting the airspaces and epithelial cells against oxidants in the extracellular milieu (Droge et al. 1994; Rahman et al. 2000). Compared to plasma levels

that typically range from 1-10 mM, GSH is present in greater concentration in the epithelial lining fluid and is also involved in maintaining lung epithelial cell membrane integrity against the deleterious effects of ROS (Haddad 2004; Rahman 2005; Rahman et al. 2000). Depletion of GSH in epithelial cells is associated with loss of barrier function and increased permeability (Li et al. 1994; Li et al. 1996).

The synthesis of GSH requires the enzymes γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase as well as the amino acids glycine, cysteine, and glutamic acid. The GSH tripeptide is formed by the consecutive actions of γ-GCS and GSH synthetase (Rahman et al. 2000). Acting either intra- or extracellularly, GSH scavenges lipid peroxidases, H_2O_2, and other radicals in a reaction catalyzed by glutathione peroxidase (GPx). Ultimately, this reaction generates an oxidized form of GSH called glutathione disulphide (GSSG). Once formed, GSSG can then be reduced back into GSH by the enzyme GSH reductase (GSR) as part of the GSH redox couple. Alternatively, resynthesis of GSH may also take place on the outer cell surface via γ-glutamyl transpeptidase (γ-GT), an enzyme found in the plasma membrane and, in particular, on lung epithelial cells. γ-GT cleaves extracellular GSH into a γ-glutamyl moiety along with its constituent amino acids and is transferred to an appropriate amino acid receptor where they are transported into the cell and salvaged into reformed GSH. It has also been suggested that plasma GSH may undergo direct uptake into type II epithelial cells but the mechanism remains controversial (Deneke et al. 1989; Rahman et al. 1996).

1.5 – Resveratrol

Resveratrol (*trans*-3,4‟,5-trihydroxystilbene) is a non-flavanoid, polyphenolic compound found in a variety of plant species present in the human diet, and specifically can be found in relatively at high concentrations in grapes (*Vitis* spp.), berries (*Vaccinium* spp.), and peanuts (*Arachis* spp.) (Wood et al. 2010). Known to be an active constituent in at least 72 plant species, resveratrol is also a phytoalexin, synthesized in response to fungal, viral, and bacterial attacks, and/or exposure to ultraviolet (UV) radiation (Ignatowicz et al. 2001; Pervaiz 2003; G. J. Soleas et al. 2001; Wood et al. 2010). Recently, resveratrol has been of intense focus in a number of studies, demonstrating an array of beneficial effects in diseases of nervous, gastrointestinal, and respiratory systems, and it has also been shown to have cardio-protective and anti-cancer properties (Fremont 2000; Pervaiz 2003).

1.5.1 – Structure & Synthesis

Resveratrol is made up of two phenol rings linked by a styrene double bond, with *cis*- and *trans*-isomersization facilitated by UV exposure (**Figure 2**) (Wood et al. 2010). Of these two isomers, trans-resveratrol displays greater steric stability when protected from high pH and light, and for this reason much less is known about the pharmacological effects of the *cis*-resveratrol isomer (Leiro et al. 2004).

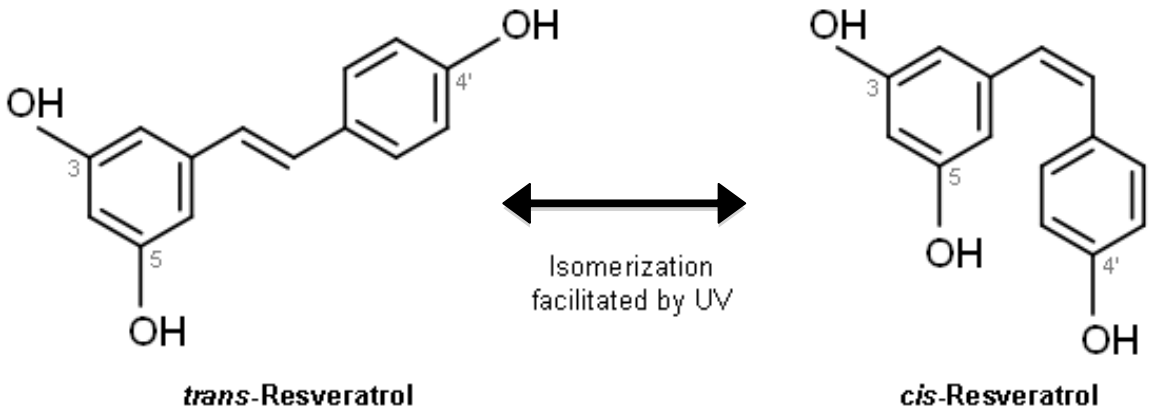

Figure 2.The structure of resveratrol in both of its isometric forms.

Synthesis of resveratrol takes place in the leaf epidermis and stalks of the plant, as well as the kernels and skin (pericarp) of the grape berries, but not in the flesh itself (Creasy 1988; Langcake 1976). The amount of resveratrol in the skin of fresh grapes is heavily dependent upon the species variety, but typically ranges from 50-100µg/g (Jeandet 1991). The higher content of the stilbene in red wines is a consequence of longer contact time (several days) between the berry skin and the must, compared to white wine where the must is immediately removed from the berry residue (Daniel et al. 1999). Consequently, the concentration of resveratrol in red wines is in the range of 1.5-3mg/L (Goldberg 1995; Romero-Perez et al. 1996), with lower levels also being found in white and rose wines (Romero-Perez et al. 1996; Vrhovsek et al. 1995). In grape juice, resveratrol is found in substantially lower amounts, typically ranging from 3-15 µg/L (G. Soleas et al. 1995).

Indeed, the consumption of resveratrol in many red wines is believed to be linked to the paradoxical observation of low mortality rates due to coronary heart disease in France despite a high incidence of risk factors including smoking, a diet rich in saturated fats, and a general lack of exercise (Soleas et al. 1997b). This phenomenon is referred to

as the “French paradox”, which suggests that moderate amounts of red wine (consuming 150-300 mL/day) is the root of this paradox (Renaud et al. 1992). The possible mechanisms for resveratrol cardioprotective activity involve its effect on lipid metabolism and platelet function, and the inflammatory mediators, alteration of eicosanoid synthesis, or inhibition of activated immune cells, such as neutrophils and monocytes/macrophages (Hao et al. 2004; Ignatowicz et al. 2001).

1.5.2 – Pharmacokinetics & Bioavailability

To date, much of the information surrounding the pharmacokinetics and bioavailability of resveratrol is inconclusive and contradictory. *In vivo* studies using mice, rats, and dog models, however, suggest that resveratrol is absorbed satisfactorily in the gastrointestinal tract, with significant concentrations being found in the blood and a number of internal organs (Bertelli et al. 2001). The molecule itself has a relatively short half-life of approximately 8-14 minutes (Baur et al. 2006; Marier et al. 2002) and is rapidly metabolized by extensive first-pass metabolism where it becomes sulphated in the liver and intestinal epithelial cell (Bertelli et al. 2001; Goldberg 1995; Soleas et al. 2001; Vitrac et al. 2003; Yu et al. 2009). Indeed, this process may be important for the biological effects of resveratrol as studies by Kaldas et al. (2003), which showed that sulphate conjugation was imperative for resveratrol transport in the human intestinal epithelial cell line Caco-2. Moreover, resveratrol administered intravenously was found to be converted into a sulphate conjugate within 30 minutes in humans (Walle et al. 2004). The same study also found that the serum half-life of total resveratrol was ~9.2 hours, suggesting that exposure to modified forms of resveratrol may be higher compared to the original molecule.

In another study of resveratrol oral bioavailability an average of 77.6 μM resveratrol was measured in the urine of healthy volunteers following the intake of a 25mg (110 μM) resveratrol supplement (Walle et al. 2004), suggesting that much of the resveratrol taken up orally is excreted from the body. Therefore, it is more reasonable to suggest that in terms of our study, and at a clinical level, the doses of resveratrol needed to exert the effects here would require alternate routes of administration.

New therapeutic strategies are currently under development to help bypass metabolic breakdown of resveratrol. Resveratrol-containing lozenges, for example, have been found to increase buccal absorption of resveratrol (Asensi et al. 2002), and administration of resveratrol via injection may be another potential therapeutic tool in bypassing intestinal metabolism (Lu et al. 2009). Nanoparticles and liposome use are also being investigated as potential carriers of resveratrol (Santos et al. 2011).

1.5.3 – Antioxidant Properties

As previously discussed, an important relationship exists between ROS and the development of oxidative stress. Polyphenolic compounds are known to boast a variety of antioxidant effects stemming the redox properties associated with their phenolic hydroxyl groups and the resulting potential for electron delocalization within the chemical structure (Alarcon de la Lastra et al. 2007; Lopez-Velez et al. 2003). Current evidence suggests that in addition to acting as a free radical scavenger (Soleas et al. 1997a), resveratrol promotes the activity of several endogenous antioxidant defense enzymes. Indeed, in the non-small-cell lung carcinoma cell line, A549, resveratrol was shown to induce transcription of GPx, thioredoxin reductase (TrxR), and mitochondrial SOD (Hu et al. 2007).

1.5.4 – Anti-inflammatory Properties

Many of the beneficial effects reported for resveratrol are attributed to an anti-inflammatory effect observed in relation to a variety of inflammatory diseases. During inflammation, vascular permeability allows for the extravasation of leukocytes and inflammatory mediators out of capillaries and into the tissue. Resveratrol, however, has been shown to reduce vascular leakage induced by TNF-α (Fulgenzi et al. 2001). Resveratrol has also been found to down-regulate cytokine release in lung epithelial A549 cells, including IL-8 and GM-CSF (Donnelly et al. 2004). This is partially due to the fact that resveratrol can modulate activity of specific transcription factors, including nuclear factor-kappa (NF-κB) and activator protein (AP)-1, associated with inflammation (Donnelly et al. 2004; Wood et al. 2010).

Other potential anti-inflammatory effects of resveratrol include inhibition of synthesis and release of pro-inflammatory mediators, modification of eicosanoid synthesis, inhibition of active immune cells, and inflammatory enzymes such as iNOS and COX-2 through its inhibitory effect on NF-κB and AP-1 (Alarcon de la Lastra et al. 2007). Although the effects of resveratrol have been studied in several models of inflammation including LPS stimulation (Ferrero et al. 1998; Manna et al. 2000), viral infections, and allergies (Wood et al. 2010), no studies have explored its effects on inflammation in a model of bacterial infection.

2 – Objectives

Rationale:

- *Pseudomonas aeruginosa* causes serious lung infection in immunosuppressed patients.
- Infection with *P. aeruginosa* will increase ROS production and inflammatory markers in infected lung epithelial cells *in vitro*.
- During the infectious process, oxidative stress and inflammation are interrelated and therefore a decrease in ROS levels by antioxidants will have an inhibitory effect on inflammatory pathways.

Research Hypothesis

Resveratrol will demonstrate anti-inflammatory effects in a model of inflammation caused by *P. aeruginosa* as a result of its antioxidant properties.

Objectives:

1. Determine an appropriate range of resveratrol pre-treatment conditions for an epithelial cell line to assess its effect in an *in vitro* model of *P. aeruginosa* infection. This can be done by measuring cell viability following pre-treatment with resveratrol.

2. Determine which concentration of resveratrol demonstrates the strongest antioxidant effect by the assessing intracellular ROS-production resulting from *P. aeruginosa*-induced oxidative stress.

3. Examine the anti-apoptotic effect of resveratrol.

4. Examine the anti-inflammatory effect of resveratrol.

5. Examine the effect of resveratrol on select molecular markers of the cellular antioxidant and innate immune systems.

3 – Materials and Methods

3.1 – Cell Lines and Bacterial Strains

The A549 human type II alveolar cell line (ATCC # CCL-185) was used between passage numbers of 5-15. A549 cells were maintained in Dulbecco‟s modified Eagle‟s medium (DMEM) nutrient mixture F-12 Ham (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% heat inactivated fetal bovine serum (FBS, SAFC Biosciences, Lenexa, KS) and 1% L-glutamine (Gibco, Carlsbad, CA) without antibiotics. Cells were grown at 37°C with 5% CO_2 and seeded every 2-3 days when confluency neared 80%. A549 cells were seeded in T-25 cm^2 flasks (Corning Incorporated, NY, USA) for 48 hours, which corresponded approximately to 2.4×10^6 cells/flask, unless otherwise stated.

Pseudomonas aeruginosa strain K (PAK) was kindly provided by Dr. RJ Irvin, University of Alberta, Edmonton. Bacterial cultures were maintained on sterile Luria Burtani (LB) medium (Fischer Scientific, Fair Lawn, NJ) with 1% agar (LBA). Unless otherwise stated, infection times were used based on experimental conditions previously optimized in our lab. Generally, shorter infection times (i.e. 4 hours or less) were used when measuring markers involved in cellular signaling, while longer infection times (i.e. 18 hours) were needed when measuring protein structures that required more time for actual assemblage.

3.2 – Preparation of *P. aeruginosa* for experiments

A single colony of PAK was grown overnight in sterile LB medium at 37°C on a shaking platform at 150 rpm. Cultures were diluted by a factor of 20 into fresh LB medium and allowed to grow for approximately 1 hour or until mid-log phase when optical density at 600 nm (OD_{600}) reached 0.3. Bacteria were then centrifuged at 3500 ×

g, for 20 minutes at 4°C and washed twice in sterile phosphate-buffered saline (PBS, pH 7.4). Following the final resuspension, bacteria were diluted to an OD_{600} of 0.30 in sterile serum-free DMEM F-12 Ham corresponding approximately to 2×10^8 CFU/mL as confirmed by serial dilutions and drop plating on LBA. From this stock, bacteria were added to cells at a multiplicity of infection (MOI) of either 50:1 or 100:1.

3.3 – Pre-treatment with resveratrol

A549 cells were grown in sterile serum-supplemented media for 48 hours to achieve 80% confluency. The medium was removed and the cells were washed with sterile PBS. *Trans*-Resveratrol (Sigma-Aldrich, Oakville, ON) was dissolved in 95% ethanol to produce a 60 mM stock solution and then was added to adherent A549 cells in serum-free media to achieve 100 µM, which was deemed to exert the strongest antioxidant effect after preliminary optimizational studies. The cells were incubated in the presence of resveratrol for the indicated time (37°C, 5% CO_2), and then washed twice with PBS, and used for experiments. Treatment with resveratrol did not have an effect on cell viability as determined using the Trypan blue exclusion assay run either with the ViCell XR Cell Viability Analyzer (Beckman Coulter, Mississauga, ON, CA) or by manual counting using a Nikon eclipse 50i phase-contrast microscope (Nikon Canada, Winnipeg, CA) and a Phase Micro 3200 Bright-Line hemocytometer (Hausser Scientific, Horsham, PA, USA). Resveratrol stock solutions were stored at -20°C in the dark.

3.4 – Trypan Blue Exclusion Assay of Cell Viability

The Trypan blue exclusion method was used to measure both viability and number of A549 cells; it is based on the principle that in dead or dying cells a loss of membrane integrity allows for entry of the stain. Viable cells are seen in a microscope

with a clear cytoplasm while membrane-compromised cells have a distinct blue colour in the cytoplasm. Viable cell counts were performed via the Trypan Blue Exclusion assay using a Vi-Cell XR Cell Viability Analyzer (Beckman Coulter), wherein 0.6 mL of diluted cell suspensions were mixed with Trypan Blue reagent. Cell counts were automatically assessed via 50 individual sub-samples measured using the Vi-Cell XR Cell Viability Analyzer software, and the total viable cell count was used when passaging or standardizing cell samples for experimentation.

3.5 – Reactive oxygen species Indicator Assay

To detect intracellular ROS, we used CM-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester), a fluorescent due that diffuses passively through the cell membrane and is retained in viable cells. As oxidation occurs in the cell, esterase cleavage of the lipophilic blocking groups yields a charged form of the dye that is more effectively retained in the cell compared to the parent compound. As well, the thiol-reactive chloromethyl deriviative of H_2DCFDA used here boasts enhanced covalent binding to intracellular components, permitting even longer retention of the fluorescent adduct within the cell (as described by Invitrogen).

To measure intracellular ROS production, A549 cells were seeded onto flat-bottom 6 well plates (Corning) and grown until 80% confluency. Adherent cells were pre-treated with resveratrol as previously described and/or infected with *P. aeruginosa* at a MOI of 50:1 or 100:1 for 1 hour as these conditions had been previously optimized in our lab (Hawdon et al. 2010). Cells were washed and stained with 500 µL of 100 µM CM-H_2DCFDA (Invitrogen Molecular Probes, Eugene, OR), and incubated for 30 minutes at 37°C in the dark, following the manufacturer‟s protocol. After incubation,

cells were washed with sterile 37°C PBS and 1 mL of fresh serum-free media was added, followed by another incubation for 30 minutes at 37ºC in the dark. Stained cells were detached from the plate surface using disposable sterile cell scrapers (Fisher Scientific), washed twice, and resuspended in 500 µL PBS followed by flow cytometric analysis. The BD FACSCalibur Flow Cytometer (BD Biosciences) together with BD CellQuest Pro Software were used in the assessment of fluorescent probes via flow cytometry. For each experiment a minimum of 10,000 gated events were acquired per trial. The mean fluorescent intensity (MFI) in the FL-1 Channel was deemed to be directly proportional to levels of intracellular ROS.

3.6 – Measurement of ICAM-1 Expression

Confluent A549 cells were pre-treated with resveratrol as described, and either stimulated for 18 hours with 10 ng/mL TNF-α or infected with *P. aeruginosa* at a MOI of 50:1 for 1 hour followed by incubation with medium containing 10 µg/mL polymyxin B (Sigma-Aldrich) for 17 hours to kill the bacteria as per previously established methods (Hawdon et al. 2010). The expression of ICAM-1 was determined using immunostaining with phycoerythrin (PE)-conjugated monoclonal antibody (mAb) against ICAM-1 (Mouse anti-human CD54, BD Pharmingen, Mississauga, ON). After 18-hour long incubation, cells were washed and detached using 0.5% Trypsin-EDTA (Gibco, Eugene, OR). The cells were resuspended in 100 µL of 0.1% BSA-PBS containing antibodies at a dilution of 1:50 and incubated for 1 hour at 4°C. Following incubation, cells were washed twice with PBS and analyzed by flow cytometry (FL-2 channel). The data were expressed as relative mean fluorescence intensity (MFI), which was calculated by normalizing all fluorescence values to the untreated, labeled control.

3.7 – Active Caspases-3/7-Based Apoptosis Detection Assay

For analysis of apoptosis, adherent A549 cells were infected with *P. aeruginosa* at an MOI of 50:1 for 2 hours, then detached and washed as described above. Apoptosis was measured by the presence of active caspase-3 and caspase-7, detected by the CaspaTag caspase-3,7 *in situ* assay kit (Chemicon International, Temecula, CA) according to the manufacturer‟s protocol and previously established methods (Hawdon et al. 2010). In this approach, a cell-permeable, carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor, fluorochrome inhibitors of caspases (FLICA), covalently binds to a reactive cysteine residue that resides on the large subunit of the active caspase heterodimer, thereby inhibiting further enzymatic activity. The bound labeled reagent is retained within the cell, while any unbound reagent diffuses out of the cell and is washed away. The resulting green fluorescent signal is measured via flow cytometry on the FL-1 channel. The data were expressed as % caspase-positive cells, with gating of cell populations based on their forward and side scatter properties.

3.8 – Measurement of Fas Receptor Expression

Confluent cells were infected with *P. aeruginosa* at a MOI of 50:1 for 1 hour followed by incubation with medium containing 10 µg/mL polymyxin B (Sigma-Aldrich) for 17 hours to kill the bacteria, following the infection conditions outlined above. The cell-surface expression of FasR was determined using immunostaining with FITC mAb against FasR (Mouse anti-human CD95, BD Pharmingen, Mississauga, ON). After infection, cells were washed and detached using 0.5% Trypsin-EDTA (Gibco, Eugene, OR). The cells were resuspended in 100 µL of 0.1% BSA-PBS containing antibodies at a dilution of 1:50 and incubated for 18 hours at 4°C. Following incubation, cells were washed twice with PBS and analyzed by flow cytometry (FL-1 channel). The data were

expressed as relative mean fluorescence intensity (MFI), which was calculated by normalizing all fluorescence values to the untreated, labeled control.

3.9 – Total RNA Isolation

A549 cells were seeded onto T-25 cm^2 tissue culture flasks, grown for 48 hours to ~80% confluency, and incubated in serum- and antibiotic-free medium in the presence of 100 μM resveratrol for 5 hours, followed by infection with *P. aeruginosa* at a MOI of 50:1 in serum-free media for 3 hours. This infection time for our model was based on optimization studies showing a stronger effect on gene expression levels compared to longer or shorter infection times. Adherent cells were washed once with sterile 37°C PBS and detached via trypsinization. Cells were collected and centrifuged at 1000 × *g* for 5 min at 4°C, resuspended in 1.5 mL PBS and placed on ice. RNA isolation was performed using the Aurum Total RNA Mini kit (Bio-Rad) in accordance with the manufacturer‟s instructions using certified RNase-free barrier tips (Ambion, Foster City, CA, USA). Cells were lysed using the provided lysis buffer and total RNA was extracted using a silica membrane spin column placed within RNase-free microfuge tubes. To avoid contamination by genomic DNA, salts, and other cellular protein components, DNase I digest was added to the columns at room temperature followed by several washes. A volume of 40 μL of low ionic strength elution buffer was used to collect the pure RNA from the column. Of the total extracted volume, 5μL was placed into RNase-free 100μL tubes and used immediately to quantify extracted RNA while the remaining aliquots were stored at -80°C for further use.

3.10 – RNA Quantification and Purity Analysis

The concentration and integrity of extracted RNA was assessed using the Experion RNA StdSens kit (Bio-Rad) on an Experion Automated Electrophoresis Station (Bio-Rad) supported by Experion software (Bio-Rad) in accordance with the manufacturer‟s instructions. Briefly, 1µL aliquots of denatured RNA samples and ladder were loaded onto an Experion RNA StdSens chip where 18 and 28 S rRNA peaks were measured. Only high-quality (i.e. 9+ RNA quality index, as per automatic Experion software calculation) RNA samples were used for subsequent gene expression analysis.

3.11 – cDNA Synthesis

First strand complementary DNA (cDNA) synthesis reactions were performed using the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, Flamborough, ON, Canada) in accordance with the manufacturer‟s instructions. To help preserve the quality of the RNA during cDNA synthesis the kit contained two important features: a reverse transcriptase with a point mutation that prevents RNA hydrolysis by RNase H, and RNase inhibitor to further protect against RNA degradation. Briefly, RNA samples were mixed with oligo(dT)18 primers and the other kit components and incubated at 42°C for 1 hour before terminating the reaction by heating at 70°C for 5 minutes. The resultant products were either stored on ice and used immediately or placed at -20°C for short-term storage.

3.12 – Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time PCR (qPCR) was performed using primers for several genes as noted in Table 1 (SABiosciences, Frederick, MD, USA) and SYBR Green RT^2 qPCR Master Mix (SABiosciences) with iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) in accordance with the manufacturer‟s instructions. Briefly, 8 µL of

nuclease-free ddH_2O was pipetted into the wells of a sterile 96-well PCR plate (Bio-Rad), followed by 1 µL of primer, and 1 µL of cDNA template. Due to its photosensitive nature, 10 µL of the 2× Master Mix was added to the wells last and the plate was sealed using Microseal „B" Film (Bio-Rad). Following iQ5 calibration, qPCR analysis of triplicate samples was performed using a two-step cycling program involving an initial single cycle of 95°C for 10 minutes to activate the DNA polymerase, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Following the qPCR reaction, a first derivative dissociation curve was performed as a quality control measure. Briefly, the reaction was heated to 95°C for 1 min, cooled to 65°C for 2 min, then ramped from 65 to 95°C at a rate of 2°C per minute. The formation of a single peak at temperatures greater than 80°C indicated the presence of a single PCR product in the reaction mixture. Gene expression was normalized to the reference gene GAPDH, which was found to be more consistent than β2-microglobulin.

Gene name	UniGene #	RefSeq Accession #	Band Size (bp)	Reference Position	Source
NOD1	Hs.405153	NM_006092	144	4060	SABiosciences
NOD2	Hs.592072	NM_022162	141	4424	SABiosciences
Human β-defensin 2	Hs.105924	NM_004942	92	170	SABiosciences
Human Glutathione peroxidase 1	Hs.76686	NM_000581	169	772	SABiosciences
Human GAPDH	Hs.592355	NM_002046	175	1287	SABiosciences
β2-microglobulin	Hs.534255	NM_004048	114	381	SABiosciences

Table 1.Quantitative real-time PCR primers. These primer sets were used as per manufacturer‟s instructions, as described above.

3.13 – Statistical Analysis

Data were presented as mean ± standard error of the mean (SEM) and are representative of n ≥ 3 independent experiments. In studies involving gene expression analysis, comparison of sample means was completed using one-way ANOVA with post hoc Tukey‟s test, otherwise one-tailed Student‟s t-test was applied. A p-value < 0.05 was considered significant. GraphPad Prism 5.01 (GraphPad Software Inc, San Diego, CA) was used for the analysis. The use of asterisks denotes significant according to the following scheme: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4 – Results

4.1 – Resveratrol has no effect on A549 cell viability, but is sensitive to serum

To optimize the conditions of resveratrol pretreatment, A549 cells were treated with 5, 10, 50, 100 µM resveratrol for 1, 4, 5, 12, and 24 hours. Following the treatment, cell viability was assessed using the Trypan blue exclusion assay either through automated trypan blue staining (**Figure 3**) or manual hemocytometer counting (**Figure 4**) to ensure accuracy of both techniques. For all time points and concentrations used, viability of A549 cells remained above 90% suggesting that resveratrol exerted no harmful or cytotoxic effects.

To further optimize our model, A549 cells were pre-treated with resveratrol in serum-containing and serum-free media (**Figure 5**). In our experiments, we observed a significant increase in intracellular ROS levels in A549 cells following 1 hour of PAK infection. Resveratrol pre-treatment in serum-containing media caused a significant decrease in ROS produced by A549 cells, while cells pre-treated in serum-free media displayed an even stronger decrease in ROS production. Indeed, serum-free pre-treatment with resveratrol resulted in a 50% decrease in ROS produced by A549 cells. For this reason subsequent pre-treatments were carried out in serum-free conditions.

4.2 – Resveratrol down-regulates *P. aeruginosa*-induced oxidative stress

ROS production by infected cells have been implicated in both inflammation and tissue damage during *P. aeruginosa* infection (Ciofu et al. 2005). In agreement with previous findings in our lab (Hawdon et al. 2010), we observed a significant increase in ROS levels in A549 cells (**Figures 6 & 7**) following 1 hour of PAK infection. Resveratrol pre-treatment for 4 hours at concentrations of 100 µM and 200 µM, but not

10 μM caused a significant decrease in post-infection ROS production in A549 cells (**Figure 6**). A stronger inhibitory effect towards ROS production, however, was noted after 5 hours of resveratrol pre-treatment (**Figure 7**). As well, an increased MOI of 100:1 was found to yield increased resolution in terms of ROS production. These findings suggest that under the conditions of *P. aeruginosa* infection, resveratrol pre-treatment was able to protect A549 cells from oxidative stress.

4.3 – Resveratrol down-regulates *P. aeruginosa*-induced apoptosis in A549 cells

P. aeruginosa can induce apoptosis of infected airway epithelial cells via activation of the pro-apoptotic death receptor Fas/CD95 (Grassme et al. 2000) in a caspase-8 dependent mechanism culminating in the activation of executioner caspases-3 and -7 (Lakhani et al. 2006; Mizuta et al. 2008). Thus, surface expression of the death receptor Fas/CD95,and levels of active caspases-3 and -7 were used as measures of apoptosis. Following methods previously optimized in our lab (Hawdon et al. 2010), surface expression of Fas/CD95 was up-regulated after 1 hour of PAK infection with a 17 hour polymyxin B co-incubation (**Figure 8**). After 2 hours, infected A549 cells contained significant levels of active caspases-3/7 (**Figure 9**). Resveratrol pre-treatment prior to infection caused a significant decrease in both Fas/CD95 surface expression (**Figure 8**) and caspase activity (**Figure 9**). Hence, our results show that pre-treatment of A549 cells with resveratrol can decreased apoptosis of cells caused by *P. aeruginosa*.

4.4 – Resveratrol regulates ICAM-1 surface expression on A549 cells infected with *P. aeruginosa*

Under conditions of inflammation and oxidative stress during *P. aeruginosa* infection, airways epithelial cells increase the expression of cellular adhesion molecules, like ICAM-1, that can facilitate leukocyte adhesion (Aldallal et al. 2002; Hawdon et al. 2010). In our experiments, 1 hour *P. aeruginosa* infection with 17 hour polymyxin B co-incubation caused a significant increase in surface ICAM-1 expression on A549 cells (**Figure 10**). As a positive control, A549 cells were also stimulated with TNF-α, a known inducer of ICAM-1 expression on airway epithelial cells (Krunkosky et al. 2000), at a concentration of 10 ng/mL for 18 hours. Resveratrol pre-treatment prior to *P. aeruginosa* infection or TNF-α stimulation caused a significant decrease in ICAM-1 expression. These findings suggest that infection with *P. aeruginosa* may stimulate inflammatory responses, like ICAM-1 expression, following a similar mechanism as TNF-α and that resveratrol down-regulates these inflammatory responses.

4.5 – Resveratrol up-regulates mRNA expression of glutathione peroxidase

Oxidative stress has been shown to retard antioxidant activity of enzymes like glutathione peroxidase (GPx), which is used by glutathione to catalyze the reaction of hydrogen peroxide into water (Rahman et al. 2000). Thus, our next goal was to determine if resveratrol could indirectly boost cellular antioxidant defense via the effect on GPx gene expression. No change in GPx expression was observed in A549 cells infected for 3 hours with *P. aeruginosa* (**Figure 11**). However, GPx gene expression was significantly up-regulated in both untreated control and resveratrol-pre-treated cells. Hence, our data indicate that pre-treatment of *P. aeruginosa*-infected A549 cells with resveratrol can increase cellular antioxidant defense.

4.6 – Resveratrol down-regulates mRNA expression of antimicrobial peptides

Expression of the antimicrobial peptide, human beta-defensin-2 (HBD-2), can be rapidly induced during a PAK-stimulated inflammatory response (Diamond et al. 2000; Harder et al. 2000; Huang et al. 2007; Wehkamp et al. 2006). Indeed, we observed a significant increase in HBD-2 mRNA expression in A549 cells following 3 hours of infection with *P. aeruginosa* (**Figure 12**). Resveratrol pre-treatment caused significant decrease in HBD-2 mRNA transcription post-infection in A549 cells, suggesting that resveratrol may also impact innate immunity during infection.

4.7 – Resveratrol up-regulates mRNA expression of intracellular pathogen recognition receptors

Nod-like receptors, including Nod1 and Nod2, are intracellular pattern recognition receptors that regulate inflammatory and innate immune responses by responding to specific peptidoglycan motifs present in the bacterial cell wall (Delbridge et al. 2007). Nod1 and Nod2 respond to specific peptidoglycan motifs within the bacterial cell in a fashion similar to Toll-like receptor (TLR)-mediated cellular activation.

In our experiments, infection with *P. aeruginosa* did not have any noticeable effect on Nod1 and Nod2 mRNA expression (**Figure 13 & 14**). Pre-treatment with resveratrol prior to infection was found to induce increases in both Nod1 and Nod2 mRNA expression. While the effect of resveratrol on Nod-like receptor activity had not been reported previously, it appears that resveratrol may modulate the expression of these receptors during *P. aeruginosa* infection.

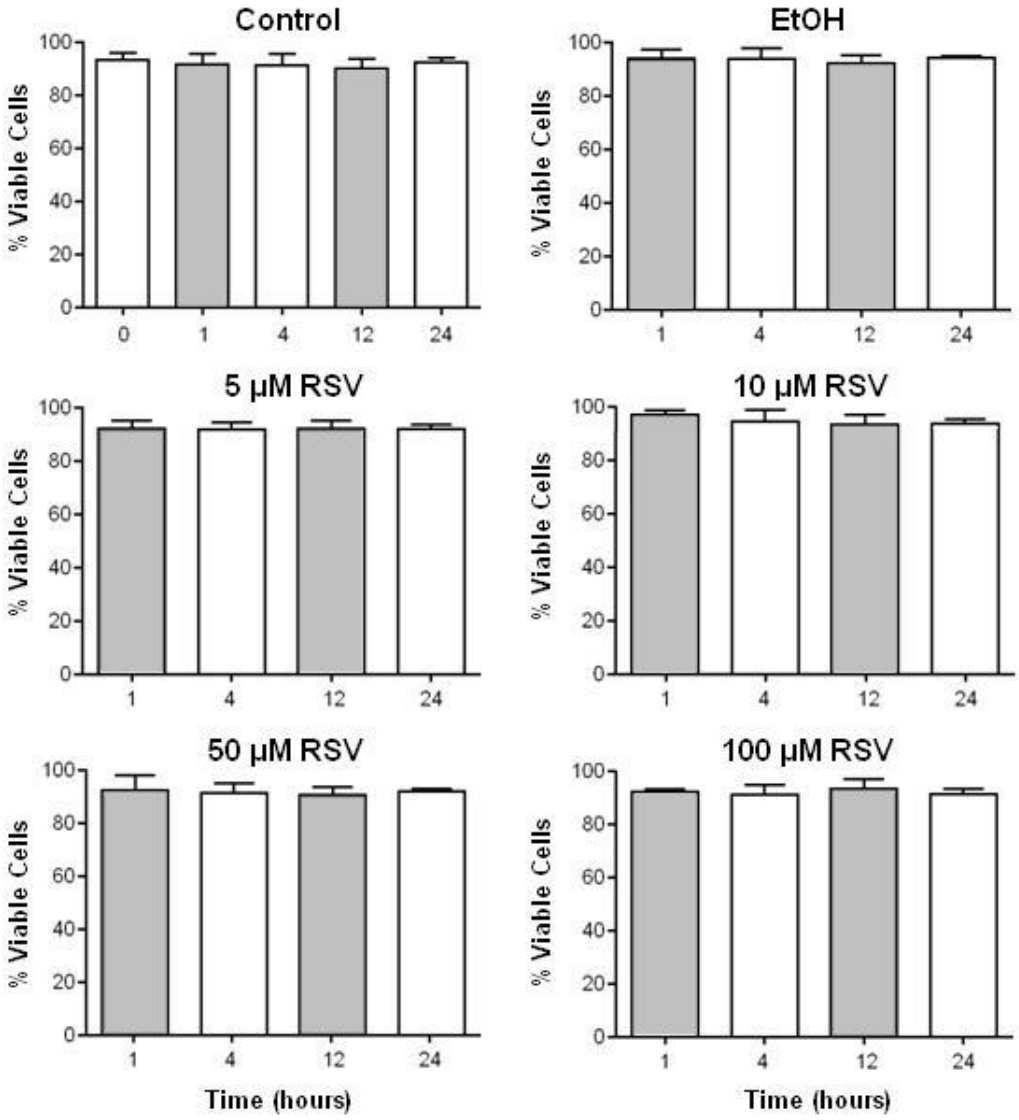

Figure 3. Effect of resveratrol on A549 cell viability. Viability of A549 cells was assessed via automated Trypan blue assay and counting. Cells were seeded onto 6-well plates, grown overnight to ~80% confluence, and incubated in serum- and antibiotic-free medium in the presence of resveratrol for 1, 4, 12 or 24 hours. Bars represent mean ± SEM of 3 independent experiments. No statistical significance between samples was found.

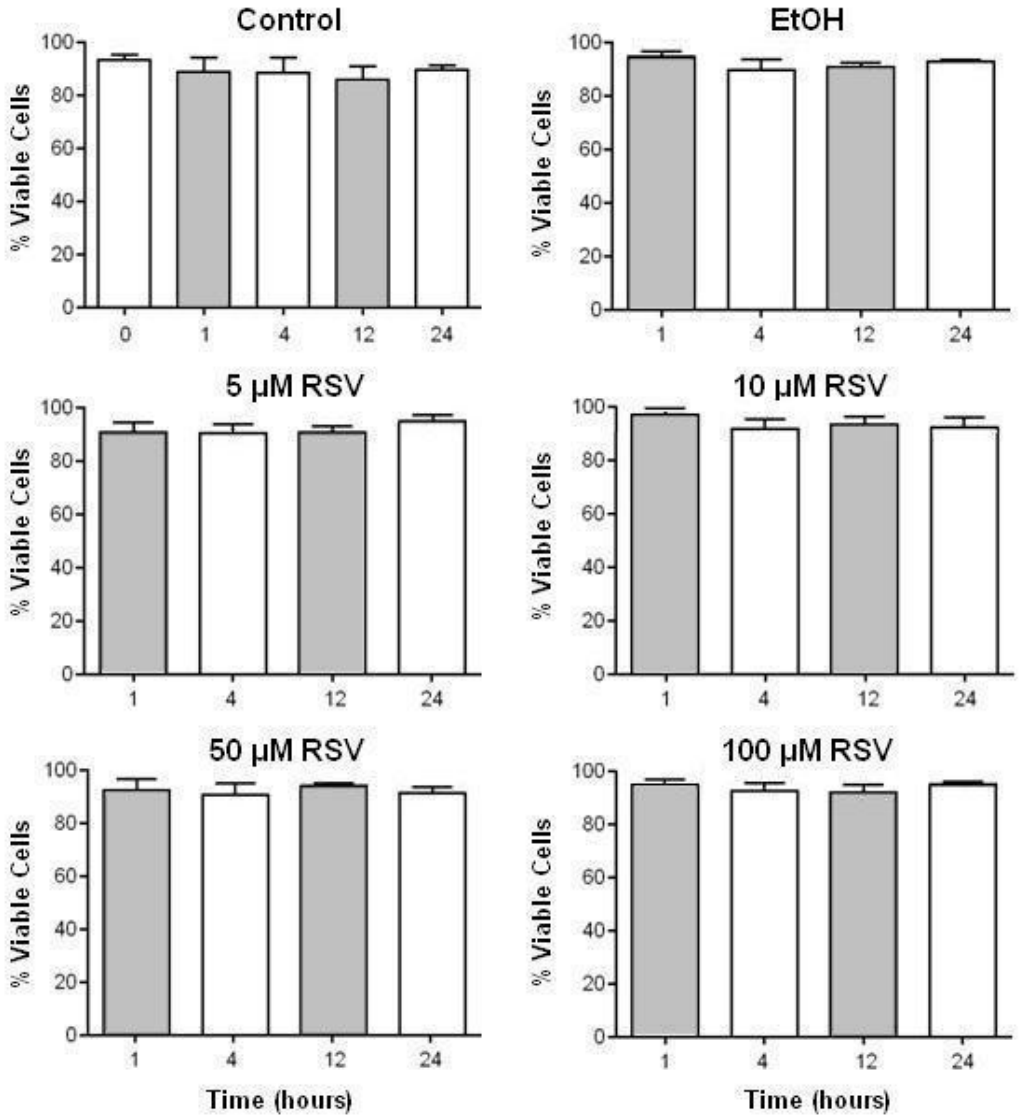

Figure 4. Effect of resveratrol on A549 cell viability. Viability of A549 cells was assessed via Trypan blue assay and manual counting with a haemocytometer. Cells were seeded onto 6-well plates, grown overnight to ~80% confluence, and incubated in serum-free medium in the presence of resveratrol for 1, 4, 12 or 24 hours. Bars represent mean ± SEM of 3 independent experiments. No statistical significance between samples was found.

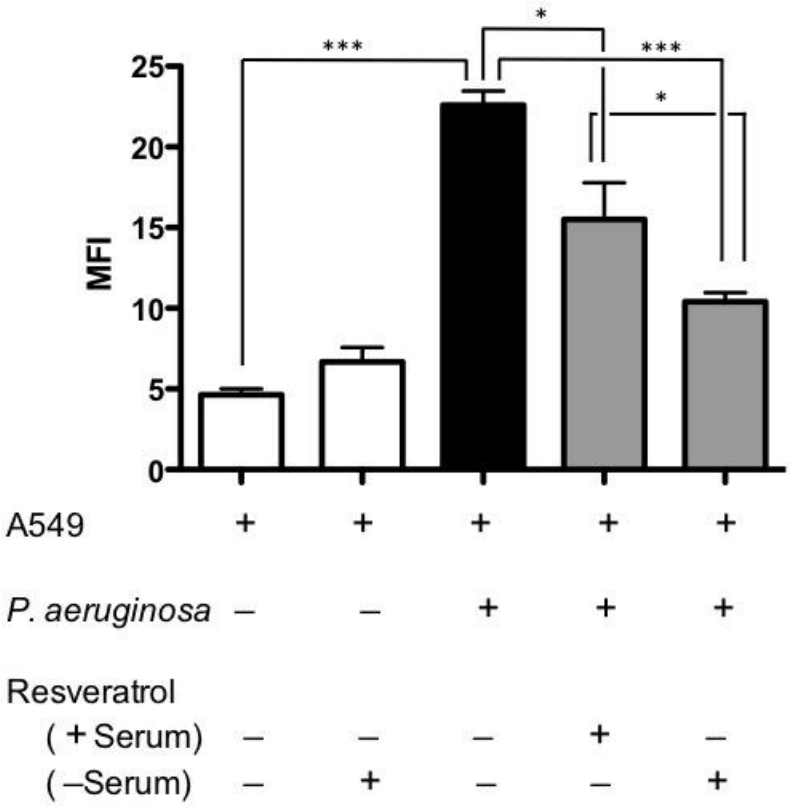

Figure 5. Effect of serum on the antioxidant capacity of resveratrol. Intracellular ROS levels in A549 cells were assessed using the CM-H_2DCFDA assay. Cells were seeded onto 6-well plates, grown overnight to ~80% confluence, and pre-treated with 100 μM resveratrol in either serum-containing or serum-free media for 4 hours followed by infection with *P. aeruginosa* MOI 50:1 for 1 hour. Cells were then incubated with the fluorescent label for 30 minutes in order to ascertain the final redox environment of the cells, and a minimum of 1×10^4 gated events were analyzed via flow cytometry (FL-1 Channel). Bars represent mean ± SEM of 3 independent experiments. (* *p<0.05*, *** *p<0.001*; MFI, mean fluorescence intensity)

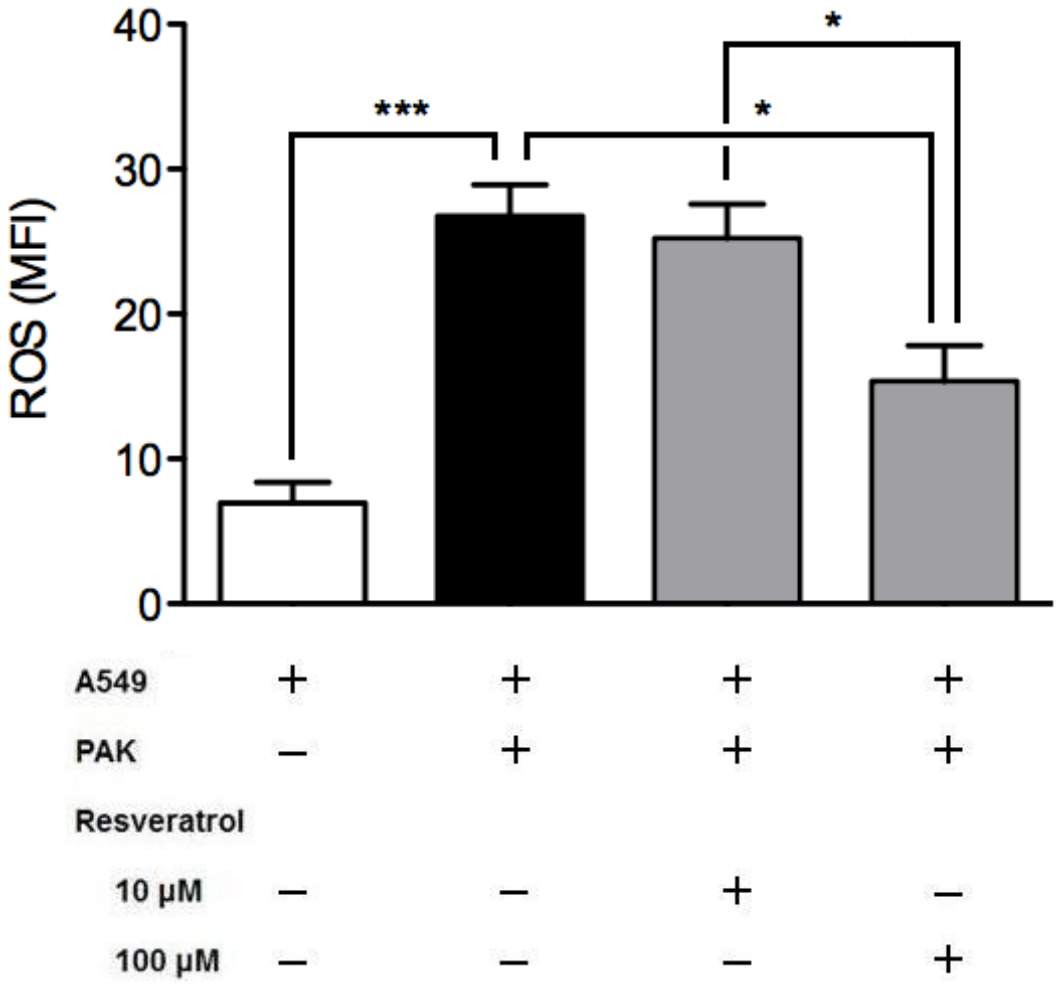

Figure 6. Effect of resveratrol on intracellular ROS levels in A549 cells infected with *P. aeruginosa*. Intracellular ROS levels in A549 cells were assessed using the CM-H_2DCFDA assay. Cells were seeded onto 6-well plates, grown overnight to ~80% confluence, and pre-treated with 10 or 100 µM resveratrol in serum-free media for 4 hours followed by infection with *P. aeruginosa* MOI 50:1 for 1 hour. Cells were then incubated with the fluorescent label for 30 minutes, and a minimum of 1×10^4 gated events, as determined using forward and side scatter properties, were analyzed via flow cytometry (FL-1 Channel). Bars represent mean ± SEM of 3 independent experiments. (* $p<0.05$, *** $p<0.001$; MFI, mean fluorescence intensity)

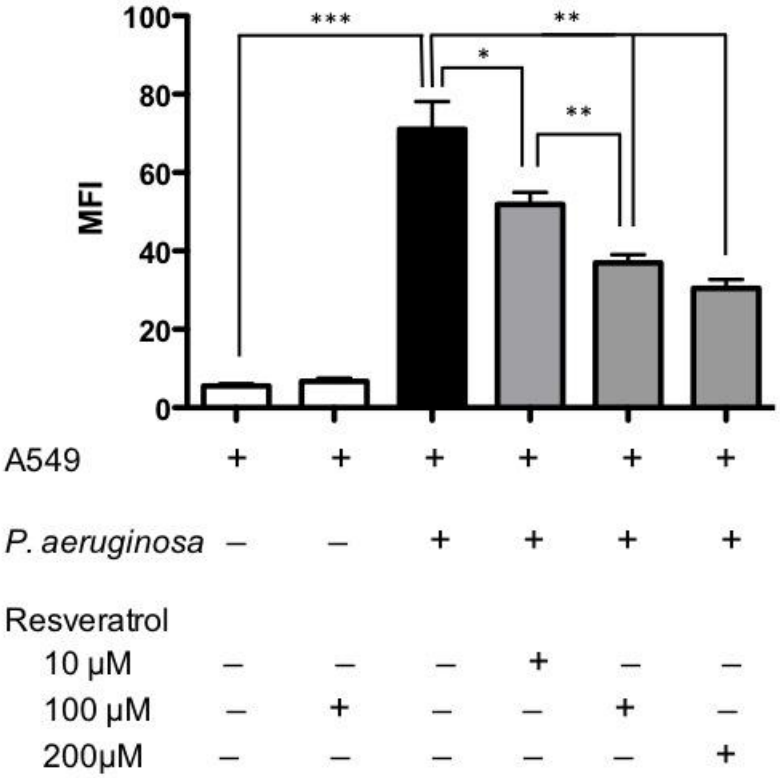

Figure 7. Effect of resveratrol pre-treatment on intracellular levels of bacteria-induced ROS. Intracellular ROS levels in A549 cells were assessed using the CM-H_2DCFDA assay. Cells were seeded onto 6-well plates, grown overnight to ~80% confluence, and pre-treated with 10, 100, or 200 µM resveratrol in serum-free media for 5 hours followed by infection with *P. aeruginosa* MOI 100:1 for 1 hour. Cells were then incubated with the fluorescent label for 30 minutes, and a minimum of 1×10^4 gated events were analyzed via flow cytometry (FL-1 Channel). Bars represent mean ± SEM of 3 independent experiments. (* *p<0.05*, ** *p<0.01*, *** *p<0.001*; MFI, mean fluorescence intensity)

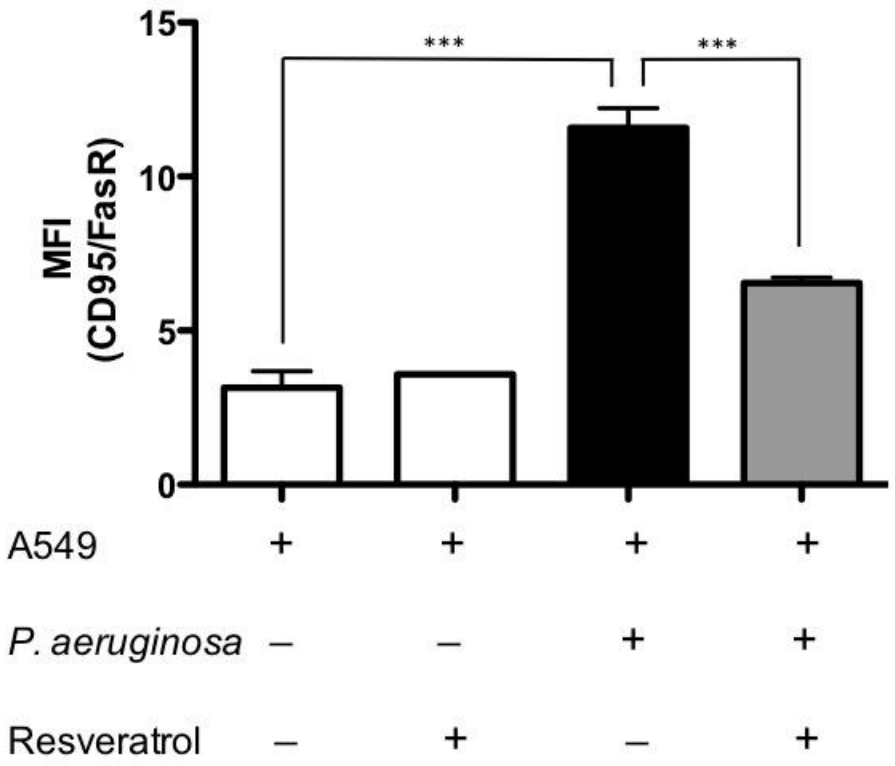

Figure 8. Effect of resveratrol on FasR/CD95 expression. Analysis of cell surface expression of Fas/CD95 was assessed via immunostaining with fluorescein isothiocyanate-conjugated mAb to CD95 (DX2) and analyzed via flow cytometry. A549 cells were seeded onto T-25cm^2 flasks for 48 hours to ~80% confluence, and incubated in serum-free media in the presence of 100 μM resveratrol for 5 hours, followed by a 1 hour infection with *P. aeruginosa* MOI 50:1 and a 17 hour co-incubation with 50 mg/mL polymyxin B. Cells were then immunostained with antibodies against CD95 and a minimum of 1 × 10^4 gated events were analyzed via flow cytometry (FL-1 Channel). Bars represent mean ± SEM of 3 independent experiments. (*** *p<0.001*; FasR, Fas receptor; MFI, mean fluorescence intensity).

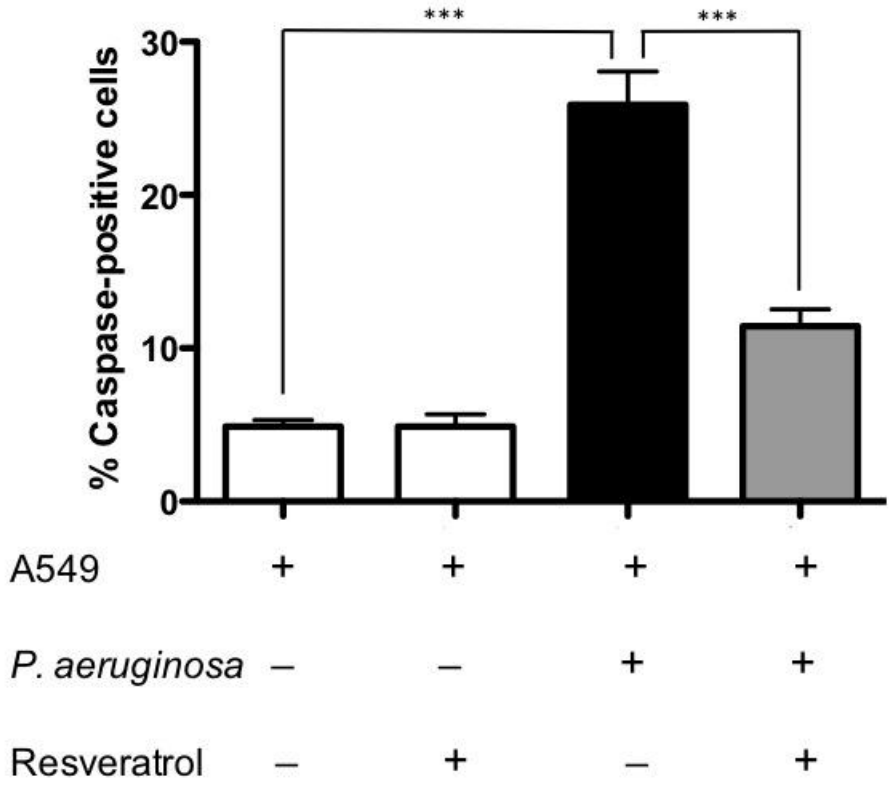

Figure 9. Effect of resveratrol on *P. aeruginosa*-induced apoptosis. The effect of resveratrol on apoptosis in A549 cells infected with *P. aeruginosa* was assessed via fluorescent detection of active caspases-3 and -7 (CaspaTag Caspase 3,7 In Situ Assay Kit). Cells were seeded onto T-25cm^2 flasks for 48 hours to ~80% confluence, and incubated in serum-free media in the presence of 100 μM resveratrol for 5 hours followed by infection with *P. aeruginosa* MOI 50:1 in serum-free media for 2 hours. Cells were incubated with fluorescent inhibitors of caspases (FLICA) for 1 hour, and a minimum of 1×10^4 gated events were analyzed via flow cytometry (FL-1 Channel). Bars represent mean ± SEM of 3 independent experiments. (*** *p<0.001*).

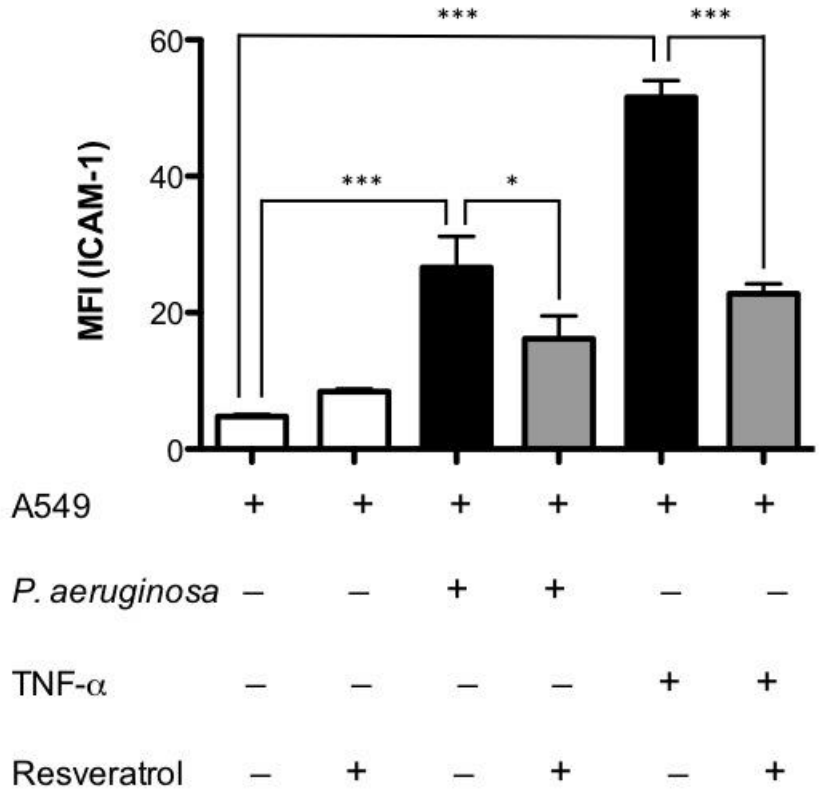

Figure 10. Effect of resveratrol on ICAM-1 expression. Analysis of cell surface expression of ICAM-1 was assessed via immunostaining with phycoerythrin-conjugated mAb to ICAM-1 (HA58) and analyzed via flow cytometry. A549 cells were seeded onto T-25cm^2 flasks for 48 hours to ~80% confluence, and incubated in serum-free media in the presence of 100 μM resveratrol for 5 hours, followed by either an 18 hour stimulation with 10 ng/mL TNF-α or 1 hour infection with *P. aeruginosa* MOI 50:1 and a 17 hour co-incubation with 50 mg/mL polymyxin B. Cells were then incubated with antibodies against ICAM-1 and a minimum of 1 × 10^4 gated events were analyzed via flow cytometry (FL-2 Channel). Bars represent mean ± SEM of 3 independent experiments. (* *p<0.05*, *** *p<0.001*; MFI, mean fluorescence intensity)

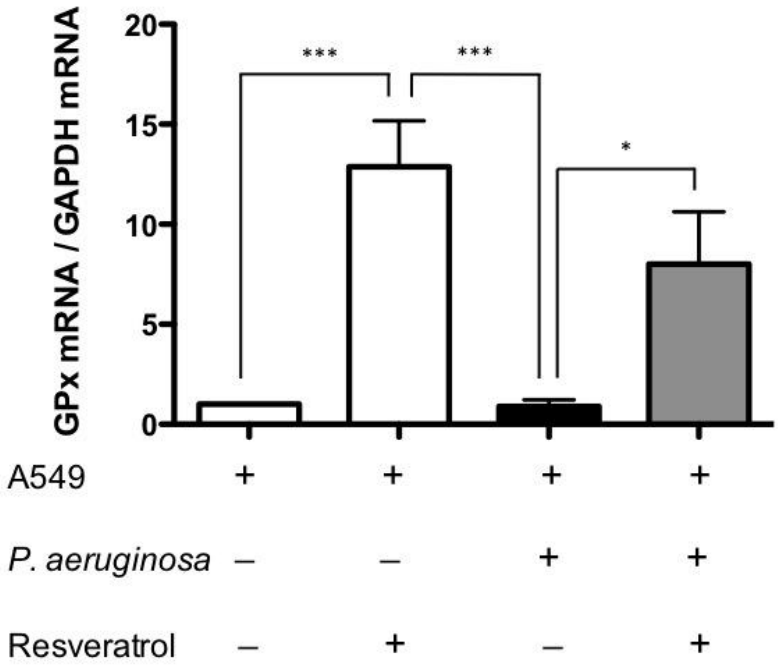

Figure 11. Effect of resveratrol on GPx mRNA expression in A549 cells infected with *P. aeruginosa*. The effect of resveratrol on GPx mRNA expression in A549 cells infected with *P. aeruginosa* was assessed via qPCR. A549 cells were seeded onto T-25 cm^2 tissue culture flasks, grown for 48 hours to ~80% confluency, and incubated in serum-free medium in the presence of 100 μM resveratrol for 5 hours, followed by infection with *P. aeruginosa* MOI 50:1 in serum-free media for 3 hours. Cells were lysed and their RNA was isolated via silica membrane spin column. RNA quantity and integrity was assessed via automated electrophoresis. cDNA was generated via oligo-dT-primed reverse transcription. Real-time PCR was performed via a two-step cycling program in accordance with the DNA polymerase and primer specifications. Results were normalized to GAPDH. Bars represent mean ± SEM of 3 independent experiments. (* $p<0.05$, *** $p<0.001$; MOI, multiplicity of infection; qPCR, quantitative PCR)

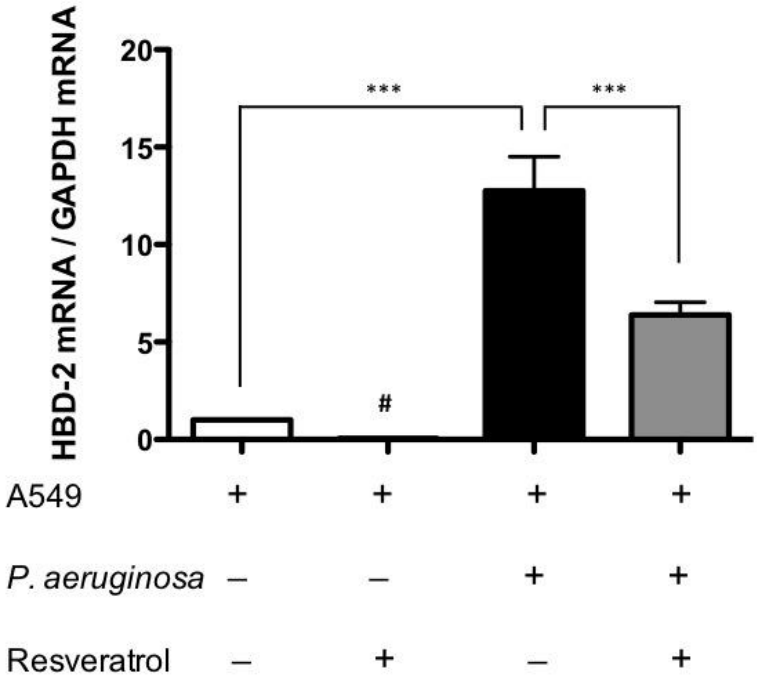

Figure 12. Effect of resveratrol on HBD-2 mRNA expression in A549 cells infected with *P. aeruginosa*. The effect of resveratrol on HBD-2 mRNA expression in A549 cells infected with *P. aeruginosa* was assessed via qPCR. A549 cells were seeded onto T-25 cm^2 tissue culture flasks, grown for 48 hours to ~80% confluency, and incubated in serum-free medium in the presence of 100 μM resveratrol for 5 hours, followed by infection with *P. aeruginosa* MOI 50:1 in serum-free media for 3 hours. Cells were lysed and RNA was isolated via silica membrane spin column. RNA quantity and integrity was assessed via automated electrophoresis. cDNA was generated via oligo-dT-primed reverse transcription. Real-time PCR was performed via a two-step cycling program in accordance with the DNA polymerase and primer specifications. Results were normalized to GAPDH. Bars represent mean ± SEM of 3 independent experiments. (*** *$p<0.001$*; # resveratrol control = 0.05430 ± 0.001577; MOI, multiplicity of infection; qPCR, quantitative PCR)

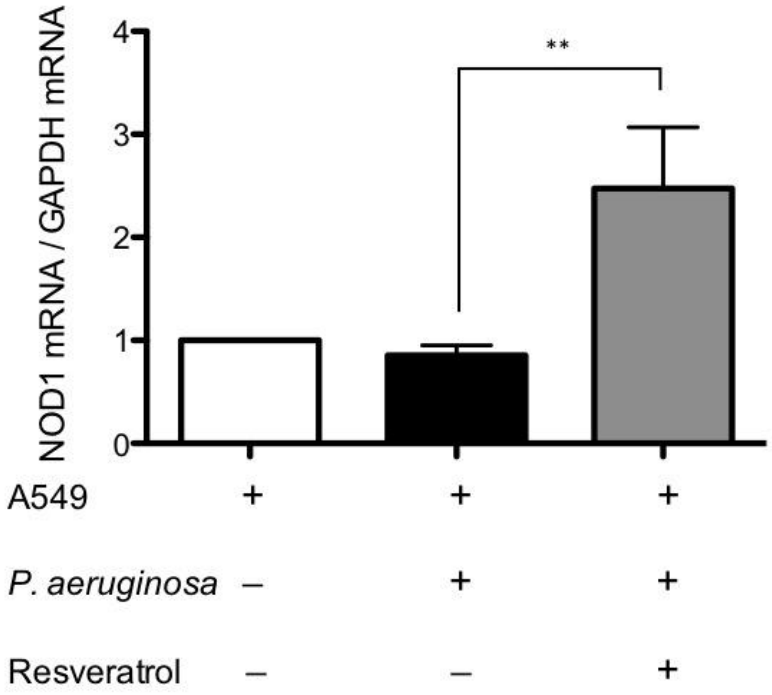

Figure 13. Effect of resveratrol on Nod1 mRNA expression in A549 cells infected with *P. aeruginosa*. The effect of resveratrol on Nod1 mRNA expression in A549 cells infected with *P. aeruginosa* was assessed via qPCR. A549 cells were seeded onto T-25 cm^2 tissue culture flasks, grown for 48 hours to ~80% confluency, and incubated in serum- and antibiotic-free medium in the presence of 100 μM resveratrol for 5 hours, followed by infection with *P. aeruginosa* MOI 50:1 in serum-free media for 3 hours. Cells were lysed and their RNA was isolated via silica membrane spin column. RNA quantity and integrity was assessed via automated electrophoresis. cDNA was generated via oligo-dT-primed reverse transcription. Real-time PCR was performed via a two-step cycling program in accordance with the DNA polymerase and primer specifications. Results were normalized to GAPDH. Bars represent mean ± SEM of 3 independent experiments. (** $p<0.01$; MOI, multiplicity of infection; qPCR, quantitative PCR)

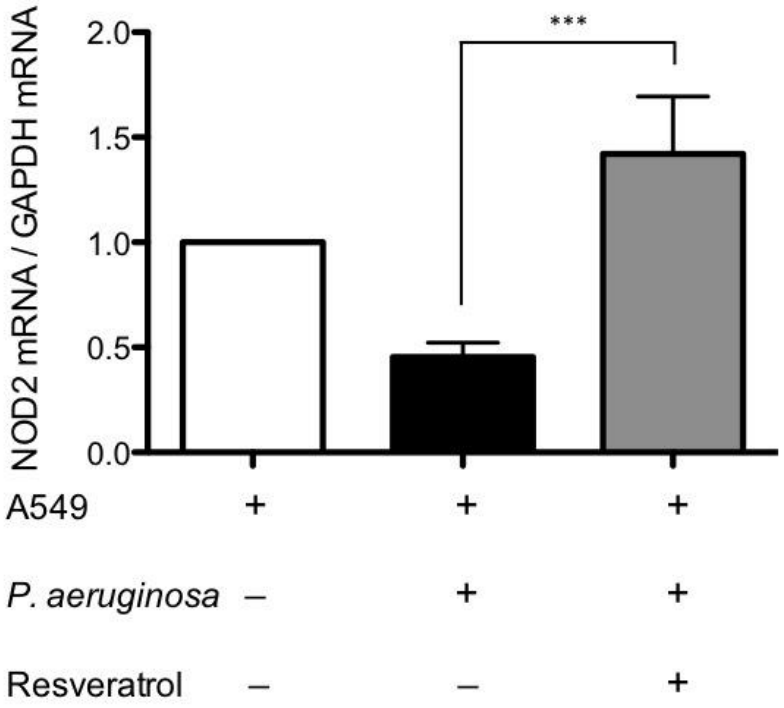

Figure 14. Effect of resveratrol on Nod2 mRNA expression in A549 cells infected with *P. aeruginosa*. The effect of resveratrol on Nod2 mRNA expression in A549 cells infected with *P. aeruginosa* was assessed via qPCR. A549 cells were seeded onto T-25 cm^2 tissue culture flasks, grown for 48 hours to ~80% confluency, and incubated in serum- and antibiotic-free medium in the presence of 100 μM resveratrol for 5 hours, followed by infection with *P. aeruginosa* MOI 50:1 in serum-free media for 3 hours. Cells were lysed and their RNA was isolated via silica membrane spin column. RNA quantity and integrity was assessed via automated electrophoresis. cDNA was generated via oligo-dT-primed reverse transcription. Real-time PCR was performed via a two-step cycling program in accordance with the DNA polymerase and primer specifications. Results were normalized to GAPDH. Bars represent mean ± SEM of 3 independent experiments. (*** $p<0.001$; MOI, multiplicity of infection; qPCR, quantitative PCR)

5 – Discussion

Respiratory tract infections caused by *P. aeruginosa* provoke a robust inflammatory response in the lung leading to severe tissue damage. Many of these cellular responses are the result of important pathogen-host interactions, which when triggered, signal the activation of pro-inflammatory transcription factors (i.e. NF-κB and AP-1) that in turn initiate the transcription and release of pro-inflammatory mediators. As a consequence, activated immune cells swarm the inflamed tissue and release proteases, myeloperoxidase, and reactive oxygen species leading to oxidative stress and contributing to lung parenchymal tissue damage seen in CF (Doring et al. 1986; Hull et al. 1997), COPD (Murphy et al. 2008; Stockley 2002), and acute lung injury (Chabot et al. 1998). It is not surprising then that a great focus has been placed on understanding the underlying mechanisms between oxidative stress and inflammation.

Resveratrol, an antioxidant compound currently believed to be at least partly responsible for the beneficial properties attributed to red wine, has been shown to exert anti-inflammatory properties in a variety of *in vitro* and *in vivo* models. To our knowledge, however, the protective effect of resveratrol in a model of *P. aeruginosa* lung infection has never been addressed. Therefore, in this study, we examined whether lung epithelial cellular responses induced by interactions with *P. aeruginosa* could be mitigated or even prevented with resveratrol pre-treatment *in vitro*. Indeed, we observed that infection of A549 lung adenocarcinoma cells with *P. aeruginosa* led to an up-regulation of intracellular ROS production. Several virulence factors are believed to contribute to this, including the redox-active compound pyocyanin (Lau et al. 2004; Schwarzer et al. 2008) and the exotoxin (Exo)U (Saliba et al. 2006), as well as pili, LPS, and flagella, which are known to be critical inducers of inflammation (Feldman et al.

1998; Pier 2007; Saiman et al. 1993). We have also found that pre-treatment with resveratrol successfully decreased ROS production. Interestingly, we also found that the presence of serum inhibited the antioxidant effect of resveratrol. This is likely due to the binding of resveratrol to some serum proteins. In agreement with our findings, resveratrol can be bound by protein carriers in the blood and transported in the circulation to its target tissue where it can then exert its biological effect (Jannin et al. 2004; Lancon et al. 2004).

ROS has been shown to play a critical role in the regulation of apoptosis (Simon et al. 2000), as have pro-inflammatory cytokines including TNF-α and IL-1β(Manna et al. 2000; Rath et al. 1999; Shakibaei et al. 2005).In the case of *P. aeruginosa* infection, airway epithelial cells undergo apoptosis via both death-receptor associated (extrinsic) and mitochondrial (extrinsic) pathways (Grassme et al. 2000; Jendrossek et al. 2001). By detecting expression levels of the death receptor Fas/CD95, involved in the extrinsic pathway, and the detection of active executioner caspases-3 and -7, involved in both pathways, we found that *P. aeruginosa* induced apoptosis in infected cells, and that pre-treatment with resveratrol decreased both markers of apoptosis post-infection. The anti-apoptotic effect may be due to up-regulation of pro-survival Bcl-2 family members (Shakibaei et al. 2008), and a decrease in nuclear translocation of NF-κB as a result of inhibited IkBa degradation and p65 activity (Csaki et al. 2009). These reports are in stark contrast to several studies that have found resveratrol to be an inducer of both caspase-3/7 activation (Dorrie et al. 2001), and Fas ligand/CD95L pathway activation (Clement et al. 1998; Ko et al. 2011; Tsan et al. 2000). However, these studies used longer resveratrol pre-treatment times and different models including acute lymphoblastic leukemia cells,

breast carcinoma cells, human monocytes, and anaplastic large-cell lymphoma cells, respectively.

Indeed, the intrinsic apoptosis pathway involves ROS-induced depolarization of the mitochondria followed by the release of cytochrome *c* (Alaoui-El-Azher et al. 2006). In the extrinsic pathway, Fas/CD95 activation triggers aggregation the FADD adapter resulting in membrane raft clustering and receptor internalization and activation of the caspase cascade (Barnhart et al. 2003; Delmas et al. 2003; Guicciardi et al. 2009). Previous work in our lab demonstrated a significant correlation between ROS generation and active caspase-3/7 expression levels in infected A549 cells (Hawdon et al. 2010). While evidence supports the role of ROS in *P. aeruginosa*-induced apoptosis (Alaoui-El-Azher et al. 2006), our results suggest a protective role of resveratrol in cell death.

Acute lung infection caused by *P. aeruginosa* causes a rapid and severe inflammatory response characterized by increased surface expression of the cell-surface adhesion molecule ICAM-1, which is critical in the recruitment of inflammatory cells to the infected tissue, and pro-inflammatory mediator release (i.e. cytokines and antimicrobial peptides) (Harder et al. 2000; Sadikot et al. 2005; Wehkamp et al. 2006). Thus, we chose to examine the effect of resveratrol on inflammation caused by *P. aeruginosa* infection by measuring ICAM-1 and human beta-defensin-2 (HBD-2) expression. In agreement with the literature, we found that both ICAM-1 (Aldallal et al. 2002) and HBD-2 (Harder et al. 2000; Wehkamp et al. 2006) expression were induced by *P. aeruginosa* in A549 cells. Studies with resveratrol demonstrated a decrease in both ICAM-1 and HBD-2, confirming that resveratrol possesses anti-inflammatory properties in our model of acute lung infection.

Indeed, promoter regions for the antimicrobial peptide, HBD-2, have been found to contain binding sites for pro-inflammatory transcription factors, including NF-κB and AP-1(Harder et al. 2000). As a part of the innate immune response, HBD-2 is released by airway epithelial cells infected with *P. aeruginosa*, and works on either epithelial cell surfaces or synergistically within phagocyte phagolysosomes to kill bacteria (Harder et al. 2000; Schroder et al. 1999). Despite the inflammatory properties of HBD-2, antimicrobial peptide suppression may increase the likelihood of sustained infection (Schroder et al. 1999). Thus, in relation to our findings, we suggest here that resveratrol may decrease the innate immune response to *P. aeruginosa* infection.

Nod proteins (nucleotide binding, oligomerization domains) act as intracellular sentinels of bacterial products and can activate NF-κB directly (Martin et al. 2005). While Nod1 is ubiquitously expressed and recognizes muramyl tripeptides in the cell walls of Gram-negative bacteria, Nod2 expression is more cell-type specific and recognizes muramyl dipeptides found in both Gram-positive and Gram-negative bacterial cell walls (Franchi et al. 2008). In our experiments, however, *P. aeruginosa* had no effect on Nod1 or Nod2 gene expression levels, however pre-treatment with resveratrol caused an increase in these receptors. Further study is needed to clarify the effect of antioxidants, like resveratrol, on NLRs.

Emerging evidence implicates resveratrol as an inducer of antioxidant enzymes, conferring mostly indirect cellular protection against oxidative stress (Hu et al. 2007; Kode et al. 2008; Robb et al. 2008). Currently, studies by others suggest that resveratrol can up-regulate levels of catalase, thioredoxin, thioredoxin reductase, and mitochondrial superoxide dismutase (MnSOD), as well as glutathione (GSH) and glutathione

peroxidase (GPx) (Hu et al. 2007; Kode et al. 2008; Robb et al. 2008; Ungvari et al. 2007). Indeed, GSH is widely considered the master antioxidant of the cell and works in concert with GPx to convert lipid peroxides, H_2O_2, and other radicals into less toxic forms (Rahman et al. 2000). In our results, expression levels of GPx in A549 cells infected with *P. aeruginosa* remained unchanged compared to control. Resveratrol pre-treatment, however, initiated a sharp increase in GPx mRNA expression correlating with the previously mentioned studies. This is interesting because expression of the death receptor Fas/CD95 is regulated by intracellular GSH levels (Chiba et al. 1996), and Fas/CD95 has been found to decrease cellular GSH levels (van den Dobbelsteen et al. 1996). These studies also suggest that resveratrol can exert beneficial antioxidant effects earlier than 18 – 24 hours as previously reported (Donnelly et al. 2004; Kode et al. 2008; Ungvari et al. 2007).

Our study is limited, however, by the scope of our model. First and foremost, one must be careful in interpreting data from an *in vitro* model, as *in vivo* there are many more cell types involved in the pathophysiology of lung inflammation and bacterial infection. As well, while the signaling pathways explored in our model (i.e. apoptosis and inflammation) may be activated independently, their signaling pathways are not mutually exclusive, and can often feedback into each other. Moreover, *P. aeruginosa* infection may activate inflammatory pathways in microenvironments not yet experiencing oxidative stress. It is this convergence, however, which leads to a vicious cycle of tissue damage and runaway inflammation.

With this in mind there are still many avenues of study needed to expand the ideas presented here. An important question left unaddressed is the direct effect of resveratrol

on *P. aeruginosa* viability. As a phytoalexin, resveratrol can be produced in several plant species as a part of a defensive strategy during bacterial or fungal attack (Donnelly et al. 2004), and while it was reported that resveratrol (ranging at concentrations of 171-342 μg/ml) inhibited the growth of *Enterococcus faecalis*, *Staphylococcus aeurus*, *Pseudomonas aeruginosa*, and several dermatophyte species (Chan 2002), it remains to be seen if and/or how resveratrol retains these microbicidal effects in the *in vitro* and *in vivo* settings. An interesting way to measure this with respect to our model would be to determine the effect of resveratrol on the internalization of *P. aeruginosa* into the lung epithelial cell. Alive or not, *P. aeruginosa* virulence factors may still initiate inflammatory responses mediated through TLR-activation (Sadikot et al. 2005), thus in terms of our findings it is unlikely that resveratrol exerts its anti-inflammatory effects via only antibacterial means.

To confirm the anti-apoptotic properties of resveratrol it would be helpful to measure any changes in mitochondrial membrane potential. As well, measuring levels of active caspase-9 would help clarify the mechanism used by resveratrol to prevent cell death. Continuing downstream, measuring the direct activation of both NF-κB and AP-1 would also be pivotal markers in confirming the effect of resveratrol in this model. Lastly, while the use of immortalized cell lines like A549 are both invaluable and practical, a natural improvement would be to test the anti-inflammatory and anti-oxidative effects of resveratrol in primary human lung epithelial cells. Such studies would help pave the way for clearer clinical interpretations.

6 – Conclusion

To summarize, we have established that the antioxidant resveratrol can protect against pathogen-induced potentially damaging cellular responses during infection of type II lung epithelial A549 cells with *P. aeruginosa in vitro* by unburdening the cell from oxidative stress, decreasing inflammatory responses implicated in tissue damage, and possibly influencing innate immune responses. Despite intense focus on the putative mechanisms underlying the diverse activities of resveratrol in different tissues, contradictory results emerge, making complete understanding of underlying mechanisms difficult. Regardless, understanding the preventative and therapeutic relationships between natural antioxidants and bacterial-induced inflammation remains a worthwhile endeavor.

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