Philadelphia College of Osteopathic Medicine

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2010 – June 30, 2011

Formula Grant Overview

The Philadelphia College of Osteopathic Medicine received $17,036 in formula funds for the grant award period January 1, 2009 through December 31, 2012. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Dissecting the Apoptosis Pathway Affected by C. pneumoniae in Alzheimer's Disease - Alzheimer's disease (AD) is thought to be the leading cause of dementia in the elderly population, and is recognized clinically in early-onset (familial) and more prevalent late-onset forms, both of which manifest similar neuropathologies. This project will examine the detailed events that lead to neuronal cell death in late-onset AD, specifically the role in initiating these events played by the bacterium *Chlamydia pneumoniae* (Cpn), a common pathogen that infects the respiratory system and has been found in the autopsied brains of late-onset AD patients. We focus on the apoptotic process that has been implicated in the death of neurons in AD. Understanding these mechanisms may lead to treatment strategies for the growing numbers of AD patients.

Anticipated Duration of the Project

1/1/2009 – 12/31/2012

Project Overview

There is increasing evidence to suggest that apoptosis plays a role in neurological disorders such as Alzheimer's disease (AD). Recent developments in understanding the mechanisms leading to neuronal cell loss observed in AD have implicated apoptosis as being the link interconnecting amyloid and tau pathologies. The apoptosis hypothesis proposes that β-amyloid activates caspases that cleave tau as has been shown in recent studies. In addition, β-amyloid induced neuroinflammation is recognized as a major factor in AD etiology. Consistent with these data, we hypothesize that a pathogen such as *Chlamydia pneumonia*e (Cpn) stimulates β-amyloid processing or production in neuronal cells, leading to synaptic dysfunction, neuroinflammation and apoptosis in neighboring cells, while the infected host is paradoxically protected from apoptosis.

We have preliminary data suggesting that neuronal cells infected with Cpn have increased levels of β-amyloid, specifically oligomeric forms which may be amyloid β-derived diffusible ligands, or ADDLs, shown to disrupt synaptic function in neurons. In our most recent publication we have demonstrated that Cpn can sustain a chronic infection by inhibiting aspects of the apoptotic process in neuronal cells.

Within the scope of this project, we will further analyze the cellular mechanisms by which Cpn inhibits apoptosis. It is our aim to examine the mitochondria cytochrome c pathway since mitochondrial dysfunction has been implicated in AD. It is our hypothesis that Cpn mediates a cellular process that blocks the mitochondrial cytochrome c release and thus inhibits the biochemical process of apoptosis. Our approach will include immunocytochemistry and flow cytometry to analyze for mitochondria cytochrome c release following an infection with Cpn in human SHSY-5Y human neuronal cells from ATCC (American Type Culture Collection). Specific antibodies will be used to screen for the Cpn infection and the cytochrome c in this paradigm. Additionally, a cytochrome c inhibitor, methazolamide will be used to determine if an alternate pathway is being utilized by Cpn in the neuronal cell. This research will add to our knowledge of factors affecting the apoptotic cellular mechanism observed in AD.

Principal Investigator

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Other Participating Researchers and Employers

None

Expected Research Outcomes and Benefits

Apoptosis, a form of cell death, is being investigated as one of the primary mechanisms leading to the death of neurons in Alzheimer's disease (AD). Several risk factors have been identified in the development of sporadic AD such as neuroinflammation and mitochondrial dysfunction. Furthermore, the environmental pathogen *C pneumoniae* (Cpn) has been associated with AD, having been found in cerebrospinal fluid (CSF) and brains of patients diagnosed with sporadic AD. Cpn infection has been shown to cause mitochondrial dysfunction and to inhibit apoptotic pathways in other diseases such as atherosclerosis. In this regard, Cpn infection could underlie aspects of mitochondrial dysfunction through the modulation of apoptotic process seen in sporadic AD.

We have presented evidence that Cpn can sustain a chronic infection by inhibiting apoptosis in neuronal host cells. Our data suggest that Cpn may be a key factor in modulating neuronal cell death. Since the mitochondrial dysfunction is suspect in AD etiology and pathogenesis, the cytochrome *c* apoptotic pathway is thus a sensible target for investigation. The knowledge gained

from this project may be useful in targeting the key cellular alterations identified for the development of clinical therapeutic modalities aimed at treating or preventing AD.

Summary of Research Completed

The previous data generated through the support of this grant demonstrated that *Chlamydia pneumoniae* may be involved in blocking cytochrome c release and/or protect the mitochondria from cellular damage in neuronal cells at 72hr post infection. These data also indicated that an infection with *Chlamydia pneumoniae* at 72hrs in neuronal cells either directly or indirectly inhibits a cascade of catalytic activation of caspase at the onset of apoptosis. Therefore, since these data demonstrated from analysis of protein regulation/processing that *Chlamydia pneumoniae* has a physiological relevance in the apoptotic cascade, it was relevant to design studies that would evaluate molecular events that occur in order to understand the pathology associated with Alzheimer's disease.

Neuronal cell death in Alzheimer's disease may occur through several mechanisms and in some instances; cells may activate the autophagy pathway instead of, or before, initiating apoptosis. Modulation of the apoptotic process may be regulated by molecular mechanisms stimulated by autophagy. An increase in the number of autophagic vacuoles (AVs) was identified in neurons from AD brains, implicating autophagy as a pathological process in AD. Autophagy in AD pathogenesis has been linked to the endosomal-lysosomal system, which has been shown to play a role in amyloid processing. Studies have found that the activity of the lysosomal system is enhanced in patients with AD by observing an increase in the production of hydrolases present in lysosomes. The lysosomal system is also related to the endosomal pathway since early endosomes that are formed will fuse with late endosomes or lysosomes. Neurons from AD brains have been found to exhibit enlarged early endosomes. This is significant in AD because early endosomes take in proteins such as apolipoprotein E and APP, and it has been determined that Aβ is formed in early endosomes.

The focus of the current studies was to determine if there is a relationship between the molecular mechanisms interconnecting apoptosis and autophagy following *Chlamydia pneumoniae* infection in neuronal cells that could lead to the pathologies observed in AD.

Neuronal cells (SKNMC obtained from ATCC) were infected with AR39 strain of *C. pneumoniae* at a multiplicity of infection (MOI)=1 for 24 to 72hrs and analyzed using real-time polymerase chain reaction (PCR) microarrays (SABiosciences) specific for apoptosis and autophagy markers. All experimental profiles were performed in triplicate. At 24hrs, neuronal cells infected with *C. pneumoniae* resulted in down-regulation of apoptosis and autophagy gene regulation. Neuronal cells infected at 72hrs with *C. pneumoniae* up-regulated the expression of several genes associated with autophagy and apoptosis. For example, BECN1 and ATG4C, both prominent genes in the autophagy pathway were 3.66 to 25.77 fold down-regulated at 24hrs and increased 1.53 to 1.73 fold 72hrs post-infection, respectively. Similarly, apoptosis gene expression such as that for BAK1 was increased from -1.15 fold at 24hrs to 2.09 fold at 72hrs post-infection.

Typically, apoptosis and autophagy are common pathways by which infected cells, incapable of eliminating the infectious agent, undergo cell death. Intriguingly, these pathways appear to be altered in *C. pneumoniae* infected neuronal cells. Since both autophagic and apoptosis dysfunction have been been implicated in the neurodegeneration associated with Alzheimer's disease (AD), these studies are an attempt to identify the defects in the regulation of genes associated with apoptosis and autophagy as observed in AD following an infection by *C. pneumoniae.*

Our studies will continue to analyze C*hlamydia pneumoniae's* pathogenic effects on the mechanisms that regulate apoptotic factors in neuronal cells. Our findings continue to suggest that inhibition of apoptosis and now the interaction of autophagic factors may be key elements in regulating neuronal cell death as a pathogenic mechanism by which a C*hlamydia pneumoniae* infection can mediate the development of chronic diseases such as AD.

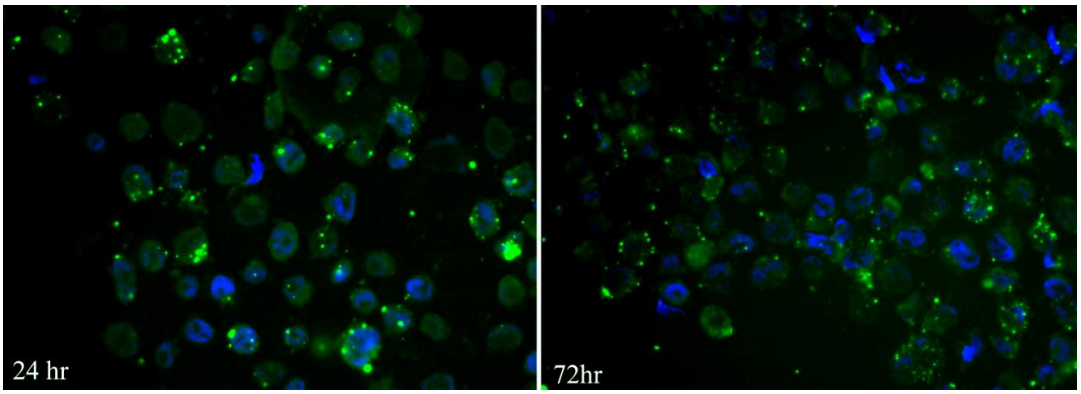
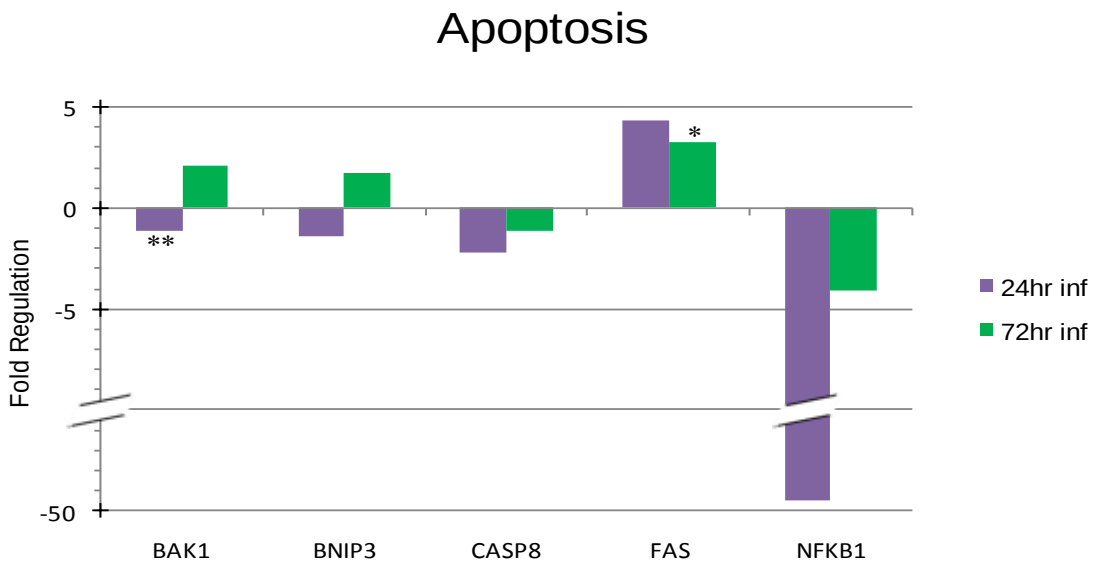


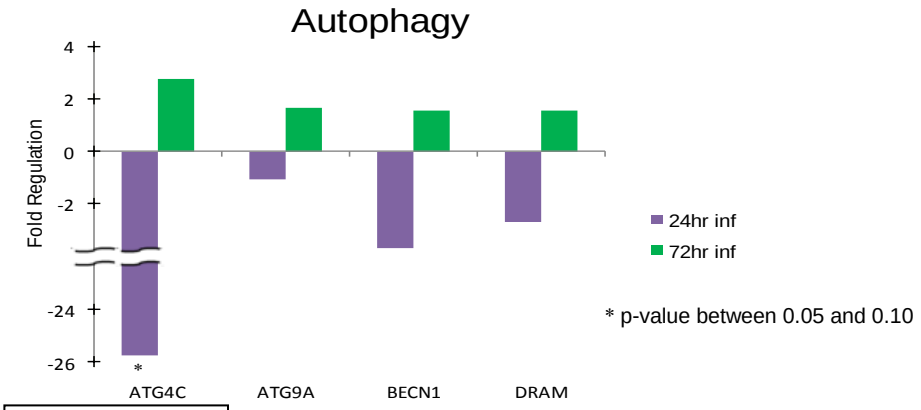
Figure 1. Representative neuronal cells infected with *Chlamydia pneumoniae* at an MOI of 1 for 24hr. and 72hr. The rate of infection was between 50% at 24hrs and 75% at 72hrs. The Chlamydia was immunolabeled with FITC conjugated Chlamydial antibody 61C75A. Green represents the *C. pneumoniae* and blue is the nuclei of the cell.



Panel A

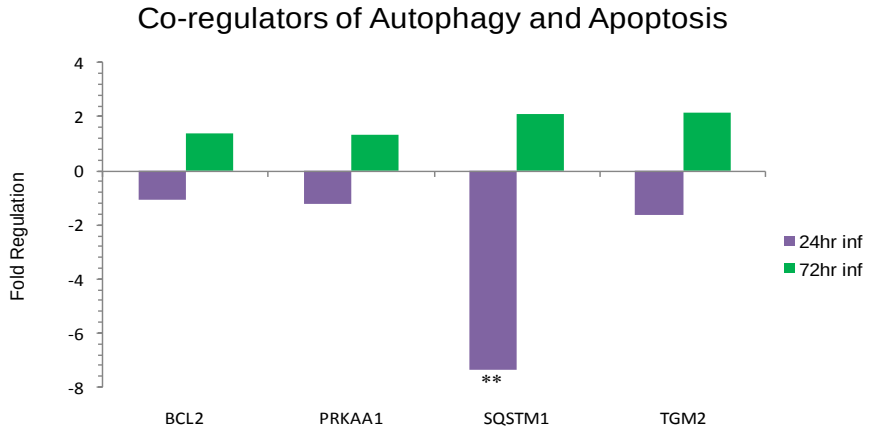
* p-value between 0.05 and 0.10

** p-value less than 0.05



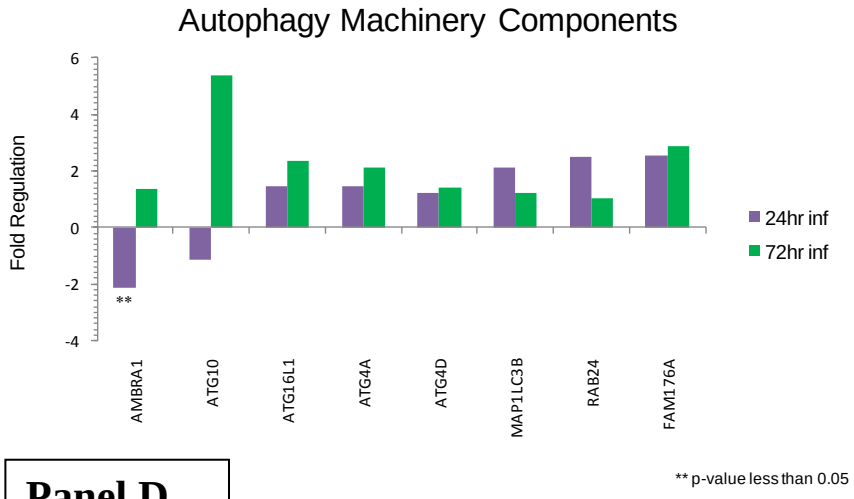
* p-value between 0.05 and 0.10

Panel B



** p-value less than 0.05

Panel C



Panel D

Figure 2. Fold regulation for the key genes involved in apoptosis (panel A), autophagy (panel B) the co-regulation of autophagy and apoptosis (panel C) and autophagy machinery components (panel D) following infection of neuronal cells with *C. pneumoniae* for 24hrs and 72hrs. Genes with significant p-values are indicated with asterisks.

Table 1. Functions of the gene products of each gene that was discussed in the results section.

Gene	Functions of Gene Products
BAK1	Contributes to the mitochondrial pathway of apoptosis (37)
BNIP3	Contributes to the mitochondrial pathway of apoptosis (37)
CASP8	Initiator caspase that induces apoptosis via the death receptor pathway (37)
FAS	Death receptor that induces apoptosis via the death receptor pathway (38)
NFKB1	Inhibits apoptosis and is a nuclear transcription factor (17)
ATG4C	Important for autophagosome formation (39, 40)
ATG9A	Important for autophagosome formation (39, 40)
BECN1	Important for autophagosome formation (39, 40)
DRAM	Lysosomal protein that is a damage-regulated autophagy modulator (41)
AMBRA1	Controls protein turnover (38); Interacts with Beclin 1-Class III PI3K complex (18)
ATG10	E2-like enzyme that is involved in the conjugation of Atg12 and Atg5 for autophagosome formation (38)
ATG16L1	Part of large protein complex necessary for autophagy (39)
ATG4A	Cysteine protease that is necessary for autophagy (38)
ATG4D	Cysteine protease that is necessary for autophagy (38)
MAP1LC3B	Subunit of the neuronal microtubule-associated MAP1A and MAP1B proteins (39)
RAB24	GTPase protein that is implicated in the regulation of intracellular protein trafficking (38)
FAM176A	Lysosome and endoplasmic reticulum-associated membrane protein (44)
BCL2	Inhibition of apoptosis autophagy pathways (18)
PRKAA1	Subunit of AMPK that can sense the cell's energy levels (38)
SQSTM1	Binds ubiquitin; regulates activation of the NFKB signaling pathway (38)
TGM2	Transglutaminase enzyme that seems to be involved in apoptosis (38)