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# Chronic Acetaminophen Treatment Influences Indices of Reactive Oxygen Species Accumulation in the Aging Fisher 344 X Brown Norway rat Aorta

Sarath Meduru

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**Chronic Acetaminophen Treatment Influences Indices of Reactive Oxygen Species Accumulation in the Aging Fisher 344 X Brown Norway rat Aorta**

By
Sarath Meduru

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Committee:

_____ Dr. Eric R Blough, Thesis adviser

_____ Dr. David Mallory, Committee member

_____ Dr. Guo- Zhang Zhu, Committee member

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# Abstract

We have previously reported that aging in the Fisher 344 X Brown Norway (FBN) rat aorta is characterized by increased levels of ROS and alterations in cell signaling. Acetaminophen was found to scavenge free radicals in recent ischemia-reperfusion studies. Here we examined if chronic treatment with a therapeutic dose of acetaminophen attenuates age-associated increase in aortic ROS accumulation and signaling. FBN rats (27 month old; n=8) were subjected to 6 months of treatment with a therapeutic dose of acetaminophen (30mg/kg/day), with age-matched untreated FBN rats as controls. Protein oxidation levels were altered in control and treated aortae compared to aortae from 6 month animals. Immunoblotting analysis revealed that activated levels of c- Jun-N-Terminal kinase (JNK), Erk1/2 and AMPK levels were altered with aging and treatment. Activated p38-MAPK levels were altered with aging. Our data suggest that chronic acetaminophen treatment alters age associated ROS signaling in FBN rat aorta. [148 words]

**Key words:** aging; aorta; acetaminophen; ROS; cell signaling

# Acknowledgements

# LIST OF SYMBOLS

| | |
|---|---|
| AAALAC | Association for Assessment and Accreditation of Laboratory Animal Care |
| AMPK | AMP-Activated Protein Kinase |
| ANOVA | One-way Analysis of Variance |
| APAP | Acetaminophen |
| BSA | Bovine Serum Albumin |
| ECL | Enhanced chemiluminiscence |
| ERK | Extra cellular Signal Regulated Kinase |
| F344/N X BN | Fisher 344 Brown Norway hybrid |
| JNK | c- Jun-N-terminal Kinase |
| KRB | Krebs-Ringers Buffer Solution |
| MAPK | Mitogen Activated Protein Kinase |
| PBS | Phosphate Buffered Saline |
| PBST | Phosphate Buffered Saline with 0.5% Tween |
| PKB | Protein Kinase B |
| ROS | Reactive Oxygen Species |
| SAPK | Stress Activated Protein Kinase |
| SDS-PAGE | Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis |
| TBS | Tris Buffered Saline |
| TBST | Tris Buffered Saline with 0.5% Tween |
| VSMC | Vascular Smooth Muscle Cells |

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# Chapter 1

## <u>Introduction</u>

Life expectancy has nearly doubled since the beginning of the 20th century with the fastest growing segment of the United States population being those aged 65 years and older. Indeed, those > 65 years constitute 14% of the population and will account for > 20% in the next half century [1]. Heart disease, comprising ischemic heart disease and congestive heart failure, is not only the leading cause of death and lost life expectancy in most western countries but also one of the most important causes of morbidity and health care costs. Over two- thirds of current health care costs are for treating chronic illnesses; among older Americans, almost 95% of health care expenditure is for chronic diseases [2]. Alzheimer's disease alone, for example, costs the nation more than $50 billion each year in Medicare and Medicaid expenditures. As the population ages and this devastating disease becomes more prevalent, these costs are projected to rise by as much as 54% by 2010 [2]. The development of interventions designed to mitigate the problems and costs associated with heart disease is required to prevent the burden of health care costs from increasing exponentially in the coming years [3].

The blood vessel wall is divided into three regions, the adventitia, media and intimal layers. The intrinsic aging change in thickness on the arterial wall is predominantly in the media. Atherosclerosis, in contrast, is an intimal disease. Throughout life, elastin fibers undergo progressive disorientation, fragmentation, and degeneration, with subsequent collagen deposition, calcification, and/or cystic degeneration [4]. As a result, the central elastic arteries dilate and become tortuous. Age-related increases in arterial stiffness, which results in a doubling of pulse wave velocity in the aorta, a quadrupling of ascending aorta impedance, and a progressive rise in systolic pressure [4] greatly increasing the risk of heart disease.

Aging, defined as a gradual decline of the fighting ability of the organism against stress, damage and diseases, is intuitively considered invincible; however recent studies are attempting to shed new light on some of the possible mechanisms of aging [5-7]. One of the most accepted theories on aging is the oxidative stress theory which postulates that the overproduction of free radicals damage lipids, proteins, and DNA [6, 7]. Since Harman (1956) proposed the free radical theory of aging a half century ago, there has been growing evidence that suggests reactive oxygen species may play a crucial role in aging [8, 9]. This conclusion has been supported by many studies, but two most important questions still remain unanswered: (1) How aging processes progresses in completely healthy humans who do not suffer from any pathological disorders? (2) And if we agree that free radicals play a major role in aging, then how free radicals initiate aging processes in healthy organisms under physiological conditions is not much known.

Recent data indicate that increased oxidative stress caused by the enhanced production of reactive oxygen species (ROS) is an important contributor to vascular dysfunction in heart disease and that ROS levels are an independent predictor of future cardiovascular events [10]. Why ROS increases the risk of heart disease is not well understood. A critical component of eukaryotic signal transduction is the activation of protein kinases which phosphorylate a host of cellular substrates, including transcription factors which control the induction of various genes [11]. Mitogen activated protein kinases (MAPK) are an evolutionarily conserved family of enzymes that form a highly integrated network required to achieve specialized cell functions including cell differentiation, cell proliferation, and cell death [12]. In addition to the MAPK proteins, another ROS sensitive molecule is the AMP-activated protein kinase (AMPK). The AMPK is viewed as an energy sensor that acts to modulate glucose uptake and fatty acid

oxidation in skeletal muscle. Recent study has shown that a strong association exists between the amount of ROS and aging in the F344/NXBN rat aorta and that this increased ROS was correlated with the altered regulation of several proteins thought to be associated with cellular signaling cascades [13]. Whether these differences in protein signaling play a role in regulating age associated cardiovascular dysfunction is not clear.

Acetaminophen is a widely used drug that is a standard antipyretic and analgesic agent for the treatment of mild to moderate pain. Acetaminophen is a phenol, that may have antioxidant properties [14, 15]. For example Recent ischemia - reperfusion studies suggested that acetaminophen has antioxidant properties that are cardio protective and antiarrhythmic under conditions of increased free radical production [16]. Whether acetaminophen exhibits similar functions, when given chronically and in the aging animal has not been investigated.

**Purpose of study**

        The purpose of this study is to determine how aging and acetaminophen treatment alters multiple, previously identified markers of oxidative stress in the F344NXBN rat aorta. The working hypothesis of this study is that chronic treatment with therapeutic dose of acetaminophen will decrease the age-associated increases in oxidative stress and alter the expression and/or regulation of age-associated indices of oxidative stress.

**Specific Aims and Hypotheses**

*Specific Aim 1*

To determine if aging in the F344/NXBN aorta is associated with increased ROS.

*Hypothesis*

We hypothesize that aging will be associated with increased indices of aortic ROS.


*Specific Aim 2*

To determine how aging affects multiple, previously identified markers of oxidative stress in the F344/NXBN rat aorta.

*Hypothesis*

We hypothesize that age-associated increases in ROS will be strongly associated with indices of oxidative stress in the aging F344/NXBN aorta.


*Specific Aim 3*

To determine how chronic acetaminophen treatment alters the regulation of previously identified markers of oxidative stress in the F344/NXBN rat aorta.

*Hypothesis*

We hypothesize that acetaminophen treatment will alter the regulation of previously identified markers of oxidative stress in the aging F344/NXBN rat aorta.

# Chapter 2

## Review of Literature

## Introduction

A review of the pertinent literature concerning the present study will be presented in the following chapter. The following areas will be addressed:  1) Effects of aging on aortic structure and function, 2) Aging and oxidative stress and 3) Indices of Reactive Oxygen levels and 4) Acetaminophen.

## Effects of aging on aortic structure and function

It is estimated that by the year 2035, nearly one in four individuals will be 65 years of age or older [17]. In the United States, cardiovascular disease e.g., atherosclerosis and hypertension, is the leading cause of mortality, accounting for over 40 percent of deaths in those aged 65 years and above [17]. One symptom of aging is the gradual impairment of vascular function in arteries. There is a substantial set of people experiencing the enlargement of aorta over the age of 60 years [18]. Arterial distensibility decreases with aging and hypertension in both humans and rats [19]. Mechanical and structural properties of arteries have been studied extensively with respect to the development and treatment of hypertension. Aging is associated with an increase in wall thickness of medium and large-sized arteries [20]. This increase in wall thickness is due to smooth muscle hypertrophy, which results in a thickening of the intima and medial layer [21].

Age-related increases in arterial wall thickness are observed in the absence of atherosclerosis and hypertension in Fisher 344 X Brown Norway (F344XBN) rats [21], but the exact mechanisms of arterial wall thickening have not been determined. The aortic wall thickness also increases with age in the male rats [13, 22]. Gerontologists have developed a considerable amount of quantitative data on the structural and functional changes in the vasculature of apparently healthy human populations. Principal among their findings are age-associated increases in vessel wall thickness [23] mainly due to intima medial thickening [24], with luminal dilation and reduced compliance [25]. Few studies have directly addressed age-associated changes in vessel contractility in humans, although there is evidence of endothelial dysfunction with aging [26] with attenuation of endothelium-dependent dilation due to altered nitric oxide (NO) synthase activity and increased formation of reactive oxygen species [27].


## Aging and oxidative stress

Reactive oxygen species (ROS), including superoxide ($\cdot O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl anion ($OH^-$), and reactive nitrogen species, such as nitric oxide (NO) and peroxynitrite ($ONOO^-$), are biologically important $O_2$ derivatives that are increasingly recognized to be important in vascular biology through their oxidation/reduction (redox) potential [28]. ROS are known to come from two major categories including endogenous and exogenous sources. The exogenous factors include $\gamma$- and UV-irradiation, ultrasound, food, drugs, pollutants, xenobiotics and toxins while endogenous factors are comprised of neutrophils, enzymes, and mitochondria [29]. A natural consequence of aerobic metabolism is the production of reactive oxygen species (ROS), including free radicals, which are capable of oxidizing vital cellular components. There are three major types of ROS: superoxide anions, hydrogen peroxide

and lipid peroxides. The amount of ROS in cells and tissues are normally held in check by a system of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), respectively). Reactive oxygen species from mitochondria and other cellular sources were traditionally considered as injurious cellular by-products with the potential to damage lipids, proteins and DNA [29]. However, there is now convincing evidence that ROS are not only toxic consequences of cellular metabolism but also essential participants in cell signaling and regulation [30-33]. Oxidative stress in tissues has been a topic of intense investigation both in the basic science laboratory and in clinical research in recent years [34, 35]. Taking basic discoveries in oxidative stress to a clinical application, i.e., translational research, is of significant contemporary interest [36, 37].

Reactive oxygen species are implicated in many intracellular signaling pathways leading to changes in gene transcription and protein synthesis and consequently in cell function. Under physiological conditions, ROS are produced in a controlled manner at low concentrations and function as signaling molecules regulating vascular smooth muscle cell (VSMC) contraction–relaxation and VSMC growth [38-41]. However, under pathological conditions increased ROS production has been associated with endothelial dysfunction, alterations in vessel contractility, VSMC growth and apoptosis, monocyte migration, lipid peroxidation, inflammation, and increased deposition of extracellular matrix proteins [38]. These findings are significant as each of these processes is thought to be risk factors for the development of cardiovascular disease. In addition, systemic and vascular ROS production lead to reduced endothelial NO bioactivity, increased expression of cell surface adhesion molecules, and inflammatory changes that contribute to microvascular and macrovascular damage [42-46]. ROS mediate the proliferation of VSMCs, which can lead to thickening of the aorta [38, 47-49].

Oxidative stress occurs when the antioxidant system is unable to cope with the production of ROS, resulting in cell damage that may underlie numerous pathological conditions. Oxidative stress is believed to play an important role in the aging process [9, 50]. Age-related damage from oxidative stress could be elicited through increased ROS, decreased antioxidant enzyme activity, or a combination of both. Free radical oxidation of proteins also increases with age [51]. Several investigations have shown an increase in production of ROS in various tissues of older animals [52, 53]. In contrast, age-associated reduction of antioxidant enzymes has been demonstrated in many tissues [48, 54, 55]. Although there are many approaches to evaluate the existence of ROS in biochemical reactions, the only direct ways to measure ROS are electron spin resonance that measures relatively stable radicals, and the spin trapping method [56]. However, due to the difficulty of direct ROS detection, the quantification of oxidative damage markers known as the fingerprint method is often utilized e.g. the introduction of carbonyl groups to proteins [51, 57]. Additionally, excessive superoxide and nitric oxide can produce highly reactive peroxynitrite and can disturb homeostatic regulation by interacting with macromolecules [56].

Recent data suggest that active oxygen species may play a role in the progression of atherosclerosis and development of restenosis following balloon angioplasty. Epidemiological studies show a correlation between antioxidant therapy and a decreased incidence of coronary events in both men and women [58, 59]. The beneficial effects of antioxidants may be related to altered vessel redox, as demonstrated by findings that aortas from hyperlipidemic rabbits[60] and coronary arteries from balloon-injured pigs[61] generate increased levels of $O_2^-$ compared with control vessels. The effects of aging-associated increases in ROS may affect VSMC independently by causing lipid peroxidation and DNA damage. These modifications may

ultimately contribute to VSMC dysfunction and disease progression [62]. Whether antioxidant

therapy is able to decrease age-associate increases in ROS levels is not well understood.

## Indices of Reactive Oxygen levels

Cells respond to extracellular stimuli by activating signal transduction pathways, which culminate in changes in gene expression. The particular genetic program activated determines, in large part, the response of the cell (e.g., growth versus growth arrest versus apoptosis; differentiation versus dedifferentiation). A critical component of eukaryotic signal transduction is the activation of protein kinases which phosphorylate a host of cellular substrates, including transcription factors which control the induction of various genes [11]. Mitogen activated protein kinases (MAPK) are an evolutionarily conserved family of enzymes that form a highly integrated network required to achieve specialized cell functions including cell differentiation, cell proliferation, and cell death [12]. On activation of MAPK, transcription factors present in the cytoplasm or nucleus are phosphorylated and activated which lead to the expression of target genes resulting in a biological response. The multiple interactions between the different MAPK cascades serve to integrate responses and to moderate outputs. Indeed, it has been demonstrated that MAPK have overlapping substrate specificities and that the phosphorylation of regulatory sites is shared among multiple protein kinases [63-65]. All eukaryotic cells possess multiple MAPK pathways, each of which is preferentially recruited by distinct sets of stimuli, thereby allowing the cell to respond coordinately to multiple divergent inputs [12].

MAPKs can be activated by a wide variety of different stimuli, but in general, ERK1 and ERK2 are preferentially activated in response to growth factors, phorbol esters, exogenous $H_2O_2$ and by endogenously generated ROS in SMCs [49]. c-Jun N-terminal kinases (JNKs) and p38 MAPK, are also sensitive to redox modulation [66] however these molecules are more responsive to stress stimuli such as osmotic shock and ionizing radiation to cytokine stimulation

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[67]. Activation of ERKs has also been implicated in vascular endothelial growth factor (VEGF)–induced EC survival [68]. In contrast to ERKs, JNKs and their downstream target c-Jun, have been implicated in $H_2O_2$ and other stress-induced apoptosis of ECs [69]. Moreover, p38 MAPK has been implicated in EC upregulation of intercellular adhesion molecule-1 and, therefore, endothelial dysfunction. In SMCs, redox-sensitive activation of p38 MAPK mediates Angiotensin II–induced hypertrophy and has also been implicated in SMC migration [70-72]. It is known that ROS-mediated MAPK activation is involved in smooth muscle cell hypertrophy and that the inhibition of MAPK by antioxidants may affect signal transduction [73].

Because both $O_2^-$ and $H_2O_2$ cause VSMC growth yet only $O_2^-$ activates MAPK, it is intriguing to speculate regarding the site(s) of action of these reactive oxygen species. More research is needed to further address this question. In addition to the MAPK proteins, another ROS sensitive molecule is AMP-activated protein kinase (AMPK). The AMPK is viewed as an energy sensor that acts to modulate glucose uptake and fatty acid oxidation in skeletal muscle. Given that protein synthesis is a high energy-consuming process, it may be transiently depressed during cellular energy stress. AMPK responds to changes in the ratio of ATP/AMP as well as phosphocreatine/ creatine [74, 75]. Changes in the cellular energy state activate AMPK through various mechanisms involving allosteric regulation of AMPK, activation by an upstream AMPK kinase, and diminished activity of phosphatases [76]. AMPK activation increases glucose uptake and fatty acid oxidation in muscle [77] as well as up-regulates expression of various metabolic genes. There has been no agreement as to the direction that AMPK regulates cell proliferating pathways. Some investigators showed that AMPK upregulates p38 MAPK activity in a rat liver cell line (clone 9 cells) [78], whereas others showed that it downregulates Akt/PKB in skeletal muscle cells [79]. Conversely, Fryer et al., [80] reported that the p38 MAPK pathway is not

affected by AMPK in skeletal muscle cells, and We [81] and Ido et al., [82] have shown that Akt signaling is upregulated by AMPK in endothelial cells. The reasons for discrepancies between studies are not clear but could be due to differences in the methods or materials used for investigation.

AMPK activation has been shown to inhibit skeletal muscle protein synthesis [79], likely because protein synthesis is a bioenergetically expensive process. Suppression of protein synthesis by AMPK suggests that this signaling protein may also regulate skeletal muscle hypertrophy. There is some indication of such a role for AMPK in cardiac muscle, where mutations in the gene encoding the gamma subunit of AMPK are associated with pathological hypertrophy [83], and AMPK activation has been shown to inhibit cultured cardiac myocyte growth [84]. AMPK expression differs in vascular smooth muscle compared with striated muscles and that activation and inactivation after metabolic stress occur rapidly and are associated with signaling pathways that may regulate smooth-muscle contraction [85]. Igata et al., (2005) reported that activation of AMPK effectively suppressed cell cycle progression in primary human VSMCs and isolated rabbit aortas [86]. Several investigators have shown that AMP-activated protein kinase (AMPK) is also a redox-sensitive enzyme [87-89]. Kukidome et al., (2006) reported that AMPK activation decreases hyperglycemia-induced mitochondrial ROS production [90]. A recent study from our lab suggests that vascular aging in the F344/NXBN rat is associated with an increase in superoxide which is highly correlated with changes in the expression and/or regulation of AMPK-α [13]. Therefore it is of interest to examine the redox-sensitive AMPK activation as a good indicator of ROS levels.

Programmed cell death is a genetically controlled response of individual cells that are no longer needed to commit suicide. High levels of ROS can lead to necrotic cell death, whereas

low levels of ROS have been shown to induce apoptotic cell death [91, 92]. Apoptosis generally involves changes in the expression of pro-apoptotic and anti-apoptotic genes and gene products including both the tumor suppressor gene p53 as well as the proapoptotic protein of the Bcl-2 family Bcl-2 associated protein X (Bax) [93-95]. Programmed cell death is regulated through different mechanisms including the expression of the members of the Bcl2 protein family consisting of pro- and antiapoptotic peptides interacting with each other by forming homo- and heterodimers. Bcl-2 is thought to act as negative regulator of apoptosis and has been thought to protect cells from ROS, although the mechanism by which this latter event occurs remains unclear. Conversely, Bax has been implicated to promote or accelerate cell death with recent data showing that Bax may be able to induce apoptosis by both caspase-dependent and -independent mechanisms [96]. It has been proposed that cell viability may depend on the ratio of the level of Bcl-2 to that of Bax [97]. Previous data from our lab suggests that Bcl-2 and Bax are upregulated with aging to a similar degree. Given the role that Bcl-2 may play in protecting the cell against elevated ROS [98], it is conceivable that age-related increases in Bcl-2 may be a mechanism employed with aging to protect the cell against elevated $O_2^-$. As the ratio of Bcl-2 to Bax is thought to regulate cell viability, our findings that the regulation of Bax parallel to that of Bcl-2 suggest that this strategy may act as a means to ensure constancy of the Bcl-2 to Bax ratio. Such a mechanism, if present, may act to minimize unnecessary cell death.

Taken together, these data suggest that ROS generated within VSMCs and ECs can either induce cell growth or death, thereby leading to vascular dysfunction. The available literature suggest that smooth muscle cells proliferation and hypertrophy are due, at least in part, to the increased ROS [71]. Whether or how alterations in ROS may be associated with age-associated vascular dysfunction is not well understood.

**Acetaminophen**

Acetaminophen is one of the most commonly used over-the-counter analgesics and antipyretics. Acetaminophen was introduced into Western medicine more than 100 years ago, and its pain-relieving and temperature-lowering actions have been under investigation for several decades [99, 100]. For more than 50 years in the United States, acetaminophen, when used appropriately at therapeutic doses, has been shown to be extremely safe in virtually all patient populations. Despite this remarkable time-tested safety profile, in recent years, some healthcare providers have questioned the safety of acetaminophen use by alcoholic patients and patients who drink alcohol [99]. These initial, presumably well-intentioned yet scientifically unproven concerns spread rapidly in both the medical literature and the lay press. This unsubstantiated concern that therapeutic doses of acetaminophen, less than or equal to 4 g/day, may cause life-threatening hepatotoxicity and fulminant hepatic failure led to an unfounded belief that alcoholic patients and patients who drink alcohol should avoid acetaminophen. When taken in overdose, particularly large intentional overdose, acetaminophen can cause fulminant hepatic failure and death. In recent years, retrospective case reports and case series have suggested that alcoholic patients may be at risk of acetaminophen-induced hepatic injury and even death following the ingestion of recommended therapeutic doses of acetaminophen [101].

Potential cardiovascular properties of acetaminophen have gone undiscovered, in part, because no one has made an effort to do the experiments. This might have been influenced by standard textbooks of pharmacology that report that acetaminophen lacks efficacy in the cardiovascular system of mammals [102]. Increased use of acetaminophen by an aging population justifies a more indepth exploration into the unknown mechanisms of action of this

compound. The recent literature, however, suggests that investigators are beginning to fill this void. For example, Nakamoto et al. [103] reported beneficial effects of acetaminophen against gastric mucosal injury caused by ischemia-reperfusion in the rat. Farquhar et al. [104] reported reduced renal dysfunction in the stressed human kidney in the presence of acetaminophen versus ibuprofen, while Colletti et al., (1999) found that ibuprofen caused significantly greater renal arterial vasoconstriction than acetaminophen in sodium-depleted dogs [104, 105]. Merril (2002) and others [100, 106, 107] reported that acetaminophen has antioxidant and cardiovascular protective properties. For example, it has been reported that acetaminophen has cardioprotective properties in the isolated guinea pig heart exposed to hypoxia and reoxygenation [107]. In addition, Merril et al., [16, 108] reported that acetaminophen has antiarrhythmic properties in isolated hearts of guinea pigs and dogs. During regional myocardial ischemia and reperfusion in dogs, acetaminophen markedly and significantly reduced tissue necrosis and infarct size [109]. Merrill [100] have reported that the content of protein carbonyls in the injured myocardium treated with acetaminophen was significantly less during ischemia than was seen in vehicle-treated hearts and concluded that the isolated, perfused guinea pig heart exposed to low-flow global myocardial ischemia and reperfusion, acetaminophen is cardioprotective. Golfetti et al., [110] have reported that acetaminophen-treated hearts were more functional at the end of reperfusion than hearts treated either with uric acid or vehicle. In the other study with chronic administration of acetaminophen for 10 days Golfetti et al., (2003) [106] found that the release of creatine kinase is reduced, the production of peroxynitrite/ its precursors is attenuated, and that reperfusion-induced mechanical failure is less evident or absent in the presence of acetaminophen. It should be noted that all of these studies were acute in nature. Whether chronic acetaminophen treatment exhibits similar cardiovascular effects is unknown. Further, whether

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acetaminophen is capable of attenuating age-associated increases in ROS has not been investigated.

# Chapter 3

**Chronic Acetaminophen Treatment Influences Indices of Reactive Oxygen Species**

**Accumulation in the Aging Fisher 344 X Brown Norway rat Aorta**

**Abstract:**

Age associated cardiovascular disease is thought to be caused in part by the gradual oxidative damage to biomolecules. We have previously reported that aging in the Fisher 344 X Brown Norway (FBN) rat aorta is characterized by increased levels of ROS and alterations in cell signaling. Acetaminophen was found to scavenge free radicals in recent ischemia-reperfusion studies. It remains unknown if chronic acetaminophen administration influences ROS accumulation and signaling in the aging aorta. Here we examine if chronic treatment with a therapeutic dose of acetaminophen attenuates age-associated increase in aortic ROS accumulation and signaling. FBN rats (27 month old; n=8) were subjected to 6 months of treatment with a therapeutic dose of acetaminophen (30 mg/kg/day), with age-matched untreated FBN rats as controls (n=8). Immunoblotting and immunohistochemical analysis were used to examine protein oxidation, protein nitration and various indices of oxidative stress in the aorta. Protein oxidation levels were $30.25 \pm 1.33\%$ lower in treated aortae as compared to controls. Immunoblotting analysis revealed that activated levels of cJun-N-Terminal kinase (JNK) were $74.73 \pm 3.15\%$ and $87.39 \pm 3.14\%$ respectively in control and treated aortae. Phospho-Erk1/2 levels were $78.7 \pm 0.49\%$, $99.45 \pm 1.85\%$, activated p38-MAPK levels were $65.03 \pm 1.65\%$, $67.65 \pm 1.96\%$ and activated AMPK levels were $88.62 \pm 2.26\%$, $106.86 \pm 3.89\%$ respectively in aortae from control and treated animals. Our data suggest that chronic acetaminophen treatment alters age associated ROS signaling in FBN rat aorta. (237 words)

**Key words:** aging; aorta; acetaminophen; ROS; cell signaling

**Introduction:**

It is estimated that by year 2035, nearly one in four individuals will be 65 years of age or older [17]. Cardiovascular diseases, such as coronary artery disease, Atherosclerosis and hypertension, and resultant chronic heart failure [17] reach epidemic proportions among older persons. An emerging hypothesis described as the free radical theory of aging suggests that aging occurs through the gradual accumulation of free radical damage to biomolecules [111]. Inherent to this theory is that oxidative stress increases with advancing age. It is thought that oxidative stress both promotes and is induced by vascular disease and risk factors that lead to vascular disease. Earlier reports from our laboratory have reported that aging in the F344/N X BN aorta is characterized by increased levels of ROS and alterations in metabolic stress indicators (AMPK), apoptotic regulators (Bax and Bcl-2) and MAPK signal transduction pathways [13].

Acetaminophen (APAP) is one of the most widely used of all drugs, with a wealth of experience clearly establishing it as the standard antipyretic and analgesic for mild to moderate pain states. APAP is a phenol, and many phenols have antioxidant properties [14, 15]. Recent ischemia - reperfusion studies suggested that APAP has antioxidant properties that are cardio protective and might be antiarrhythmic under selected conditions of the generation and release of free radicals [16]. It seems reasonable, therefore, to speculate that in the presence of increased oxidative stress, APAP might offer preservation of tissue structure and function.

The function and activity of specific mitogen activated protein kinases (MAPKs), such as p38 kinase, extracellular signal regulated kinase 1/2 (ERK 1/2-p44/p42), as well as the c-Jun N-terminal kinase (JNK) have been found to be regulated by ROS in vascular tissues [12, 73, 112]. Src family kinases are also activated by reactive oxygen species [113]. Already identified other

markers of oxidative stress like AMP- activated protein kinase (AMPK), apoptotic regulators like Bcl-2 and BAX are also altered with aging and reactive oxygen species [13, 87]. APAP is also involved in the regulation of MAPKs and Apoptotic regulators in hepatocytes [114-116]. But how APAP regulates these molecules in the aging aorta is not well studied.

The purpose of this study is to determine how aging and acetaminophen treatment alters the production of ROS and activation of the markers of oxidative stress in the aging F344NXBN rat aorta. Here we hypothesize that chronic treatment with therapeutic dose of APAP would decrease the age associated increase in ROS and alters age associated changes in signaling pathways.

## Materials and Methods:

## Animals

All procedures were performed in accordance with the Marshal University Institutional Animal Care and Use Committee (IACUC) guidelines, using the criteria outlined by the American Association of Laboratory Animal Care (AALAC) as proclaimed in the Animal Welfare Act (PL89-544, PL91-979, and PL94-279). Fischer 344/NNiaHSD x Brown Norway/BiNia (FBN) rats aged 6 and 27 months, were purchased from the National Institute on Aging colony at Harlan. Animals were housed two per cage in an AALAC approved vivarium with a 12 hour light-dark cycle and temperature maintained at $22 \pm 2°C$, and fed *ad libitum*. All animals were allowed to acclimatize for 2 weeks before initiation of any treatment or procedures. All animals were examined for precipitous weight loss, failure to thrive or unexpected gait alterations and animals with apparent abnormalities or tumors were removed from the study. Periodic weight measurements were taken throughout the duration of the study.

## Materials

Acetaminophen tablets or pure compound used in the study was provided by McNeil Pharmaceuticals (Fort Washington, PA). Antibodies against Bax, Bcl-2, and mouse IgG, goat IgG and rabbit IgG antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against AMPK-α, p44/42(ERK1/2-MAPK), p38-MAPK, SAPK/JNK - MAPK, p-AMPK-α $^{(Thr\ 172)}$, p-p44/42 $^{(Thr\ 202/Try\ 204)}$, p-p38 $^{(Thr\ 180/Try\ 182)}$, p-SAPK/JNK $^{(Thr\ 183/Tyr\ 185)}$, biotinylated protein ladder, mouse and rabbit IgG antibodies were purchased from Cell Signaling Technology (Beverly, MA). Precast 10% SDS-PAGE gels were procured from Cambrex

Biosciences (Baltimore, MD) and enhanced chemiluminescence (ECL) western blot detection reagent was acquired from Amersham Biosciences (Piscataway, NJ). Restore western blot stripping buffer was obtained from Pierce (Rockford, IL) and 3T3 cell lysates were from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Sigma (St Louis, MO).

## APAP Treatment:

FBN rats (27 month old; n=8) were subjected to 6 months (Upto 33 months of age) of treatment with a therapeutic dose of acetaminophen i.e.,30 mg/kg/day administered through drinking water. Age-matched FBN rats were maintained under the same environmental conditions without any drug treatment as controls (n=8).

## Vessel collection:

Rats were anesthetized with a ketamine-xylazine (4:1) cocktail (50 mg/kg IP) and supplemented as necessary for reflexive response. In a sterile aseptic environment, the ventral surface of the thorax was shaved and the superficial musculature was exposed by means of a transverse incision through the skin distal to the thoracic cavity. After midline laparotomy and perforation of the heart, the aorta was isolated and removed from the left ventricle to the renal arch and placed in Krebs-Ringer bicarbonate buffer (KRB) containing; 118mM NaCl, 4.7mM KCl, 2.5mM $CaCl_2$, 1.2mM $KH_2PO_4$, 1.2mM $MgSO_4$, 24.2mM $NaHCO_3$ and 10mM a-D-glucose, (pH 7.4) equilibrated with 5% $CO_2$ / 95% $O_2$ and maintained at 37°C. Isolated aortae were cleaned of connective tissue, weighed and immediately snap frozen in liquid nitrogen.

## Histology and oxidative fluorescent microscopy:

Aortic specimens were serially sectioned (8 μm) using an IEC Microtome cryostat and collected on poly-lysine coated slides. After fixing in acetone, (22°C for 2 min) sections were stained with hematoxylin and eosin, mounted and cover slipped. Morphometric evaluation was performed with the use of a computerized imaging analysis system (Image J). Medial thickness in micrometer was calculated from the average of eight different points of cross section. Hydroethidine (HE), an oxidative fluorescent dye, was used to visualize superoxide production in situ [117, 118]. HE is freely permeable to cells and in the presence of $O_2^{\cdot-}$ is oxidized to Et bromide, where it is trapped by intercalating with the DNA [119]. Because Et is impermeable to cell membranes, extracellular $O_2^{\cdot-}$ would not be expected to significantly contribute to the observed cellular fluorescence [120]. Neither hydroxyl radical, $^{\cdot}NO$, peroxynitrite, $H_2O_2$, hypochlorite, nor singlet $O_2$ significantly oxidizes HE, as such, an increase in Et fluorescence is thought to specifically indicate $O_2^{\cdot-}$ generation within the fluorescing cell. Briefly, aortic sections were incubated for 30 min at 37°C with 5mM HE. After extensive washing with PBS and mounting (permount) tissue was visualized under fluorescence using an Olympus fluorescence microscope (Melville, NY) and analyzed using imaging software (AlphaEaseFC). The intensity of fluorescent Et-stained nuclei as calculated by digitizing images and then determining the average pixel intensity of six randomly positioned regions (1000 $mm^2$) per arterial cross section. Six images per vessel were analyzed with ≥500 nuclei per vessel examined.

**Immunoblot analysis:**

Tissues were pulverized in liquid nitrogen using a mortar and pestle until a fine powder was obtained. After washing with ice cold PBS, pellets were lysed on ice for 15 minutes in T-PER (2mL/1g tissue weight) (Pierce, Rockford, IL) and centrifuged for 10 minutes at 2000 X g to pellet particulate matter. Protein concentrations of homogenates were determined in triplicate via the Bradford method (Pierce) using bovine serum albumin as a standard. Samples were diluted to a concentration of 2.0µg/µl in SDS loading buffer and boiled for 5 min. 40 µg of total protein for each sample was separated on a 10% SDSPAGE gel. Transfer of protein onto nitrocellulose membranes was performed using standard conditions [121]. To verify transfer of proteins and equal loading of lanes the membranes were stained with Ponceau S. For immunodetection, membranes were blocked in 5% Milk TBST for 1 h at room temperature and then incubated with the appropriate primary antibody overnight. After washing in TBST, the membranes were exposed to horseradish peroxidase-labeled IgG secondary antibody for 1 h and protein bands were visualized with ECL (Amersham Biosciences). Exposure time was adjusted to keep the integrated optical densities (IODs) within a linear and nonsaturated range. Band signal intensity was quantified by densitometry using a flatbed scanner (Epson Pefection 3200 PHOTO) and Imaging software (AlphaEaseFC). Molecular weight markers (cell signaling) were used as molecular mass standards and NIH 3T3 cell lysates were included as positive controls. A total of three SDS-PAGE gels were run for each experimental set to evaluate changes in dependent variable tissue content and basal phosphorylation where applicable. Immunoblots were stripped with restore western blot stripping buffer as described by the manufacturer to obtain direct comparisons between expression and phosphorylation levels of different signaling molecules. After verifying the absence of residual HRP activity by treating the membrane with

the ECL reagent, membranes were washed and reprobed. To minimize potential experimental error associated with membrane stripping, the order of antibody incubation was randomized between experiments.

## OxyBlot™ Analysis:

To identify carbonyl groups that are introduced into the amino acid side chain after oxidative modification of proteins, 2D-oxyblot analysis was performed, as previously described [122]. The level of protein oxidation was determined by an Oxidized Protein Detection Kit (Oxyblot, Chemicon Cat# S7150-Kit).  Oxyblot kit derivatizes carbonyl groups to a 2, 4-dinitrophenylhydrazone (DNP) moiety. The DNP moiety can then be detected using anti-DNP antibodies and is a method to assay for one form of oxidative damage to a protein. The proteins are derivatized as per the protocol given in the kit. These proteins are separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membrane. After the transfer, membranes were blocked with 2.5% BSA (in Tris Buffered Saline [TBS] with 0.2% Tween-20) for 1 h at room temperature. The nitrocellulose membrane was exposed to a primary rabbit anti-DNPH protein antibody from Chemicon Oxyblot (1:200 working dilution) for 1 hour, and then to a secondary antibody (Goat Anti-Rabbit IgG (HRP-conjugated) diluted in the blocking solution 1:500 for 1 h at room temperature. Membranes were washed after every step in washing buffer (TBS with 0.2% Tween-20). Protein bands were visualized with ECL (Amersham Biosciences). Band signal intensity was quantified by densitometry using a flatbed scanner (Epson Pefection 3200 PHOTO) and Imaging software (AlphaEaseFC).

**Results:**

**Aortic wall thickness, $^{\cdot}O_2^{-}$ and protein oxidation are altered with aging**

The tunica media thickness in aortae from 33-month control is $38.48 \pm 7.23\%$ more than aortae from 6-month animals *(p≤0.05)* (Fig 1). Ethidium (Et) fluorescence was seen throughout the aortic cross section with prominent signal in both the endothelial and medial portions (Fig 2). Levels of superoxide anion $O_2^{\cdot -}$, as determined by oxidation of HE to Et and the quantification of Et fluorescence were $91.81 \pm 31.08\%$ more in 33-month control aortae compared to the levels determined in the 6-month aortae *(p ≤0.05)* (Fig 2). To estimate the levels of oxidized proteins in the aging aortae we performed an OxyBlot assay. We found that when compared to 6 month aortae the level of protein oxidation was $7.09 \pm 2.31\%$ higher in 33-month control aortae *(p ≤0.05)* (Fig. 3).

**Phosphorylation of the metabolic sensor protein AMPK-α is altered with aging**

To determine whether aging influenced the total amount of AMPK-α present in the aorta, gel electrophoresis and immunoblot analysis using antibodies that recognize both the unphosphorylated and phosphorylated forms of AMPK-α was performed. There were no differences between 6- and 33-month control aortae in the total content of AMPK- α (Data not shown). Because AMPK- α is activated by phosphorylation it is important to determine that whether aging is characterized by changes in the basal phosphorylation level of AMPK- α. Compared to 6-month control animals, the basal phosphorylation levels of AMPK- α was $11.38 \pm 6.73\%$ lower in 33-month control *(p ≤0.05)* (Fig. 4).

## Total content and basal phosphorylation of the signaling molecules, p44-p42 (ERK 1/2)-, p38-, and JNK-MAPK altered with aging

The MAPK proteins play an important role in propagating the external stimuli into the cytoplasm and nucleus. The p38-MAPK content was 19.12 ±7.45% less in 33-month control aorta and when compared to aortae from 6-month animals ($p < 0.05$) (Fig. 5). No change in the total level of the p44 (ERK 1) - MAPK was observed with aging, but the p42 (ERK 2) - MAPK content was 11.79 ± 3.35% lower in 33-month control, compared to 6- month aortae ($p ≤0.05$) (Fig. 5). The JNK-1 MAPK levels in 33- month control aortae were 19.03 ± 9.12% less than 6-month aortae ($p ≤0.05$) (Fig 5) while JNK-2 MAPK levels were 29.12 ± 5.41% lower in 33-month control when compared to 6 month aortae. The JNK-3 MAPK levels in 33-month aortae were not significantly different from 6- month levels (Fig 5).

The phosphorylated p38- MAPK content in 33-month control aortae was 43.19 ± 12.30% less than that observed in 6-month aortae ($p ≤0.05$) (Fig. 6). The activated levels of p44 (ERK 1) - MAPK and p42 (ERK 2) - MAPK of 33-month control aortae were 21.30 ± 3.27% and 26.96 ± 2.11% lower than 6-month aortae, respectively ($p ≤0.05$) (Fig. 6). Compared to 6-month control animals the phosphorylated levels of JNK- 1 and JNK- 3 MAPKs were 25.26 ± 5.67% and 22.64 ± 3.34% less than that found in 33-month control animals ($p ≤0.05$) (Fig.7).

## Aging alters aortic content of the apoptotic regulator Bax, but not Bcl-2

Bax and Bcl-2 are important regulators of apoptosis. With aging the level of expression of Bax was decreased by 28.42 ± 9.96% in 33-month control compared to 6-month aortae ($p ≤0.05$) (Fig. 8). Bcl-2 levels are not altered with aging.

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## Treatment alters aortic wall thickness, $\cdot O_2^-$ and protein oxidation

The tunica media thickness in the treated aortae was $12.52 \pm 4.09\%$ less than aortae from 33-month control animals *(p≤0.05)* (Fig. 1). No significant differences in the levels of superoxide anion ($O2^{\cdot\ -}$) were found between 33-month control and APAP treated aortae (Fig. 2). The levels of oxidized proteins determined by OxyBlot assay, were $30.25 \pm 1.33\%$ less in 33- month APAP aortae *(p ≤0.05)* (Fig. 3).

## Treatment alters total content and basal phosphorylation of metabolic and signaling molecules AMPK-α, p44-p42 (ERK 1/2)-, p38-, and JNK-MAPK

There were no differences in the total content of AMPK- α between 33-month control and 33- month APAP treated aortae (Data not shown). Compared to 33- month control aortae the basal phosphorylation levels of AMPK- α were $20.59 \pm 6.16\%$ more in the 33-month APAP aortae *(p ≤0.05)* (Fig. 4). The MAPK proteins play an important role in propagating the external stimuli into the cytoplasm and nucleus. The p38- MAPK content in the 33-month control aortae was $24.42 \pm 8.06\%$ less than aortae from APAP treated animals *(p ≤0.05)* (Fig. 5). The p44 and p42- MAPK levels are $20.83 \pm 7.27\%$ and $18.94 \pm 2.85\%$ lower in 33 month APAP, compared to 33-month control aortae, respectively *(p ≤0.05)* (Fig. 5). There was no significant difference in the expression of total JNK-1 MAPK levels between 33-month control and 33-month APAP aortae (Fig. 5). The JNK-2 MAPK levels in APAP treated aortae were $22.84 \pm 4.45\%$ higher than 33-month control aortae *(p ≤0.05)*. The JNK-3 MAPK levels in APAP treated aortae were $14.89 \pm 4.56\%$ lower than in 33-month control aortae *(p ≤0.05)* (Fig. 5).

The phosphorylated p38- MAPK content in 33-month APAP aortae were 20.37 ± 7.81% more than in 33-month control aortae *(p ≤0.05)* (Fig. 6). The activated levels of p44 (ERK 1) - MAPK and p42 (ERK 2) - MAPK were 26.37 ± 2.36% and 15.90 ± 2.71% higher with treatment, respectively *(p ≤0.05)* (Fig. 6). Compared to 33-month control animals the phosphorylated levels of JNK- 1 and JNK- 3 MAPKs were 16.93 ± 6.30% and 41.75 ± 3.15% higher in APAP treated aortae *(p ≤0.05)* (Fig. 7).

## **Aortic content of the apoptotic regulator Bcl-2 was altered with treatment, but not Bax**

With treatment the level of Bcl-2 protein was decreased by 13.51 ± 4.38% in 33-month APAP compared to 33-month control *(p ≤0.05)* (Fig. 8). Bax levels were not altered with treatment.

## Discussion:

Previous work from our lab has suggested that aging is associated with increased levels of oxidative stress [13]. Here we investigate if acetaminophen treatment affects the regulation of previously identified indices of oxidative stress in the aging F344XBN rat aorta. Our findings suggest that chronic treatment with a therapeutic dose of acetaminophen alters aortic morphology and the level of aortic oxidative stress.

## Alterations in Aortic tunica media thickness and ROS

It has been generally accepted that increases in vascular ROS are linked to VSMC proliferation and hypertrophy [38, 47-49, 71, 123] and therefore, may act as a hypertrophic/hyperplastic effector to thicken the arterial wall. Similar to previous reports tunica media thickness was found to significantly increase with age in the present study [124, 125]. This age-associated increase in aortic media thickness is believed to reflect the smooth muscle cell hypertrophy. APAP treatment appeared to significantly decrease age-related increases in aortic wall thickness (Fig. 1). The mechanism(s) underlying this response are not clear but could be due to the antioxidant properties of acetaminophen [100, 105-107].

In present study we used dihydroethidium (HE) staining of the aortic cross sections to examine the efficacy of acetaminophen treatment in attenuating aortic ROS levels [126]. Our data suggest that aging in F344XBN rat aorta is characterized by a marked elevation of super oxide anion and that the amount of superoxide anion is unaltered with acetaminophen treatment (Fig. 2). In addition to superoxide levels, other ROS indices include the level of protein oxidation. Peroxynitrite because of its highly diffusible nature across phospholipid membranes

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[127] is known to initiate oxidative modification of proteins. In our present study protein oxidation in the rat aorta determined by OxyBlot™ analysis found to be elevated with age. This elevation of protein oxidation with age is consistent with the previous data [128]. The elevation in the protein oxidation was decreased with acetaminophen treatment (Fig. 3). Why acetaminophen decreases protein oxidation levels but not the amount of superoxide is unclear. We suspect that decreases in protein oxidation with acetaminophen treatment can be attributed to the antioxidant properties of the acetaminophen molecule as it may function as a ROS scavenger [16].

## Alterations in AMPK activity with age and APAP treatment

AMP-activated protein kinase (AMPK) is a stress-activated protein kinase that works as a metabolic sensor of cellular ATP levels. Several investigators have shown that AMP-activated protein kinase (AMPK) is also a redox-sensitive enzyme [87, 129]. AMPK is thought to be activated by phosphorylation when the ratio of ATP: ADP falls. Once activated, AMPK switches on catabolic pathways that generate ATP while switching off ATP-consuming processes such as protein synthesis [130]. Our data suggest that aging is associated with a decrease in AMPK-α (Thr 172) phosphorylation. The decrease in AMPK-α (Thr 172) phosphorylation we show with aging is similar to the change in AMPK phosphorylation one would expect to see in cells undergoing hypertrophy [131]. With acetaminophen treatment AMPK-α (Thr 172) phosphorylation appears to be increased (Fig. 4). This increase in phosphorylation of AMPK-α (Thr 172) would be expected to decrease protein synthesis and may help to explain why acetaminophen treatment is associated with decreased age-associated increases in aortic medial thickness.

## Alterations in ERK 1/2, p38-, and JNK-MAPK with acetaminophen treatment

The mitogen-activated protein kinase (MAPK) family has been shown to be activated by exogenous $H_2O_2$ and by endogenously generated ROS in VSMCs stimulated with growth factors [49]. The extracellular signal-regulated kinase (ERK)-MAPK proteins play a major role in cell proliferation and differentiation as well as survival mediated by various growth factors [132]. The ROS sensitivity of MAPK proteins has been subject to controversy, since some groups have found these proteins to be sensitive [133, 134] while others have found them to be insensitive to ROS [135, 136]. In our present study the amount of phosphorylated ERK1/2-, p38, and c-Jun N-terminal kinase (JNK) –MAPK were lowered with age but increased with acetaminophen treatment (Figs. 6, 7). The reason(s) why acetaminophen may increase the phosphorylation of MAPK proteins in the aging FBN aorta is not known. Because increases in MAPK phosphorylation have been implicated in causing cell apoptosis it is possible that the treatment induced decrease in aortic wall thickness we observe may be due to increased levels of age-related VSMC apoptosis.

## Effect of ROS and APAP on apoptotic regulators Bcl-2 and Bax

To investigate the possibility that acetaminophen diminishes age-associated aortic wall thickening by causing increased levels of apoptosis we examined how acetaminophen treatment affected the ratio of Bcl-2 to Bax. Bcl-2 is an evolutionary conserved protein that blocks apoptosis [137]. Conversely, Bax is a pro-apoptotic signaling protein of Bcl-2 family this has been proposed to promote cell death by dimerizing with anti-apoptotic proteins. It is thought that the susceptibility of a cell to apoptotic signals is regulated by the ratio of anti- to pro-apoptotic

proteins [138, 139]. In the present study we observed that acetaminophen treatment decreased the amount of Bcl-2 which is consistent with an increase in apoptosis.

In summary, our data indicate that acetaminophen treatment decreases age-associated increases in aortic wall thickening and protein oxidation. These changes were associated with alterations in AMPK and MAPK signaling and are consistent with the anti-oxidant properties of acetaminophen. Further experiments will be necessary to determine the exact mechanisms through which acetaminophen acts to improve vascular health during aging.

# Chapter 4

## <u>Conclusions</u>

1. Aging increased aortic wall thickness however this process appeared to be attenuated with acetaminophen treatment.

2. Age associated increase in protein oxidation is decreased with acetaminophen treatment.

3. Acetaminophen treatment increased the levels of phosphorylated AMPK

4. APAP treatment is associated with increased phosphorylation of the ERK1/2-, p38- and JNK-MAPK proteins.

5. The Bcl-2 to Bax ratio is also altered with APAP treatment.

6. Although not measured directly, we suspect that acetaminophen-induced decreases in age-related aortic wall thickening are associated with decreases in vascular smooth muscle cell protein synthesis and increased apoptosis.

7. These alterations in vascular structure and signaling with acetaminophen treatment may be due to the antioxidant properties of acetaminophen.

## Future directions

Future directions for research based on this study should focus on the mechanisms underlying the alterations in age- associated aortic wall thickening and protein oxidation with acetaminophen treatment. Recent data has suggested that acetaminophen has antioxidant and cardioprotective properties[16, 100, 106, 108, 110, 140]. In the present study we found decreased protein oxidation with acetaminophen treatment without having effect on the superoxide production. The exact mechanism underlying this is unknown. Future research concerning how APAP is reducing the protein oxidation and why it is not effective on superoxide production would be very helpful.

Our study suggests that chronic acetaminophen treatment decreases age-associated increases in aortic wall thickness. This decrease in wall thickness may be due to decreased protein synthesis or increased apoptosis, or a combination of each. In the present study, acetaminophen was associated with increased AMPK- α phosphorylation which is associated with decreases in protein synthesis. The mechanism underlying this finding is not known. To answer this, further investigation of different protein synthesis pathways would be helpful. This can be done by studying the expression and activation of the p70s6k, mTOR and Akt signaling pathways.

In the present study we found a decrease in the expression of anti- apoptotic signaling protein Bcl-2 with acetaminophen treatment without any change in the expression of the pro-apoptotic protein Bax. This change has been found to be permissive for apoptosis and is consistent with our finding of acetaminophen-associated decreases in aortic wall thickness. Further study of apoptosis in the aorta with acetaminophen treatment would be helpful. This can

be studied employing TUNEL staining of the aortic sections. Further study of expression and activation of caspases would be helpful in understanding the apoptosis, if present, that may be associated with acetaminophen treatment in the aorta. Along with caspases, the study of the expression and activation of different pro- and anti- apoptotic signaling proteins of Bcl-2 family would also be helpful in this investigation.

# Appendix

## Appendix A

### Figure legends

### Figure 1. Aortic medial thickness is altered with aging and APAP treatment

The medial thickness of aortae from 6 month, 33 month control and 33 month APAP treated rats.

Data are presented as means ± SE. *Insets*: representative H & E stained aortic sections. (*)

indicates significant difference from adult (6 month) value (*P<0.05*). (†) indicates significant

difference from 33 month control value (*P<0.05*). n = 8 for all groups. Scale Bar = 50 µm.

### Figure 2. Alterations in ROS with aging and APAP treatment

Detection of vascular superoxide by dihydroethidium (hydroethidine) with aging in the aortae of

6-month, 33-month control and 33-month APAP treated F344/N X BN rat. The increase in

superoxide involves all layers within the vessel wall. Scale Bar = 50µm. Quantification of aortic

ROS as determined by intensity of fluorescent Et-stained nuclei. Results are expressed as percent

of the 6 month integrated optical density (IOD) value. An asterisk (*) indicates significant

difference from the 6 month value (*P<0.05*), (†) indicates significant difference from 33 month

control value (*P<0.05*). n = 8 for all groups.

### Figure 3. Alterations in Protein oxidation with aging and APAP treatment

Protein isolates from the aortae excised from 6-month, 33-month control and 33-month APAP

treated F344/N X BN rats were analyzed by immunoblotting for changes in Protein oxidation.

Data are presented as means ± SE. *Insets*: representative blots for Protein oxidation. An asterisk

(*) indicates significant difference from the 6 month value, p < 0.05, (†) indicates significant difference from 33 month control value (*P<0.05*).  n = 8 for all groups.


**Figure 4.** **Aging and APAP treatment alters phosphorylation status of signaling protein AMPK α**

Protein isolates from the aortae excised from 6-month, 33-month control and 33-month APAP treated F344/N X BN rats were analyzed by immunoblotting for changes in phosphorylated AMPK α. Data are presented as means ± SE.  *Insets*: representative blots for phosphorylated AMPK α. An asterisk (*) indicates significant difference from the 6 month value, p < 0.05, (†) indicates significant difference from 33 month control value (*P<0.05*).  n = 8 for all groups.


**Figure 5.** **Aging and APAP treatment differentially affects the concentration of proteins involved in aortic signaling**

Protein isolates from the aortae excised from 6-month, 33-month control and 33-month APAP treated F344/N X BN rats were analyzed by immunoblotting for changes in total p44/p42 (ERK 1/2), p38 and Jnk protein expression. Data are presented as means ± SE. *Insets*: representative blots for total p44/p42 (ERK 1/2), p38 and Jnk. An asterisk (*) indicates significant difference from the 6 month value, p < 0.05, (†) indicates significant difference from 33 month control value (*P<0.05*).  n = 8 for all groups.

**Figure 6.** **Aging and APAP treatment alters phosphorylation status of signaling proteins p38 and p44/p42 (ERK 1/2)**

Protein isolates from the aortae excised from 6-month, 33-month control and 33-month APAP treated F344/N X BN rats were analyzed by immunoblotting for changes in phosphorylated p38 and p44/p42 (ERK 1/2) protein expression. Data are presented as means ± SE. *Insets*: representative blots for phosphorylated p44/p42 (ERK 1/2), p38 and Jnk. An asterisk (*) indicates significant difference from the 6 month value, p < 0.05, (†) indicates significant difference from 33 month control value (*P<0.05*).  n = 8 for all groups.

**Figure 7**. **Aging and APAP treatment alters phosphorylation status of signaling protein Jnk-MAPK**

Protein isolates from the aortae excised from 6-month, 33-month control and 33-month APAP treated F344/N X BN rats were analyzed by immunoblotting for changes in phosphorylated Jnk-MAPK protein expression. Data are presented as means ± SE. *Insets*: representative blots for phosphorylated Jnk-MAPK. An asterisk (*) indicates significant difference from the 6 month value, p < 0.05, (†) indicates significant difference from 33 month control value (*P<0.05*).  n = 8 for all groups.

**Figure 8**. **Aging and APAP treatment alters basal levels of apoptotic regulators BAX and Bcl-2**

Protein isolates from the aortae excised from 6-month, 33-month control and 33-month APAP treated F344/N X BN rats were analyzed by immunoblotting for changes in BAX and Bcl-2 protein expression. Data are presented as means $\pm$ SE. *Insets*: representative blots for BAX and Bcl-2. An asterisk (*) indicates significant difference from the 6 month value, $p < 0.05$, ($\dagger$) indicates significant difference from 33 month control value (*P<0.05*). n = 8 in all groups.

**Figure 1. Aortic medial thickness is altered with aging and APAP treatment in F344/N X BN rat**
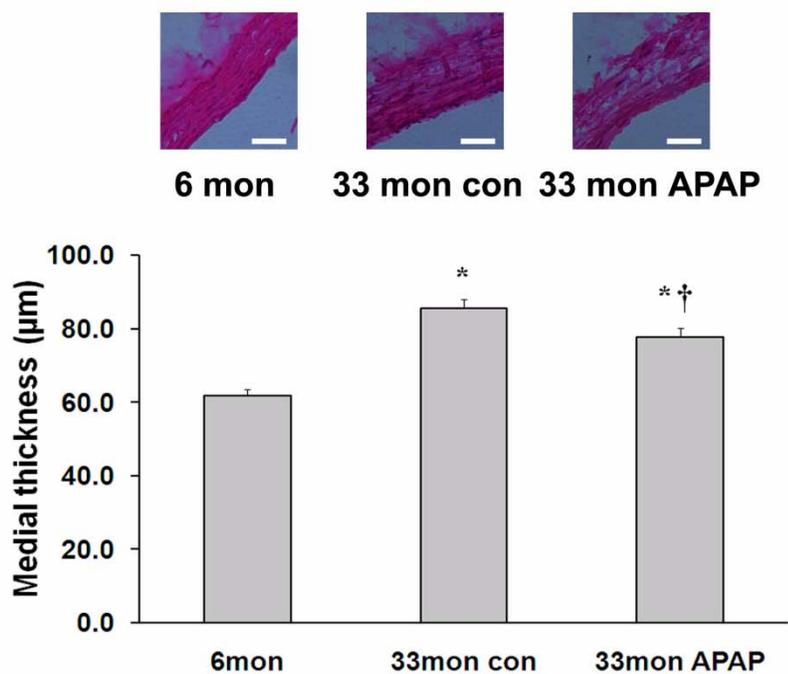
**Figure 2. Alterations in ROS with aging and APAP treatment in F344/N X BN rat Aorta**
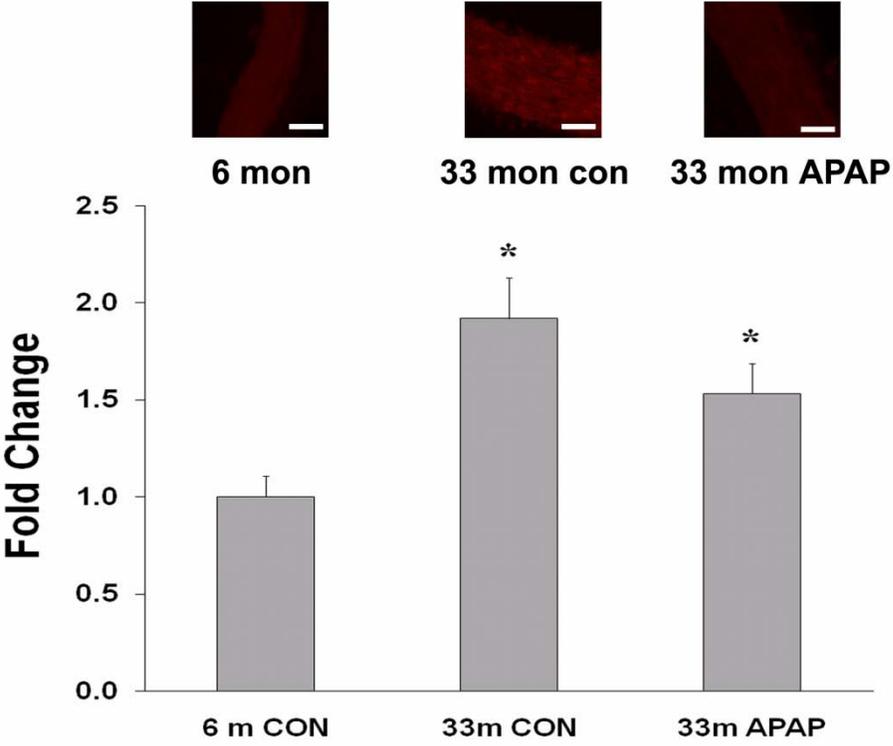
**Figure 3. Alterations in Protein oxidation with aging and APAP treatment in F344/N X BN rat Aorta**
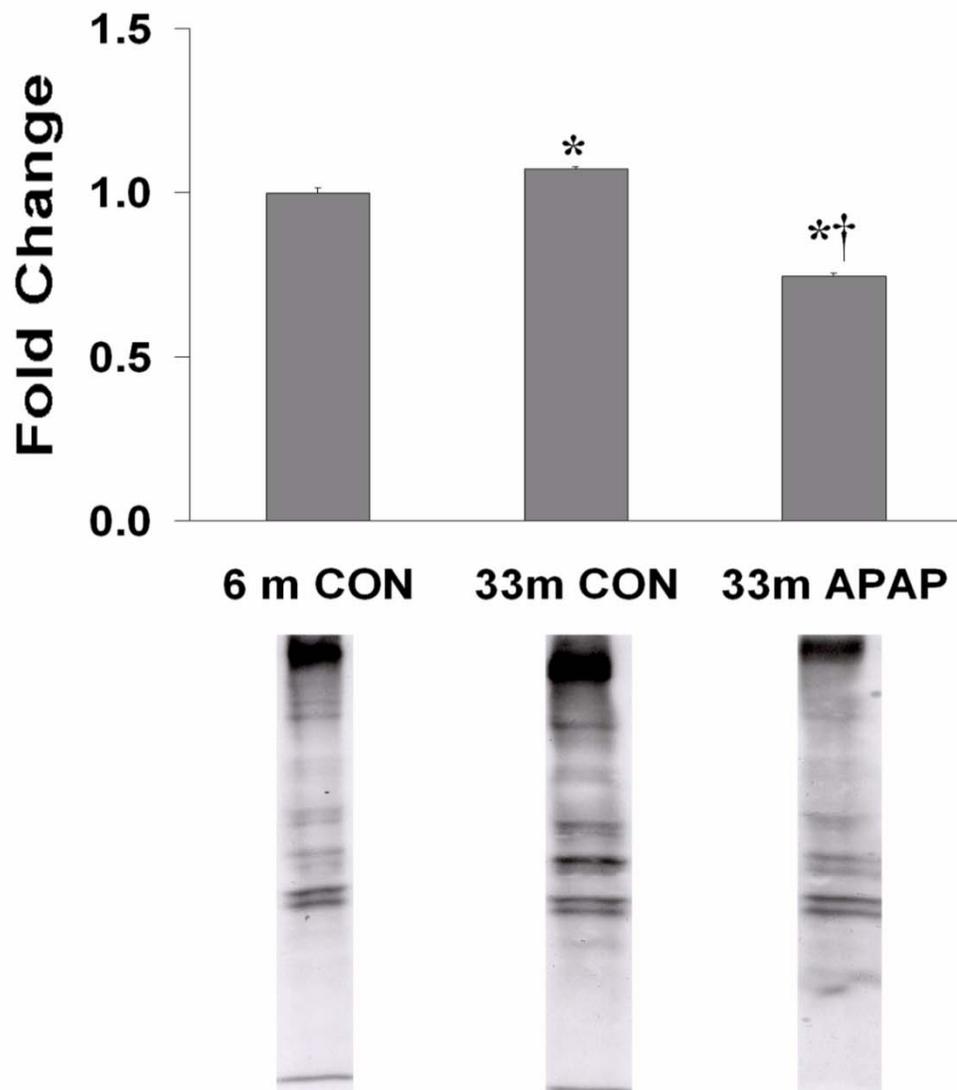
**Figure 4. Aging and APAP treatment alters phosphorylation status of signaling protein AMPK α in F344/N X BN rat Aorta**
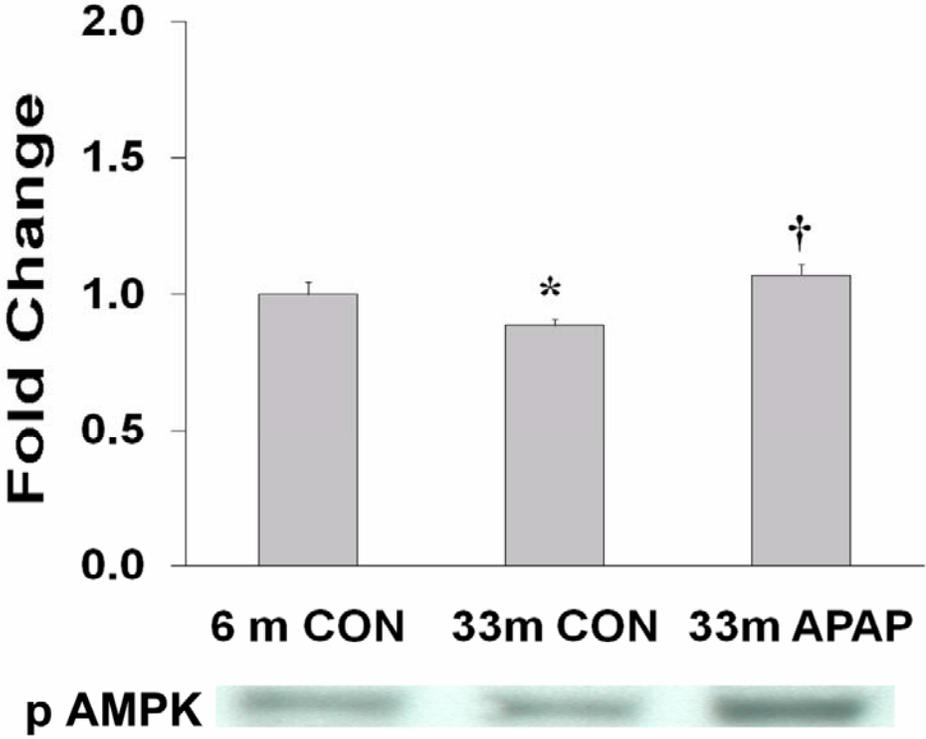
**Figure 5. Aging and APAP treatment differentially affects the concentration of proteins involved in aortic signaling**
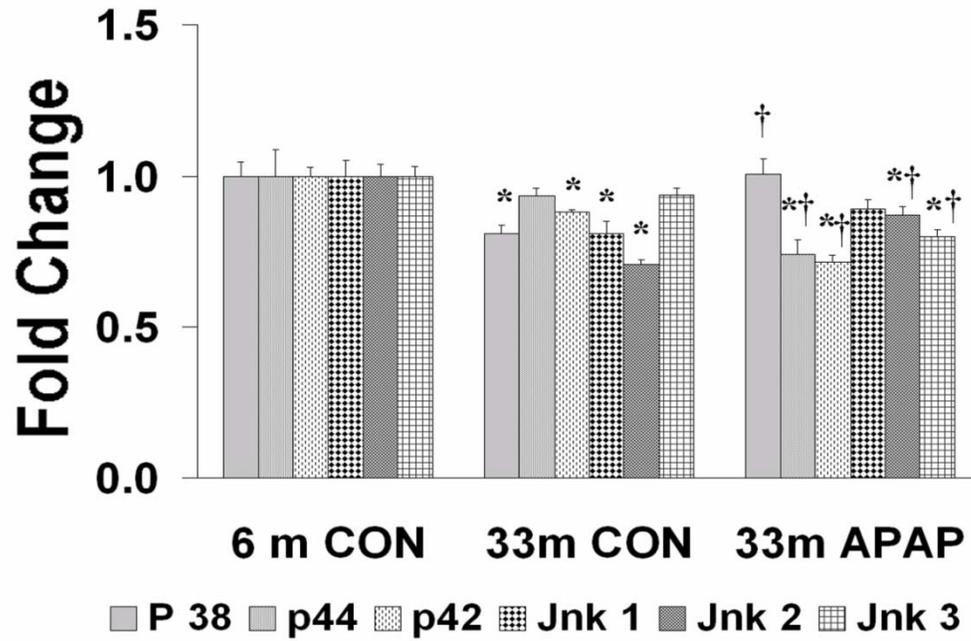
**Figure 6. Aging and APAP treatment alters phosphorylation status of signaling proteins p38 and p44/p42 (ERK 1/2) in F344/N X BN rat Aorta**
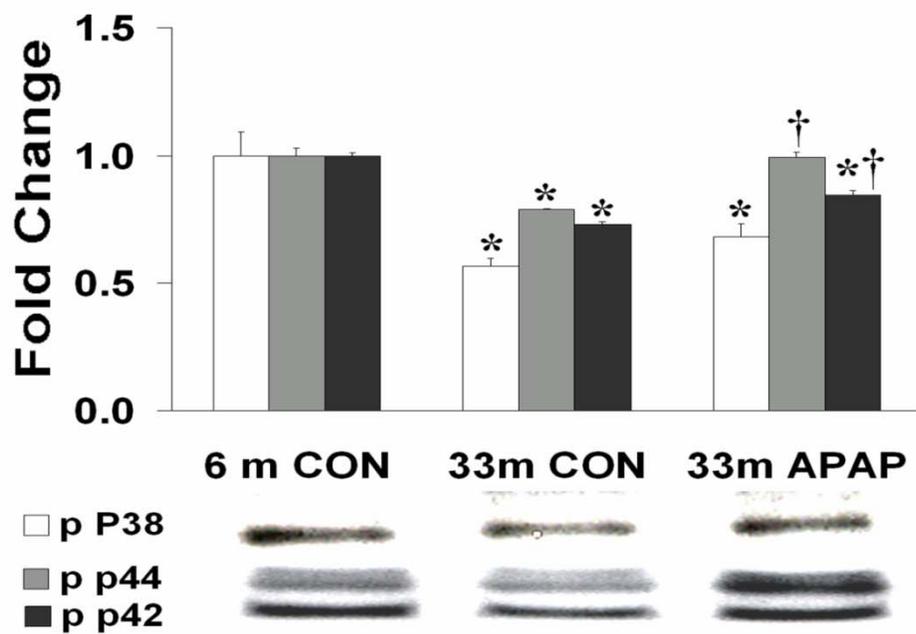
**Figure 7. Aging and APAP treatment alters phosphorylation status of signaling protein Jnk- MAPK in F344/N X BN rat Aorta**
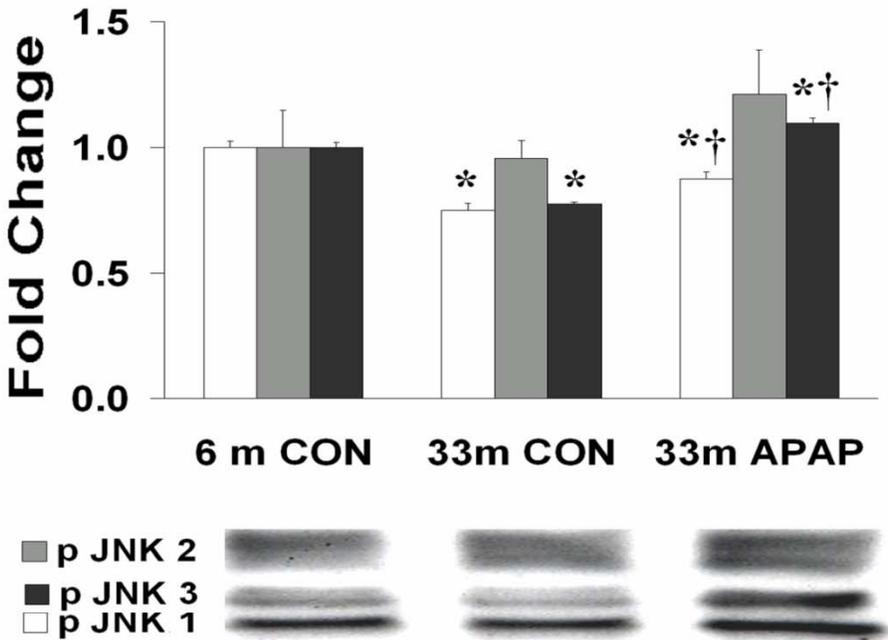
**Figure 8. Aging and APAP treatment alters basal levels of apoptotic regulators BAX and Bcl 2 in F344/N X BN rat Aorta**
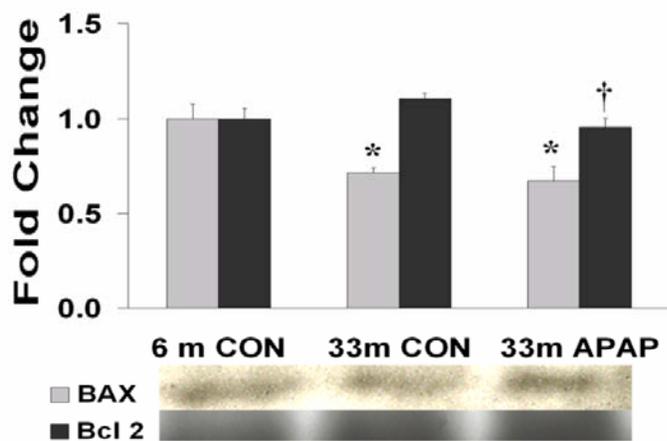
**Appendix B**

Laboratory of Molecular Physiology

# Laboratory of Molecular Physiology

## Film Properties Report

| | |
|---|---|
| Experimenter | Sarath Meduru |
| Date | 2006/09/20 |
| Project | McNeil |
| Tissue/ cell line/ etc. | Aorta |
| Report Number | |
| Protien Concentration | 30 µg (1.5µg / µl X 20µl) |
| Gel type | 10% Tris - Glycine Gel |

Electrophoresis Voltage 125 volts   Duration _____   Transfer Voltage 24 volts   Duration 45 min

### Primary Antibody
Type phospho AMPK   Dilution 1:500
Media 5% BSA in TBST
Incubation time Over Night

### Secondary Antibody
Type Anti Rabbit   Dilution 1:500
Media 5% Milk in TBST
Incubation time 1 hr

Lane # 1  Biotin Ladder (8µl+8µl)
Lane # 2  Color Marker (8µl)
Lane # 3  6 mon Control (20µl)
Lane # 4  33 mon Control (20µl)
Lane # 5  33 mon APAP (20µl)
Lane # 6  6 mon Control (20µl)
Lane # 7  33 mon Control (20µl)
Lane # 8  33 mon APAP (20µl)
Lane # 9  6 mon Control (20µl)
Lane # 10  33 mon Control (20µl)
Lane # 11  33 mon APAP (20µl)
Lane # 12  Positive Control (4µl+4µl+4µl+4µl)



Place Scan of Film Here

### Properties
Exposure time 5 min   Molecular Wieght 60 k Da   ☑ Used For Analysis
Location of Scan  \\Blough-raid\exp-data\Project folder\McNeil Aorta\Films
Location of Excell Data  \\Blough-raid\exp-data\Project folder\McNeil Aorta\Excel

Notes

_____
Your Signature

email Report

Laboratory of Molecular Physiology, Marshall University, Science Bldg. Suite 311, 1 John Marshall Drive, Huntington, WV, 25755 * Phone: 304-696-3267 Fax: 304-696-7136
http://www.science.marshall.edu/blough
Shipping adress Marshall University, 201 21st, Huntington, WV, 25755
MURC billing address: 401 11th St., Suite 1400 Huntington, Wv 25701 304-696-6203 Fax 304-697-3861
University Billing: Marshall University, 1 John Marshall Drive, Huntington, WV, 25755

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This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

Phospho AMPK in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 25.4 | 26.49524 | 33.42388 |
| %C | 31.2 | 23.78646 | 31.33971 |
| %C | 31.4 | 27.42639 | 27.94329 |
| %C | 25.7 | 26.15665 | 33.50107 |
| %C | 29.3 | 24.20971 | 32.49758 |
| %C | 31.8 | 26.83384 | 28.09767 |
| N | 6 | 6 | 6 |
| Mean | 29.13333 | 25.81805 | 31.13387 |
| SD | 2.907691 | 1.476882 | 2.535672 |
| SEM | 1.300359 | 0.660482 | 1.133987 |
|  |  |  |  |
| reletive expression level | 1 | 0.886203 | 1.068668 |
| SEM | 0.044635 | 0.022671 | 0.038924 |
|  |  |  |  |
| %RE | 100 | 88.6203 | 106.8668 |
| SE | 4.463475 | 2.2671 | 3.892404 |

**Statistics**

**One Way Analysis of Variance**

**Data source:** Data 9 in McNeil Aorta ROS (Normalized)

**Normality Test:**     Passed (P = 0.070)

**Equal Variance Test:**     Passed (P = 0.364)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 29.133 | 2.908 | 1.187 |
| 33m CON | 6 | 0 | 25.818 | 1.477 | 0.603 |
| 33m APAP | 6 | 0 | 31.134 | 2.536 | 1.035 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 86.502 | 43.251 | 7.603 | 0.005 |
| Residual | 15 | 85.327 | 5.688 | | |
| Total | 17 | 171.830 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.005).

Power of performed test with alpha = 0.050: 0.851

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| **33m APAP vs. 33m CON** | **5.316** | 3 | 5.459 | 0.004 | Yes |
| 33m APAP vs. 6 m CON | 2.001 | 2 | 2.055 | 0.167 | No |
| **6 m CON vs. 33m CON** | **3.315** | 2 | 3.405 | 0.030 | Yes |

# Laboratory of Molecular Physiology

## Film Properties Report

| | |
|---|---|
| Experimenter | Sarath Meduru |
| Date | 2006/11/08 |
| Project | McNeil |
| Tissue/ cell line/ etc. | Aorta |
| Report Number | |
| Protien Concentration | 30 μg (1.5μg / μl X 20μl) |
| Gel type | 10% Tris - Glycine Gel |

Electrophoresis Voltage 125 volts    Duration ___    Transfer Voltage 24 volts    Duration 45 min

### Primary Antibody

Type ERK 1/2    Dilution 1:500

Media 5% BSA in TBST

Incubation time Over Night

### Secondary Antibody

Type Anti Rabbit    Dilution 1:500

Media 5% Milk in TBST

Incubation time 1 hr

Lane # 1    Biotin Ladder (8μl+8μl)

Lane # 2    Color Marker (8μl)

Lane # 3    6 mon Control (20μl)

Lane # 4    33 mon Control (20μl)

Lane # 5    33 mon APAP (20μl)

Lane # 6    6 mon Control (20μl)

Lane # 7    33 mon Control (20μl)

Lane # 8    33 mon APAP (20μl)

Lane # 9    6 mon Control (20μl)

Lane # 10    33 mon Control (20μl)

Lane # 11    33 mon APAP (20μl)

Lane # 12    Positive Control (4μl+4μl+4μl+4μl)



Place Scan of Film Here

### Properties

Exposure time 30 sec    Molecular Wieght 42, 44 k Da    ☑ Used For Analysis

Location of Scan    \\Blough-raid\exp-data\Project folder\McNeil Aorta\Films

Location of Excell Data    \\Blough-raid\exp-data\Project folder\McNeil Aorta\Excel

Notes

Your Signature

email Report

*Raw data*

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

ERK 1 in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 37.1 | 30.21981 | 20.91887 |
| %C | 24 | 33.26719 | 28.32925 |
| %C | 34.2 | 30.30446 | 23.15742 |
| %C | 39.6 | 27.51103 | 21.45921 |
| %C | 25.6 | 31.7435 | 28.48363 |
| %C | 35.2 | 30.05051 | 22.61708 |
| N | 6 | 6 | 6 |
| Mean | 32.61667 | 30.51609 | 24.16091 |
| SD | 6.348674 | 1.921186 | 3.384119 |
| SEM | 2.839214 | 0.85918 | 1.513424 |
|  |  |  |  |
| reletive expression level | 1 | 0.935598 | 0.740754 |
| SEM | 0.087048 | 0.026342 | 0.0464 |
|  |  |  |  |
| %RE | 100 | 93.55979 | 74.07536 |
| SE | 8.704794 | 2.634176 | 4.640033 |

**Statistics**

**One Way Analysis of Variance**

**Data source:** Data 1 in McNeil Aorta ROS

**Normality Test:**    Passed (P = 0.687)

**Equal Variance Test:**    Passed (P = 0.169)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 32.617 | 6.349 | 2.592 |
| 33m CON | 6 | 0 | 30.516 | 1.921 | 0.784 |
| 33m APAP | 6 | 0 | 24.161 | 3.384 | 1.382 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 232.601 | 116.300 | 6.292 | 0.010 |
| Residual | 15 | 277.244 | 18.483 | | |
| Total | 17 | 509.845 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = 0.010).

Power of performed test with alpha = 0.050: 0.756

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| **6 m CON vs. 33m APAP** | **8.456** | **3** | **4.818** | **0.010** | **Yes** |
| 6 m CON vs. 33m CON | 2.101 | 2 | 1.197 | 0.411 | No |
| **33m CON vs. 33m APAP** | **6.355** | **2** | **3.621** | **0.022** | **Yes** |

*Raw data*

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

ERK 2 in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 36.6 | 29.62727 | 21.84517 |
| %C | 32.2 | 29.45797 | 25.55036 |
| %C | 33 | 29.79657 | 24.62406 |
| %C | 35.8 | 30.30446 | 21.99955 |
| %C | 31 | 29.96587 | 25.93631 |
| %C | 33.6 | 29.20402 | 24.62406 |
| N | 6 | 6 | 6 |
| Mean | 33.7 | 29.72603 | 24.09659 |
| SD | 2.138224 | 0.387604 | 1.76187 |
| SEM | 0.956243 | 0.173342 | 0.787932 |
|  |  |  |  |
| reletive expression level | 1 | 0.882078 | 0.715032 |
| SEM | 0.028375 | 0.005144 | 0.023381 |
|  |  |  |  |
| %RE | 100 | 88.20779 | 71.50322 |
| SE | 2.837515 | 0.514367 | 2.338078 |

# Statistics

**One Way Analysis of Variance**

**Data source:** Data 2 in McNeil Aorta ROS(Normalized)

**Normality Test:**    Passed (P = 0.452)

**Equal Variance Test:**    Passed (P = 0.094)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 33.700 | 2.138 | 0.873 |
| 33m CON | 6 | 0 | 29.726 | 0.388 | 0.158 |
| 33m APAP | 6 | 0 | 24.097 | 1.762 | 0.719 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 279.417 | 139.709 | 53.553 | <0.001 |
| Residual | 15 | 39.132 | 2.609 | | |
| Total | 17 | 318.549 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| 6 m CON vs. 33m APAP | 9.603 | 3 | 14.564 | <0.001 | Yes |
| 6 m CON vs. 33m CON | 3.974 | 2 | 6.027 | <0.001 | Yes |
| 33m CON vs. 33m APA | 5.629 | 2 | 8.537 | <0.001 | Yes |

# Laboratory of Molecular Physiology

| | |
|---|---|
| Experimenter | Sarath Meduru |
| Date | 2006/10/04 |
| Project | McNeil |
| Tissue/ cell line/ etc. | Aorta |
| Report Number | |
| Protien Concentration | 30 μg (1.5μg / μl X 20μl) |
| Gel type | 10% Tris - Glycine Gel |

## Film Properties Report

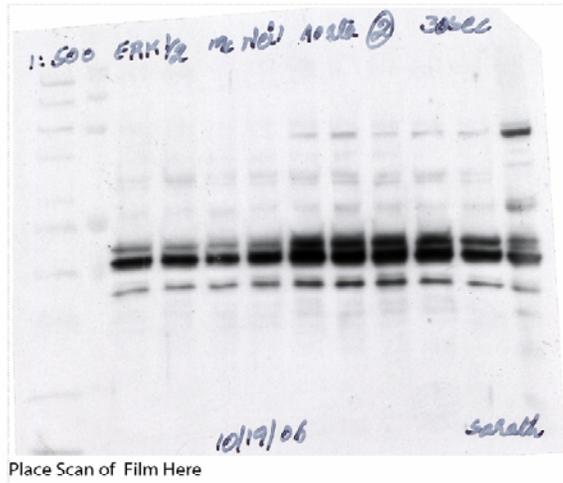Electrophoresis Voltage  125 volts    Duration _____    Transfer Voltage  24 volts    Duration  45 min

### Primary Antibody

Type  SAPK/Jnk        Dilution  1:500

Media  5% BSA in TBST

Incubation time  Over Night

### Secondary Antibody

Type  Anti Rabbit        Dilution  1:500

Media  5% Milk in TBST

Incubation time  1 hr

| | |
|---|---|
| Lane # 1 | Biotin Ladder (8μl+8μl) |
| Lane # 2 | Color Marker (8μl) |
| Lane # 3 | 6 mon Control (20μl) |
| Lane # 4 | 33 mon Control (20μl) |
| Lane # 5 | 33 mon APAP (20μl) |
| Lane # 6 | 6 mon Control (20μl) |
| Lane # 7 | 33 mon Control (20μl) |
| Lane # 8 | 33 mon APAP (20μl) |
| Lane # 9 | 6 mon Control (20μl) |
| Lane # 10 | 33 mon Control (20μl) |
| Lane # 11 | 33 mon APAP (20μl) |
| Lane # 12 | Positive Control (4μl+4μl+4μl+4μl) |



Place Scan of Film Here

### Properties

Exposure time  5 min        Molecular Wieght  46, 49, 54 k Da        ☑ Used For Analysis

Location of Scan  \\Blough-raid\exp-data\Project folder\McNeil Aorta\Films

Location of Excell Data  \\Blough-raid\exp-data\Project folder\McNeil Aorta\Excel

Notes

_____

_____

Your Signature

email Report

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*Raw data*

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

JNK 1 in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 27 | 29.96587 | 29.02397 |
| %C | 36.3 | 26.49524 | 25.08721 |
| %C | 34.3 | 25.81805 | 27.17138 |
| %C | 28.3 | 28.01893 | 29.87308 |
| %C | 34.5 | 24.04041 | 28.63801 |
| %C | 32.6 | 21.92418 | 31.95725 |
| N | 6 | 6 | 6 |
| Mean | 32.16667 | 26.04378 | 28.62515 |
| SD | 3.712501 | 2.84687 | 2.34307 |
| SEM | 1.660281 | 1.273159 | 1.047853 |
|  |  |  |  |
| reletive expression level | 1 | 0.809651 | 0.889901 |
| SEM | 0.051615 | 0.03958 | 0.032576 |
|  |  |  |  |
| %RE | 100 | 80.96512 | 88.9901 |
| SE | 5.161496 | 3.958007 | 3.257573 |

# Statistics

**One Way Analysis of Variance**

**Data source:** Data 4 in McNeil Aorta ROS(Normalized)

**Normality Test:**       Passed (P = 0.462)

**Equal Variance Test:**       Passed (P = 0.554)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 32.167 | 3.713 | 1.516 |
| 33m CON | 6 | 0 | 26.044 | 2.847 | 1.162 |
| 33m APAP | 6 | 0 | 28.625 | 2.343 | 0.957 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 113.391 | 56.696 | 6.213 | 0.011 |
| Residual | 15 | 136.887 | 9.126 | | |
| Total | 17 | 250.278 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = 0.011).

Power of performed test with alpha = 0.050: 0.749

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| **6 m CON vs. 33m CON** | **6.123** | **3** | **4.965** | **0.008** | **Yes** |
| 6 m CON vs. 33m APAP | 3.542 | 2 | 2.872 | 0.061 | No |
| 33m APAP vs. 33m CON | 2.581 | 2 | 2.093 | 0.160 | No |

*Raw data*

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

JNK 2 in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 34.6 | 25.2255 | 27.48014 |
| %C | 37.3 | 24.04041 | 26.47665 |
| %C | 31.3 | 22.93997 | 32.11163 |
| %C | 29.4 | 25.31015 | 31.41691 |
| %C | 35.8 | 22.60137 | 28.86959 |
| %C | 33.9 | 23.27857 | 29.79589 |
| N | 6 | 6 | 6 |
| Mean | 33.71667 | 23.89933 | 29.35847 |
| SD | 2.91439 | 1.162709 | 2.194554 |
| SEM | 1.303355 | 0.519979 | 0.981435 |
|  |  |  |  |
| reletive expression level | 1 | 0.708828 | 0.870741 |
| SEM | 0.038656 | 0.015422 | 0.029108 |
|  |  |  |  |
| %RE | 100 | 70.88284 | 87.07405 |
| SE | 3.865609 | 1.542203 | 2.910829 |

# Statistics

**One Way Analysis of Variance**

**Data source:** Data 5 in McNeil Aorta ROS(Normalized)

**Normality Test:**       Passed (P = 0.860)

**Equal Variance Test:**       Passed (P = 0.252)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 33.717 | 2.914 | 1.190 |
| 33m CON | 6 | 0 | 23.899 | 1.163 | 0.475 |
| 33m APAP | 6 | 0 | 29.358 | 2.195 | 0.896 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 290.352 | 145.176 | 29.705 | <0.001 |
| Residual | 15 | 73.308 | 4.887 | | |
| Total | 17 | 363.661 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| 6 m CON vs. 33m CON | 9.817 | 3 | 10.878 | <0.001 | Yes |
| 6 m CON vs. 33m APAP | 4.358 | 2 | 4.829 | 0.004 | Yes |
| 33m APAP vs. 33m CON | 5.459 | 2 | 6.049 | <0.001 | Yes |

*Raw data*

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

JNK 3 in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 30.6 | 30.38911 | 25.78193 |
| %C | 34.2 | 29.37332 | 24.00653 |
| %C | 33.4 | 27.00314 | 26.86261 |
| %C | 30 | 30.81236 | 25.93631 |
| %C | 33.8 | 31.06631 | 22.69428 |
| %C | 28.8 | 30.47376 | 27.17138 |
| N | 6 | 6 | 6 |
| Mean | 31.8 | 29.853 | 25.40884 |
| SD | 2.280351 | 1.511095 | 1.730477 |
| SEM | 1.019804 | 0.675782 | 0.773893 |
|  |  |  |  |
| Relative expression level | 1 | 0.938774 | 0.79902 |
| SEM | 0.032069 | 0.021251 | 0.024336 |
|  |  |  |  |
| %RE | 100 | 93.87736 | 79.90201 |
| SE | 3.206931 | 2.125102 | 2.433625 |

## Statistics

**One Way Analysis of Variance**

**Data source:** Data 6 in McNeil Aorta ROS(Normalized)

**Normality Test:**       Passed (P = 0.084)

**Equal Variance Test:**       Passed (P = 0.241)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 31.800 | 2.280 | 0.931 |
| 33m CON | 6 | 0 | 29.853 | 1.511 | 0.617 |
| 33m APAP | 6 | 0 | 25.409 | 1.730 | 0.706 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 128.777 | 64.388 | 18.435 | <0.001 |
| Residual | 15 | 52.390 | 3.493 | | |
| Total | 17 | 181.166 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = <0.001).

Power of performed test with alpha = 0.050: 0.999

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| **6 m CON vs. 33m APAP** | **6.391** | 3 | 8.377 | <0.001 | Yes |
| 6 m CON vs. 33m CON | 1.947 | 2 | 2.552 | 0.091 | No |
| **33m CON vs. 33m APAP** | **4.444** | 2 | 5.825 | 0.001 | Yes |

# Laboratory of Molecular Physiology

## Film Properties Report

| | |
|---|---|
| Experimenter | Sarath Meduru |
| Date | 2006/09/21 |
| Project | McNeil |
| Tissue/ cell line/ etc. | Aorta |
| Report Number | |
| Protien Concentration | 30 µg (1.5µg / µl X 20µl) |
| Gel type | 10% Tris - Glycine Gel |

Electrophoresis Voltage 125 volts     Duration ____     Transfer Voltage 24 volts     Duration 45 min

### Primary Antibody

Type p38          Dilution 1:500

Media 5% BSA in TBST

Incubation time Over Night

### Secondary Antibody

Type Anti Rabbit          Dilution 1:500

Media 5% Milk in TBST

Incubation time 1 hr

| | |
|---|---|
| Lane # 1 | Biotin Ladder (8µl+8µl) |
| Lane # 2 | Color Marker (8µl) |
| Lane # 3 | 6 mon Control (20µl) |
| Lane # 4 | 33 mon Control (20µl) |
| Lane # 5 | 33 mon APAP (20µl) |
| Lane # 6 | 6 mon Control (20µl) |
| Lane # 7 | 33 mon Control (20µl) |
| Lane # 8 | 33 mon APAP (20µl) |
| Lane # 9 | 6 mon Control (20µl) |
| Lane # 10 | 33 mon Control (20µl) |
| Lane # 11 | 33 mon APAP (20µl) |
| Lane # 12 | Positive Control (4µl+4µl+4µl+4µl) |

Place Scan of Film Here

### Properties

Exposure time 1 min          Molecular Wieght 38 k Da          ☑ Used For Analysis

Location of Scan          \\Blough-raid\exp-data\Project folder\McNeil Aorta\Films

Location of Excell Data          \\Blough-raid\exp-data\Project folder\McNeil Aorta\Excel

Notes

email Report

_____
Your Signature

*Raw data*

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

p38 in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 28.2 | 26.74919 | 31.03095 |
| %C | 27.3 | 22.00883 | 36.04839 |
| %C | 33.3 | 25.81805 | 27.94329 |
| %C | 29.7 | 26.072 | 30.49061 |
| %C | 30.2 | 22.51672 | 33.34669 |
| %C | 35.4 | 25.7334 | 26.39946 |
| N | 6 | 6 | 6 |
| Mean | 30.68333 | 24.81636 | 30.87657 |
| SD | 3.095427 | 2.016302 | 3.511997 |
| SEM | 1.384317 | 0.901717 | 1.570613 |
|  |  |  |  |
| relative expression level | 1 | 0.80879 | 1.006298 |
| SEM | 0.045116 | 0.029388 | 0.051188 |
|  |  |  |  |
| %RE | 100 | 80.87897 | 100.6298 |
| SE | 4.511625 | 2.938786 | 5.118782 |

**Statistics**

**One Way Analysis of Variance**

**Data source:** Data 8 in McNeil Aorta ROS(Normalized)

**Normality Test:**       Passed (P = 0.671)

**Equal Variance Test:**       Passed (P = 0.545)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 30.683 | 3.095 | 1.264 |
| 33m CON | 6 | 0 | 24.816 | 2.016 | 0.823 |
| 33m APAP | 6 | 0 | 30.877 | 3.512 | 1.434 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 142.369 | 71.185 | 8.220 | 0.004 |
| Residual | 15 | 129.906 | 8.660 | | |
| Total | 17 | 272.276 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = 0.004).

Power of performed test with alpha = 0.050: 0.884

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| **33m APAP vs. 33m CON** | **6.060** | **3** | **5.044** | **0.008** | **Yes** |
| 33m APAP vs. 6 m CON | 0.193 | 2 | 0.161 | 0.911 | No |
| **6 m CON vs. 33m CON** | **5.867** | **2** | **4.883** | **0.004** | **Yes** |

# Laboratory of Molecular Physiology

## Film Properties Report

| | |
|---|---|
| Experimenter | Sarath Meduru |
| Date | 2006/10/19 |
| Project | McNeil |
| Tissue/ cell line/ etc. | Aorta |
| Report Number | |
| Protien Concentration | 30 µg (1.5µg / µl X 20µl) |
| Gel type | 10% Tris - Glycine Gel |

Electrophoresis Voltage 125 volts    Duration _____

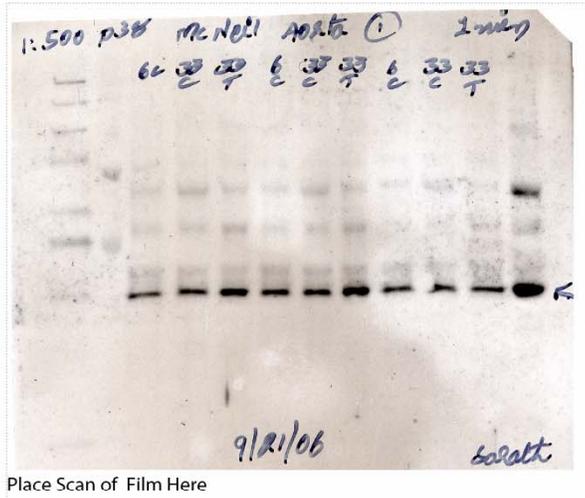Transfer Voltage 24 volts    Duration 45 min

### Primary Antibody

Type phospho Erk    Dilution 1:500

Media 5% BSA in TBST

Incubation time Over Night

### Secondary Antibody

Type Anti Mouse    Dilution 1:500

Media 5% Milk in TBST

Incubation time 1 hr

| | |
|---|---|
| Lane # 1 | Biotin Ladder (8µl+8µl) |
| Lane # 2 | Color Marker (8µl) |
| Lane # 3 | 6 mon Control (20µl) |
| Lane # 4 | 33 mon Control (20µl) |
| Lane # 5 | 33 mon APAP (20µl) |
| Lane # 6 | 6 mon Control (20µl) |
| Lane # 7 | 33 mon Control (20µl) |
| Lane # 8 | 33 mon APAP (20µl) |
| Lane # 9 | 6 mon Control (20µl) |
| Lane # 10 | 33 mon Control (20µl) |
| Lane # 11 | 33 mon APAP (20µl) |
| Lane # 12 | Positive Control (4µl+4µl+4µl+4µl) |



Place Scan of Film Here

### Properties

Exposure time 1 min    Molecular Wieght 42, 44 k Da    ☑ Used For Analysis

Location of Scan    \\Blough-raid\exp-data\Project folder\McNeil Aorta\Films

Location of Excell Data    \\Blough-raid\exp-data\Project folder\McNeil Aorta\Excel

Notes

_____

email Report

Your Signature

*Raw data*

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

Phospho ERK 1 in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 32.5 | 24.20971 | 30.10465 |
| %C | 32.1 | 24.29436 | 30.25903 |
| %C | 28 | 24.54831 | 33.19231 |
| %C | 32.9 | 24.04041 | 29.87308 |
| %C | 31.5 | 24.71761 | 30.33623 |
| %C | 29.5 | 24.97155 | 31.72567 |
| N | 6 | 6 | 6 |
| Mean | 31.08333 | 24.46366 | 30.91516 |
| SD | 1.925011 | 0.346959 | 1.292351 |
| SEM | 0.860891 | 0.155165 | 0.577957 |
|  |  |  |  |
| relative expression level | 1 | 0.787035 | 0.99459 |
| SEM | 0.027696 | 0.004992 | 0.018594 |
|  |  |  |  |
| %RE | 100 | 78.70346 | 99.45896 |
| SE | 2.769623 | 0.49919 | 1.859379 |

**Statistics**

**One Way Analysis of Variance**

**Data source:** Data 10 in McNeil Aorta ROS(Normalized)

**Normality Test:**       Passed (P = 0.728)

**Equal Variance Test:**       Passed (P = 0.200)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 31.083 | 1.925 | 0.786 |
| 33m CON | 6 | 0 | 24.464 | 0.347 | 0.142 |
| 33m APAP | 6 | 0 | 30.915 | 1.292 | 0.528 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 170.941 | 85.470 | 46.652 | <0.001 |
| Residual | 15 | 27.481 | 1.832 | | |
| Total | 17 | 198.422 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| **6 m CON vs. 33m CON** | **6.620** | 3 | 11.980 | <0.001 | Yes |
| 6 m CON vs. 33m APAP | 0.168 | 2 | 0.304 | 0.833 | No |
| **33m APAP vs. 33m CON** | **6.452** | 2 | 11.675 | <0.001 | Yes |

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

Phospho ERK 2 in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 35.1 | 25.2255 | 27.09419 |
| %C | 33.7 | 25.0562 | 28.32925 |
| %C | 33.1 | 23.44787 | 30.25903 |
| %C | 34.3 | 25.3948 | 27.48014 |
| %C | 33.7 | 24.88691 | 28.48363 |
| %C | 32.8 | 24.04041 | 29.95027 |
| N | 6 | 6 | 6 |
| Mean | 33.78333 | 24.67528 | 28.59942 |
| SD | 0.830462 | 0.764192 | 1.279376 |
| SEM | 0.371394 | 0.341757 | 0.572155 |
|  |  |  |  |
| relative expression level | 1 | 0.730398 | 0.846554 |
| SEM | 0.010993 | 0.010116 | 0.016936 |
|  |  |  |  |
| %RE | 100 | 73.03981 | 84.65541 |
| SE | 1.09934 | 1.011614 | 1.6936 |

## Statistics

**One Way Analysis of Variance**

**Data source:** Data 11 in McNeil Aorta ROS(Normalized)

**Normality Test:**    Passed (P = 0.847)

**Equal Variance Test:**    Passed (P = 0.463)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 33.783 | 0.830 | 0.339 |
| 33m CON | 6 | 0 | 24.675 | 0.764 | 0.312 |
| 33m APAP | 6 | 0 | 28.599 | 1.279 | 0.522 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 250.457 | 125.228 | 129.081 | <0.001 |
| Residual | 15 | 14.552 | 0.970 | | |
| Total | 17 | 265.009 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| 6 m CON vs. 33m CON | 9.108 | 3 | 22.651 | <0.001 | Yes |
| 6 m CON vs. 33m APAP | 5.184 | 2 | 12.892 | <0.001 | Yes |
| 33m APAP vs. 33m CON | 3.924 | 2 | 9.759 | <0.001 | Yes |

# Laboratory of Molecular Physiology

## Film Properties Report

Experimenter: Sarath Meduru
Date: 2006/09/20
Project: McNeil
Tissue/ cell line/ etc.: Aorta
Report Number:
Protien Concentration: 30 µg (1.5µg / µl X 20µl)
Gel type: 10% Tris - Glycine Gel

Electrophoresis Voltage: 125 volts    Duration:

Transfer Voltage: 24 volts    Duration: 45 min

### Primary Antibody

Type: phospho Jnk    Dilution: 1:500
Media: 5% BSA in TBST
Incubation time: Over Night

### Secondary Antibody

Type: Anti Rabbit    Dilution: 1:500
Media: 5% Milk in TBST
Incubation time: 1 hr

Lane # 1: Biotin Ladder (8µl+8µl)
Lane # 2: Color Marker (8µl)
Lane # 3: 6 mon Control (20µl)
Lane # 4: 33 mon Control (20µl)
Lane # 5: 33 mon APAP (20µl)
Lane # 6: 6 mon Control (20µl)
Lane # 7: 33 mon Control (20µl)
Lane # 8: 33 mon APAP (20µl)
Lane # 9: 6 mon Control (20µl)
Lane # 10: 33 mon Control (20µl)
Lane # 11: 33 mon APAP (20µl)
Lane # 12: Positive Control (4µl+4µl+4µl+4µl)


Place Scan of Film Here

### Properties

Exposure time: 3 min    Molecular Wieght: 46, 49, 54 k Da    ☑ Used For Analysis
Location of Scan: \\Blough-raid\exp-data\Project folder\McNeil Aorta\Films
Location of Excell Data: \\Blough-raid\exp-data\Project folder\McNeil Aorta\Excel

Notes

Your Signature

email Report

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*Raw data*

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

Phospho Jnk 1 in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 32.9 | 21.58558 | 32.11163 |
| %C | 34.7 | 26.15665 | 26.55385 |
| %C | 31.4 | 26.57989 | 28.63801 |
| %C | 33.8 | 22.00883 | 31.03095 |
| %C | 35.5 | 25.81805 | 26.32227 |
| %C | 30.7 | 26.57989 | 29.25555 |
| N | 6 | 6 | 6 |
| Mean | 33.16667 | 24.78815 | 28.98538 |
| SD | 1.869403 | 2.338171 | 2.33049 |
| SEM | 0.836022 | 1.045662 | 1.042227 |
|  |  |  |  |
| relative expression level | 1 | 0.747381 | 0.873931 |
| SEM | 0.025207 | 0.031527 | 0.031424 |
|  |  |  |  |
| %RE | 100 | 74.73813 | 87.39309 |
| SE | 2.52067 | 3.15275 | 3.142393 |

# Statistics

**One Way Analysis of Variance**

**Data source:** Data 12 in McNeil Aorta ROS(Normalized)

**Normality Test:** Passed (P = 0.388)

**Equal Variance Test:** Passed (P = 0.932)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 33.167 | 1.869 | 0.763 |
| 33m CON | 6 | 0 | 24.788 | 2.338 | 0.955 |
| 33m APAP | 6 | 0 | 28.985 | 2.330 | 0.951 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 210.599 | 105.299 | 21.948 | <0.001 |
| Residual | 15 | 71.964 | 4.798 | | |
| Total | 17 | 282.563 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| 6 m CON vs. 33m CON | 8.379 | 3 | 9.370 | <0.001 | Yes |
| 6 m CON vs. 33m APAP | 4.181 | 2 | 4.676 | 0.005 | Yes |
| 33m APAP vs. 33m CON | 4.197 | 2 | 4.694 | 0.005 | Yes |

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

Phospho Jnk 2 in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 15.8 | 20.23119 | 46.54642 |
| %C | 33.2 | 29.88122 | 24.39249 |
| %C | 31.9 | 26.74919 | 28.09767 |
| %C | 15.4 | 21.07769 | 46.08327 |
| %C | 33.2 | 29.96587 | 24.2381 |
| %C | 32.6 | 27.34174 | 27.01699 |
| N | 6 | 6 | 6 |
| Mean | 27.01667 | 25.87448 | 32.72916 |
| SD | 8.857182 | 4.25599 | 10.62946 |
| SEM | 3.961052 | 1.903337 | 4.753638 |
|  | 6 m CON | 33m CON | 33m APAP |
| relative expression level | 1 | 0.957723 | 1.211443 |
| SEM | 0.146615 | 0.07045 | 0.175952 |
|  | 6 m CON | 33m CON | 33m APAP |
| %RE | 100 | 95.77229 | 121.1443 |
| SE | 14.66151 | 7.045047 | 17.5952 |

**Statistics**

**One Way Analysis of Variance**

**Data source:** Data 13 in McNeil Aorta ROS(Normalized)

**Normality Test:** Passed (P = 0.294)

**Equal Variance Test:** Passed (P = 0.580)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 27.017 | 8.857 | 3.616 |
| 33m CON | 6 | 0 | 25.874 | 4.256 | 1.738 |
| 33m APAP | 6 | 0 | 32.729 | 10.629 | 4.339 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 161.848 | 80.924 | 1.159 | 0.341 |
| Residual | 15 | 1047.742 | 69.849 | | |
| Total | 17 | 1209.590 | | | |

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.341).

Power of performed test with alpha = 0.050: 0.069

The power of the performed test (0.069) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

*Raw data*

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

Phospho Jnk 3 in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 28.2 | 23.02462 | 34.50456 |
| %C | 30.5 | 24.20971 | 31.57129 |
| %C | 30.9 | 22.34743 | 33.03792 |
| %C | 28 | 23.27857 | 34.27299 |
| %C | 31.5 | 23.87111 | 31.10814 |
| %C | 30.9 | 22.51672 | 32.88354 |
| N | 6 | 6 | 6 |
| Mean | 30 | 23.20803 | 32.89641 |
| SD | 1.507315 | 0.734875 | 1.375149 |
| SEM | 0.674092 | 0.328646 | 0.614985 |
|  |  |  |  |
| relative expression level | 1 | 0.773601 | 1.096547 |
| SEM | 0.02247 | 0.010955 | 0.0205 |
|  |  |  |  |
| %RE | 100 | 77.36009 | 109.6547 |
| SE | 2.246973 | 1.095486 | 2.049951 |

# Statistics

**One Way Analysis of Variance**

**Data source:** Data 14 in McNeil Aorta ROS(Normalized)

**Normality Test:**    Passed (P = 0.738)

**Equal Variance Test:**    Passed (P = 0.519)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 30.000 | 1.507 | 0.615 |
| 33m CON | 6 | 0 | 23.208 | 0.735 | 0.300 |
| 33m APAP | 6 | 0 | 32.896 | 1.375 | 0.561 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 296.770 | 148.385 | 94.652 | <0.001 |
| Residual | 15 | 23.515 | 1.568 | | |
| Total | 17 | 320.285 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| 33m APAP vs. 33m CON | 9.688 | 3 | 18.954 | <0.001 | Yes |
| 33m APAP vs. 6 m CON | 2.896 | 2 | 5.666 | 0.001 | Yes |
| 6 m CON vs. 33m CON | 6.792 | 2 | 13.287 | <0.001 | Yes |

# Laboratory of Molecular Physiology

# Film Properties Report

| | |
|---|---|
| Experimenter | Sarath Meduru |
| Date | 2006/12/14 |
| Project | McNeil |
| Tissue/ cell line/ etc. | Aorta |
| Report Number | |
| Protien Concentration | 30 μg (1.5μg / μl X 20μl) |
| Gel type | 10% Tris - Glycine Gel |

Electrophoresis Voltage 125 volts    Duration ____

Transfer Voltage 24 volts    Duration 45 min

## Primary Antibody

Type phospho p38    Dilution 1:200

Media 5% BSA in TBST

Incubation time Over Night

## Secondary Antibody

Type Anti Mouse    Dilution 1:500

Media 5% Milk in TBST

Incubation time 1 hr

Lane # 1    Biotin Ladder (8μl+8μl)

Lane # 2    Color Marker (8μl)

Lane # 3    6 mon Control (20μl)

Lane # 4    33 mon Control (20μl)

Lane # 5    33 mon APAP (20μl)

Lane # 6    6 mon Control (20μl)

Lane # 7    33 mon Control (20μl)

Lane # 8    33 mon APAP (20μl)

Lane # 9    6 mon Control (20μl)

Lane # 10    33 mon Control (20μl)

Lane # 11    33 mon APAP (20μl)

Lane # 12    Positive Control (4μl+4μl+4μl+4μl)



Place Scan of Film Here

## Properties

Exposure time 2 min    Molecular Wieght 38 k Da    ☑ Used For Analysis

Location of Scan    \\Blough-raid\exp-data\Project folder\McNeil Aorta\Films

Location of Excell Data    \\Blough-raid\exp-data\Project folder\McNeil Aorta\Excel

Notes

____

Your Signature

email Report

*Raw data*

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

Phospho p38 in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 32.5 | 24.20971 | 30.02746 |
| %C | 39.3 | 20.40049 | 28.25206 |
| %C | 32.2 | 25.0562 | 29.48712 |
| %C | 47.4 | 20.90839 | 21.5364 |
| %C | 32.7 | 24.12506 | 30.02746 |
| %C | 50.6 | 18.62285 | 21.15045 |
| N | 6 | 6 | 6 |
| Mean | 39.11667 | 22.22045 | 26.74682 |
| SD | 8.16466 | 2.592399 | 4.237169 |
| SEM | 3.651347 | 1.159356 | 1.894919 |
|  | 6 m CON | 33m CON | 33m APAP |
| relative expression level | 1 | 0.568056 | 0.683771 |
| SEM | 0.093345 | 0.029638 | 0.048443 |
|  | 6 m CON | 33m CON | 33m APAP |
| %RE | 100 | 56.80558 | 68.37706 |
| SE | 9.334504 | 2.963842 | 4.844276 |

**Statistics**

**One Way Analysis of Variance**

**Data source:** Data 21 in McNeil Aorta ROS(Normalized).SNB

**Normality Test:**    Passed (P = 0.214)

**Equal Variance Test:**    Passed (P = 0.108)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 39.117 | 8.165 | 3.333 |
| 33m CON | 6 | 0 | 22.220 | 2.592 | 1.058 |
| 33m APAP | 6 | 0 | 26.747 | 4.237 | 1.730 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 917.966 | 458.983 | 15.076 | <0.001 |
| Residual | 15 | 456.679 | 30.445 | | |
| Total | 17 | 1374.645 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = <0.001).

Power of performed test with alpha = 0.050: 0.996

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| **6 m CON vs. 33m CON** | **16.896** | **3** | **7.501** | **<0.001** | **Yes** |
| **6 m CON vs. 33m APAP** | **12.370** | **2** | **5.491** | **0.002** | **Yes** |
| 33m APAP vs. 33m CON | 4.526 | 2 | 2.009 | 0.176 | No |

# Laboratory of Molecular Physiology

| | |
|---|---|
| Experimenter | Sarath Meduru |
| Date | 2006/10/06 |
| Project | McNeil |
| Tissue/ cell line/ etc. | Aorta |
| Report Number | |
| Protien Concentration | 30 µg (1.5µg / µl X 20µl) |
| Gel type | 10% Tris - Glycine Gel |

## Film Properties Report

Electrophoresis Voltage  125 volts    Duration ___    Transfer Voltage  24 volts    Duration  45 min

### Primary Antibody

Type  phospho Src 416    Dilution  1:500

Media  5% BSA in TBST

Incubation time  Over Night

### Secondary Antibody

Type  Anti Rabbit    Dilution  1:500

Media  5% Milk in TBST

Incubation time  1 hr

Lane # 1   Biotin Ladder (8µl+8µl)

Lane # 2   Color Marker (8µl)

Lane # 3   6 mon Control (20µl)

Lane # 4   33 mon Control (20µl)

Lane # 5   33 mon APAP (20µl)

Lane # 6   6 mon Control (20µl)

Lane # 7   33 mon Control (20µl)

Lane # 8   33 mon APAP (20µl)

Lane # 9   6 mon Control (20µl)

Lane # 10  33 mon Control (20µl)

Lane # 11  33 mon APAP (20µl)

Lane # 12  Positive Control (4µl+4µl+4µl+4µl)



Place Scan of Film Here

### Properties

Exposure time  1 min    Molecular Wieght  60 k Da    ☑ Used For Analysis

Location of Scan   \\Blough-raid\exp-data\Project folder\McNeil Aorta\Films

Location of Excell Data   \\Blough-raid\exp-data\Project folder\McNeil Aorta\Excel

Notes

Your Signature

email Report

*Raw data*

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

Phospho Src 416 in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 36.9 | 24.04041 | 26.86261 |
| %C | 38.2 | 19.13075 | 30.33623 |
| %C | 43.5 | 23.53252 | 22.15394 |
| %C | 33.8 | 23.61717 | 29.6415 |
| %C | 39.8 | 18.8768 | 29.25555 |
| %C | 44.1 | 23.36322 | 21.84517 |
| N | 6 | 6 | 6 |
| Mean | 39.38333 | 22.09348 | 26.6825 |
| SD | 3.952425 | 2.404995 | 3.812245 |
| SEM | 1.767578 | 1.075546 | 1.704888 |
|  | 6 m CON | 33m CON | 33m APAP |
| relative expression level | 1 | 0.560985 | 0.677507 |
| SEM | 0.044881 | 0.02731 | 0.04329 |
|  | 6 m CON | 33m CON | 33m APAP |
| %RE | 100 | 56.09855 | 67.75074 |
| SE | 4.488138 | 2.730968 | 4.328958 |

**Statistics**

**One Way Analysis of Variance**

**Data source:** Data 17 in McNeil Aorta ROS(Normalized)

**Normality Test:**  Passed (P = 0.400)

**Equal Variance Test:**  Passed (P = 0.451)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 39.383 | 3.952 | 1.614 |
| 33m CON | 6 | 0 | 22.093 | 2.405 | 0.982 |
| 33m APAP | 6 | 0 | 26.682 | 3.812 | 1.556 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 962.619 | 481.309 | 40.177 | <0.001 |
| Residual | 15 | 179.694 | 11.980 | | |
| Total | 17 | 1142.313 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| 6 m CON vs. 33m CON | 17.290 | 3 | 12.236 | <0.001 | Yes |
| 6 m CON vs. 33m APAP | 12.701 | 2 | 8.988 | <0.001 | Yes |
| 33m APAP vs. 33m CON | 4.589 | 2 | 3.248 | 0.037 | Yes |

# Laboratory of Molecular Physiology

| | |
|---|---|
| Experimenter | Sarath Meduru |
| Date | 2006/10/06 |
| Project | McNeil |
| Tissue/ cell line/ etc. | Aorta |
| Report Number | |
| Protien Concentration | 30 µg (1.5µg / µl X 20µl) |
| Gel type | 10% Tris - Glycine Gel |

## Film Properties Report

Electrophoresis Voltage  125 volts    Duration _____

Transfer Voltage  24 volts    Duration  45 min

### Primary Antibody

Type  phospho Src 527    Dilution  1:500

Media  5% BSA in TBST

Incubation time  Over Night

### Secondary Antibody

Type  Anti Rabbit    Dilution  1:500

Media  5% Milk in TBST

Incubation time  1 hr

| | |
|---|---|
| Lane # 1 | Biotin Ladder (8µl+8µl) |
| Lane # 2 | Color Marker (8µl) |
| Lane # 3 | 6 mon Control (20µl) |
| Lane # 4 | 33 mon Control (20µl) |
| Lane # 5 | 33 mon APAP (20µl) |
| Lane # 6 | 6 mon Control (20µl) |
| Lane # 7 | 33 mon Control (20µl) |
| Lane # 8 | 33 mon APAP (20µl) |
| Lane # 9 | 6 mon Control (20µl) |
| Lane # 10 | 33 mon Control (20µl) |
| Lane # 11 | 33 mon APAP (20µl) |
| Lane # 12 | Positive Control (4µl+4µl+4µl+4µl) |



Place Scan of Film Here

### Properties

Exposure time  5 min    Molecular Wieght  60 k Da    ☑ Used For Analysis

Location of Scan    \\Blough-raid\exp-data\Project folder\McNeil Aorta\Films

Location of Excell Data    \\Blough-raid\exp-data\Project folder\McNeil Aorta\Excel

Notes

_____

Your Signature

email Report

*Raw data*

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

Phospho Src 527 in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 33.3 | 29.37332 | 24.70125 |
| %C | 37.7 | 25.31015 | 25.08721 |
| %C | 34.2 | 28.95007 | 24.3153 |
| %C | 32.5 | 29.71192 | 25.08721 |
| %C | 36 | 25.98735 | 25.70474 |
| %C | 34.3 | 29.03472 | 24.2381 |
| N | 6 | 6 | 6 |
| Mean | 34.66667 | 28.06126 | 24.85564 |
| SD | 1.891736 | 1.900184 | 0.552337 |
| SEM | 0.84601 | 0.849788 | 0.247013 |
|  | 6 m CON | 33m CON | 33m APAP |
| relative expression level | 1 | 0.809459 | 0.716989 |
| SEM | 0.024404 | 0.024513 | 0.007125 |
|  | 6 m CON | 33m CON | 33m APAP |
| %RE | 100 | 80.94593 | 71.69895 |
| SE | 2.440414 | 2.451312 | 0.712536 |

**Statistics**

**One Way Analysis of Variance**

**Data source:** Data 18 in McNeil Aorta ROS(Normalized)

**Normality Test:**     Passed (P = 0.576)

**Equal Variance Test:**     Passed (P = 0.361)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 34.667 | 1.892 | 0.772 |
| 33m CON | 6 | 0 | 28.061 | 1.900 | 0.776 |
| 33m APAP | 6 | 0 | 24.856 | 0.552 | 0.225 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 300.328 | 150.164 | 60.110 | <0.001 |
| Residual | 15 | 37.472 | 2.498 | | |
| Total | 17 | 337.800 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| 6 m CON vs. 33m APAP | 9.811 | 3 | 15.205 | <0.001 | Yes |
| 6 m CON vs. 33m CON | 6.605 | 2 | 10.237 | <0.001 | Yes |
| 33m CON vs. 33m APAP | 3.206 | 2 | 4.968 | 0.003 | Yes |

# Laboratory of Molecular Physiology

## Film Properties Report

| | |
|---|---|
| Experimenter | Sarath Meduru |
| Date | 2006/11/08 |
| Project | McNeil |
| Tissue/ cell line/ etc. | Aorta |
| Report Number | |
| Protien Concentration | 30 µg (1.5µg / µl X 20µl) |
| Gel type | 10% Tris - Glycine Gel |

Electrophoresis Voltage 125 volts    Duration _____    Transfer Voltage 24 volts    Duration 45 min

### Primary Antibody

Type Bcl-2                      Dilution 1:500

Media 5% BSA in TBST

Incubation time Over Night

### Secondary Antibody

Type Anti Mouse            Dilution 1:500

Media 5% Milk in TBST

Incubation time 1 hr

| | |
|---|---|
| Lane # 1 | Biotin Ladder (8µl+8µl) |
| Lane # 2 | Color Marker (8µl) |
| Lane # 3 | 6 mon Control (20µl) |
| Lane # 4 | 33 mon Control (20µl) |
| Lane # 5 | 33 mon APAP (20µl) |
| Lane # 6 | 6 mon Control (20µl) |
| Lane # 7 | 33 mon Control (20µl) |
| Lane # 8 | 33 mon APAP (20µl) |
| Lane # 9 | 6 mon Control (20µl) |
| Lane # 10 | 33 mon Control (20µl) |
| Lane # 11 | 33 mon APAP (20µl) |
| Lane # 12 | Positive Control (4µl+4µl+4µl+4µl) |

Place Scan of Film Here

### Properties

Exposure time 5 min          Molecular Wieght 26 k Da          ☑ Used For Analysis

Location of Scan          \\Blough-raid\exp-data\Project folder\McNeil Aorta\Films

Location of Excell Data          \\Blough-raid\exp-data\Project folder\McNeil Aorta\Excel

Notes

Your Signature

email Report

89

*Raw data*

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

Bcl-2 in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 27.1 | 33.77509 | 25.39597 |
| %C | 27.1 | 29.71192 | 29.17835 |
| %C | 23.8 | 32.50535 | 29.10116 |
| %C | 33.7 | 31.48955 | 22.4627 |
| %C | 30.8 | 29.71192 | 26.32227 |
| %C | 26.6 | 29.88122 | 29.33274 |
| N | 6 | 6 | 6 |
| Mean | 28.18333 | 31.17917 | 26.96553 |
| SD | 3.502808 | 1.707876 | 2.764559 |
| SEM | 1.566504 | 0.763785 | 1.236348 |
|  | 6 m CON | 33m CON | 33m APAP |
| relative expression level | 1 | 1.106298 | 0.95679 |
| SEM | 0.055583 | 0.027101 | 0.043868 |
|  | 6 m CON | 33m CON | 33m APAP |
| %RE | 100 | 110.6298 | 95.67901 |
| SE | 5.558262 | 2.71006 | 4.386807 |

**Statistics**

**One Way Analysis of Variance**

**Data source:** Data 20 in McNeil Aorta ROS(Normalized)

**Normality Test:**     Passed (P = 0.169)

**Equal Variance Test:**     Passed (P = 0.636)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 28.183 | 3.503 | 1.430 |
| 33m CON | 6 | 0 | 31.179 | 1.708 | 0.697 |
| 33m APAP | 6 | 0 | 26.966 | 2.765 | 1.129 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 56.426 | 28.213 | 3.707 | 0.049 |
| Residual | 15 | 114.146 | 7.610 | | |
| Total | 17 | 170.572 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = 0.049).

Power of performed test with alpha = 0.050: 0.446

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| 33m CON vs. 33m APAP | 4.214 | 3 | 3.742 | 0.046 | Yes |
| 33m CON vs. 6 m CON | 2.996 | 2 | 2.660 | 0.080 | No |
| 6 m CON vs. 33m APAP | 1.218 | 2 | 1.081 | 0.457 | No |

# Laboratory of Molecular Physiology

| | |
|---|---|
| Experimenter | Sarath Meduru |
| Date | |
| Project | McNeil |
| Tissue/ cell line/ etc. | Aorta |
| Report Number | |
| Protien Concentration | 30 µg (1.5µg / µl X 20µl) |
| Gel type | 10% Tris - Glycine Gel |

## Film Properties Report

Electrophoresis Voltage 125 volts   Duration _____   Transfer Voltage 24 volts   Duration 45 min

### Primary Antibody

Type Bax   Dilution 1:500
Media 5% BSA in TBST
Incubation time 1 hr

### Secondary Antibody

Type Anti Rabbit   Dilution 1:500
Media 5% Milk in TBST
Incubation time 1 hr

Lane # 1  Biotin Ladder (8µl+8µl)
Lane # 2  Color Marker (8µl)
Lane # 3  6 mon Control (20µl)
Lane # 4  33 mon Control (20µl)
Lane # 5  33 mon APAP (20µl)
Lane # 6  6 mon Control (20µl)
Lane # 7  33 mon Control (20µl)
Lane # 8  33 mon APAP (20µl)
Lane # 9  6 mon Control (20µl)
Lane # 10  33 mon Control (20µl)
Lane # 11  33 mon APAP (20µl)
Lane # 12  Positive Control (4µl+4µl+4µl+4µl)


Place Scan of Film Here

### Properties

Exposure time 5 min   Molecular Wieght 23 k Da   ☑ Used For Analysis
Location of Scan   \\Blough-raid\exp-data\Project folder\McNeil Aorta\Films
Location of Excell Data   \\Blough-raid\exp-data\Project folder\McNeil Aorta\Excel

Notes

email Report

Your Signature

*Raw data*

This section represents the raw data tables produced from spot densitometry of the

immunoblot films.

Bax in Aorta

Raw IOD values (relative percentage)

|  | 6 m CON | 33m CON | 33m APAP |
|---|---|---|---|
| %C | 36.3 | 26.74919 | 24.77844 |
| %C | 29.3 | 25.3948 | 31.41691 |
| %C | 42 | 29.45797 | 17.90841 |
| %C | 39.8 | 24.12506 | 24.46968 |
| %C | 29.5 | 24.97155 | 31.72567 |
| %C | 43.9 | 27.34174 | 18.44875 |
| N | 6 | 6 | 6 |
| Mean | 36.8 | 26.34005 | 24.79131 |
| SD | 6.264822 | 1.926896 | 5.99483 |
| SEM | 2.801714 | 0.861734 | 2.680969 |
|  |  |  |  |
| relative expression level | 1 | 0.715762 | 0.673677 |
| SEM | 0.076134 | 0.023417 | 0.072852 |
|  |  |  |  |
| %RE | 100 | 71.57623 | 67.36769 |
| SE | 7.613353 | 2.341669 | 7.285243 |

**Statistics**

**One Way Analysis of Variance**

**Data source:** Data 15 in McNeil Aorta ROS(Normalized)

**Normality Test:**    Passed (P = 0.662)

**Equal Variance Test:**    Passed (P = 0.082)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 6 m CON | 6 | 0 | 36.800 | 6.265 | 2.558 |
| 33m CON | 6 | 0 | 26.340 | 1.927 | 0.787 |
| 33m APAP | 6 | 0 | 24.791 | 5.995 | 2.447 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 2 | 512.036 | 256.018 | 9.735 | 0.002 |
| Residual | 15 | 394.495 | 26.300 | | |
| Total | 17 | 906.530 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = 0.002).

Power of performed test with alpha = 0.050: 0.939

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

| Comparison | Diff of Means | p | q | P | P<0.050 |
|---|---|---|---|---|---|
| **6 m CON vs. 33m APAP** | **12.009** | **3** | **5.736** | **0.003** | **Yes** |
| **6 m CON vs. 33m CON** | **10.460** | **2** | **4.996** | **0.003** | **Yes** |
| 33m CON vs. 33m APAP | 1.549 | 2 | 0.740 | 0.609 | No |

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