Regulation of NF-κB in response to age-related energy stress in human skin fibroblasts

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DEDICATIONS

This thesis is dedicated to my parents Professor Satyanarayana Yalamanchili and Ms. Sujatha Yalamanchili. I am grateful for the life you have provided me.

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LIST OF ABBREVIATIONS

ATP: Adenosine-5'-triphosphate

DNA/RNA: Deoxy ribonucleic acid/ Ribonucleic acid

ROS: Reactive Oxygen Species

NF-κB: Nuclear Factor Kappa B

GROα: growth-related oncogene alpha

PI3K: Phosphatidylinositol 3-kinase

Akt/PKB: serine/threonine protein kinase

p53: protein 53

PDL: Population Doubling Level

pRB: retinoblastoma protein

p16INK4a: Cyclin-dependent kinase inhibitor 2A

mTOR: The mammalian target of rapamycin

GSK3β: Glycogen synthase kinase 3 beta

eIF4E: eukaryotic initiation factor 4E

e-EF2: Elongation factor 2

Atg: Autophagy protein

CQ: chloroquine

LC3/MAP1LC3B: Microtubule-associated proteins 1A/1B light chain 3B

p62/SQSTM1: Nucleoporin p62 (p62) - a protein complex associated with the nuclear envelope

Baf: bafilomycin

IKK: IκB kinase

ABSTRACT

Regulation of NF-κB in response to age-related energy stress in human skin fibroblasts

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Aging is associated with cumulative damage to nuclear and mitochondrial genomes, misfolding and oxidization of proteins, and organelle dysfunction. It encompasses not only the accumulation of damage over time, but also protective/adaptive responses regulated by complex signaling processes. Whereas the loss of molecular fidelity and cellular damage has been, in large part, deciphered and quantified, no clear picture has emerged on the relative importance, interconnectivity and sequence of events that characterize the adaptive response to cellular aging. Our previous work has implicated activation of inflammatory pathways as an adaptive mechanism to cellular aging. Specifically, we observed that fibroblasts from donors at advanced age exhibit a gene expression signature *in vitro* reflective of an inflammatory cell stress response. We found that these age-associated changes in transcriptional patterns were cell autonomous and accompanied by an enhanced NF-κB DNA binding activity (Kriete, Mayo et al. 2008). The central objective of this thesis project was to identify pathways and mechanisms involved in NF-κB activation of aging fibroblasts. Since mitochondrial dysfunction has been implicated in aging we first investigated the basal levels of ROS and intracellular

ATP in pre-senescent fibroblasts from young and old donors (22 to 92 years old). Though there was no noticeable change in the intracellular ROS levels, we found an overall decrease in the total basal ATP levels with aging. Based on this observation, we explored a potential link between cellular energy levels and increased NF-κB p65 DNA binding activity in fibroblasts.

In our approach we subjected 'young' fibroblasts to experimental conditions (glycolysis reduction, disruption of oxidative phosphorylation) that mimicked reduced basal ATP levels in ‘old’ fibroblasts. These treatments triggered increased NF-κB p65 DNA binding activity. Activation of NF-κB under these conditions occurred through the canonical NF-κB pathway and was independent of PI3K/Akt and p53 pathways. Further analysis revealed autophagy induction in parallel to energy deprivation-triggered NF-κB. Autophagy, a fundamental cellular ‘housekeeping’ process and usually pro-survival in response to metabolic stress, might be induced presumably as an adaptive response. Pharmacological inhibition of autophagy reduced energy deprivation-triggered NF-κB indicating its crucial role as a mediator of NF-κB activation in response to reduced cellular energy levels in ‘young’ fibroblasts. These findings motivated us to probe for autophagy in pre-senescent fibroblasts aged *in vivo*. In doing so, we found evidence that supports the involvement of autophagy. However, further studies are required to elucidate additional intracellular signal transduction pathways that may contribute to NF-κB regulation in aging. While the aging process is inherently driven by damage accumulation and multiple pathways in response to various cell stressors over time, our studies establish a new mechanism of whereby 'chronic inflammation' as evidenced by enhanced

NF-κB activity may be induced in response to energy stress increasing during the normal aging process.

1 INTRODUCTION

1.1 Introduction and Rationale

Aging is characterized by the accumulation of changes that affect the normal functioning of cell, tissues and the organism over time. Cell-specific changes during aging have been described for the regulation of protein synthesis, metabolism, cell repair and maintenance, post-translational modifications and protein turnover leading to damage buildup (Rattan 2010). Furthermore, the biology of aging includes up-regulation of cell-survival and adaptive mechanisms in response to the age-related changes in order to maintain cell homeostasis (Jarrett and Boulton 2005). When protective mechanisms, for instance anti-oxidant and DNA repair systems, are compromised due to advancing chronological age, increasing occurrence of age-related diseases such as cardiovascular maladies, metabolic disorders, cancers, and neurodegenerative diseases are observed (Walker 2002; Lombard, Chua et al. 2005; Lou and Chen 2006). Therefore, understanding the adaptive mechanisms and molecular pathways in aging process are expected to more accurately forecast the likelihood of diseases, prevent the onset of age-related pathologies and thereby support healthy aging.

Molecular events associated with the cellular aging process have long been studied *in vitro* using human dermal fibroblasts as a model system. Since the classical description of replicative senescence in cultured mammalian fibroblasts (Hayflick 1968), most studies on aging have utilized this *in vitro* model to better understand somatic aging *in vivo*. The advent of microarray technology has further enhanced insights into the molecular processes associated with cellular senescence (Shelton, Chang et al. 1999; Zhang, Pan et

al. 2003). These studies indicated increasing errors in the mitotic machinery of dividing cells with age (Ly, Lockhart et al. 2000; Geigl, Langer et al. 2004). Evidence gathered from recent studies (Coppe, Desprez et al. 2010) has shown DNA damage to be a primary mediator responsible for cellular aging in this model. Damage to DNA maybe induced by a number of intrinsic and extrinsic stressors that the cell has experienced acutely or over time and has not repaired properly due to limited resource allocation as predicted by Disposable Soma Theory of Aging (Kirkwood, Kapahi et al. 2000). DNA damage can be induced and exacerbated by physical and chemical factors such as exposure to sources of radiation like UV, X-rays and chemo-therapy during therapeutic interventions (Pillai, Oresajo et al. 2005). As fibroblasts play a crucial role in photo aging-triggered connective tissue damage, they have been used extensively to model effects of acute and chronic UV exposure in order to comprehend the underlying mechanisms (Sander, Chang et al. 2002; Pillai, Oresajo et al. 2005). Other important mechanisms are telomere uncapping, which is the final step in shortening of telomeres resulting from repeated cell divisions (replicative senescence); mitochondrial degradation; p53-triggered senescence; increase in reactive oxygen species (ROS); expression of oncogenes and chromatin disruption etc (Aubert and Lansdorp 2008; Dimauro and David 2009; Kuilman, Michaloglou et al. 2010; Wheaton 2011). While all these aspects and models contribute to the aging process they provide different opportunities to study age-related deterioration of physiological functions in response to various genetic and environmental factors.

The main objective of this research was to investigate underlying adaptive mechanisms in response to a gradual decline in various physiological functions as part of normal aging process. Several studies have used fibroblasts cultured *in vitro* as a model system

primarily because fibroblasts endure physiological changes as a function of age and fibroblast cell type itself can be easily established in culture. For the most part, replicative senescent fibroblasts have been extensively used as a cellular model to study aging mechanisms (Chen, Hales et al. 2007). Replicative cellular senescence is a phenomenon that involves generation of senescent cells *in vitro* by serial passaging, culminating in reduction and eventual loss of replicative potential. However, under homeostatic conditions, senescent cells may represent only a small sub-population in non-proliferative tissues of aging organisms including the connective tissue (Dimri, Lee et al. 1995; Paradis, Youssef et al. 2001; Martin and Buckwalter 2003). These considerations motivated the investigation of age-associated changes in pre-senescent fibroblasts derived from donors representing a range of ages who were members of the Baltimore Longitudinal Study of Aging (BLSA). Special care was taken to ensure that the population doublings for the fibroblast cultures at the time of the experiments were less than 50% of their total *in vitro* life span, in order to avoid the senescent phenotype. Since the quality and replicative lifespan of fibroblasts is influenced by the individual's health status and biopsy conditions, it was equally important to only consider cell cultures from a highly controlled study such as BLSA (Cristofalo, Allen et al. 1998). In this study, the entire process starting from selection of volunteers and skin biopsy site followed by biopsy procedures to expansion of adherent cultures were performed according to highly standardized, quality-controlled BLSA protocols. Furthermore, we considered that phenomena associated with exponentially growing cultures do not necessarily reflect the state of mostly non-dividing fibroblasts in multicellular organisms. Under normal physiological conditions in tissues *in vivo*, most fibroblasts do not proliferate for

extended periods of time and, for that reason, have not achieved replicative senescence akin to that of cultured fibroblasts. Throughout our studies, we investigated molecular markers, pathways and transcriptional profiles in non-proliferating, quiescent fibroblasts from donors aged 22 to 92 years. Quiescence may be introduced by various methods *in vitro*, such as contact inhibition, loss of adhesion and mitogen deprivation, with most transcriptional changes occurring within the first 24 hours after the introduction of quiescence (Coller, Sang et al. 2006). In the present study, we used mitogen deprivation to induce quiescence, and investigated messenger RNA and protein activity in steady-state.

Our studies of primary changes in transcription patterns with homogeneous cell cultures revealed a low-grade chronic inflammatory response and constitutive activation of NF-κB in 'old' fibroblasts. This is in line with higher activity of NF-κB and inflammatory markers that have been observed in many aging tissues in both human and animal studies in which gene expression analysis had been performed, such as in brain (Lu, Pan et al. 2004), lung (Aoshiba and Nagai 2007), liver (Kelder, Boyce et al. 2007), cartilage (Kato, Matsumine et al. 2007) and coronary arteries (Csiszar, Ungvari et al. 2003). The role of inflammation is considered protective by mediating cell/tissue repair in acute phases of infection, injuries, toxins, or ionizing radiation. The transcription factor NF-κB plays a crucial role in upregulating inflammatory signals at the transcriptional level and hence is considered an important inflammatory marker. Potential inducers of NF-κB signaling pathway include inflammatory signals themselves, impaired mitochondrial function, cellular redox state and DNA damage (Jazwinski 2000; Adler, Kawahara et al. 2008). Furthermore, NF-κB has been investigated as a mechanism associated with the induction

of a senescent state (Rovillain, Oncogene, 2011). However, at present the molecular mechanisms underlying chronic inflammation in pre-senescent cells, associated with elevated NF-κB activity during aging, is unclear. It has been hypothesized that the NF-κB transcription factor is activated in response to mitochondrial dysfunction, akin of the retrograde response in yeast (Srinivasan, Kriete et al. 2010; Jazwinski and Kriete 2012). In support of this theory we suspected a relationship of NF-κB with compromised metabolic activity. The investigation of NF-κB regulation in response to changes in energy metabolism provides a novel opportunity to unlock the origin and adaptive mechanisms involving inflammation in aging process.

1.2 Project Description

The overall aim of this project was to explore and understand adaptive pathways and mechanisms involved in cellular aging by investigating pre-senescent fibroblasts from donors of different age. Based on the gene expression data from human skin fibroblasts aged *in vivo*, we have described a cell-autonomous inflammatory cell stress response associated with elevated NF-κB DNA binding activity. The central objective of this project is to identify the pathways/mechanisms involved in NF-κB activation in these aging fibroblasts. To this end, we searched for measurable changes in the basal levels of different molecular and physiological parameters, which are potential inducers of NF-κB in the ‘old’ fibroblasts, as a function of age. Another important aspect of this project was to simulate these basal changes in the ‘young’ fibroblasts in order to explore a causal relationship to NF-κB activation as it relates to aging.

1.3 Specific Aims and Hypotheses

Overall, we focused on changes in physiological and molecular markers as a function of age, followed by evaluating a causal relationship using energy deprivation model. The specific aims were as follows:

Specific Aim 1: To define the transcriptome of biologically aged fibroblasts.

Hypothesis: The chronic inflammatory transcriptome in biologically aged fibroblasts is associated with increased NF-κB p65 DNA binding activity.

Specific Aim 2: To determine age-dependent energy alterations and its correlations with NF-κB profile

Hypothesis: The constitutive NF-κB activation as it relates to chronic inflammation is associated with altered cellular redox state, diminished mitochondrial respiratory capacity (a.k.a. mitochondrial dysfunction) and changes in cellular functions.

Specific Aim 3: To investigate a potential link between energy decline and elevated NF-κB activity using energy deprivation

Hypothesis: Reduced total intracellular ATP levels in response to energy stress induce NF-κB p65 DNA binding activity.

Specific Aim 4: To identify pathways regulating NF-κB in aged human fibroblasts

Hypothesis: Increase in nuclear import of NF-κB p65 in response to energy deprivation is a result of canonical NF-κB pathway activation. Akt and its downstream effectors play a

role in regulating the NF-κB activity as a result of serum and glucose starvation. Energy stress may induce NF-κB activation and is dependent on stress-sensor p53 pathway. Energy deprivation may also involve autophagy mediating the NF-κB signaling pathway.

2 BACKGROUND

2.1 Chronic Inflammation and Aging

Inflammation is a critical host response to either infection due to pathogens or tissue damage caused by chemicals or trauma. Acute inflammatory responses are usually localized, help to remove the damaged tissue, prevent the spread of infection by assisting the body's repair mechanisms and hence are protective in nature. Age-associated chronic inflammatory states are distinct from inflammation triggered by infection. Chronic inflammation may occur in response to cellular stress and dysfunction over time and may contribute to age-related deterioration of cell and tissue function. It is characterized by the production and secretion of specific cytokines that may elicit a response different from the expression patterns described for acute inflammation. Chronic inflammation and alterations in protein homeostasis have been implicated in many age-associated conditions (Chung, Kim et al. 2001; Longo and Finch 2003; McGeer, Klegeris et al. 2005; Wellen and Hotamisligil 2005; Figaro, Kritchevsky et al. 2006; Sarkar and Fisher 2006). Inflammatory profiles have been reported in other age-related human and animal studies in which gene expression analysis had been performed such as in brain (Lu, Pan et al. 2004), lung (Aoshiba and Nagai 2007), liver (Kelder, Boyce et al. 2007), cartilage (Kato, Matsumine et al. 2007) and coronary arteries (Csiszar, Ungvari et al. 2003). Caloric restriction, known to extend lifespan in different model organisms, has been associated with repression of chronic inflammation, and to this extent aging has been framed into an "inflammation hypothesis" (Chung, Kim et al. 2001).

Chronic inflammation associated with the aging process has been termed ‘inflamm-aging’ (Franceschi, Bonafe et al. 2000) and is implicated in a host of degenerative disease states including osteoarthritis, atherosclerosis, type-2 diabetes and even cancer (Yung 2000; Licastro, Candore et al. 2005; Sarkar and Fisher 2006). It is presently unclear to what extent chronic inflammatory states in older individuals represent autoimmune processes caused by deregulation of the innate immune system (Franceschi, Capri et al. 2007; Salminen, Huuskonen et al. 2007). Alternatively, these states may arise as a consequence of an increased cell stress response in old cells triggered by molecular damage incurred over a lifetime. Constitutive activation of inflammatory mediator and redox-sensitive transcription factor NF-κB has been documented to be associated with inflammatory pathogenesis in many aging studies (Adler, Sinha et al. 2007; Csiszar, Wang et al. 2008; Pierce, Lesniewski et al. 2009; Real, Martinez-Hervas et al. 2010). In support of cell-autonomous causes for age-associated inflammation, expression of inflammatory markers such as cytokines, has been observed in cells subjected to replicative senescence *in vitro* caused by serial passaging (Shelton, Chang et al. 1999; Krtolica and Campisi 2002; Zhang, Pan et al. 2003; Mariotti, Castiglioni et al. 2006). Furthermore, it has been shown that reactive oxygen species (ROS) production triggers the nuclear translocation of the inflammatory mediator NF-κB, where it activates pro-inflammatory genes (Sarkar and Fisher 2006). However, as outlined above, molecular events observed during replicative senescence *in vitro* may not mirror important characteristics that occur during 'physiological' aging, occuring in a dramatically different time frame. Moreover, these studies were limited to continuously proliferating epidermis,

but not fibroblasts in the underlying dermis that typically do not actively divide *in vivo* (Boukamp 2005).

2.2 NF-κB and Inflamm-Aging

An important consequence of all inflammation-associated conditions, regardless of the triggering factors, is the activation of the NF-κB transcription factor (Karin 2009). NF-κB is primarily involved in stress-triggered immune or inflammatory responses and apoptosis and thereby its deregulation may lead to cancer, infectious diseases, inflammatory and autoimmune diseases (Perkins 2007). NF-κB is a family of dimeric transcription factors composed of different subunits, which can form multiprotein DNA-bound complexes (Wolberger 1998). NF-κB proteins are homo- and/or hetero-dimers and share the DNA binding/dimerization domain called Rel homology domain (RHD) in their N-terminus. Phosphorylation of RHD results in regulation of NF-κB activity and modulates effects on target gene expression. Based on the NF-κB protein structure, there are two classes of NF-κB family proteins. Class I includes NF-κB1 and NF-κB2 and class II includes RelA/p65, RelB and c-Rel (Courtois 2005; Karin 2009). NF-κB is in its inactive form in the cytoplasm bound to the inhibitory proteins IκBs. To date, two NF-κB activation pathways are characterized, and they are the canonical (classical) and the non-canonical (alternative) pathway (Courtois 2005; luo 2005; Gilmore 2006). The activation of the canonical pathway is induced by inflammatory cytokines such as TNF or IL-1 (Dan 2008). The alternative pathway is activated by Lymphotoxin B (LTβ), B-cell activating factor (BAFF) and CD40 receptor signals (Gilmore 2006). In the canonical activation

pathway, IκB is phosphorylated by IκB kinase (IKK) in response to various stimuli, subsequently ubiquitinated and degraded (Wang 2002; Hayden 2008) releasing the active NF-κB complex, which translocates to the nucleus. NF-κB hetero-dimers p65-p50 and p65-c-Rel are the transcriptional activators that bind to DNA consensus sequences and transcribe various genes involved in a variety of physiological processes (luo 2005). p65-p50 hetero-dimers are ubiquitous, found in many different cell-types, and responsible for controlling many of the transcription functions of NF-κB. Studies show up-regulation of NF-κB activity as a pivotal mediator of aging-related inflammation (inflamm-aging) in rodent and human tissues (Adler, Sinha et al. 2007) including mouse brain, muscle (Helenius, Hanninen et al. 1996; Korhonen, Helenius et al. 1997; Kim, Kim et al. 2000; Bar-Shai, Carmeli et al. 2005) and human endothelial tissues (Donato, Eskurza et al. 2007). More recently it has also been shown that inhibition of NF-κB in tissues can reverse signs of aging (Adler, Kawahara et al. 2008).

2.3 Potential inducers of NF-κB and Inflamm-Aging

Among the many damage and/or nutrient sensing stress signaling pathways/mechanisms involved in regulation of NF-κB are mitochondrial reactive oxygen species (ROS) generation, the phosphoinositide 3-kinase (PI3K)/Akt pathway, p53 activation and autophagy. The activation of these upstream effectors/pathways is not unique to regulation of NF-κB; however they do play critical roles in regulating cellular responses to stress signals as it relates to aging and age-related pathologies.

2.3.1 Mitochondrial dysfunction

Mitochondria are cell organelles located in the cytoplasm of eukaryotic cells and their main function is to produce most of the cellular energy in the form of adenosine tri-phosphate (ATP) apart from being involved in other cellular processes. Mitochondrial dysfunction is a consequence of damage to mitochondrial proteins by either exogenous stimuli such as environmental toxins or endogenous stressors. Endogenous stressors include reactive oxygen species (ROS) that are byproducts of mitochondrial energy generation and the aging process itself and may lead to oxidative stress. Depending on the level of ROS production, the resulting oxidative stress either triggers an inflammatory response through NF-κB activation or induces cell death (Gloire, Legrand-Poels et al. 2006). It has been recognized, that mitochondrial dysfunction accompanies the aging process , even though the exact mechanisms for mitochondrial dysfunction discussed, for example mtDNA mutations in vertebrates, remain controversial (Trifunovic, Wredenberg et al. 2004; Lim, Jeyaseelan et al. 2005; Khrapko, Kraytsberg et al. 2006; Kujoth, Bradshaw et al. 2007). However, there is agreement that mitochondrial damage may impair oxidative metabolism and energy production with age (Nicholls 2004). Despite accumulating evidence that alterations in mitochondrial respiratory function can stimulate inflammatory signals, it remains to be answered what molecular mechanisms regulate such a response (Bulua, Simon et al. 2011; Rubartelli, Gattorno et al. 2011; Tschopp 2011). Extensive studies have been done in yeast, in which mtDNA mutations occur at much higher frequency than in vertebrates, and the term ‘retrograde response’ has been coined to describe the nuclear transcriptional response to mitochondrial dysfunction (Parikh, Morgan et al. 1987; Butow and Avadhani 2004; Liu and Butow 2006). It has

been suggested that this mitochondria-to-nucleus crosstalk represents a compensatory mechanism in response to the functional decline of mitochondria (Kirchman, Kim et al. 1999), serving to adjust metabolic activities and extend lifespan. While most of the work on retrograde signaling and longevity has been done in yeast (Epstein, Waddle et al. 2001; Jazwinski 2002), altered expression of nuclear genes in response to experimentally induced mitochondrial dysfunction in mammalian cells (p0 cells) has also been shown (Marusich, Robinson et al. 1997; Miceli and Jazwinski 2005). Interestingly, activation of NF-κB has been discussed in relation to mitochondrial retrograde signaling (Biswas, Anandatheerthavarada et al. 2003; Biswas, Tang et al. 2008; Srinivasan, Kriete et al. 2010; Jazwinski and Kriete 2012; Tang, Roy Choudhury et al. 2012). The same group has demonstrated that mitochondrial respiratory dysfunction triggered a metabolic shift that culminated in increased glucose uptake, glycolysis and resistance to apoptosis (Guha, Srinivasan et al. 2007). Moreover, compensatory mechanisms exist that conceal the mitochondrial deficits in certain diseases by way of increased mitochondrial volume and/or increased glycolysis in order to maintain intracellular ATP homeostasis (Krahenbuhl and Reichen 1992; Ben Sahra, Laurent et al. 2010; Oliveira 2010). Therefore, in order to understand the age-related alterations in cellular metabolism, it seems important to consider separately the two important sources of ATP production namely mitochondrial oxidative phosphorylation and glycolysis.

2.3.2 Role of oncoprotein Akt in NF-κB regulation

Akt, also known as Protein Kinase B (PKB), is a serine/threonine protein kinase activated downstream of many growth factor receptors (Florentine, Sanchez et al. 1997). It is an important effector of PI3K pathway and participates in the control of cell growth and survival by inhibiting apoptosis. It also plays a critical role in many other cellular processes involving glucose metabolism and transcription by regulating the phosphorylation of downstream targets such as mTOR, NF-κB, BAD and GSK-3. In some cancer cells, its function is de-regulated resulting in aggressive tumor growth and hence it is a potential target for anti-cancer therapies (Mitsiades, Mitsiades et al. 2004). Oncogenic cell transformation associated with activated NF-κB is a well-studied consequence of Akt activation in cancer research (Kane, Shapiro et al. 1999; Madrid, Mayo et al. 2001; Agarwal, Das et al. 2005; Jeong, Pise-Masison et al. 2005; Li, Cheung et al. 2007; Bai, Ueno et al. 2009). Age-related deterioration in Akt activity has been reported in rat hepatocytes, mouse skeletal muscle and muscles of very old rats (Li, Li et al. 2003; Shay and Hagen 2009; Wu, Katta et al. 2009). In addition, increased Akt activity was found to be associated with *in vitro* aged primary cultured human endothelial cells indicating its negative role in regulating the lifespan (Miyauchi, Minamino et al. 2004). Nevertheless, it remains to be investigated what role Akt plays in human aging associated with increased NF-κB, given its strong well-established anti-apoptotic function in response to a wide range of stress conditions.

One of the major down-stream targets of Akt is GSK-3 (Glycogen synthase kinase 3), which plays a key role in glucose uptake and glycogen synthesis. GSK3 is serine/threonine kinase composed of two isoforms, GSK-3α and GSK-3β (Woodgett

1990). The GSK-3 activity is inhibited by phosphorylation of GSK-3α and GSK-3β at Ser-21 and Ser-9 respectively, which is important to mediate cell survival (Endo, Nito et al. 2006; Ohori, Miura et al. 2008; Song, Lai et al. 2010). The effects of GSK-3 on glucose uptake and GLUT1 expression are mediated by TSC2/mTOR pathway downstream of GSK-3 (Buller, Loberg et al. 2008). Akt/GSK-3, as part of the mTOR pathway, play a crucial role in muscle development of rats subjected to mechanical overloading; conversely, aging appears to impair the efficacy of the Akt/mTOR/GSK-3 pathway (Hwee and Bodine 2009). On the other hand, phosphorylation of GSK-3 downstream of Akt is known to be involved in the regulating NF-κB at the transcriptional level and promote cell survival (Steinbrecher, Wilson et al. 2005; Graham, Tullai et al. 2010). Kwon et al suggest that phosphorylations of Akt1 and GSK-3β in response to ionizing radiation can partially contribute to the transcriptional transactivation of NF-κB in ATM-deficient fibroblasts (Kwon, Kim et al. 2008).

2.3.3 Role of tumor suppressor protein p53 in NF-κB regulation

The tumor suppressor p53 plays a critical role in the cellular response to a variety of cellular processes and stressors that include ROS, metabolic challenges, aging, senescence, autophagy, DNA damage and repair (Zheltukhin and Chumakov 2010). Most widely studied role of p53 is in tumor suppression through induction of apoptosis and senescence following DNA damage; loss or deregulation of p53 promotes cancer progression and is associated with an inflammatory response (Zuckerman, Wolyniec et al. 2009; Coppe, Desprez et al. 2010). NF-κB and p53 are known to be involved in

maintaining the balance of normal cell metabolism as well as metabolic reprogramming in response to stress (Finkel and Holbrook 2000; Mauro, Leow et al. 2011; Sen, Satija et al. 2011). Based on the severity of mitochondrial dysfunction or DNA damage, the generated reactive oxygen species (ROS) and oxidative stress activate both p53 and NF-κB pathways, which accordingly regulate the cell-cycle progression, inflammation or apoptosis (Ryan, Ernst et al. 2000; Del Rio and Velez-Pardo 2002; Aleyasin, Cregan et al. 2004; Liu and Sun 2010; Yin, Chen et al. 2011). Alternatively, some reports have revealed antagonistic roles of p53 and NF-κB (Webster and Perkins 1999; Puszynski, Bertolusso et al. 2009; Ak and Levine 2010; Gudkov, Gurova et al. 2011) and interestingly enough a study has revealed reduced transcriptional activity of p53 with age (Feng, Hu et al. 2007). These studies, therefore, reveal a complex, and potentially interdependent relationship between p53 and NF-κB.

2.3.4 Autophagy and its role in NF-κB regulation

Aging is characterized by cumulative molecular damage to cells/tissues coupled with increased vulnerability to genetic/environmental challenges and decreased efficiency of various damage counter-acting mechanisms resulting in age-related diseases (Rajawat, Hilioti et al. 2009; Vellai 2009). Autophagy is an important cellular process known to be involved in keeping the aging cell “clean” by degradation of the damaged cellular proteins and organelles in the autolysosomes. It is a lysosome-based catalytic process triggered in response to nutrient deprivation or metabolic stress (Klionsky and Emr 2000; Mathew, Karantza-Wadsworth et al. 2007). Apoptotic or pro-survival role of autophagy

depends on the stimulus, cellular context and stress conditions. It has been demonstrated that TNF-induced NF-κB activity suppresses autophagy in Ewing sarcoma cells via mTOR (Djavaheri-Mergny, Amelotti et al. 2006). In another study involving intestinal epithelial cells, TNF-α induced a long term activation of NF-κB, which was accompanied by autophagy dependant degradation of IκBα (Colleran, Ryan et al. 2011). Meng et al demonstrated that inhibition of autophagy in the mediobasal hypothalamus of mice caused metabolic changes and development of obesity via activation of pro-inflammatory NF-κB pathway (Meng and Cai 2011). Defects in autophagic mechanisms are implicated in several age-related diseases such as cancer, neurodegenerative disorders, and inflammatory myopathies (Cuervo, Bergamini et al. 2005; Rubinsztein, DiFiglia et al. 2005; Nishino 2006; Bergamini, Cavallini et al. 2007; Maycotte and Thorburn 2011; Sridhar, Botbol et al. 2012).

2.4 Overview of autophagy and its regulation

Autophagy is an evolutionarily conserved catabolic process wherein cytoplasmic components are degraded by lysosomes (Ravikumar, Futter et al. 2009). The purpose of autophagy is not only clearance of misfolded or ubiquitinated proteins and damaged organelles, formed as a result of normal or pathological processes, but also to supply vital nutrients to the cell during periods of starvation by recycling intracellular content. When this maintenance system for cellular homeostasis is abrogated, it results in several disease conditions. Autophagy has recently been implicated in many physiological and pathological conditions such as aging, cancer, neurodegenerative disorders, infectious,

muscular and liver diseases (Cuervo, Bergamini et al. 2005; Rubinsztein, DiFiglia et al. 2005; Nishino 2006; Bergamini, Cavallini et al. 2007; Maycotte and Thorburn 2011; Sridhar, Botbol et al. 2012). It serves dual roles as either a cell-survival or cell-death mechanism depending on the physiological conditions or non-physiological cellular insults.

To date, three types of autophagy have been identified based on the mode of delivery of the intracellular components to the lysosomes for degradation and subsequent recycling. They are macroautophagy, chaperone-mediated autophagy and microautophagy and among these macroautophagy, generally referred to as autophagy, is the most extensively studied and characterized process (Esclatine, Chaumorcel et al. 2009; He and Klionsky 2009; Fimia and Piacentini 2010). Macroautophagy is characterized by formation and elongation of double-membraned vesicles called autophagosomes that sequester the cytoplasmic organelles and macromolecules and fuse with lysosomes to form autolysosomes. The contents in the autolysosomes are degraded via hydrolases and then transferred back to the cytosol for reuse with the help of lysosomal permeases (Klionsky 2007).

The serine/threonine kinase mTOR (mammalian target of rapamycin) is a key molecule in nutrient-sensing signaling pathways, and is known to play important roles in regulating cell survival metabolism, cellular life-span, protein synthesis and autophagy (Yoshimori 2004; Hands, Proud et al. 2009). mTOR is a multi-domain protein kinase that consists of two types of complexes, mTORC1 and mTORC2 (Guertin 2009; Foster 2010). mTORC1 is known to regulate autophagy in response to nutrients, growth-factors and/or energy

starvation (Liu, Thoreen et al. 2009). The mTORC1 inhibitor rapamycin is known to induce autophagy (Harrison, Strong et al. 2009; Ravikumar, Futter et al. 2009). mTOR activity has been associated with aging and life-span studies (Kaeberlein, Powers et al. 2005; Powers, Kaeberlein et al. 2006). The mTOR pathway coordinates a variety of cellular processes that include protein translation, autophagy, survival and metabolism in response to nutrient, hormonal, and energy signals (Meijer 2004). Glucose, amino acids, and growth factors stimulate protein synthesis and inhibit autophagy. This process is mediated by the activation of mTOR via PI3K (phosphatidylinositol 3 kinase) and Akt and the inactivation of tuberous sclerosis complexes TSC1 and TSC2. mTOR phosphorylates S6 kinase and increases the translation of mRNAs that encode ribosomal and other proteins involved in translation. Subsequently translation is initiated following phosphorylation of 4EBP1 (eIF4E-binding protein 1), an inhibitor of initiation, causing its dissociation from eIF4E (eukaryotic initiation factor 4E) (Sabatini 2006). Active eIF4 promotes cell proliferation by increasing translation of cyclin D1, c-Myc, and vascular endothelial growth factor. mTOR and S6kinase also release the cellular check on peptide elongation by phosphorylating and inactivating eEF-2 kinase, an enzyme whose activity is known to be increased in cancer. Conversely, in the absence of nutrients, cell growth is down-regulated to reduce energy demand and autophagy is induced to enable adaptation and survival. ATP limitation, as a result of nutrient/growth-factor/glucose deprivation, induces autophagy by inhibiting mTOR and its downstream target p70S6 kinase and 4EBP1 (Hait 2006). Furthermore, mTOR is a downstream effector in the primary energy-sensing pathway regulated by AMPK (Shackelford and Shaw 2009). Increased ratios of

AMP to ATP due to energy depletion activate AMPK, which is known to inhibit mTORC1, thus promoting autophagy.

In mammals, autophagy is induced by formation of an active Atg1 complex (ULK complex), which is dependent on nutrient/growth-factor signaling and stress response pathways via mTORC1 (Kamada, Yoshino et al. 2010; Chen and Klionsky 2011). Although there are mTORC1-independent pathways regulating ULK complexes, current knowledge in human cells suggests Atg1/ULK complexes negatively regulate mTORC1 (Lee, Kim et al. 2007; Scott, Juhasz et al. 2007). The autophagy induction is followed by vesicle nucleation, which requires the activation of a class III phosphoinositide 3-kinase complex, which includes Beclin 1 (Thorburn 2008; Djavaheri-Mergny 2010). Beclin1 is a mammalian homolog of yeast Vps30/Atg6 that is required for both vacuolar transport and autophagy. During nucleation, proteins and lipids are recruited for the formation of autophagosomes. Autophagosome formation is a *de novo* process where vesicles expand and sequester the cytosolic components. Atg12 binds covalently with Atg5 and accrues on the isolation membrane triggering the elongation of the autophagosomes. The conjugation of Atg12 to Atg5 requires Atg7 and Atg10. Atg12-Atg5 conjugate binds with Atg16 forming a trimeric complex that oligomerizes and localizes on the external surface forming the autophagosomes. Atg12-Atg5-Atg16 signals the recruitment of Atg8 (known as LC3 in mammalian cells) and forms Atg8/LC3-PE (phosphatidylethanolamine) and Atg12-Atg5 conjugates. Atg12-Atg5 conjugate dissociates from the autophagosomes membrane upon completion of the autophagosome formation. Thus formed autophagosomes fuses with the lysosomes to form autolysosomes, where the sequestered contents are degraded. Protein 1 light chain 3 (LC3) is the unprocessed form and when

proteolytically processed, it is referred to as LC3-I. It is further modified to PE conjugated form LC3-II. This active form of LC3 remains attached to the membrane of autolysosomes (Kadowaki 2006; Longatti 2009; Ravikumar 2009). Therefore, LC3-II is a reliable protein marker indicating completion of autophagosome formation in autophagy. P62/SQSTM1 is another protein marker as it binds to LC3 and is sequestered into the completed autophagosomes and thereby degraded in autolysosomes (Pankiv, Clausen et al. 2007). Recent studies have shown that autophagy induction correlates with decreased levels of p62 suggesting its importance in determining autophagy status (Bjorkoy, Lamark et al. 2005; Mizushima and Hara 2006; Komatsu, Wang et al. 2007).

3 AIM 1 – DEFINE THE TRANSCRIPTOME OF PRE-SENESCENT FIBROBLASTS

3.1 Introduction

Here we summarize the design, analysis and results of our recently published findings (Kriete, Mayo et al. 2008). Only few studies report gene expression profiling of fibroblasts aged *in vivo* and these are largely focused on age-associated changes in cell cycle progression of dividing cells (Ly, Lockhart et al. 2000; Geigl, Langer et al. 2004). In contrast, in the present study we found 'inflammatory signatures', i.e. changes in gene expression patterns previously implicated in inflammatory states.

3.2 Materials and Methods:

3.2.1 Primary Cell Cultures

Human fibroblast cultures, established from skin samples derived from young and old donors, were obtained from the NIA Aging Cell Repository (Coriell Institute for Medical Research, Camden, NJ). These adherent cell cultures originated from 2 mm punch biopsies taken from the donors' medial aspect of the upper arm. The donors were members of the Baltimore Longitudinal Study of Aging (BLSA), who were medically examined and certified "healthy" at the time of the study. We selected a set of early PDL fibroblast cultures, which were cultured for until less than 50% of their total *in vitro* life span, and that had normal karyotypes for our study. Coriell catalog numbers of these fibroblast cultures for the group of young donors were AG10803 (22 yrs), AG0454 (29 yrs-I yrs), AG04441 (29-II yrs), AG13153 (30 yrs) and AG04438 (33 yrs) and for the

group of middle-age and old donors were AG04456 (49 yrs), AG04659 (65 yrs), AG13369 (68-I yrs), AG14251 (68-II yrs), AG11243 (74 yrs), AG09156 (81 yrs), AG13349 (86 yrs), AG13129 (89 yrs) and AG04064 (92 yrs). Among these cell cultures we had 2 isogenic pairs (biopsy from the same donor at different ages), which were AG04456 (49 yrs), AG14251 (68-II yrs) and AG04659 (65 yrs), AG11243 (74 yrs). These cells were propagated and cell stocks frozen according to Coriell's standard procedures. When fibroblasts were propagated for experiments, confluent cells were split and re-plated at a cell density of 1.2×10^4 cells per cm^2. The cells were fed with warm growth media every 3 days. The number of population doublings was calculated as log [(TVC / seed) × 3.32], where TVC is the total viable cells after trypsinizing the confluent cell culture, and seed is the number of cells that are used to passage cells to a new flask.

3.2.2 Design of the Experimental System

Human skin fibroblasts were cultured in growth medium that consisted of MEM (Minimum Essential Medium, Invitrogen, 51200-038) supplemented with 15% FBS (Fetal Bovine Serum) and 1% L-glutamine at 37 °C and 5% CO_2 without antibiotics. The cells were plated at sub-confluence (1.2×10^4 cells per cm^2) in growth medium to prevent contact inhibition. To exclude confusion by *in vitro* aging, cells at less than 50% of their total *in vitro* life span were used for all the experiments. An early passage number is important not only to avoid replicative senescence, but also because prolonged cell culturing introduces a bias towards the most robust, proliferative cells, diminishing the relationship between donor age and replicative vigor (Cristofalo, Allen et al. 1998). After

24 hours, the growth medium was replaced with serum-free medium (MEM supplemented with 0.2% BSA (Bovine Serum Albumin) and 1% L-glutamine). At this point, any experimental treatments were included in the starvation medium and then the cells were incubated for another 24 hours. On the day of sample collection, cells were washed once with ice-cold 1×PBS and then appropriate methods for the downstream applications were followed as described below. Cell cycle distribution was determined by PI staining followed by FACS analysis. Percentage of cells in S-phase before starvation was generally >10% and after serum starvation <1%. It was important to use starvation media so as to introduce quiescent state which is more physiologically relevant steady-state of cells.

3.2.3 Sample Collection for RNA Isolation

350µl of lysis buffer (Buffer RLT, Qiagen) was used per T25 flask and cells were scrapped and collected in sterile eppendorf tubes. These cell lysates were then snap frozen in a dry ice/isopropanol bath and stored at -80 °C until use. RNA was isolated from the cell lysates using Qiagen RNeasy mini kit according to the manufacturer's instructions.

3.2.4 Microarray Analysis

Gene expression analysis was performed using the Codelink human bioarray containing single-stranded 30-mer oligonucleotide probes (Applied Microarrays, Tempe, AZ) and

chips were run in duplicate. Details of this platform are available on the vendor's homepage. Characteristics of the Codelink platform have been evaluated by us (Young, DiSilvestro et al. 2003), and as part of the microarray quality control (MAQC) assessment (Canales, Luo et al. 2006). Sample preparation and hybridization followed procedures described in (Young, DiSilvestro et al. 2003). Slides were scanned at 5 µm resolution with a ScanArray 4000xl (Perkin Elmer, Waltham, Ma) and analyzed with the CodeLink Analysis Software, providing an integrated optical density (IOD) value for each hybridization spot, which is a measurement of an integrated background intensity value subtracted from the total pixel intensities within the area of the spot. The data was uploaded onto the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). Expressions of characterized genes related to immunity and inflammation were identified, replicate readouts were averaged, outliers detected and differential expressions determined. A variance filter trimmed the resulting list ($p<0.15$). We used a correlative approach to search for similarities between the NF-κB profile and expression of genes related to inflammation. In modification of previously used rank correlation for template matching of phenotypical markers (Kriete, Anderson et al. 2003; Boyce, Kriete et al. 2005), we used Pearson correlation as a measure of similarity, because the NF-κB values of the samples from young donors were close and within error margins, which can lead to low correlation values if ranked wrongly. Finally, the data was clustered by a dendrogram, using single linkage analysis and a Canberra distance metric (J-Express, Molmine AS, Norway).

3.3 Results:

3.3.1 Gene expression

Microarrays included 55,000 single-stranded oligonucleotide probes (GE-Healthcare/Applied Microarrays, Tempe, AZ) corresponding to 16,220 characterized genes. All arrays were run in duplicate. We determined differential expression of genes between the young and old group, and the resulting list was trimmed by an outlier detection and statistical significance threshold. We found 549 genes (3.4%) differentially up-regulated > 2-fold between fibroblast cultures from old compared to young donors, and 327 genes (2.0%) down-regulated <-2-fold. We used gene ontologies (GEO) to broadly classify genes, and DAVID (http://david.abcc.ncifcrf.gov/) to seek pathway associations.

The ontological classification of molecular function of up-regulated genes included cytokines, peptidase and hydrolase activities, as well as ion binding and calcium binding proteins. Prevailing groups of genes with down-regulated expression included ribosomal proteins as well as other RNA binding proteins, indicative of reduced protein synthesis. Considerable variation in expression of these transcripts in the older group possibly reflects variability in the cell panel used. As expected, we did not observe significant differences in transcripts related to cell cycle, including cyclins. Furthermore, the profile was different than previously described for cells that have undergone replicative senescence (Shelton, Chang et al. 1999).

Thirty-one genes constituted an inflammatory signature in these cells (Table 1). The dendrogram, as seen in Figure 3.a, reproduced from (Kriete, Mayo et al. 2008), shows

hierarchical ordered clusters using expression values normalized for each gene. One group of genes represents a strong correlation with NF-κB, including HLA-G, a class I histocompatibility antigen; PTGIS, a protein involved in prostaglandin I2 (prostacyclin) synthase; HSPA9B, a mitochondrial heat shock 70kDa protein 9B (mortalin-2), and PPBP, a pro-platelet chemokine (C-X-C motif) ligand 7. Elevated expression levels in cells from older individuals were observed for inflammatory cytokines including interleukin 1 (IL1A), cardiotrophin-like cytokine (CLC), a member of the IL-6 family, TNF-α (TNFAIP6), a tumor necrosis factor receptor (TNFRS13B) and an interleukin 8 receptor (IL8RA). Expression of mRNA encoding proteins involved in the immune responses *in vivo* was elevated including MAGEA5, BAGE, FOSL1, CD3D and STAG3, as well as another member of prostaglandins, PTGES. Prostaglandins (PGs), members of the eicosanoid family, are intimately linked to inflammation and act as local hormones that bind to specific cellular receptors. Elevated transcripts of several chemokine ligands could be found, including CXCL2, CXCL3 and CXCL6, consistent with the potential of aged fibroblasts to chemo attract immune cells. Another remarkable group of genes with potential roles in the inflammatory response were elements of both the classical (C1R) and alternative (DF/adipsin) complement cascades.

Another cluster proteins induced by interferons (IFIT1, IFITM1, G1P2, IFI35, IFIH1, MX1) and a TNF-stimulated protein TNFAIP6 were identified. Next to HLA-G, as mentioned above, fibroblasts from aged individuals contained higher message levels for another MHC class I related gene, HLA-F, with a role in antigen presentation, and, potentially, autoimmunity. Collectively, these results indicate that several genes involved

in inflammation and innate or acquired immune response pathways are expressed at significantly higher levels in older donors, contributing to a moderate inflammatory state.

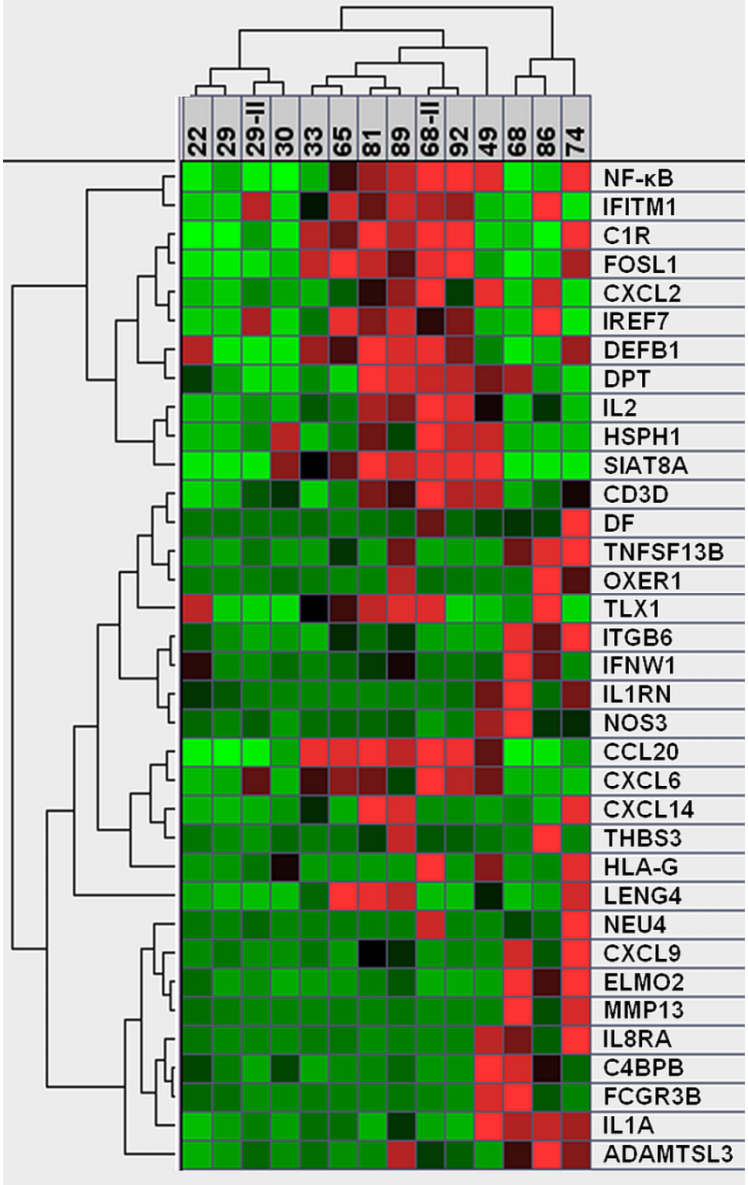


Figure 3.a: Hierachical clustering of genes encoding inflammatory mediators and effectors in cultured fibroblasts of normal individuals of increasing age, showing up regulation (coded red) in donors of middle and old age (Kriete, Mayo et al. 2008).

The transcriptional profiles are further indicative of functional alterations in organelles like peroxisomes, golgi, endosomes and lysosomes. Peroxisome proliferator activated receptors (PPARs), known to mediate inflammatory stress, such as PPAR alpha, showed enhanced activity. Transcripts of GTP-ases belonging to the RAS family, such as RABGAP1, RAS7A and RAB14 involved in endosomal and lysosomal degradation processes, as well as cathepsin S (CTSS), were elevated. However, we also observed changes supporting the idea that aging can be associated with catabolic malfunction (Terman and Brunk 2004). For instance, RAB7, essential for endosome and lysosomal function, is expressed at lower levels (Bucci, Thomsen et al. 2000). Furthermore, we observed down-regulation of heat shock proteins, lower levels of the H+ transporting lysosomal ATPase (ATPASE), essential for preserving an acidic lysosomal environment, as well as lower transcripts of galactosidase (GLB1), required for cleavage of the terminal beta-galactose from glycoconjugates. Therefore our data supports that mechanisms of degradation are increased, but not necessarily sufficiently enhanced. Moreover, down-regulated gene transcripts directly involved in transcriptional regulation, such as TP53 inducible protein indicate further age-associated deregulations. This is in line with another study that shows decline in transcriptional activity of p53 function as a function of age (Feng, Hu et al. 2007).

3.3.2 NF-κB and Correlation with Gene Expression

The transcription factor Nuclear Factor kappa B (NF-κB) plays a key role in regulating inflammatory responses (Perkins 2007). Therefore, we analyzed nuclear import and the DNA binding activity of NF-κB p65 using a sensitive chemiluminescent DNA binding assay, which demonstrated variable but generally higher NF-κB activity levels in older cells as shown in Figure 3.b, reproduced from (Kriete, Mayo et al. 2008). To address whether differential gene expression as assessed by microarrays was associated with NF-κB activity, we determined the correlation between the NF-κB activity profile as shown in Figure 3.b and each gene, expressed by a Pearson correlation coefficient R between +1 and -1, as shown in Table 1 for inflammatory markers. We identified a group of 103 genes that were expressed at high levels in old cells and correlated positively with NF-κB activity ($R > 0.7$). Conversely, a group of 63 genes with reduced expression relative to young fibroblasts was negatively correlated ($R < -0.7$) with the NF-κB profile. This suggested a central role of NF-κB in the inflammatory signature in older fibroblasts.

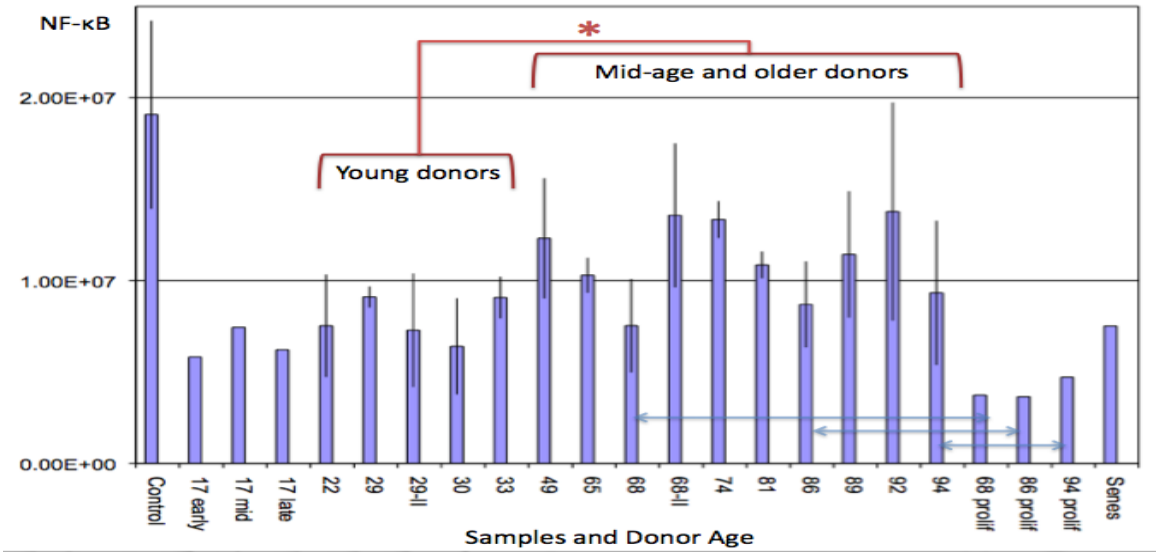


Figure 3.b: Steady-state DNA binding of the NF-κB p65 transcription factor in pre-sensecent fibroblasts dervied from donors of different ages. Binding activity is independent of PDL as shown for the sample from a 17 year old donor. Elevations in the group of older donors in quiescence (p = 0.04, independent two-tailed test) are indicative of NF-κB being a mediator of the inflammatory profile observed in the microarray analysis. NF-κB binding activity is lower in proliferating cells as shown for 68-, 86- and 94-year old samples and also lower in the senescent sample derived from a 66 year-old donor at right. The positive control on the left represents TNFα treated HeLa cells.

Table 1: Correlation of inflammatory markers and NF-κB profiles in quiescent fibroblasts derived from donors at different biological ages.

ACCN#	fold	R	Description
NM_002127	4.3	0.92	HLA-G histocompatibility antigen, class I, G (HLA-G)
NM_005438	3.4	0.86	FOS-like antigen 1 (FOSL1)
NM_002090	2.7	0.85	chemokine (C-X-C motif) ligand 3 (CXCL3)
NM_000961	3.0	0.82	prostaglandin I2 (prostacyclin) synthase (PTGIS)
NM_017595	3.8	0.80	NF-κB inhibitor interacting Ras-like 2 (NKIRAS2), transcript variant 2
NM_004591	3.0	0.79	chemokine (C-C motif) ligand 20 (CCL20)
NM_000732	2.6	0.79	CD3D antigen, delta polypeptide (TiT3 complex) (CD3D)
NM_001937	3.8	0.77	dermatopontin (DPT)
NM_002089	3.9	0.77	chemokine (C-X-C motif) ligand 2 (CXCL2)
NM_002993	4.9	0.76	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2) (CXCL6)
NM_001733	3.3	0.73	complement component 1, r subcomponent (C1R)
NM_004134	4.6	0.69	heat shock 70kDa protein 9B (mortalin-2) (HSPA9B), nuclear gene encoding mitochondrial protein
NM_002704	3.0	0.62	pro-platelet basic protein (chemokine (C-X-C motif) ligand 7) (PPBP)
NM_018950	7.7	0.60	major histocompatibility complex, class I, F (HLA-F)
			D component of complement (adipsin)

4 AIM 2 – DETERMINE AGE-DEPENDENT ENERGY ALTERATIONS AND ITS CORRELATION WITH NF-κB PROFILE

4.1 Introduction

Our initial studies of primary changes in transcription patterns in steady-state homogeneous fibroblast cell cultures revealed a low-grade chronic inflammatory signature, which provided a novel opportunity to unlock the origin and mechanisms of inflammation and age-related diseases. Aging can also be defined as a condition in which chronic stress when not counteracted by adequate protective mechanism may lead to development of age-related maladies. A number of studies have indicated that aging presents increased vulnerability to the chronic oxidative stress or inflammation (Joseph, Shukitt-Hale et al. 2005; Khansari, Shakiba et al. 2009). Among the different theories of aging, the "mitochondrial theory of aging" has managed to gather most attention and suggests that age-related accumulation of oxidative stress is accountable for cellular damage, dysfunctional mitochondria and altered cellular energetics ultimately leading to age-related diseases (Cadenas and Davies 2000; Gianni, Jan et al. 2004; Tosato, Zamboni et al. 2007).

4.2 Objective

With the activity of NF-κB already determined as being significantly different in fibroblasts from young donors when compared to their older counterparts, we sought to investigate possible variations in cellular energy levels. The underlying hypothesis tested in this section is that the constitutive NF-κB activation as it relates to chronic

inflammation is associated with altered cellular redox state, diminished mitochondrial respiratory capacity and alteration of cellular functions. The proposed methods were implemented on a set of 14 fibroblast cell lines derived from young, mid-age and old donors, in which we previously determined gene expressions of inflammatory markers and NF-κB binding activity.

4.3 Materials and Methods:

Human fibroblast cultures at biological ages ranging from 22 years to 92 years were plated for the experiments as described in sections 3.2.1 and 3.2.2

4.3.1 Measurement of ROS levels

Intracellular ROS production was detected using CM-H_2-DCFDA (Molecular Probes, C6827); a cell-permeant fluorescent dye that passively diffuses into the cells. It was dissolved in serum-free medium that contained no phenol red and used at a final concentration of 10μM. After a wash cycle, the cells were trypsinized and the cell pellets collected in 5ml FACS tubes and subsequent steps were followed as per the manufacturer's instructions. Finally, the cell-pellet was re-suspended into the medium containing the probe and the control cells with DMSO and serum-free medium. The cells are then incubated for 30mins in dark at 37°C. After two wash cycles, the cells were re-suspended in staining buffer and fluorescence intensities are measured at excitation and

emission, in nm: 492-495/517-527, in nm using BD FACSCalibur System and expressed as mean fluorescence intensities.

4.3.2 Determination of cell-viability

CellTiter-Fluor™ Cell Viability Assay (Promega) is based on the principle that fluorogenic and cell-permeant peptide substrate GF-AFC (glycylphenylalanyl-aminofluorocoumarin) enters only intact cells, and cleaved by the live-cell protease activity. This in-turn generates a fluorescent signal proportional to the number of only viable cells after experimental manipulation.

4.3.3 Determination of intracellular ATP content

CellTiter-Glow® Luminescent Cell Viability Assay (Promega) was used to measure the cellular ATP levels and these measurements were normalized by the number of viable cells at the time of the experiment by performing CellTiter-Fluor™ Cell Viability Assay (Promega) preceding ATP measurement as described in section 4.3.2.

CellTiter-Glow® Luminescent Cell Viability Assay was used to generate a stable "glow-type" luminescence, which is proportional to the ATP concentration present, following cell lysis by CellTiter-Glo® reagent. The generation of bioluminescence is based on the principle that the oxidation of luciferin is catalyzed by luciferase enzyme in the presence of Mg2+, ATP and molecular oxygen. More specifically, cells were seeded in a 96-well plate in triplicates with 100µl of growth media/well and after 24 hours, the cells were

starved for another 24 hours. Assays were carried out as per manual instructions and first the fluorescence generated by addition of substrate GF-AFC was measured using a fluorescence reader set up at 380-400nm excitation, 505nm emission, sensitivity '125' and optics position 'bottom'. Following this, addition of CellTiter-Glo® reagent resulted in luminescent signal that was measured using a Veritas luminometer.

4.3.4 Statistical Analysis of ATP concentration data

Data analysis was performed using SAS 9.3 statistical program and the Generalized Estimating Equation (GEE) through the "Proc GenMod" SAS procedure. GEE is similar to simple linear regression but accounts for repeating the experiment three times on the same individuals.

4.4 Results

4.4.1 ROS levels in *in vivo* aged fibroblasts

High ROS levels are known to inflict damage on cellular organelles and macromolecules and subsequently lead to activation of the redox sensitive NF-κB transcription factor (Kregel and Zhang 2007). Therefore, to determine if oxidative stress and associated metabolic alterations played a role in aging, we first measured the basal ROS levels in the *in vivo* aged fibroblast cultures.

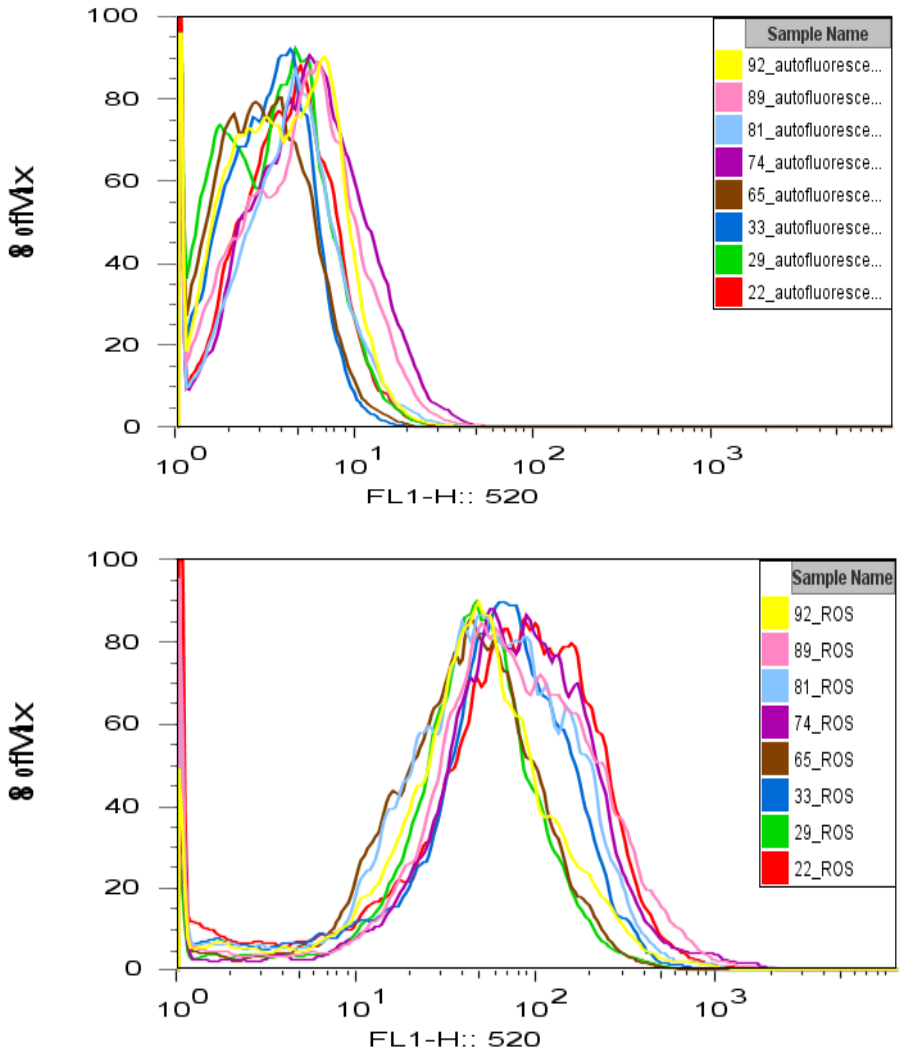


Figure 4.a: Steady-state ROS levels in fibroblasts of different biological ages. Fluorescence intensities from untreated (autofluorescence levels on the top) and treated with ROS probe (ROS levels at the bottom) *in vivo* aged fibroblasts and expressed as mean fluorescence intensities.

We did not find any significant change in ROS production as it relates to increasing age of fibroblast donors as shown in Figure 4.a and Table 2. However, we observed a subtle increase in cell intrinsic autofluorescence in the older fibroblasts. Cellular autofluorescence is an intrinsic property of the cells, where in certain endogenous

molecules become fluorescent when excited in the spectral range of 500-600nm, mostly originating from mitochondria and lysosomes, and may suggest alterations in the metabolic state of the stressed cells (Monici 2005). Therefore, in order to understand the role of metabolism in aging, we measured intracellular ATP in the pre-senescent human skin fibroblasts.

Table 2: Values representing mean fluorescence intensities in Figure 4.a

cell-line	biological-age	Autofluorescence (mean-intensity)	ROS (mean-intensity)
PI00892A	22	19.07	131.63
PI00924	29	21.84	68.47
AG04438	33	19.88	95.73
AG04659	65	23.53	66.09
AG11243	74	20.37	130.14
AG09156	81	19.97	103.12
AG13129	89	19.64	137.61
PI00931	92	19.86	83.04

4.4.2 Intracellular ATP content and its correlation with NF-κB p65 DNA binding activity

Following the unforeseen result that basal ROS levels were comparable across age groups in *in vivo* aged fibroblasts, we asked if there were any alterations in the cellular energetics

and determined total intracellular ATP levels. We found that the total intracellular ATP levels decreased as a function of age as depicted in Figure 4.b.

Statistical analysis was performed using GEE and various models were tested. The best model resulted in a p-value of 0.0447, and the following equation:

$$\hat{y} = 24.615 - Age * 0.0653 + Exp1 * 6.1743 - Exp2 * 6.4170$$

The additional terms adjust for fluorescent intensity differences between the experiments. These results indicate a small but statistically significant energy limitation in aging fibroblasts that may result in low-grade chronic energy stress.

We went on to address whether the decline in the ATP levels was associated with increase in NF-κB activity. We therefore determined the correlation between the NF-κB activity data as depicted in Figure 3.b and ATP data (second experiment) as shown in Figure 4.b, expressed by a Pearson correlation coefficient R between +1 and -1. The ATP data inversely correlated with NF-κB activity data ($R = -0.6$, $p = 0.039$). This raised the question whether a functional relationship exists between ATP decline and elevated NF-κB in the older fibroblasts.

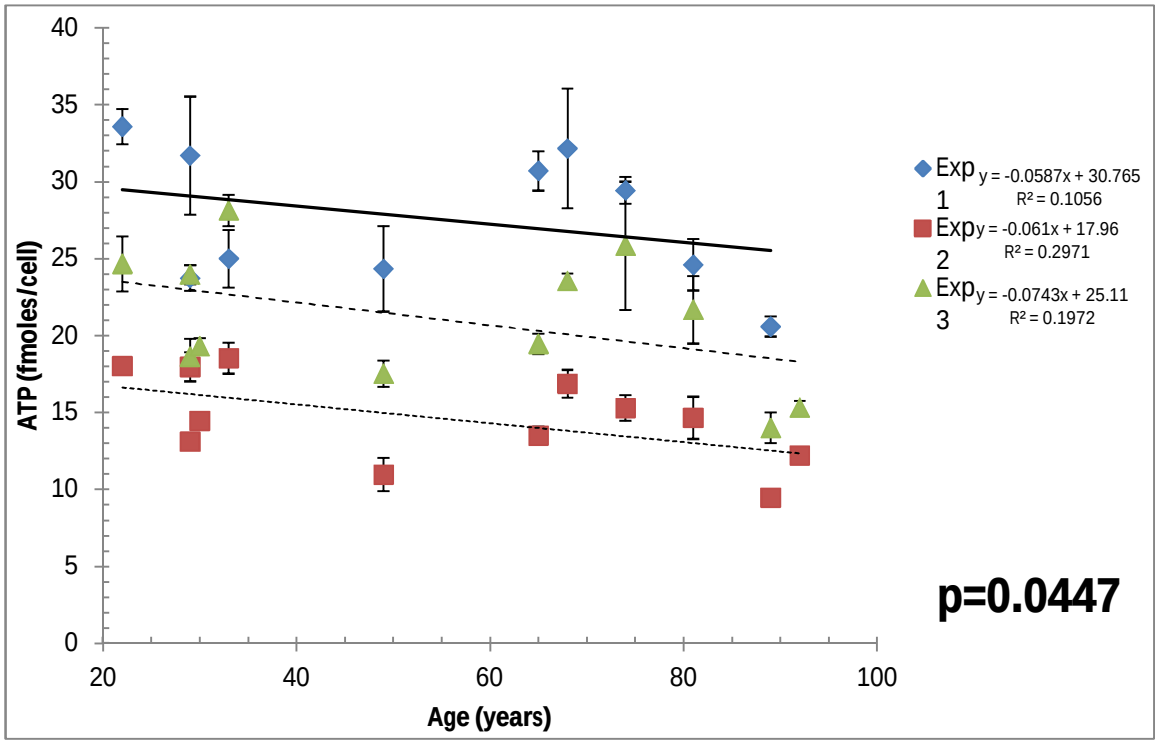


Figure 4.b: Decline in intracellular ATP concentration (p = 0.0447, used Generalized Estimating Equations on data from three independent experiments) in human skin fibroblasts derived from individuals of different ages. Each point represents three measurements made in the same experiment. Regression analysis for each experiment is shown and standard deviation is plotted.

5 AIM 3 – CONNECTIVITY OF AGE-DEPENDENT ENERGY DECLINE AND ELEVATED NF-κB ACTIVITY USING ENERGY DEPRIVATION MODEL

5.1 Introduction

We reported a reduction in the total cellular ATP levels in *in vivo* aged fibroblasts and its inverse correlation with NF-κB activity as a function of age. These subtle but statistically significant findings motivated us to explore a probable connection between them. In line with our findings, two research groups have independently reported similar reductions in ATP levels and mitochondrial respiratory capacity in aging fibroblasts (Greco, Villani et al. 2003; Miyoshi, Oubrahim et al. 2006).

5.2 Objective

The purpose of this aim was to find a possible link between the reduced total ATP levels and elevated NF-κB activity as it relates to aging. To achieve this particular aim, we induced different amounts of energy depletion in the 'young' fibroblasts as described in the sections to follow and observed the effects of this treatment on the NF-κB activity.

5.3 Materials and Methods

Fibroblasts from a 22-year old donor were plated for the experiments as described in sections 3.2.1 and 3.2.2. In order to mimic the reduced ATP levels in old fibroblasts; we combined drugs that target both major sources of ATP generation in the cell. Mitochondrial respiration inhibitors to uncouple the respiration chain from the

phosphorylation reaction resulting in inhibition of mitochondrial ATP synthesis and glycolysis inhibitor to inhibit aerobic glycolysis. Uncoupling agents 2, 4-Dinitrophenol (DNP) and Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) were used at working concentrations of 5mM and 10µM respectively. DNP (Sigma, D198501) and FCCP (Sigma, C2920) were dissolved in 95% ethanol to prepare 833mM and 50mM stock concentration in that order. Glucose analog 2-Deoxy-D-glucose (2DG) was used at a final concentration 5mM and 10mM resulting in reduced cellular ATP levels. 2DG (Sigma, D6134) was dissolved in water and sterilized by filtration to prepare 1M stock solution.

5.3.1 Determination of intracellular ATP content

CellTiter-Glow® Luminescent Cell Viability Assay (Promega) was used to measure the cellular ATP levels as described in section 4.3.3.

5.3.2 Sample collection for preparation of nuclear and cytoplasmic extracts

$1 – 2 \times 10^6$ cells were plated and trypsinized at a constant incubation time (3 minutes at 37°C) followed by centrifugation at 1200 rpm for 10 minutes at 4°C. The sample containing tubes were inverted on a stack of paper towels to get rid of the supernatant and cell pellet was carefully resuspended in 1ml ice cold 1×PBS, transferred to 2ml tubes and then centrifuged at 500×g (rcf) for 10 minutes. The supernatant was removed once more by inverting the tube on a stack of paper towels and centrifuged again. This time the

supernatant was carefully pipetted out to leave the cell pellet as dry as possible. The samples were snap frozen in a dry ice/isopropanol bath and stored at -80°C until subsequent extraction of nuclear and cytoplasmic fractions. The nuclear and cytoplasmic fractions were prepared using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Product No. 78833, Rockford, IL) according to the manufacturer's instructions. The protein concentration was estimated using a BCA Protein Assay kit (Pierce) and the extracts were preserved at –80°C until use.

5.3.3 NF-κB p65 transcription factor assay

The DNA-binding activity of NF-κB p65 protein was measured using the EZ-Detect chemiluminescent transcription factor assay kit (Pierce, 89859, Rockford, IL) as per the manufacturer's instruction. The kit was provided in a 96-well format and consisted of streptavidin-coated plates with bound NF-κB binding-consensus sequence. 10µg of nuclear extracts (prepared as described in section 5.3.2) were incubated with 50µl of binding buffer for an hour with mild agitation at room temperature. Assay was performed in triplicates with thorough washes between each step. The bound "active form" of NF-κB was detected by NF-κB p65 primary antibody followed by HRP-conjugated secondary antibody incubations for an hour each with no agitation at room temperature. Chemiluminescence, generated by adding equal amounts of Luminol/Enhancer Solution and Stable Peroxide Solution, was immediately measured using a Veritas Microplate Luminometer with an integration time of 8 seconds. Luminometric data were expressed in relative light units and/or converted to fold change relative to the control samples.

5.4 Results:

5.4.1 Depletion in cellular ATP levels with single and combined DNP, FCCP and 2DG treatments on 'young' fibroblasts

Initial experiments were done using either DNP or FCCP at concentrations that did not affect cell viability. Though the ATP synthesis from mitochondria may have reduced greatly, the total intracellular ATP levels decreased only marginally. The fibroblasts seem to efficiently compensate for the induced energy crisis by up-regulating glycolysis, which was confirmed with increased lactate levels in the cell culture medium.

However, by combining both mitochondrial respiration and glycolysis inhibitors (DNP/FCCP and 2DG) and treating the 'young' fibroblasts for 24 hours, we obtained a massive reduction in total intracellular ATP levels as shown in the Figure 5.a.

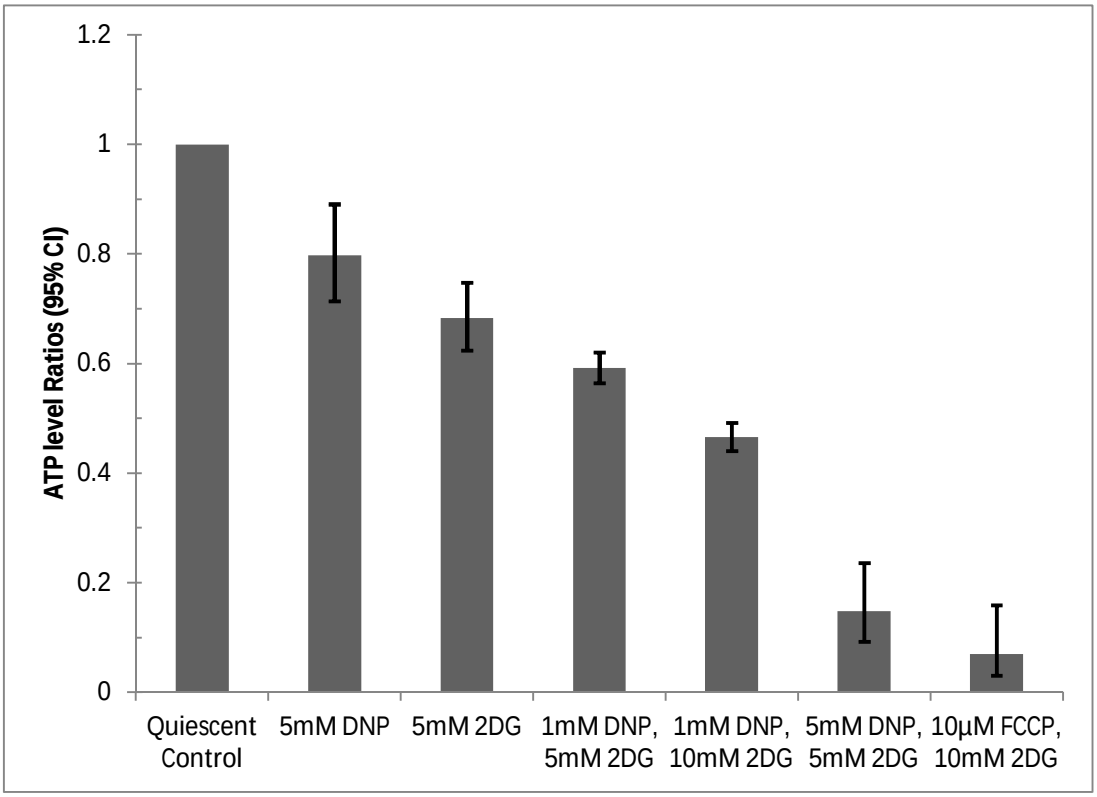


Figure 5.a: Reduced intracellular ATP levels following treatment with different concentrations of mitochondrial uncouplers (DNP, FCCP) and/or glycolysis inhibitor (2DG) in the young fibroblasts. Agents were used either alone or in combination at the concentrations indicated. Strong reductions of ATP levels were noted following combined treatments. Standard errors are plotted from 3 independent experiments.

5.4.2 Increase in NF-κB p65 DNA binding activity with single and combined DNP, FCCP and 2DG treatments on the young fibroblasts

We noticed a considerable increase in NF-κB p65 DNA binding activity on combining higher concentrations of mitochondrial respiration and glycolysis inhibitors unlike single treatments or by combining lower concentrations of both the inhibitors as shown in Figure 5.b & Figure 5.c.

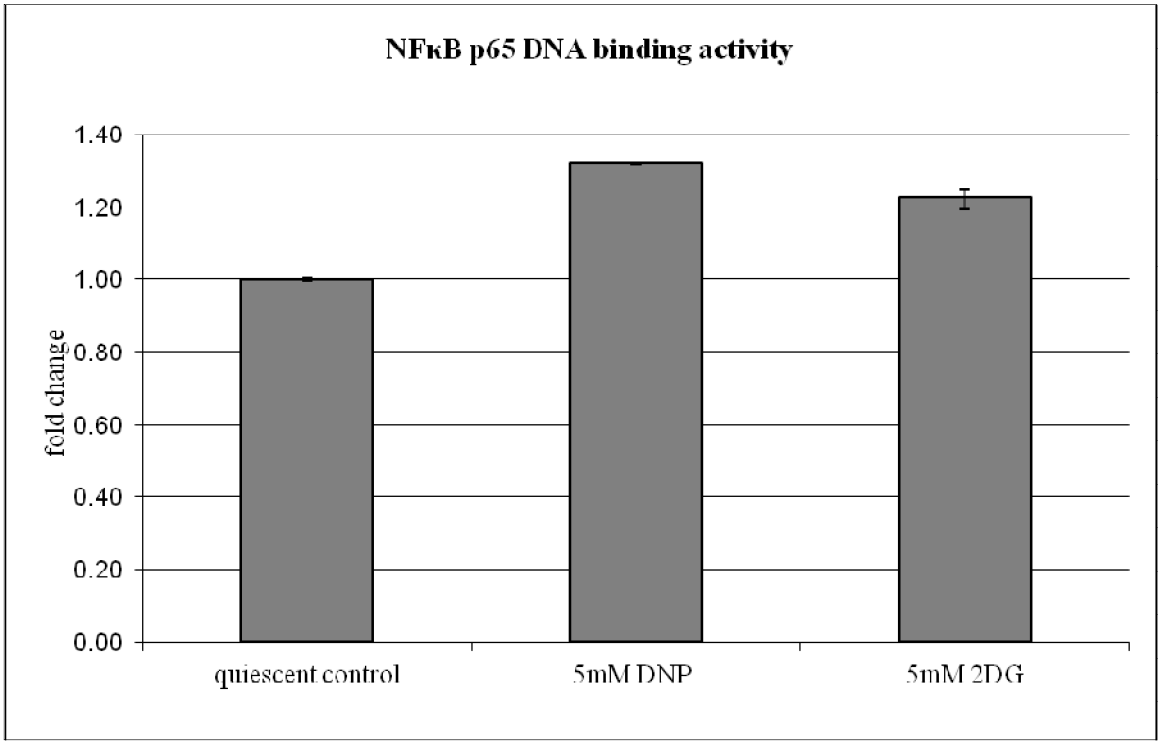


Figure 5.b: Slightly elevated NF-κB p65 DNA binding activity following treatment with mitochondrial uncoupler - DNP and glycolysis inhibitor - 2DG separately in the young fibroblasts. (Error bars represent standard deviation – very little variation is observed for quiescent control and 5mM DNP treatment)

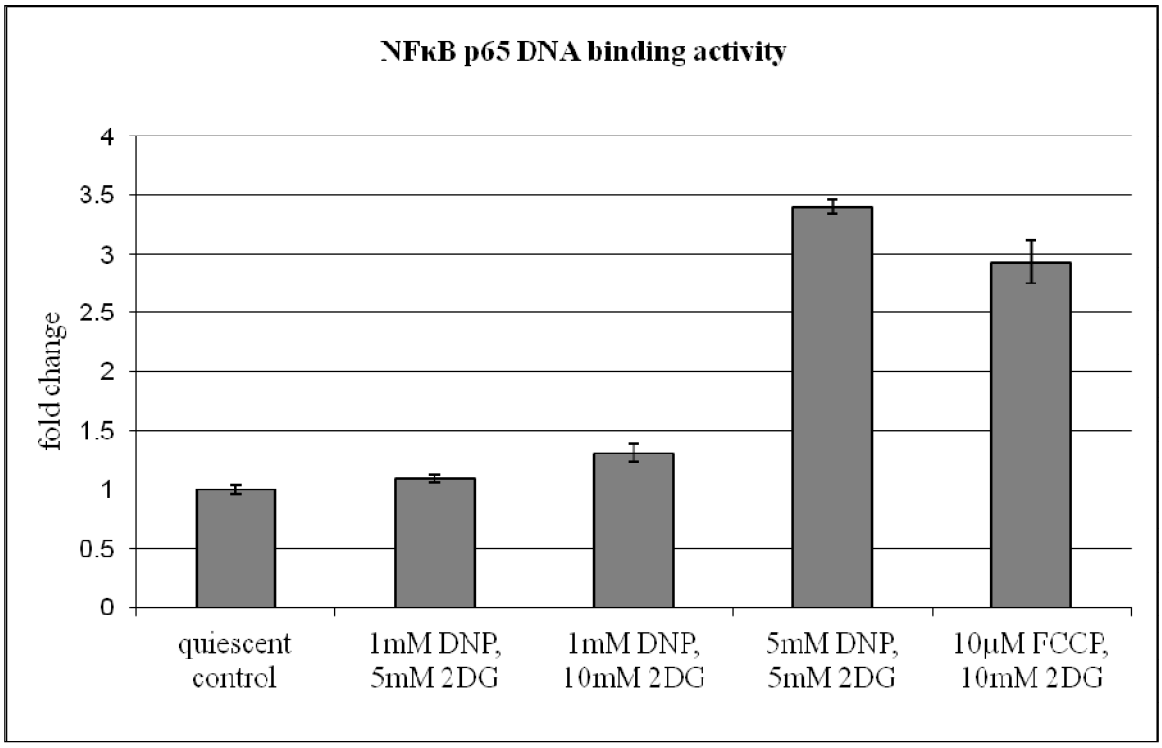
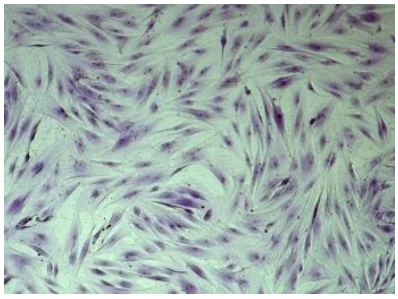


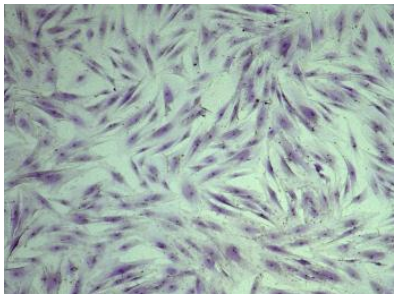
Figure 5.c: Marked increase in NF-κB p65 DNA binding activity following combined treatment of mitochondrial uncouplers (DNP/FCCP) and glycolysis inhibitor (2DG) in the young fibroblasts. (Error bars represent standard deviation)

Thus, similar to the effect of mitochondrial respiration and/or glycolysis inhibitors on the ATP content, either of the single treatments did not significantly increase NF-κB. By contrast, we observed a marked increase in NF-κB p65 DNA binding activity by combining both mitochondrial respiration and glycolysis inhibitors at higher concentrations. These results are consistent with the notion that ATP depletion as a consequence of mitochondrial OXPHOS inhibition in addition to glucose deprivation induced NF-κB substantially in the 'young' fibroblasts. We further noticed morphological changes in the fibroblasts with the double treatments. We observed cell

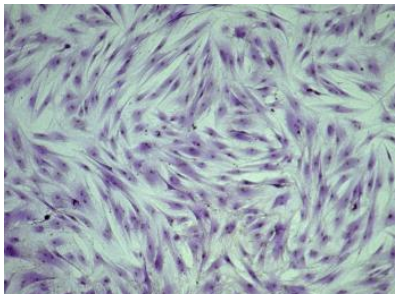
shrinkage, only by combining higher concentrations of both mitochondrial respiration and glycolysis inhibitors and treating the fibroblasts for 24 hours as shown in Figure 5.d.



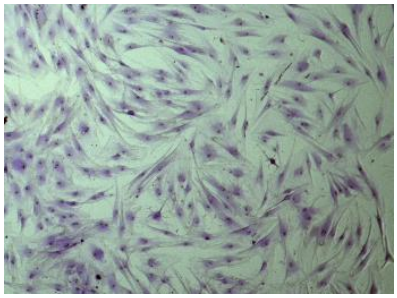
Quiescent fibroblasts



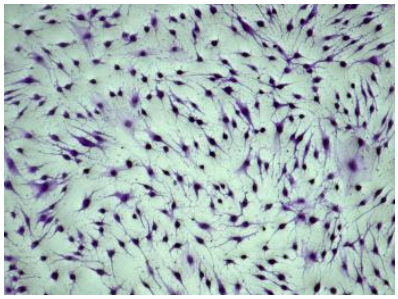
Quiescent fibroblasts with vehicle



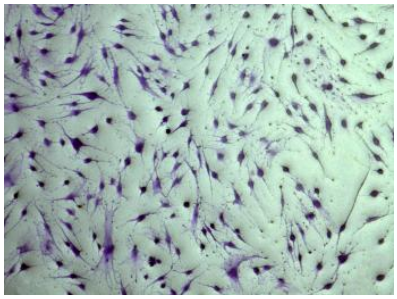
Quiescent fibroblasts with 1mM, 5mM – DNP, 2DG



Quiescent fibroblasts with 1mM, 10mM – DNP, 2DG



Quiescent fibroblasts with 5mM, 5mM – DNP, 2DG



Quiescent fibroblasts with 10uM, 10mM – FCCP, 2DG

Figure 5.d: Images representing 'cell shrinkage' as visualized by crystal violet stain in response to different concentrations of inhibitors for 24 hours.

5.4.3 The increase in the NF-κB p65 DNA binding activity and cell shrinkage due to energy depletion is a reversible process

Metabolic inhibitors were present for 24 hours in the cell culture medium, followed by replacement with inhibitor-free medium for another 24 hours. We achieved complete recovery from stress by changing the medium to inhibitor-free medium in terms of reverting both, the increased NF-κB p65 DNA binding activity to normal basal levels and cell shrinkage to normal morphology as shown in Figure 5.e & Figure 5.f.

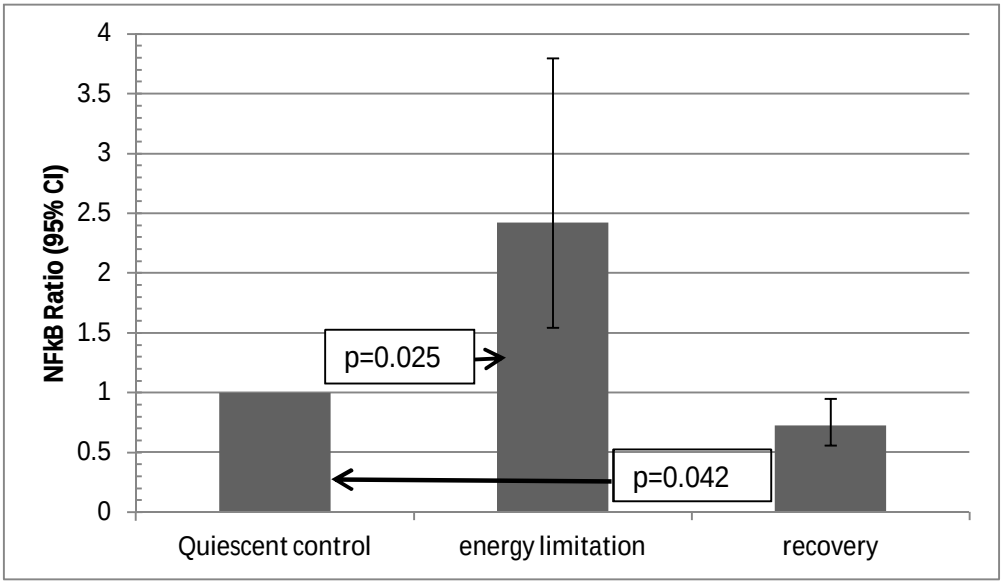
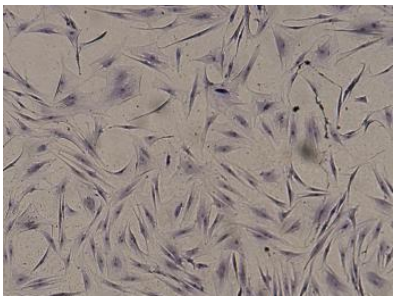
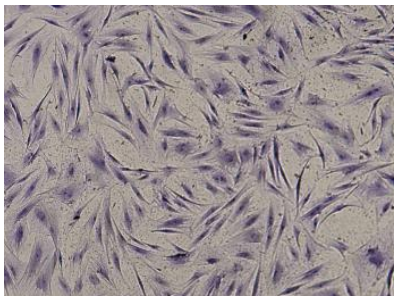


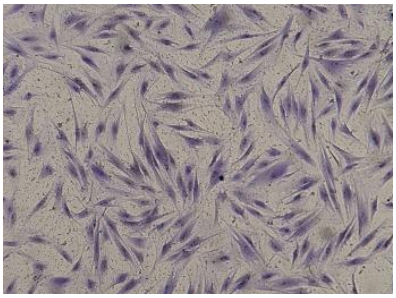
Figure 5.e: NF-κB p65 DNA binding activity reverts back to basal levels after removal of energy stress in the young fibroblasts (from two independent experiments). Standard errors are plotted.



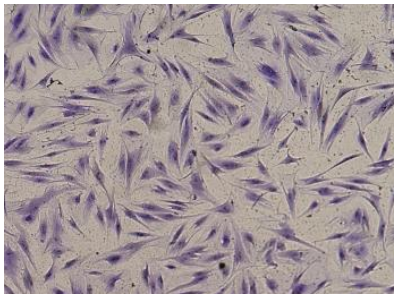
Quiescent fibroblasts



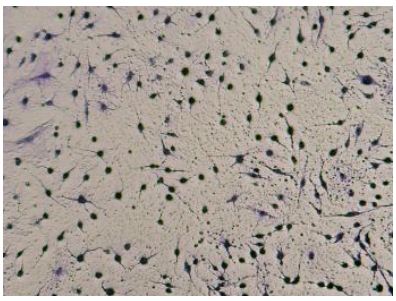
Quiescent fibroblasts with vehicle



Quiescent fibroblasts with 1mM, 5mM – DNP, 2DG

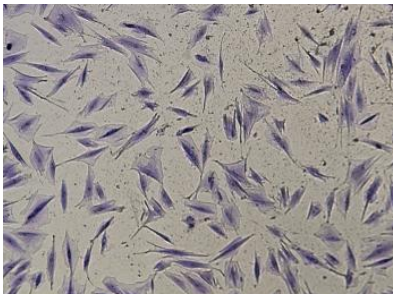


Quiescent fibroblasts with 1mM, 10mM – DNP, 2DG

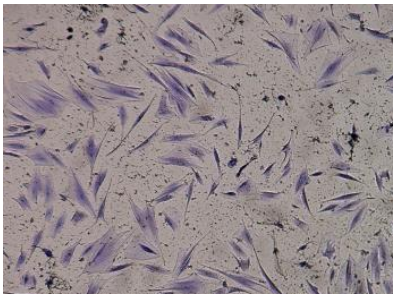


Quiescent fibroblasts with 5mM, 5mM – DNP, 2DG

Quiescent fibroblasts with 10uM, 10mM – FCCP, 2DG



Recovered fibroblasts from 5mM, 5mM – DNP, 2DG



Recovered fibroblasts from 10uM, 10mM – FCCP, 2DG

Figure 5.f: Images representing cell shrinkage morphology using crystal violet stain in response to different concentrations of inhibitors for 48 hours followed by a complete recovery on switching to inhibitor-free medium for 24 hours.

6 AIM 4 – PATHWAYS REGULATING NF-κB IN AGING HUMAN FIBROBLASTS

6.1 Introduction

In order to induce energy deprivation, we inhibited both mitochondrial respiration and glycolysis. As a result of this treatment regimen involving both serum and glucose deprivation in the 'young' fibroblasts, we reported an increase in NF-κB p65 DNA binding activity as shown in Figure 5.c. We therefore explored the status of the nutrient sensing Akt/mTOR and autophagy pathways and DNA damage sensing p53 pathway using the energy deprivation model previously introduced in section 5.

6.2 Objective

We sought to investigate molecular pathways contributing to the increased NF-κB p65 DNA binding activity in response to metabolic starvation. Our primary aim was to find if the increase in NF-κB p65 translocation into the nucleus was a result of canonical NF-κB pathway activation. Additionally, we sought to explore if p53 and Akt and its downstream effectors in addition to autophagy played a role in regulating the NF-κB activity as a result of serum and glucose starvation.

6.3 Materials and Methods

6.3.1 Acetone precipitation

The cytoplasmic extracts that were obtained from nuclear and cytoplasmic fraction extraction (as described in section 5.3.2) were subjected to acetone precipitation to eliminate substances that interfered with subsequent downstream immunoblotting. Cold (-20°C) acetone, a volume four times that of the cytoplasmic extracts to be precipitated was added. The acetone-protein mixture was then vortexed and incubated for an hour at -20°C. Following centrifugation for 10 minutes at 15000 × g, the supernatant containing the acetone soluble interferences was discarded. After 30 minutes of air-drying at room temperature, the protein pellet was reconstituted in 60µl of lysis buffer (added protease inhibitor to laemmli buffer at a ratio 1:25). Total protein concentration was quantified from the sample using BCA protein assay (microplate format).

6.3.2 Immunoblot analysis

30-45µg of protein from the acetone precipitated cytoplasmic extracts (as described in section 6.3.1) were loaded into the 4 - 20% gradient gels and separated according to molecular weight using gel electrophoresis. A constant voltage of 120 V was applied for approximately 90 minutes. In order to detect the antibodies of interest, the proteins were transferred from the gel onto a nitrocellulose membrane. Non-specific proteins were blocked to prevent their interactions with the antibody used for detection of the target protein. The detection process involved probing the membrane with primary antibodies against phospho-Akt (Ser473), Akt, phospho-GSK3β (S9), IκB-α, Phospho-Bad (Ser136)

(D25H8) and LC3B from cell signaling (cat # 9271, 9272, 9336, 9242, 4366 and 2775 respectively). Other primary antibodies include anti-NF-κB p65 (phospho S276) from Abcam (ab30623), p62 (BML-PW9860 Enzo Life Sciences), p53 (sc-263 santa cruz biotechnology, inc.) and GSK3β (610201 BD trans. Lab.). The membrane was exposed to X-ray film in a dark room for the required time. The blots were stripped and re-probed 3 to 4 times. All the blots were probed with α-tubulin (from CP06 calbiochem) to check for equal loading of the samples.

6.4 Results:

6.4.1 Activation of canonical NF-κB pathway in response to energy deprivation in 'young' human skin fibroblasts

Nuclear fractions from the 'young' fibroblasts that were subjected to a combined treatment of mitochondrial uncoupler and glycolysis inhibitor were assayed for NF-κB p65 DNA binding activity. As reported in section 5.4.2, we found increased NF-κB DNA binding activity in response to energy deprivation. This increase in NF-κB was associated with reduced IκBα expression in cytoplasmic extracts from the 'young' fibroblasts shown in

Figure 6.a consistent with the activation of canonical NF-κB signaling pathway. Phosphorylation of serine 276 in the transactivation domain of p65 NF-κB is known to be necessary for its transcriptional activity (Okazaki, Sakon et al. 2003). Therefore, endogenous levels of NF-κB p65 phosphorylated at serine 276 were probed in the nuclear fractions of the energy deprived 'young' fibroblasts. We noticed increase in the nuclear

protein levels of phosphorylated NF-κB p65 (phospho S276) as shown in Figure 6.a indicating p65-dependent cellular responses to energy deprivation.

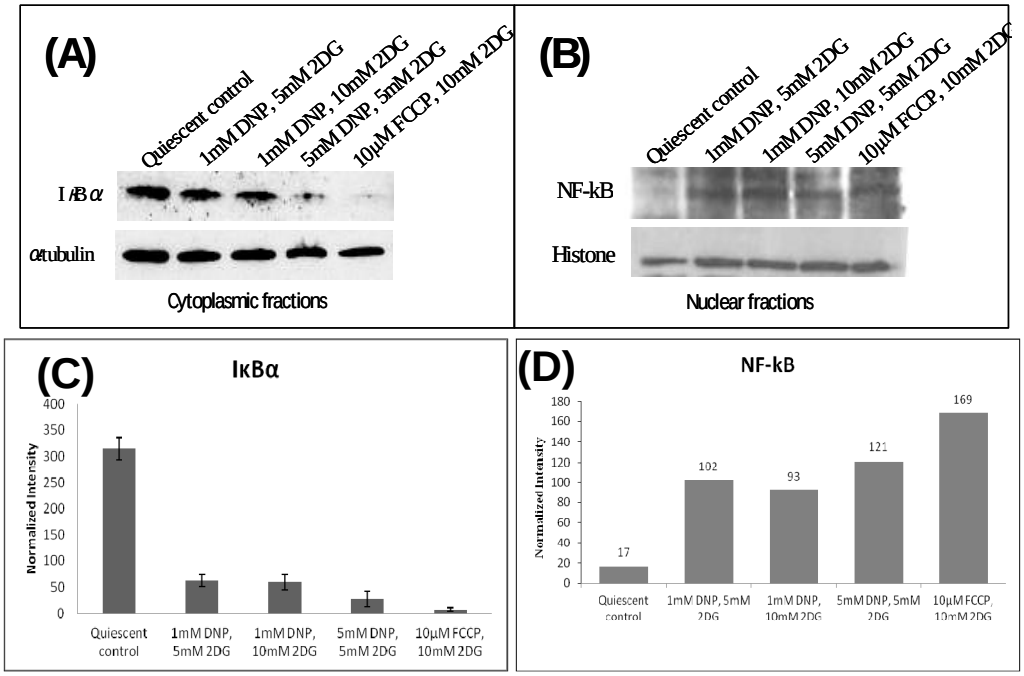
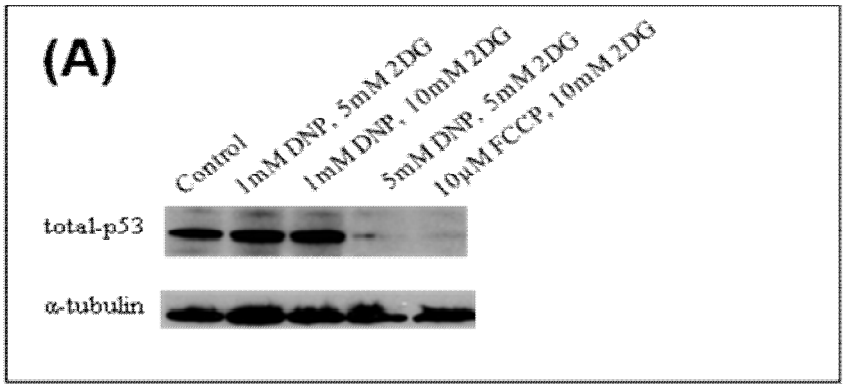


Figure 6.a: Energy limitation activated canonical NF-κB pathway. (A) Decrease in IκBα expression levels in the cytoplasmic fractions. (B) Increase in NF-κB p65 (phospho S276) levels in the nuclear fractions. (C) Western blot band intensities normalized to α-tubulin for cytoplasmic fractions. Standard deviations are plotted. (D) Densitometric analysis of immunoblot results normalized to loading control histone for nuclear fractions.

6.4.2 Activation of NF-κB p65 transcription factor is independent of p53 signaling pathway.



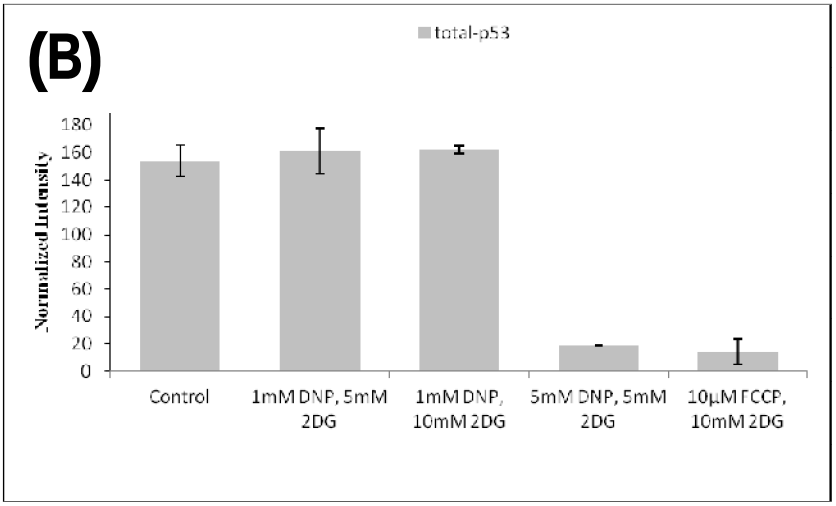


Figure 6.b: (A) Decrease in p53 expression levels with increasing energy limitation occurring simultaneously with increased NF-κB p65 DNA binding activity as shown in Figure 5c. (B) Densitometric analysis of immunoblot results normalized to loading control α-tubulin. Standard deviations are plotted.

p53 is a known apoptosis activator depending on the nature and extent of cell stresses. Recent studies have implicated p53 pathway in the consequence of energy starvation, we therefore investigated if the energy limitation led to stress-induced stabilization of p53 protein. We measured p53 expression in the whole-cell lysates prepared from 'young' fibroblasts subjected to energy deprivation. We noticed reduced p53 expression as shown in Figure 6.b suggesting p53-independent mechanism in the cell stress response.

6.4.3 Energy deprivation led to activation of Akt signaling pathway.

Energy deprivation attained by inhibiting both mitochondrial respiration and glycolysis resulted in a marked increase of NF-κB p65 DNA binding activity in the nuclear fractions as illustrated in Figure 5.c.

Since, Akt plays a major role in regulating glycolysis and cell growth/survival, we probed for phosphorylation of Akt at serine 473 in the cytoplasmic fractions obtained from energy-deprived 'young' fibroblasts as shown in Figure 6.c. Subsequently, we determined the phosphorylation states of select Akt substrates to confirm its functional state. Specifically, we determined levels of GSK3β phosphorylated at serine 9; GSK3 β is known to be involved in regulation of glucose metabolism and cell survival. In addition, we determined expression of another Akt target, i.e. phospho-Bad (serine 136) as shown in Figure 6.d. The increase in phosphorylation of both GSK3β (S9) and Bad (S136) proteins confirmed their roles in sensing the serum/glucose starvation and pro-survival signaling.

In order to investigate, the status of protein synthesis and translation during the intense energy starvation conditions, we probed for phosphorylation levels of p70 S6 Kinase at threonine 389 and eIF2α at serine 52, which are downstream effectors in the Akt/mTOR pathway. The decrease in phospho-p70S6 (Thr389) and increase in phospho-eIF2α (S52) as shown in Figure 6.d indicate a hold and stress on protein translation machinery respectively.

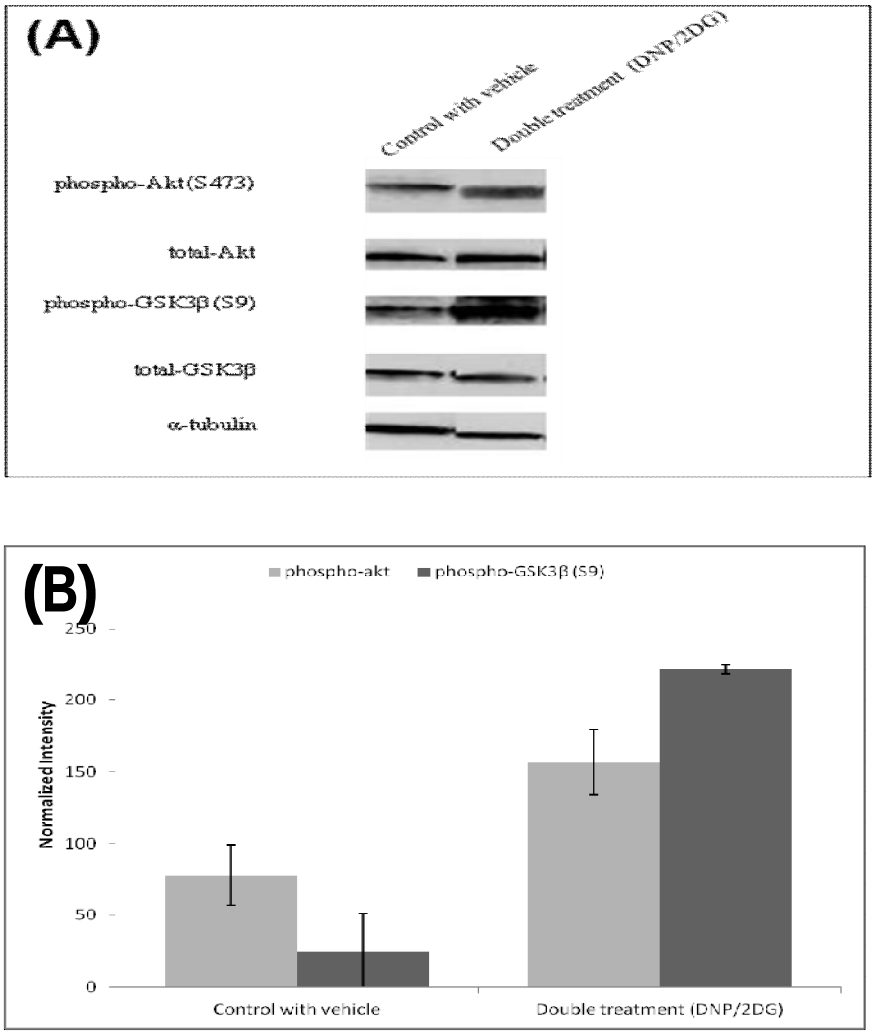


Figure 6.c: (A) Increase in phospho-Akt (S473) and phospho-GSK3β (S9) in the cytoplasmic fractions on inducing energy starvation (5mM DNP and 5mM 2DG). (B) Western blot band intensities normalized to loading control α-tubulin. Standard deviations are plotted. The bands are from the same gel run at the same time (part of a larger experiment).

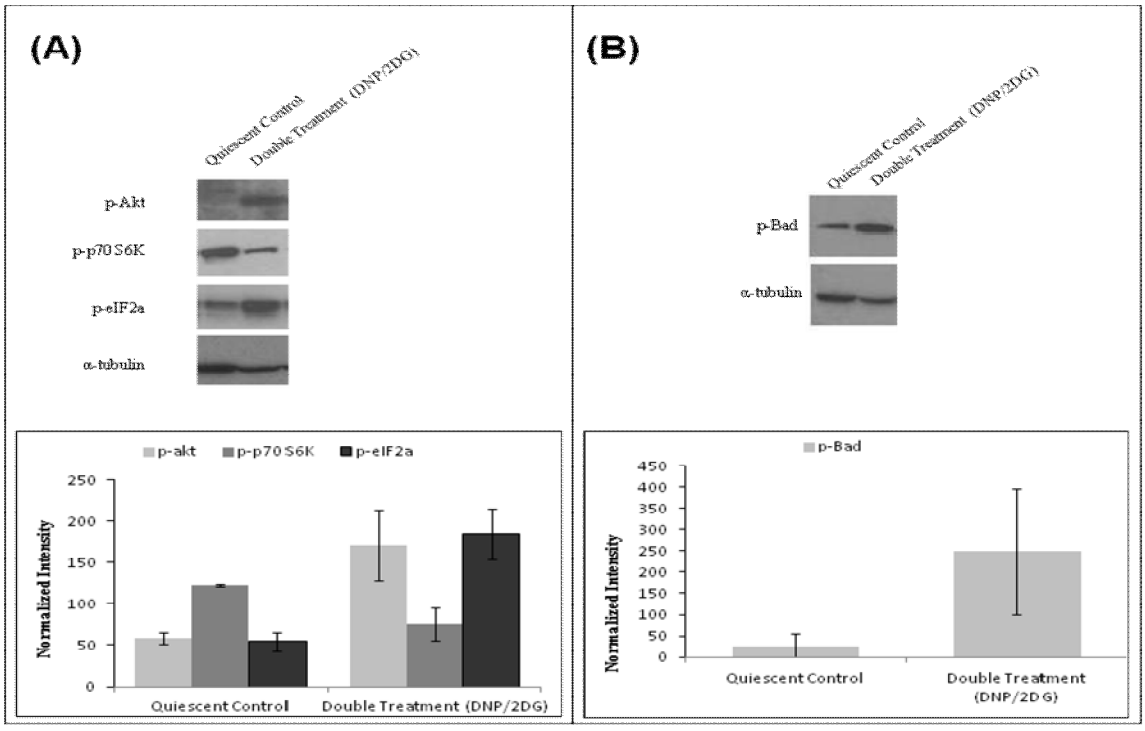


Figure 6.d: (A) Decrease in phospho- p70S6K and increase in phospho-eIF2α for 24 hours of energy limitation. Western blot band intensities normalized to loading control α-tubulin is plotted below. (B) Increase in phosphorylation of Bad protein at serine 136 for 24 hours of energy limitation. Western blot band intensities normalized to loading control α-tubulin is plotted below. Standard deviations are plotted.

6.4.4 Induction of autophagy in response to energy limitation in 'young' human skin fibroblasts.

Autophagy is a prevalent self-digestion process that the cell activates in response to energy deprivation or oxidative stress.

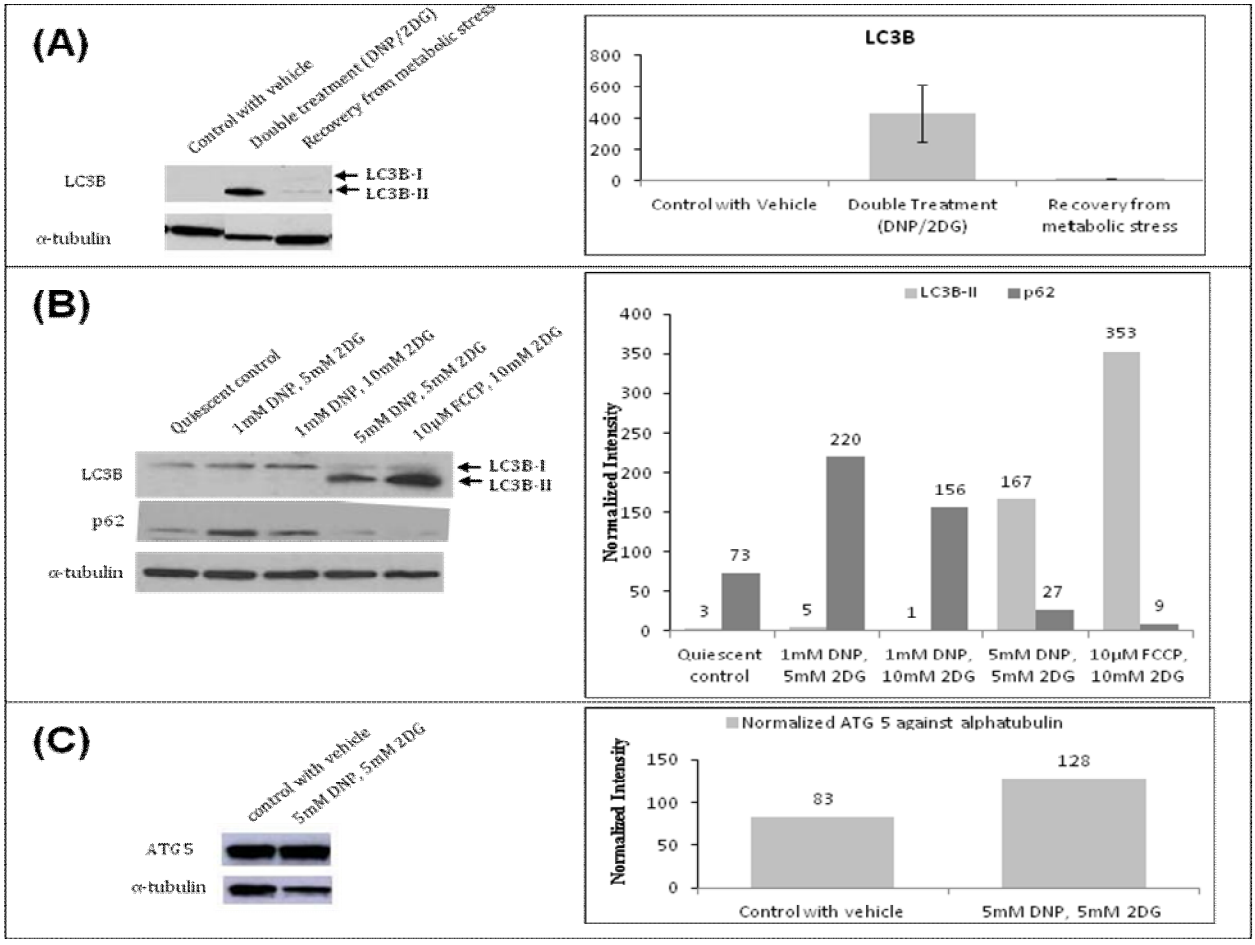


Figure 6.e: Autophagy induced by energy limitation. (A) Increased conversion of autophagy marker LC3B I to LC3B II on limiting energy, which revert back to normal levels upon removal of the energy stress. The bands are from the same gel run at the same time (part of a larger experiment). Western blot band intensities are shown on the right normalized to loading control α-tubulin with standard deviations plotted. (B) Increased conversion of autophagy marker LC3B I to LC3B II and degradation of p62 in the cytoplasmic fractions as a result of increasing amount of energy limitation. Western blot band intensities normalized to loading control α-tubulin is plotted on the right. (C) Increase in ATG5 protein expression upon energy deprivation. Western blot band intensities normalized to loading control α-tubulin is plotted on the right.

We detected an induction in autophagy following energy limitation attained by a combined treatment of DNP and 2DG. The conversion of LC3B-I to LC3B-II, a marker of autophagy is illustrated in Figure 6.e. Additionally, we report an increase in ATG5 expression level and p62 degradation shown in Figure 6.e, both proteins involved in autophagy machinery and point to autophagy induction.

6.4.5 Energy deprivation triggered an autophagy activated NF-κB signaling pathway

Next, we sought to investigate if induction of autophagy influenced the NF-κB activation. Autophagy inhibition was accomplished by using three different pharmacological inhibitors namely 3-Methyladenine (3-MA), Chloroquine (CQ) and Bafilomycin-A1 (Baf). 3-MA is known to inhibit class III PI3K involved in the autophagosome formation and the other two inhibitors halt the fusion of autophagosomes to the lysosomes. Though they are not highly specific, they are the most commonly used autophagy inhibitors to date in most cells.

Autophagy inhibition resulted in decrease of NF-κB p65 DNA binding activity as shown in Figure 6.f, which indicated that autophagy induction was one of the cellular processes that contributed to NF-κB activation. The autophagy inhibitors did inhibit the energy deprivation triggered autophagy and this can be confirmed by p62 accumulation and flux changes in the conversion of LC3 I to LC3 II shown in Figure 6.f.

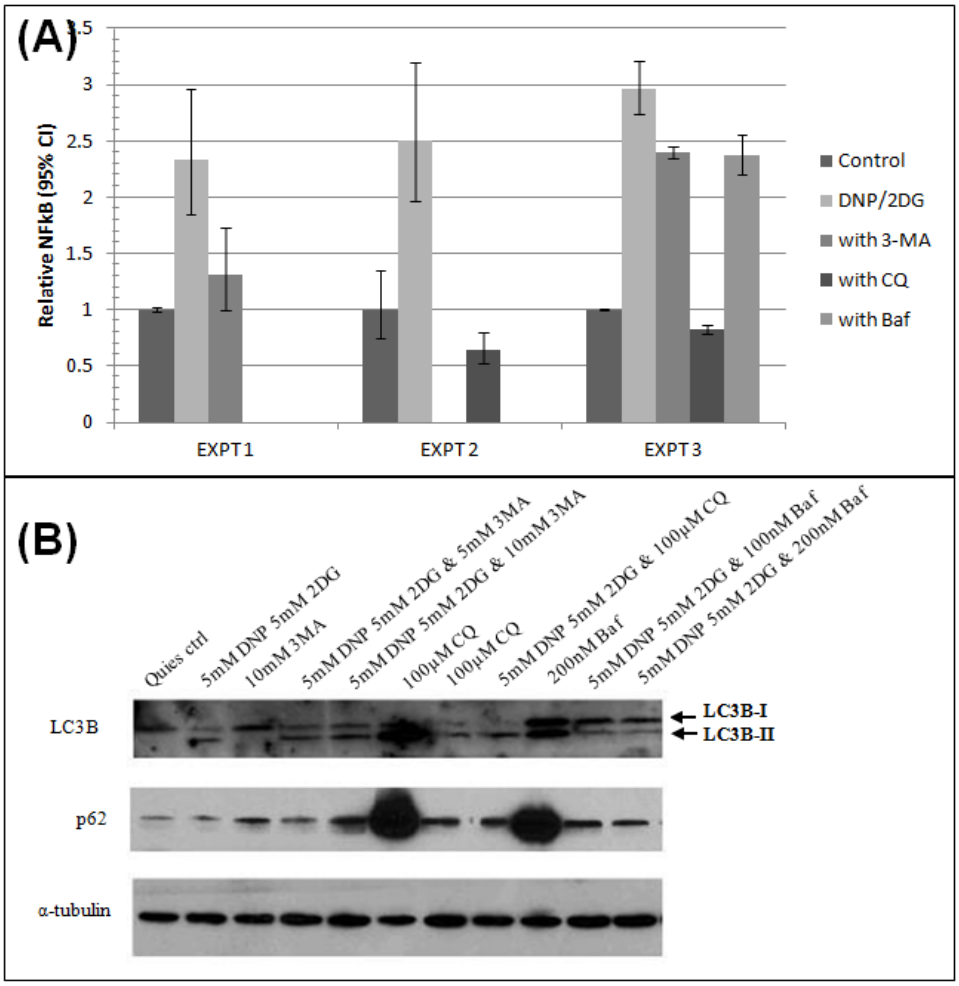


Figure 6.f: Energy limitation triggered NF-κB p65 DNA binding activity is dependent on autophagy. (A) Reduction in NF-κB p65 DNA binding activity following treatment with autophagy inhibitors 3-methyladenine (3-MA), chloroquine (CQ) and Bafilomycin-A1 (Baf) in the presence of mitochondrial uncoupler - DNP and glycolysis inhibitor - 2DG in the 'young' fibroblasts. Standard Errors are plotted. (B) Western blot analysis of whole cell lysates prepared using 'young' fibroblasts that were subjected to ATP limitation and/or autophagy inhibition and probed for autophagy proteins LC3B and p62. α-tubulin was used as a loading control.

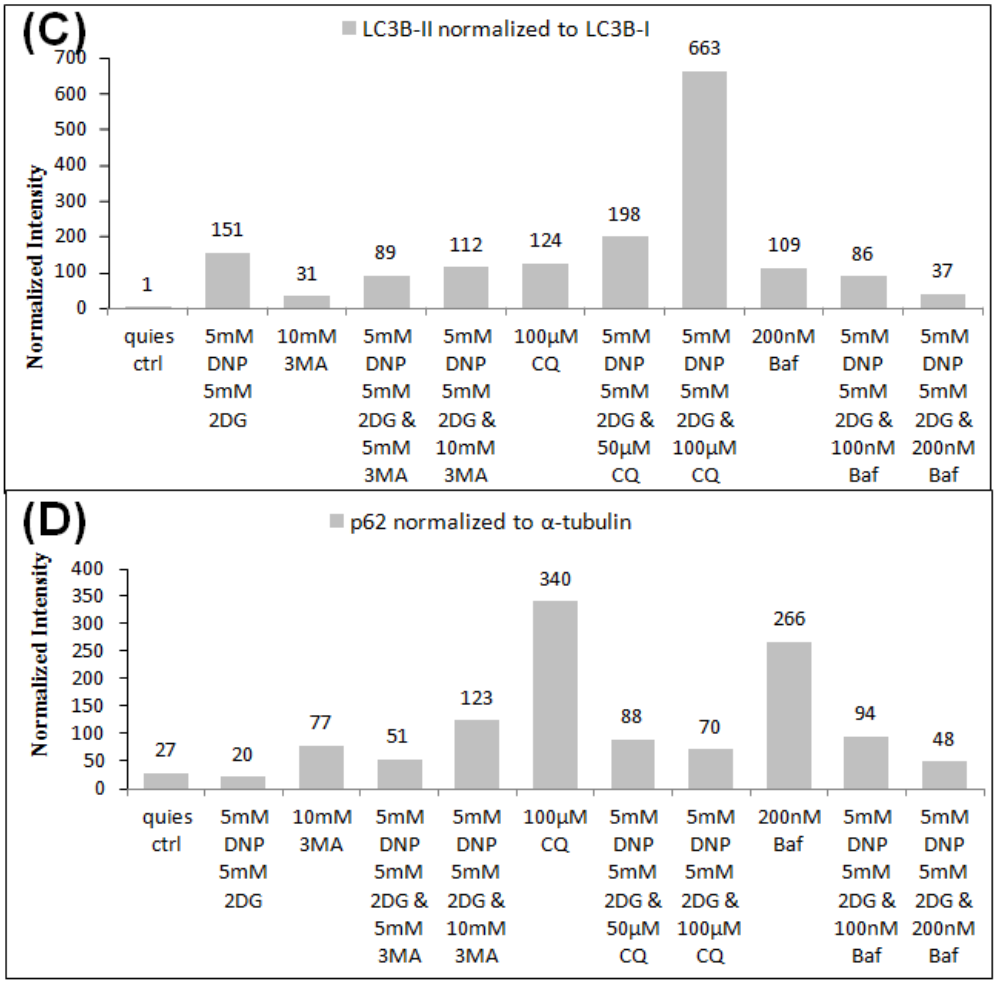


Figure 6.f (Continued): (C) Intensities of LC3-II bands normalized to LC3-I. (D) Intensities of p62 bands normalized to α-tubulin.

6.4.6 Autophagy induction in *in vivo* aged fibroblasts.

Based on the energy deprivation model results reported in the previous sections, we decided to probe for LC3B marker in the cytoplasmic extracts of 'old' fibroblasts. We found increased LC3B-I to LC3B-II conversion with increasing biological age as shown in Figure 6.g. Thus, we found evidence for autophagic cell response in 'older' human fibroblasts confirming the involvement of autophagy in human aging.

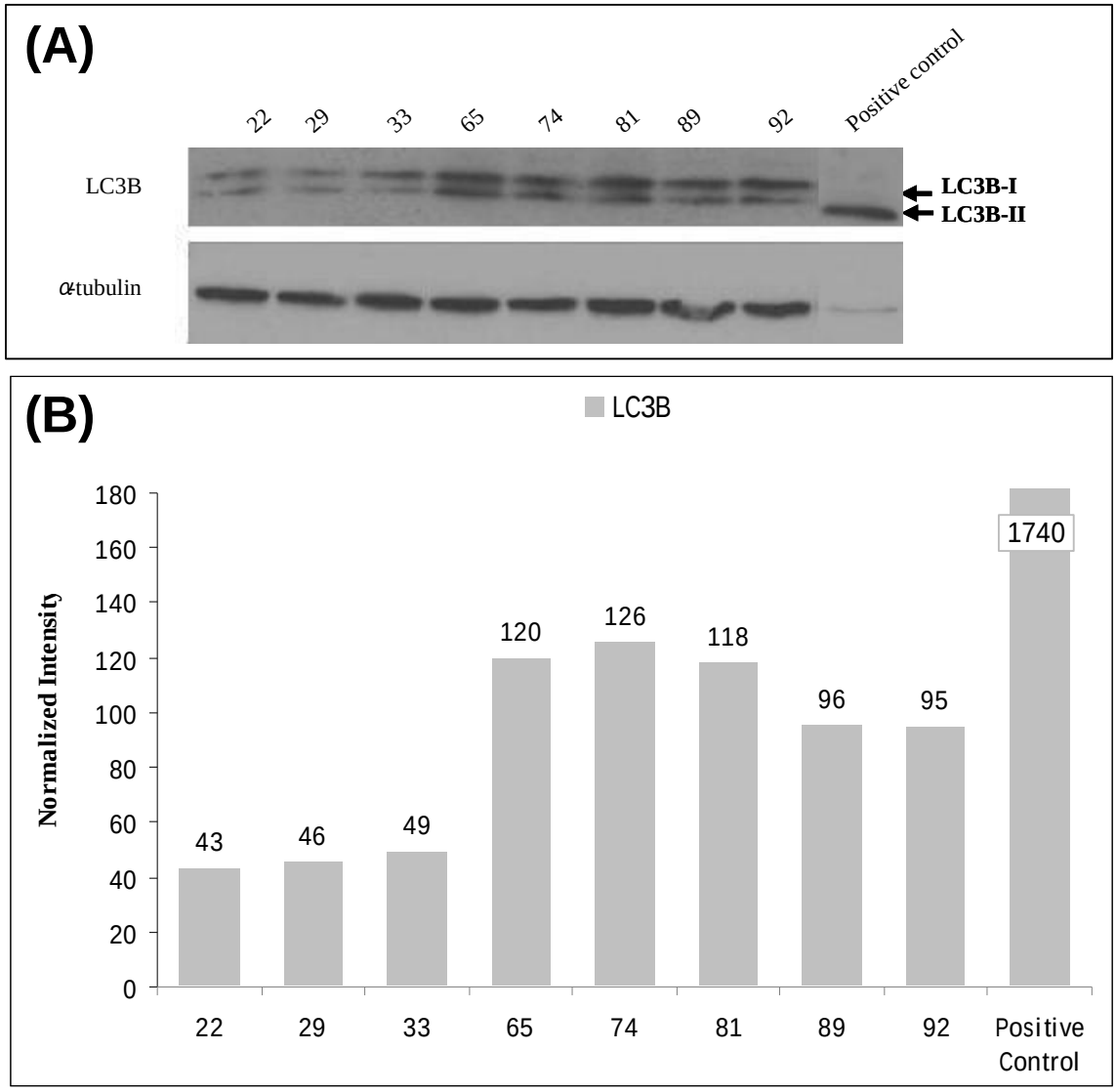


Figure 6.g: (A) Western blot analysis of cytoplasmic fractions prepared using human skin fibroblasts from biologically aged donors. The increased conversion of LC3-I to LC3-II with increasing age confirms involvement of autophagy as a potential link between age-dependent energy decline and elevated NF-κB p65 DNA binding activity profile in the 'older' fibroblasts. The control band is from the same gel run at the same time (part of a larger experiment). (B) Western blot band intensities normalized to loading control α-tubulin.

7 DISCUSSION

7.1 Introduction:

The advent of DNA microarray technology in the mid-1990s has benefitted the field of aging. Use of microarrays made possible the study of many candidate genes for a given condition and the application of advanced computational and statistical analysis tools provides valuable insights. We began our pilot study using 14 human skin fibroblast cultures derived from biologically aged donors, who were certified healthy at the time of biopsy. We cultured these pre-senescent fibroblasts using standard fibroblast culture procedures and prepared samples to run the microarray chips. Subsequently, gene expression analysis was done and this data formed a critical stepping stone to our research in the field of aging. We found, specifically in aged fibroblasts, a chronic low-grade inflammatory signature associated with a constitutive activation of NF-κB DNA binding in a cell-autonomous manner (Kriete, Mayo et al. 2008).

During the course of our research focusing on potential inducers of NF-κB in aging, we were able to successfully demonstrate a subtle but statistically significant reduction in total intracellular ATP levels in ‘old’ human fibroblasts. Therefore, we hypothesized that an age-related energy decline is a contributing factor to the constitutive NF-κB activation and the chronic inflammatory phenotype.

The uniqueness of this project was in confirming the connectivity between intracellular ATP levels and NF-κB activity levels by studying the effects of energy deprivation on ‘young’ human fibroblasts. Major challenges were encountered throughout our project that involved probing for biological age-associated subtle changes. Thus, the novelty of

this project lies not only in using our well established pre-senescent fibroblast model system, which is considerably relevant to study *in vivo* aging process, but also by applying a means of energy deprivation that helped us amplify the subtle changes. Many studies have been carried out to decipher the involvement of different pathways in regulating the energy status and inflammation but mostly in cancer cells or other organisms. Here, for the first time, we used healthy pre-senescent 'young' fibroblasts and subjected them to varying amounts of energy stress and demonstrated its link to NF-κB activity.

Using energy deprivation, we were able to study potential signaling pathways mediating the cellular energy limitation and inflammation. We found autophagy to be a potentially important mediator of NF-κB activation in energy-deprived fibroblasts. This motivated us to probe for autophagy in the previous samples from our pilot study and consequently confirmed the involvement of autophagy in *in vivo* aged fibroblasts. In summary, using the energy deprivation model, we were able to begin to understand how autophagy may affect NF-κB activation states of pre-senescent 'young' and, by implication, old fibroblasts. It appears that the pre-senescent *in vivo* aged fibroblast system in conjunction with energy deprivation model may be extended to further investigate underlying pathways and their roles in regulating NF-κB as it relates to aging.

7.2 Cell autonomous expression of inflammatory genes in biologically aged fibroblasts associated with elevated NF-κB activity

Previous studies using fibroblasts aged *in vivo* have largely focused on age-associated changes in cell cycle progression of dividing cells (Ly, Lockhart et al. 2000; Geigl,

Langer et al. 2004). In contrast, we report ‘inflammatory signatures’ i.e. changes in gene expression patterns of serum-deprived, quiescent fibroblasts derived from 14 healthy donors aged 22 to 92 years. The analyses were performed on quiescent cells to mimic the situation in adult individuals in which fibroblasts embedded in connective tissue seldom divide except in wound healing or regeneration. Samples originated from medial upper arm biopsies and were available from the NIA Aging Cell Repository (Coriell Institute for Medical Research, Camden, NJ). Pre-senescent fibroblasts from older donors expressed an inflammatory gene signature characterized by increased levels of transcripts representing cytokines, chemokines, the complement cascade proteins and MHC molecules. This gene expression pattern was accompanied by enhanced NF-κB activity. Importantly, this low-grade inflammation pattern was cell-autonomous, since no inflammation inducing compound or environment was used. These results are consistent with the view that low-grade inflammation, a hallmark of many age-associated diseases, is part of the aging process in fibroblasts. This is in line with higher activity of NF-κB and inflammatory markers observed in many aging tissues in both human and animal studies in which gene expression analysis had been performed, such as in brain (Lu, Pan et al. 2004), lung (Aoshiba and Nagai 2007), liver (Kelder, Boyce et al. 2007), cartilage (Kato, Matsumine et al. 2007) and coronary arteries (Csiszar, Ungvari et al. 2003). More recently it has also been shown that inhibition of NF-κB in tissues can reverse signs of aging (Adler, Kawahara et al. 2008). The complex relationships of inflammatory mediators in tissues and organs and their interaction with the immune system in aging has been explored with a systemic computer modeling (Salvioli, Capri et al. 2006). Since inflammation represents an attempt to return the cell to homeostasis and mediate tissue

repair, *in vivo* responses of tissues to oxidative stress have features that depend on cell communication and may not be fully observed in cell cultures. However, in many studies the primary causes of inflammation have not been addressed (NIA 2004 summary report of a 2004 on "Inflammation, Inflammatory Mediators and Aging" available on-line at: www.nia.nih.gov/ResearchInformation/ConferencesAndMeetings/NIA+WorkshoponInflammation.htm) and the relationship between aging and inflammation remains elusive. We suggest that our initial studies of primary changes in transcription patterns in homogeneous cell cultures of non-immune cells (e.g. fibroblasts), which revealed a low-grade chronic inflammatory response; provide a novel opportunity to unlock the origin and mechanisms of inflammation and age-related diseases.

7.3 Energy decline in biologically aged human skin fibroblasts

In order to investigate the molecular mechanisms that contribute to NF-κB activity associated with aging, we first focused on changes in cellular redox state. ROS production and NF-κB activation have been implicated as an important contributing factor in biological aging (De Benedictis, Carrieri et al. 2000; Radak, Chung et al. 2004). We did not observe any significant change in the ROS levels in the 'old' fibroblasts relative to the 'young' fibroblasts. Nevertheless, similar studies in aging fibroblasts have indicated slightly compromised mitochondrial function with potential consequences for a subtle reduction of ATP synthesis (Greco, Villani et al. 2003; Miyoshi, Oubrahim et al. 2006). We therefore probed for total intracellular ATP levels and noted a small but significant reduction in the fibroblast ATP content in 'old' fibroblasts. Acute or chronic

alterations in ATP content can have major effects on cellular functions and the aim was to investigate if such metabolic changes occurred in the aging process. Our finding was consistent with the work of two other groups that used fibroblasts to address different aspects of human aging. A large-scale study, using skin-derived primary fibroblasts (1 – 103 years), concludes that increasing donor age compromised mitochondrial processes that regulate the capacity of cellular OXPHOS (Greco, Villani et al. 2003). They particularly measured rate of mitochondrial protein synthesis, respiration rate, control of respiration by mitochondrial membrane potential and efficiency of ADP phosphorylation, all of which were less efficient in aged cells. In a relatively small-scale study on primary human fibroblasts from donors of different ages (17 – 80 years), Miyoshi and Oubrahim have shown that aged-related increase in oxidatively modified proteins correlated with reduced mitochondrial ATP generation implying that the energy decline may perhaps deteriorate cellular processes regulating cell defense and repair mechanisms (Miyoshi, Oubrahim et al. 2006). Along similar lines, we found a reduction of total intracellular ATP levels with increasing donor age of the human fibroblasts. Next we asked if a correlation exists between the energy decline and the increase in NF-κB activity. We found that the intracellular ATP content negatively correlated with NF-κB activity (Pearson correlation co-efficient $R = -0.6$, pvalue = 0.039) in the fibroblasts derived from biologically aged donors. This strong correlation led us to explore a mechanistic cause and effect relationship between these parameters.

7.4 Energy Deprivation Model using 'young' human skin fibroblasts

In order to investigate if the reduced ATP content in 'old' fibroblasts influenced their NF-κB activity, we decided to mimic this hypothetical cause in the 'young' fibroblasts. To this end, we decreased the ATP levels of 'young' fibroblasts by using different pharmacological inhibitors of mitochondrial respiration. We failed to achieve marked decreases in ATP at concentrations of these inhibitors compatible with cell survival, which is probably due to the increase in glycolysis as a compensatory metabolic shift in response to oxidative stress in both healthy and diseased cells (Krahenbuhl and Reichen 1992; Wu and Wei 2012). We therefore decided to inhibit both, mitochondrial OXPHOS and glycolysis with the aim to reduce total intracellular ATP levels significantly. A mitochondrial respiration inhibitor in addition to glycolysis inhibitor, at concentrations that induced no considerable loss in cell survival for 24 hours, led to a marked decrease in total ATP content and modulation of NF-κB activity in the 'young' fibroblasts. In addition, small decreases in ATP content of 'young' fibroblasts (for 24 hours), which are more robust to cell stressors, do not replicate the slow accumulation of consequences as observed in aging *in vivo* (for years). We, therefore, decreased the ATP levels more dramatically in 'young' fibroblasts with no compromise in cell survival for a period of 24 hours to attain a sub-acute steady-state. Consequently, we found observed 'steady-state' levels of NF-κB in response to the energy limitation. We therefore were successful in developing an energy deprivation model that (1) allowed us to magnify the small but significant ATP changes found in biologically aged pre-senescent fibroblasts and (2) retained relevance to the chronic adaptive state, which is distinct from acute responses triggered by a sudden dip in the ATP levels. Reduction of ATP levels by approximately

50% (as compared to control cells) did not change the morphology of the fibroblasts. However, ATP limitation greater than 80% resulted in cell shrinkage. Cell shrinkage is one of the characteristics of apoptosis. However, cell shrinkage associated with ATP deprivation was completely reversible without apparent loss of cell viability. Specifically, upon exchange of inhibitor medium with inhibitor-free medium for 24 hours, we noticed a complete recovery in the NF-κB activity levels as well as the cell morphology. Thus, reduction in intracellular ATP content of 'young' fibroblasts though dramatic aids our purpose of investigating aging process and we were able to establish a causal link between cellular energy levels and NF-κB activity using energy deprivation method in pre-senescent human fibroblasts.

7.5 Pathways and NF-κB regulation

Our energy deprivation model allowed us to investigate molecular pathways that potentially link energy metabolism and NF-κB activation. A block diagram illustrating the potential mediators of NF-κB activation is shown in Figure 7.a.

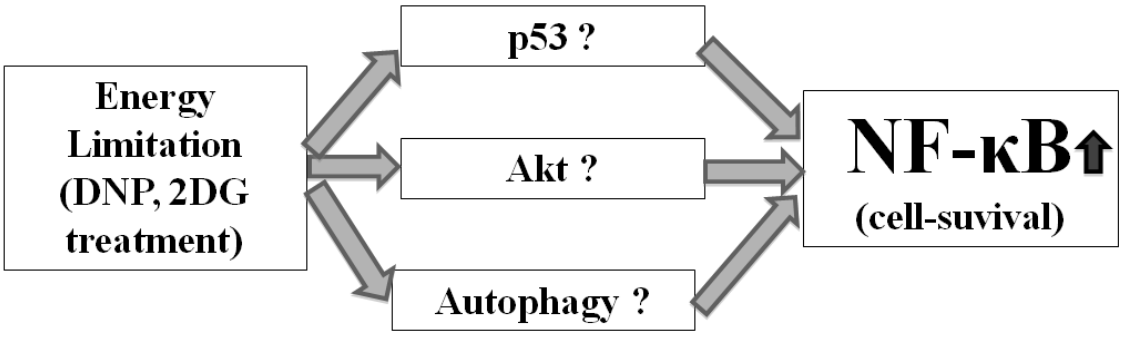


Figure 7.a: Potential Inducers of NF-κB associated with ATP restriction

7.5.1 Canonical NF-κB signaling pathway is stimulated

NF-κB is one of the "fast-acting" primary transcription factors regulating multiple pathways in response to various stimuli and cellular stressors. Potential activators of NF-κB pathway include inflammatory signals, oxidative stress, ROS and DNA damage (Adler, Kawahara et al. 2008). Activation of NF-κB is associated with aging of mammalian cells (Helenius 1996; Gilmore 2006; Salminen 2009). We report that elevated NF-κB p65 DNA binding activity in 'young' fibroblasts is in response to energy deprivation-stimulated degradation of IκBα. Upon energy deprivation-induced stimulus, the inactive form of NF-κB is rendered active by enabling its release from the inhibitor of κB, by phosphorylation and subsequent degradation of IκBα through known ubiquitin-proteasome pathway. The active form of NF-κB translocates into nucleus, binds to DNA and subsequently regulates gene expression. This indicates activation of canonical NF-κB pathway. Therefore, we suppose that energy deprivation-triggered NF-κB activation is through the classical NF-κB signaling pathway. Figure 7.b summarizes all of the players involved thus far in a diagram as investigated by our study, discussed in more detail below.

7.5.2 Stress-induced p53-independent pro-survival mechanism

It is known that DNA-damaging and pro-oxidant conditions activate p53, a well-known stress-activated DNA binding protein that regulates gene expression and cellular processes involved in DNA repair, cell-cycle and apoptosis.

We demonstrate that the energy starvation induced NF-κB activation is not dependent on p53 function in the 'young' fibroblasts. This indicates that our studies are temporally placed in the age-relevant pro-survival response mode. Also, it has been reported that p53 inactivation is utilized by cells as an adaptive mechanism in response to stress involving a vital role of GSK-3β (Qu, Huang et al. 2004). Interestingly enough, reduced transcriptional activity of p53 with age may potentially favor tumorigenesis (Feng, Hu et al. 2007). Furthermore, it has been reported that mitochondrial dysfunction that results in impaired ATP synthesis reduces the steady-state levels of p53 protein (Compton, Kim et al. 2011). Our energy deprivation model that mimics the reduced ATP production in aging fibroblasts clearly illustrates decreased expression of the p53 protein in response to the energy stress. In other words, the energy starvation-triggered down regulation of p53 may reflect the declining fidelity of p53-mediated apoptosis and senescence in response to age-associated stress signals and thus contributing to age-associated pathologies. This is consistent with the gene expression data where we report down regulation of TP53 inducible protein. The results are further consistent with a model of competing activity levels between p53 and NF-kappaB (Ak and Levine 2010).

7.5.3 Akt/GSK-3/Bad phosphorylation provides anti-apoptotic effect

Akt is known to be phosphorylated at serine 473 and threonine 308 as a part of activating canonical Akt pathway that results in downstream activation of glucose uptake and the protein synthesis machinery via mTOR pathway. Akt further forms a critical part of the anti-apoptotic PI3K-Akt-Bad pathway. Activated Akt phosphorylates Bad at serine 132

thereby preventing association of Bad with the anti-apoptotic BCL-2 family members BCL-X_L or BCL-2 and hence inhibiting apoptosis susceptibility (Datta, Dudek et al. 1997). GSK-3β is phosphorylated by Akt at Ser-9, leading to its inactivation and thereby further contributing to anti-apoptotic effects of Akt (Li, Wang et al. 2000; Loberg, Vesely et al. 2002). It is known that Akt/GSK-3 signaling axis acts as a glucose sensor in response to changes in basal levels of glucose and insulin (Clodfelder-Miller, De Sarno et al. 2005). Furthermore, chronic inhibition of GSK-3 activity is known to increase glucose uptake and GLUT1 expression only in the presence of functional TSC2/mTOR pathway independent of acute insulin signaling (Buller, Loberg et al. 2008). The TSC2/mTOR pathway most importantly regulates translation, cell growth and survival in response to intracellular ATP levels (Inoki, Zhu et al. 2003). We found that Akt and GSK-3β were phosphorylated upon reduction of intracellular ATP levels in the fibroblasts. In addition, the Akt substrate Bad was similarly phosphorylated. Together, our findings suggest that though phosphorylation of Akt and GSK-3 act as glucose sensor, the non-functional TSC2/mTOR pathway due to energy starvation blocks glucose uptake. Therefore, Akt-mediated Bad phosphorylation and inhibitory phosphorylation of GSK-3 only play a role in cell survival in response to energy deprivation conditions. Our study not just depicts the importance of the Akt/GSK-3 as a sensor to the presence of non-metabolized glucose but also pinpoints a survival dependence on this signaling axis. Inhibition of functional Akt in energy deprived ‘young’ fibroblasts did not reveal any significant change to the already increased NF-κB DNA binding activity and the autophagy marker (LC3) expression levels. We infer that the Akt and NF-κB/autophagy pathways function

independently and in parallel to enhance the cell-survival in response to the energy decline.

7.5.4 Autophagy induction

Autophagy is a tightly regulated catabolic process that helps maintain cellular homeostasis by balancing synthesis and lysosomal degradation of cytoplasmic contents. Therefore, low-grade basal autophagy occurs in the presence of growth-factors/nutrients; however, it is rapidly up regulated in response to starvation stresses like growth-factor, glucose or energy withdrawal. It is known that growth-factor deprivation-triggered ATP limitation induces autophagy via inhibition of mTOR pathway and subsequent decrease in the phosphorylation of p70S6 kinase and 4EBP1 (eIF4E-binding protein 1) (Hait, Jin et al. 2006). This mechanism helps transfer the nutrients from not so important processes to more vital ones by producing amino acids for de novo protein synthesis or maintenance of intracellular ATP levels. Though mTOR is considered to be a central regulator of autophagy, recent work studies have shown that there are mTOR-independent types of regulation via c-Jun N-terminal kinase (JNK) and ROS (Meijer and Codogno 2006). Recent studies have demonstrated involvement of ROS in the autophagy regulation (Chen, Azad et al. 2009), however, it does not appear limiting in autophagy induction (Jiang, Maeda et al. 2011). It is known that the mTOR pathway is down-regulated by caloric restriction, which reduces NF-κB signaling via dampening of PI3K/Akt pathway (Vaughan and Jat 2011). Clearly, pathways involved in the regulation of autophagy and NF-κB depends on the type and degree of stimulus. We report here that catabolic

autophagy controls NF-κB activity in energy deprived 'young' fibroblasts via degradation of IκBα and inhibiting cell-death pathway. In a study involving treatment of B-cell lymphoma cells with bortezomib led to proteasome down-regulation and accumulation of poly-ubiquitinated proteins (Jia, Gopinathan et al. 2012). Importantly they discuss that the autophagy adapter protein p62 recruits LC3-II and ubiquitinated proteins including IκBα and transports for subsequent degradation to autophagosomes and not the proteasomes. Therefore, it appears a similar mechanism might be involved in our energy deprivation model as to degradation of IκBα by autolysosomes in addition to the ubiquitin-proteasome degradation system. In summary, our findings demonstrate the crucial role of starvation-induced autophagy in mediating the activation of canonical NF-κB pathway.

Furthermore, recognizing the connectivity of autophagy and NF-κB signaling in energy deprived 'young' fibroblasts, we went on to probe for autophagy marker in the cytoplasmic extracts from 'old' fibroblasts. An increased conversion of LC3 I to LC3 II (autophagy marker) as a function of age confirmed the presence of autophagy in pre-senescent fibroblasts. Given its primary role in removal of damaged organelles in order to maintain cellular homeostasis, one may speculate that autophagy would limit the level of cellular oxidants by clearing the damaged and excess mitochondria. However, these findings seem to contradict the common belief that increased autophagy and mTOR inhibition in particular reduce the inflammatory phenotype. Furthermore, cell studies of age-related disease often referred to as 'pathological aging' do demonstrate a reduced autophagy (Rubinsztein, Marino et al. 2011). In support of this, we report gene expression data indicative of poorly performing lysosomes in *in vivo* aged cells.

Therefore, further studies are needed to confirm the activity of autophagy as a mediator in this process. Figure 7.b summarizes all of the players involved thus far in a detailed pathway diagram.

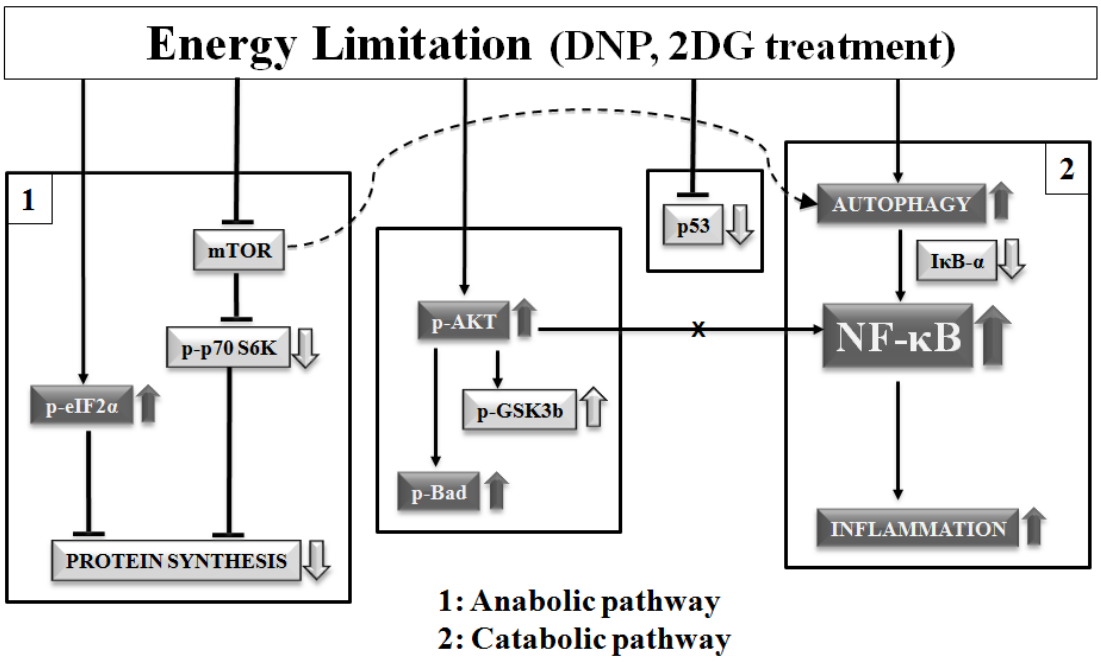


Figure 7.b: Detailed pathway diagram

8 CONCLUSION AND FUTURE WORK

Our studies of primary changes in transcription pattern with homogeneous cell cultures revealed a low-grade chronic inflammatory response and constitutive activation of NF-κB. The data presented here are the first comprehensive assessment of steady-state transcriptional differences indicative of an inflammatory state as it relates to cellular aging. Concurrent findings show up-regulation of NF-κB activity as a pivotal mediator of aging related inflammation in rodent and human tissues (Adler, Sinha et al. 2007) including mouse brain, muscle (Helenius, Hanninen et al. 1996; Korhonen, Helenius et al. 1997; Kim, Kim et al. 2000; Bar-Shai, Carmeli et al. 2005) and human endothelial tissues (Donato, Eskurza et al. 2007), which suggests that similar processes as those observed here play a role in many other cell types and tissues. The observation that such changes occur in cell lines irrespective of other cell types or the *in vivo* context highlights that the inflammatory state is intrinsic to the fibroblasts under investigation. Another important corollary of this study is the observation that inflammatory gene expression patterns were obvious in quiescent cells and, thus, are not an artifact of rapid cell cycle progression unique to *in vitro* studies with questionable relevance to the *in vivo* state. At the same time, we recognize that a study *in vitro* cannot capture the more complex exogenous processes *in vivo*, involving other cellular participants and components of the immune system which may provoke, mediate, and amplify the transcriptional response. This would include cells that have entered a senescent state, previously known to be the prime contributors of age-related inflammation and suspected to harbor preneoplastic mutations in their microenvironment (Campisi 2005). However, cells in aging tissues

chronically inflamed and in a pro-survival state, may be more prone to accumulate additional damage and provoke a senescent phenotype, as recent research has revealed that activation of NF-κB promotes cellular senescence (Rovillain, Mansfield et al. 2011).

The ATP data determined in human fibroblasts derived from different aged donors is in accordance with other experimental reports under similar cell culture conditions. In order to find if energy decline is linked to NF-κB regulation, we developed a model to mimic low energy levels of 'old' donors in the 'young' fibroblasts and thereby confirmed the connection. Though the treatments seem dramatic and 24 hours time scale does not precisely reflect chronic *in vivo* aging, we measure sub-acute 'steady-state' NF-κB activity and thus retain its relevance to biological aging process. Additionally, by developing the energy deprivation model in 'young' fibroblasts, we were able to amplify the subtle changes in ATP levels and NF-κB activity occurring in the 'old' fibroblasts.

Many studies in cancer research have demonstrated that most tumor cells are characterized by increased NF-κB activity and a metabolic switch from oxidative phosphorylation to aerobic glycolysis. We observed elevated NF-κB levels and decrease in the cellular ATP content in 'old' fibroblasts relative to 'young' fibroblasts. Since we use 2DG to inhibit glycolysis in 'young' fibroblasts, we are not able to quantify the compensatory effect of glycolysis using the energy deprivation model. Therefore, the energy deprivation model does not allow us to estimate the relative contribution of ATP from mitochondrial oxidative phosphorylation and glycolysis. On the other hand, the

possibility of intracellular lactate levels stimulating NF-κB can be ruled out. In conclusion, our model suggests that reduction in cellular energy levels regulate NF-κB activity and could be a contributing factor to the inflammatory phenotype ubiquitously observed in aging tissues.

An apparent disparity in Akt activation following serum and glucose starvation led to its functional ambiguity; however many studies have shown its central role in cell-survival (Ly, Pelech et al. 2008). Based on our results, Akt and NF-κB/autophagy induction are instrumental in the survival of serum and glucose deprived human skin fibroblasts. The energy limitation conditions induce parallel survival pathways and not necessarily sequential events in the same pathway. Many studies involving different cell systems and conditions indicate the interaction of Akt and its downstream target GSK3β and their combined effect on NF-κB transcription factor. Therefore a step-by-step investigation with a battery of specific Akt and GSK3β inhibitors can help better understand the Akt/GSK-3 signaling axis on NF-κB activity. Our results illustrating inhibitory phosphorylation of GSK3β at serine 9 and simultaneous loss of p53 stabilization in the cytoplasmic fractions may indicate an additional mechanism in adaptation to energy stress. Qu and Huang (Qu, Huang et al. 2004) have shown that nuclear translocation of GSK3β to physically bind to p53, and subsequent translocation of p53 to cytoplasm followed by degradation results in a cell stress adaptive response. Determining whether or not loss of p53 stabilization depends on interaction of GSK3β and p53 in the nucleus will require further studies but the basis that this is resulting in cells adapting to the

energy stress is attractive. Additionally, a study involving p53-deficient cancer cells, however, maintained higher ATP levels by way of autophagy induction and in another study autophagy impairment led to ROS and p53 dependent induction of senescence in fibroblast cell culture (Tasdemir, Maiuri et al. 2008; Kang, Lee et al. 2011). These studies point towards our future studies involving the effect of p53 activation under the currently imposed starvation conditions. In conclusion, an energy starvation triggered p53-independent responses in our studies may indicate a cell-survival mechanism.

Autophagy is not the only process responsible for aging. The causal role of this process in regulating NF-κB activity and aging has been determined in our work using well-known pharmacological inhibitors of autophagy. There exists an obvious advantage of using RNA interference method over pharmacological inhibitors and that is specificity. There is an opportunity to efficiently down-regulate autophagy in our primary fibroblasts and further demonstrate its causal role in NF-κB regulation by using lenti-viral delivery system targeted against the essential autophagy regulators ATG5 and ATG7 (Kang, Lee et al. 2011). Nevertheless, our choice of inhibitors targeted two different stages of autophagy machinery. 3-methyladenine is known to inhibit class III PI3K pathway, whose activation is crucial for autophagy induction. Bafilomycin-A1 and chloroquine inhibit fusion of autophagosomes to lysosomes which otherwise would form autolysosomes where degradation occurs. Though these three inhibitors are not necessarily specific and highly effective in down-regulating autophagy, their common effect in reducing NF-κB DNA binding activity is more supportive that autophagy does

play a causal role in NF-κB signaling. Thus we conclude that autophagy is one of the activators of NF-κB in the energy-deprived 'young' fibroblasts. Subsequently, the cytoplasmic fractions from 'old' fibroblasts were probed for the autophagy marker and we report an increase in the conversion of LC3-I to LC3-II. This then implicates that autophagy may play a role in elevating NF-κB levels in 'old' fibroblasts. However, a re-visit to the gene expression data is necessary to uncover autophagy-related genes as it relates to aging. Thus, autophagy as a consequence of the reduced ATP content is one of the activators of NF-κB associated with age-dependent increase in inflammatory markers.

The emerging picture of this investigation reveals a stronger role of inflammation in cellular aging than previously anticipated, being an integral part of a broad cellular survival response. While acute inflammation is a critical host response against harmful internal and external stimuli, in the context of aging this once beneficial response becomes constitutively active and potentially detrimental. Inflammation, when chronic, may affect susceptibility to a variety of age-related diseases including cardiovascular diseases, cancer, arthritis, pulmonary and autoimmune diseases, as well as metabolic disorders. Our results, therefore, suggest when a cell-survival response (chronic inflammation/ NF-κB associated with aging) tips the balance, may lead to age-associated degenerative diseases. Though, we do not have indications of active cell-death mechanisms with the extreme treatments, we attribute this to the resilience of 'young' fibroblasts and hence helpful in magnifying the age-related energy limitation of 'old' fibroblasts. Clearly aging-process introduces conditions that may favor induction of age-related diseases and in addition reduces the resilience of the 'old' cells to buffer any extreme stresses unlike the 'young' cells. Therefore, we suggest that our studies of

including the fibroblast system with the energy deprivation model, which revealed an autophagic response involved in enhanced NF-κB activity, provides a unique opportunity to unravel mechanisms and molecular pathways of NF-κB and age-related diseases.

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