UNIVERSITY OF CALIFORNIA, SAN DIEGO

AMP Kinase Attenuates Biomechanical Injury Induced Endoplasmic Reticulum Stress and Catabolic Responses in Articular Chondrocytes

A thesis submitted in partial satisfaction of the requirements for the degree

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by

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2012

The Thesis of Freyr Petursson is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2012

DEDICATION

I would like to dedicate this to my girlfriend, Megan, and my family in Iceland.

EPIGRAPH

“Good judgment is the result of experience; experience is the result of bad judgment.”

Mark Twain

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ABSTRACT OF THE THESIS

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Osteoarthritis (OA) is a chronic disease characterized by a degradation of the articular cartilage, increases in catabolic responses, and inflammation. Osteoarthritis is a leading cause of disability the world, and an enormously

expensive health problem. Biomechanical injury or excess joint use is a known risk factor for the development of osteoarthritis. To identify chondrocyte abnormalities that provide a basis upon which OA is accelerated we studied the unfolded protein response (UPR), a fundamental means by which cells normally resolve stress.

The UPR restores equilibrium to the ER via a reprogrammed proteome rich in chaperones and protein folding catalyst. Three UPR signaling cascades are triggered by dissociation of ER membrane proteins from the chaperone GRP78, which normally dampens the UPR, and limits apoptosis. Each UPR specific cascade promotes terminal expression of CHOP, with unsuccessful resolution of the UPR promoting oxidative stress, inflammation, and apoptosis. Chondrocytes stimulated with sub-lethal cyclic compressive stress demonstrated rapid increase in the UPR over 1-7 days in both as indicated by increased expression of both GRP78 and CHOP.

However, our lab has previously shown that AMP-activated protein kinase (AMPK) a regulator of energy homeostasis and cellular inflammatory cytokines. We therefore stimulated chondrocytes with pharmacological activators of AMPK and tested the effects of AMPK activators on UPR expression and chondrocyte catabolic activity. Catabolic responses, and activation of the UPR were significantly decreased post injury. Targeted activation of AMPK may have the potential to protect articular cartilage from degradation caused by biomechanical injury, and slow the progression of OA.

INTRODUCTION

Osteoarthritis

Osteoarthritis (OA) is a chronic disease in which the pathogenesis is not clearly understood. Although related to aging, it is not necessarily caused by aging; other factors involved in the pathogenesis of OA include genetics, mechanical injury, and/or joint instability. Osteoarthritis is a leading cause of disability in the world and is an extremely common and enormously expensive health problem (Haq et al., 2003). Clinical symptoms of osteoarthritis include loss of mobility, stiffness, and severe joint pain (Pearson-Ceol, 2007). The articular joint is an organ composed of many different tissues, cartilage, synovial membrane, ligaments, tendons, and the subchondral bone, all of which are affected in osteoarthritis. The main characteristics of OA include reduced cartilage cellularity, degradation of the articular cartilage, and inflammation of the synovium. Further pathological abnormalities include thickening of the subchondral bone and fibrosis of the bone marrow. Over time, there is a severe loss of articular cartilage to the point of bone on bone contact, resulting in severe pain, loss of functionality of the whole joint, and subsequently disability (Husa et al. 2010).

Chondrocytes are the only cells found within the cartilage. They are specialized cells that are responsible for maintaining the cartilage, and are found embedded within the articular cartilage that lines the end of long bones (Katz et al., 2010). These cells live in a nonvascularized hypoxic environment,

and rely on diffusion for nutrients (Husa et al. 2010) The cartilage is formed from an extracellular matrix (ECM) that consists of mostly water (70%) and a Type II collagen matrix that is interwoven with proteoglycans (PG's) and glycosaminoglycans (GAG's), such as aggrecan (GAG) and chondroitin (PG) (Haq et al., 2003). The extracellular matrix acts as an absorber of shock, allows for friction free movement between the joints, and the Type II collagen provides tensile strength. In addition to the collagen and proteoglycans, chondrocytes secrete a number of matrix metalloproteinases (MMPs) that are responsible for matrix breakdown and turnover (Katz et al. 2010). In normal cartilage tissue there is a delicate balance between matrix breakdown and synthesis, and OA can be considered an imbalance between catabolic breakdown and anabolic synthesis (Husa et al., 2010).

Although OA has been traditionally considered a disease that results from changes in cartilage metabolism, recent evidence suggests that OA might be a part of a larger group of metabolic and inflammatory diseases (Katz et al. 2010). Chondrocytes are frequently exposed to a variety of stresses, including oxidative and biomechanical stress (Wuertz et al. 2007 and Sutipornpalangkul et al. 2009). Chondrocytes must be able to withstand these stresses and adapt to them in order to maintain homeostasis and a healthy extracellular matrix. Ways in which cells cope and resolve these stresses include autophagy and the unfolded protein response (Boot-Handford et al. 2010 and Caramés et al. 2010).

OA is related to aging, but recent evidence suggests that it is not necessarily caused by aging, rather a cultivation of genetic and environmental factors including biomechanical injury, and obesity (Valdez et al. 2010).

Osteoarthritis and Biomechanical Injury

A common cause of OA is joint trauma or biomechanical injury. Biomechanical injury can be a result of instability of the joint, caused by occupational hazards such as frequent knee bending, obesity, excessive sport participation, ligament tear, and/or quadriceps weakness (Das, 2008). Currently, approximately 12% of all OA cases are a result of trauma to the joint (Anderson et al. 2011). For example, patients with knee ligamentous and meniscal injuries have been shown to have a 10-fold increased risk of OA when compared with non-injured controls (Gillquist., and Messner, 1999). Furthermore, articular fractures have been shown to increase the risk of OA 20 fold, even with surgical intervention to re-stabilize joint biomechanics (Anderson et al. 2011). In addition, post traumatic OA often affects a younger patient population in which treatment modalities such as joint replacements are not a desirable option.

There are several molecular mechanisms that have been indicated in the progressive degeneration of cartilage post injury. For example, human in-vivo studies have shown that following joint injury, levels of aggrecan fragments and type II collagen cross-linked peptides increase in the synovial fluid. In addition, pro-inflammatory cytokines, Tumor Necrosis Factor-α (TNF-

α), Interleukin-1β (IL-1β), IL-6, and IL-8 have also been shown to be elevated for several weeks following injury (Lu et al, 2011). Furthermore, in-vitro studies have shown that following injury, chondrocytes undergo increased cell death/apoptosis, increased release of pro-inflammatory cytokines, nitric oxide production, increased levels of oxygen free radicals, matrix metalloproteases (MMPs), aggrecanases, all leading to chondrocyte damage and matrix degradation (Anderson et al. 2011, D'Lima et. 2001 and Kisday et al. 2009). Studies have also shown that mechanical injury induces the activation cell signaling cascades, such as the MAP kinase pathway or the stress related p38 pathway (Ding et al. 2010 and Bajaj et al. 2010).

Regardless of the huge need, there is currently no cure or disease modifying agents for OA and/or injury induced OA. Current research however suggests that joint injury induces a series of molecular events that cause progressive degeneration of the joint. There is also evidence emerging that suggests that molecular and cellular interventions could possible mitigate these events and prevent the progressive degeneration post injury, and even possibly promote healing. For example, it has been shown that treatment with caspase inhibitors, antioxidants, or superoxide inhibitors such as rotenone can decrease mechanically induced chondrocyte apoptosis and matrix degradation (Anderson et al. 2011 and D'Lima et. 2001). Conversely, current treatment modalities focus primarily on pain management, and do not prevent the initiation or progression of the osteoarthritis post injury. Current therapeutic

approaches are inadequate and there is a gap of knowledge behind the molecular mechanisms resulting in biomechanical injury and OA pathogenesis. Increased knowledge of the molecular mechanisms of biomechanical injury leading to OA pathogenesis will be critical for the development of OA therapeutics.

Biomechanical Injury and the Unfolded Protein Response

The endoplasmic reticulum (ER) is the dynamic membrane-bound organelle responsible for all synthesis and folding of proteins destined for the membrane and/or extracellular matrix. In addition to trafficking, quality control and post-translational modification of all ECM proteins, it is the site of calcium storage, steroid, cholesterol and lipid synthesis (Hotamisligil, 2010). The ER is an oxidative environment different than that of the cytosol, topologically equivalent to the extracellular space. There are a substantial number of protein folding chaperones, protein disulfide isomerases, foldases, and glycolating enzymes all which participate in the folding and post-translational modification of proteins in an ATP dependent matter (Naidoo, 2009). The ER can be considered an energy sensing organelle, and is closely linked with cellular metabolism (Hotamisligil, 2010). Proteins are found in the ER at a very high concentration, which increases the likelihood of protein aggregation. This issue is resolved via a dynamic mechanism called the unfolded protein response (Naidoo, 2009).

An accumulation of unfolded proteins in the ER can be a result of a variety of metabolic and oxidative changes, including glucose/energy deprivation, changes in calcium homeostasis, oxidative changes, pathogens, hypoxia, and/or gene mutation that impairs proper protein folding (Ellgaard et al., 2003). The accumulation of unfolded proteins activates the unfolded protein response (UPR), and the UPR in turn also interacts with several inflammatory and stress signaling systems, including NF-kB IkB kinase (IKK), and oxidative stress signaling pathways. When chronically sustained, these pathways can be detrimental to the cell and eventually lead to programmed cell death, also known as apoptosis (Hotamisligil, 2010 and Boot-Handford et al., 2010).

There are three canonical branches of the UPR, mediated by three ER membrane proteins, IRE-1 (inositol signaling enzyme), PERK (PKR-like eukaryotic initiation factor 2α kinase), and ATF6 (activating transcription factor-6). Normally, these proteins are found in association with chaperone protein Glucose Regulatory Protein 78 (GRP78/BiP/Hsp5A) and are therefore inactivated. Under conditions of stress, an increase in the unfolded protein substrates forces GRP78 to dissociate from the membrane to assist in protein folding, subsequently releasing other effectors of the UPR such as PERK, IRE-1 and ATF6. This results in the activation of a complex downstream signaling pathway which includes upregulation of ER chaperone proteins such as GRP78, attenuation of protein translation, ubiquitination and degradation of

unfolded proteins mediated via the ERAD (ER associated degradation) pathway. These pathways are normally protective. However, sustained activation leads to activation of inflammatory pathways and subsequently cell death (Naidoo, 2009 and Lin et al., 2008, and Hotamisligil).

Specifically, PERK activation leads to its homodimerization and auto-phosphorylation, and subsequently a halt in most protein translation via its phosphorylation of translation initiator factor eIF2α. The translation of UPR related proteins actually increases since they have internal ribosomal entry sites (IRES) that do not rely on translation initiator factor eIF2α. PERK activation also leads to the phosphorylation of transcription factor Nuclear Factor Like-2 (Nrf2), which is involved in the transcription of anti-oxidant response genes. On the other hand, when IRE-1α is activated, it gains an intrinsic endonuclease activity. This allows it to cleave the mRNA encoding for X-box binding protein 1 (XBP-1), forming the alternatively spliced variant XBP-1s which subsequently acts as a transcription factor involved in stimulating the transcription of protein folding, export and degradation related genes. Activation of the third arm, ATF-6 leads to its proteolytic cleavage and subsequent translocation into the nucleus where it can stimulate the transcription of UPR related proteins (Naidoo, 2009 and Lin et al., 2008, and Hotamisligil, 2010). The overall response is an inhibition of protein synthesis, and upregulation of proteins involved in protein folding and degradation.

Sustained UPR activation as a result of failure to resolve ER stress leads to an activation of several apoptotic pathways, including the induction of C/EBP homologous protein (CHOP/GADD153) through the PERK pathway (Naidoo, 2009). High amounts of ER stress allow the translation of ATF4, a transcription factor that subsequently activates the transcription of CHOP. CHOP mediates apoptosis through the intrinsic pathway by causing the down regulation of the anti-apoptotic protein Bcl-2, and up regulation of the pro apoptotic protein Bim (Rasheva et al., 2009). Strong evidence to support this is that CHOP deficient mice show increased protection against ER stress induced apoptosis (Zinszner et al., 1998). CHOP may also promote apoptosis by upregulating a thiol reductase, Ero-1. This protein normally promotes protein folding, however it also generates reactive oxygen species (ROS) in the process, which may further contribute to the apoptotic process (Rasheva et al., 2009). In addition to CHOP, pro-apoptotic proteins Bax and Bak, are switched on by IRE-1α (Homatmisligil), which also activates the c-Jun N-terminal Kinase (JNK) signaling pathway, a part of a superfamily of mitogen activated kinases. The JNK pathway induces ER stress-related apoptosis through the activation of ASK1 (apoptosis signal-regulating kinase) (Lin et al., 2008, and Rasheva et al., 2009).

It has been shown previously that inducing ER stress in chondrocytes has resulted in decreased chondrocyte cell growth, decreased matrix accumulation, decreased expression of type II collagen, decreased aggrecan

synthesis, and ultimately apoptosis (Yang et al., 2005). Moreover, previous studies have shown that GRP78 is up-regulated in advanced OA (Nugent et al., 2009). Despite its obvious importance, little or no knowledge exists about the relationship between the UPR and biomechanical injury in chondrocytes. Previous research on biomechanical injury in chondrocytes has focused mostly on cytokine-mediated inflammation and changes in chondrocyte metabolism. For example, in vitro biomechanical studies have shown that continuous biomechanical and high hydrostatic pressure can lead to an increase in the catabolic and inflammatory response. This response is measured by a decrease in proteoglycan synthesis, increased nitric oxide synthesis (a marker for cytokine mediated inflammation) and increased matrix metalloproteinases synthesis, DNA damage, and ultimately apoptosis. (Fioravanti et al. 2010, Hashimoto et al., 2009, and Honda et al., 2000). On the other hand, studies have shown that low pressure, intermittent biomechanical compression effectively attenuates cytokine mediated inflammation, and increases matrix synthesis, including increased collagen type II and proteoglycan release (Chowdhury et al., 2010).

Interestingly, it has been shown using RT-PCR that several markers of extracellular matrix synthesis increase within the first 8 hours of 50% compression of cartilage explants, but decrease subsequently beyond 8 hours compression (Fitzgerald et al., 2004). It is therefore not unreasonable to propose that as an adaptation to excessive mechanical forces an aberrant

increase in protein synthesis results in ER stress in chondrocytes, and subsequently an attenuation of protein synthesis that, if sustained, results in cell death. However, as stated previously, there is a gap in knowledge behind the molecular mechanisms mediating biomechanical injury, and the UPR in chondrocytes. Elucidating these mechanisms and answering questions such as whether manipulation of the UPR can be used to treat and prevent both biomechanical injury and OA will be important for the development of OA therapeutics.

Moreover, the UPR is implicated in multiple tissue diseases other than OA, including type II diabetes, neurodegenerative diseases such as ALS, and becomes dysregulated with aging (Hotamisligil, 2010). Chondroytes synthesize and secrete large quantities of protein, and healthy UPR is required for healthy matrix synthesis (Yang et al., 2005). Is it is therefore reasonable to propose that chondrocytes maybe highly susceptible to ER stress. One of the hypotheses of this project is that the UPR becomes dysregulated as an adaptation to biomechanical injury and inflammatory stress in aging articular cartilage chondrocytes, and therefore promotes the development of the chondrocyte dedifferentiation, hypertrophy, stimulation of apoptotic pathways, and subsequently OA. Currently, there is a gap in knowledge regarding the molecular mechanisms mediating the relationship between the UPR and stresses such as biomechanical stress. Elucidating these mechanisms will be important in the development of osteoarthritic preventative drugs, and

preventing biomechanical injury related OA. We will approach this hypothesis by studying biomechanical injury in vitro, and assay for UPR markers. We will assay the role pharmacological modulators of the UPR, as well as investigate the relationship between master metabolic regulator AMPK and the UPR in the context of biomechanical injury.

Biomechanical Injury, Inflammation and AMP Kinase

Nearly every cellular process requires the hydrolysis of ATP either directly or indirectly. It is therefore important that cells maintain supply with demand, and generate adequate supplies of ATP. Adenosinemonophosphate-activated protein kinase (AMPK) is a master metabolic regulator and sensor of energy homeostasis that is activated in response to AMP in addition to ADP, and is responsible for activation of ATP generating catabolic pathways, and inhibition of ATP consuming anabolic pathways. AMPK is most notably known for its effects on metabolism and energy, but has in recent years been shown to have many other functions, including cell survival, mitochondrial biogenesis, autophagy, cell polarity, growth and proliferation. AMPK has also been implicated in various other diseases including cancer, metabolic diseases such as diabetes, obesity, cardiovascular disease, and even viral infections (Hardie 2011 and Carling et al. 2011).

AMPK is a heterotrimeric protein composed of an alpha catalytic subunit, and 2 noncatalytic regulatory beta and gamma subunits. There are a total of seven genes encoding AMPK, two isoforms of α (α1 and α2), two of β,

(β1 and β2), and three of γ (γ1, γ2, and γ3). All 3 subunits are required for a stable and fully functional AMPK. The α subunit contains a serine/threonine kinase domain at the N terminus that when phosphorylated by upstream kinases at a conserved threonine residue within the activation loop (Thr 172 in human α1) increases several thousand fold in activity (Hardie 2011 and Tekeltaub et al. 2011 and Carling et al. 2011). The γ subunits contain the regulatory adenine nucleotide-binding sites divided into four segments of CBS domains corresponding to the four potential nucleotide binding sites which bind AMP, ADP or ATP interchangeably. The occupancy at these sites depends on the intracellular concentration of the different adenine nucleotides, reflecting the energy state of the cell (Carling et al. 2011).

AMPK is activated via allosteric binding of AMP and by the phosphorylation of α subunit (Thr172) via the upstream kinases serine/threonine kinase 11 (LKB1), Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ),and transforming growth factor-β-activated kinase 1. In addition, there are several pharmacological activators of AMPK including 5-aminoimidazole-4-carboxamide riboside (AICAR), A769662 (a small-molecule AMPK activator), metformin, thiazolidinediones, statins, and natural products such as resveratrol from red wine, epigallocatechin gallate from green tea, or the Chinese medicinal compound berberine (Carling et al 2011, and Hardie 2011). The mechanism of activation varies between compounds or is unknown. A769662, for example, appears to activate AMPK directly, AICAR is

transported into cells by adenosine transporters and converted by adenosine kinase into monophosphorylated ZMP, an AMP mimetic (Hardie 2011). Metformin, on the other hand, works by inhibiting the AMP deaminase enzyme, thereby increasing AMP concentration (Ouyang et al. 2011). The natural activators seem to have evolved as plant defense products and work primarily by inhibiting mitochondrial ATP synthesis, therefore increasing the AMP:ATP ratio.

In addition to pharmacological activation, recent studies have shown that AMPK is also under the control of endocrine and autocrine control. AMPK has been shown to be activated by the adipocyte derived hormones (adipokines), adiponectin and leptin. On the contrary, AMPK activity is decreased in response to increases in concentrations of adipokine, resistin, or inflammatory cytokines TNF-α, or IL-1β (Viollet and Andreelli 2011, and Terkeltaub et al. 2011). This suggests that it plays an important role in inflammatory diseases, and/or disease related to obesity, such as type II diabetes. Recent studies have shown a correlation between obesity and osteoarthritis; however, non-weight bearing joints such as hands also show an increased prevalence of osteoarthritis in obese patients (Cicuttini 1996). This data suggests that adipose tissue plays a role in the development of osteoarthritis, possibly through adipokines and modulation of AMPK.

AMPK is also activated by a variety of metabolic stresses such as glucose deprivation, hypoxia, ischemia, or processes that accelerate ATP breakdown such as muscle contraction (Hardie, 2011). AMPK signaling also plays a key role in cell survival during stress by its ability to maintain metabolic homeostasis. There is emerging evidence that AMPK can suppress the activation of nuclear factor-κB (NF-κB) system, an important regulator of inflammation and innate immunity (Salminen et al, 2011). It has been observed that treatment with inflammatory cytokine TNF-α, or pro-inflammatory lipopolysaccharides (LPS) reduced the activity of AMPK, partly by upregulating the expression of protein phosphatase 2C, an inhibitor of AMPK signaling (Salminen et al, 2011).

Several targets of AMPK have been shown to play a role in the inhibition of NF-κB, including Forkhead box O family (FoxO), Type III deacetylase SIRT1, and peroxisome proliferator-activated receptor co-activator 1α (PGC-1α), all of which have been shown to suppress inflammatory factors in-vitro. AMPK appears to activate SIRT1 by increasing the levels of NAD^+. SIRT1 then, in a positive feedback loop, increases the activity of AMPK upstream kinase LKB1, further enhancing AMPK activity (Salminen et al, 2011). SIRT1 has the ability to decreases inflammation by deacetylating the p65 subunit of NF-κB, thereby decreasing its transcriptional activity, and enhancing its ubiquitylation and degradation (Yang et al. 2010).

AMPK has been shown to directly phosphorylate a key regulator of energy metabolism PGC-1α, which when phosphorylated leads to its activation via SIRT1-mediated deacetylation. Activated PGC-1α is known to increase mitochondrial biogenesis, and enhance energy production via glycolysis (Alvarez-Guardia et al. 2010). PGC-1α lesser studied role is its part in inflammation. Although studies are limited, it has been shown that overexpression of PGC-1α can inhibit the TNF-α induced NF-kB activity, conversely, overexpression of TNF-α was shown to downregulate PGC-1α expression (Alvarez-Guardia et al. 2010, Kim et al. 2007, and Salminen et al, 2011).

Another mechanism of AMPK directed inhibition of NF-kB and inflammation is through the direct phosphorylation and activation of transcription factor p53; p53 has been shown to inhibit NF-kB by indirect mechanisms, one of which is through its inhibition of glycolysis. During times of increased glycolytic activity, the IκB kinase-beta (IKKβ) gets O-glycosylated. This modification blocks the inhibitory phosphorylation site on IKKβ which enhances its activity and therefore enhances activation of NF-kB (Kawauchi et al. 2008). Another family of transcription factors that have been shown to be under the control of AMPK is the FoxO family of transcription factors. A particularly important one involved in inflammation is FOXO3a. AMPK can directly phosphorylate and activate FOXO3a at several regulatory sites which then leads to transcription of several FOXO3a directed genes (Salminen et al,

2011). Lin and colleagues have shown that deficiency of FOXO3a in mice results in exacerbation of the inflammation process in several tissues, increased activation of T-helper cells, and increased production of cytokines. Moreover, they showed FOX3a inhibited NF-kB activity in T cells (Lin et al. 2004).

There are also numerous studies that elucidate AMPK's ability to suppress oxidative stress. For example, Xia et al. has shown that AMPK increased the expression of mitochondrial uncoupling protein-2 (UCP-2) which is known to reduce superoxide radicals (Xia et al. 2008). Furthermore, by inhibition of NF-kB, AMPK downregulates expression of NAD(P)H oxidase, and therefore ROS production, and oxidative stress. In addition, through activation of FOX3a, AMPK increases the expression of thioredoxin(Trx), a disulfide reductase, and inhibitor of oxidative stress caused by cysteine oxidation. Trx has been shown to indirectly inhibit the activation of the inflammasomal multiprotein (Salminen et al. 2011).

Currently, there is limited knowledge about the role of AMPK in chondrocytes and its role in the development of osteoarthritis, and no knowledge at this date exists about the role AMPK plays in biomechanical injury in articular chondrocytes. However, it can be hypothesized that since chondrocytes live in an nutritionally deficient, avascular and hypoxic environment and must meet challenging metabolic demands in response inflammation, aging, and biomechanical stress, that AMPK plays a significant

role in chondrocytes and in osteoarthritis development post injury (Pfander and Gelse 2007, Terkeltaub et al. 2010). AMPK has also been shown to be highly expressed in articular and growth plate chondrocytes, and is activated in a hypoxia inducible-1α dependent manner (HIF-1α) (Bohensky et al. and Terkeltaub et al. 2010).

In a revealing study done by Terkeltaub and associates, it was shown that AMPK phosphorylation and its expression is decreased in human osteoarthritic knee cartilage. They found that phosphorylation of AMPK is decreased in response to challenge with inflammatory cytokines IL-1β, and TNF-α. In addition, it was shown that pre-treatment with pharmacological activators of AMPK, AICAR and A769662, prevented a number of pro-inflammatory catabolic responses to IL-1β, and TNF-α, such as nitric oxide release (NO), glycosaminoglycan (GAG) release, and release of matrix metalloproteases (MMP-3 and MMP-13). Lastly, they found that proinflammatory cytokine–induced procatabolic responses were exacerbated in in chondrocytes with knockdown of AMPKα_1. Taken together, these data suggest that AMPK plays an important role modulating the development of osteoarthritis, particularly in response to inflammatory cytokines. As aforementioned, it is well established that biomechanical injury to chondrocytes is known to increase inflammatory cytokines and pro-catabolic responses (GAG, NO, MMP's), reactive oxygen species, and oxidative stress. It is therefore not unreasonable to propose that activation of AMPK prior to

injury could potentially offer a protective effect, and attenuate inflammation, oxidative stress, and catabolic responses that are commonly seen in biomechanical injury or traumatic osteoarthritis.

Biomechanical Injury, Oxidative Stress, UPR and AMPK.

There is increasing evidence suggesting that reactive oxygen species affects protein folding, and that redox status could directly or indirectly alter protein folding, and ER homeostasis, and thus increases in oxidative stress can lead to activation of the UPR (Malhotra and Kaufman 2007). Conversely, several studies have shown AMPK activation can attenuate ER stress (Dong et al. 2010, Terai et al. 2005, and Thériault et al. 2011). Recent work by Dong et al. has elucidated at least one mechanism in which AMPK is able to suppress ER stress. First, they showed that knockdown or deletion of AMPK results in increased expression of the UPR; however, this effect was attenuated by treatment with several antioxidant compounds or by the intracellular Ca^{2+} chelator BAPTA. They also found that AMPK deficiency led to increases in intracellular Ca^{2+}, which is known to cause aberrant protein folding and increases in ER stress. For example, thapsigargin, an inhibitor the the sarcoplasmic/endoplasmic reticulum Ca(2+) ATPase (SERCA) pump normally responsible for pumping Ca^{2+} into the ER and therefore out of the cytoplasm, is a well-known inducer of ER stress.

They discovered that AMPK deficiency leads to oxidation and therefore inhibition of the SERCA pump. In addition, they found oxidation of the SERCA

pump could be restored by treatment with pharmacological activators or AMPK such as AICAR or by viral transfection of constitutively active AMPK (Dong et al 2010). Taken together this data suggest that AMPK is able to protect against ER stress by maintain the reductive state of the cell and preventing oxidation of the SERCA calcium pump and therefore preventing calcium leakage into the cytosol and thereby UPR activation.

It is well established that biomechanical injury leads to increases in reactive oxidative species and oxidative stress (Martin et al. 2009 and Goodwin et al. 2010). It is therefore not unreasonable to propose that biomechanical injury via its induction of oxidative stress activates the UPR. Also it can be hypothesized that pre-activation of AMPK with pharmacological activators could potentially attenuate the effects of biomechanically induced oxidative stress, and therefore increases in UPR activity. Furthermore, since AMPK appears to modulate the UPR by activity on cell redox states, we should in theory be able to suppress UPR activation via pre-treatment with antioxidant compounds such as NAC. We will test the hypothesis that the UPR is activated response to biomechanical injury by looking at increases in UPR mediators GRP78, and CHOP. We will test the secondary hypothesis that AMPK activation modulates aberrant ER stress in response to biomechanical injury.

MATERIALS AND METHODS

CHONDROCYTE 3-D EMBEDDING

Articular cartilage chondrocytes were isolated from a mature bovine knee, subsequently digested overnight in 2mg/mL type II collagenase solution. Cells were isolated and resuspended in a sterile/endotoxin free solution containing 2% alginate (Novamatrix) in Hanks Buffered Saline Solution (HBSS) at a cell density of 8 million cells/mL. Chondrocyte–alginate suspensions were injected into a custom designed mold consisting two Ca2+ permeable membrane filter papers. The suspensions were gelled via Ca2+ diffusion using 102 mM $CaCl_2$ solution for 30 minutes. Afterwards, the alginate slab was washed 3 times in Dulbecco's Phosphate Buffered Saline to remove excess Calcium. The chondrocyte–alginate slabs were removed from the mold and punched into circular constructs measuring 6mm in diameter and 3mm in thickness using a biopsy punch (Sklar). Alginate cell-constructs were cultured in DMEM media containing 10% FBS and 1% penicillin-streptomycin in a 37° 5% CO_2 incubator for a minimum of 20 days before compression to allow for extracellular matrix production.

BIOMECHANICAL INJURY

Following culture the construct thickness were measured using digital precision calipers (Mitutoyo). Constructs were then subjected to a

biomechanical injury using custom made mechanical testing apparatus housed inside a 37° 5% CO2 incubator. Each specimen was subjected to continuous dynamic unconfined compression, at 24% strain, 12% amplitude, 0.5hz for 16 hours. After injury cells were either isolated from alginate constructs using a 50mM EDTA/PBS solution to dissolve the alginate and isolate the free cells, or re-cultured in free swelling conditions for later isolation. Constructs were harvested post compression at 0 hours, 24 hours, 48 hours, 3 days and 5 days after injury. Cells were lysed and RNA or protein was isolated. The Conditioned Media was also isolated at the aforementioned time points.

NITRIC OXIDE ASSAY

Media was harvested post injury at 0hr, 24hr, 48hr, 3d and 5d for injury and non-injury control group. IL-1β was used as a positive control at a concentration of 1ng/mL for 24 hours (Data not shown). 50ul of each cultured medium was mixed with the same volume of the Griess reagent [1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H3PO4]. The reaction is followed by a colorimetric detection of nitrite as a product of the Griess reaction. The nitrite concentration was determined by measuring the absorbance at 540 nm with a 96-well microplate spectrophotometer and was calculated by comparing the data to a standard curve reference of sodium nitrite generated with known concentrations.

GAG RELEASE ASSAY

Media was harvested post injury at 0hr, 24hr, 48hr, 3 days and 5 days for injury and non-injury control group. IL-1β was used as a positive control at a concentration of 1ng/mL for 24 hours (Data not shown). The colorimetric assay used to quantitate proteoglycan release in cell media post compression was based on the binding of the basic dye 1,9 dimethylmethylene blue to the negatively charged sulfated GAGs, resulting in a color shift. The absorbance of the solution was read at 535 nm on a colorometric plate reader, and proteoglycan concentration determined by use of a standard curve of a known shark chondroitin sulfate concentration.

LIVE-DEAD ASSAY

Constructs were sectioned in half to expose a full thickness plane. A small section was removed from the core and sections were incubated at room temperature in 100 µl PBS containing 4 µM calcein AM and 2 µM ethidium homodimer-1 (Invitrogen, California) for 25 min to stain viable and non-viable cells, respectively, and then washed in PBS for 10 min. Cores were sectioned in half to expose a full-thickness plane and imaged using fluorescent microscopy.

QUANTITATIVE REAL TIME PCR

RNA was isolated from cells using standard protocol provided by RNA Easy Kit (QIAGEN, Germany). RNA was quantified and then 100ng of RNA was used for reverse transcription using the First Strand Synthesis Kit (Roche, Germany) using both random hexamer, and oligoDT primers. The cDNA was then used for quantitative RT-PCR on Roche LightCycler 480, using SYBR green provided by Roche. GAPDH was used as an internal control, the primers used are described in Table 1.

WESTERN BLOT ANALYSIS

Western blot was performed according to previously published protocol with minor modifications. Antibodies were obtained from Cell Signaling, (pAMPK, Total AMPK, GRP78, and Caspase-3), Biolegend (CHOP), Abcam (β-Actin), and epitomics (MMP-3).

GELATIN ZYMOGRAPHY

Gelatin zymography was performed by using 10% acrylamide, 0.1% gelatin zymogram gel. In brief, 20µl conditioned media were mixed with non-reducing sample buffer and loaded without boiling. After electrophoresis, the gel were soaked in 2.5% Triton X-100 to renature enzyme, rinsed, and incubated for 24 h at 37℃ in developing buffer (50 mM Tris-HCl, pH 8.5, 0.2 M NaCl, 5 mM CaCl2, 0.02% Brii35). After incubation, gels were stained with

0.5% Coomassie blue and then destained in 20% methanol, 30% acetic acid. Clear bands on the zymogram were indicative of gelatinase activity**.**

HYDROGEN PEROXIDE ASSAY

The levels of hydrogen peroxide in the conditioned media post injury were measured using a Fluorometric Hydrogen Peroxide Assay Kit (Biovision). In the presence of Horse Radish Peroxidase (HRP), the OxiRed Probe reacted with H2O2 to produce product with color (λmax = 570 nm) and red-fluorescent (Ex/Em=535/587 nm). Samples were read on a florescent microplate reader, and Hydrogen peroxide in media was detected and quantified in relation to standard curve.

STATISTICAL ANALYSIS

Data are presented as the mean±SD values. Comparisons between Control and Injury treated groups were made by two-way ANOVA. Bonferroni correction post tests were used as a post hoc analysis during multiple comparisons. Statistical significance was accepted with $p<0.05$. All statistical analyses were performed by using Graphpad Prism v5.0.

RESULTS

Novel In-Vitro Injury System Induces Delayed Cell Death and Apoptosis

In order to validate the new in-vitro biomechanical injury system which was custom built from de-novo, and in order to find a condition representative of osteoarthritic injury several experiments were performed. First the alginate molds were subjected to various percentages of deformation to test the deformation break point of the alginate. It was found that alginate frequently cracked above a deformation percentage of 25% (results not shown). We therefore decided to settle on a maximum compressive percentage of 24% total alginate height. Next, in order to find a condition representative of typical traumatic joint injury, and noting that apoptosis occurs after an ordered sequence of cellular events, and is typically associated with the progressive activation of caspases; we desired an injury condition which markedly induced delayed yet significant apoptosis, with minimal immediate necrosis.

Since the alginate deformation is limited, we tested various lengths (in time) of compression. Lengths of compression test varied from 2 hours (results not shown), 8 hours, 16 hours, and 24 hours of continuous dynamic compression at 24% percent, 12% amplitude. After the injury the alginate molds were sliced and cell death was measured using Invitrogen live/dead assay. It was found that after 2 hours of injury (results not shown) there were no significant increases in cell death. Furthermore, 8 hours injury there was no

noted increases in cell death (**Fig.3A**). On the other hand, with sixteen hours of compression there was a delayed but marked cell death 1 day or 24 hours after completion of the injury (**Fig.3B**). Quantitative analysis of the live-dead assay showed that cell death continued to increase 2 days or 48 hours after injury (**Fig.4A**). Furthermore, these analysis showed that there was no significant increase in cell death immediately after the 16 hour compression. These results were further verified in a western blot for capasse-3 expression, which showed no increase in capase-3 immediately after the injury, however one day or 24 hours after injury had stopped there was a significant increase in caspase-3 expression. These results corroborated well with previous published studies, which have shown that after traumatic injury apoptosis does not start to increase until between 6 and 24 hours after injury, and can continue to increase up to 7 days post injury (D'Lima et al. 2001). We also attempted to increase the injury length even further to 24 hours straight; however it was found that 24 hours of compression induced a rather significant number of apoptotic cells immediately after the injury (**Fig.3A**). We therefore decided to make the 16 hours of compression at 24% strain and 12% amplitude the standard injury condition for all further experiments.

Novel In-Vitro Injury System Induces Significant increases in Catabolic Responses (NO, GAG, and MMP) Expression and Activity.

In order to further validate our system and make sure our injury condition corroborated with previously published studies showing increases in

pro-catabolic responses with injury, we tested for GAG, and nitric oxide release after the injury. Immediately after the injury the levels of GAG within the media were not significantly increased, but became significant when compared with controls 24 hours after the injury, and continued to increase at 48 hours after injury (**Fig.5A**). On the other hand, colorimetric detection of Nitrate in a media, a byproduct of the degradation of nitric oxide, was significantly increased immediately following the injury, and continued to increase 24 and 48 days after the injury (**Fig.5B**). Quantitative and real time PCR was performed to indicate expression of various catabolic and anabolic genes. Results from both the real time, and reverse transcriptase PCR showed there were significant increases in various catabolic markers, including MMP-3, MMP-13, and ADAMTS5. Furthermore, results also showed there was a significant decrease in type 2 collagen, the primary collage in normal cartilage; however there was a slight but not significant decrease in aggrecan expression (**Fig.6 and Fig.7**).

To confirm the results of the PCR, additional experiments were conducted to test the amount of MMP-3 released into the conditioned media via western blot, and also test the activity of the MMP's specifically MMP-9 via gelatin zymography. There was a strong robust release of MMP-3 into the conditioned media after injury, and corroborating with qPCR results. Similar to the qPCR results, we noted no significant increase in MMP-3 immediately after the injury, but rather 24 hours and 48 hours after injury, and tapering off at 5

days after injury (**Fig.7A**). Gelatin zymography also revealed increases in MMP-9 activity (**Fig.7B**), however no particular pattern was noted over the time course. It is possible that the gelatin zymography was not sensitive enough to reveal any expression patterns.

A supplementary study was conducted to further test the whether our system induced free radical release in agreement with previously published studies. An experiment measuring the release of hydrogen peroxide in to the conditioned media was conducted. Results revealed an early release of hydrogen peroxide into the media immediately following the injury, and 1 day after the injury, however after 1 day levels were not significantly different than the controls (**Fig.9**).

Biomechanical Injury Induces a Marked Increase in UPR activity, and attenuation of AMPK Activity.

To test our hypothesis of whether the UPR was involved with biomechanical injury, we performed a series of western blots measuring early UPR marker GRP78, and late UPR marker CHOP. Both UPR markers were markedly increased following injury, following a similar pattern as the pro-catabolic responses, and cell death studies, with the largest increases 24 hours and 48 hours following injury (**Fig.10A and Fig.10B**). Next, phosphorylation of AMP Kinase was assessed, it was revealed that the phosphorylation was prominently decreased following injury starting 24 hours after injury and further decreasing at 48 hours after the injury, following an

pattern inverse of the aforementioned catabolic responses (**Fig.11**). The levels of total AMPK were not significantly affected.

To test the hypothesis that activated AMP Kinase could modulate the UPR after injury we decided we pretreated the chondrocytes for 24 hours with AMP kinase activators AICAR and A769662. Results showed that pretreatment prevented the loss the AMPK phosphorylation after injury. Pre-treatment also dampened the activity of the UPR, and apoptosis, as reveled by western blot CHOP and GRP78, and capase-3 expression (**Fig.12**). Additionally, activation of AMPK without mechanical injury was not found to affect basal CHOP and GRP78 or capase-3 expression (results not shown).

Pharmacological Activation of AMPK Markedly Suppressed Several Catabolic Markers (NO, GAG, MMP) and Caspase-3 Activity

To test the hypothesis that activated AMPK could modulate and lessen the pro-catabolic responses to injury; chondrocytes were pre-treated for 24 hours with AMPK activators A769662 and AICAR before injury. Following injury, the GAG release and NO release was measured. Pretreatment with AICAR and A769662 was able to reduce both GAG and NO release immediately following injury (day 0 on graph), and 24 and 48 hours following injury (day 1 and day 2 on graph) **(Fig.13A and Fig.13B**). Further qPCR analysis revealed that pharmacologic activation of AMPK decreased the mRNA expression levels of both MMP-3 and MMP-13 following injury when compared to injury alone (Fig.14A and Fig.14B)

DISCUSSION

Several premises and hypothesis were validated in the presented studies. For one, we showed that our newly developed in-vitro biomechanical system of prolonged supra-physiological compression resulted chondrocyte apoptosis, and a substantial increases in several pro-catabolic responses, activation of the UPR, and a destruction of AMPK activity. These cellular responses to our injury model help to substantiate previously published studies. In addition, they show that besides the single blunt traumatic force system used by many previous studies, a more physiological, yet prolonged compression can also cause the cellular responses commonly associated with post traumatic osteoarthritis.

Additionally, we were able to demonstrate that catabolic responses and apoptosis do not occur or reach their maxima immediately following the injury; rather these cellular events do not occur or peak until 24 and 48 hours following the injury. This finding is important for several reasons. It suggests that there is complex cell signaling, and perhaps transcription events that occur after the injury account for the delay. Furthermore, this suggests there is a small “window of opportunity” following injury, and that these cell signaling events could be halted via specific intervention in order to improve patient outcomes following joint injury.

The implications of the early nitric oxide and hydrogen peroxide release are not clearly understood. However, NO has been shown to induce apoptosis in chondrocytes by activating caspase-3, which can be attenuated with the use of the nitric oxide synthase inhibitor N-monomethyl L-arginine (Blanco et al. 1995). This suggests that perhaps the generation of nitric oxide is an early cell signaling event post injury, preceding the apoptosis activation. NO has been shown to activate apoptosis via activation of the tyrosine kinase pathway. However, it has been shown that NO by itself cannot initiate apoptosis and that the production of superoxide radical ($O_2^{\cdot-}$) by NADPH oxidase complexes is required; these two react together to form peroxynitrate ($ONOO^-$) (Del Carlo and Loeser, 2002). Peroxynitrate has been shown to increase expression of MMP-3 and MMP-13, and decrease the expression of matrix proteins, Type II collagen, and aggrecan (Henrotin et al. 2003). Taken together, this suggests that as an early event to biomechanical injury, nitric oxide and reactive oxygen species are generated, activating a series of signaling cascades leading to apoptosis, and increased catabolic gene expression and decreased anabolic gene expression. These discoveries are consistent with our findings which showed an increase in hydrogen peroxide, and nitric oxide increases immediately following injury (**Fig.9 and Fig.5B**).

It remains to be determined what component of mechanical stress is causing the activation of the nitric oxide and ROS production. Several cytokines have been shown to increase ROS and nitric oxide production in

chondrocytes (Henrotin et al. 2003). However, during these studies we were unable to detect IL-1β or TNF-α in the conditioned media, which suggests that either the NO and ROS are activated via other cytokines or another mechanism altogether. This may have been due to technical difficulties in detecting the cytokines as we were working with primarily bovine cells in which manufactured ELISA kits are limited in supply. We were able to detect increases in IL-1β mRNA following injury (data not shown); however whether this translates to increased IL-1β release is not yet known, and these experiments will have to be repeated in order to get a clear answer. A further limitation of this study is that we did not directly measure peroxynitrate or other free radicals. We instead measured nitrate and hydrogen peroxide, both byproducts of the generation of ROS. Previous studies have shown that ROS can activate MAP kinase pathways (Henrotin et al. 2003), which as previously mentioned, are pathways activated after injury. Taken together this suggests that these pathways play an important role following injury. These studies were limited in the fact that we did not investigate the activation or implications of these pathways in our system, and whether these pathways play a role in the decreased activity of AMPK.

Another premise validated in these studies was that the UPR in increased following injury. We found injury activated the unfolded protein response, as revealed by expression of GRP78 and CHOP. This corroborates well with previous accounts that have shown the UPR is upregulated in

advanced OA (Nugent et al. 2009) and leads decreases expression of Type II collagen and aggrecan, and ultimately apoptosis (Yang et al. 2005). Interestingly, activation of the ER has also been shown to stimulate transcription of MMP-13; this can be attenuated by inhibiting p38 MAP kinase pathway (Hamamura K, et al. 2009), which suggests there is a cross talk between the p38 MAP kinase pathway and the UPR. In these studies we found that the activation of the UPR correlated with the activation of MMP-13. Interestingly, qPCR data showed there was a significantly greater and earlier activation of MMP-3. There are many implications of these findings, for one we have revealed a new potential therapeutic target for post traumatic OA. In addition, our studies suggest that the UPR may play a role in the activation of the catabolic responses commonly associated with osteoarthritis and joint trauma.

However, this study is limited in the fact that we did not further investigate the exact cell signaling mechanism that caused the activation of the UPR. One could postulate that the increases in ROS and nitro radicals could modify proteins by oxidation, nitrosylation, and nitration of specific amino acids leading to changes in protein structure and accumulation of damaged proteins, and disruption in protein folding, leading to activation of the UPR as previous studies have shown (Malhotra and Kaufman 2007 and Henrotin et al. 2003). However, the exact mechanism of activation remains to be determined

and will surely prove important in the development of therapeutics to biomechanical injury.

Another premise validated in this study was the fact that injury seemingly decreased activity of AMPK. As previously mentioned, recent work done by Terkeltaub et al. has revealed that stimulation with inflammatory cytokines can decrease activity of AMPK in articular chondrocytes. This suggests that during injury there are increases in inflammatory cytokines. This study is limited in the fact we did not directly measure cytokine production. However, several of our findings suggest the presence of cytokines or activation of cytokine cell signaling pathways such as p38 and NF-kB, for example, NO, GAG release, and MMP expression, all of which are commonly associated with cytokine, and activation of NF-kB.

In our studies, we showed that pretreatment with AMPK activators could suppress many of the catabolic responses such as NO, GAG, and MMP release following injury. This supports the previous work done by Terkeltaub et al. which showed activation of AMPK could suppress cytokine mediated catabolic responses. There is limited information of the role of AMPK in biomechanical injury, however in recent work done by Lu et al. 2011, found that treatment with dexamethasone attenuated NO release, GAG loss and restored proteoglycan biosynthesis following injury. Interestingly, they found that the dexamethasone mediated suppression of catabolic responses appeared to be regulated primarily at the translational level since they found

that the dexamethasone did not significantly alter the mRNA levels of iNOS or aggrecanses, however protein levels appeared to be effected. These studies corroborate well with our findings since it is known that dexamethasone is strong activator of AMPK in other cell types (Kewalramani et al. 2009), which suggest that perhaps the protective effects of dexamethasone may be in part mediated through activation of AMPK. We also showed that activation of AMPK decreased caspase-3 activation. It has been shown AMPK can phosphorylate the pro-apoptotic protein Bad, preventing cytochrome c release and attenuating caspase-3 activation (Kewalramani et al. 2009).

We have brought up many of the implications of these findings; however there still exist a gap of knowledge in how chondrocytes fundamentally sense aberrant mechanical forces. There have been numerous studies in various cell types showing how cells sense force through multiple mechanisms, including but not limited to, cytoskeleton, integrins, G proteins, receptor tyrosine kinases, mitogen-activated protein kinases, and stretch-activated ion channels (Wang and Thampatty 2006). Novel experimentation, and further research will have to be done to elucidate the type and magnitude of the forces experienced, and identify force sensors/receptors in the context of chondrocytes and aberrant mechanical stress. Some interesting recent studies have potentially implicated primary cilium in the sensing of mechanical forces in chondrocytes. Electron microscopy has revealed that primary cilium is ubiquitously found in chondrocytes and projects into the rectify ECM.

Mechano-sensing proteins such as integrins have been found to be present on chondrocyte cilium. Moreover, studies found that mechanical loading modulates chondrocyte primary cilia incidence and length (Muhammad et al. 2012) Suggesting a possible role for cilia in mechanosensing of chrondrocytes. Further understanding will surely prove fundamentally important in the development of novel therapeutics in the treatment of post traumatic osteoarthritis.

FIGURES

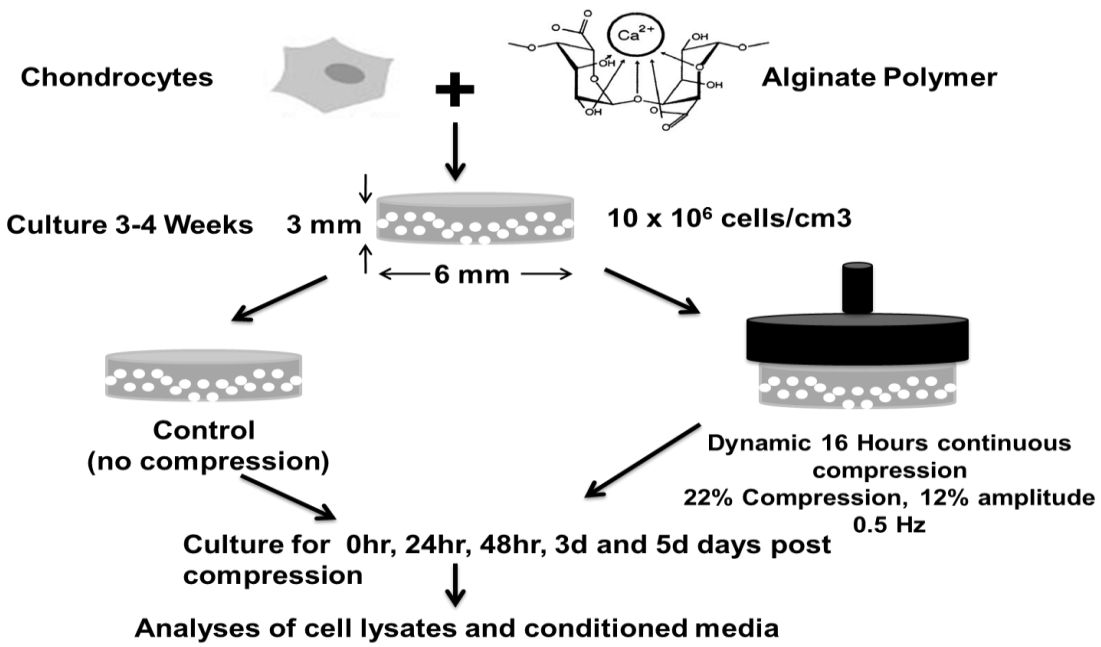


Figure 1. Experimental Outline. Chondrocytes were embedded in 3D scaffolds consisting of polymer alginate. Chondrocytes were allowed to be cultured for 3-4 weeks to allow for extracellular matrix (ECM) deposition within the 3D scaffold. Alginate molds were then either subjected to 22% dynamic strain for 16 hours or left in free-swelling conditions. Cells were further isolated from the ECM and alginate scaffold and harvest at pre-determined times post injury.

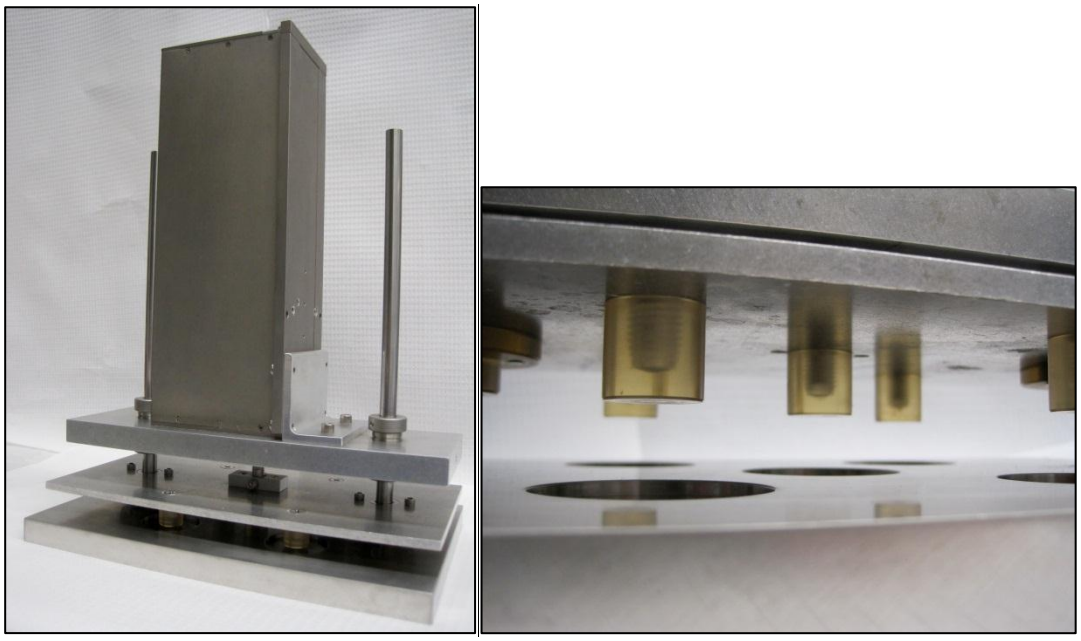


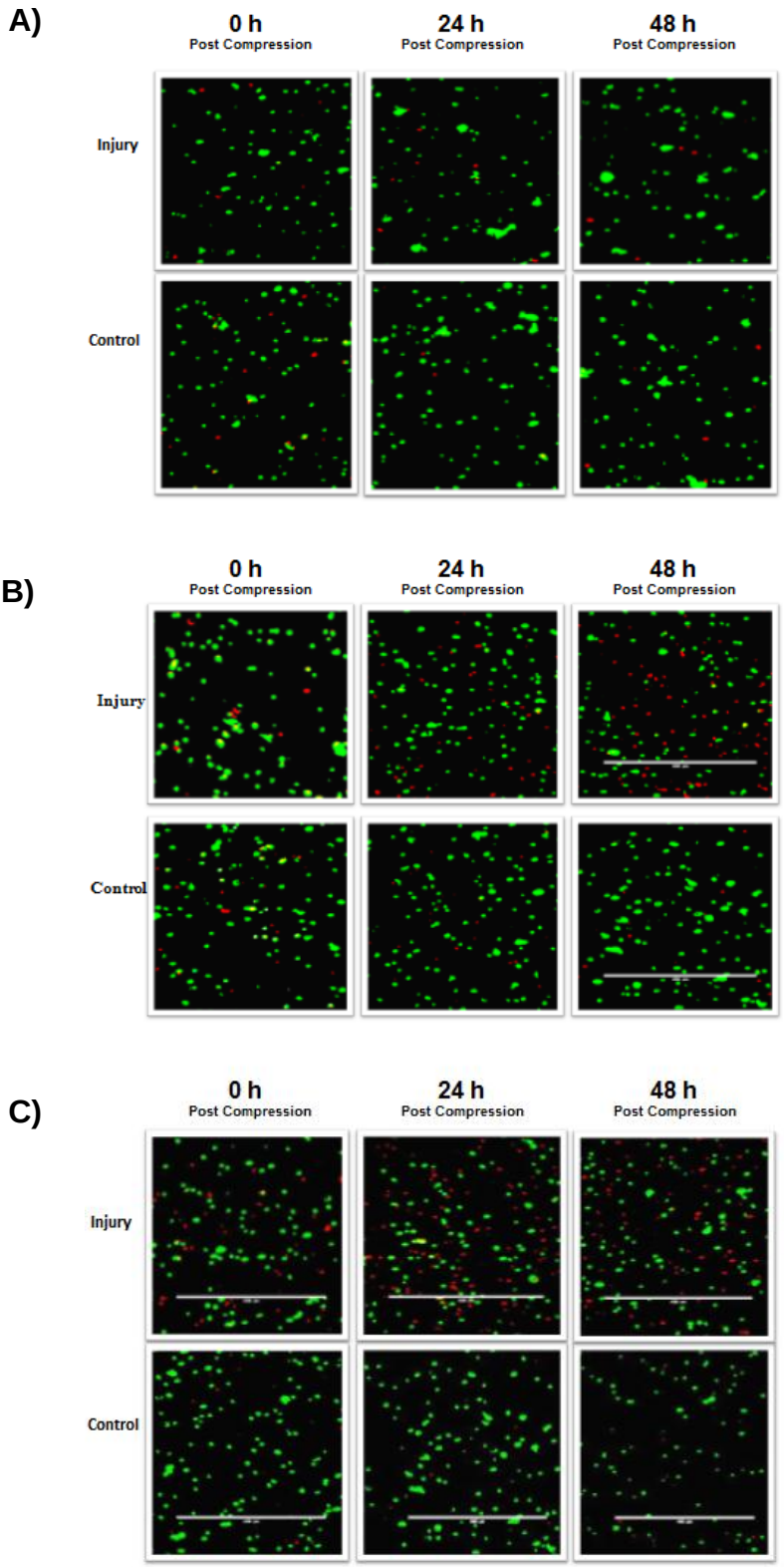
Figure 2. Custom Made Bioreactor Custom made bioreactor is housed inside a 37°C incubator maintained at 5% carbon dioxide. Samples are contained inside custom made semi-enclosed dishes capable of holding 3D molds along with 2mL of media. Machine is capable of 5 samples per run and modifiable variables include compression %, amplitude %, cycle frequency, and time.

Figure 3. Continuous Compression for 16 hours Induces Moderate and Delayed Cell Death (Below)

(A) Results from a calcein AM and ethidium homodimer live dead assay after 8 hours of continuous dynamic compression at 22%, 12% amplitude 0.5 Hz. Live cells are represented in green, apoptotic cells are represented in red.

(B) Live dead results after 16 hours of dynamic compression at 22%, 12% amplitude 0.5 Hz

(C) Live dead results after 24 hours of continuous dynamic compression 22%, 12% amplitude 0.5 Hz


A)
0 h
Post Compression
24 h
Post Compression
48 h
Post Compression
Injury
Control
B)
0 h
Post Compression
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C)
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Post Compression
Injury
Control

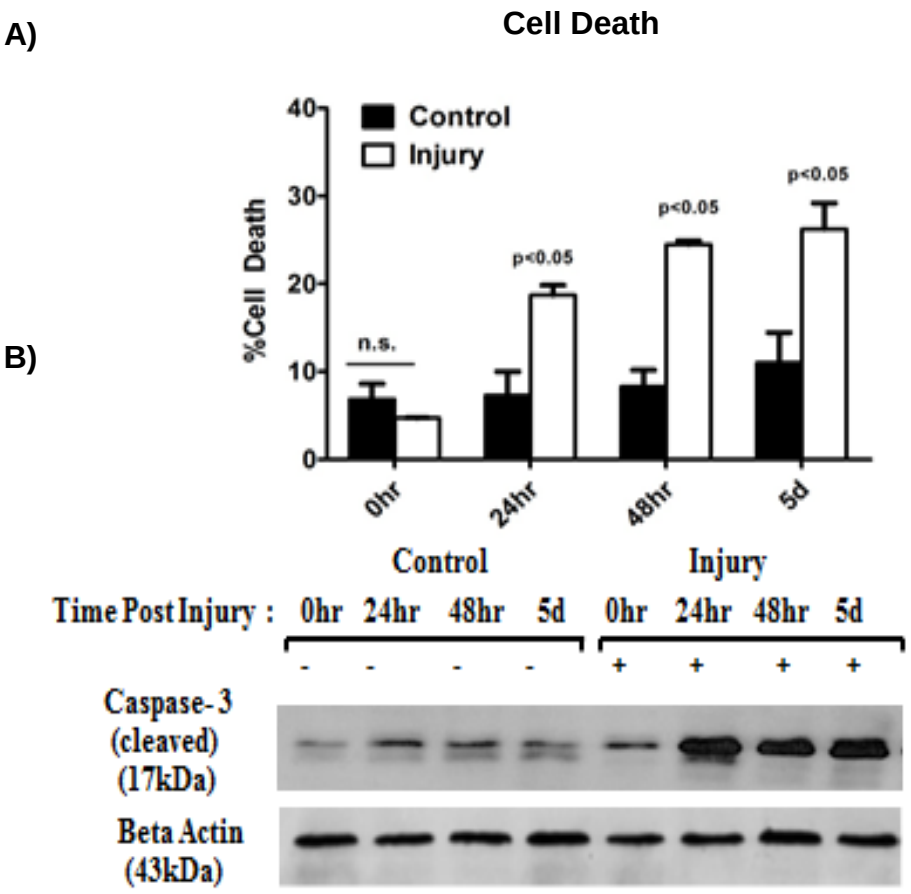

Figure 4. Quantification of Live Dead Assay and Expression of Apoptotic Markers.

(A) Quantification of the previously shown live dead assay from 16 hours of continuous injury. Solid black bars represent non-injured controls, white bars represent injured cells. Standard error bars are shown and represent results from 3 different experiments.

(B) Western blot showing expression of apoptotic marker caspase-3 after 16 hours continuous injury.

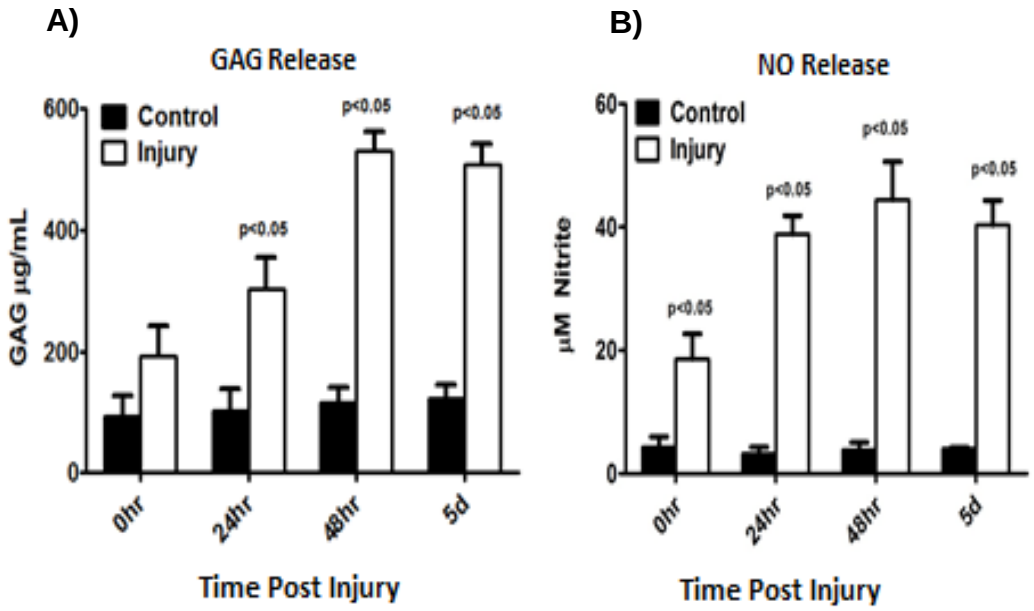

Figure 5. Injury Induced Increased Pro-Catabolic Responses

(A) Results from the colorimetric detection of glycosaminoglycans (GAGs) in the conditioned media after 16 hours of continuous injury. Non-injured controls are represented in the solid black bars, white bars represent injured samples. Standard error bars are shown and represent results from 3 different experiments.

(B) Results from the colorimetric detection of nitrite in the conditioned media after 16 hours of continuous injury. Non-injured controls are represented in the solid black bars, white bars represent injured samples. Standard error bars are shown and represent results from 3 different experiments.

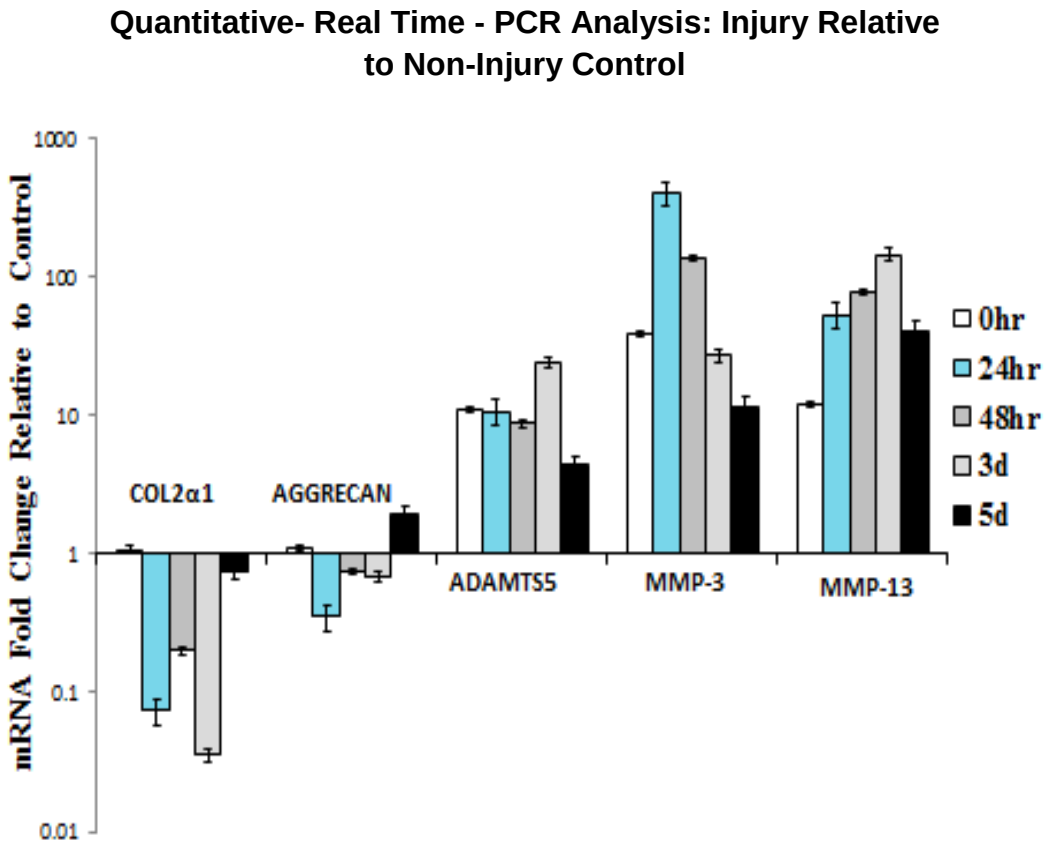

Figure 6. Quantitative Expression of Catabolic and Anabolic Markers Post Injury. Results show levels of anabolic and catabolic gene expression post injury. Results shown are mRNA fold change of the injured samples relative to the non-injured control. Analysis was done according to the well-established ΔΔCt method (Insert ref). An mRNA fold change of 1 represents no change relative to control. Error bars represent error between triplicates of one experiment.

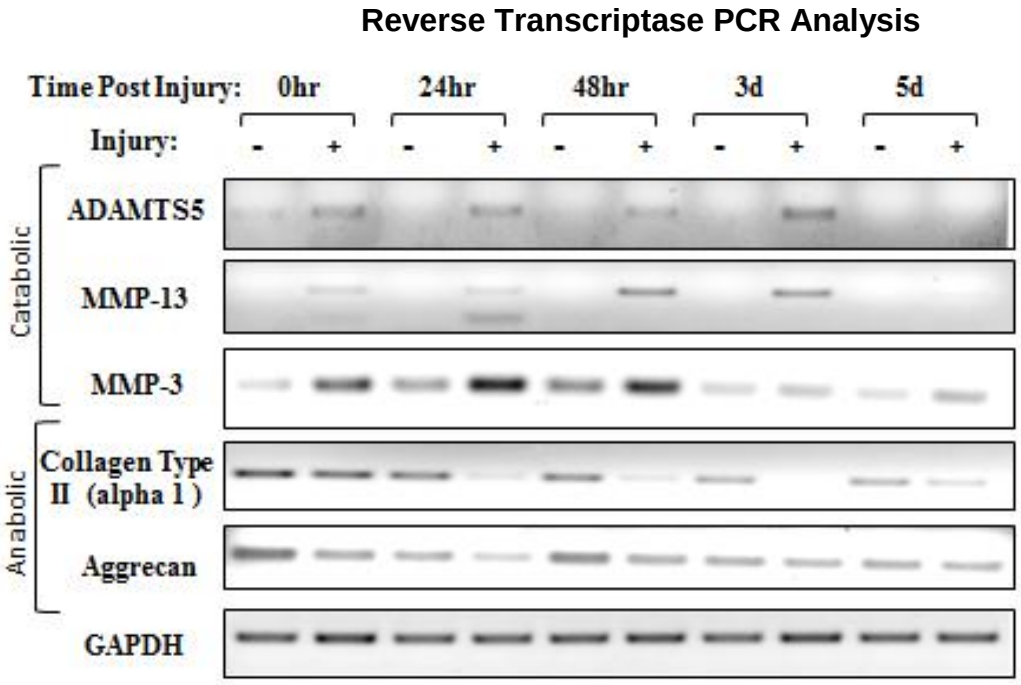

Figure 7. Expression of Catabolic and Anabolic Markers Post Injury Via Reverse Transcriptase Results show levels of anabolic and catabolic gene expression post injury relative to housekeeping gene GAPDH.

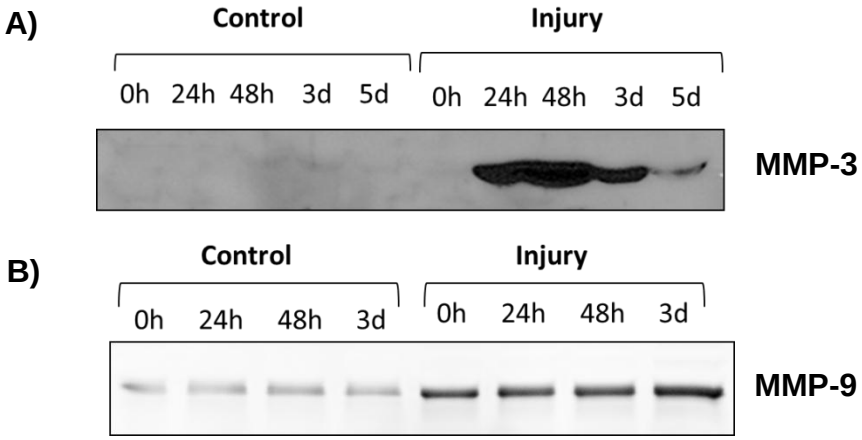

Figure 8. Matrix Metalloprotease Activity and Expression Levels

(A) Western blot expression of MMP-3 in the conditioned media post injury.

(B) Gelatin zymography of the conditioned media post injury.

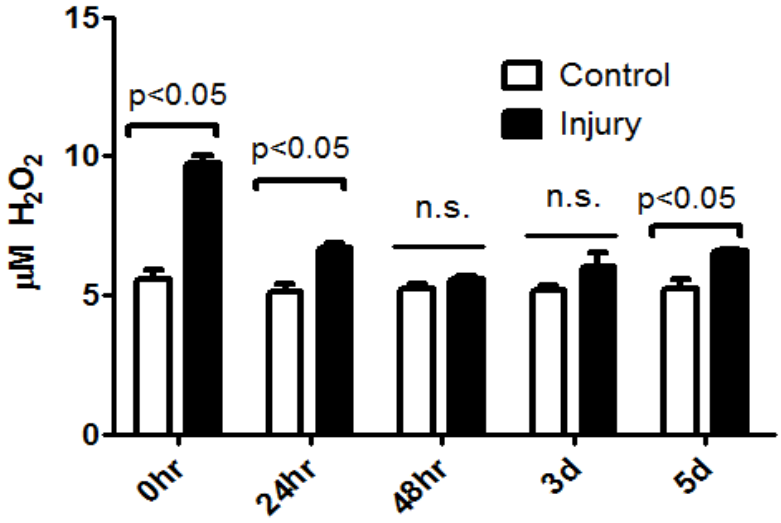

Figure 9. Injury Induced Early Increases in Hydrogen Peroxide Hydrogen peroxide release into the media after 16 hours of continuous injury. Standard error is mean of 2 different experiments.

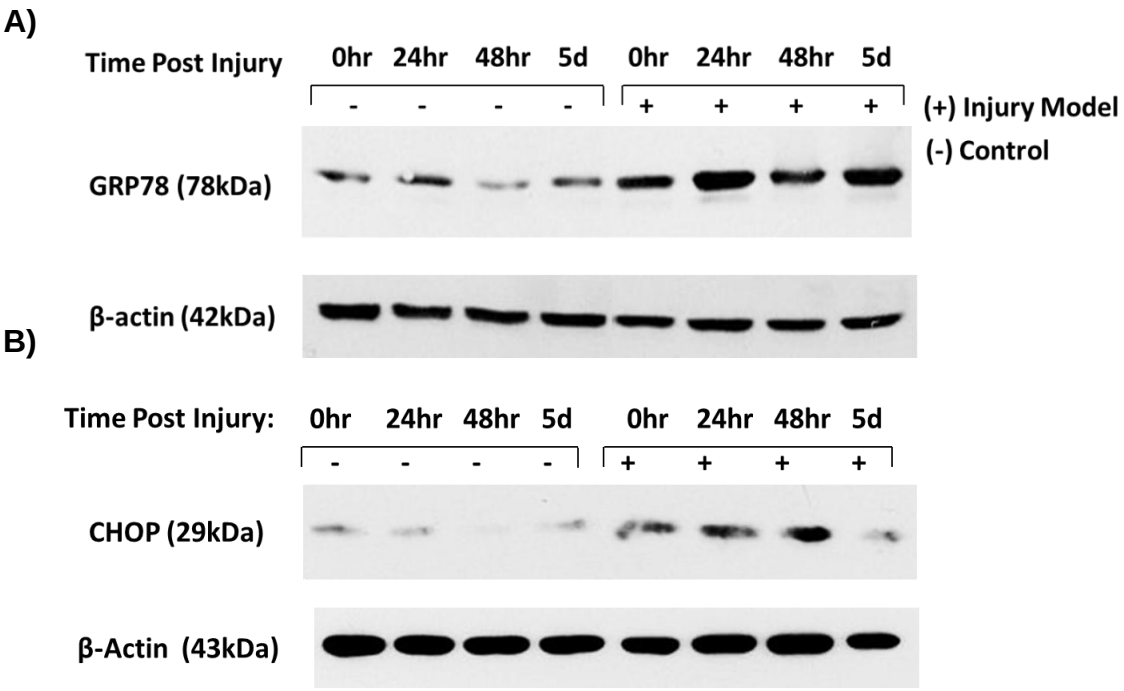

Figure 10. Expression of UPR Markers Post injury

(A) Western Blot Analysis of GRP78 expression post injury.

(B) Western Blot Analysis of CHOP expression post injury.

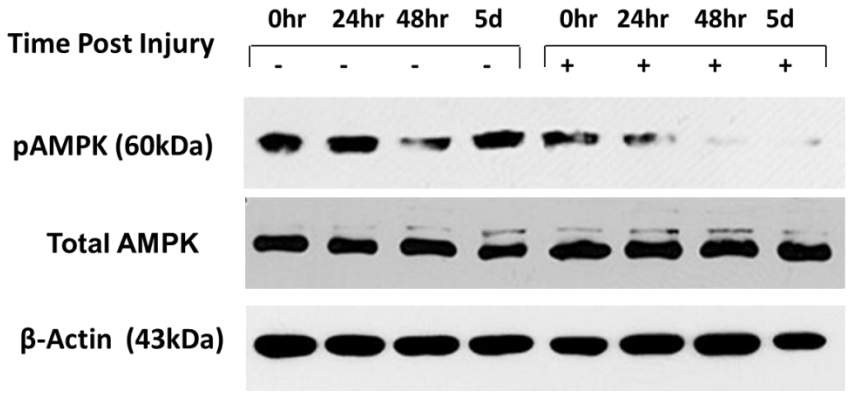

Figure 11. AMPK Phosphorylation Post Injury Western blot assaying the levels of AMPK phosphorylation and total AMPK levels post injury.

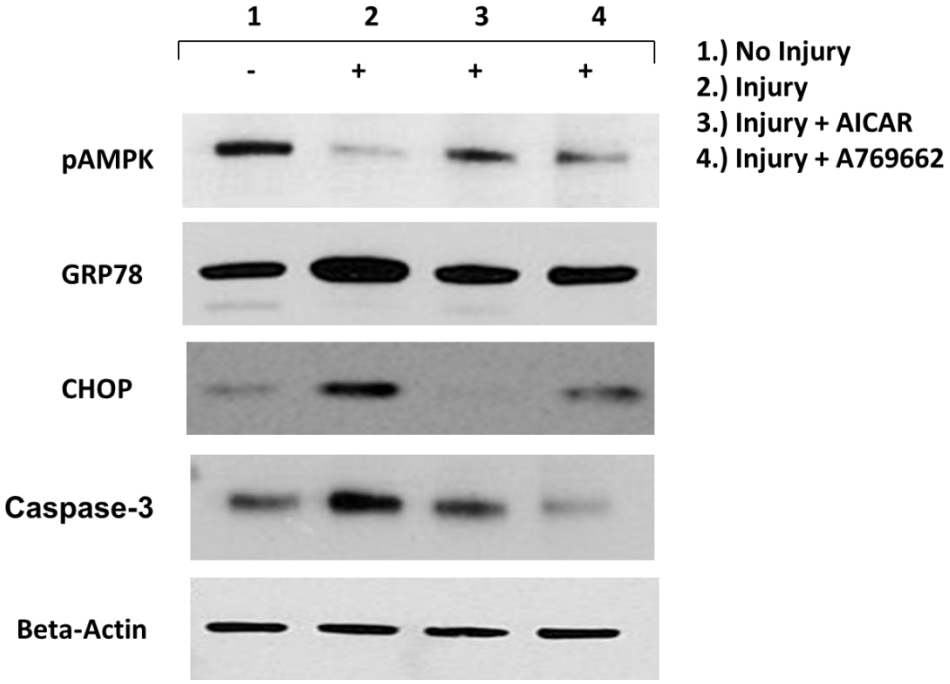

Figure 12. Pretreatment of with AMPK activators Results shown after 24 hours of pretreatment with AMPK activators AICAR and A769662. Samples were collected

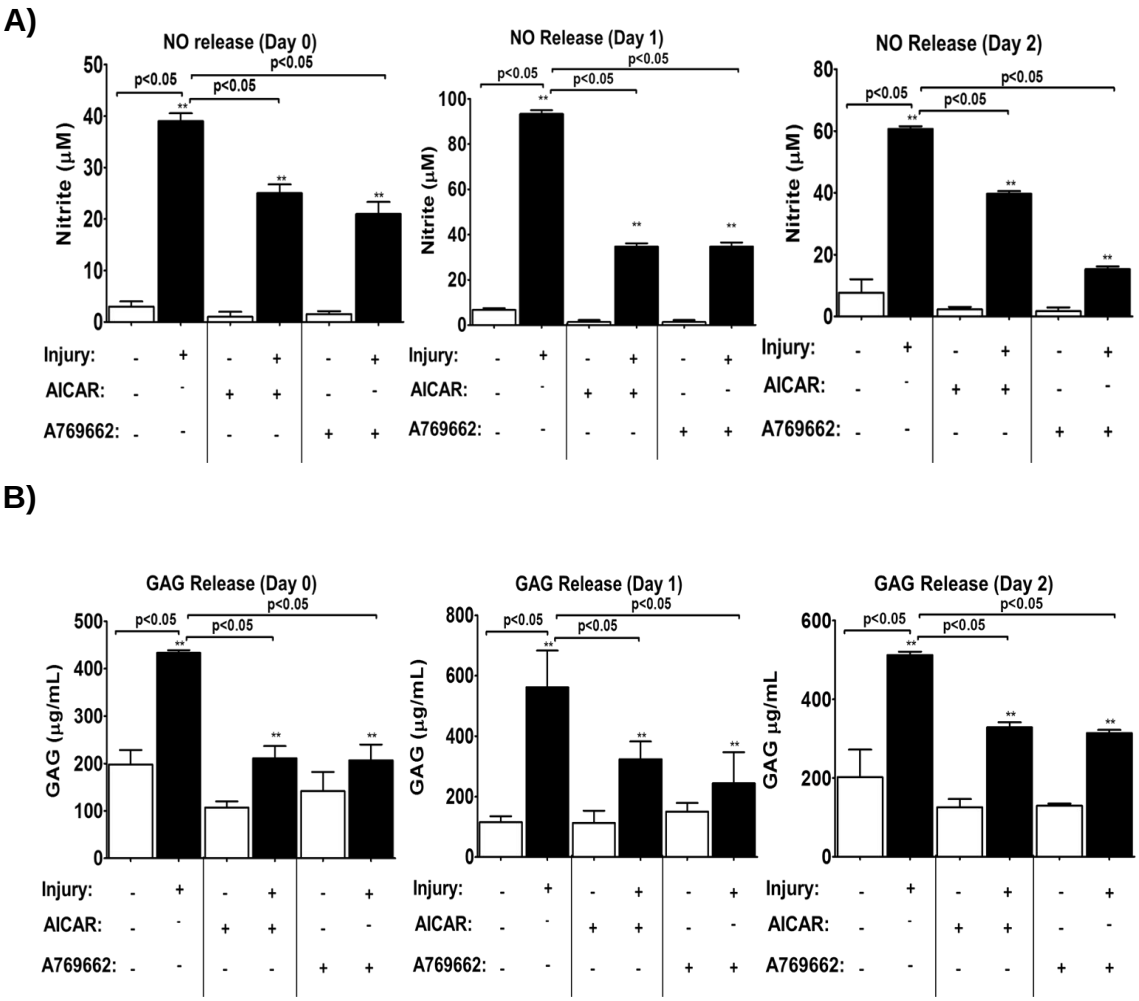

Figure 13. Pro-Catabolic Responses after Pretreatment with AMPK Activators

(A) Results from the colorimetric detection of nitrite in the conditioned media after 16 hours of continuous injury with or without 24 hour pre-treatment with AICAR or A769662. Non-injured controls are represented in the solid black bars, white bars represent injured samples. Standard error bars are shown and represent results from 3 different experiments.

(B)Results from the colorimetric detection of glycosaminoglycans (GAGs) in the conditioned media after 16 hours of continuous injury with or without 24 hour pre-treatment with AICAR or A769662. Non-injured controls are represented in the solid black bars, white bars represent injured samples. Standard error bars are shown and represent results from 3 different experiments.

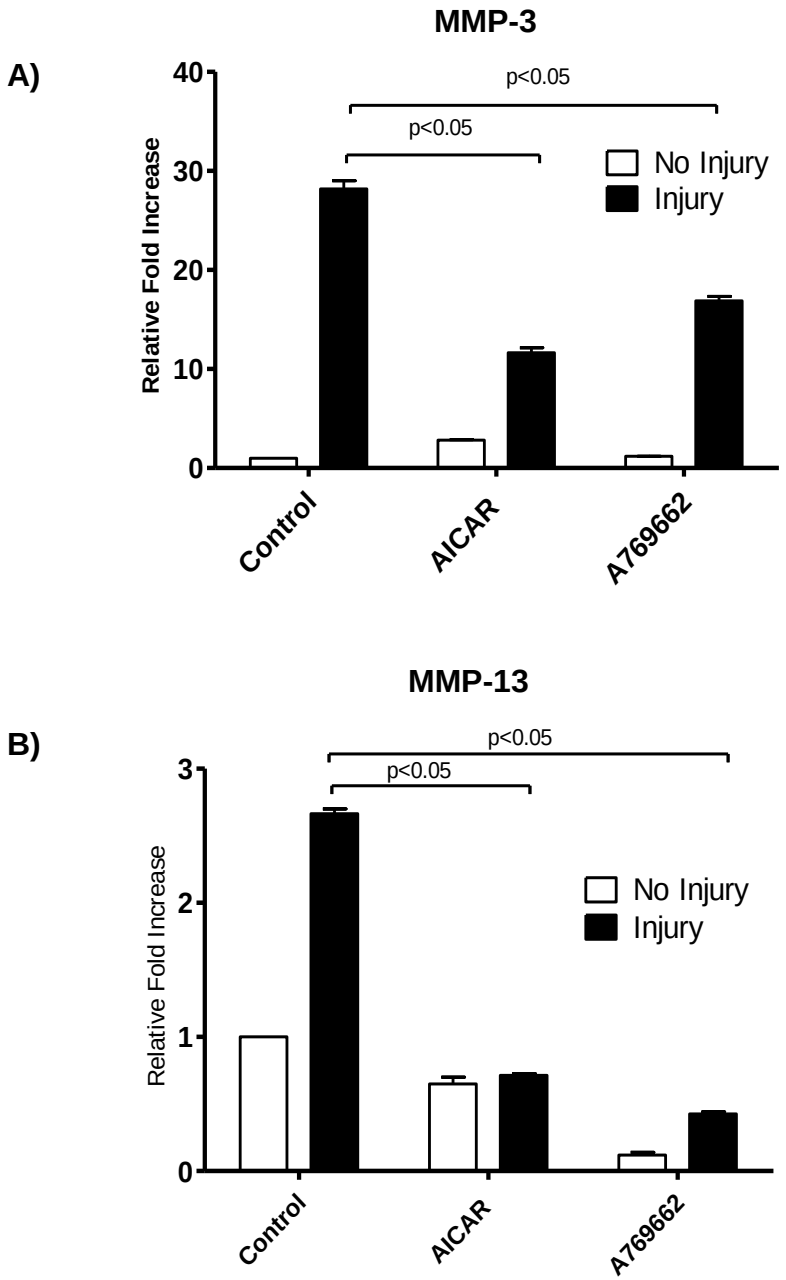

Figure 14. Quantitative Real Time PCR of MMPs Post injury Results shown represent quantitative real time PCR expression of MMPs from RNA that was harvested 24 hours after 16 hours of continuous dynamic compression with or without pretreatment with A769662 or AICAR.

(A) Relative fold change of MMP3 compared to non-injured controls

(B) Expression of MMP13 compared to non-injured controls

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