ADAPTIVE RESPONSES OF MURINE SKELETAL MUSCLE TO CONTRACTILE ACTIVITY: THE EFFECT OF AGE AND GENDER

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Abstract

Exposure of cells to a wide range of stresses results in a highly synchronised genetically determined response. This is initiated by detection of the stress, leading to a regulatory response that involves the elevated synthesis of specific proteins (such as antioxidant defence enzymes and Heat Shock Proteins). This adaptation helps to re-establish cellular homeostasis. Previous data indicate that the response of many cells from all ageing species to stress is severely reduced.

The aim of this study was to characterise the extent and time course of production of antioxidant defence enzymes and HSPs in skeletal muscles of adult and aged B6XSJL mice following non-damaging isometric contractions and to examine the mechanisms responsible for attenuation of the stress response in skeletal muscle of aged mice following contractions.

Adult and aged, male and female B6XSJL mice were subjected to a 15-minute period of isometric contractions. Mice were killed at different time points and skeletal muscles from non-exercised and exercised mice were analysed for the activities of antioxidant defence enzymes, heat shock protein content, transcription factor binding activity and gene expression using spectrophotometric techniques, SDS-PAGE electrophoresis, western blotting, electrophoresis mobility shift assay (EMSA), northern blotting and cDNA microarrays.

Data demonstrated that the contraction protocol did not result in overt damage to muscles of adult or aged mice. The catalase and superoxide dismutase (SOD) activities and HSP content of muscles from adult male mice increased significantly following the contraction protocol. This response was not evident in muscles of aged male mice. In contrast, the catalase and SOD activities and HSP content of muscles from adult or aged female mice were not significantly altered following the contraction protocol.

In muscles of aged male mice, binding of HSF1 to the Heat Shock Element (HSE) binding domain was not grossly altered, although the heat shock protein and mRNA content (e.g. HSP25) of muscles of aged mice were significantly attenuated compared with that of muscles of adult mice following the contraction protocol. In contrast, no increase in the binding of NF-κB and AP-1 to the NF-κB and AP-1 binding domains was seen in muscles from aged male mice.

Data suggest that muscles from adult male mice adapt rapidly following a period of mild, non-damaging isometric contractions and that this adaptation is attenuated with age. The attenuated binding of NF-κB and AP-1 to an idealised DNA sequence suggests that this may be, at least in part, the mechanism by which production of antioxidant defence enzymes is altered in muscles of aged male mice following the contraction protocol. This attenuation in the adaptive response may play a major role in the development of age – related functional deficits in muscle.

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Abbreviations

µg	microgramme
µl	microlitre
µM	micromolar
µmol	micromole
µm	micrometer
ADP	adenosine diphosphate
AP-1	activator protein 1
APS	ammonium persulphate solution
AT	anterior tibialis
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
°C	degrees Celsius
CAT	catalase
CK	creatine kinase
cm	centimetre
CR	caloric restriction
Cu/Zn SOD	copper/zinc superoxide dismutase
dH_2O	distilled water
DNA	deoxyribonucleic acid
DTNB	5-5'-dithiobis- (2-nitrobenzoic acid)
DTT	dithiothreitol
DVL	deep vastus lateralis
Ec-SOD	extracellular superoxide dismutase
EDL	extensor digitorum longus
EDTA	ethylene diamine tetra-acetic acid
EMSA	electrophoresis mobility shift assay
ER	endoplasmic reticulum
Fe-SOD	iron superoxide dismutase
g	grammes
g	centrifugal force

GPX	glutathione peroxidase
GSH	reduced glutathione
GSSG	oxidised glutathione
h	hours
HSE	heat shock element
HSF	heat shock factor
HSP	heat shock protein
H_2O_2	hydrogen peroxide
kDa	kilodaltons
kg	kilogramme
M	Molar
mg	milligramme
min	minute
ml	millilitre
mM	millimolar
mmol	millimole
Mn-SOD	manganese superoxide dismutase
mRNA	messenger RNA
msec	millisecond
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa B
nm	nanometre
$NO^{\bullet}$	nitric oxide
NOS	nitric oxide synthase
$O_2^{\bullet -}$	superoxide radical
$OH^{\bullet}$	hydroxyl radical
$ONOO^-$	peroxynitrite
PBS	phosphate-buffered saline
PMSF	phenylmethylsulphonyl fluoride
RNA	ribonucleic acid
RNS	reactive nitrogen specoes
R-O/N-S	reactive oxygen/nitrogen species

ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SE	standard error
sec	second
-SH	thiol or sulphydryl groups
SOD	superoxide dismutase
SSA	sulphosalicylic acid
TEMED	NNN'N'- tetramethylethylene-diamine
Tween	polyoxyethylene-sorbitan monolaurate
UV	ultraviolet light
XO	xanthine oxidase
>	greater than
<	less than

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Dedication

To my Dad

(Στη λατρεια μου...)

CHAPTER 1

INTRODUCTION

1.1. SKELETAL MUSCLE

Skeletal muscle comprises 40-50% of the human body. The muscle bulk is composed of several kinds of tissue including muscle tissue, nerves, blood vessels and various types of connective tissue (Powers and Howley, 1994). Skeletal muscles are connected to the bones in the legs, arms and spine by tendons and they generate rapid movements by contracting quickly. Skeletal muscles are responsible for posture, as well as complex coordinated activities such as running or swimming.

1.1.1. Structure of skeletal muscle

Skeletal muscles consist of long parallel bundles of multinucleated cells called muscle fibres (Figure 1.1a), which are formed during development by the fusion of numerous precursor cells known as myoblasts. The plasma membrane of each muscle fibre is called the sacrolemma.

Each muscle fibre is, in turn, composed of parallel bundles of approximately one thousand myofibrils of 1 to 2 μm in diameter (Figure 1.1b), which may extend the full length of a fibre (Voet and Voet, 1990). Myofibrils are the contractile elements of the muscle cell.

Each myofibril can be further subdivided into alternating light and dark bands that run along the length of the muscle cell. The dark bands, called *A* bands are divided by a dark line, the *M* line, while the light bands are divided by a different dark line, the *Z* disc (Figure 1.1c, Figure 1.1d). The segment from one *Z* disc to the next is termed a sacromere (Alberts *et al*, 1994).

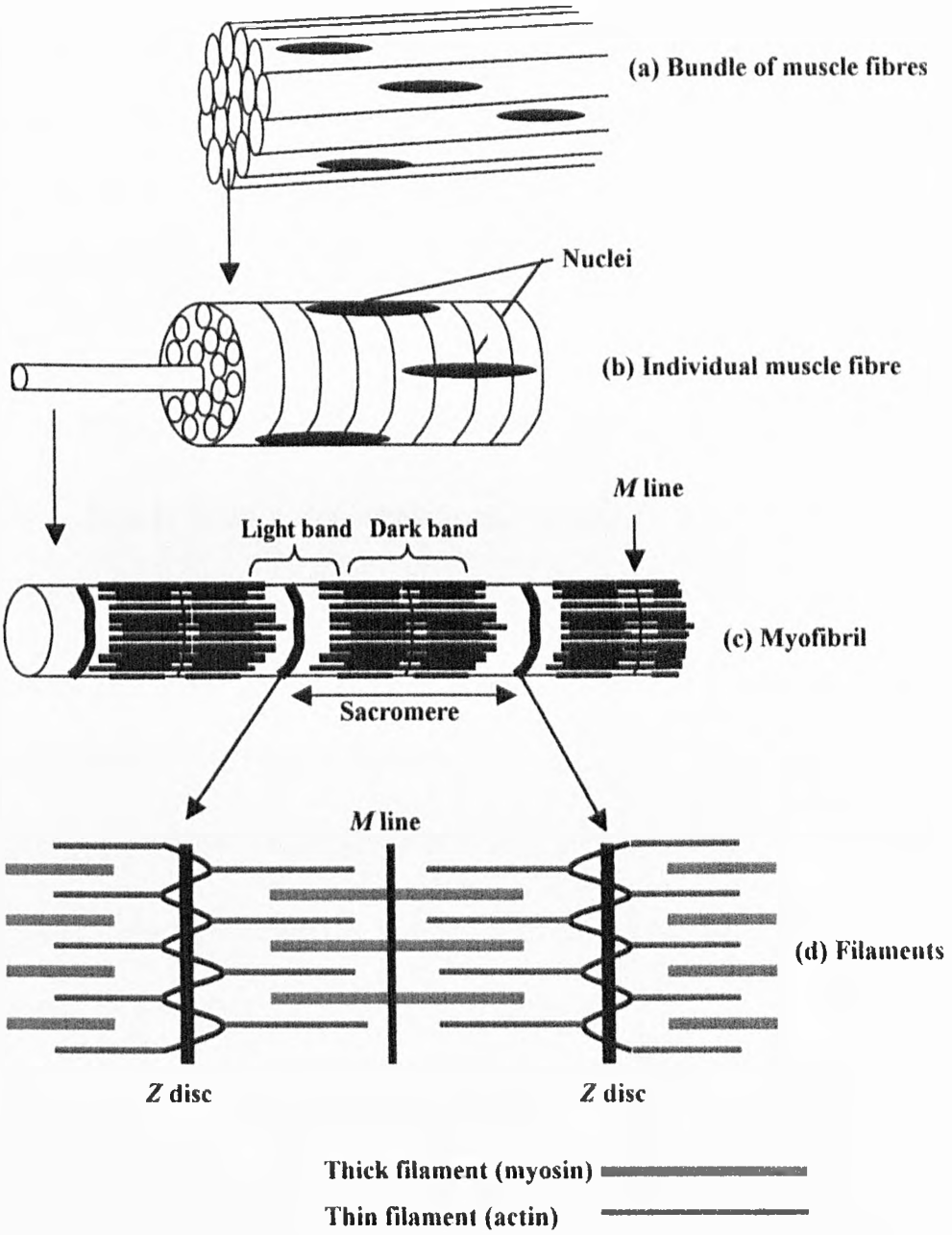


Figure 1.1. Organisation of skeletal muscle.

Each sacromere contains two types of protein filaments: thick filaments composed of the protein myosin and thin filaments composed of the protein actin (Figure 1.1d). Thin filaments are also associated with two additional proteins, troponin and tropomyosin. These proteins play an important role in the regulation of muscle contraction.

Myosin filaments are located primarily within the dark band of the sacromere whereas actin filaments are located in the light band of the sacromere. However, actin filaments overlap the myosin filaments in the *A* band (Lodish *et al*, 1996). The arrangement of these two protein filaments gives skeletal muscle its striated appearance.

1.1.2. Definitions of terms used to describe contractile characteristics

Force: Muscle mass x Acceleration due to gravity (g). Force is measured in Newtons (N).

Specific force: The maximum force generation per cross-sectional area. Specific force is usually measured in kN/mm^2.

Muscular strength: The ability of muscle or a group of muscles to generate force.

Tension: This term is usually used to describe the resting force value i.e. resting tension. Hence, tension is measured in Newtons.

Fatigue: Fatigue is defined as an inability to maintain a power output or force during repeated muscle contractions.

Definitions were taken from Gibson and Edwards (1985), Jones and Round (1990) and Powers and Howley (1994).

1.1.3. Contraction of skeletal muscle - the sliding filament hypothesis

Muscular contraction is a complex process based on the sacromere shortening by the sliding of myosin filaments past the actin filaments with no change in the length of either type of filaments (Figure 1.2; Alberts *et al*, 1994). This sliding filament theory was first proposed in 1954 by Huxley and Niedergerke and was crucial to understanding the contractile mechanism.

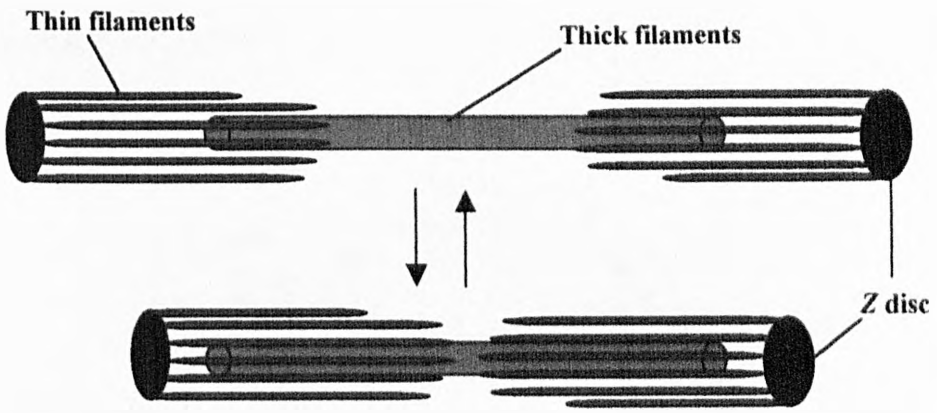


Figure 1.2. The sliding filament hypothesis of muscle contraction; the actin and myosin filaments slide past one another without shortening (Reproduced from Alberts *et al*, 1994).

During muscular contraction, numerous cross-bridges extend out from myosin and make contact with adjacent actin filaments. These cross-bridges are myosin heads, which contain an actin-binding site and a catalytic site that hydrolyses ATP (Ganong, 1993). The myosin heads face in opposite directions on either side of the bare central region of a myosin filament. Thus, when the heads attach to actin on either side of the sacromere they can pull the actin filaments from each side towards the centre. This movement results in muscle shortening and force generation, which is proportional to the overlap between the two filament systems (Lodish *et al*, 1996).

Skeletal muscle contraction is triggered by motor nerve impulses. When a nerve impulse arrives to the sacroplasmic reticulum, it causes the release of calcium (Schmalbruch, 1985). The calcium binds to troponin with the resulting conformational change causing tropomyosin to move, thereby exposing the myosin head-binding sites of actin. Hydrolysis of ATP to ADP + P_i by the ATPase enzyme, releases energy that is used to "cock" the myosin cross-bridges, which in turn pull the actin molecules over myosin and thus shorten the muscle (Ganong, 1993; Figure 1.3).

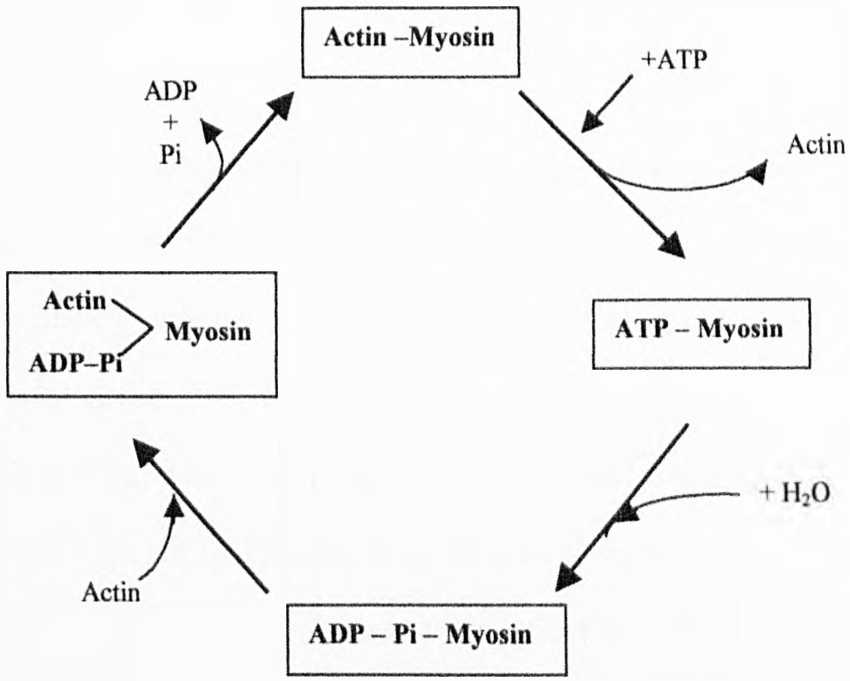


Figure 1.3. The hydrolysis of ATP drives the cyclic association and dissociation of actin and myosin (Reproduced from Voet and Voet, 1990).

In the absence of nerve impulse, calcium is taken up by the sacroplasmic reticulum. This removal of calcium causes tropomyosin to return and cover the binding sites on actin, resulting in the termination of muscle contraction (Powers and Howley, 1994).

1.1.4. Fibre types of skeletal muscle

Skeletal muscle fibres have been grouped into two main types according to the performance and biochemical characteristics of the individual muscle cells: type I (or slow-twitch) and type II (intermediate or fast-twitch).

Type I fibres (also called slow oxidative fibres) contain larger numbers of mitochondria and higher concentrations of the red pigment myoglobin. Therefore, muscles containing many type I fibres are also called red muscles due to their darker colour. Red muscles contract relatively slowly, have a high capacity for aerobic metabolism and higher resistance to fatigue (Powers and Howley, 1994; Ganong, 1993).

Type II fibres are divided into several sub-groups including type IIA and type IIB. Type IIB fibres (also called fast-glycolytic fibres) contain smaller numbers of mitochondria, have a limited capacity for aerobic metabolism and less resistance to fatigue. However, type IIB fibres contain large amounts of glycolytic enzymes providing them with a high capacity for anaerobic metabolism. (Powers and Howley, 1994). Muscles containing many type IIB fibres are known as white muscles; these muscles have short twitch duration and are specialised for fine, rapid and precise movements (Ganong, 1993).

Type IIA fibres (also called fast-oxidative fibres) have physiological properties that fall between type I and type IIB fibres. They are relatively fatigue resistant with intermediate levels of glycolytic activity. The properties of the three main fibre types in skeletal muscle are summarised in Table 1.1.

Table 1.1. **Classification of fibre types in skeletal muscles (Modified from Powers and Howley, 1994).**

	TYPE I	TYPE IIB	TYPE IIA
Other names	**Slow oxidative; red**	**Fast glycolytic; white**	**Fast oxidative**
Twitch speed	Slow	Fast	Fast
ATPase activity	Low	High	High
Glycolytic activity	Moderate	High	Moderate to high
Myoglobin content	High	Low	High
Resistance to fatigue	High	Low	Intermediate
Predominant energy system	Aerobic	Anaerobic	Combination

1.1.5. Types of contraction of skeletal muscle

When a single stimulus is given to a muscle, the muscle responds with a simple twitch (Figure 1.4a). This twitch is divided into three phases; a latent period prior to the beginning of contraction, a contraction period and a relaxation period. The duration and the maximum force generated by the twitch varies with the type of the muscle. Thus, type II fibres contract in a shorter time period when stimulated than type I fibres (Figure 1.4.a). With rapidly repeated stimulations (Figure 1.4b), individual responses fuse in one continuous contraction prior to any relaxation. This response is known as tetanus during which, maximum force is produced (Figure 1.4c). Muscular contractions that occur during normal body movements are primarily tetanic contractions (Ganong, 1993).

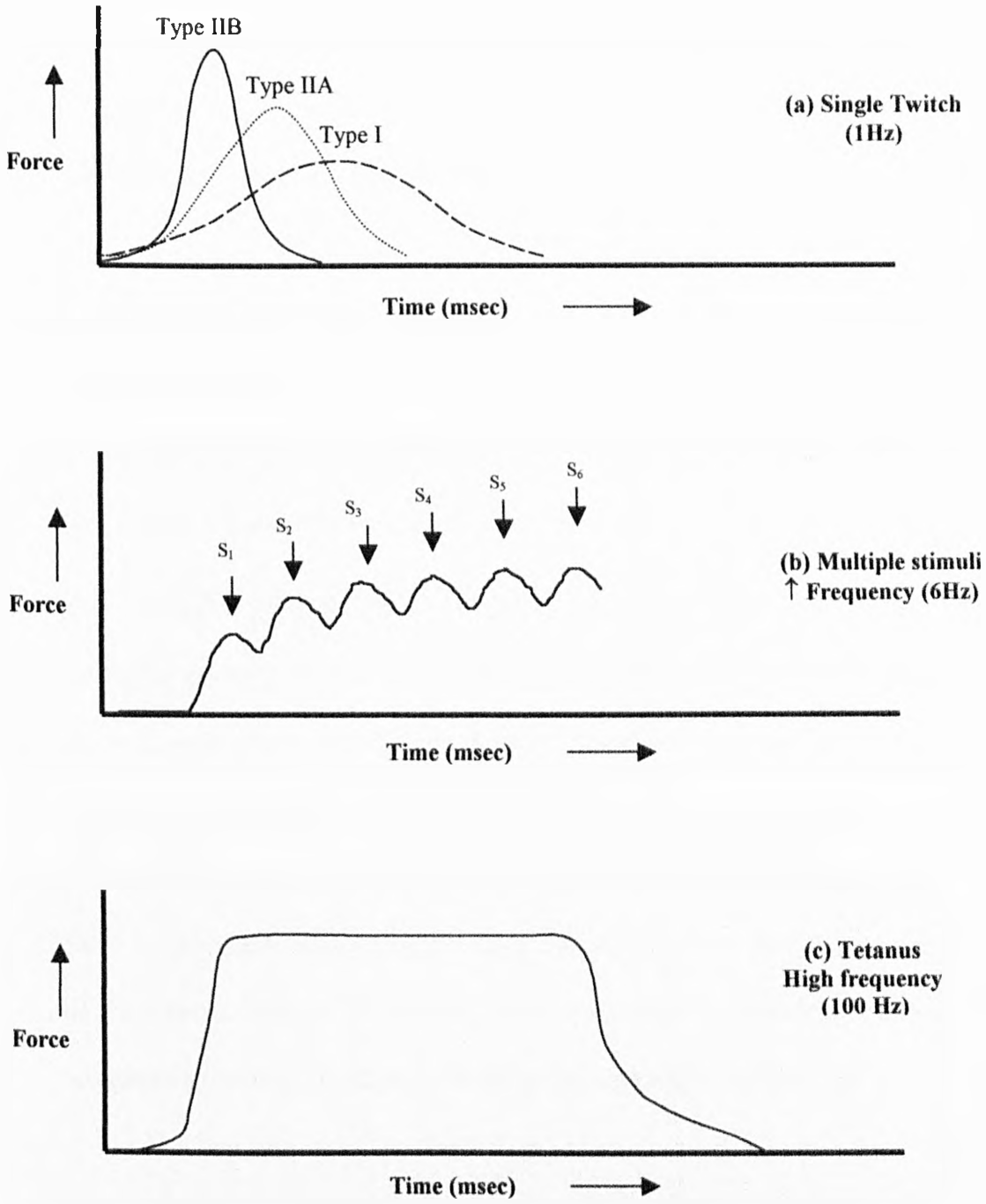


Figure 1.4. Schematic diagram of muscle contraction. (a) Maximum force produced by a single twitch in three different muscle fibres (b) Increasing the frequency of the stimulus results in a summation of twitches (c) High frequency results in tetanus.

Activation of muscle movement involves three types of contraction:

a) Shortening, concentric or dynamic contractions, where muscle allows shortening of the contractile elements during activation. Shortening contractions are common in most types of exercise.

b) Isometric or static contractions, where muscle contracts without an appreciable change in the length of the whole muscle. Clenching of fists or tensing of muscles are common examples where muscles undergo isometric contractions, in which pairs of contracting muscles work to oppose each other and thus cancel out any movement (Lobish *et al*, 1996).

c) Lengthening, eccentric or pliometric contractions, where the muscle lengthens during activation. It has been documented that lengthening contractions cause considerably more damage to skeletal muscle than shortening or isometric contractions (Faulkner *et al*, 1990b). The main difference between lengthening contractions and shortening and isometric contractions is that more tension per fibre is generated during lengthening contractions, since the cross-sectional area of the fibre is reduced (Armstrong, 1991). However, the mechanisms underlying lengthening contraction-induced damage are not clearly understood.

1.2. FREE RADICAL PRODUCTION BY SKELETAL MUSCLE

The cardiovascular benefits of regular muscular exercise have been well documented. However, physical exercise is associated with a dramatic increase in oxygen intake. Most of the oxygen is utilised in the mitochondrial electron transport chain, where it is reduced to water (Ji, 1995). However, about 2 to 3% of oxygen may be converted into highly reactive intermediates called free radicals. Free radicals are defined as atoms or compounds capable of independent existence and having at least one unpaired electron in the outer orbital (Halliwell, 1994). Free radical species released from skeletal muscle include reactive oxygen species (ROS) such as $O_2^{\bullet -}$ as well as reactive nitrogen species (RNS) such as $NO^{\bullet}$ (Table 1.2).

Table 1.2. **Examples of reactive oxygen/nitrogen species in skeletal muscle and their major sources (Modified from Lawler and Powers, 1998).**

R-O/N-S	PRIMARY SOURCE
Hydroxyl radical ($^{\bullet}OH$)	$O_2^{\bullet -}$ H_2O_2
Superoxide anion ($O_2^{\bullet -}$)	Electron transport chain; Xanthine oxidase; NAD(P)H oxidase
Nitric oxide ($NO^{\bullet}$)	Nitric oxide synthases
Peroxynitrite ($ONOO^-$)	$O_2^{\bullet -}$+ $NO^{\bullet}$
Hydrogen peroxide (H_2O_2)	$O_2^{\bullet -}$

1.2.1. Reactive oxygen species

1.2.1.1. Superoxide anion radical

The superoxide anion radical results from the one electron reduction of oxygen. A major cellular source of $O_2^{\bullet-}$ is the mitochondria where it is produced as a by-product of the mitochondrial electron transport chain (Davies, 1982; Halliwell *et al*, 1992). Superoxide radical can also be produced enzymatically by cytochrome P450 oxidase and by xanthine oxidase (XO) produced by xanthine dehydrogenase due to changes in calcium homeostasis (Ji, 1999; Reid, 2001). Other sources of $O_2^{\bullet-}$ include NAD(P)H oxidase activity, autoxidation reactions in which compounds such as catecholamines and reduced flavins react directly with O_2 to form $O_2^{\bullet-}$ and the nitric oxide synthases (NOS; Halliwell, 1998; Reid, 2001). The superoxide anion radical is highly reactive and undergoes electron exchange reactions that result in the production of hydrogen peroxide, hydroxyl radicals and other redox active derivatives and it has been claimed to inactivate the NADH dehydrogenase complex of the mitochondrial transport chain (Halliwell, 1992).

In skeletal muscle, superoxide radicals are also found in the extracellular fluid and are produced at a relatively low rate with a significant increase during contractile activity (Reid, 1992; McArdle *et al*, 2001).

1.2.1.2. Hydroxyl radical

One of the most reactive free radicals is the hydroxyl radical ($^{\bullet}OH$). Initially, many studies *in vitro* suggested that $O_2^{\bullet-}$ and H_2O_2 react to form $^{\bullet}OH$ according to a reaction known as the iron-catalysed Haber-Weiss reaction. The Haber-Weiss reaction is a sum of the following two reactions:

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$$

Net:

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{Fe\text{-}catalyst} {}^{\bullet}OH + OH^- + O_2$$

However, the rate constant for this reaction in aqueous solution is virtually zero (Halliwell *et al*, 1992; Halliwell and Gutteridge, 1999).

In vivo, hydroxyl radical generation might occur via several pathways, such as by Fenton-type chemistry according to the following reaction:

$$Fe^{2+} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$$

Copper ions may also react with H_2O_2 to form $^{\bullet}OH$.

Hydroxyl radical reacts at a diffusion-controlled rate with almost all molecules in living cells (Halliwell, 1998). Hydroxyl radicals have an extremely short half-life and are not thought to migrate any significant distance within a cell. They damage any molecule present in their immediate vicinity, including proteins, carbohydrates, DNA and lipids (Halliwell, 1994; Michiels *et al*, 1994).

1.2.1.3. Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is not a free radical. *In vivo*, H_2O_2 is mainly produced enzymatically by the dismutation of $O_2^{\bullet-}$ according to the following reaction:

$$2O_2^{\bullet-} + 2H^+ \xrightarrow[Dismutase]{Superoxide} H_2O_2 + O_2$$

Hydrogen peroxide is highly diffusible between and within cells and it is a weak oxidising agent, less reactive than the superoxide radical (Reid, 2001). However, H_2O_2 can inactivate some enzymes directly by oxidation of essential thiol (-SH) groups at the active site; H_2O_2 can also oxidise certain keto-acids such as pyruvate (Halliwell and Gutteridge, 1999). Furthermore, H_2O_2 can lead to $^{\bullet}OH$ generation (section 1.2.1.2). Thus, despite its relatively weak reactivity, hydrogen peroxide is cytotoxic (Halliwell, 1998).

1.2.1.4. Nitric oxide

Nitric oxide is generated continuously in skeletal muscle. Resting muscles produce low levels of $NO^{\bullet}$ and the production of $NO^{\bullet}$ is increased during contractile activity (Balon and Nadler, 1994; Reid, 1996).

Nitric oxide is largely synthesised *in vivo* from the amino acid L-arginine by the nitric oxide synthases (NOS), a family of enzymes expressed in skeletal muscle fibres. The NOS family consists of three different isoforms; the neuronal-type NOS isoform (nNOS) localised on or near the cell membrane, the endothelial- type NOS (eNOS), which is associated with mitochondria and the inducible-type NOS isoform (iNOS). Both nNOS and eNOS are constitutively expressed, however their activity varies among muscles. Generally, nNOS and eNOS activities are higher in fast-twitch muscles than in slow (Reid, 1996; Reid, 2001).

In contrast to the harmful effects of other ROS, such as $^{\bullet}OH$, increased generation of $NO^{\bullet}$ is thought to be useful. Nitric oxide is a vasodilator and an anti-thrombotic agent, as well as a neurotransmitter (Halliwell, 1998).

Excess levels of nitric oxide can be cytotoxic and over-production is thought to be involved in several inflammatory diseases (Halliwell, 1998). Furthermore, nitric oxide can also react with $O_2^{\bullet-}$ to form peroxynitrite ($ONOO^-$), a free radical species that is far more reactive than either of the parent radicals (Reid, 1996). A scheme of proposed oxidant generation by skeletal muscle is shown in Figure 1.5.

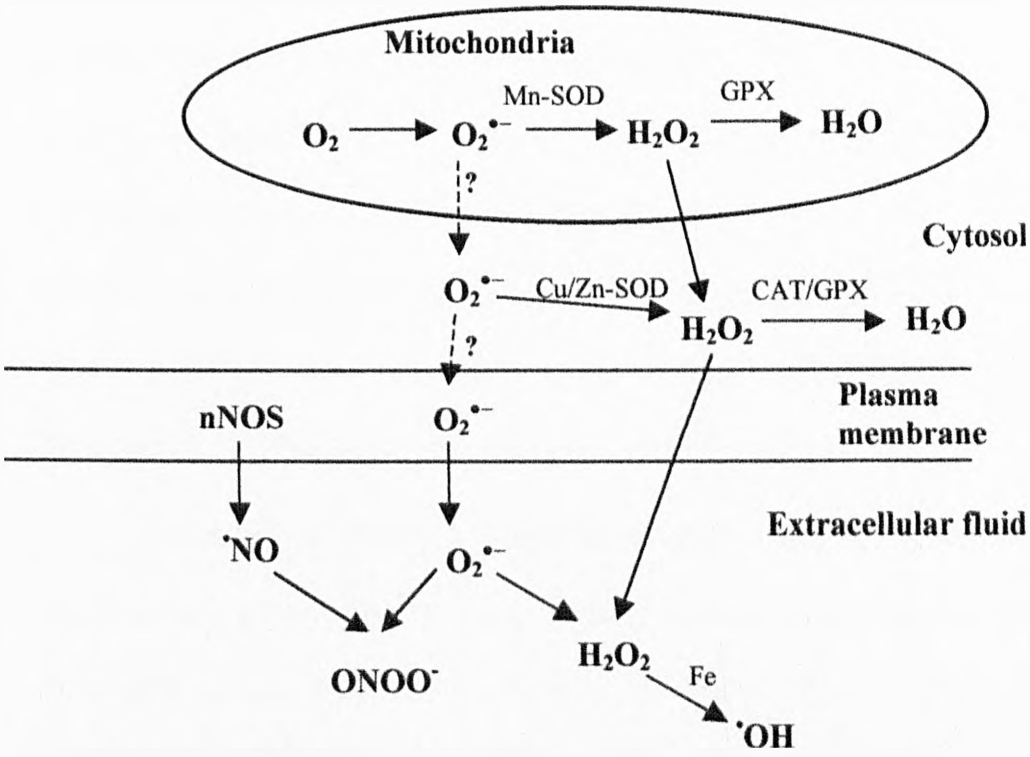


Figure 1.5. Pathways of ROS formation in skeletal muscle. $O_2^{\bullet-}$: superoxide radical; $^{\bullet}OH$: hydroxyl radical; H_2O_2: hydrogen peroxide; $NO^{\bullet}$: nitric oxide; $ONOO^-$: peroxinitrite anion; nNOS: neuronal-type nitric oxide synthase; Mn-SOD: Manganese superoxide dismutase; Cu/Zn-SOD: Copper/Zinc superoxide Dismutase; CAT: catalase; GPX: glutathione peroxidase (Jackson, personal communication).

1.2.2. Reactive oxygen species and exercise

A number of researchers have attempted to identify whether exercise results in increased ROS generation. Davies *et al*, (1982) and Jackson *et al* (1985) found an increase in the electron paramagnetic resonance (EPR) signals in contracting rat muscles compared with resting muscles. Reid *et al* (1992) have reported that isolated strips of rat diaphragm released superoxide anion radicals into the external medium during isometric contraction. In an *in situ* model, O'Neill *et al* (1996) demonstrated that isometric contracting cat skeletal muscle generated $^{\bullet}OH$ radicals that were detectable in the muscle microvasculature. More recently, our laboratory has shown that fifteen minutes of isometric contractile activity induced a rapid release of superoxide anion radicals from mouse skeletal muscle *in vivo* and studies using contracting cultured primary skeletal muscle myotubes confirmed that this release was from muscle cells rather than other cell types present within the muscle (McArdle *et al*, 2001).

In summary, evidence suggests that increased contractile activity results in increased production of ROS. Many authors have suggested that this increased production of free radicals is responsible for exercise-induced muscle damage. However, a comparison of the patterns of oxidant generation and muscle damage argues against this; the oxygen flux through the mitochondria is considerably greater during shortening and isometric contractions, whereas by far the most damaging form of contractile activity is by lengthening contractions (Newham *et al*, 1983) during which, oxygen flux through the mitochondria is relatively low.

The reason why these sudden changes in ROS production do not result in skeletal muscle damage may be due to the fact that skeletal muscle cells have a

complex antioxidant defence mechanism to provide protection against fluctuations in the production of ROS at resting conditions and during increased activity.

1.3. ADAPTIVE RESPONSES IN SKELETAL MUSCLE

During oxidative stress, ROS can oxidise virtually all macromolecules, including proteins, nucleic acids, carbohydrates and lipids. However, cells have developed mechanisms to respond to the increased ROS generation as an adaptation to protect themselves against potential subsequent damaging results. This complex machinery involves the transcription of antioxidant defence enzymes, such as catalase and superoxide dismutase, as well as the production of a family of proteins known as stress or heat shock proteins (HSPs). The production of these proteins is termed the "stress response".

1.3.1. Antioxidant defences of skeletal muscle

Cellular antioxidant defences are conventionally classified into two categories; enzymatic defences and non-enzymatic antioxidants. The first includes antioxidant defence enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) that are capable of neutralising reactive oxygen metabolites (Powers *et al*, 1999). The second includes low molecular weight molecules such as glutathione, vitamin C, vitamin E and β-carotene. Table 1.3 summarises the cellular location and function of each of the antioxidant defence enzymes. Table 1.4 summarises the cellular location and properties of important non-enzymatic antioxidants.

Table 1.3. **Action and cellular location of antioxidant defence enzymes (Modified from Powers and Lennon, 1999).**

ENZYMES	CELLULAR LOCATIONS	ANTIOXIDANT PROPERTIES
Mn-SOD	Mitochondria	Dismutates superoxide anion radicals
Cu/Zn-SOD	Cytosol	Dismutates superoxide anion radicals
Ec-SOD	Extracellular fluid	Dismutates superoxide anion radicals
CAT	Mitochondria and cytosol	Removes H_2O_2
GPX	Mitochondria and cytosol	Removes H_2O_2 and organic hydroperoxides

Table 1.4. **Properties and cellular location of non-enzymatic antioxidants (Modified from Powers and Lennon, 1999).**

NON-ENZYMATIC ANTIOXIDANT	CELLULAR LOCATIONS	PROPERTIES
GSH	Mitochondria and cytosol	Interacts with a variety of radicals by donating H
Vitamin C	Cytosol	Quenches a wide variety of aqueous-phase ROS
Vitamin E	Membranes	Major lipid peroxidation chain-breaking antioxidant
Carotenoids	Primarily in membranes	Reduce lipid peroxidation

1.3.1.1. Superoxide dismutase

Superoxide dismutase (SOD) was discovered by McCord and Fridovich in 1969. SOD represents a family of metalloenzymes found in all oxygen-utilizing organisms. SODs play an important role in the cell because they catalyse the dismutation of superoxide anion radicals into oxygen and hydrogen peroxide, according to the following reaction:

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Thus, they constitute a defence against oxygen toxicity (Crapo *et al*, 1978).

There are three types of SOD, depending on the metal ion bound to its active site; Copper and zinc-containing SOD (Cu/Zn-SOD or *Sod1*), manganese-containing SOD (Mn-SOD or *Sod2*) and iron-containing SOD (Fe-SOD) found only in bacteria (Ji and Hollander, 2000). Mammalian skeletal muscle contains both Cu/Zn- and Mn-SOD.

Cu/Zn-SOD is a dimer, has a molecular weight (MW) of 32,000 and is located in the cytosol, whereas Mn SOD is a tetramer with a much larger MW of 88,000 and is found in mitochondria (Ji and Hollander, 2000, Powers and Lennon, 1999). According to Ohno *et al* (1994) both enzymes catalyse the dismutation of superoxide anion radicals with similar efficiency.

The distribution of the two isoforms varies from tissue to tissue. In skeletal muscle, 65-85% total SOD activity exists in cytosol, whereas 15-35% total SOD activity is in the mitochondria (Ji *et al*, 1990). Furthermore, muscles with high percentage of type I fibres have a greater SOD activity than muscles with high percentage of type IIB fibres (Powers *et al*, 1994).

Mammalian species also contain extracellular SOD (Ec-SOD). Ec-SOD belongs to the Cu/Zn SOD family, is a tetramer with a large molecular weight and

the primary function is to remove $O_2^{\bullet-}$ generated outside the cell membrane (Ji and Hollander, 2000).

1.3.1.2. Catalase

The hydrogen peroxide formed by superoxide dismutase can potentially cause oxidative damage and so is scavenged by catalase (CAT), a ubiquitous heme (Fe^{3+}) protein that catalyses the dismutation of hydrogen peroxide into water and molecular oxygen according to the following reaction:

$$2H_2O_2 \xrightarrow{Catalase} 2\ H_2O + O_2$$

Thus, catalase plays an important role in cytoprotection (Ji, 1998).

Catalase is a tetramer with a relatively high molecular weight of 240,000. Heme binds to the active site of the enzyme and is a required co-factor for catalytic function (Ji and Hollander, 2000).

Catalase is widely distributed in the cell, however the highest concentrations are found in both peroxisomes and cytosol. In skeletal muscle, type I fibres display the highest catalase activity, followed by type IIA; type IIB muscle fibres have the lowest catalase activity (Ji and Hollander, 2000).

While there is overlap between the function of catalase and glutathione peroxidase, the two enzymes differ in their affinity for H_2O_2; GPX has a greater affinity for H_2O_2 at low concentrations and therefore, when the cellular levels of H_2O_2 are low GPX is more active than CAT in removing hydrogen peroxide from the cell (Powers and Lennon, 1999). However, CAT activity increases enormously with an increase in H_2O_2 concentration (Ji, 1998).

1.3.1.3. Glutathione Peroxidase

Glutathione peroxidase (GPX) catalyses the reduction of hydrogen peroxide or organic hydroperoxides to H_2O and alcohol respectively, using reduced glutathione (GSH) as an electron donor (Lawler and Powers, 1998):

$$2GSH + H_2O_2 \xrightarrow{GPX} GSSG + 2H_2O$$

or

$$2GSH + ROOH \xrightarrow{GPX} GSSG + ROH + H_2O$$

By donating a pair of hydrogen ions, GSH is oxidised to glutathione disulfide (GSSG). Reduction of GSSG is catalysed by glutathione reductase, which uses NADPH as a reductant (Ji and Hollander, 2000).

GPX is a homotetramer with each 22kDa subunit bound to a selenium atom, a required co-factor for catalytic function (Ji and Hollander, 2000).

GPX is located in both the cytosol and mitochondria. In skeletal muscle approximately 45% of GPX activity is found in the cytosol and the remaining 55% is found in the mitochondria. GPX activity varies across muscle fibre types; muscles with high oxidative capacity (i.e. high percentage of type I fibres) have greater GPX activity, whereas muscles with low oxidative capacity (i.e. high percentage of type IIB fibres) possess the lowest activity (Lawler and Powers, 1998).

1.3.1.4. Glutathione and thiols

Glutathione is a thiol-containing tripeptide consisting of glycine, glutamine and cysteine (L-γ-glutamyl-L-cysteinyl-glycine). Glutathione is one of the most important cellular antioxidants found in virtually all animal and plant cells as well as in some bacteria (Powers *et al*, 1999).

Glutathione plays a multifunctional role in protecting tissues from oxidative damage and keeping the intracellular environment in the reduced state:

a) It reduces hydrogen- and organic- peroxides via a reaction catalysed by glutathione peroxidase (Section 1.3.1.3)

b) It serves as a scavenger of $^{\bullet}OH$ and singlet oxygen ($^1O_2$)

c) It reduces tocopherol radicals, either directly, or indirectly thereby preventing free radical chain reaction and lipid peroxidation (Ji, 1995).

Much of the *de novo* synthesis of glutathione occurs in the liver, which supplies approximately 90% of the glutathione under physiological conditions (Deveke and Fanburg, 1989). Glutathione is transported from liver to the blood plasma where it is utilised by metabolically active tissues such as heart, lung and skeletal muscle (Leeuwenburgh and Ji, 1996). Although much of glutathione synthesis occurs in the liver, many other tissues can also synthesise glutathione *de novo* (Ji, 1995).

Concentrations of glutathione vary between tissues. The liver and the lens of the eye have the highest concentrations of glutathione. Muscles with high percentage of type I fibres contain six-fold higher glutathione content than muscles with high percentage of type IIB fibres. However, the GSH:GSSG ratio appears remarkably consistent across various fibre types (Powers *et al*, 1999).

1.3.1.5. Vitamin C

Vitamin C (ascorbate) is a water-soluble antioxidant found in the cytosol and extracellular fluid (Ji, 1999). The antioxidant roles of vitamin C are multiple. Vitamin C can interact directly with $O_2^{\bullet-}$ and $^{\bullet}OH$ as well as lipid hydroperoxide radicals (Powers and Lennon, 1999). In addition, vitamin C reduces vitamin E

radicals by donating an electron. While donating an electron, vitamin C is oxidised to a semidehydroascorbate (SDA) radical, which is a less reactive compound (Ji and Hollander, 2000). This radical can be reduced back to vitamin C by the enzyme semidehydroascorbate reductase using nicotinamide adenine dinucleotide (NADH) as the reductant or cellular thiols such as GSH (Ji and Hollander, 2000).

1.3.1.6. Vitamin E

Vitamin E is a lipophilic compound found in the cell membranes. Vitamin E can scavenge $O_2^{\bullet-}$ and $^{\bullet}OH$ (Powers and Lennon, 1999). Interaction of vitamin E with ROS results in the formation of a vitamin E radical, which can be reduced by several other antioxidants including vitamin C (Section 1.3.1.5)

In skeletal muscle, vitamin E content is relatively low (20-30 nmol/g depending on the fibre type) compared with other tissues that contain ~60-70 nmol/g (Ji and Hollander, 2000). The importance of vitamin E is best illustrated in studies with vitamin E deficient animals. For example, Davies *et al* (1982) have reported that rats with vitamin E deficiency demonstrated mitochondrial dysfunction and increased lipid peroxidation following an acute bout of exhaustive exercise compared with vitamin E adequate rats. In addition, a study by Jackson *et al* (1983) also demonstrated that skeletal muscle from vitamin E deficient mice and rats are more susceptible to damage during isometric contractions. Thus, vitamin E appears to be an essential compound for normal cell function during exercise.

1.3.1.7. Carotenoids

Carotenoids are lipid-soluble antioxidants found in cellular membranes (Powers and Lennon, 1999). β-carotene, a major carotenoid precursor of vitamin A, is capable of combining with several forms of ROS, such as the singlet oxygen in order to form less active radicals (Goldfarb and Sen, 1994). β-carotene can also inhibit lipid peroxidation initiated by free radicals (Ji and Hollander, 2000).

1.3.2. Exercise and the antioxidant response in skeletal muscle

Evidence suggests that a period of exercise results in a rapid increase in the production of ROS (Section 1.2.2). However, this does not lead directly to muscle damage. This seems to be due to the fact that the antioxidant capacity of mammalian tissues is highly evolved to protect against changes in ROS production and antioxidant defence systems of muscle cells can adapt in response to exposure to oxidants (Powers and Lennon, 1999). Since the rates of ROS production are highly correlated with the levels of O_2 consumption, aerobic exercise should upregulate the activity of antioxidant defence enzymes in muscle in order to reduce the risk of free radical damage to muscle tissue.

Indeed, several studies have shown that an acute bout of exercise can increase the activities of different antioxidant defence enzymes. Khassaf *et al* (2001) found that the SOD activity in human vastus lateralis muscles was increased following a single bout of non-damaging maximal isometric exercise. Ji *et al* (1992) found that an acute bout of exercise significantly increased the activities of CAT and GPX in the deep vastus lateralis (DVL) muscles from rats. McArdle *et al* (2001) have shown that a fifteen-minute period of isometric

contractile activity leads to an increase in the activities of catalase and superoxide dismutase in muscles of mice.

Longer term exercise training also appears to result in an increase in the activity of several antioxidant defence enzymes. Leeuwenburgh *et al* (1994) found that a 10-week training programme resulted in increased activities of GPX and SOD in DVL muscles of rats. In another study Leeuwenburgh *et al* (1997) have shown that GSH content was increased by 33% in DVL muscles from rats following training and that trained rats showed a 62% and 27% higher GPX and SOD activity respectively compared with muscles of non-trained rats. In addition, Powers *et al* (1994) showed an increase in GPX activity in red gastrocnemius muscles from rats following a 10-week training programme. In the same study, the authors demonstrated an increase in SOD activity in rat soleus and red gastrocnemius muscles following training, whereas SOD activity in the white gastrocnemius muscles was not significantly altered. These data suggest that training-induced changes in muscle antioxidant defence enzymes are fibre type specific, but also muscles composed of highly oxidative fibres such as soleus (primarily type I fibres) and red gastrocnemius (primarily type IIA fibres) appear to be more responsive to oxidative stress.

The field is a little confused since different studies have not only examined different muscles in rodents at various ages, but the contraction protocols used in different studies range from damaging lengthening contractions to mild, non-damaging contraction protocols. In addition, the nature of the exercise varies from treadmill running, rodent wheel running or electrically-stimulated contraction protocols. Thus, the general consensus is that the production of ROS during non-

damaging exercise may act as a signal to muscle cells to adapt to provide protection against further and possibly damaging insults.

1.3.3. The stress response in skeletal muscle

A second cytoprotective response in skeletal muscle and other tissues is the increased production of a family of proteins known as stress or heat shock proteins (HSPs).

HSPs are so called because their induction was first observed in response to hyperthermia or "heat shock". It is now known that the cellular content of HSPs is increased following a variety of stresses including oxidative stress, viral infection, changes in pH and incorporation of amino acid analogues into proteins. The induction of HSPs following stress has been termed as "the cellular stress response" (Locke, 1997).

Although the synthesis of HSPs is induced following heat and other stresses, some family members are constitutively expressed in cells, which indicates that HSPs fulfil important tasks in cells under normal conditions (Feige and Polla, 1994).

In an unstressed cell, HSPs act as molecular chaperones, necessary for facilitating protein folding, for blocking non-productive protein-protein interactions, for safely transporting newly synthesized proteins to their correct site of action and prevention of mislocation or aggregation (For a review see McArdle *et al*, 2002a; Figure 1.6). As a result, HSPs play fundamental roles in maintaining cellular homeostasis.

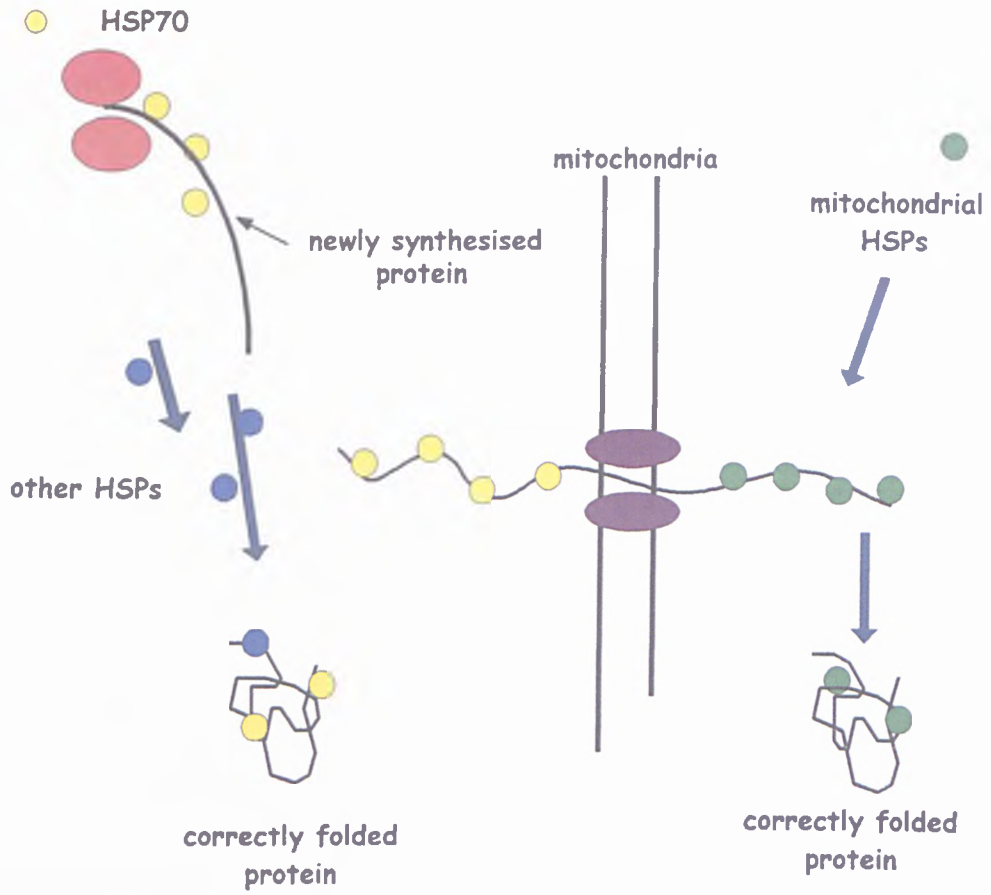


Figure 1.6. A schematic representation of the role of HSPs in the unstressed cell (reproduced from Jackson *et al*, 1998).

1.3.4. The role of HSPs in the unstressed cell

HSPs are usually named according to their molecular weight and include the small HSPs such as HSP25, mitochondrial HSP60, the HSP70 family (which consists of the constitutively expressed HSC70 and highly inducible HSP70), and the larger HSPs such as HSP90 and HSP100 (Gething, 1997; Table 1.5).

Many other stress proteins have been identified in mammalian cells however, information regarding their role and expression in most tissues is limited. Since the greater number of exercise-related studies has focused on the expression of certain HSPs such as HSP25, HSP60, HSP70 and HSP90, these are the HSPs that will be discussed in this section.

Table 1.5. **Size, name, function and cellular location of HSPs (Modified from Moseley, 1997).**

SIZE, kDa	NAME	CELLULAR LOCALISATION	MAJOR FUNCTION
Small HSPs (<35)	Hsp25/27 or αB crystallin	Cytosol and nucleus	Stabilisation of microfilaments Cytokine signal transduction
60	HSP60	Mitochondria	Protein assembly
70-73	HSP72 or HSP70i HSP73 or HSC70	Cytosol, nucleus, ER and mitochondria	Protein folding and translocation
90	HSP90 HSP90α HSP90β GRP94	Cytosol, nucleus, ER	Protein translocation Receptor regulation
100-110	HSP100 HSP104 HSP110	Cytosol	Protein folding Disaggregation

1.3.4.1. Small HSPs (HSP25, α or β crystallin)

HSP25 (HSP27 in human cells) is expressed at low levels in unstressed cells. HSP25/27 has been shown to be present within the cytoplasm but relocates into the nucleus after heat shock (Welch, 1992). Although the exact function of HSP25 remains unknown, it has been shown to be involved in signal transduction, differentiation and growth and stress-induced expression of HSP25 appears to be complex and considerably tissue-specific (Locke, 1997). HSP25 is rapidly phosphorylated at two serine residues in response to stress as a result of activation of the MAP kinase activated protein kinase-2 (Gaestel and Buchner, 1997).

HSP25 can act as a molecular chaperone *in vitro*, preventing thermal aggregation of citrate synthase and assisting in refolding of chemically denatured β-glucosidase (Jacob *et al*, 1993). Furthermore, HSP25, HSC70 and HSP110 can interact with each other to form large complexes (Wang *et al*, 2000). Baek *et al*

(2000), have demonstrated that overexpression of HSP25 in transfected murine fibroblastoid cells not only provided protection against ionising radiation, but it also resulted in a dramatic increase in cellular glutathione levels.

HSP25 has significant sequence homology with αB crystallin (22kDa), a member of the small HSPs (Thomason and Menon, 2002). αB crystallin is a major structural protein in the lens of the eye, but is also expressed in other tissues, especially tissues with high oxidative capacity, including heart and type I skeletal muscle fibres (Neufer and Benjamin, 1996). Like HSP25, αB crystallin can act as a molecular chaperone *in vitro*, preventing aggregation of denatured proteins in response to stress and facilitating protein refolding upon removal of stress (Jacob *et al*, 1993; Neufer and Benjamin, 1996).

1.3.4.2. HSP60

The majority (95%) of HSP60 is located within the mitochondria. HSP60 is encoded by a nuclear gene, is synthesized in the cytoplasm and is then translocated into the mitochondria (Welch, 1992). HSP60 is expressed constitutively under normal conditions and plays an important role in maintaining normal cell function by mediating the correct folding of polypeptides that enter the mitochondria and facilitating protein transport across intracellular membranes.

HSP60 is also involved in the correct assembly of oligomeric protein complexes. In addition, HSP60 together with HSP10 have been shown to interact with newly synthesised mitochondrial proteins and are thought to be able to rescue proteins that denature spontaneously within the mitochondria (Martinus *et al*, 1995). As a result, HSP60 has been termed a molecular chaperone or

chaperonin (Locke, 1997). Under stress conditions, HSP60 is involved in the stabilisation of pre-existing proteins.

1.3.4.3. The HSP70 family

The HSP70 family is large, with most organisms having multiple members. In skeletal muscle, four major forms of HSP70 have been identified; the two glucose-regulated proteins GRP75 and GRP78, HSC70 and HSP70 proteins. All share the common property of binding both adenosine triphosphate (ATP) and polypeptides (Welch, 1992).

GRP75 and GRP78 have a molecular mass of 75kDa and 78kDa respectively, and they are induced by glucose deprivation, calcium influx or agents that perturb glycosylation (Liu and Steinacker, 2001). GRP75 is located in the mitochondria and involved in the translocation of precursor proteins across mitochondrial membranes, their stabilisation and their correct folding within the mitochondria. GRP78 is located in the sacroplasmic/endoplasmic reticulum, is constitutively expressed and may have a general role in the assembly of secretory proteins (Liu and Steinacker, 2001).

The two most extensively studied proteins in the HSP70 family are the cognate isoform HSC70 and the highly inducible isoform HSP70. The two proteins have extremely high sequence homology (~95%) although they are encoded by different genes and they seem to have similar biological properties; they have been shown to be involved in transport, folding, protein synthesis, disassembly, prevention of protein aggregation and restoring the function of damaged proteins following stress (Welch, 1992; Locke, 1997).

HSC70 has a molecular mass of 73kDa, is constitutively expressed in most cells, and is only mildly inducible. Under non-stress conditions it is located in the cytoplasm. During stress, HSC70 migrates to the nucleus and the nucleolus. It has been shown that HSC70 plays a role in the transport of proteins into the nucleus as well as the mitochondrion and the endoplasmic reticulum and that absence of HSC70 slows down ribosome translocation, thus slowing the rate of protein synthesis (Welch, 1992; Liu and Steinacker, 2001).

In general, in the unstressed cell HSP70 is present in low or undetectable quantities, however it is highly induced during episodes of stress. It is synthesised in the cytoplasm where it binds to proteins and other macromolecules (Locke and Noble, 1995).

Both cytosolic forms of HSP70 act as molecular chaperones, facilitating the early steps of protein maturation. Such interaction appears to be transient; the newly synthesized proteins remain complexed with HSP70 (HSP70/HSC70) only for short time (15-30 mins) after their synthesis. This interaction of the nascent polypeptide chain with HSP70 prevents its premature folding until its translation has been completed (Welch, 1992).

It is not known why cells require both the inducible and the constitutive forms of HSP70 since they are so closely related. However one major difference between HSC70 and HSP70 is the presence of intervening sequences in the HSC70 and their absence in the HSP70 gene. The lack of intervening sequences in the HSP70 gene may facilitate rapid transcription of HSP70 during stress (Locke, 1997).

1.3.4.4. The HSP90 family

The HSP90 family consists of three proteins; the glucose-regulated protein GRP94 located in the endoplasmic reticulum and two closely related cytoplasmic isoforms HSP90α and HSP90β (Locke, 1997).

HSP90 is one of the most abundant cellular proteins found in both stressed and unstressed cells and appears to be involved in the general folding of various proteins. HSP90 binds to nascent proteins at a level of one HSP90 dimer to one or two substrates (Jakob & Buchner, 1994; Pratt *et al*, 1996). HSP90 is frequently found in complexes with other chaperones such as HSP70.

HSP90 is also associated with unoccupied steroid hormone receptors such as oestrogen, progesterone and androgen receptors. In the absence of the hormone, HSP90 is thought to bind to the receptor and maintain it in an inactive form. When the hormone is present, the receptor-HSP90 complex dissociates, allowing receptor binding to DNA (Locke, 1997).

1.3.5. The role of HSPs in the stressed cell

Several studies have shown that the cellular content of HSPs is increased following a variety of stresses such as incorporation of non-native amino acid analogues (Welch *et al*, 1983), alterations in pH (Whelan and Hightower, 1985), hypoxia (Heacock and Sutherland, 1990), as well as oxidative stress (Welch, 1992; Locke, 1997).

Early studies in the heart demonstrated that exposure of cardiac muscle to stress results in the increased production of HSPs. Salo *et al* (1991) demonstrated that acute exhaustive exercise resulted in an increase in the HSP70 mRNA levels

in cardiac muscle of rats. In addition, Locke *et al* (1995) reported an increase in the HSP70 mRNA levels after heat shock and exercise in cardiac muscle from rats. Most importantly, recent studies have demonstrated that this increase in the HSP production is associated with myocardial protection. The development of transgenic mouse models overexpressing HSPs has demonstrated a direct protective effect by HSPs. For example, Marber *et al* (1995) have reported that overexpression of HSP70 in transgenic mice increases the resistance of the heart to ischemic injury.

The ability of prior induction of the stress response to protect cells from damage has also been examined by several workers. Studies have shown that an initial mild stress, sufficient enough to induce HSPs, can provide protection against subsequent more severe stress (Figure 1.7).

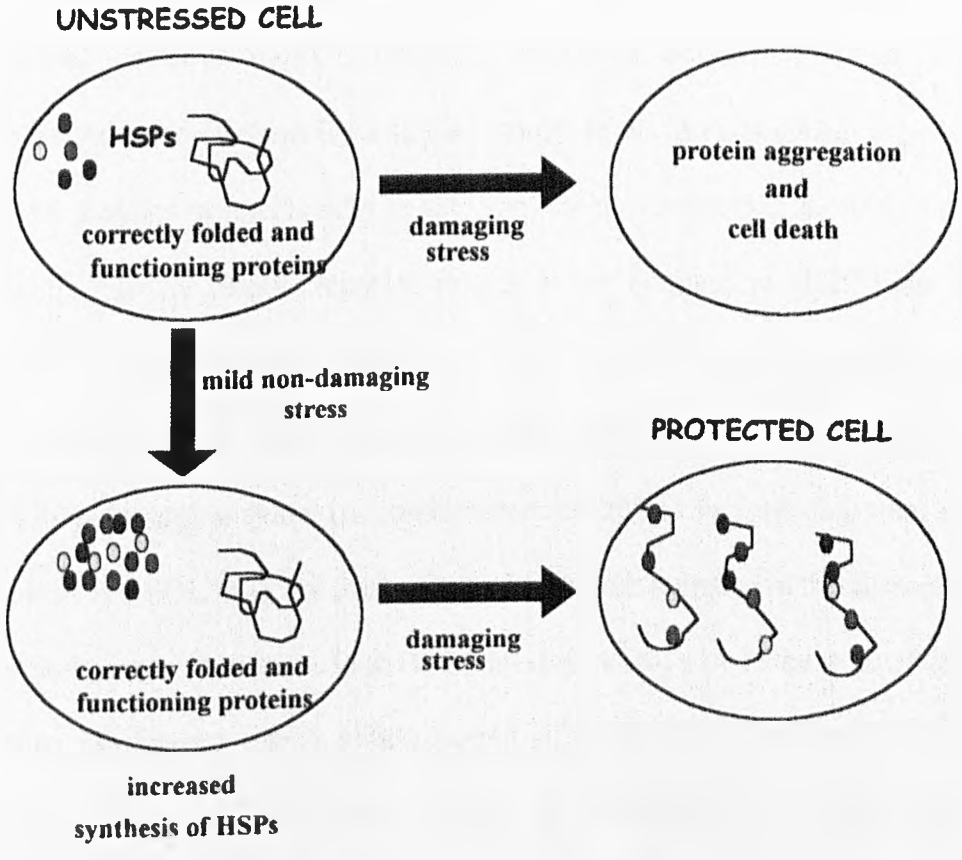


Figure 1.7. Cytoprotective function of HSPs (reproduced from McArdle *et al*, 2002a).

Garramone *et al* (1994) and Lepore *et al* (2000) have demonstrated that a prior heat stress in rats, which resulted in increased muscle content of HSP70, provided protection to skeletal muscle against necrosis induced by ischemia-reperfusion. Furthermore, Suzuki *et al* (2000) have shown that prior heat shock of muscle cells provides considerable protection against cell death following hypoxia and re-oxygenation *in vitro*. It is thought that an increased cellular content of HSPs protects the cell by associating with misfolded cellular proteins during stress and facilitating the refolding of these proteins when conditions become more favourable. Thus, in this case, the induced HSPs act in a manner analogous to their molecular chaperone function (Figure 1.7).

1.3.6. Expression of HSPs in skeletal muscle following exercise

The possibility that HSPs provide cytoprotection to skeletal muscle following oxidative stress is receiving increasing attention. Salo *et al* (1991) showed that acute exhaustive exercise results in an increased HSP expression in skeletal muscles of rats. Neufer *et al* (1996) demonstrated that chronic stimulation of rabbit anterior tibialis muscles results in the increase in HSP70 and HSP60 expression, demonstrated by changes in protein levels, mRNA and transcription rate. McArdle *et al* (2001) have also demonstrated that a 15-minute period of mild, non-damaging isometric contractions resulted in increased content of HSPs in soleus and EDL muscles from mice, whereby the increase in the soleus muscles appeared greater than that of the EDL muscles. A study by Khassaf *et al* (2001) in humans has shown that a single period of exhaustive, non-damaging aerobic exercise resulted in a significant increase in the HSP70 and HSP60 contents of vastus lateralis muscles at 3 to 6 days following the exercise protocol.

Interestingly, the authors observed a rapid increase in the HSP70 content in muscles with relatively low resting HSP70 content, whereas muscles with relatively high resting content of HSP70 did not respond to the same extent to the exercise protocol.

A number of workers have also examined the effect of endurance training on HSP content of muscle. Ornatsky *et al* (1995) demonstrated that chronic contraction at a frequency of 10Hz for 10 days resulted in an increase in the GRP75, HSP70 and HSP60 contents of anterior tibialis muscles from rats. Samelman (1999) showed an increase in the HSP70/HSC70 and HSP60 expression in soleus muscles from rats that were trained for 16-20 weeks on a motorised treadmill. In addition, Gonzalez *et al* (2000) demonstrated that treadmill running for 3 months increased the HSP70, GRP75 and GRP78 content in rat soleus muscles.

The HSP production in skeletal muscle appears to be fibre type specific since data from several studies suggest that, in general, the levels of HSPs in resting skeletal muscles composed predominantly of type I fibres are higher than that of muscles composed of type II fibres. Furthermore, the production of some HSPs appears to be greater following exercise in the soleus muscles (type I) compared with the production in the EDL muscles (type II; McArdle *et al*, 2001; McArdle and Jackson, 2002).

Evidence suggests that this increased content of HSPs provides protection to skeletal muscle. McArdle *et al* (1995) have demonstrated that a short period of non-damaging isometric exercise in mice leads to a rapid increase in HSP content and subsequent protection of skeletal muscle against a period of normally damaging contractile activity (McArdle *et al*, 1996). However, the direct evidence

for a role of HSP70 in providing protection against exercise-induced damage comes from studies using transgenic mice. McArdle *et al* (2002b) have demonstrated that overexpression of HSP70 in transgenic mice provided protection against damage and resulted in an increase in the rate of recovery in EDL muscles following damaging lengthening contractions compared with muscles from wild-type mice.

In summary, it is now clear that enhanced production of ROS results in the increased production of antioxidant defence enzymes such as catalase and SOD, as well as in the increased production of HSPs. This ability of cells to respond rapidly and appropriately to stress is essential for their survival.

Current evidence indicates that the increase in the production of antioxidant defence enzymes and HSPs is due to activation of transcription factors responsible for the enhanced expression of genes encoding these antioxidant defence enzymes and HSPs. Therefore, the next section will focus on the mechanisms of activation of three transcription factors, NF-κB, AP-1 and HSF1.

1.4. TRANSCRIPTION FACTORS AND ACTIVATION OF THE ADAPTIVE RESPONSE

Cells can respond to acute or chronic increases in R-O/N-S production by increased production of antioxidant defence enzymes and HSPs. In recent years, much attention has been focused on the cellular mechanisms that initiate the adaptive response. In the vast majority of cases when a protein is produced in a tissue or in response to a signal, this is achieved by control processes that ensure that the corresponding gene is transcribed only in that specific tissue or in response to the specific signal. In mammalian cells, the induction of antioxidant defence enzymes and HSPs is usually associated with the activation of transcription factors.

Transcription factors are proteins that control gene expression and are required to initiate or regulate transcription in eukaryotes. Generally, a stimulus such as an increase in the free radical production will trigger a signal transduction pathway and activate transcription factors that are then able to bind to the promoter region of target genes and initiate transcription. Furthermore, the rate of transcription initiation can be increased or decreased in response to this particular stimulus.

Cells contain several redox-sensitive transcription factors. Heat Shock Factor 1 (HSF1), Nuclear Factor kappa B (NF-κB) and activator protein-1 (AP-1) are three of the most well characterised transcription factors that play important roles in the response of cells to stress. These transcription factors will be discussed in more detail in the following sections.

1.4.1. NF-κB

Nuclear Factor kappa B (NF-κB) was first described in 1986 in B-cells (Sen and Baltimore, 1986), however it soon became apparent that NF-κB was not restricted to B-cells but is ubiquitous. It is now known that NF-κB is activated in cells by a great variety of stimuli that represent internal or external stress situations including pathological stimuli such as bacteria, viruses, hypoxia and inflammatory mediators as well as internal cellular stress such as endoplasmic reticulum overload and oxidative stress (Mueller and Pahl, 2000).

In higher eukaryotes, NF-κB is a multisubunit transcription factor. The currently known DNA-binding subunit members of the NF-κB family are p50, p52, p65 (RelA), c-Rel and Rel-B (Bowie and O'Neill, 2000). These subunits can homo- and heterodimerise in various combinations. The classical, and predominant form of NF-κB is a heterodimer consisting of the p50 and p65 (RelA) subunits (Mueller and Pahl, 2000).

In unstimulated resting cells NF-κB resides within the cytoplasm in an inactive form where is controlled by a family of inhibitory proteins called IκBs. To date five IκB proteins are known: IκBα, β, γ, δ and ε (Mueller and Pahl, 2000). However, most work has focused on the p50/p65 dimer and its association with IκBα.

The mechanism by which stimulants lead to the activation of NF-κB is relatively well understood. In general, NF-κB activation is initiated by the phosphorylation of IκB proteins by IκB kinase complex consisting of at least two proteins, IKKα and IKKβ (Bowie and O'Neill, 2000). External stimuli initiate a signal transduction cascade that leads to the activation of IκB-kinase complex that

specifically phosphorylates IκBα on serine-32 and serine-36. Phosphorylation of these sites leads to ubiquitination of IκBα and subsequent degradation (Bowie and O'Neill, 2000). Dissociation of IκBα results in unmasking of the nuclear localisation signal of NF-κB dimers, which subsequently translocate to the nucleus and activate target genes (Mueller and Pahl, 2000; Figure 1.8). NF-κB DNA binding and transcriptional activation properties within the nucleus may be further modulated by phosphorylation (see Jackson *et al*, 2002 for a review). The genes activated by NF-κB are diverse and include many used by the immune system. However NF-κB is also in part responsible for the induction of antioxidant enzymes such as Mn SOD (Zhou *et al*, 2001; Section 1.4.5)

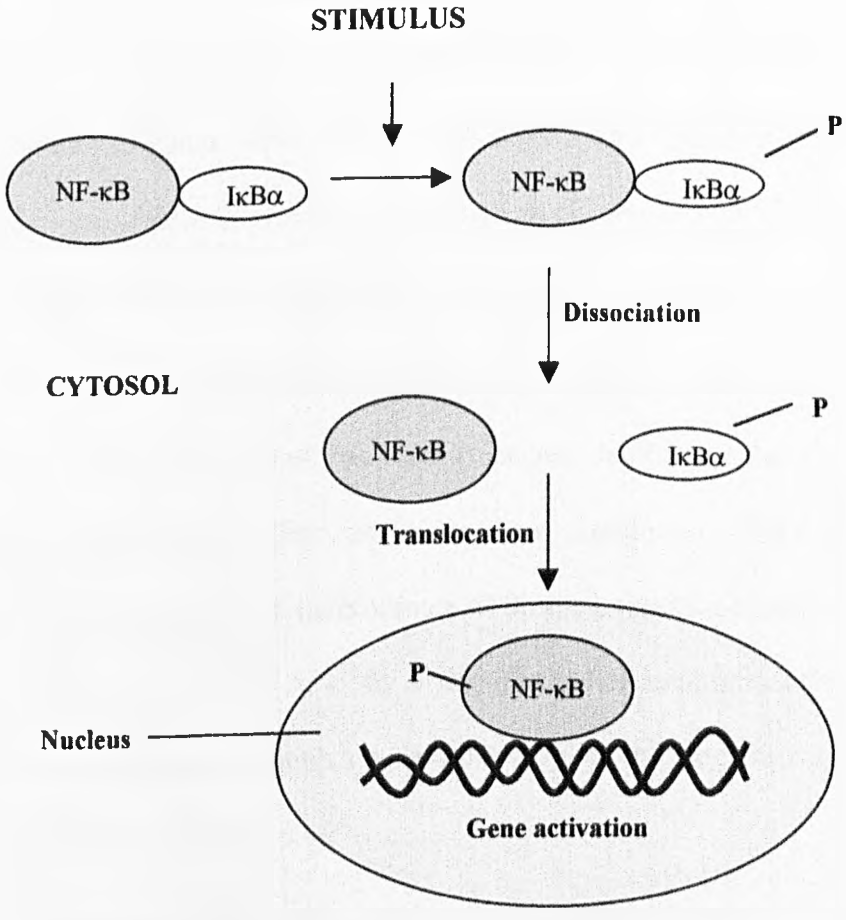


Figure 1.8. Schematic diagram of NF-κB activation.

A substantial body of experimental data links NF-κB activity to cellular oxidative status. It has been shown that NF-κB can be activated by H_2O_2 in some cell lines in the absence of any physiological stimulus and that NF-κB binding to DNA can be inhibited by a variety of antioxidants (Flohe *et al*, 1997). In addition, agents that can activate NF-κB tend to trigger the formation of ROS or are oxidants themselves, such as the superoxide anion radical (Flohe *et al*, 1997). These observations have led to a general agreement that NF-κB activation is at least facilitated by some oxidative reactions.

1.4.2. AP-1

Like NF-κB, activator protein 1 (AP-1) is a well-characterised and ubiquitously expressed transcription factor capable of inducing expression of a large number of genes. AP-1 regulates the expression of genes associated with the control of proliferation, growth, differentiation of cells as well as stress-inducible genes such as the Mn SOD gene (Latchman, 1995; Mueller and Pahl, 2000).

AP-1 is a dimer composed of two DNA-binding subunits, c-Jun and c-Fos, which belong to the Jun and Fos proto-oncogene families. AP-1 can be either a Jun-Jun homodimer or a Fos-Jun heterodimer (Latchman, 1995). The classical form of AP-1 is the Fos-Jun heterodimer. With the exception of pre-existing c-Jun homodimers, induction of AP-1 by a stimulus relies predominantly on *de novo* synthesis of its subunits, which are controlled by pre-existing transcription factors (Zhou *et al*, 2001; Figure 1.9).

Activation of AP-1 also involves phosphorylation of the c-Jun and c-Fos proteins, which influences its DNA binding and transcriptional regulatory

properties (Xanthoudakis and Curran, 1996; Figure 1.9). Once activated, the AP-1 complex is then capable of self-induction by binding to promoter regions within the c-Jun and c-Fos genes (For a review see Jackson *et al*, 2002).

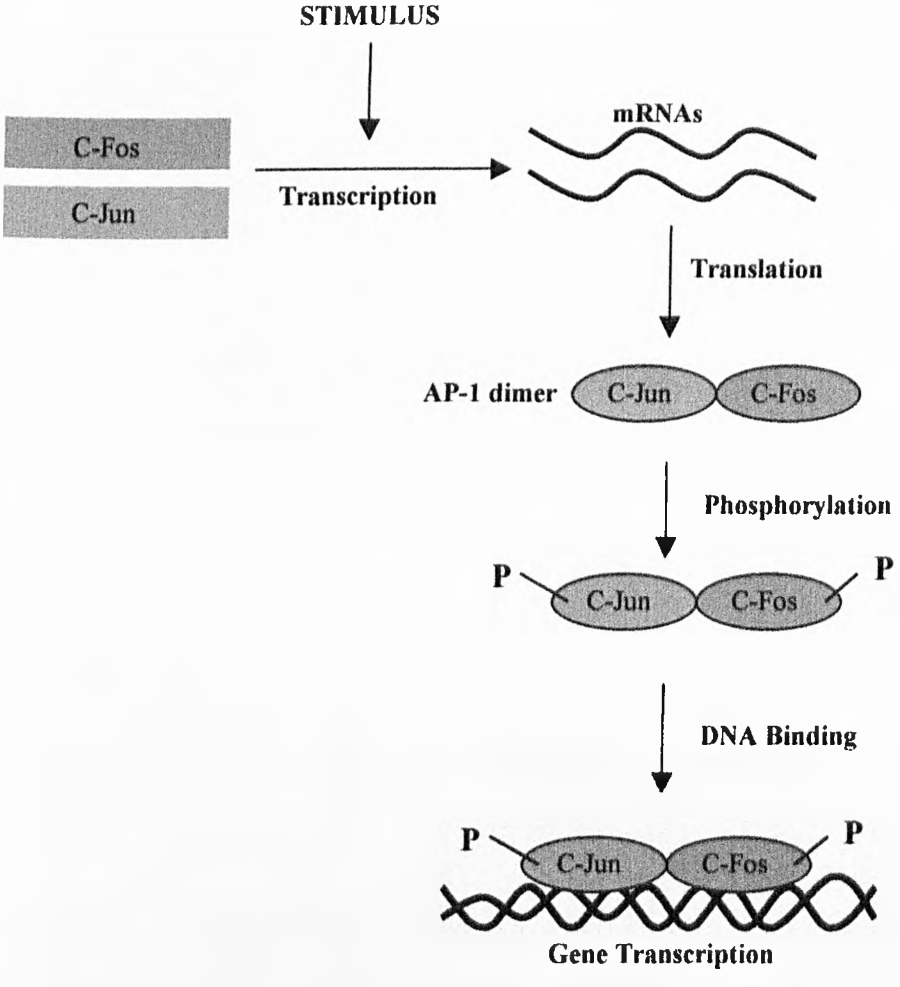


Figure 1.9. Schematic diagram of AP-1 activation (Reproduced from Jackson *et al*, 2002).

In addition to increased subunit synthesis, oxidative stress induces AP-1 mediated transcription by enhancing DNA binding activity (Zhou *et al*, 2001). Like NF-κB, the response of AP-1 to oxidative stress is cell type specific (Zhou *et al*, 2001). It has been shown that low concentrations of hydrophilic antioxidants can inhibit the redox-sensitive activation of AP-1 however, when the levels of

intracellular antioxidants are increased above a certain level, they can act as a stimulus for AP-1 activation (Jackson *et al*, 1998).

1.4.3. The HSF family

The inducible HSP expression in eukaryotic cells is regulated by the heat shock transcription factors (HSFs). In response to various inducers such as elevated temperatures, viral infections and oxidative stress, most HSFs acquire DNA binding activity thereby mediating transcription of the heat shock genes, which result in accumulation of HSPs (Pirkkala *et al*, 2001).

Several different HSFs have been isolated from various species; a single HSF from yeasts and Drosophila, two (HSF1 and HSF2) from mouse, three (HSF1, HSF2, HSF3) from chicken and three (HSF1, HSF2 and HSF4) in human cells (Morimoto, 1998). The role and the function of these HSFs are still incomplete; however, it appears that different HSFs respond to different stimuli in a different ways.

Both HSF1 and HSF2 act through a highly conserved upstream response element (heat shock element, HSE), which is located within the HSP gene promoters and is composed of a series of pentameric units arranged as inverted adjacent arrays of the sequence 5'-nGAAn-3' (Leppa and Sistonen, 1997). Furthermore, HSF1and HSF2 share a common feature of adopting a trimeric form upon activation, however, the non-DNA binding forms of the two factors differ since HSF1 is a monomer and HSF2 is either a homodimer or a heterodimer (Leppa and Sistonen, 1997). In addition, HSF1 undergoes rapid activation in response to a multitude of stress conditions, whereas HSF2 appears to be an important control mechanism during developmental and cellular differentiation

processes (Pirkkala *et al*, 2001). In response to stress, HSF1 activation involves translocation to the nucleus and hyperphosphorylation on serine residues, whereas HSF2 appears not to undergo any changes in the phosphorylation upon activation (Leppa and Sistonen, 1997).

HSF3 is expressed ubiquitously in avian cells. Like HSF1, HSF3 is a stress responsive transcription factor and has many characteristics similar to those of HSF1 such as sequence specific binding to the HSE and activation to a nuclear trimer; however, HSF3 in its non-DNA binding form is an inert dimer (Cotto and Morimoto, 1999). Furthermore, it has been shown that the amount of HSF3 increases after severe heat shock, whereas HSF1 levels diminish suggesting that HSF3 has a role during severe and persistent stress in avian cells. The fact that no HSF3 homologue has been found in other than avian cells raises the possibility that the mechanisms responsible for activation of the stress response may be organism specific (Pirkkala *et al*, 2001).

The most recently discovered mammalian HSF is HSF4. This is found in human cells and appears to be preferentially expressed in the human brain, heart, skeletal muscle and pancreas. HSF4 binds to DNA constitutively but lacks the properties of a transcriptional activator (Santoro, 2000).

1.4.4. Regulation of the stress response

The mechanism by which the stress results in an increased cellular content of HSPs is relatively well understood. In the unstressed cells HSF1 is present in the cytoplasm as a monomer, associated with HSP70 or 90. Within seconds of the initiating stress, HSF1 is activated (Morimoto *et al*, 1996; Figure 1.10). Activation of HSF1 is linked to the appearance of non-native proteins and the requirement for

molecular chaperones (such as HSP70 and HSP90) to prevent the appearance of misfolded proteins. During stress, HSP70 and HSP90 have a greater binding affinity for destabilized proteins, releasing HSF1 and allowing it to migrate to the nucleus.

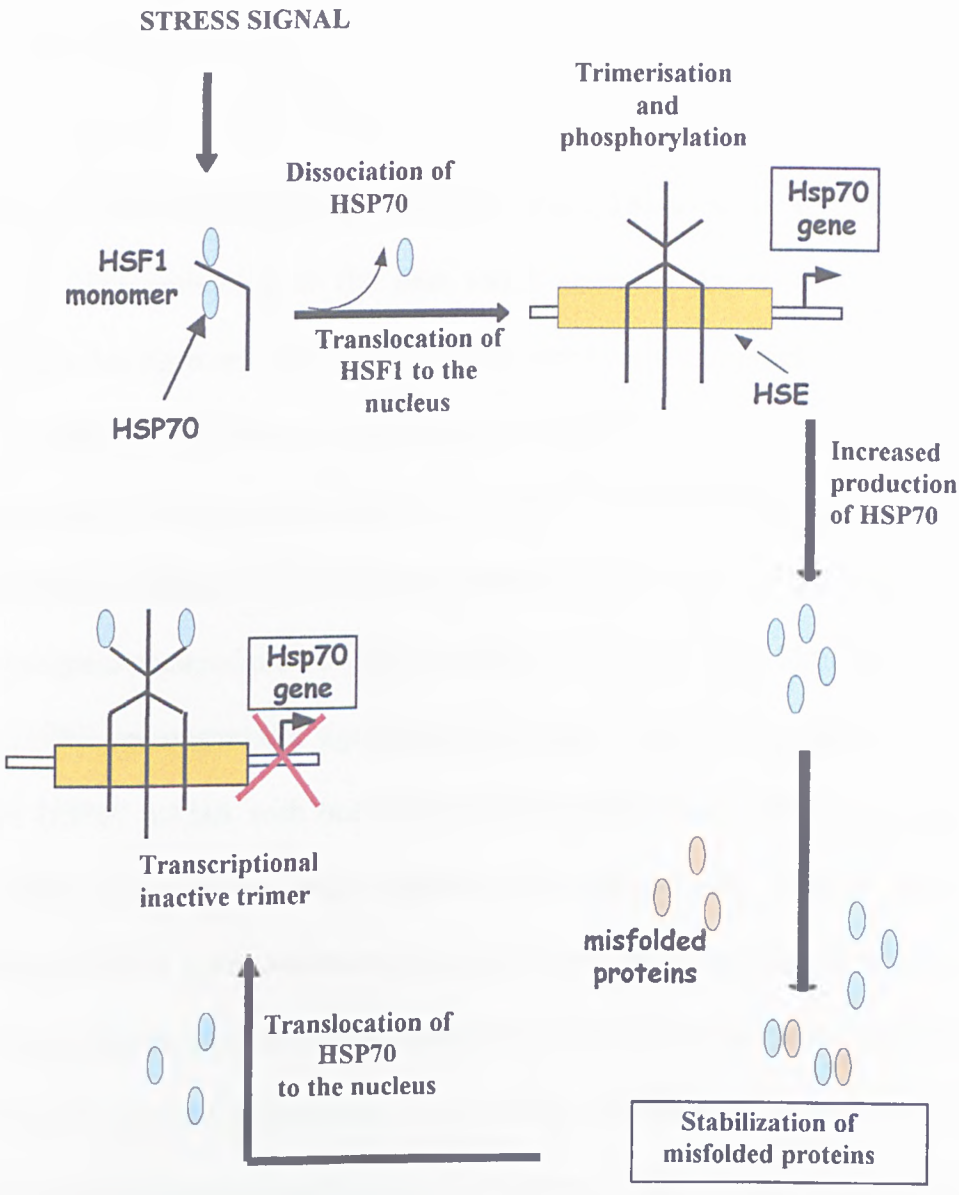


Figure 1.10. Activation of HSF1 following stress.

In the nucleus, HSF1 monomers oligomerise to a trimeric state, bind to the Heat Shock Element (HSE) of the heat shock protein gene promoters and become

inducibly phosphorylated at serine residues (Figure 1.10). This results in increased transcription of the heat shock genes and subsequently, an increase in cellular content of HSPs. As the synthesis of HSPs increases, HSP70 and other molecular chaperones translocate to the nucleus where they are free to re-associate with HSF1. This negative feedback results in the inactivity of HSF1 (Morimoto, 1998; Santoro, 2000).

1.4.5. Transcription factors, oxidative stress and skeletal muscle

The activation of the heat shock genes in response to environmental stresses has been studied extensively in several tissues. A numbers of studies have reported that ROS play an important role in HSF1 activation as well as HSP gene expression. For example, Locke *et al* (1995) have reported that a single bout of treadmill running is a sufficient stimulus to activate HSF1 and cause the subsequent accumulation of HSP70 mRNA in rat heart. In addition, Nishizawa *et al* (1999) demonstrated a significant activation of HSF1 and appearance of HSP70 and HSP90 mRNA with both ischemia-reperfusion and with H_2O_2 perfusion in isolated hearts. It has been suggested that one pathway through which ROS activate HSF-1 is via oxidation of protein thiols. Work by McDuffee *et al* (1997) showed that the oxidative stress generated by menadione (a compound that redox-cycles to generate superoxide) results in the formation of non-native disulfides followed by protein destabilization, activation of HSF-1, accumulation of HSP70 mRNA and increased synthesis of HSP70 in human hepatoma and chinese hamster lung cells.

In skeletal muscle, McArdle *et al* (2001) have demonstrated that an increased muscle content of HSPs following a short protocol of isometric

contractions is associated with the generation of superoxide anion radicals during the contractions and a subsequent, transient, non-damaging fall in the thiol content of cellular proteins. Interestingly, there was no effect of the contraction protocol on muscle temperature, thus hyperthermia was not the main signal for activation of HSF1. Although the study did not investigate the activation of HSF1 following exercise, the data suggests an involvement of mild oxidative stress in the activation of HSF1, which results in the increased content of HSPs is the exercised muscles. In addition, activation of HSF1 in skeletal muscle has been shown following thermal stress (Locke and Tanguay, 1996b). However the effect of exercise on the HSF-1 activation remains uncharacterised.

The possibility that either NF-κB or AP-1 might be involved in the regulation of antioxidant defence enzyme induction in response to oxidative stress was based on the presence of NF-κB and AP-1 response elements in the promoter regions of genes encoding a number antioxidant defence enzymes (Zhou *et al*, 2001). Both NF-κB and AP-1 binding sites are present in the promoter of the mammalian Mn SOD gene and oxidative stress has been shown to activate their binding (Hollander *et al*, 2000). In addition, Zhou *et al* (2001) has demonstrated that both NF-κB and AP-1 are important mediators of redox-responsible gene expression in skeletal muscle and that NF-κB is actively involved in the upregulation of glutathione peroxidase and catalase in response to oxidative stress. These findings, together with the increasing evidence that NF-κB and AP-1 are redox responsive, suggest that combinations of these two transcription factors and possibly other redox-sensitive transcriptional regulators may determine which antioxidant defence enzymes are induced and to what extent, depending upon the tissue specific regulation.

1.5. SKELETAL MUSCLE AND AGEING

Ageing is usually defined as "the progressive loss of function accompanied by decreasing fertility and increasing mortality with advancing age" (Kirkwood & Austad, 2000). It is a complex physiologic process involving morphologic and biochemical changes in single cells and in the whole organism. However, the ageing process is as yet poorly understood.

During the last few years, the effect of ageing on skeletal muscle has been increasingly studied. Ageing of mammals leads to a decrease in the total skeletal muscle mass. This reduction in muscle mass (also known as muscle atrophy) is the main cause of the age-related decrease in muscle strength and power (Porter *et al*, 1995). In addition, it has been shown that the reduction of muscle mass is accompanied by a replacement with connective tissue and fat (Lexell, 1995).

1.5.1. Age-related changes in size and number of muscle fibres

In order to understand the causes of muscle atrophy during ageing, numerous attempts have been made to examine the morphology of muscles from ageing individuals. The earliest studies on ageing and human muscle reported alterations in the proportions of type I and type II muscle fibres, which results in an increase of the percentage of type I fibres with ageing (Larsson & Karlsson, 1978; Larlsson, 1978).

However, more recent studies now contradict these early findings. Grimby and Saltin (1983) examined the fibre distribution in the vastus lateralis (VL) muscles of 66- to 100 years old individuals and found no age-related changes in type I fibre distribution. Sato *et al* (1984) reported that the total volume of type I

fibres in the minor pectoral muscle of women did not change with age. In addition, Lexell *et al* (1986) measured the fibre type distribution of the VL muscle and found that the type I distribution was 49% for 24-year old men and 52% and 51% for men in their 50s and late 70s respectively. These findings suggest that type I fibres are little affected by ageing.

In contrast, it has been shown that a large proportion of the age-related muscle atrophy is the result of the reduction in type II muscle fibre size (Lexell *et al*, 1988). Lexell and associates (1988) have also reported a significant decrease in the total number of fibres with age, which is accompanied by a greater loss of contractile material of fast twitch type than of slow twitch type.

Studies on animals have shown similar findings. For example, Caccia *et al* (1979) have reported an age-related decrease in the relative proportion of type II fibres in rat soleus muscles. Furthermore, Holloszy *et al* (1991) has demonstrated a 37% decrease in the average cross-sectional area of the type IIB fibres in plantaris muscle of aged rats.

Thus, the reduction in muscle mass during ageing has been postulated to be due to a decrease in fibre number, fibre size, or both (Thomson, 1994). As a result, muscle of older individuals is weaker (Kirkendall & Garrett, 1998).

1.5.2. Age-related changes in mechanical characteristics of skeletal muscle

Loss of muscle mass results in a loss of total force production (Kirkendall & Garrett, 1998). Since muscle mass declines with ageing, there is a concomitant loss of total force production. Young *et al* (1984) reported that the isometric forces of the quadriceps femoris muscles from women demonstrated a 35%

reduction with ageing. McDonagh *et al* (1984), have also demonstrated that the maximal isometric force of the triceps surae muscles from old men demonstrated a decrease of 40% compared with the maximal isometric force of the triceps surae muscles from young men. Studies on whole skeletal muscles of small rodents have shown an age-related decrease in strength and power due to the loss in muscle mass (Faulkner *et al*, 1990a). The maximum force developed by both slow and fast muscles of old mice and rats is 20-30% less than the force developed by muscles of adult animals (Brooks and Faulkner, 1988; Carlson and Faulkner, 1988).

Another interesting phenomenon of ageing is that the ability of muscle to resist fatigue is reduced with ageing. Studies on extensor digitorum longus (EDL) muscles of young, adult and old mice have shown that not only are the muscles of old mice more fatigable than those of young mice, but those of adult mice are also more fatigable than muscles of young mice (Faulkner *et al*, 1990b).

1.5.3. Susceptibility of muscles of older individuals to damage

During lengthening contractions the force per unit of active fibre area is greater, leading to the possibility of muscle fibre injury (Kirkendall & Garrett, 1998). A study by Zebra *et al* (1990) involving lengthening contractions of skeletal muscles from mice has shown that the muscles of old mice are more susceptible to damage than those of young and adult mice. Brooks and Faulkner (1996) have also demonstrated that the magnitude of the injury induced by stretches of muscle fibres from rats and mice is increased with age and suggest

that the increased susceptibility of muscles from old rodents to contraction-induced injury resides in part within the myofibrils.

1.5.4. Recovery of muscles of older individuals from damage

The ability of muscle to repair damage efficiently is critical to its survival. Repair occurs by activation and differentiation of stem cells from within the muscle bulk. However, this ability of muscle to repair declines considerably with age (Brooks and Faulkner, 1990; McBride *et al*, 1995).

Brooks and Faulkner (1990) have demonstrated that following contraction-induced injury, muscles from young and adult mice recover fully by 28 days following a severely damaging protocol, whereas muscles from old mice have not recover completely by up to 60 days following the same protocol. The pattern of injury and recovery in mouse muscles is similar to that reported from human muscles (Jones *et al*, 1986). Interestingly, Carlson and Faulkner (1989) have demonstrated that the ability of muscle to regenerate successfully is dependant on the "environment" of that muscle. When muscle is transplanted from an old rat into a young rat, then the muscle regenerates at an equivalent rate to young muscle in contrast to the impaired regeneration in muscles of old rats. This suggests that there is no inherent difference between the ability of the stem cells from muscle of old rats to regenerate and that the environment of the muscle plays an important role.

In summary, several studies have shown that ageing results in a reduction in muscle mass due to a decrease in muscle fibre number and fibre size and that

skeletal muscles of older individuals are also weaker, are more susceptible to contraction-induced damage and take longer to recover from damage.

1.5.5. Reactive oxygen species and ageing

It has been hypothesized that a main cause of the ageing process and the development of chronic disease in older people may be the cumulative damage to lipids in cell membranes, DNA, and sub-cellular membranes and structures, by reactive oxygen species. This theory, also known as the free radical theory of ageing, was first described by Harman in 1956 and is currently one of the most popular explanations for how ageing occurs at the biological level.

It has been observed that the radical levels increase with age in species such as the housefly, rats and humans (Koward & Kirkwood, 1994). In skeletal muscle, studies on animals have provided evidence of an age related increased production of ROS. For example, Lass *et al* (1998) have demonstrated that the rate of superoxide radical generation in skeletal muscle increases with age.

An increase in free radical production can lead to changes in the redox state of muscle cells with potentially serious effects on muscle. Proteins with a high percentage of sulphydryl groups such as myosin and creatine kinase can be oxidised and therefore they will not function efficiently. In addition, transcription factors containing redox-sensitive sites will be particularly susceptible to damage.

Furthermore, numerous studies have shown that ageing cells accumulate increased levels of oxidant-damaged mitochondrial DNA. Increasing damage to mitochondrial DNA inevitably leads to abnormal mitochondrial function and integrity. Damaged mitochondria are thought to release more ROS that in turn

leads to more DNA damage. Large mitochondrial deletions have been found to be increased as much as 10,000 fold with age in several human tissues including skeletal muscle (De Flora *et al*, 1996). In addition, oligonucleotide array analysis of resting skeletal muscle of aged mice has demonstrated an age-related decrease in the expression of genes involved in oxidative phosphorylation due to mitochondrial dysfunction in aged animals (Lee *et al*, 1999).

Thus, a pathological increase in production of free radicals would result in an accumulation of oxidation products and an accumulation of abnormal mitochondria. In return, dysfunctional mitochondria produce significant amounts of free radicals that may play a major role in the physiological and structural changes seen in skeletal muscle of aged mammals.

A number of studies have focused on the role that calorie-restricted feeding plays during ageing. It has been demonstrated that caloric restriction (CR) not only extends the average and maximum life span of rodents but it also retards the accrual of markers of oxidative damage such as the tissue concentration of peroxidised lipids, protein carbonyls and oxidative damage to bases in genomic and mitochondrial DNA (for a review see Merry, 2002). In addition, rodents subjected to CR show attenuation of age-related increases in rates of mitochondrial $O_2^{\bullet-}$ and H_2O_2 generation and delayed loss of membrane fluidity (Sohal and Weindruch, 1996). In skeletal muscle, Lass *et al* (1998) have demonstrated that oxidative damage to mitochondrial proteins and lipids as well as the rate of superoxide anion radical generation was significantly increased with age in ad libitum-fed mice, whereas the CR mice demonstrated no age-associated increase in mitochondrial protein or lipid oxidative damage, or in superoxide anion radical generation. Thus, this data suggests that CR can prevent age-

associated accrual of oxidative damage to skeletal muscle mitochondria. However, the mechanisms induced by CR feeding that results in reduced ROS generation and reduced oxidative damage to mitochondria are still unresolved.

1.5.6. Antioxidant defence enzymes and ageing in resting skeletal muscle

Although this area of research is somewhat confusing, there seems to be some evidence of an attempt, particularly in skeletal muscle, to adapt to an age-related chronic increase in the production of ROS by adaptation of antioxidant defence enzymes.

Ji *et al* (1990) have shown that there was an increase in the specific activities of both mitochondrial and cytosolic SOD in rat skeletal muscle during ageing. CAT and GPX activities were also significantly higher in DVL muscle of aged versus young rats. Leeuwenburgh *et al* (1994) found similar increases in the activities of all antioxidant enzymes with ageing in DVL muscles in rats.

However, the response of the antioxidant defences to ageing appears to be highly tissue specific. Leeuwenburgh *et al* (1994) have shown a 37% increase in GSH content in soleus muscles of aged rats, however DVL muscles from the same rats showed no significant alterations in GSH content with ageing. Lawler *et al* (1993) reported a significant increase in GPX but not SOD activity in the soleus and gastrocnemius muscles of aged rats. In addition, Oh-Ishi *et al* (1995) reported an age- related increase in the activity and content of Cu/Zn SOD in rat soleus and extensor digitorum longus (EDL) muscles, whereas those of Mn SOD showed no difference between young and aged rats. Moreover, GPX activity indicated age-related increases only in soleus muscles with no significant differences in EDL muscles.

These observations indicate that age-related adaptation of cellular antioxidant defences is evident but seems to be muscle fibre specific, with the most prominent increases found in type I muscles such as soleus, followed by type IIA muscles such as DVL, whereas type IIB muscle fibres show little effect (Leeuwenburgh *et al*, 1994; Oh-Ishi *et al*, 1995).

1.5.7. Influence of exercise on ROS production and antioxidant defence enzyme activity in tissues of aged mammals

Although skeletal muscles of young/adult mammals rapidly adapt to acute bouts of exercise by increasing their antioxidant activity (Section 1.3.2), muscles of aged mammals appear to be unable to adapt. Ji *et al* (1990), found no significant alterations in most antioxidant defence enzymes in the skeletal muscles from aged male rats following exercise. Furthermore, Lawler *et al* (1993) found no increase in the SOD and GPX activities in the gastrocnemius and soleus muscles from aged female rats after 40 mins treadmill running.

A few studies have shown that endurance training has some effect in the increase of some antioxidant defence enzyme activities in skeletal muscle of aged mammals, however this increase appears to be specific. For example, Ji *et al* (1991) reported an increase in the GPX activity in skeletal muscles from aged rats, whereas CAT and SOD activities remained unchanged with training. In addition, Leeuwenburgh *et al* (1994) have shown that training has little effect on the antioxidant defence enzyme activity in both DVL and soleus muscles from aged male rats.

Aged muscles already have higher activities of antioxidant defence enzymes and GSH levels may also influence their response to exercise. Due to the complex methods necessary to measure the production of free radicals directly,

there are few reports examining whether the altered activity of the antioxidant defence enzymes in muscles of aged rodents is sufficient enough to cope with any age-related increase in the production of ROS. However, Bejma and Ji (1999) have demonstrated that the production of ROS by muscle homogenates of aged mice remained elevated. This is supported by unpublished observations from our laboratory suggesting that the $O_2^{\bullet-}$ production of contracting muscle of aged mice was elevated compared with that of young mice.

The inability of muscle from aged mammals to adapt following exercise may be associated with the age-related muscle weakness, their increased susceptibility to damage, as well as their poor recovery from damage. However, the cellular mechanisms responsible for this age-related decline in skeletal muscle function remain unclear.

1.5.8. HSPs, skeletal muscle and ageing

A growing body of literature indicates that ageing is associated with a reduced ability to express HSPs in several tissues following stress. In 1990, Fargnoli *et al* reported an age-related decline in the expression of HSP70 in lung and skin fibroblasts of aged rats following thermal stress. Wu *et al* (1993) demonstrated that ageing resulted in a decrease in the ability of hepatocytes from rats to synthesise HSP70 in response to hyperthermia. In addition, Locke and Tanguay (1996a) showed that following heat stress, hearts from aged rats demonstrated a reduction in Hsp70 mRNA and a reduction in Hsp70 protein content, compared with hearts from adult rats.

We have previously shown that the production of HSP70 in response to a period of mild, non-damaging contractile activity was severely blunted in the

gastrocnemius muscles from aged rats at 24 hours following the contraction protocol (Vasilaki *et al*, 2002). However, a study by Locke (2000) demonstrated no significant differences in the HSP70 content in both soleus and gastrocnemius muscles between adult and aged rats following whole-body hyperthermia. In this case, it appears that the nature of the stress may be important and that the mechanisms responsible for activation of the stress response following hyperthermia or exercise may not be entirely comparable and may be differentially affected by age.

The ability of muscles of aged rodents to adapt in response to training appears to be fibre type specific. Naito *et al* (2001) have demonstrated that exercise training of rats on a treadmill for ten weeks resulted in a similar exercise-induced accumulation of HSP70 in highly oxidative skeletal muscles (such as soleus) of young and aged animals whereas, in fast skeletal muscles (such as white gastrocnemius), HSP70 expression was blunted with ageing.

These observations indicate that the blunted HSP expression in skeletal muscles of aged individuals seems to be muscle fibre specific as well as stress specific.

1.5.9. Transcription factor activation and ageing

As discussed above, enhanced ROS production seems to be an important contributor to the ageing process (Section 1.5.5). The possibility that age-dependent changes in the cellular redox status due to the increased production of reactive oxygen intermediates and accumulation of oxidatively modified proteins can affect the function and regulation of the transcription factors has received increased attention.

There is now considerable evidence that ageing affects transcription factor activation in several tissues. For example, Fawcett *et al* (1994) demonstrated that while the levels of HSF1 remained constant as a function of ageing, HSF1 in the adrenals of aged rats exhibited a decreased ability to bind DNA following neurohormonal stress. Liu *et al* (1996) also reported a decrease in HSF1 DNA binding activity in aged human diploid lung fibroblasts following heat shock due to the reduced ability of heat shock to promote trimerisation of HSF1. Furthermore, Locke and Tanguay (1996) demonstrated a 47% reduction in HSF1 activation in hearts from aged rats compared to hearts from young rats following thermal stress while myocardial HSF1 protein content was similar between the two experimental groups. In addition, a study by Heydari *et al* (2000) showed that the DNA binding activity of HSF1 from rat hepatocytes was decreased significantly following heat shock.

In skeletal muscle, a study by Locke (2000) demonstrated that heat shock activates HSF1 in soleus (fast) and white gastrocnemius (slow) muscles from aged rats. However, the effect of exercise on HSF1 activation in skeletal muscle of aged mammals has not been studied in detail.

The influence of ageing on the activation of NF-κB and AP-1 has also been examined. Helenius *et al* (1996a), reported an up-regulation of NF-κB binding activity during ageing in the quiescent mouse heart whereas, AP-1 binding activity was not affected by age. A different study by the same group also demonstrated an age-associated activation of NF-κB in the liver, heart, kidney and brain from aged rats and mice, whereas AP-1 binding activity showed an age-related decrease (Helenius *et al*, 1996b). In response to oxidative stress, Pahlavani and Harris (1998) demonstrated that reactive oxygen species generated by

xanthine-xanthine oxidase treatment resulted in a decrease in the DNA binding activity of NF-κB in T cells from aged rats compared to untreated T cells from aged rats.

In skeletal muscle, Hollander *et al* (2000) reported that DNA binding of both NF-κB and AP-1 was significantly decreased with age in the gastrocnemius, soleus and superficial vastus lateralis muscles of aged rats compared with the DNA binding of NF-κB and AP-1 in the gastrocnemius, soleus and superficial vastus lateralis muscles of young rats. Whether or not long term exercise during ageing can affect the activation of these transcription factors requires further investigation.

1.6. AIMS OF THE STUDY

The aims of this study were to examine:

1. the oxidative status of skeletal muscles from adult and aged B6XSJL mice
2. the relationship of (1) to the ability of the muscle to adapt following a period of non-damaging contractile activity
3. the extent and time course of attenuation in the stress response in skeletal muscles of aged mice following contractile activity
4. the mechanisms which may be responsible for any attenuation detected
5. whether there are any gender-specific differences in the stress response of adult and aged mice following contractile activity

CHAPTER 2

MATERIALS AND METHODS

2.1. MICE AND EXERCISE PROTOCOL

25 adult (average age: 12 months old) and 24 aged (average age: 30 months old) male B6XSJL mice as well as 19 adult (average age: 13 months old) and 22 aged (average age: 30 months old) female B6XSJL mice were used in this study. A proportion of mice from each group was subjected to a period of non-damaging isometric contractions. Animals were fed on a standard laboratory diet and subjected to a 12 hours light-dark cycle.

Exercise protocol:

Adult and aged B6XSJL mice were anaesthetised with either 65mg/kg or 20mg/kg sodium pentobabitone respectively. Both hindlimbs were subjected to a 15-minute period of electrical contractions via surface electrodes using a 100 H_z pulse train at 60 Volts for 0.5sec every 5sec with a pulse width of 0.1msec. Mice were killed immediately, 4 hrs, 12 hrs and 24 hrs following the exercise protocol by cervical dislocation and gastrocnemius and anterior tibialis (AT) muscles were removed and frozen in liquid nitrogen. Muscles were stored at -70°C until analysis.

Blood samples were also removed from the aorta and centrifuged at 10,000g for 10mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.). Serum was analysed for activity of creatine kinase (CK) as an index of muscle damage.

2.2. SAMPLE PREPARATION

A schematic representation of sample preparation is shown in Figure 2.1.

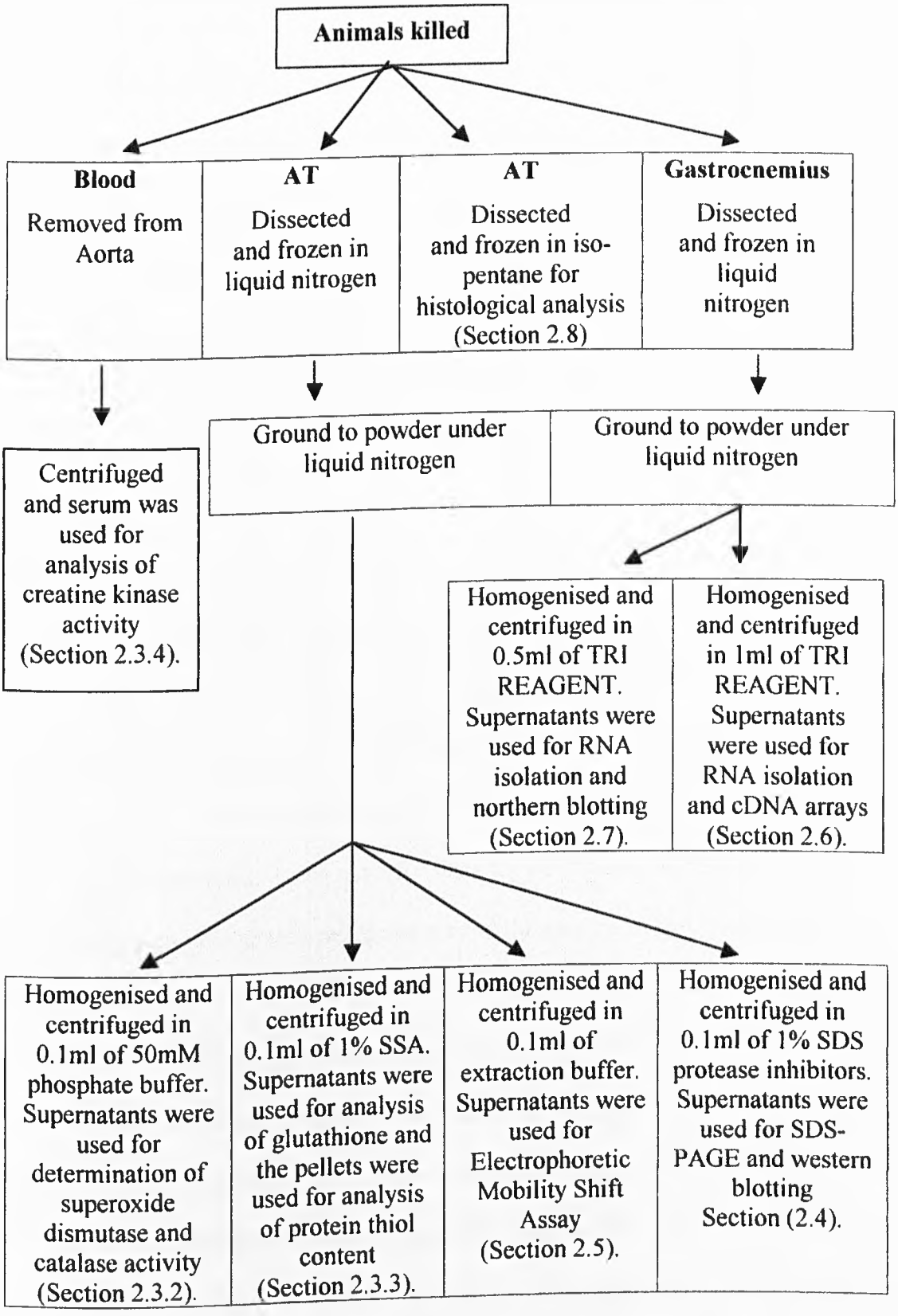


Figure 2.1. Flow diagram of sample preparation.

2.2.1. Preparation of samples from anterior tibialis muscles

Anterior tibialis (AT) muscles (approximately 60-70mg) from non-exercised (control) and exercised adult and aged male and female mice at 4, 12 and 24 hours following the contraction protocol were ground under liquid nitrogen and the resulting powder was equally divided as follows:

a) Analysis of antioxidant defence enzymes:

One aliquot was placed into 100µl of 50mM phosphate buffer, pH 7.0. The samples were homogenised (TRI-R Instruments, Model K43, Rockville Centre, N.Y.) and centrifuged at 10,000g for 10mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.) and supernatants were used for the determination protein content by the BCA method (Section 2.3.1) and for determination of superoxide dismutase (Section 2.3.2.1) and catalase (Section 2.3.2.2) activity.

b) Analysis of glutathione and protein thiol content:

One aliquot was placed into 100µl of 1% SSA. The samples were homogenised (TRI-R Instruments, Model K43, Rockville Centre, N.Y.) and centrifuged at 10,000g for 10mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.) and supernatants were used for analysis of glutathione (Section 2.3.3.1), and the protein-precipitated pellets were used for analysis of protein thiol content (Section 2.3.3.2).

c) Analysis of HSPs by SDS-PAGE and western blotting:

One aliquot was placed into 100µl of 1% SDS containing protease inhibitors (Section 2.4.2) The samples were homogenised (TRI-R Instruments, Model K43, Rockville Centre, N.Y.) and centrifuged at 10,000g for 10mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.) and supernatants were used for detection of HSPs by SDS-PAGE (Section 2.4.3).

In addition, AT muscles (approximately 60-70mg) from non-exercised adult and aged male mice and mice killed immediately following the contraction protocol were ground in liquid nitrogen and the resulting powder was equally divided as follows:

d) Detection of transcription factor DNA binding activity using EMSA:

One aliquot was placed into 100µl of extraction buffer (Section 2.5.1). The samples were homogenised (TRI-R Instruments, Model K43, Rockville Centre, N.Y.) and centrifuged at 10,000g for 10mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.) and supernatants were analysed for Heat Shock Factor 1 (HSF1), Activator protein-1 (AP-1) and Nuclear Factor κB (NF-κB) DNA binding activity (Section 2.5).

e) Analysis of transcription factors by SDS-PAGE and western blotting:

One aliquot was placed into 100µl of 1% SDS containing protease inhibitors (Section 2.4.2) The samples were homogenised (TRI-R Instruments, Model K43, Rockville Centre, N.Y.) and centrifuged at 10,000g for 10mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.) and supernatants were used for detection of transcription factors by SDS-PAGE (Section 2.4.3).

2.2.2. Preparation of samples from gastrocnemius muscles

a) Isolation of RNA for cDNA expression arrays:

Approximately 100mg of gastrocnemius muscle tissues from non-exercised and exercised adult and aged male mice killed immediately, 4 and 12 hours following the contraction protocol were ground and centrifuged in 1ml of TRI REAGENT (Section 2.6.1) at 12,000g for 10mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.). Supernatants were used for total RNA isolation (Section 2.6.1) and cDNA microarrays (Section 2.6).

b) Isolation of RNA for northern blotting:

Approximately 50mg of ground muscle from non-exercised adult and aged mice and mice killed immediately following the contraction protocol were placed in 500µl of TRI REAGENT (Section 2.6.1) The samples were centrifuged at 12,000g for 10mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.) and supernatants were used for isolation of RNA (Section 2.6.1) and northern blotting (Section 2.7).

2.3. BIOCHEMICAL ANALYSES

2.3.1. Analysis of protein content of samples using the bicinchoninic acid (BCA) method for protein determination.

Where specified, the protein content of the samples was measured using the bicinchoninic acid (BCA) protein assay kit (Sigma Co., Dorset, U.K.). This was based on the method developed by Smith *et al* (1985).

Reagents:

- Reagent A: Bicinchoninic acid (BCA) solution, containing: 25mM BCA-Na, 160mM $NaCO_3.H_2O$, 7.0mM Na_2 tartrate, 0.1mM NaOH and 0.95% $NaHCO_3$, pH 11.25 (Sigma Immunochemicals, Dorset, U.K.)
- Reagent B: 160mM $CuSO_4.5H_2O$ (Sigma Immunochemicals, Dorset, U.K.)

Protocol:

A range of standards between 0-250µg/ml was prepared from a stock solution of 1mg/ml Bovine serum albumin (BSA) in 0.15M NaCl with 7.7mM NaN_3. Reagent C was prepared immediately before use by the addition of 500µl of Reagent B to 25ml of reagent. A 20µl of the standard, blank or sample and 200µl of Reagent C were placed in a 96 well microtitre plate, mixed and incubated at 50°C for 30mins. Samples were then cooled at room temperature. The absorbance of standards and samples was measured at 570nm using a microplate reader (Benchmark, Biorad, U.K.). The protein content of each sample was calculated from the standard curve.

2.3.2. Spectrophotometric assays for superoxide dismutase and catalase activities

2.3.2.1. Spectrophotometric assay for superoxide dismutase activity based on the reduction of cytochrome c:

The assay is based on the production of superoxide radical by xanthine/xanthine oxidase. These radicals react with the oxidised form of cytochrome c to produce a reduced form of cytochrome c that absorbs light at 550nm. Thus, xanthine/xanthine oxidase is used as a source of superoxide and cytochrome c is used as the indicating scavenger for the radical. SOD quenches this reaction; thus, the greater the activity of SOD, the greater the quenching. This assay is based on the method developed by Crapo *et al* (1978).

Reagents:

All reagents were prepared in 50mM potassium phosphate containing 0.1mM EDTA, pH 7.8.

- 0.1mM Ferricytochrome c (Sigma Immunochemicals, Dorset, U.K.)
- 0.5mM xanthine (Sigma Immunochemicals, Dorset, U.K.)
- Stock xanthine oxidase (12.425 units/ml, Sigma Immuno-chemicals, Dorset, U.K.)
- 50mM potassium phosphate containing 0.1mM EDTA, pH 7.8

Protocol:

Fifty microlitres of cytochrome c, 50µl of xanthine and 400µl of the buffer were placed in a cuvette. The reaction was initiated by the addition of 5µl of stock xanthine oxidase diluted to give a rate of increase in absorbance at 550nm and

25°C of 0.020 AU per minute The reaction was monitored using a spectrophotometer (CECIL CE594/ Double Beam Spectrophotometer, Cambridge, U.K.). Another reaction mixture was then prepared in which 50µl of the sample to be assayed replaced an equal volume of the buffer, and the rate was again recorded after addition of xanthine oxidase. The ability of the sample to quench the production of superoxide radical, and so the reduction of cytochrome c was determined.

2.3.2.2. Spectrophotometric assay for catalase activity

The spectrophotometric assay of catalase, originally developed by Beers and Sizer (1952), was employed (Claiborne, 1985). The enzyme catalyses the decomposition of hydrogen peroxide as follows:

$$2H_2O_2 \rightarrow 2\,H_2O + O_2 \quad (1)$$

The decomposition of hydrogen peroxide catalysed by catalase (Equation 1) can be followed by ultraviolet spectroscopy, due to the absorbance of hydrogen peroxide in this region (Claiborne, 1985). At 240nm, the molar extinction coefficient for H_2O_2 is $43.6M^{-1}cm^{1}$.

Reagents:

- 30% hydrogen peroxide (Sigma Immunochemicals, Dorset, U.K.).
- 50mM potassium phosphate buffer, pH 7.0

Protocol:

Since H_2O_2 degrades rapidly upon storage, the concentration of the stock hydrogen peroxide solution (normally 8.8 to 9.1M) was measured by diluting hydrogen peroxide in distilled water (1/800 dilution). The absorbance at 240nm of

the diluted peroxide sample was then recorded (CECIL CE594/ Double Beam Spectrophotometer, Cambridge, U.K.) against a matched quartz cuvette containing distilled water. The concentration of the stock hydrogen peroxide was calculated from the molar extinction coefficient of $43.6 M^{-1} cm^{1}$.

A solution of 19mM hydrogen peroxide was prepared in 50mM potassium phosphate buffer, pH 7.0. Five hundred microlitres of this solution and 5µl of sample were placed into a quartz cuvette and the decrease in absorbance (at 240nm, 25°C) was monitored. All measurements were repeated in triplicate.

The specific activity of catalase at 25°C is defined in terms of micromoles of hydrogen peroxide consumed per minute per milligram of protein. The conversion of maximum velocity to specific activity of catalase is made as follows:

$$\text{Specific Activity (units/mg)} = \frac{\Delta A_{240nm} \text{ per minute x } 1000}{43.6 \text{ x } \frac{\text{mg protein}}{\text{ml reaction mix}}}$$

2.3.3. Determination of total glutathione and sulphydryl (thiol) content of muscles

2.3.3.1. Glutathione assay

Glutathione was analysed using a kinetic enzymatic re-cycling assay (Anderson, 1985) based on the reaction of glutathione with DTNB.

Reagents:

- 1% (w/v) sulphosalicylic acid (SSA)
- Glutathione reductase (1346 units/ml, Sigma Immunochemicals, Dorset, U.K.) diluted 1 in 20 in stock buffer
- Glutathione (oxidized form, Sigma Immunochemicals, Dorset, U.K.)
- Glutathione (reduced form, Sigma Immunochemicals, Dorset, U.K.)
- Stock buffer: 143mM sodium phosphate (Na_2HPO_4), pH 7.5
- 6.3mM Tetrasodium EDTA (Na_4EDTA)
- Daily buffer: 3.5mg NADPH (Sigma Immunochemicals, Dorset, U.K.) in 14ml stock buffer
- DTNB solution: 11.9mg 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in 5ml stock buffer

Protocol:

A range of standards between 10.4 and 0.081mM and a blank was prepared from a stock solution containing 16mg glutathione (oxidised form) and 16mg glutathione (reduced form) in 10ml of 1% SSA. A cocktail solution

containing 14ml of daily buffer, 2ml of DTNB solution, 2ml of distilled water and 200µl of glutathione reductase was prepared immediately before use. A 20µl of the standard or sample and 200µl of the cocktail solution were placed in a 96 well microtitre plate and mixed. The absorbance of standards and samples was measured at 415nm for 10mins using a microplate reader (Benchmark, Biorad, U.K.). 1% SSA was used as blank.

2.3.3.2. Sulphydryl group (protein thiol) assay

The sulphydryl content of the AT muscles was measured using the assay developed by Di Monte *et al* (1984).

Reagents:

- 0.5M Tris/HCl buffer, pH 7.6
- 1.1mM DTNB in Tris/HCl buffer, pH 7.6
- 1% (w/v) SSA
- Glutathione (reduced form) Sigma Immunochemicals, Dorset, U.K.)

Protocol:

A range of standards between 50 and 0.4 µM was prepared from a 5mM stock solution containing 3mg glutathione (reduced) in 2ml of Tris/HCl buffer, pH 7.6. The samples had previously been homogenised in 1ml of SSA (Section 2.2), centrifuged and their pellet was resuspended in 1ml of Tris/HCl buffer, pH 7.6. A 200µl of the standard or sample and 20µl of DTNB solution were placed in a 96 well microtitre plate, mixed and incubated at room temperature for 20mins. The

absorbance of standards, samples and blank (Tris/HCl buffer) was measured at 415nm using a microplate reader (Benchmark, Biorad, U.K.).

2.3.3.3. Lowry assay for protein content

Reagents:

- 1M KOH
- 0.9% (w/v) NaCl
- Bovine Serum Albumin (BSA, Sigma Immunochemicals, Dorset, U.K.)
- Folin and Ciocalteu's phenol reagent (diluted 1 to 1 with distilled water, Sigma Immunochemicals, Dorset, U.K.)
- Solution A: 20g of Na_2CO_3 in 0.1M NaOH
- Solution B: 0.5g of $CuSO_4.5H_2O$ in 1% trisodium citrate

Protocol:

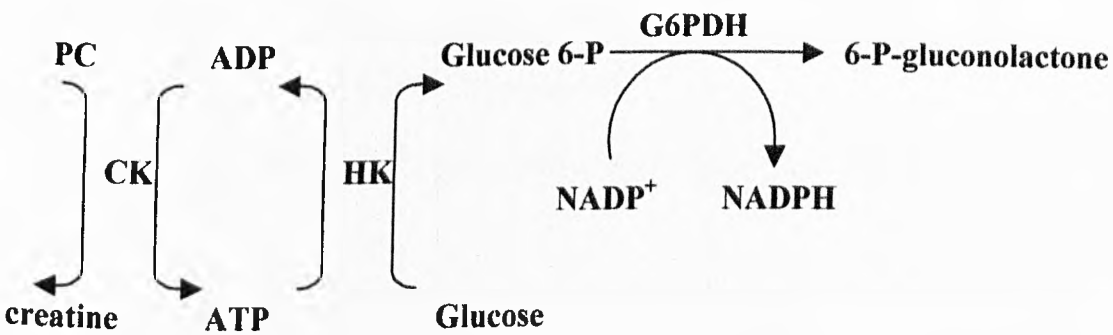
Samples were diluted 1 in 3 in 1M KOH and incubated for 3 hours at 37°C. A range of standards between 0 - 250µg/ml was prepared from a stock solution of 0.25mg/ml Bovine serum albumin (BSA) in 0.9% NaCl. Solution C was prepared immediately before use by the addition of 50ml of Solution A to 1ml of Solution B. 500µl of Solution C and 50µl of Folin solution were added in 100µl of standard or sample, mixed and allowed to stand at room temperature for 2 hours. The absorbance of standards and samples was measured using a glass cuvette at 750nm (CECIL CE594/ Double Beam Spectrophotometer, Cambridge, U.K.) against a blank.

2.3.4. Analysis of serum creatine kinase (CK) activity

CK catalyses the reversible formation of adenosine triphosphate (ATP) and creatine from adenosine diphosphate (ADP) and creatine phosphate (PC) according to the following reaction:

$$PC + ADP \xleftrightarrow{CK} ATP + creatine$$

Activity of CK is calculated by the conversion of $NADP^+$ to NADPH as described below, where one unit of CK activity is equivalent to the conversion of 1μmol of creatine phosphate substrate per minute at 20°C. The method was modified from Jones *et al*, 1983.



HK: hexokinase
G6PDH: glucose-6-phosphate-dehydrogenase

Reagents:

- Cocktail solution: 10mM $MgCl_2$

  2mM DL- Dithiothreitol (DTT)

  10mM Adenosine 5'-diphosphate (ADP)

  20mM Adenosine 5'-monophosphate (AMP)

  40mM phosphocreatine (PC)

  2mM β-Nicotinamide adenine dinucleotide phosphate (β-NADP)

  5mM glucose (all from Sigma Immunochemicals, Dorset, U.K.)

  5.4 U/l HK/G6PDH (Hexokinase/Glucose-6-Phosphate Dehydro-genase, Boehringer Manheim, Sussex, UK)

  40mM TEA buffer, pH 7.2

- 10mM NADPH (Sigma Immunochemicals, Dorset, U.K.)

Protocol:

A range of NADPH standards between 10 and 0.078 mM was prepared from a stock solution containing 16.6mg of NADPH in 2ml of TEA buffer, pH 7.2. 200μl of the cocktail solution were added to 20 microlitres of serum. The absorbance of samples was measured at 340nm for 10mins using a microplate reader (Benchmark, Biorad, U.K.). Two hundred microlitres of standards were placed in a microplate and the absorbance of the range of standards was measured at 340nm using a microplate reader (Benchmark, Biorad, U.K.). TEA buffer was used as blank. The CK activity was calculated based on the production of NADPH.

2.4. ANALYSIS OF HSP CONTENT OF SKELETAL MUSCLE TISSUES BY SODIUM DODECYL SULPHATE POLY-ACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND WESTERN BLOTTING

2.4.1. Preparation of polyacrylamide gradient gels

Reagents:

- Stock acrylamide solution: 30% acrylamide, 0.8% bisacrylamide cross-link, in dH_2O (Protogel, National Diagnostics, USA)

- Gel buffer: 1.5M Tris/HCl, 0.384% SDS, pH 8.8. (Protogel buffer, National Diagnostics, USA)

- Stacking buffer: 0.5M Tris/HCl, 0.4% SDS, pH 6.8. (Protogel stacking buffer, National Diagnostics, USA)

- 12% acrylamide solution (100ml):

  40ml stock acrylamide solution

  26ml gel buffer

  32.9ml dH_2O

- 4% stacking gel solution (100ml):

  13ml stock acrylamide solution

  25ml stacking buffer

  61ml dH_2O

Protocol:

12% solution of acrylamide with 0.8% bisacrylamide cross-link was prepared as described above. Gel formation was catalysed by the addition of 0.1ml of 10% aqueous ammonium persulphate solution (APS) and 10µl of NNN'N'-tetramethylethylene-diamine (TEMED) to 10ml of the 12% acrylamide solution. The gel solution was poured immediately between gel plates (8 x 10cm) with 2mm spacers and allowed to set for 10 to 20 mins. A 4% stacking gel solution was made as described above. Fifty microlitres of 10% aqueous APS and 10µl of TEMED were added to 10ml of the 4% gel solution. The 4% stacking gel solution was poured on top of the 12% gel to form a 1-1.5cm stacking gel and a well comb placed in position to facilitate sample loading.

2.4.2. Preparation of samples for SDS-PAGE and western blotting

Reagents:

- Homogenisation buffer: 1mM iodoacetimide, 1mM benzithonium chloride, 5.7mM Phenylmethylsulphonyl fluoride (PMSF) in 1% (w/v) SDS (lauryl sulphate)

- Laemmli buffer: 46.03 mg/ml SDS, 20.9% glycerol, 2.1% (v/v) β-mercaptoethanol, 0.052 mg/ml bromophenol blue in 0.128M Tris/HCl buffer, pH 6.8.

Protocol:

Muscle powder was placed into buffer containing 1% SDS and a range of protease inhibitors. Tissues were then homogenised (TRI-R Instruments, Model

K43, Rockville Centre, N.Y.) on ice and centrifuged at 10,000g for 10mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.). Protein content of supernatant was determined as described is Section 2.3.1. One hundred microgrammes of total cellular protein was boiled for 5mins in a water bath in an equal volume of Laemmli buffer. The cooled sample was then applied to a 12% polyacrylamide gel.

2.4.3. Electrophoresis of proteins

Reagents:

- Electrophoresis buffer: 10X Tris/ Glycine/ SDS (0.025M Tris, 0.192M glycine, 0.1% (w/v) SDS; National Diagnostics, Hessle Hull, UK)

Protocol:

Electrophoresis was carried out using an Anachem Electrophoresis tank with an LKB Power Pack cooled with H_2O at a constant current of approx. 20 mA per gel until the samples had run through the 4% gel (stacking gel). The current was then increased to 40 mA until the visible bromophenol dye was approx. 1cm above the bottom of the gel (approx. 1.5-2 hr). The separated proteins were then transferred from the gel onto a nitrocellulose membrane by western blotting.

2.4.4. Western blotting of separated proteins

Reagents:

- Anode 1 buffer: 0.3M Tris in a 20% methanol solution, pH 10.4.
- Anode 2 buffer: 25mM Tris in a 20% methanol solution, pH 10.4.
- Cathode buffer: 40mM 6-amino n hexanoic acid in a 20% methanol solution, pH 7.6.

Protocol:

The Multiphor II discontinuous blotting system (Pharmacia, Milton Keynes, UK.) consists of two graphite plate electrodes with Whatman No 1 filter paper used as a buffer reservoir (shown in Figure 2.2).

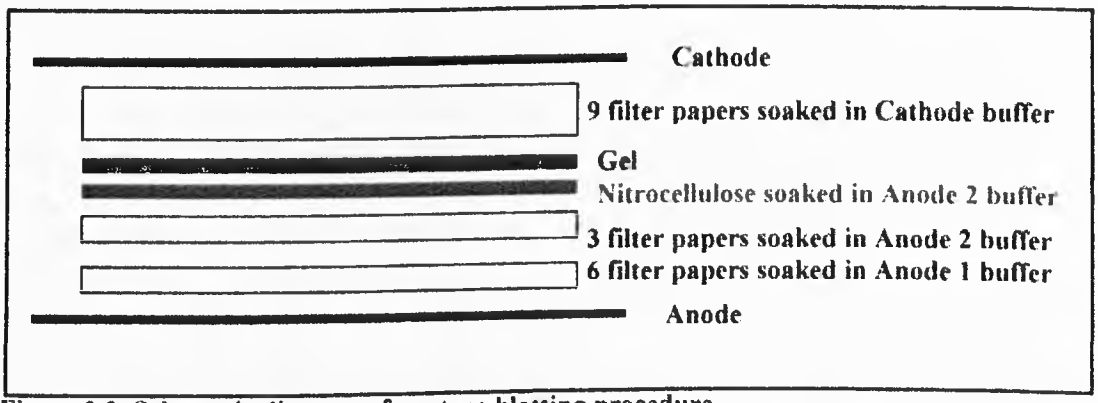


Figure 2.2. Schematic diagram of western blotting procedure.

Following electrophoresis, the gel was removed from the glass plates, placed on top of nitrocellulose (0.45µM pore size; Anderman & Co. Ltd., Surrey, UK.) and this was sandwiched between the electrodes as shown in Figure 2.2. A constant current density of 0.8 mA/cm^2 was applied to the system for a minimum time of 1.5hr at room temperature. Post-blotting staining of gels has revealed that this was sufficient to produce maximal transfer of proteins to the nitrocellulose.

2.4.5. Processing and development of the nitrocellulose membrane

Reagents:

- PBS solution: 0.05M KH_2PO_4, 0.05M Na_2HPO_4, 1.3M NaCl in dH_2O, pH 7.2.

- PBS solution: 0.05M KH_2PO_4, 0.05M Na_2HPO_4, 1.3M NaCl in dH_2O, pH 6.0.

- PBS/Tween solution: 0.05% (v/v) polyoxyethylene-sorbitan monolaurate (Tween 20) in PBS solution.

Protocol:

Following electroblotting, the nitrocellulose was removed and placed in 100ml of a blocking solution of 5g powdered milk/100ml PBS for either 1 hr at room temperature or overnight at 4°C. The membrane was then washed in a solution of PBS/Tween. The nitrocellulose membrane was then analysed for HSP60, HSC70, HSP70 and HSP25 using a panel of monoclonal or polyclonal antibodies. Some membranes were also analysed for c-Fos, c-Jun, p-50, p-65 and HSF1 content. The nitrocellulose was agitated for an hour in 10ml of a solution of PBS/Tween containing the primary antibody at the concentration listed in Table 2.1. The membrane was then washed in PBS/Tween solution for 3x5mins and placed into the appropriate peroxidase labelled secondary antibody solution in PBS/Tween containing 25% FCS as a non-specific blocking agent 1 hour at room temperature. The membrane was then washed for 2x5mins, 1x15mins in PBS/Tween and 10mins in PBS, pH 6.0 (the optimal pH for peroxidase activity).

The membrane was developed using the ECL chemiluminescent detection kit (Amersham International, U.K.). The membrane was placed between acetate sheets and analysed using a Biorad Chemi-Doc System (Biorad, Hercules, USA) or exposed to chemiluminescence film (Hyperfilm ECL, Amersham International, U.K.), for 20sec-1min. The film was developed in a 10% solution of developer (Ilford PQ Universal, Ilford, U.K.) and fixed in a 25% solution of fixer (Ilford Hypam, Ilford, U.K.).

Table 2.1. Anti-HSP antibody concentrations

			PRIMARY ANTIBODY	SECONDARY ANTIBODY
ANTIBODY	SOURCE	SPECIES	DILUTION	DILUTION
HSP25	Stressgen Inc., Canada	Rabbit	1: 2500	1: 1000
HSP60	Stressgen Inc., Canada	Mouse	1: 5000	1: 1000
HSC70	Sigma, Dorset, U.K.	Mouse	1: 5000	1: 1000
HSP70	Calbiochem, San Diego, USA	Mouse	1: 500	1: 1000
c-Fos	Autogen Bioclear, Calne, UK	Mouse	1: 1000	1: 1000
c-Jun	Autogen Bioclear, Calne, UK	Rabbit	1: 1000	1: 1000
NF-κB p50	Autogen Bioclear, Calne, UK	Mouse	1: 1000	1: 1000
NF-κB p65	Autogen Bioclear, Calne, UK	Rabbit	1: 1000	1: 1000
IκB-α	Autogen Bioclear, Calne, UK	Rabbit	1: 1000	1: 1000
HSF1	Lab Vision Co., Suffolk, UK	Rat	1: 1000	1: 1000

Primary antibodies:

HSP25

Rabbit polyclonal Anti-Heat Shock protein 25 (Stressgen Inc., Canada). The antibody supplied as whole rabbit serum. It is shipped lyophilised and contains 100µl rabbit anti-serum.

HSP60

Mouse Monoclonal Anti-Heat Shock protein 60 (Stressgen Inc., Canada). This antibody was purified from mouse ascites fluid and has been previously referenced as clone LK-2. Catalogue Number SPA-807. 200µl lyophilised protein in reconstituted in 200µl sterile distilled water.

HSC70

Mouse Monoclonal Anti-Heat Shock protein cognate 70 (Sigma Immunochemicals, Dorset, U.K.). This was derived from the BRM-22 hybridoma produced by the fusion of mouse myeloma cells and splenocytes from BALB/c mice immunised with purified bovine brain HSC70. Antibody supplied as mouse ascites fluid. Clone BRM-22.

HSP70

Mouse monoclonal Anti-70kD Heat Shock protein (Calbiochem, San Diego, USA). This antibody is of the IgG_1 class, and is purified by ion-exchange chromatography. The antibody is suitable for detecting the inducible form of the HSP70 family (HSP72).

c-Fos

c-Fos Mouse Monoclonal IgG_1 Antibody (Autogen Bioclear, Calne, UK). This antibody is raised against a bacterial expressed GST fusion protein construct mapping between amino acids 139-211 (leucine zipper) of c-Fos p62 of human origin. This antibody detects a 55-62kDa protein, corresponding to the apparent molecular mass of c-Fos.

c-Jun

c-Jun Rabbit Polyclonal Antibody (Autogen Bioclear, Calne, UK). This affinity-purified antibody is raised against a peptide mapping within the amino terminal domain of c-Jun p39 of mouse origin. This antibody detects a 39kDa protein, corresponding to the apparent molecular mass of c-Jun.

NFκB p50

NFκB p50 Mouse Monoclonal IgG_1 Antibody (Autogen Bioclear, Calne, UK). This antibody is raised against a recombinant protein corresponding to amino acids 120-239 mapping at the amino terminus of NFκB of human origin. This antibody detects a 105kDa protein, corresponding to the apparent molecular mass of NFκB p50.

NFκB p65

NFκB p65 Rabbit Polyclonal Antibody (Autogen Bioclear, Calne, UK). This antibody is raised against a recombinant protein mapping at amino acids 1-286 sequence corresponding to the amino terminus of NFκB p65 of human origin.

The antibody detects a 65kDa protein, corresponding to the apparent molecular mass of NFκB p65.

IκB-α

IκB-α Rabbit Polyclonal Antibody (Autogen Bioclear, Calne, UK). This affinity-purified antibody is raised against a peptide mapping at the carboxy terminus of IκB-α of human origin (identical to corresponding mouse sequence). The antibody detects a 37kDa protein, corresponding to the apparent molecular mass of IκB-α.

HSF1

Heat Shock Factor 1(HSF1) Rat Monoclonal Antibody Ab-4 (Lab Vision Co, Suffolk, UK). Ab-4 antibody is a cocktail antibody (Clones 4B4+10H4+10H8) especially designed for sensitive detection of HSF1. The antibody detects a 70-85kDa protein, corresponding to the apparent molecular mass of HSF1 (depending upon the source and state of cells).

Secondary antibodies:

Peroxidase conjugated goat anti-mouse IgG monoclonal antibody (FC specific). Affinity isolated antigen specific antibody (Sigma Immunochemicals, Dorset, U.K.).

Peroxidase conjugated goat anti-rabbit IgG monoclonal antibody (whole molecule). Affinity isolated antigen specific antibody (Sigma Immunochemicals, Dorset, U.K.).

2.4.6. Removing antibodies and re-probing the nitrocellulose membrane

Reagents:

- 100mM β-mercaptoethanol (Sigma Immunochemicals, Dorset, U.K.)
- 2% (w/v) SDS
- 62.5mM Tris/HCl, pH 6.7
- PBS/Tween solution, pH 7.2
- Blocking solution containing 5g powdered milk/100ml PBS

Protocol:

Following investigation of a protein (e.g. HSP25), the antibodies can be removed and the nitrocellulose membrane can be re-analysed for other proteins. After exposure to ECL detection solution (Amersham, U.K.), the membrane was washed in PBS/Tween solution for 5mins and then incubated in a solution containing 100mM β-mercaptoethanol, 2% SDS, 62.5mM Tris/HCl, pH 6.7 for 30mins at 50°C. The membrane was then washed for 2x10mins in PBS/Tween solution at room temperature. Finally, the membrane was placed in 100ml of blocking solution for 1hr at room temperature or alternatively at 4°C overnight. The membrane was then analysed for the content of other HSPs as described in Section 2.4.5.

2.5. ELECTROPHORETIC MOBILITY SHIFT ASSAY

2.5.1. Preparation of samples for electrophoresis mobility shift assay

Reagents:

- Extraction buffer: 25% (v/v) glycerol
  0.42M NaCl
  1.5mM $MgCl_2$
  0.2mM EDTA
  0.5mM dithiothreitol (DTT)
  0.5mM Phenylmethylsulphonyl fluoride (PMSF)
  20mM HEPES (N-2-hydroxyethylpiperazine -*N*'-2-ethanesulfonic acid, pH 7.9)

Protocol:

Protein extracts were prepared according to the method described by Mosser *et al* (1988). Skeletal muscles were ground in liquid nitrogen, homogenised for 15 sec in 15 volumes of extraction buffer (TRI-R Instruments, Model K43, Rockville Centre, N.Y.) and centrifuged at 10,000g for 10mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.). The supernatants were then removed, rapidly frozen in liquid nitrogen and stored at –70°C. The protein content of supernatants was determined as described is Section 2.3.1.

2.5.2. Preparation of the ^{32}P-labeled oligonucleotide probe

Reagents:

- HSE self-complementary oligonucleotide sequence (Thermo Hybaid GmbH, Ulm, Germany):

  5'-CTAGAAGCTTCTAGAAGCTTCTAG-3'

- NF-κB oligonucleotide sequence (Promega Corporation, Madison, USA):

  5'-AGTTGAGGGGACTTTCCCAGGC-3'

  3'-TCAACTCCCCTGAAAGGGTCCG-5'

- AP-1 (c-Jun) oligonucleotide sequence (Promega Corporation, Madison, USA):

  5'-CGCTTGATGAGTCAGCCGGAA-3'

  3'-GCGAACTACTCAGTCGGCCTT-5'

- 10X kinase buffer (700mM Tris/HCl (pH 7.6), 100mM $MgCl_2$, 50mM DTT; Promega Corporation, Madison, USA)
- Redivue adenosine 5'-[γ-^{32}P]triphosphate, triethylammonium salt (9.25MBq, 250μCi , Amersham International, U.K.)
- T4 polynucleotide kinase (10U/μl, Promega Corporation, Madison, USA)
- TE buffer (10mM Tris/HCl pH 7.6, 1mM EDTA)
- 0.5M EDTA (Sigma Immunochemicals, Dorset, U.K.)

Protocol:

- ***Labeling of HSE consensus oligonucleotide:***

[^{32}P]-labeled oligo was prepared by 5' end labeling one strand with T4 kinase and [γ-^{32}P]-ATP and then annealed to the complementary strand.

Five microlitres of 10X kinase buffer, 40µl of dH_2O, 2µl of [γ-^{32}P]-ATP and 2µl of T4 kinase were added to 100ng of oligonucleotide and incubated at 37°C for 1 hour. Following incubation, 48µl of TE buffer and 2µl of 0.5M EDTA were added to the oligonucleotide. The final concentration of the kinased oligonucleotide was approximately 1ng/µl.

The radiolabeled strand was then annealed to the complementary strand by incubating 20µl of the kinased oligonucleotide with an excess (4-5X) of unlabeled strand and 10µl of TE buffer at 85°C for 5min. The mixture was allowed to cool slowly for 4hrs.

- ***Labeling of NF-κB and AP-1 consensus oligonucleotides:***

One microlitre of 10X kinase buffer, 5µl of dH_2O, 1µl of [γ-^{32}P]-ATP and 1µl of T4 kinase were added to 2µl of oligonucleotide (1.75pmol/µl) and incubated at 37°C for 10mins. Following incubation, 89µl of TE buffer and 1µl of 0.5M EDTA were added to the oligonucleotide.

The labeled oligonucleotides were separated from unincorporated nucleotides by chromatography through Micro Bio-Spin 30 columns (Biorad, Hercules, USA) equilibrated in TE buffer.

2.5.3. Electrophoresis of protein extracts

Reagents:

- Dye solution: 0.2% (w/v) bromophenol blue
  0.2% (w/v) xylene cyanol
  50% (v/v) glycerol

- AccuGel 29:1: 40% (w/v) 29:1 Acrylamide: bis-Acrylamide solution (National Diagnostics, Hessle Hull, UK)
- Ammonium persulphate (Sigma Immunochemicals, Dorset, U.K.)
- NNN'N'-tetramethylethylene-diamine (TEMED)
- 80% glycerol
- 5X binding buffer (50mM Tris/HCl (pH 7.5), 2.5mM EDTA, 20% (v/v) glycerol, 2.5mM DTT, 0.25mg/ml poly(dI-dC)•poly(dI-dC), 5mM $MgCl_2$; Promega Corporation, Madison, USA)
- 10X TBE buffer: 1M Tris
  0.9M Boric Acid
  0.01M EDTA

Protocol:

A 4% polyacrylamide solution was prepared by mixing 4ml of Accugel (29:1) with 2ml of 10X TBE buffer, 1.25ml of 80% glycerol and 32.4ml of dH_2O. Gel formation was catalysed by the addition of 300µl of 10% aqueous ammonium persulphate (APS) and 50µl of NNN'N'-tetramethylethylene-diamine (TEMED) to 40ml of the 4% polyacrylamide solution. The gel solution was poured

immediately between gel plates (8 x 10cm) with well comb placed in position to facilitate sample loading and the gel was allowed to set for at least 20 mins.

100µg of extract was mixed with 0.1ng of [^{32}P]HSE oligonucleotide in 1X binding buffer to a final volume of 20µl. Binding reactions were incubated for 30mins at 25°C. Following incubation, 2.5µl of dye solution was added to the samples and loaded onto the 4% polyacrylamide gel in electrophoresis buffer. Electrophoresis was carried out at room temperature for 2h at 200V using a Biorad DCode™ System (Biorad, Hercules, USA).

For DNA binding reactions of NF-κB and AP-1 oligonucleotides, 100µg of extract was mixed with 2µl of either NF-κB or AP-1 radiolabelled oligonucleotides in 1X binding buffer to a final volume of 20µl. The reactions were incubated at room temperature for 20mins. Following incubation, 2µl of dye solution were added to the samples and directly loaded onto the 4% polyacrylamide gel in electrophoresis buffer. Electrophoresis was carried out at room temperature for 1h at 350V using a Biorad DCode™ System (Biorad, Hercules, USA).

Gels were then wrapped in plastic membrane (Clingorap, VWR International Ltd, Lutterworth, UK) and exposed to a phosphor screen overnight (Amersham International, UK). The phosphor screen was scanned using Biorad Personal Molecular Imager FX (Biorad, Hercules, USA) and analysed using Quantity one Software (Biorad, Hercules, USA).

2.5.4. Competition and supershift experiments

In order to verify that the results from gel shift mobility assay do not arise from non-specific binding, competition experiments and supershift assays were performed.

In competition experiments, unlabelled DNA is added to the binding reaction to determine whether this effects the observed DNA-protein interaction. Competition tests take two forms: specific and non-specific. In the specific reactions, the unlabeled competitor DNA is the same sequence as the original probe. The non-specific reactions use DNA fragments or oligonucleotides with different sequences from the original probe. If the band is specific, the addition of the unlabeled specific competitor should decrease the intensity of the band. In the presence of an unlabeled non-specific competitor, the specific band should remain.

The addition of antibodies to the reaction is used to determine the identity and specificity of the binding protein. If the antibody recognizes the DNA-protein complex, it have one of 2 effects: 1) it can bind and produce a labeled band (supershift band), which migrated more slowly in the gel than the original DNA-protein complex or 2) if the antibody binds close to the DNA binding site of the transcription factor or changes the conformation of the transcription factor, binding of the transcription factor to the DNA sequence will be reduced or the band will disappear completely.

Reagents:

- c-Fos antibody (Autogen Bioclear, Calne, UK)
- c-Jun antibody (Autogen Bioclear, Calne, UK)

- NF-κB p50 antibody (Autogen Bioclear, Calne, UK)
- NF-κB p65 antibody (Autogen Bioclear, Calne, UK)
- HSF1 antibody 1 (Lab Vision Co., Suffolk, UK)
- HSF1 antibody 2 (Provided by R. Morimoto, Northwestern University, Evanston, Ill., USA)
- SP1 oligonucleotide sequence (Promega Corporation, Madison, USA):

  5'-ATTCGATCGGGGCGGGGCGAGC-3'

  3'-TAAGCTAGCCCCGCCCCGCTCG-5'

Protocol:

Competition experiments for HSF1, NF-κB and AP-1 were performed by adding 10X unlabeled HSF1, NF-κB or AP-1 probe in 100μg of extract in 1X binding buffer (to a final volume of 18μl). Unlabeled SP-1 oligonucleotide sequence was used as a non-specific competitor. Samples were incubated at room temperature for 10mins. Following incubation, 2μl of HSF1, NF-κB or AP-1 radiolabeled oligonucleotide were added to the samples and the reactions were incubated at room temperature for 20mins. Gel electrophoresis of samples was performed as described in Section 2.5.3.

Supershift assays were performed by the addition of 2μg (per 10μg of extract used) supershift specific antibodies for HSF1, p50, p65, c-Fos and c-Jun and incubated on ice for 30mins before the radiolabeled probe was added. Following addition of the probe, the samples were incubated at room temperature for 20mins. Gel electrophoresis of samples was performed as described in Section 2.5.3.

2.6. ATLAS™ cDNA EXPRESSION ARRAYS

A nucleic acid array is a new technique that allows the detection and analysis of mRNA for multiple genes simultaneously.

CLONTECH's Atlas™ cDNA Expression Arrays include hundreds of cDNAs spotted on positively charged nylon membranes. The Atlas procedure is outlined in Figure 2.3. The first step is to synthesise probes mixtures by reverse transcribing each RNA population using cDNA synthesis (CDS) Primer Mix and [a-^{32}P]dATP. Each radioactively labeled probe mix is then hybridized to separate arrays. The hybridization pattern can be analysed and quantified by phosphorimaging. The relative expression levels of a given cDNA from two different RNA sources can be assessed by comparing the signal obtained with the probe from one RNA source to that obtained with a probe from another source. Plasmid and bacteriophage DNAs are included as negative controls to confirm hybridization specificity, along with several housekeeping cDNAs as positive controls for normalizing RNA abundance.

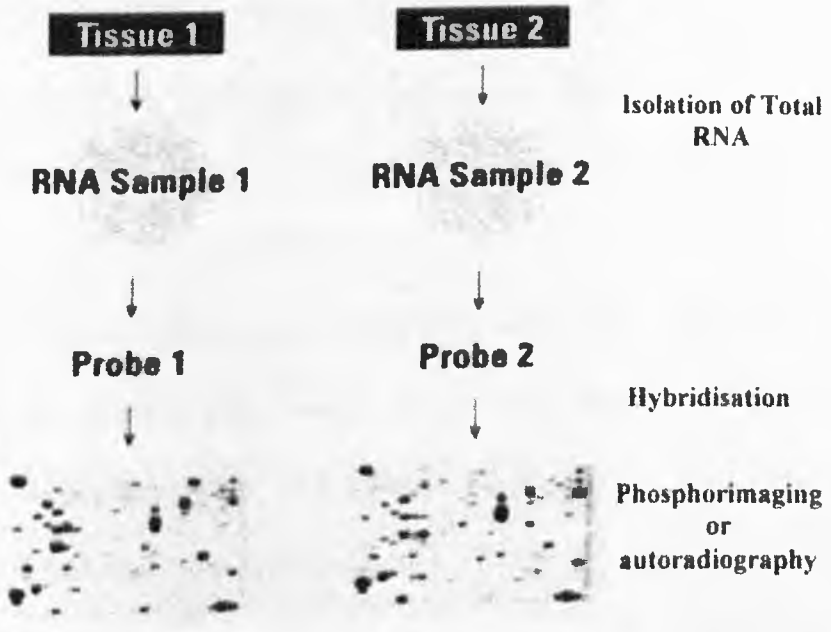


Figure 2.3. Atlas™ cDNA expression arrays procedure. Side by side hybridizations with cDNA probes prepared from two different RNA populations allow the simultaneous comparison of the expression levels of all the cDNAs on the array (CLONTECH, Palo Alto, USA).

2.6.1. RNA isolation from mouse gastrocnemius muscles

Reagents:

- TRI REAGENT™ (Sigma Immunochemicals, Dorset, U.K.)
- Chloroform
- Isopropanol
- 75% ethanol

Protocol:

Muscles were ground and placed into TRI REAGENT™ (1ml per 50-100mg of tissue) and centrifuged at 12,000g for 10mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.) to remove the insoluble material. Samples were then allowed to stand for 5mins at room temperature.

Two hundred microlitres of chloroform was then added to the supernatants, samples were shaken vigorously for 15sec and allowed to stand for 15mins at room temperature. Samples were then centrifuged at 12,000g for 15mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.). Centrifugation separated the mixtures into 3 phases: a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA).

The aqueous phase was transferred to a fresh tube and 0.5ml of isopropanol was added to each sample. The samples were allowed to stand for 5-10mins at room temperature and centrifuged at 12,000g for 10mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.).

The supernatants were then discarded and the RNA pellets were washed by adding 1ml of 75% ethanol. Samples were then centrifuged at 7,500g for

5mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.). Following centrifugation, the RNA pellets were briefly dried for 5-10mins by air-drying and 100µl of dH_2O was added to each RNA pellet. The samples were then mixed by repeated pipetting.

2.6.2. DNase treatment of total RNA from gastrocnemius muscles

Reagents:

- 10X DNase I Buffer (400mM Tris-HCl [pH 7.5], 100mM NaCl, 60mM $MgCl_2$; Clontech, Palo Alto, USA)
- DNase I (Clontech, Palo Alto, USA)
- 2M NaOAc, pH 4.5 (Clontech, Palo Alto, USA)
- 10X Termination Mix (0.1M EDTA [pH 8.0], 1mg/ml glycogen; Clontech, Palo Alto, USA)
- Chloroform (Sigma Immunochemicals, Dorset, U.K.)
- Phenol (Sigma Immunochemicals, Dorset, U.K.)
- 95% ethanol
- 80% ethanol

Protocol:

This protocol was adopted from the Clontech Atlas™ cDNA expression arrays user manual. Briefly, the total RNA from samples from the same group were pooled. Sixty microlitres of total RNA was combined with 10µl of 10X DNase buffer, 5µl of DNase I and 25µl of deionised water and the reactions were incubated at 37°C for 30mins. Following incubation, 10µl of 10X termination mix was added to the reactions.

Sixty microlitres of chloroform and 100µl of phenol were then added to the supernatants, samples were vortexed and centrifuged at 12,000g for 10mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.). The aqueous phase was transferred to a fresh tube and 110µl of chloroform was added to each sample. The samples were centrifuged at 12,000g for 10mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.).

Ten microlitres of 2M NaOAc and 275µl of 95% ethanol were then added to the supernatants, samples were vortexed and allowed to stand on ice for 10mins. Samples were then centrifuged at 12,000g for 15mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.).

Following centrifugation, the pellets were washed 100µl of 80% ethanol and centrifuged at 12,000g for 5mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.). The pellets were air dried and dissolved in 20µl of Rnase-free H_2O.

2.6.3. Analysis of RNA content of samples using the RiboGreen RNA quantitation assay

The RNA content of the samples was measured using the RiboGreen RNA quantitation assay kit (Molecular probes, Leiden, The Netherlands).

Reagents:

- RiboGreen RNA quantitation reagent (Component A), 1ml solution in DMSO
- 20X TE (Component B), 25ml of 200mM Tris-HCl, 20mM EDTA, pH 7.5 (20X TE) in DEPC-treated water.

- Ribosomal RNA standard (16S and 23S rRNA from *E. coli*; Component C), 100µg/ml in TE

Protocol:

A range of standards between 0-1µg/ml was prepared from a stock solution of 100µg/ml Ribosomal RNA standard (Component C) in 1X TE solution. A 100µl of the standard, blank or sample and 100µl of RiboGreen RNA quantitation reagent (Component A) were placed in a 96 well microtitre plate. Samples were then incubated at room temperature for 2-5mins. The fluorescence of standards and samples was measured (excitation 480nm, emission 520nm) using a fluorescence microplate reader (Fluostar optima, BMG, Germany). The RNA content of each sample was calculated from the standard curve.

2.6.4. Analysis of total RNA by gel electrophoresis

Reagents:

- Agarose
- 10X MOPS (3-[N-Morpholino]propanesulfonic acid)
- 37% Formaldehyde
- Formamide
- 0.1% DEPC-treated water (Diethyl pyrocarbonate)
- 10mg/ml ethidium bromide
- 0.1M EDTA, pH 7.5
- 0.2% (w/v) Bromophenol Blue (all from Sigma Immunochemicals, Dorset, U.K.)
- Mouse heart total RNA (1 mg/ml, Ambion Ambion Inc, Huntingdon, UK)

Protocol:

The quality of the total RNA was examined by electrophorising 1-3μg of RNA on a denaturing formaldehyde/agarose/ethidium bromide gel.

A 1% agarose solution in DEPC-treated dH_2O was prepared. The gel solution was brought to boil and then allowed to cool down for 5mins. Seven and a half millilitres of formaldehyde and 10ml of 10X MOPS were then added to the solution. The solution was poured into the gel tray and allowed to solidify for 1 hour and covered with ~800ml of 1X MOPS buffer. RNA samples were prepared by adding 1-3μg of total RNA to 10μl of loading buffer containing 45μl of formaldehyde, 45μl of formamide, 10μl of 10X MOPS buffer, 3.5μl of 10mg/ml ethidium bromide and 8μl of 0.2% bromophenol blue (in 50% glycerol). Samples were incubated at 70°C for 15mins, cooled on ice for 1min and loaded on the gel. For comparison, a set of standard using mouse control poly A^+ RNA was also prepared. Electrophoresis was carried out using a Hybaid B2 Electrophoresis tank (Owl Scientific Inc., Woburn, USA) at a constant voltage of approx. 100V for 1-2 hours. The gel was then removed form the gel tray, photographed using a Kodak DC120 Zoom Digital Camera (Kodak, Rochester, New York) and analysed using Kodak Digital 1D software (Kodak, Rochester, New York).

2.6.5. Probe synthesis using total RNA from gastrocnemius muscles

Reagents:

- Master Mix: 5X Reaction Buffer (250mM Tris-HCl [pH 8.3], 375mM KCl, 15mM $MgCl_2$; Clontech, Palo Alto, USA)
  100mM DTT (Clontech, Palo Alto, USA)
  [α-^{32}P]dATP (3,000 Ci/mmol, Amersham International, U.K.)
- CDS Primer Mix (Clontech, Palo Alto, USA)
- 10X Termination Mix (0.1M EDTA [pH 8.0], 1mg/ml glycogen; Clontech, Palo Alto, USA)
- MMLV Reverse Transcriptase (100U/µl; Clontech, Palo Alto, USA)
- 10X dNTP Mix (for dATP label, 5mM each dCTP, dGTP, dTTP; Clontech, Palo Alto, USA)

Protocol:

Two microlitres of RNA (2-5µg) were mixed with 1µl of CDS Primer Mix and incubated in a preheated PCR thermal cycler (Bio Gene Rapid cycler, Idaho Technology, Idaho Falls, USA) at 70°C for 2mins. Following incubation, the temperature was reduced to 50°C and the reactions were incubated for 2mins. During incubation, a Master Mix was prepared by mixing 2µl of 5X Reaction buffer, 1µl of 10X dNTP Mix, 3.5µl of [α-^{32}P]dATP, 1µl of MMLV reverse transcriptase and 0.5µl of DTT. After completion of the 2 min incubation at 50°C, 8µl of Master Mix was added to each reaction tube and samples were incubated at 50°C for 25mins. The reactions were then stopped by adding 1µl of 10X

Termination Mix and the labeled cDNA was purified from unincorporated ^{32}P-labeled nucleotides by column chromatography (Clontech, Palo Alto, USA).

2.6.6. Hybridization of cDNA probes to the Atlas array

Reagents:

- ExpressHyb Solution (Clontech, Palo Alto, USA)
- Sheared salmon testes DNA (Sigma Immunochemicals, Dorset, U.K.)
- C_0t-1 DNA (1mg/ml; Clontech, Palo Alto, USA)
- 20X SSC (3.0M NaCl, 0.3M Sodium Citrate)
- Wash Solution 1(2X SSC, 1% SDS)
- Wash Solution 2 (0.1X SSC, 0.5% SDS)
- Atlas Mouse Stress Arrays (Cat. #7749-1; Clontech, Palo Alto, USA)

Protocol:

Each Atlas array was placed into a hybridisation bottle containing 5ml of prewarmed (68°C) ExpressedHyb solution and 0.5mg of heated sheared salmon testes DNA and prehybridized for 30mins with continuous agitation at 68°C. During prehybridization, 5µl of C_0t-1 DNA was added to each labelled probe. Probes were incubated in boiling water for 2 mins and then on ice for 2 more minutes. Each probe was added directly into the prehybridization solution and arrays were hybridized overnight with continuous agitation at 68°C. The Atlas arrays were then washed three times (30 min each wash) with pre-warmed wash solution 1 and once (for 30 min) with pre-warmed wash solution 2 with continuous agitation at 68°C. Arrays were then washed with 2X SSC for 5 min, immediately wrapped in Clingorap (VWR International Ltd, Lutterworth, UK)

and exposed to a phosphor screen (Amersham International, UK) for 24 hours. The phosphor screen was scanned using a Biorad Personal Molecular Imager FX (Biorad, Hercules, USA) and analysed using Quantity one Software (Biorad, Hercules, USA).

2.6.7. Stripping cDNA probes from the Atlas array

Reagents:

- 0.5% SDS
- Wash Solution 1(2X SSC, 1% SDS)

Protocol:

cDNA probes were removed by boiling the arrays in 0.5% SDS for 5-10mins. The solution was then removed from heat and allowed to cool for 10mins. The Atlas arrays were then rinsed in wash solution 1, immediately wrapped in plastic membrane (Clingorap, VWR International Ltd, Lutterworth, UK) and stored at –20°C until needed.

2.7. ANALYSIS OF RNA FROM SKELETAL MUSCLE BY NORTHERN HYBRIDISATION

2.7.1. RNA isolation from mouse gastrocnemius muscles

Protocol:

The RNA isolation from mouse gastrocnemius muscles was performed as described in section 2.6.1 using TRI REAGENT™ (~50mg of ground muscle in 0.5ml of TRI REAGENT™; Sigma Immunochemicals, Dorset, UK). Following isolation, the RNA pellet was resuspended in 20µl of RNAse free water and the samples were then mixed by repeated pipetting.

2.7.2. Agarose/Formaldehyde gel electrophoresis of RNA

Reagents:

- Agarose (Sigma Immunochemicals, Dorset, UK)
- 5X MOPS (3-[N-Morpholino]propanesulfonic acid)
- 37% formaldehyde (Sigma Immunochemicals, Dorset, UK)
- 0.1% DEPC-treated water (Diethyl pyrocarbonate; Sigma Immuno-chemicals, Dorset, UK)
- RNA sample buffer (10ml formamide, 3.5ml 37% formaldehyde, 2ml 5X MOPS)
- RNA loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue)

Protocol:

A 1% agarose solution was prepared by adding 2.7g of agarose in 174ml of DEPC-treated water. The gel solution was brought to boil and then allowed to cool down for 5mins. Fifty millilitres of formaldehyde and 56ml of 5X MOPS

were then added to the solution. The solution was poured into the gel tray and allowed to solidify for 1 hour and covered with ~800ml of 1X MOPS buffer. RNA samples were prepared by mixing 15μg (1part) of RNA with 2 parts of RNA sample buffer. Samples were incubated at 65°C for 5mins, cooled at room temperature for 1min. Two microlitres of RNA loading buffer was then added to each sample and samples were loaded on the gel. Electrophoresis was carried out using a Hybaid B2 Electrophoresis tank (Owl Scientific Inc., Woburn, USA) at a constant voltage of approx. 80V for 2-3 hours.

2.7.3. Transfer of RNA to membanes

Reagents:

- 20X SSC (3M NaCl, 0.3M Sodium Citrate)
- 5X SSC
- 0.1% DEPC-treated water (Diethyl pyrocarbonate; Sigma Immuno-chemicals, Dorset, UK)
- 10mg/ml ethidium bromide (Sigma Immunochemicals, Dorset, U.K.)

Protocol:

Following electrophoresis the gel was soaked in several rinses of DEPC-treated water to remove the formaldehyde. The gel was then soaked in 20X SSC for 45 minutes. For the transfer of RNA, a nylon membrane (Hybond-N+, Amersham International, UK) was placed on top of the gel and was sandwiched between paper towels as shown in Figure 2.4. The transfer was allowed to proceed overnight at room temperature.

Following completion of RNA transfer, the membrane was removed and washed in 5X SSC for 5mins. The membrane was then placed between 2 filter papers and baked for two hours at 80°C in a vacuum oven. The transferred gel was stained in ethidium bromide to determine if the transfer was complete.

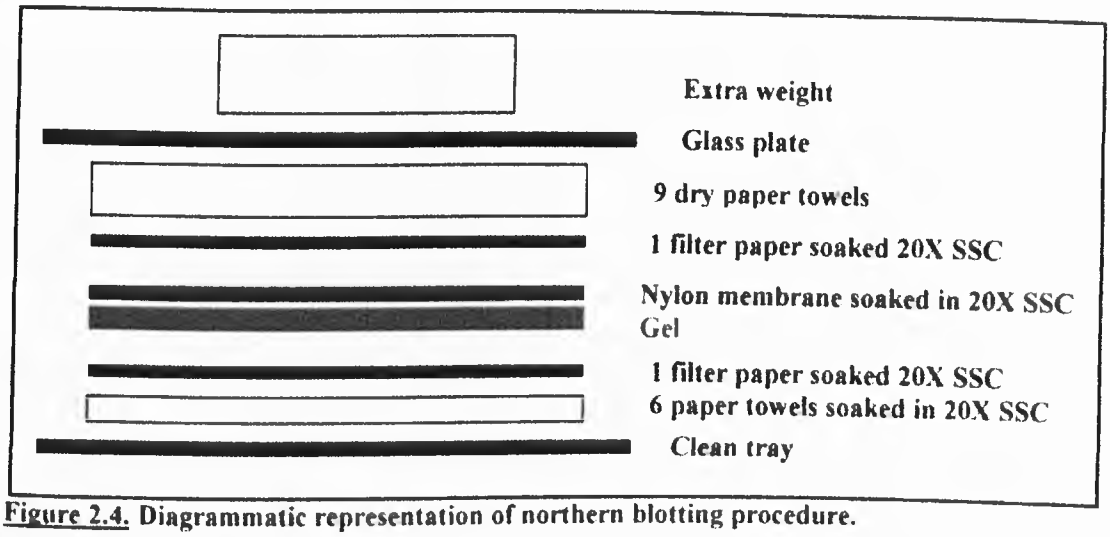


Figure 2.4. Diagrammatic representation of northern blotting procedure.

2.7.4. Probe hybridization

Reagents:

- 20X SSC (3M NaCl, 0.3M Sodium Citrate)
- 5X SSC
- ULTRAhyb hybridization buffer (Ambion Inc, Huntingdon, UK)
- Stringency wash solution I (2X SSC, 0.1% SDS)
- Stringency wash solution II (0.1X SSC, 0.1% SDS)
- 18S nucleotide sequence (Thermo Hybaid GmbH, Ulm, Germany):

  5'-CGCCTGCTGCCTTCCTTGGATGTGGTAGCCG-3'

- A 30mer nucleotide sequence, complementary to a portion of mouse HSP25 cDNA (Thermo Hybaid GmbH, Ulm, Germany):

  5'-AAGAAGGGCACGCGGCGCTCGGTCATGTTC-3'

- 10X kinase buffer (700mM Tris/HCl (pH 7.6), 100mM $MgCl_2$, 50mM DTT; Promega Corporation, Madison, USA)
- Redivue adenosine 5'-[γ-^{32}P]triphosphate, triethylammonium salt (9.25MBq, 250µCi , Amersham International, U.K.)
- T4 polynucleotide kinase (5U/µl, Promega Corporation, Madison, USA)
- 0.5M EDTA (Sigma Immunochemicals, Dorset, U.K.)

Protocol:

The [^{32}P]-labeled oligonucleotide was prepared by labeling the 5' end of the oligonucleotide with T4 kinase and [γ-^{32}P]-ATP.

Five microlitres of 10X kinase buffer, 30µl of dH_2O, 3µl of [γ-^{32}P]-ATP and 2µl of T4 kinase were added to 10 µl of oligonucleotide (10ng/µl) and incubated at 37°C for 1 hour. Following incubation, 2µl of 0.5M EDTA were added to the oligonucleotide. The final concentration of the kinased oligonucleotide was approximately 2ng/µl. The labeled oligonucleotides were separated from unincorporated nucleotides by chromatography through Micro Bio-Spin 30 columns (Biorad, Hercules, USA).

The nylon membrane was placed into a hybridisation bottle containing 10ml of prewarmed (42°C) ULTRAhyb® solution and prehybridized for 1 hour with continuous agitation at 42°C. The probe was added directly into the prehybridization solution (10ng/ml of hybridisation solution) and membrane was hybridized overnight with continuous agitation at 42°C. The membrane was then washed twice (5 min each wash) with wash solution 1 and twice (15 min each wash) with pre-warmed wash solution 2 with continuous agitation at 68°C. The membrane was then washed with 2X SSC for 10 min, immediately wrapped in

Clingorap (VWR International Ltd, Lutterworth, UK) and exposed to a phosphor screen (Amersham International, UK) for varying lengths of time. The phosphor screen was scanned using a Biorad Personal Molecular Imager FX (Biorad, Hercules, USA) and analysed using Quantity one Software (Biorad, Hercules, USA).

2.7.5. Stripping probes from the nylon membrane

Reagents:

- 6X SSC
- 50% formamide (Sigma Immunochemicals, Dorset, UK)
- 0.1% DEPC-treated water (Diethyl pyrocarbonate; Sigma Immuno-chemicals, Dorset, UK)

Protocol:

Probes were removed from the nylon membrane by incubating the membrane in 50% formamide, 6X SSC at 65°C for 1 hour. Following incubation the membrane was washed for 5mins in DEPC-treated water, wrapped in Clingorap (VWR International Ltd, Lutterworth, UK) and exposed to a phosphor screen (Amersham International, UK) overnight to confirm probe removal.

2.8. STATISTICAL METHODS

Results were expressed as mean +/- SE. Statistical significance was evaluated by ANOVA with modified Student's t-test as appropriate. A p value of less than 0.05 was considered significant.

2.9. HISTOLOGICAL ANALYSIS OF SKELETAL MUSCLE

2.9.1. Preparation of muscle blocks

Reagents:

- O.C.T. mounting compound (Merck Ltd., Dorset, U.K)
- Iso-pentane (Merck Ltd., Dorset, UK)

Protocol:

AT muscles were orientated with fibres running transversely on cork discs, surrounded by O.C.T. mounting compound and frozen in iso-pentane pre-cooled in liquid nitrogen. Blocks were stored at –70°C prior to histological analysis.

Ten micrometer sections from the centre of the muscle were taken onto glass cover slips using a cryostat (Bright Instrument Co., Huntingdon, UK) and stained with haematoxylin and eosin (Section 2.8.2).

2.9.2. Haematoxylin and eosin (H&E) staining

Reagents:

- Harris' Haematoxylin: 5% solution (Merck Ltd., Dorset, U.K)
- Eosin: 1% solution (Merck Ltd., Dorset, UK)
- DPX mountant (Merck Ltd., Dorset, U.K)

Protocol:

Sections were placed in Harris' Haematoxylin for 3-5 min, rinsed in distilled H_2O and counterstained in eosin for 30 sec. Sections were then dehydrated in 3x Absolute alcohol and cleaned in xylene. Cover slips were mounted onto microscope slides using DPX mountant.

CHAPTER 3

CHARACTERISATION OF AN ISOMETRIC CONTRACTION PROTOCOL IN SKELETAL MUSCLES OF ADULT AND AGED MICE

3.1. INTRODUCTION

Damage to skeletal muscle occurs following excessive or unaccustomed exercise, during reperfusion following a period of ischemia or a consequence of muscle disorders such as Duchenne muscular dystrophy (McArdle and Jackson, 1997). Contraction-induced muscle damage results in muscle weakness and diffuse pain. However, the cellular mechanisms by which muscle damage occurs are not fully understood. Since the production of ROS increases considerably during exercise (Section 1.2) it has been suggested that this increase in free radical production leads to exercise-induced muscle damage. However, definite evidence for a direct involvement of free radicals in contraction-induced muscle damage is lacking (Jackson and O'Farrell, 1993; van der Meulen *et al*, 1997, McArdle *et al*, 1999).

As mentioned previously (Section 1.1.5) activation of muscle movement involves three types of contraction: Shortening (also called concentric or dynamic), lengthening (also called eccentric) and isometric (also called static) contractions. Of these three types of contraction, lengthening contractions are considerably more damaging to muscle (Newham *et al*, 1983), whereas the most metabolically demanding contractions, potentially resulting in the greatest production of ROS are the isometric or shortening contractions. These forms of contraction rarely result in muscle damage *in vivo*.

Damage occurring during lengthening contractions displays a well defined course of events; Initially, the ability of muscle to generate force is reduced. At this stage, focal injury to single or groups of sacromeres is observed. This initial injury leads to a secondary injury several days later, where widespread necrosis is evident within the muscle bulk. At this point, a further reduction in the force

production is observed. The muscle then slowly regenerates and finally recovers to pre-exercise values by ~28 days following exercise (Brooks *et al*, 1995; van der Meulen *et al*, 1997; McArdle and Jackson, 2002).

Damage to skeletal muscle can be assessed using various techniques. Skeletal muscle damage is accompanied by a reduction in the ability of muscle to generate force, therefore techniques designed to measure maximal force production of muscles provide a means of assessing damage to that muscle (Newham *et al*, 1983). Furthermore, histological examination using light microscopy provides information about structural abnormalities within a muscle and allows detection of the presence of necrotic or regenerating fibres. Contraction-induced muscle damage can be also assessed by measuring the release of creatine kinase (CK) activity. CK has a molecular weight of 81kDa and it is found in 3 distinctive isoforms: CK-MM found in skeletal muscle, CK-BB found in the brain and CK-MB, which is mainly found in the heart and measurement of the release of CK activity is one of the most widely used indicator of muscle damage.

Glutathione is a non-protein thiol and one of the most important cellular antioxidants. Glutathione is a major reducing agent that is thought to protect cells against toxic effects of oxidants by keeping the environment in the cells in a reducing state (See section 1.3.1.4). Any changes in the levels of glutathione can be used as a sensitive measure of tissue oxidative stress. The most important antioxidant function of glutathione is to serve as a substrate for glutathione peroxidase to remove hydrogen peroxide and organic peroxides (Ji & Hollander, 2000). Previous data have indicated that contraction-induced muscle damage is also accompanied by oxidation of cellular glutathione and a decrease in the levels

of total glutathione within the muscle (McArdle *et al*, 1999). Data from our group suggest that the fall in glutathione content and the increase in oxidation of glutathione observed during muscle damage following lengthening contractions is associated with the presence of neutrophils and other phagocytic cells that are activated and recruited to the site of the initial damage. These infiltrating cells produce superoxide and other oxidising free radical species as part of the inflammatory response (McArdle *et al*, 1999). Oxidation of glutathione or a fall in glutathione levels in a cell can affect the function of cellular proteins, such as enzymes that maintain cellular ion homeostasis, where key thiol groups can become oxidised or form mixed disulphide with oxidised glutathione (Maglara & Griffiths, 2000).

Reduced glutathione protects the SH groups of proteins including various enzymes and the proteins that participate in protein synthesis. Thus, another indicator of oxidation is the oxidation of thiol groups on proteins. Thiol-groups are central in many biological compounds. Disulfide bonds formed from the thiol (-SH) groups of two cysteine residues have an important role in determining the tertiary structure of proteins. Molecules containing cysteine residues can easily be oxidised by transition metals or by participating in thiol-disulfide exchange (Dickinson and Forman, 2002). As with oxidation of glutathione, oxidation of protein thiols can be crucial for cell survival. Both secondary and tertiary structures of proteins are dependent on the location and number of free thiol groups as well as the number and location of the intramolecular disulphide bonds and therefore, oxidation of adjacent thiol groups to form disulphides can modify protein structure and affect enzyme activity. Previous data from our research group suggests that muscle damage is associated with a prolonged loss of protein

thiols within muscle, evident at three hours and three days following a period of damaging lengthening contractions (McArdle *et al*, 1999). In contrast, a transient, rapidly reversible loss of protein thiol groups occurs following a period of mild non-damaging isometric contractions. This fall in thiols had recovered by 2 hours following isometric contractions (McArdle *et al*, 2001). This transient fall in protein thiols may be the signal for adaptive responses.

In this study, an isometric contraction protocol was used (see Section 3.2.1). This protocol has been used previously in our laboratory (Jackson *et al*, 1985; McArdle *et al*, 2001; Vasilaki *et al*, 2002). Previous data have shown that this pattern of stimulation induced a rise in the free radical signal seen on electron spin resonance examination of skeletal muscle (Jackson *et al*, 1985). In addition, previous data using this protocol has demonstrated that the contraction protocol results in a significant rise in the superoxide level detected in muscle interstitial fluid without inducing any significant damage to skeletal muscle fibres of BALB/c mice (McArdle *et al*, 2001).

To study the adaptive responses in skeletal muscles of adult and aged mice following exercise, it was thought necessary to use a contraction protocol that was non-damaging. Therefore, the aim of this section of the study was to examine the effect of the relatively mild isometric contraction protocol that was previously used on several indices of muscle damage and oxidative stress in skeletal muscles of adult and aged male and female B6XSJL mice. Previous data indicated that this specific contraction protocol did not cause significant damage to muscles of young/adult rodents but it was necessary to evaluate whether muscles of aged mice were damaged by the protocol.

3.2. EXPERIMENTAL METHODS

3.2.1. Contraction protocol

Skeletal muscles of anaesthetised adult (12-15 months old) and aged (28-32 months old) male and female B6XSJL mice were subjected to a 15-minute period of isometric contractions via surface electrodes using a 100 Hz pulse train at 60 Volts for 0.5sec every 5sec with a pulse width of 0.1msec (Section 2.1). Mice were killed immediately and at 4 hours, 12 hours and 24 hours following the exercise protocol. Muscles were removed, frozen in liquid nitrogen and stored at -70°C until analysis. A sub-group of muscles was prepared for subsequent histological analysis and stored at -70°C until analysis (see Section 2.9.1).

3.2.2. Sample preparation

AT muscles from non-exercised and exercised adult and aged male and female mice were ground under liquid nitrogen, homogenised in 100µl of 1% SSA and centrifuged at 10,000g for 10mins at 4°C. Supernatants were used for analysis of glutathione content (Section 2.3.3.1), and the protein-precipitated pellets were used for analysis of protein thiol content (Section 2.3.3.2). Blood samples were also removed from the aorta, centrifuged and serum was analysed for activity of creatine kinase (CK) as an index of muscle damage (Section 2.3.4).

3.3. RESULTS

3.3.1. Histological analysis of muscles from adult and aged male mice following the contraction protocol

Figure 3.1 shows representative images of transverse sections through muscles of non-exercised adult male mice and muscles of adult male mice at 30 mins and 3 days following the contraction protocol. Figure 3.2 shows representative photographs of transverse sections through muscles of non-exercised aged male mice and muscles of aged male mice at 30 mins and 3 days following the contraction protocol.

Damage to muscle structure is accompanied by infiltration of a large number of phagocytic cells and widespread necrosis. An example of a transverse section through a grossly necrotic muscle at 3 days following a severely damaging lengthening contraction protocol is shown in Figure 3.3A. Occasional individual necrotic fibres were observed in muscle sections (Figure 3.3B) although this represented <0.5% of the muscle section (data not shown in detail). Similarly, regenerating fibres were occasionally observed in muscles of adult and aged mice (See Figure 3.1C).

Histological analysis of AT muscles following the contraction protocol showed no infiltration of phagocytic cells suggesting that no overt damage had occurred in muscles of either adult or aged male mice at 30 mins or 3 days following contractions.

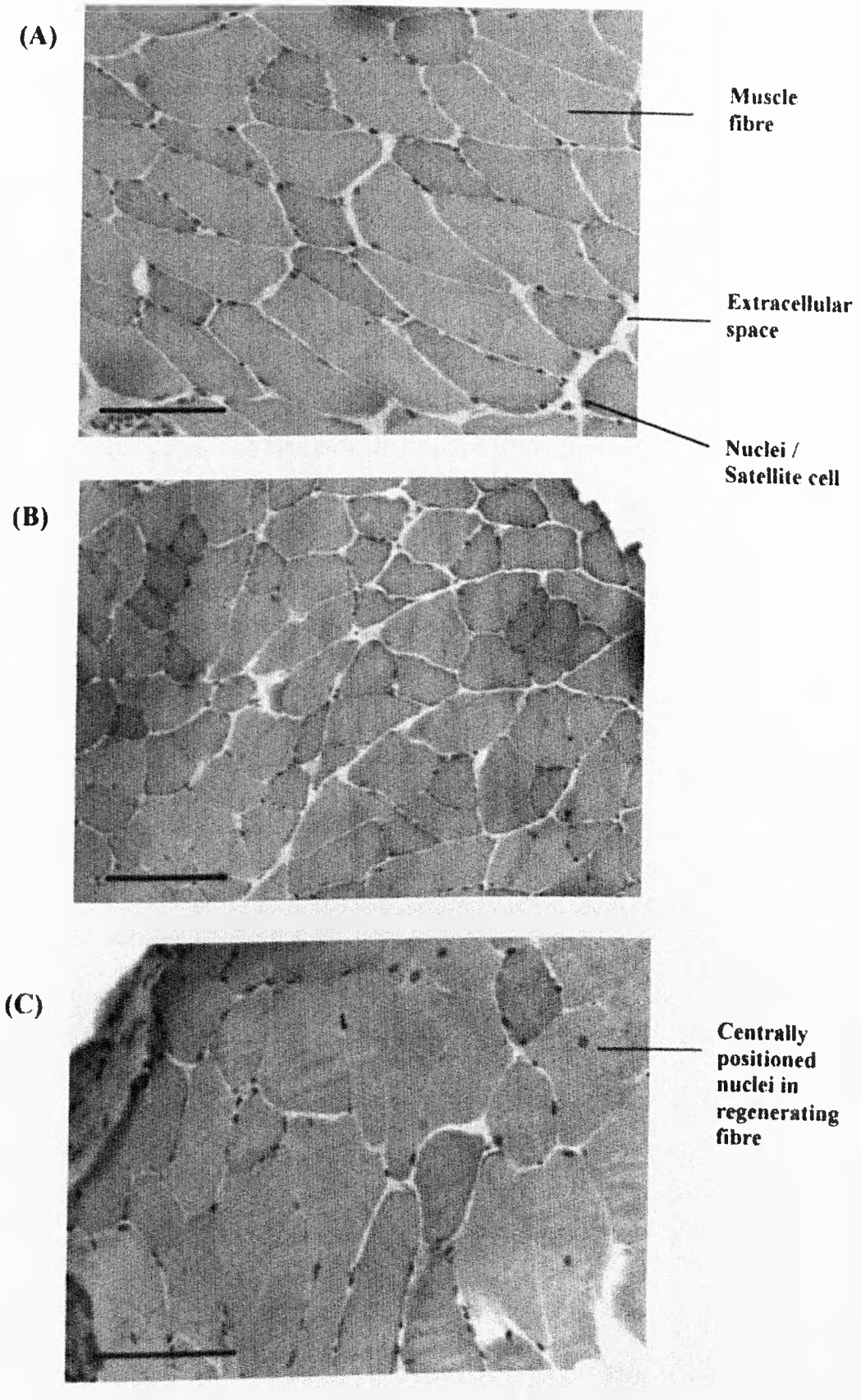


Figure 3.1. Transverse sections through AT muscles of (A) non-exercised adult male mice and muscles of adult male mice at (B) 30 mins and (C) 3 days following the contraction protocol; Bar: 100μm.

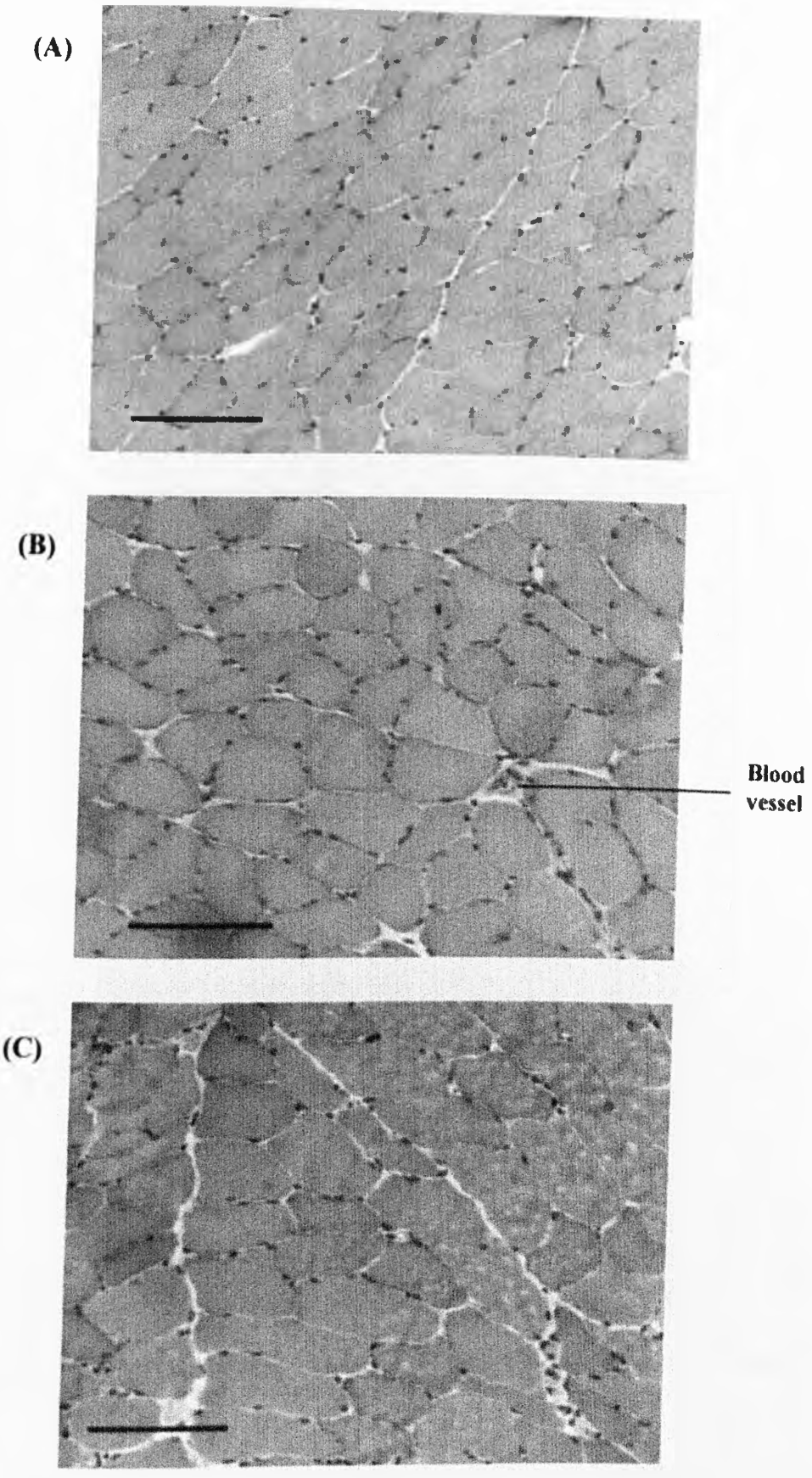


Figure 3.2. Transverse sections through AT muscles of (A) non-exercised aged male mice and muscles of aged male mice at (B) 30 mins and (C) 3 days following the contraction protocol; Bar: 100μm.

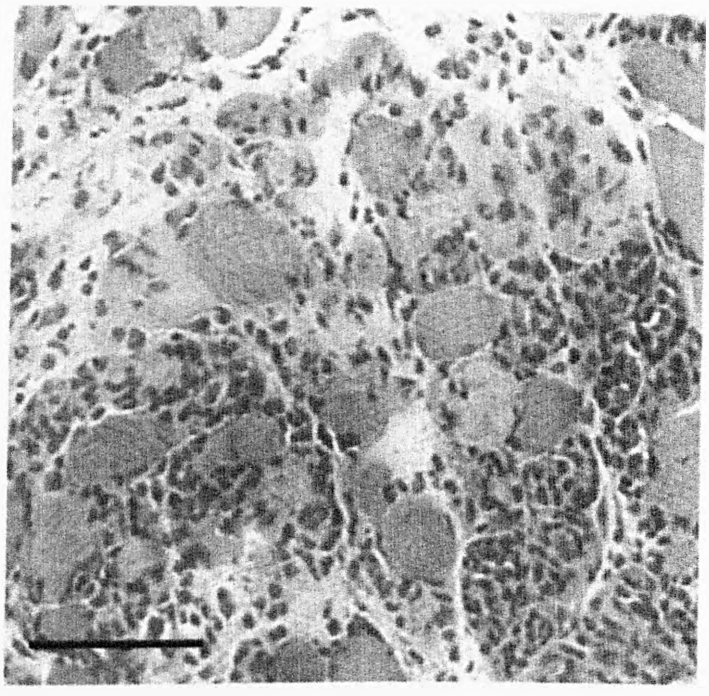


Figure 3.3A. Transverse section through EDL muscle of adult mouse at 3 days following a severely damaging contraction protocol showing infiltration of phagocytic cells; Bar: 100μm.

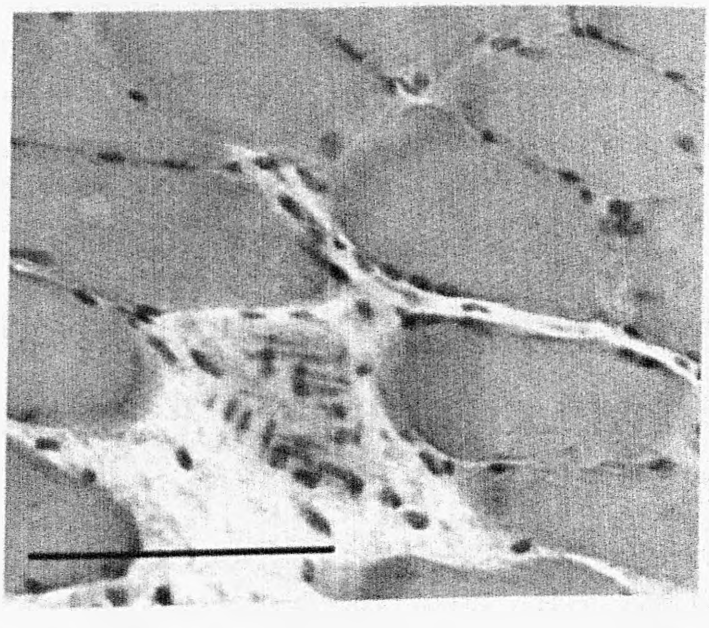


Figure 3.3B. Transverse section through AT muscle of aged mouse following the contraction protocol showing infiltration of phagocytic cells; Bar: 100μm.

3.3.2. Determination of serum creatine kinase (CK) activity as a marker of muscle damage

The serum CK activities of non-exercised adult and aged male and female mice are shown in Table 3.1. A significant difference was observed in the serum CK activity between non-exercised adult and aged male mice (Table 3.1), whereas no significant differences were observed between non-exercised adult and aged female mice.

Table 3.1. **Serum CK activity of non-exercised adult and aged male and female mice.**

	CK ACTIVITY
ADULT MALE	318.2 ± 34.1
ADULT FEMALE	301.1 ± 27.47
AGED MALE	148.8 ± 11.6[a]
AGED FEMALE	323.2 ± 83.4

[a]P<0.05 in comparison with serum CK activity from non-exercised adult male mice (n=4-6). Data is expressed as U/l.

The effect of the contraction protocol on serum CK activity in adult and aged male mice is shown in Figure 3.4. A significant rise in serum CK activity was seen in both adult and aged male mice at 4 hours following the contraction protocol. This had returned to normal levels by 12 hours following the contraction protocol.

The effect of the contraction protocol on the serum CK activity in adult and aged female mice is shown in Figures 3.5. A significant rise in serum CK activity was seen in adult female mice following the contraction protocol. This had returned to normal levels by 12 hours following the contraction protocol. The

effect of the contraction protocol on the serum CK activity in aged female mice was variable, however no significant increase was observed at any time point studied (Figure 3.5).

In addition, the increase in the serum CK activity observed in adult male mice at 4 hours following contraction was significantly greater than the increase in serum CK activity from adult female mice at the same time point (serum CK activity from adult male mice at 4hrs following contraction: 1077.1 ± 79.8 U/l; CK activity from adult female mice at 4hrs following contraction: 617.2 ± 86.4 U/l; $P<0.05$).

No significant differences were seen in the serum CK activity between male and female mice at any other time point.

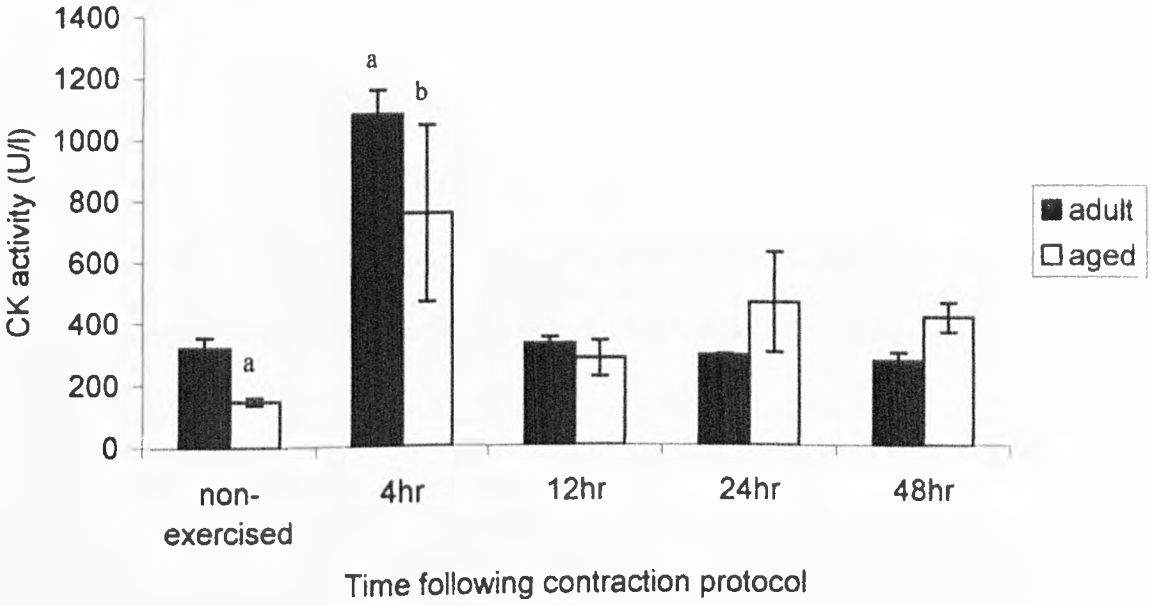


Figure 3.4. The effect of the contraction protocol on the serum CK activities of adult and aged male mice. [a]P<0.05 in comparison with serum CK activity from non-exercised adult male mice, [b]P<0.05 in comparison with the serum CK activity from non-exercised aged male mice (n=4-6).

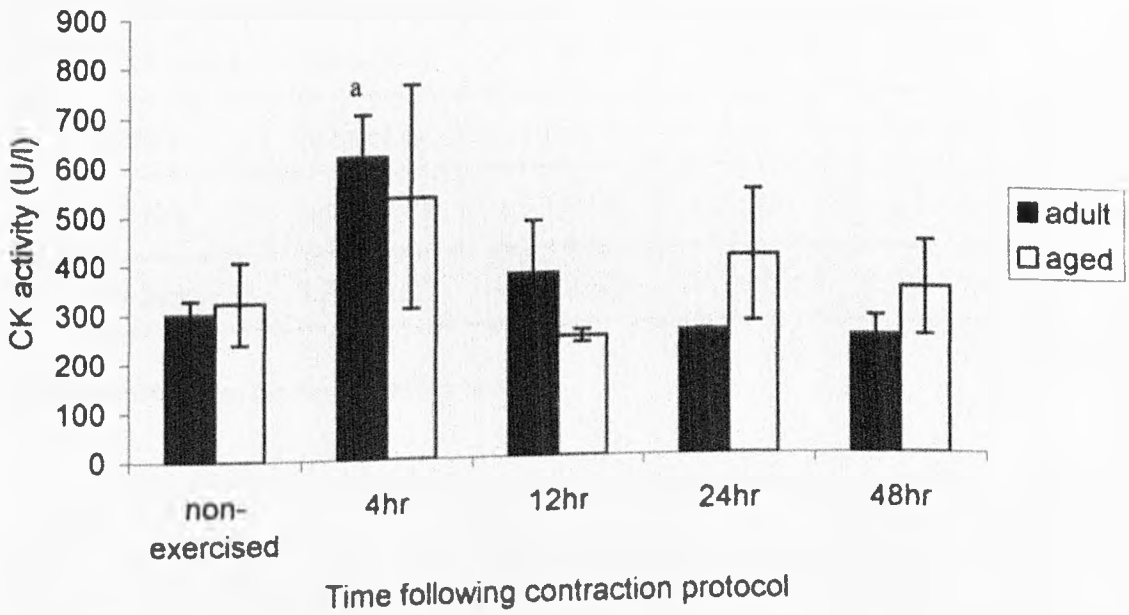


Figure 3.5. The effect of the contraction protocol on the serum CK activities of adult and aged female mice. [a]P<0.05 in comparison with serum CK activity from non-exercised adult female mice (n=4-6).

3.3.3. Determination of total glutathione content of AT muscles from adult and aged mice prior to and following isometric contractions

The total glutathione content of AT muscles from non-exercised adult and aged male and female mice and AT muscles at 4, 12 and 24 hrs following contraction is shown in Table 3.2. No significant differences in the total glutathione content were seen between any of the non-exercised groups. No effect of the contraction protocol was seen on the total glutathione content of skeletal muscles of adult or aged male or female mice.

Table 3.2. **Total glutathione content of AT muscles from non-exercised adult and aged male and female mice and from mice at different time points following the contraction protocol.**

	MALE		FEMALE	
	Adult	Aged	Adult	Aged
Non-exercised	1.8 (±0.4)	1.4 (±0.2)	1.7 (±0.7)	1.8 (±0.2)
4 hrs	3.2 (±1.0)	1.7 (±0.4)	1.7 (±0.3)	1.6 (±0.3)
12 hrs	2.0 (±0.1)	1.6 (±0.5)	0.7 (±0.3)	1.2 (±0.4)
24 hrs	1.8 (±0.3)	0.9 (±0.2)	1.3 (±0.5)	2.1 (±0.5)

Data is expressed as µmoles/g protein, (n=4-6).

3.3.4. Determination of protein thiol content of AT muscles from adult and aged mice prior to and following isometric contractions

The total thiol content of AT muscles of non-exercised adult and aged male and female mice and AT muscles at 4, 12 and 24 hrs following contractions is shown in Table 3.3. No significant differences in the protein thiol content were observed between non-exercised adult and aged male mice or between non-exercised adult and aged female mice. Data demonstrate a significant reduction in the protein thiol content of muscles from non-exercised adult female mice in comparison with muscles from non-exercised adult male mice.

No effect of age was seen in the protein thiol content were observed between skeletal muscles of male or female mice.

Table 3.3. **Protein thiol content of AT muscles from non-exercised adult and aged male and female mice and from mice at different time points following the contraction protocol.**

	MALE		FEMALE	
	Adult	Aged	Adult	Aged
Non-exercised	39.9 (± 1.4)	25.8 (±6.8)	22.4 (±5.0)[a]	33.0 (±7.9)
4 hrs	47.5 (± 29.3)	31.4 (±4.8)	21.0 (±6.1)	34.5 (±3.6)
12 hrs	35.0 (±15.2)	25.7 (±6.6)	23.8 (±6.2)	34.1 (±9.9)
24 hrs	51.3 (±15.1)	30.8 (±8.8)	37.3 (±1.5)	34.4 (±17.7)

Data is expressed as μmoles/g protein. [a]P<0.05 in comparison with muscles from non-exercised adult male mice, (n= 4-6).

3.4. DISCUSSION

In order to assess whether the contraction protocol results in damage to skeletal muscle of adult or aged mice, the serum CK activity and the total glutathione and protein thiol content from skeletal muscles were measured, in addition to histological analysis of skeletal muscles of adult mice.

Histological analysis of AT muscles from adult and aged male mice following the contraction protocol demonstrated no evidence of gross necrosis or infiltration of phagocytotic cells for up to 3 days following the protocol (See Figure 3.1; Figure 3.2) suggesting that the contraction protocol does not cause overt damage to skeletal muscle. Occasionally (<0.5% of fibres), a single necrotic or regenerating fibre was observed within a muscle section. This level of damage and regeneration is not unexpected but would not contribute significantly to any functional deficit. Evidence of the presence of regenerating fibres at approximately the same rate of incidence also suggests that the degeneration of individual fibres was not due to the contraction protocol, since regenerating fibres would not reach this level of maturity within 3 days (the maximum time studied following the contraction protocol).

Measurement of the release of CK activity is one of the most widely used indicator of muscle damage. A significantly lower CK activity was observed in non-exercised aged male mice compared with that from non-exercised adult male mice. This reduced serum CK activity may be due to a fall in muscle mass in aged male mice although this was not formally measured. No differences were observed between non-exercised adult and aged female mice or between non-exercised aged male and aged female mice.

Data demonstrated a transient but significant increase in serum CK activity following the contraction protocol in both adult and aged male and female mice. It is difficult to determine the overall significance of this transient increase in serum CK activity, as we were not able to measure activity at other times between 0 to 4 and 4 to 12 hours following the contraction protocol. The half-life of serum CK in the mouse is approximately 30 minutes (Page, 1992) and so to obtain an accurate analysis of the total CK released by the hindlimb muscles, serum samples would have to be analysed relatively frequently. Some approximate estimations of the proportion of total CK released can be made. If it is assumed that the amount of CK released between 1 and 11 hours following the contraction protocol is equivalent to the value at 4 hours (~ 1000 U/l) and based on the 30 minute half-life of CK in the serum, then the total amount of CK activity released with the serum was: 500 x 20 (twenty 30 minute periods in 10 hours) = 10,000 U/l. The total serum volume of a mouse is approximately 1 ml. Thus, the total CK released was 10U/mouse. The total hindlimb muscle mass of a mouse is approximately 4g. The CK activity of hindlimb muscle of a non-exercised adult mouse is approximately 640 mU/mg wet wt. Thus, the total CK available to be released upon damage is: 640U/g x 4g = 2560 U. Thus, a release of 10U would be equivalent to <1% of total muscle CK activity.

Although the increase in the serum CK activity does not appear to indicate gross muscle damage, it should be noted that the response to the contraction protocol differed for males and females. Data show that the serum CK release from adult male mice was significantly greater than that from adult female mice at 4 hours following the contraction protocol. Such changes have been reported previously by Amelink and Bar (1986) who showed that the CK activity in male

rats following damaging contractions was much greater that the CK activity in female rats. Amelink and Bar (1986) and Bar *et al* (1988) provided evidence for an involvement of the female hormone oestrogen in this change in response. These authors demonstrated that ovariectomised female rats exhibited a serum CK activity similar to that from male rats following exercise. In addition, pre-treatment of male rats and ovariectomised female rats with oestradiol attenuated the enzyme efflux following the contraction protocol. Therefore, these data suggest that sex differences in CK release are dependent on sex hormones and that oestrogen plays an important role in modulating CK release from skeletal muscle.

Previous studies from our laboratory cast doubt on the measure of serum CK activity as a sensitive index of muscle damage in all situations. A study examining the effect of vitamin E supplementation on muscle damage following lengthening contractions demonstrated that prior supplementation with vitamin E prevented CK efflux from muscles but did not affect the loss of force production or muscle necrosis detected by histological analysis (van der Meulen *et al*, 1997). There is increasing evidence that efflux of CK may result from membrane extrusion of cytosolic contents in an attempt to maintain cell viability by helping to reduce oedema and it is only when this fails that muscle damage occurs (Maglara *et al*, 2003).

Further lack of evidence of any gross damage following this contraction protocol has been provided by studies from Dr Jack van der Meulen at the University of Michigan, USA. Dr van der Meulen examined the ability of muscles from adult mice to generate force prior to and following an identical contraction protocol to that used here. Data demonstrated that the contraction protocol had no effect on the force generation by the gastrocnemius muscle at 3 hours following the

contraction protocol indicating that no gross muscle damage has occurred (Figure 3.6).

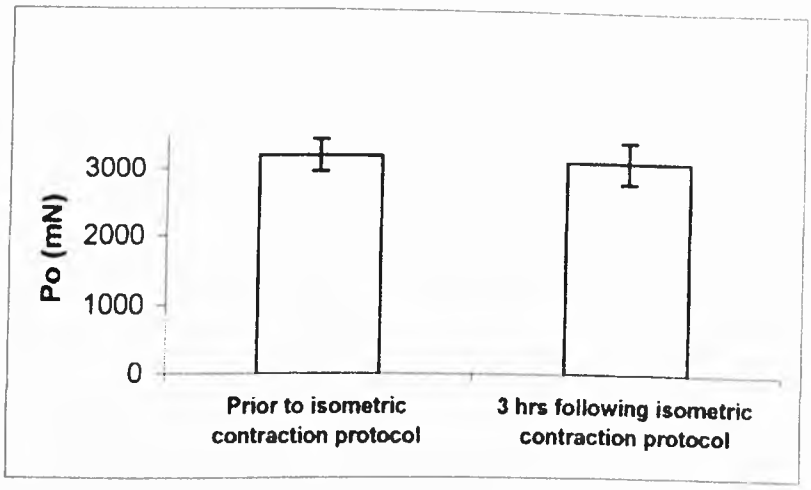


Figure 3.6. Maximum tetanic force production (Po) by gastrocnemius muscle prior to and following the isometric contraction protocol (J. H. van der Meulen, personal communications, unpublished data).

To look for evidence of oxidative damage to muscles, the glutathione and protein thiol content was examined between 4 and 24 hours following the contraction protocol. There were no significant differences in the total glutathione content between non-exercised adult and aged male or female mice and the content was not altered following the contraction protocol.

The lack of change in glutathione content following the contraction protocol was reflected in a lack of significant changes in the protein thiol content of muscles of adult or aged male or female mice following contractions. There was a significant difference in the thiol content of non-exercised adult male and adult female mice, although this was not seen between non-exercised aged male and aged female mice.

In summary, there was little evidence that the isometric contraction protocol used in this study resulted in any gross muscle damage. The contraction

protocol resulted in a small, transient increase in the serum CK activity in all mice. Total glutathione and protein thiol contents were not altered following the contraction protocol. Therefore, it was concluded that the contraction protocol used in this study would be suitable to examine the ability of muscles from adult and aged mice to respond to a period of non-damaging isometric contractions.

CHAPTER 4

ACTIVITY OF ANTIOXIDANT DEFENCE ENZYMES IN ANTERIOR TIBIALIS MUSCLES OF ADULT AND AGED MICE FOLLOWING ISOMETRIC CONTRACTIONS

4.1. INTRODUCTION

Reactive oxygen species (ROS) are produced through a number of biological reactions, usually as a consequence of aerobic metabolism. The reactivity of different ROS varies, however the majority of ROS are highly active and can initiate chain reactions, which can lead to the further production of more damaging ROS (e.g. transformation of H_2O_2 to $^{\bullet}OH$, see Section 1.2).

The dynamic balance between production and inactivation of ROS in the cellular environment determines the redox state of the cell (redox balance), which is crucial in cellular processes (Reid, 1996). Increased production of ROS can lead to damage to virtually all cellular macromolecules, including proteins, nucleic acids, carbohydrates and lipids. Damage can be direct (e.g. oxidation of thiol groups by H_2O_2), indirect or both (Halliwell *et al*, 1992).

Muscle cells have developed numerous mechanisms to respond to an increased ROS generation by adaptation to protect themselves against potential subsequent damaging insults. One of the major endogenous protective mechanisms against the potential harmful effects of ROS is the production of antioxidant defence enzymes such as superoxide dismutase and catalase (Powers *et al*, 1999).

Superoxide dismutase (SOD) is the first line of enzymatic defence against intracellular free radical production. The superoxide radical is not highly damaging in its active form (Halliwell and Gutteridge, 1999). The role of SOD is to catalyse the dismutation of superoxide radicals to hydrogen peroxide and oxygen (Figure 4.1).

The hydrogen peroxide formed by SOD can potentially cause oxidative damage. This is decomposed by catalase into water and molecular oxygen (Figure 3.1; see Section 1.3). Therefore, both SOD and catalase play an important role in *protection* against a pathological increase in ROS production.

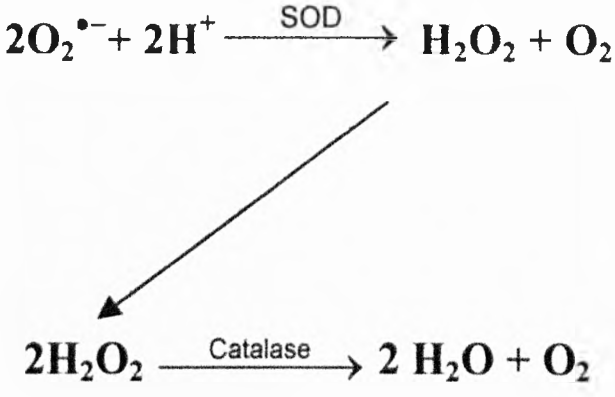


Figure 4.1. Schematic representation of superoxide dismutase and catalase functions in muscle cells.

It has been hypothesised that ageing is caused by the deleterious and accumulative effects of ROS generated throughout lifespan (Harman, 1956) and that aged organisms are exposed to a continuous oxidative environment due to higher rates of ROS production. There is some evidence that skeletal muscles of aged rodents have increased antioxidant enzyme activities (Ji *et al*, 1990; Lawler *et al*, 1993; Leeuwenburgh *et al*, 1994), suggesting that there has been some adaptation to an age-associated increase in ROS production. However, the overall ability of muscles of aged mammals to adapt following physiological stress has not been clearly defined. Various studies have been undertaken to examine the adaptation of antioxidant defence enzyme activity in muscles of aged rodents following exercise but studies are complicated due to the damaging nature of the exercise protocols used.

The aim of this study was to examine the activities of antioxidant defence enzymes in skeletal muscles from non-exercised adult and aged male and female B6XSJL mice and examine the relationship of these activities to the adaptation that occurs following a period of non-damaging isometric contractions.

4.2. EXPERIMENTAL METHODS

4.2.1. Contraction protocol

Skeletal muscles from anaesthetised adult and aged male and female B6XSJL mice were subjected to the 15-minute period of non-damaging isometric contractions described previously (Section 2.1). Mice were killed immediately, 4 hours, 12 hours and 24 hours following the contraction protocol and anterior tibialis muscles were removed and frozen in liquid nitrogen. Anterior tibialis muscles from non-exercised adult and aged male and female B6XSJL mice were also removed and frozen in liquid nitrogen. Muscles were stored at -70°C until analysis.

4.2.2. Determination of SOD and catalase activities of AT muscles from adult and aged mice

AT muscles from non-exercised and exercised adult and aged male and female mice were ground under liquid nitrogen, homogenised in 100µl of 50mM phosphate buffer and centrifuged at 10,000g for 10 mins at 4°C (Eppendorf Centrifuge 5402). The supernatants were used to determine the activity of total SOD (Section 2.3.2.1) and catalase (Section 2.3.2.2) in the samples. Data were standardized to protein concentration of the supernatant, using the bicinchoninic acid protein assay (Section 2.3.1; Sigma Immunochemicals, Dorset, UK) and data were expressed as enzyme activities (Units/mg protein).

4.3. RESULTS

4.3.1. SOD and catalase activity of AT muscles from adult and aged mice prior to and following non-damaging isometric contractions

4.3.1.1. SOD activity of AT muscles from adult and aged male mice

The total SOD activity of AT muscles from non-exercised adult and aged male mice is shown in Figure 4.2. The SOD activity of muscles of non-exercised aged male mice was significantly elevated compared with the SOD activity of muscles of non-exercised adult male mice.

The total SOD activity of AT muscles from adult and aged male mice at 4, 12 and 24 hours following the period of isometric contractions is shown in Figure 4.2. A significant rise in SOD activity was seen in muscles of adult male mice at 12 hours following the contraction protocol, whereas a significant fall in SOD activity was observed in muscles of aged male mice at this time point.

4.3.1.2. SOD activity of AT muscles from adult and aged female mice

The total SOD activity of AT muscles from non-exercised adult and aged female mice or AT muscles of adult and aged female mice at 4, 12 and 24 hours following the contraction protocol is shown in Figure 4.3. No significant differences were observed in the SOD activity between non-exercised muscles of adult and aged female mice. In addition, no significant effect of exercise was seen in muscles of adult or aged female mice (Figure 4.3).

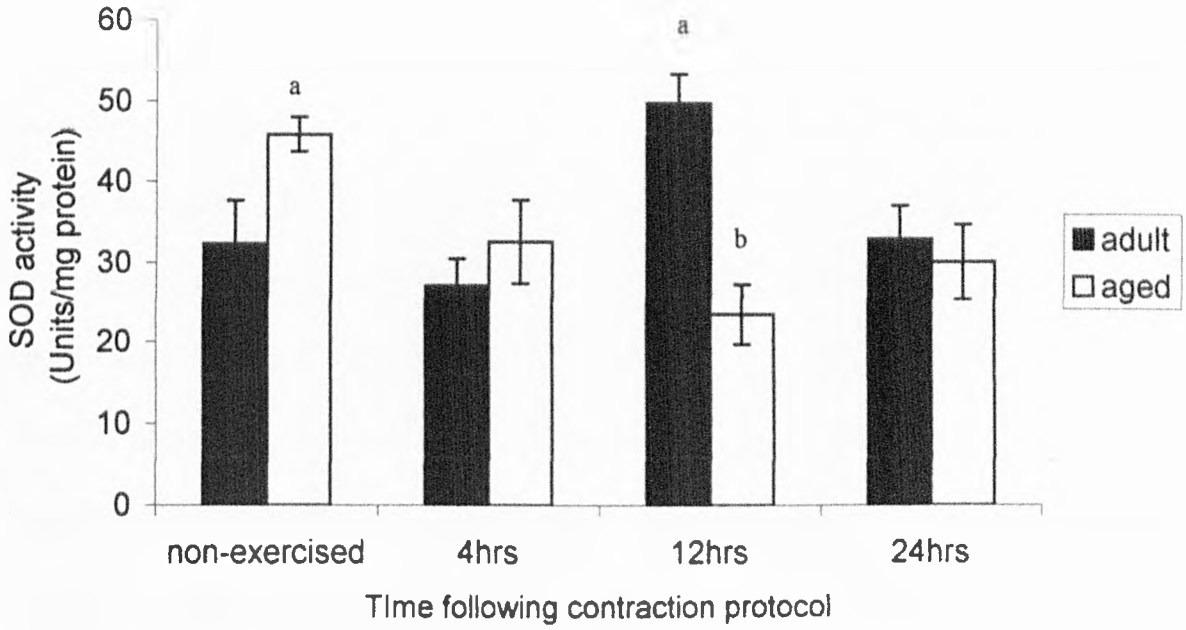


Figure 4.2. SOD activity of AT muscles from non-exercised adult and aged male mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions. [a]P<0.05 in comparison with the SOD activity of muscles from non-exercised adult mice, [b]P<0.05 in comparison with the SOD activity of muscles from non-exercised aged mice (n=4-6).

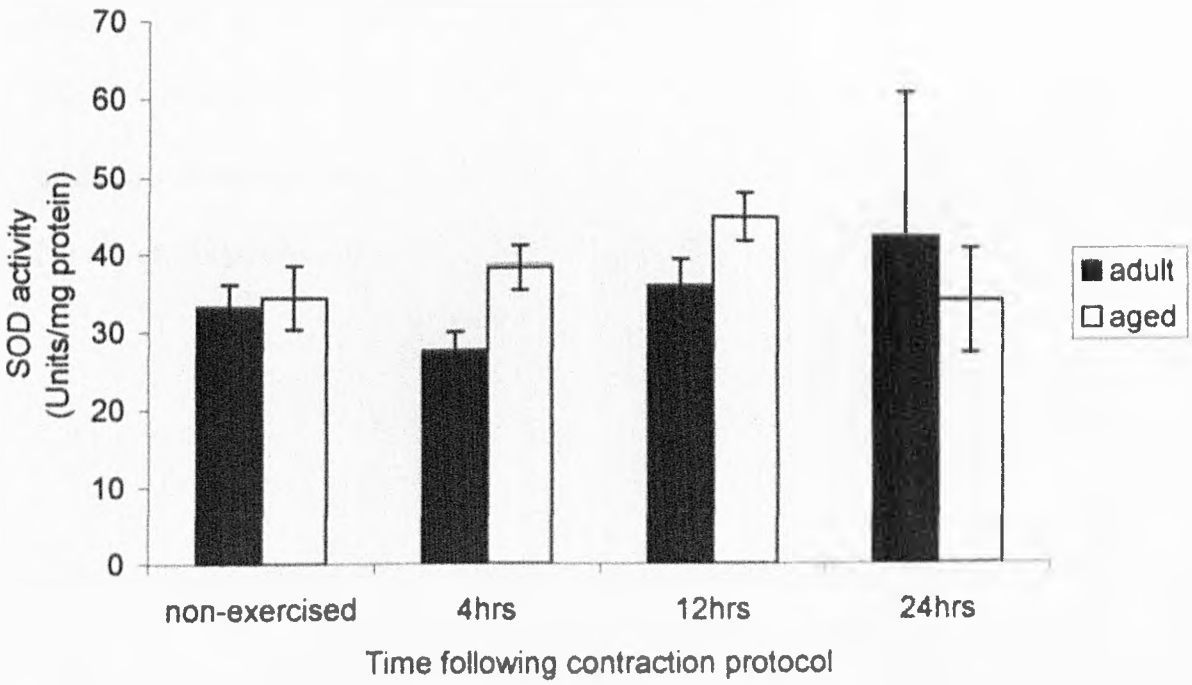


Figure 4.3. SOD activity of AT muscles from non-exercised adult and aged female mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions (n=4-6).

4.3.1.3. Catalase activity of AT muscles from adult and aged male mice

The catalase activity of AT muscles of non-exercised adult and aged male mice and AT muscles at 4, 12 and 24 hours following the contraction protocol is shown in Figure 4.4. In a similar manner to the pattern of changes in SOD activity, the catalase activity of muscles of non-exercised aged male mice was significantly elevated compared with the catalase activity of non-exercised muscles of adult male mice. A significant rise in catalase activity was seen in muscles of adult male mice at 12 hours following the contraction protocol, whereas no significant differences were observed in muscles of aged male mice following contractions.

4.3.1.4. Catalase activity in AT muscles from adult and aged female mice

The catalase activity of AT muscles of non-exercised adult and aged female mice or AT muscles at 4, 12 and 24 hours following the contraction protocol is shown in Figure 4.5. No significant differences were observed in the catalase activity between non-exercised muscles of adult and aged female mice. No effect of exercise was seen in muscles of adult or aged female mice.

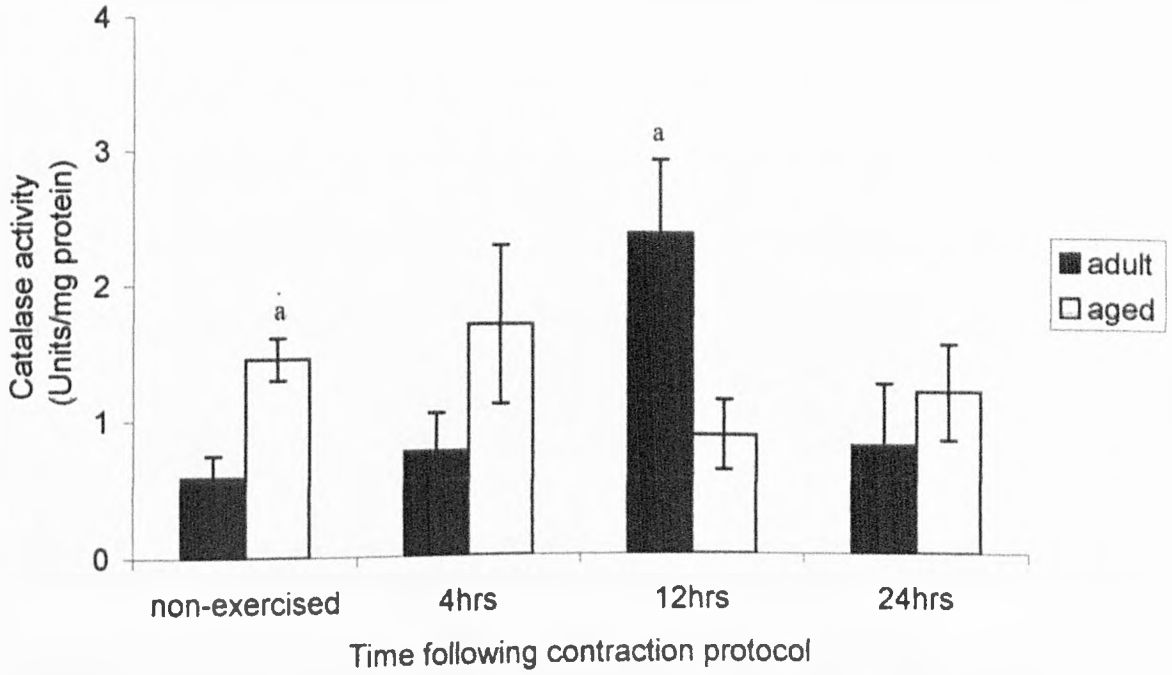


Figure 4.4. Catalase activity of AT muscles from non-exercised adult and aged male mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions. [a]P<0.05 in comparison with the SOD activity of muscles from non-exercised adult mice (n=4-6).

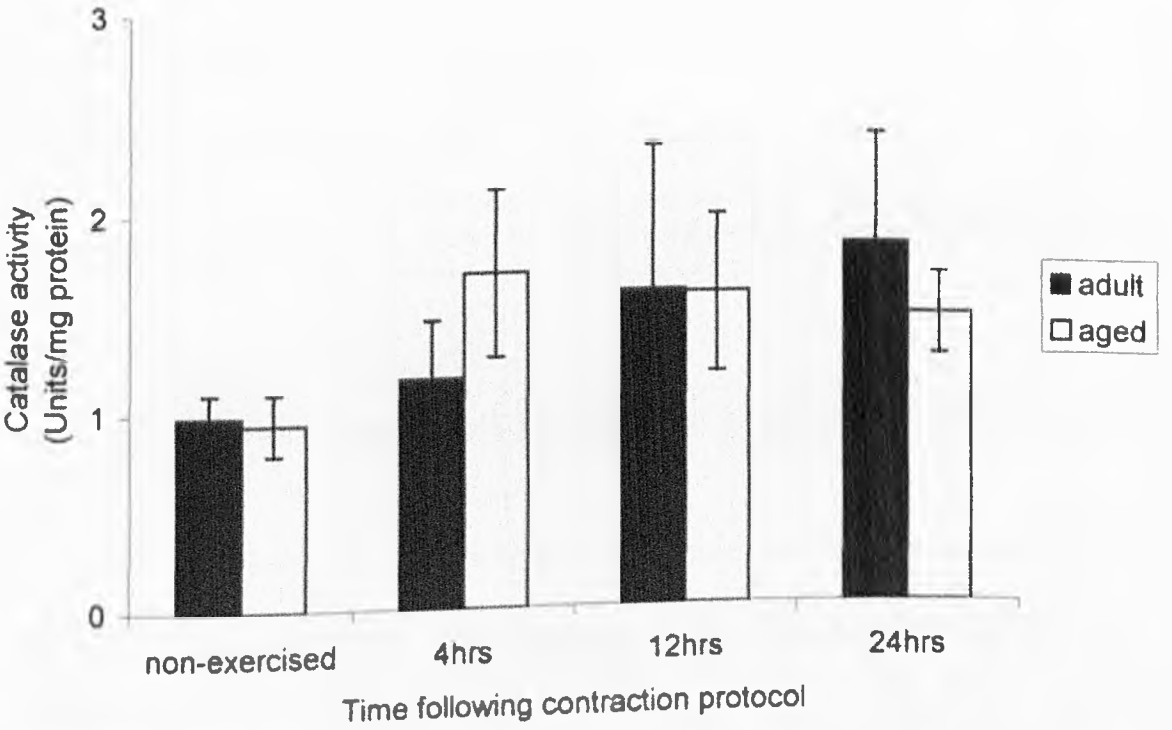


Figure 4.5. Catalase activity of AT muscles from non-exercised adult and aged female mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions (n=4-6).

4.3.1.5. Comparison of SOD and catalase activities in AT muscles of non-exercised adult and aged male and female mice

No significant differences were observed in either SOD or catalase activities when comparing muscles from adult male and adult female mice (Table 4.1). There was a significantly higher activity of SOD and catalase in muscles of aged male mice compared with those of female mice (Table 4.1).

Table 4.1. **SOD and catalase activities of AT muscles from non-exercised adult and aged male and female mice.**

	SOD (Units/mg protein)	CATALASE (Units/mg protein)
ADULT MALE	32.3 (± 5.4)	0.6 (± 0.2)
ADULT FEMALE	33.2 (± 2.9)	0.9 (± 0.1)
AGED MALE	45.8 (± 2.2) [a]	1.5 (± 0.2) [a]
AGED FEMALE	34.5 (± 4.0) [b]	0.9 (± 0.2) [b]

[a]P<0.05 in comparison with the SOD and catalase activities of muscles from non-exercised adult male mice, [b]P<0.05 in comparison with the SOD and catalase activities of muscles from non-exercised aged male mice (n=4-6).

4.3.2. Possible reasons for a lack of adaptation in muscles of adult female mice

In comparison with muscles of adult male mice, the lack of any changes in SOD and catalase activities in muscles of adult female mice may have been due to two possibilities:

a) The response in muscles of adult female mice may become attenuated at a younger age than in muscles of male mice, such that a diminished

response is already evident in muscles of female mice at 12-15 months of age.

b) The response may be delayed in muscles of female mice in comparison with the response of male mice.

In order to address these possibilities, an additional group of younger (4-6 months old) female mice were studied and this additional experiment also included a 48 hour time point.

4.3.2.1. SOD and catalase activities of AT muscles from young female mice

The SOD and catalase activities of AT muscles from non-exercised and exercised young female mice are shown in Figure 4.6 and Figure 4.7 respectively. Previous data from muscles of adult female mice are shown for comparison. No significant differences were observed in the enzyme activities between non-exercised muscles of young and adult female mice. Following the contraction protocol, there was no evidence of a change in SOD or catalase activities of muscles of young female mice at any of the time points measured.

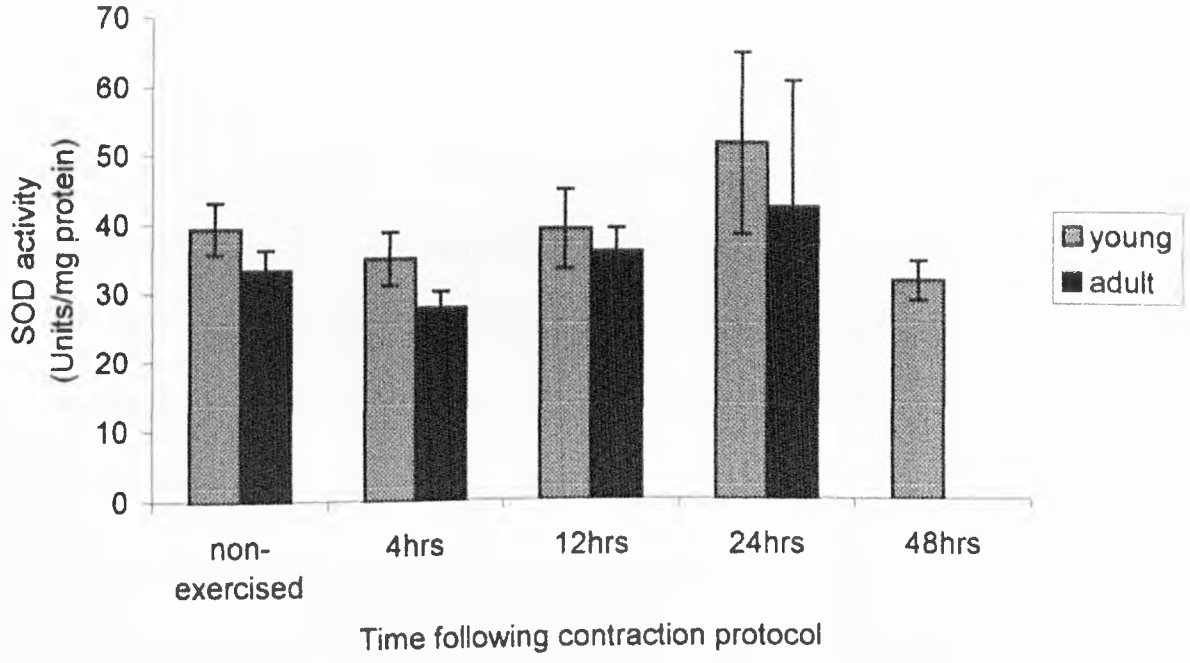


Figure 4.6. SOD activity of AT muscles from non-exercised young and adult female mice and AT muscles at 4, 12, 24 and 48 hours following a period of isometric contractions (n=4-6).

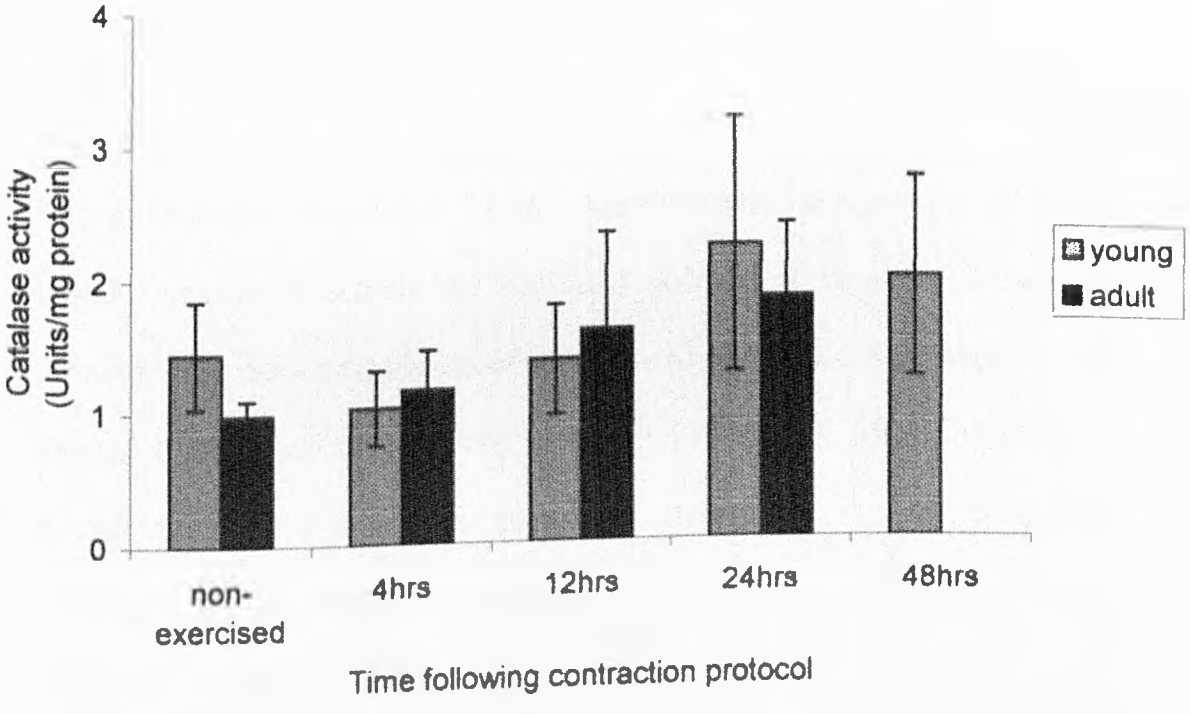


Figure 4.7. Catalase activity of AT muscles from non-exercised young and adult female mice and AT muscles at 4, 12, 24 and 48 hours following a period of isometric contractions (n=4-6).

4.4. DISCUSSION

The influence of ageing and contractile activity on the expression of total SOD and catalase activities was examined in AT muscles of adult and aged male and female mice. Data demonstrated that there was a significant increase in the SOD and catalase activities of muscles from non-exercised aged male mice compared with adult mice (Figure 4.2, Figure 4.4). In contrast, no effect of age was seen in the SOD and catalase activities of muscles from female mice (Figure 4.3, Figure 4.5).

Studies by other workers have shown a similar age-related increase in the antioxidant defence enzymes in skeletal muscle of male rodents. Ji *et al* (1990) demonstrated that Mn-SOD, Cu/Zn SOD and catalase activities were significantly higher in skeletal muscles of non-exercised aged male rats compared with the activities in skeletal muscles of non-exercised young rats. Furthermore, Leeuwenburgh *et al* (1994) have also shown a significant age-related increase in the SOD and catalase activities in skeletal muscles from non-trained male rats. The mechanisms responsible for this age-associated up-regulation of skeletal muscle antioxidant activity are not fully understood. However, it has been proposed that this up-regulation of the antioxidant defence is in response to an increased production of ROS with age (Ji and Hollander, 2000; Section 1.5.6). Increased production of ROS can be highly damaging, therefore the up-regulation of the antioxidant defence is necessary for the cells in order to maintain an appropriate oxidant/antioxidant balance during ageing.

Data also demonstrated that muscles of adult male mice respond to the contraction protocol by an increase in SOD and catalase activity following the exercise protocol (Figure 4.2, Figure 4.4). Similar data has been published by Ji *et*

al (1992), which shows that an acute period of exercise significantly increased the activity of CAT in the deep vastus lateralis (DVL) muscles from male rats immediately after the exercise protocol. Data from our laboratory shows that a single period of non-damaging exercise results in an increase in the SOD activity of skeletal muscles of humans, although no distinction was made between males and females (Khassaf *et al*, 2001). Thus, the current data suggest that skeletal muscles of adult male mice adapt to a single period of non-damaging exercise by increasing their antioxidant enzyme activity.

In contrast, muscles of aged mice showed no evidence of adaptation of these enzyme systems. There was no increase in the activity of the antioxidant defence enzymes of muscles of aged male mice following the exercise protocol (Figure 4.2, Figure 4.4). Similarly, Ji *et al* (1990) found no significant alterations in the activity of the antioxidant defence enzymes in skeletal muscles from aged rats following a single period of exercise. The cellular mechanisms responsible for this age-related decline in skeletal muscle function remain unclear. An adaptive increase in the enzyme activity would be expected to arise from an increased transcriptional activation of these proteins. The lack of increased production of antioxidant defence enzymes in muscles of aged mice following the contraction protocol may be due to alterations at the transcriptional or translational level. It may also be that the increased activity of SOD and catalase in non-exercised muscles of aged mice reflects a successful adaptation to increased oxidative stress during ageing and that this increase is sufficient to protect against the added stress of the contraction protocol. Finally, it has been shown that ageing is associated with a shift in fibre type composition of mixed fibre muscles (see Section 1.5.1). The anterior tibialis muscle is predominantly (90-95%) type II. It is feasible that

small changes in the proportion of fibre types would influence the antioxidant defence enzyme composition to some extent, although substantial changes would have to occur in order to result in the complete attenuation of the adaptive response.

No significant changes were observed in the antioxidant defence enzyme activities in muscles of adult or aged female mice following the contraction protocol (Figure 4.3, Figure 4.5) suggesting that there are major differences in the adaptive response between male and female mice. The reasons for this are unknown, although three potential reasons have been examined here:

a) the muscles may already contain a higher content of antioxidant defence enzymes in comparison with muscles of adult male mice
b) the response in muscles of adult female mice may be attenuated at a younger age than in muscles of male mice
c) the response may be delayed in muscles of female mice in comparison with the response of male mice.

No significant changes were observed in the antioxidant defence enzyme activity between skeletal muscles from non-exercised adult male and adult female mice (Table 4.1), thus ruling out (a) as a possibility.

In order to examine (b), an extra group of AT muscles from young female B6XSJL mice (4-6 months old) was analysed for SOD and catalase activity (Figure 4.6, Figure 4.7). No significant differences were observed in the antioxidant enzyme activities of muscles from non-exercised young female mice in comparison with muscles from non-exercised adult female mice. In addition, no significant differences were observed in the activities of the antioxidant defence enzymes of muscles from young female mice following contractions at any time

point studied (Figure 4.6, Figure 4.7). This ruled out the possibility of an earlier age-related attenuation in the adaptive response of female mice.

In order to detect whether there is a delayed antioxidant defence enzyme response in skeletal muscles of female mice, an extra group of young female B6XSJL mice (4-6 months old) was studied at 48 hours following the contraction protocol (Figure 4.6, Figure 4.7). However, no changes in the enzyme activities was observed in skeletal muscles of young female mice at 48 hours following contractions. These results suggest that muscles from female mice do not respond to this isometric contraction protocol by adaptation. There is little evidence that the muscles are already primed for an increased ROS production since the enzyme activities in muscles of non-exercised adult female mice are similar to those of non-exercised adult male mice. Further examination of ROS production by muscles of female mice is necessary to fully understand this sex difference, although this was not within the scope of the current work.

In summary, the activities of the antioxidant defence enzymes were significantly higher in non-exercised skeletal muscles from aged male mice compared with the enzyme activities in non-exercised skeletal muscles from adult male mice. Muscles from adult male mice demonstrated a clear increase in the production of antioxidant defence enzymes following non-damaging isometric contractions. This was not evident in muscles of aged male mice. No significant differences were observed in the enzyme activities in anterior tibialis muscles of adult and aged female mice prior to and following the contraction protocol. Thus, there are major differences in the adaptive response in muscles of male and female mice.

CHAPTER 5

HEAT SHOCK PROTEIN CONTENT OF ANTERIOR TIBIALIS MUSCLES FROM ADULT AND AGED MICE FOLLOWING ISOMETRIC CONTRACTIONS

5.1. INTRODUCTION

A second key mechanism involved in the protection of skeletal muscle during exercise is the production of a highly conserved family of proteins known as heat shock proteins (HSPs; McArdle *et al*, 2001).

There are several groups of HSPs, most often referred to on the basis of their molecular mass (e.g. HSP70 family is comprised of proteins with molecular mass in the 70 kDa range; also see Section 1.3.4.3). In unstressed skeletal muscle, HSPs are present in low levels. In this situation, they are thought to act as molecular chaperones, binding to newly synthesised proteins and ensuring that these newly synthesised proteins are folded and function correctly (McArdle and Jackson, 2002).

The synthesis of HSPs is increased following a variety of stresses, such as hyperthermia, viral infection and oxidative stress (Locke, 1997). In skeletal muscle, several workers have reported increases in the HSP production following acute bouts of exercise (Section 1.3.6). For example, a study by McArdle *et al* (2001) demonstrated that a 15-minute period of mild non-damaging isometric contractions results in an increased production of HSPs in EDL and soleus muscles from mice. In addition, the effect of endurance training on the production of HSPs in skeletal muscle has also been examined. Samelman (1999) reported that training for 16-20 weeks on a motorised treadmill resulted in an increase in the HSP70 and HSP60 expression in soleus muscles from rats.

Although the production of HSPs in skeletal muscle following exercise has been demonstrated, the mechanisms responsible for this adaptation are not clearly understood. The possibility that increased production of ROS may act as a signal for activation of the HSP response has received some attention. Skeletal muscle

generates a number of free radical species during contraction, including the superoxide anion radical, hydroxyl radical and nitric oxide. McArdle *et al* (2001) have showed that increased production of ROS is accompanied by a transient oxidation of muscle protein thiols following exercise. Oxidation of protein thiols due to oxidative stress has been found to play an important role in the signalling mechanism responsible for the activation of HSP expression in other cells (for a review see Freeman *et al*, 1999). Therefore, it was hypothesised that the increased production of HSPs during exercise may be dependent on the presence of oxidized or unfolded cellular proteins due to an increased production of ROS.

Several workers have confirmed the importance of the HSP response, particularly HSP70, using transgenic mice overexpressing HSP70. For example, Marber *et al* (1995) reported that isolated hearts from transgenic mice overexpressing HSP70 demonstrated an increased resistance to ischemic injury. Radford *et al* (1996) showed that hearts from transgenic mice overexpressing HSP70 demonstrated enhanced recovery of high-energy phosphate stores and correction of metabolic acidosis following brief periods of global ischemia. Rajdev and colleagues (2000) reported that overexpression of HSP70 in transgenic mice could markedly protect the brain against ischemic damage. In addition, workers in our laboratory have demonstrated that skeletal muscle of transgenic mice, overexpressing HSP70, is protected against lengthening contraction-induced damage (McArdle *et al*, 2000).

During ageing, skeletal muscles become smaller and weaker, such that by the age of 70, the cross-sectional area of a range of muscles is reduced by 25-30% and muscle strength by 30-40%. This age-related decline in muscle mass and strength is due to a large decrease in the total number of individual muscle fibres

within the muscle bulk and an atrophy of the remaining fibres, particularly type II fibres (Lexell *et al*, 1988). Studies in rodents have shown that the remaining muscle fibres generate less force than a similar sized muscle of a young rodent, are more susceptible to contraction-induced damage and take longer to recover from that damage (Brooks & Faulkner, 1988; Faulkner *et al* 1990).

It has been shown that the ability of cells to produce HSPs following stress is reduced in aged individuals although this appears to depend on the cell type or tissue under study (Liu *et al*, 1996). In skeletal muscle, our group (Vasilaki *et al*, 2002) have demonstrated that there was an attenuated production of HSP70 in gastrocnemius muscles of aged rats at 24 hours following a period of isometric contractions. In contrast, Locke (2000) reported a normal response in skeletal muscles of rats following a period of whole-body hyperthermia. One drawback of our previous study (Vasilaki *et al*, 2002) was that only a single time point was examined. It may be that the time course of production of HSPs is different in muscles of aged rodents or there may be a different response to different stimuli.

The aim of this section of the study was to examine the time course and extent of HSP production in skeletal muscles from adult and aged male and female B6XSJL mice following a period of non-damaging isometric contractions.

5.2. EXPERIMENTAL METHODS

5.2.1. Contraction protocol

Skeletal muscles from anaesthetised adult and aged male and female B6XSJL mice were subjected to a 15-minute period of non-damaging isometric contractions (Section 2.1). Mice were killed immediately, 4 hours, 12 hours and 24 hours following the contraction protocol and anterior tibialis muscles were removed and frozen in liquid nitrogen. Anterior tibialis muscles from non-exercised adult and aged male and female B6XSJL mice were also removed and frozen in liquid nitrogen. Muscles were stored at -70°C until analysis.

5.2.2. Analysis of HSP content of skeletal muscles by SDS-PAGE and western blotting

Anterior tibialis muscles from non-exercised and exercised adult and aged mice were ground under liquid nitrogen, and portions of ground powder were homogenised in 100µl of 1% SDS and protease inhibitors and centrifuged at 10,000g for 10mins at 4°C (Eppendorf Centrifuge 5402, London, U.K.). The supernatants were retained and analysed for protein content using the bicinconinic acid protein determination kit (Section 2.3.1; Sigma Immunochemicals, Dorset, UK). Supernatants were used for analysis of HSP content by SDS-PAGE and western blotting using various antibodies (Section 2.4).

5.2.3. Presentation of data

HSP data from non-exercised adult and aged male and young, adult and aged female mice is expressed as arbitrary units (see Tables 5.1, 5.2 and 5.3).

To allow a comparison of the HSP content of muscles of adult and aged mice with the levels in non-exercised muscles of mice of the same age, data was expressed as a percentage of non-exercised muscles of mice of the same age (see Figure 5.1a to Figure 5.12a).

In addition, to allow comparison of the absolute value of the HSP content in muscles of aged mice with that of muscles from adult mice, data was also presented as a percentage of the value for muscles of non-exercised adult mice (see Figure 5.1b to Figure 5.12b).

For clarity, data are presented separately for male and female mice.

5.3. RESULTS

5.3.1. HSP content of AT muscles from adult and aged male mice

5.3.1.1. HSP content of AT muscles from non-exercised adult and aged male mice

A comparison of the HSC70, HSP60 and HSP25 contents of AT muscles from non-exercised adult and aged male mice is shown in Table 5.1. HSP70 was not detectable in AT muscles from non-exercised adult male mice and therefore, a comparison with the HSP70 content of muscles from non-exercised aged male mice was not possible. Data are expressed as arbitrary units and so comparisons can only be made for the same protein. Values do not reflect differences between different proteins.

The HSP content of AT muscles from non-exercised aged male mice tended to be greater than that of non-exercised adult male mice, although this was only significant in the case of HSC70, where a 50% increase was detected. HSP70 was not detectable in AT muscles from non-exercised adult mice (Figure 5.2a). In contrast, HSP70 was just detectable in AT muscles of non-exercised aged male mice (Figure 5.2b).

Table 5.1. **HSC70, HSP60 and HSP25 content of AT muscles from non-exercised adult and aged male mice.**

	Male	
	Adult	Aged
HSC70	40.8 (±6.1)	65.4 (±4.3)[a]
HSP60	428.1 (±97)	590.2 (±59)
HSP25	82.0 (±13)	96.0 (±11.4)

Data are presented as arbitrary units. [a]P<0.05 in comparison with the HSC70 content of muscles from non-exercised adult male mice (n=4-5).

5.3.1.2. HSP25 content of AT muscles from adult and aged male mice following isometric contractions

The effect of the contraction protocol on the HSP25 content of male mice is shown in Figure 5.1a and Figure 5.1b. Contractions resulted in a rapid and significant rise in HSP25 content of muscles of adult male mice at 4 hours. This rise was maintained at 12 and 24 hours following the contraction protocol (Figure 5.1a, Figure 5.1b).

No effect of the contraction protocol was seen on the HSP25 content of muscles from aged male mice at any time point studied (Figure 5.1a, Figure 5.1b).

5.3.1.3 HSP70 content of AT muscles from adult and aged male mice following isometric contractions

Representative western blots of the HSP70 content of skeletal muscles from adult and aged male mice prior to and following the contraction protocol are shown in Figures 5.2a and 5.2b. Following the contraction protocol, the HSP70 content of muscles from adult male mice was highly variable, but demonstrated a detectable increase at 12 and 24 hours following contractions (e.g. Figure 5.2a). No consistent effect of the contraction protocol was seen on HSP70 content of muscles from aged male mice (Figure 5.2b).

5.3.1.4. HSC70 content of AT muscles from adult and aged male mice following isometric contractions

The contraction protocol resulted in transient but significant rise in the HSC70 content of muscles of adult male mice at 4 hours following contractions (Figure 5.3a, Figure 5.3b). The increased HSC70 content of muscles of adult male mice reached approximately the same absolute value in non-exercised aged mice

(Figure 5.3b). No significant increases were observed in the HSC70 content of muscles of aged mice following contractions (Figure 5.3a, Figure 5.3b).

5.3.1.5. HSP60 content of AT muscles from adult and aged male mice following isometric contractions

The effect of the contraction protocol on the HSP60 content of AT muscles from adult and aged male mice is shown in Figure 5.4a and Figure 5.4b. In contrast to other HSP data presented in this chapter, the contraction protocol resulted in a small but significant rise in HSP60 in muscles of adult male mice at 4 hours following contractions and a later significant rise in HSP60 content of muscles of aged mice at 12 hours following contractions (Figure 5.4a). However, when data are expressed as a percentage of male adult non-exercised muscle, there is no significant increase detectable in muscles of aged male mice (Figure 5.4b).

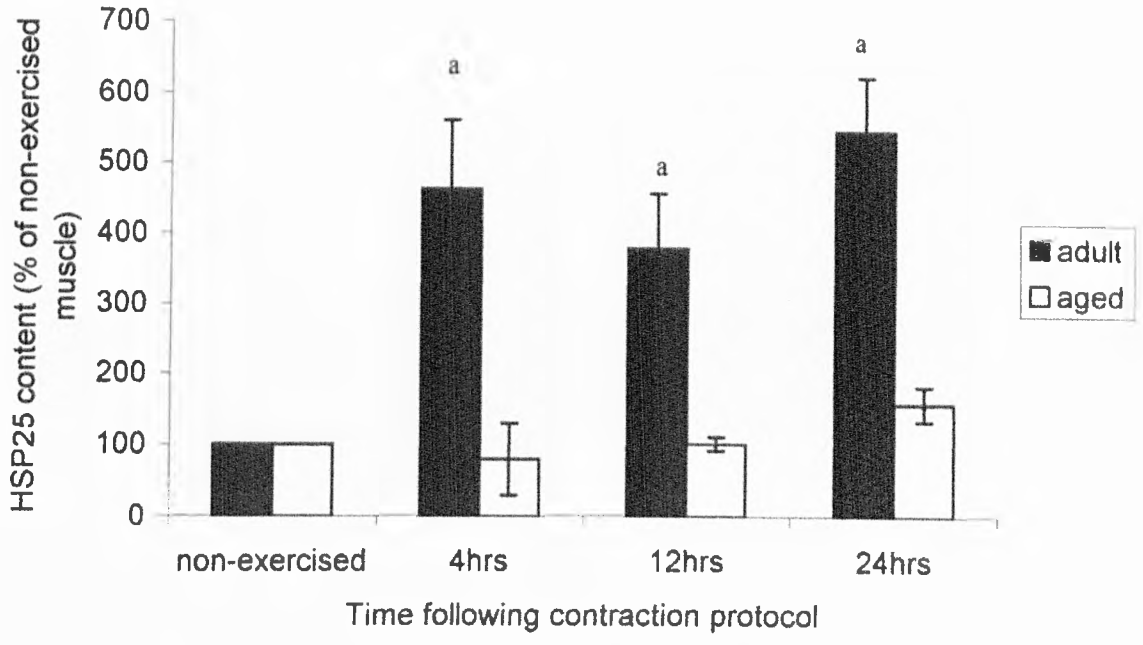


Figure 5.1a. HSP25 content of AT muscle from non-exercised adult and aged male mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions. $^{a}P<0.05$ in comparison with the HSP25 content of muscles from non-exercised adult mice (n=4-6).

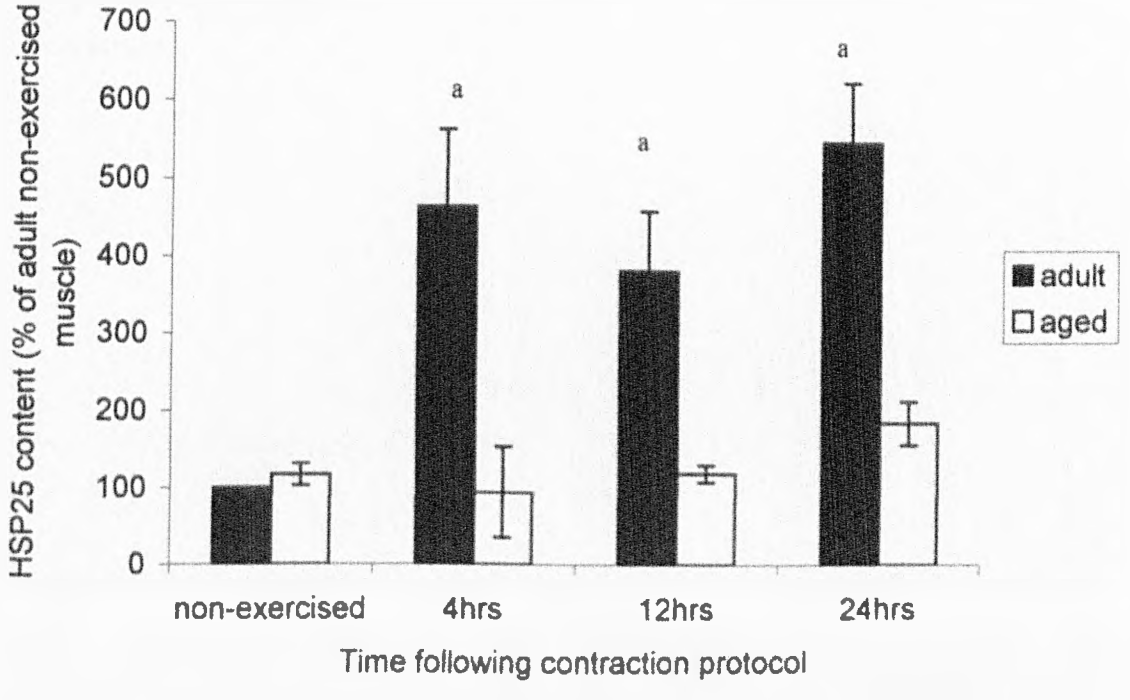


Figure 5.1b. HSP25 content of AT muscle from non-exercised adult and aged male mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions. $^{a}P<0.05$ in comparison with the HSP25 content of muscles from non-exercised adult mice (n=4-6).

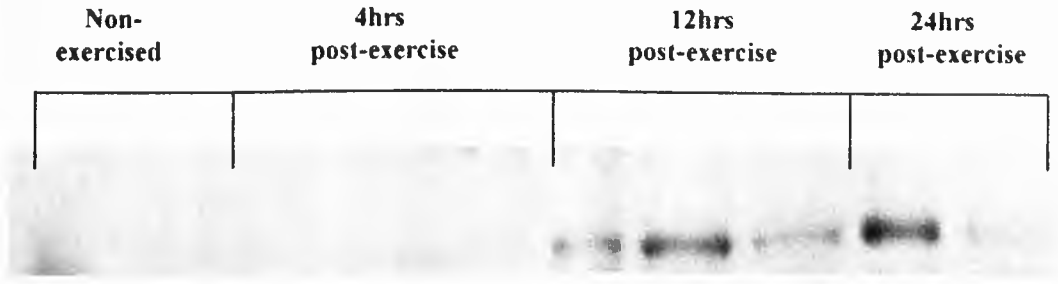


Figure 5.2a. Representative western blot showing the HSP70 content of AT muscles from non-exercised adult male mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions.

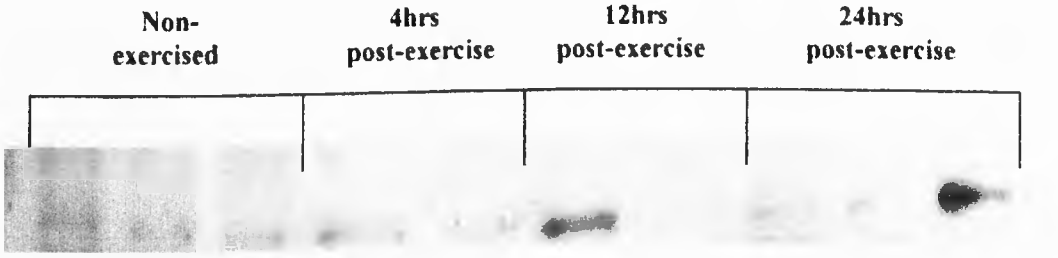


Figure 5.2b. Representative blot of the HSP70 content of AT muscles from non-exercised aged male mice and AT muscles at 4,12 and 24hours following a period of isometric contractions.

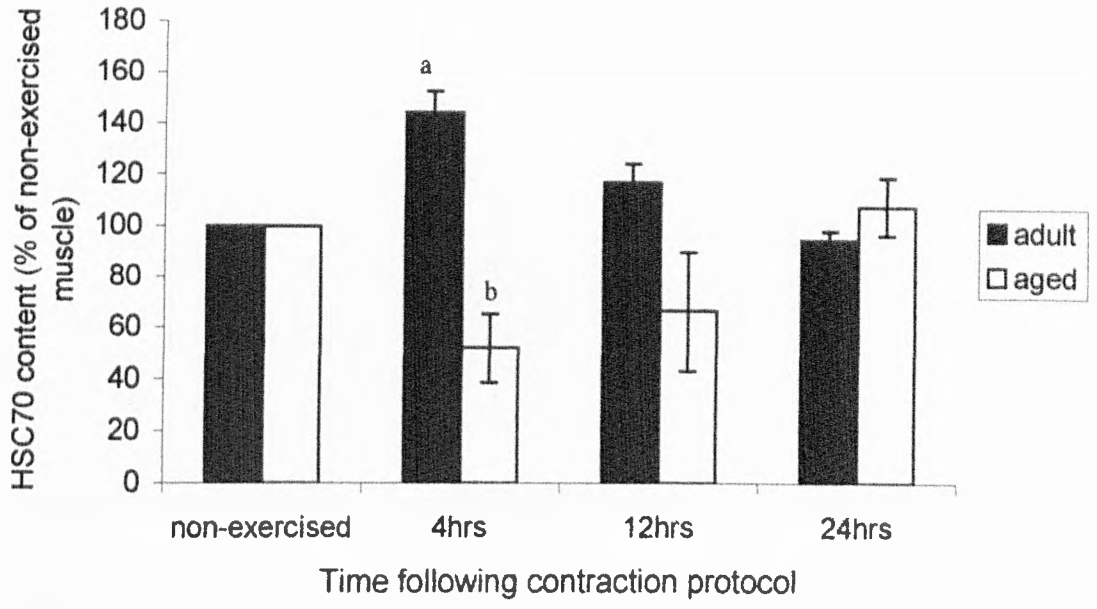


Figure 5.3a. HSC70 content of AT muscle from non-exercised adult and aged male mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions. [a]P<0.05 in comparison with the HSC70 content of muscles from non-exercised adult mice, [b]P<0.05 in comparison with the HSC70 content of muscles from non-exercised aged mice (n=4-6).

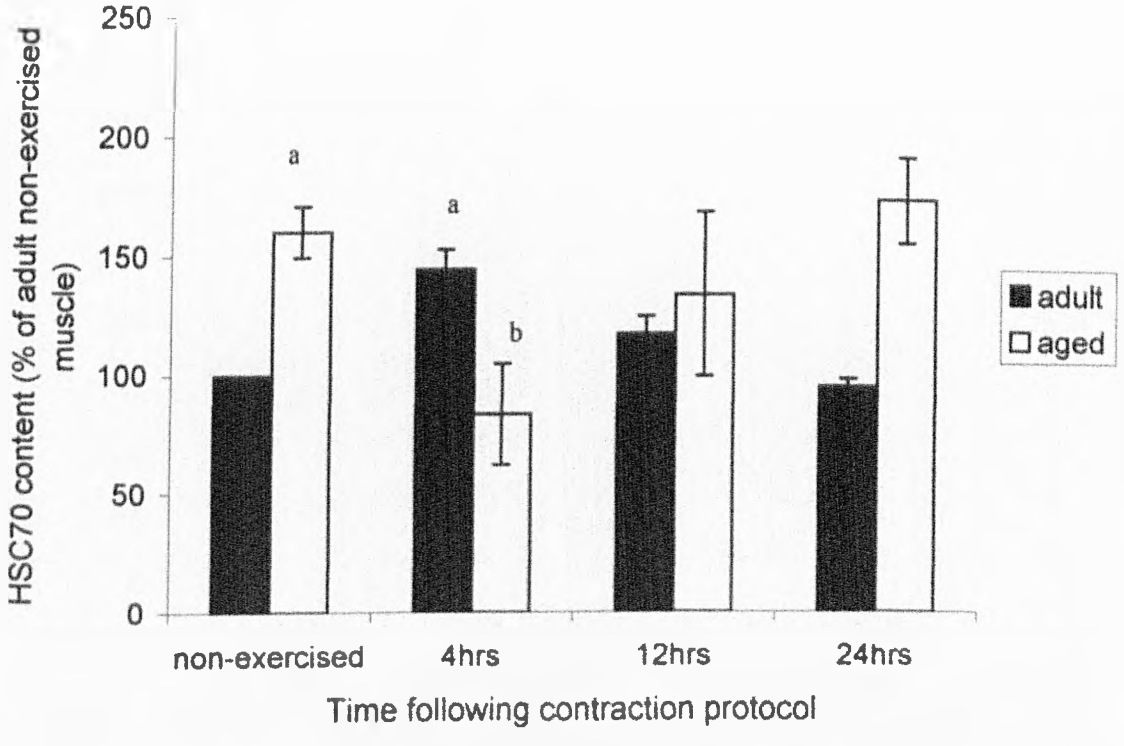


Figure 5.3b. HSC70 content of AT muscle from non-exercised adult and aged male mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions. [a]P<0.05 in comparison with the HSC70 content of muscles from non-exercised adult mice, [b]P<0.05 in comparison with the HSC70 content of muscles from non-exercised aged mice (n=4-6).

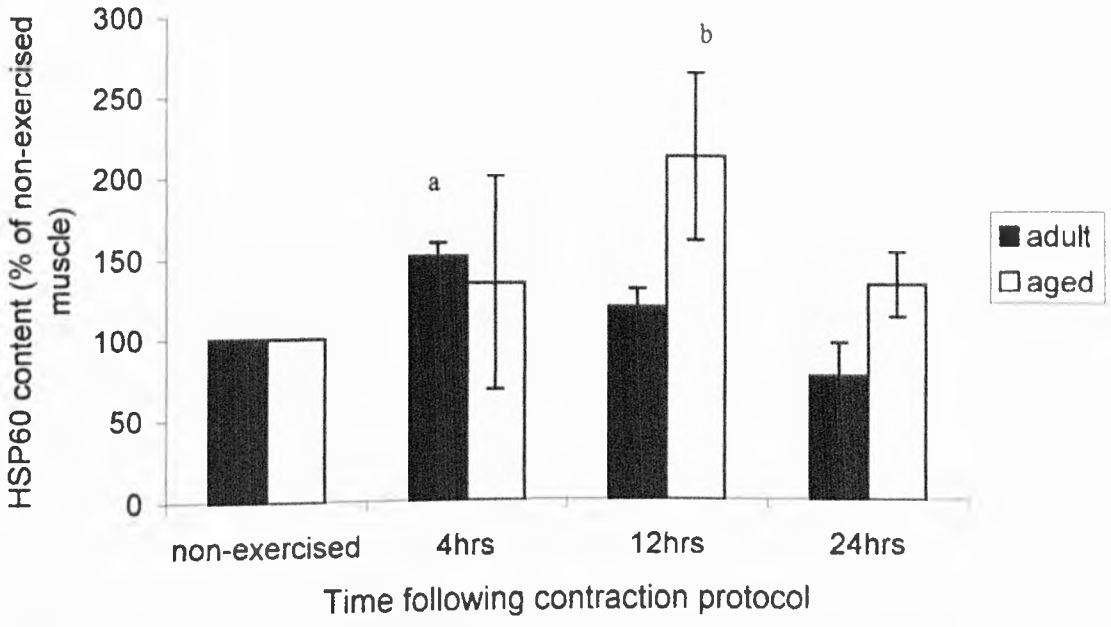


Figure 5.4a. HSP60 content of AT muscle from non-exercised adult and aged male mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions. [a]P<0.05 in comparison with the HSP60 content of muscles from non-exercised adult mice, [b]P<0.05 in comparison with the HSP60 content of muscles from non-exercised aged mice (n=4-6).

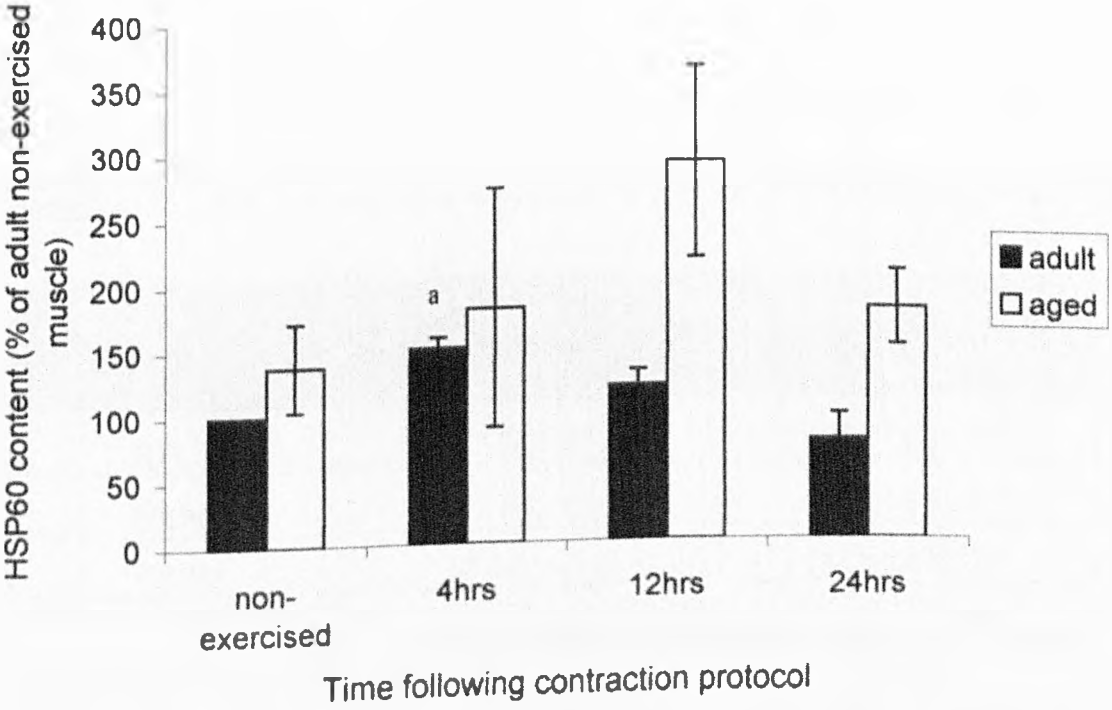


Figure 5.4b. HSP60 content of AT muscle from non-exercised adult and aged male mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions. [a]P<0.05 in comparison with the HSP60 content of muscles from non-exercised adult male mice (n=4-6).

5.3.2. HSP content of AT muscles from adult and aged female mice

5.3.2.1. HSP content of AT muscles from non-exercised adult and aged female mice

A comparison of the HSC70, HSP60 and HSP25 contents of AT muscles from non-exercised adult and aged female mice is shown in Table 5.2. A significant fall in the HSC70 and HSP60 content was seen in muscles of aged mice compared with muscles of adult mice. In contrast, a significant increase in the HSP25 content was seen in muscles of aged mice compared with muscles of adult mice (Table 5.2). Although HSP70 content in skeletal muscles of non-exercised adult female mice was detectable, the HSP70 content of skeletal muscles of non-exercised aged female mice was highly variable therefore a formal comparison at rest was not reliable.

Table 5.2. **HSP content of muscles from non-exercised adult and aged female mice.**

	Female	
	Adult	Aged
HSC70	271.2 (± 30.5)	147.1 (± 35.5)*
HSP60	427.5 (± 89.0)	139.7 (± 26.7)*
HSP25	65.6 (± 13.4)	188.6 (± 15.6)*

Data are expressed as arbitrary units. *P<0.05 in comparison to the HSP content from non-exercised adult female mice (n=4-5).

5.3.2.2. HSP25 content of AT muscles from adult and aged female mice following isometric contractions

The effect of the contraction protocol on the HSP25 content of female mice is shown in Figure 5.5a and Figure 5.5b. The protocol resulted in a significant fall in the HSP25 content of muscles from both adult and aged female mice at 4 hours following contractions (Figure 5.5a, Figure 5.5b). However, the values had normalised by 12 hours following contractions (Figure 5.5a, Figure 5.5b). When data is expressed as a percentage of the HSP25 content of non-exercised adult female mice, it is apparent that the significantly higher resting levels of HSP25 in muscles of aged female mice is maintained (Figure 5.5b).

5.3.2.3. HSP70 content of AT muscles from adult and aged female mice following isometric contractions

The HSP70 content of AT muscles from adult and aged female mice at 4, 12 and 24 hours following a period of isometric contractions is shown in Figure 5.6a and Figure 5.6b. No consistent effect of the contraction protocol was seen on HSP70 content of muscles from adult female mice at any time point studied (Figure 5.6a).

The HSP70 content of muscles from aged female mice was highly variable, but demonstrated a detectable increase at 24 hours following the contraction period (Figure 5.6b).

5.3.2.4. HSC70 content of AT muscles from adult and aged female mice following isometric contractions

The contraction protocol resulted in significant fall in the HSC70 content in muscles of adult female mice at 24 hours following contractions (Figure 5.7a, Figure 5.7b). No significant changes were observed in the HSC70 content of muscles of aged mice following contractions (Figure 5.7a, Figure 5.7b).

5.3.2.5. HSP60 content of AT muscles from adult and aged female mice following isometric contractions

The effect of the contraction protocol on the HSP60 content of AT muscles from adult and aged female mice is shown in Figure 5.8a and Figure 5.8b. No significant effect of the contraction protocol was seen in muscles of adult female mice (Figure 5.8a and Figure 5.8b). The protocol resulted in a small but significant fall in HSP60 content of muscles from aged female mice at 12 and 24 hours following contractions (Figure 5.8a). When data was expressed as % of the HSP60 content of non-exercised adult female mice, the lower resting HSP60 content of skeletal muscles of aged female mice was maintained (Figure 5.8b).

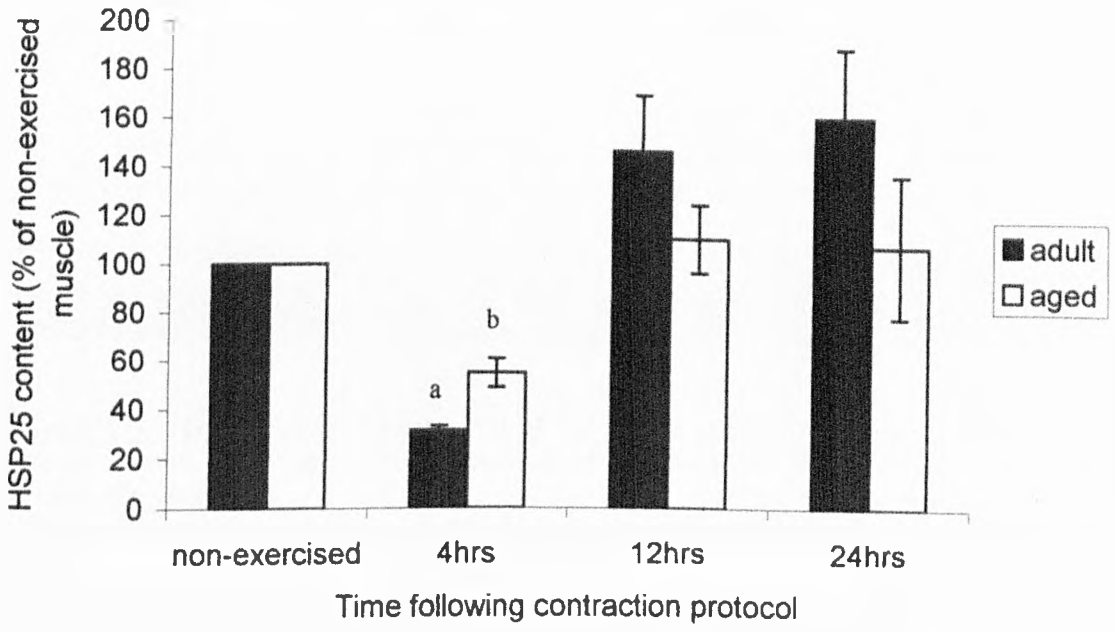


Figure 5.5a. HSP25 content of AT muscle from non-exercised adult and aged female mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions, $^{a}P<0.05$ in comparison with the HSP25 content of muscles from non-exercised adult female mice, $^{b}P<0.05$ in comparison with the HSP25 content of muscles from non-exercised aged female mice (n=4-6).

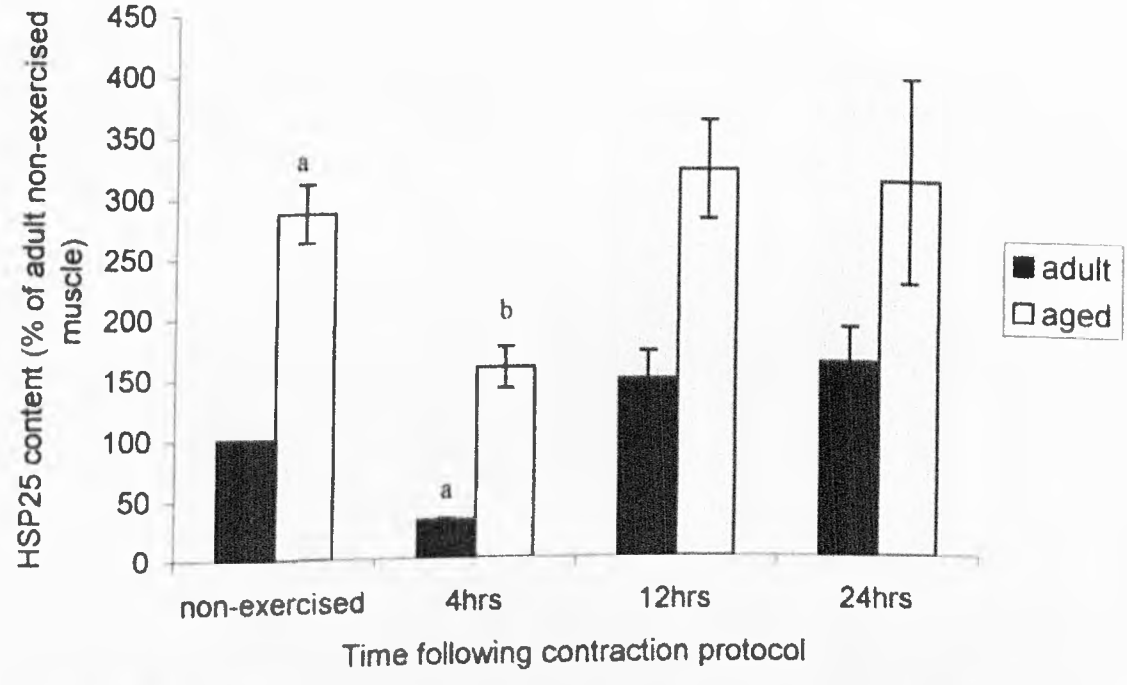


Figure 5.5b. HSP25 content of AT muscle from non-exercised adult and aged female mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions, $^{a}P<0.05$ in comparison with the HSP25 content of muscles from non-exercised adult female mice, $^{b}P<0.05$ in comparison with the HSP25 content of muscles from non-exercised aged female mice (n=4-6).

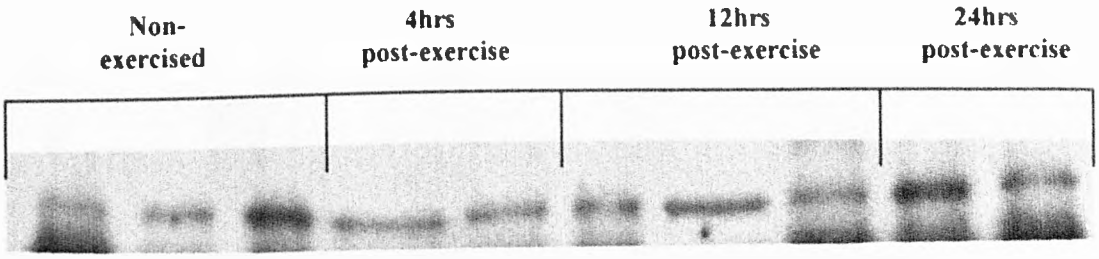


Figure 5.6a. Representative western blot of the HSP70 content of AT muscles from non-exercised adult female mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions.

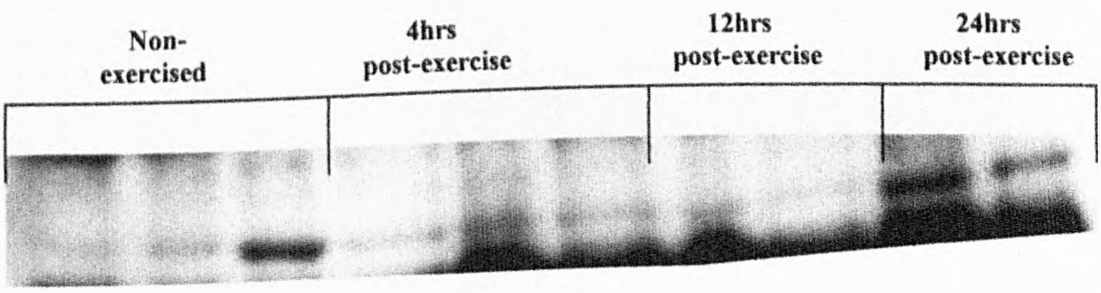


Figure 5.6b. Representative western blot of the HSP70 content of AT muscles from non-exercised aged female mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions.

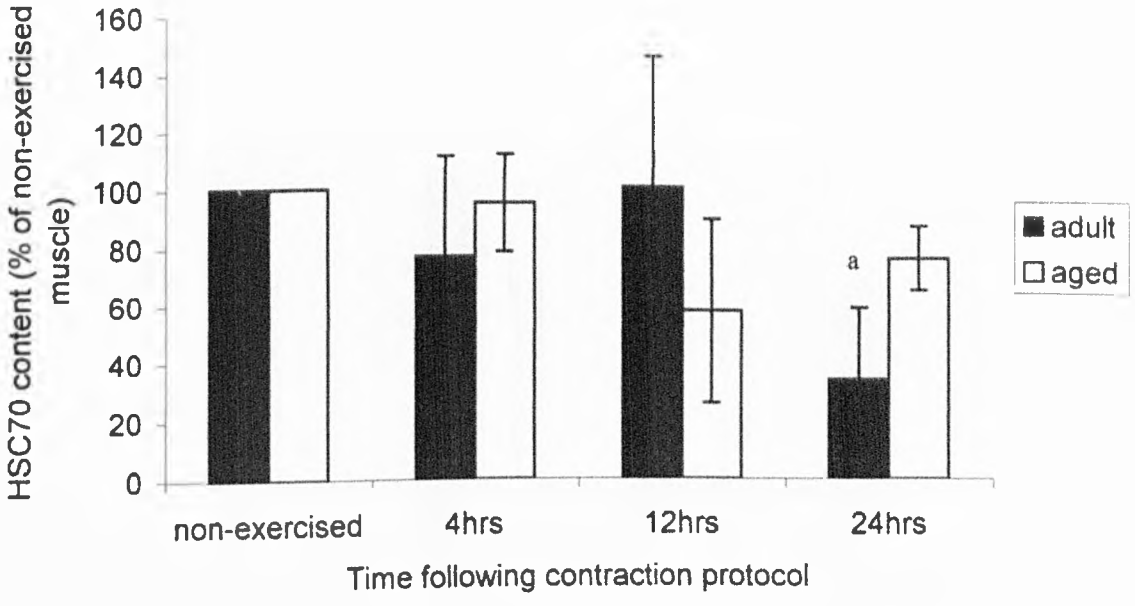


Figure 5.7a. HSC70 content of AT muscle from non-exercised adult and aged female mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions, $^{a}P<0.05$ in comparison with the HSC70 content of muscle from non-exercised adult female mice (n=4-6).

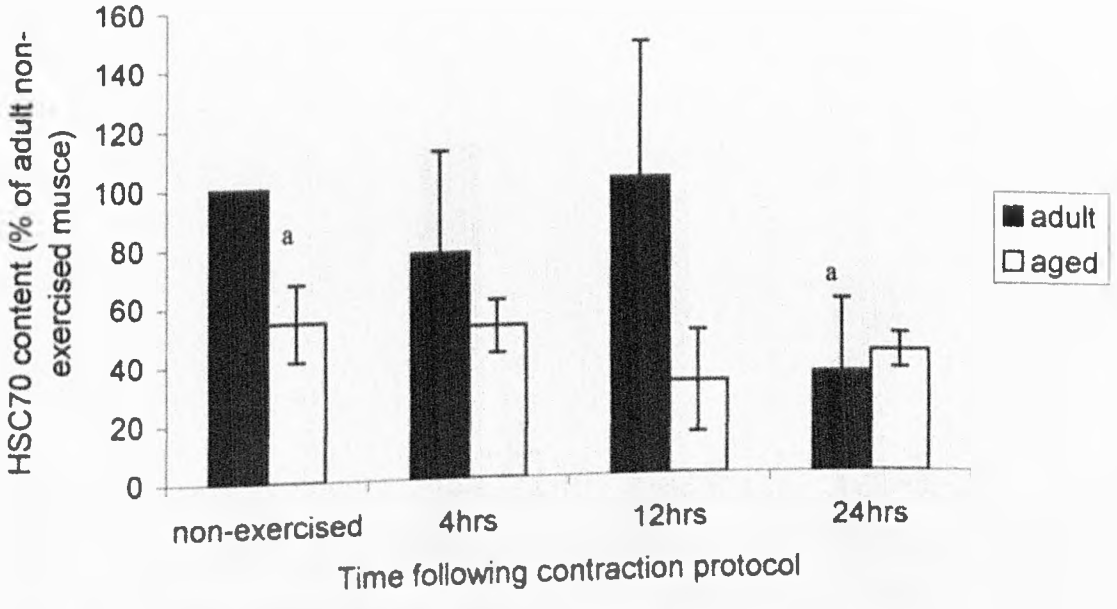


Figure 5.7b. HSC70 content of AT muscle from non-exercised adult and aged female mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions, $^{a}P<0.05$ in comparison with the HSC70 content of muscles from non-exercised adult female mice (n=4-6).

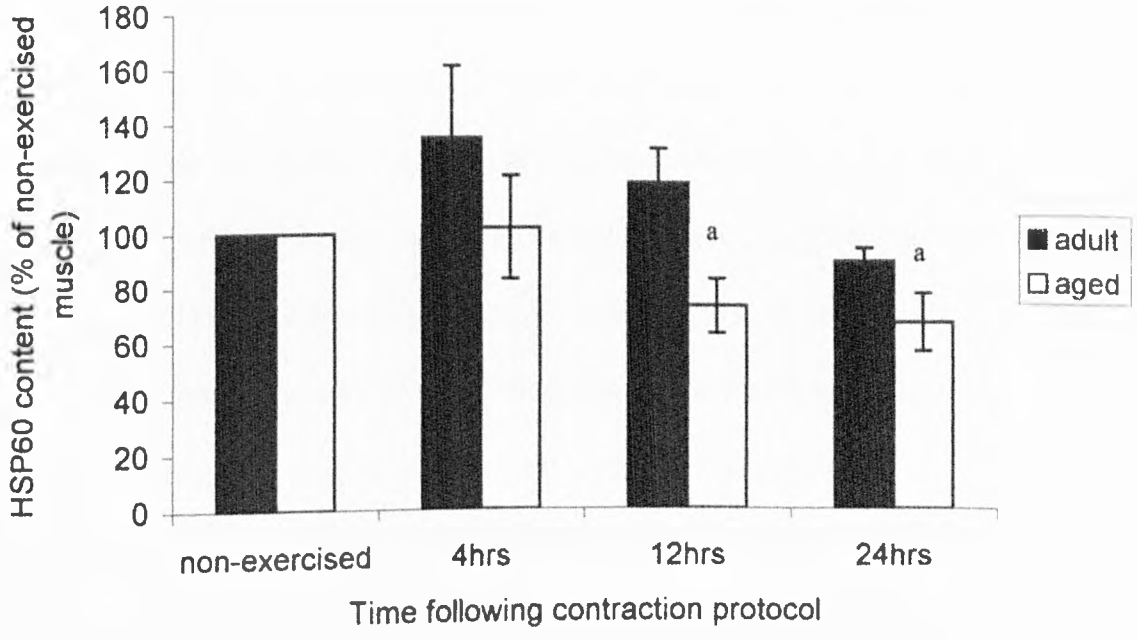


Figure 5.8a. HSP60 content of AT muscle from non-exercised adult and aged female mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions, $^{a}P<0.05$ in comparison with the HSP60 content of muscles from non-exercised aged female mice (n=4-6).

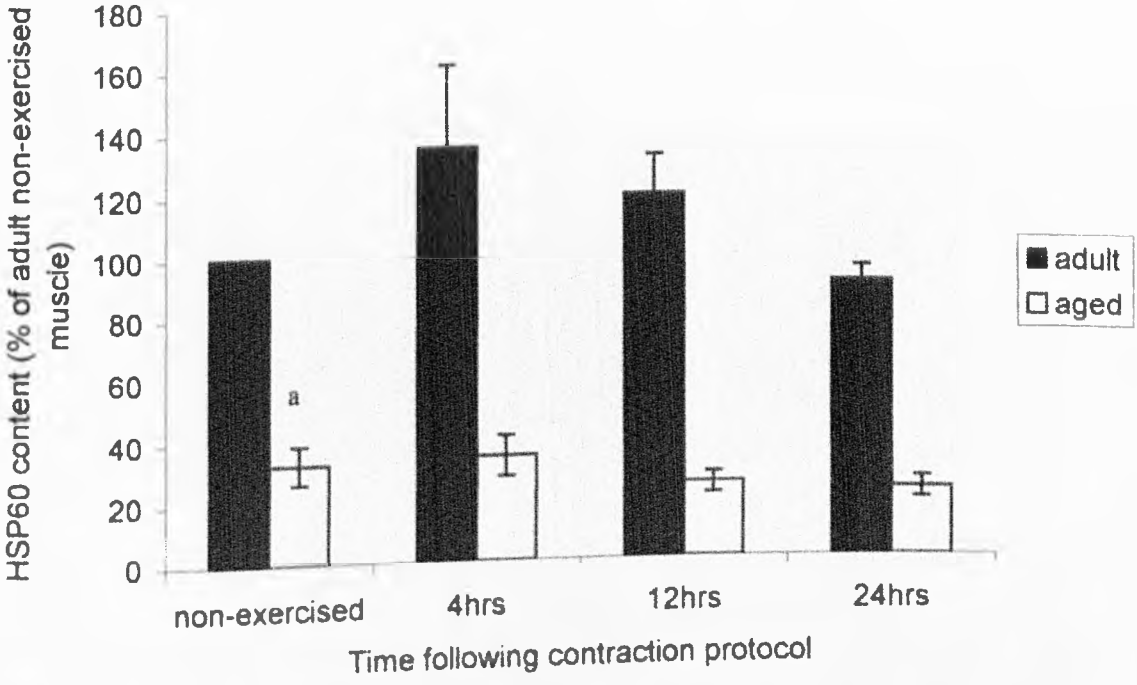


Figure 5.8b. HSP60 content of AT muscle from non-exercised adult and aged female mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions, $^{a}P<0.05$ in comparison with the HSP60 content of muscles from non-exercised adult female mice (n=4-6).

5.3.3. Examination of the possible reasons for a lack of adaptation following the contraction protocol in muscles of adult female mice

HSPs show a similar lack of adaptive response to the antioxidant defence enzyme response (Section 4.3.1) in skeletal muscles from adult female mice. This lack of adaptation in female mice may be due to several possible reasons:

a) The HSP response in muscles of female mice may be altered at a younger age than in muscles of male mice thus, the lack of adaptation in muscles of adult mice is similar to the 'aged' muscles of male mice.

b) The HSP response may be delayed (i.e. production of HSPs at a later time point) in muscles of female mice in comparison with the response of male mice.

In order to address this, an additional group of young (4-6 months old) mice were studied and this study also included a 48 hours time point following the contraction protocol. This data is presented with previous data for muscles from adult mice for clarity.

5.3.3.1. HSP content of AT muscles from non-exercised young and adult female mice

A comparison of the HSC70, HSP60 and HSP25 contents of AT muscles from non-exercised young and adult female mice is shown in Table 5.3. No significant changes where observed in the HSC70, HSP60 and HSP25 contents between skeletal muscles from non-exercised young and adult female mice (Table 5.3).

Table 5.3. **HSP content of muscles from non-exercised young and adult female mice.**

	Female	
	Young	Adult
HSC70	188.3 (± 56.2)	271.2 (± 30.5)
HSP60	372.8 (± 162.6)	427.5 (± 88.9)
HSP25	49.5 (± 2.7)	65.6 (± 13.4)

Data is expressed as arbitrary units (n=4-5).

5.3.3.2. Comparison of the HSP25 content of AT muscles from young and adult female mice

The HSP25 content of AT muscles from non-exercised young female mice and AT muscles at 4, 12, 24 and 48 hours following a period of isometric contractions is shown in Figure 5.9a and Figure 5.9b. No significant changes where observed in the HSP25 content between skeletal muscles from non-exercised young and adult female mice (Figure 5.9b).

The contraction protocol resulted in significant decrease in the HSP25 content in muscles of young female mice at 12 hours contractions (Figure 5.9a). This decrease was maintained for up to 48 hours (Figure 5.9a, Figure 5.9b).

5.3.3.3. Comparison of the HSP70 content of AT muscles from young and adult female mice

The HSP70 content of AT muscles from non-exercised young female mice and AT muscles at 4, 12, 24 and 48 hours following a period of isometric contractions is shown in Figure 5.10a. The HSP70 content of AT muscles from non-exercised and exercised adult female mice is shown for comparison (Figure 5.10b). No significant effect of the contraction protocol was seen in muscles of young female mice at any time point studied (Figure 5.10a), with no evidence of an increased content of HSP70 at 48 hours following the contraction protocol.

5.3.3.4. Comparison of the HSC70 content of AT muscles from young and adult female mice

The HSC70 content of AT muscles from non-exercised young female mice and AT muscles at 4, 12, 24 and 48 hours following a period of isometric contractions is shown in Figure 5.11a and Figure 5.11b. No significant changes where observed in the HSC70 content between skeletal muscles from non-exercised young and adult female mice (Figure 5.11b).

In a similar manner to muscles of adult female mice, the contraction protocol resulted in significant fall in the HSC70 content in muscles of young female mice at 4 hours following contractions (Figure 5.11a). This decrease was maintained for up to 48hrs (Figure 5.11a). However, when data was expressed as % of the HSC70 content from non-exercised adult female mice, no significant changes were observed in skeletal muscles of young mice following the exercise protocol (Figure 5.11b).

5.3.3.5. Comparison of the HSP60 content of AT muscles from young and adult female mice

The HSP60 content of AT muscles from non-exercised young female mice and AT muscles at 4, 12, 24 and 48 hours following a period of isometric contractions is shown in Figure 5.12a and Figure 5.12b. No significant changes where observed in the HSP60 content between skeletal muscles from non-exercised young and adult female mice at any time point (Figure 5.12b).

In addition, no effect of exercise was seen in muscles of either adult or aged female mice at any time point studied (Figure 5.12a, Figure 5.12b).

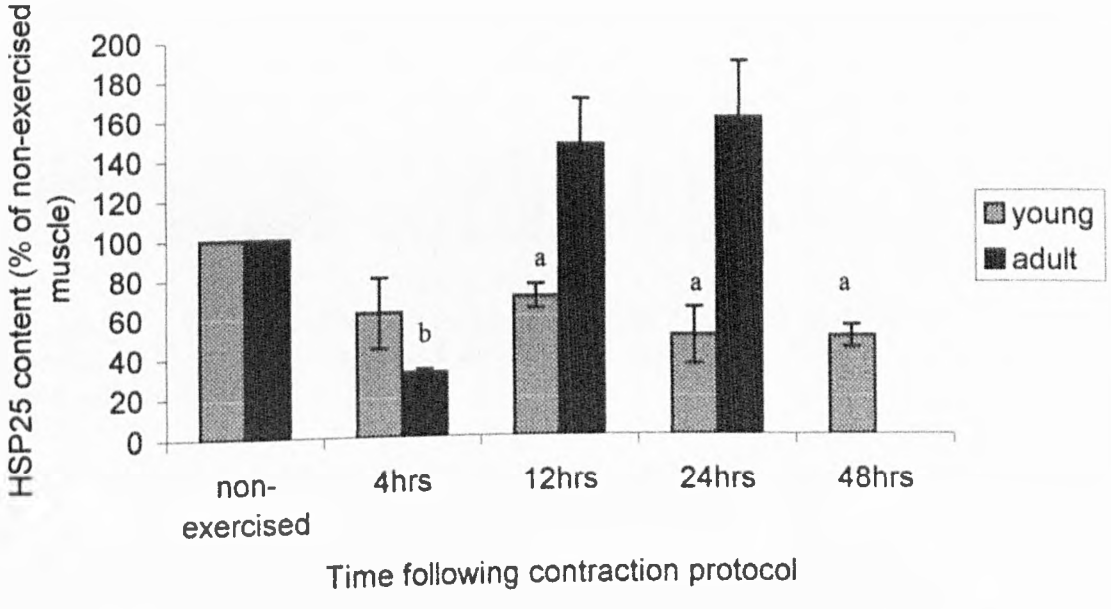


Figure 5.9a. HSP25 content of AT muscle from non-exercised young and adult female mice and AT muscles at 4, 12, 24 and 48 hours following a period of isometric contractions. [a]P<0.05 in comparison with the HSP25 content of muscles from non-exercised young female mice, [b]P<0.05 in comparison with the HSP25 content of muscles from non-exercised adult female mice (n=4-6).

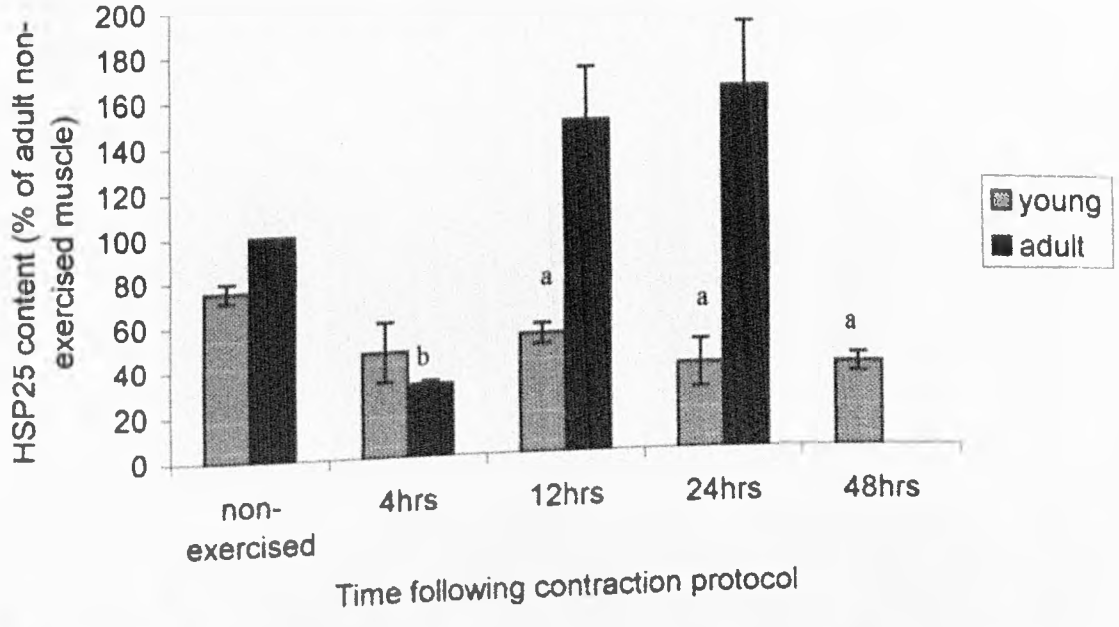


Figure 5.9b. HSP25 content of AT muscle from non-exercised young and adult female mice and AT muscles at 4, 12, 24 and 48 hours following a period of isometric contractions. [a]P<0.05 in comparison with the HSP25 content of muscles from non-exercised young female mice, [b]P<0.05 in comparison with the HSP25 content of muscles from non-exercised adult female mice (n=4-6).

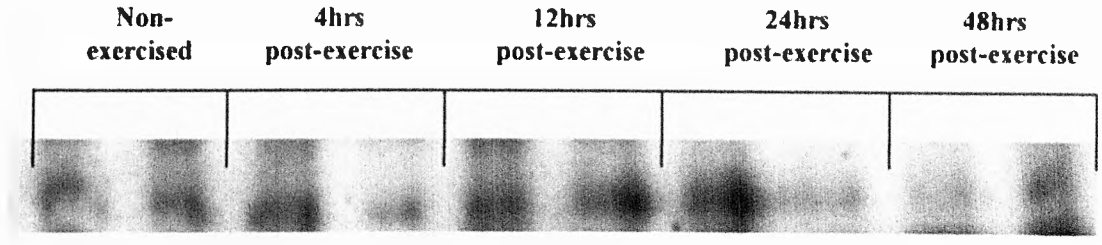


Figure 5.10a. Representative western blot of the HSP70 content of AT muscles from non-exercised young female mice and AT muscles at 4, 12, 24 and 48 hours following a period of isometric contractions.

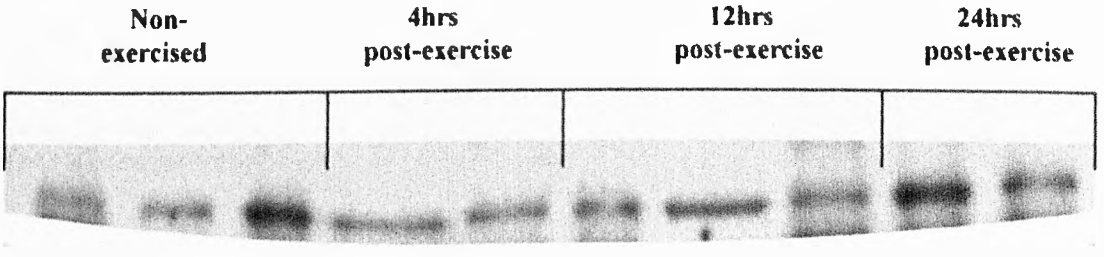


Figure 5.10b. Representative western blot of the HSP70 content of AT muscles from non-exercised adult female mice and AT muscles at 4, 12 and 24 hours following a period of isometric contractions.

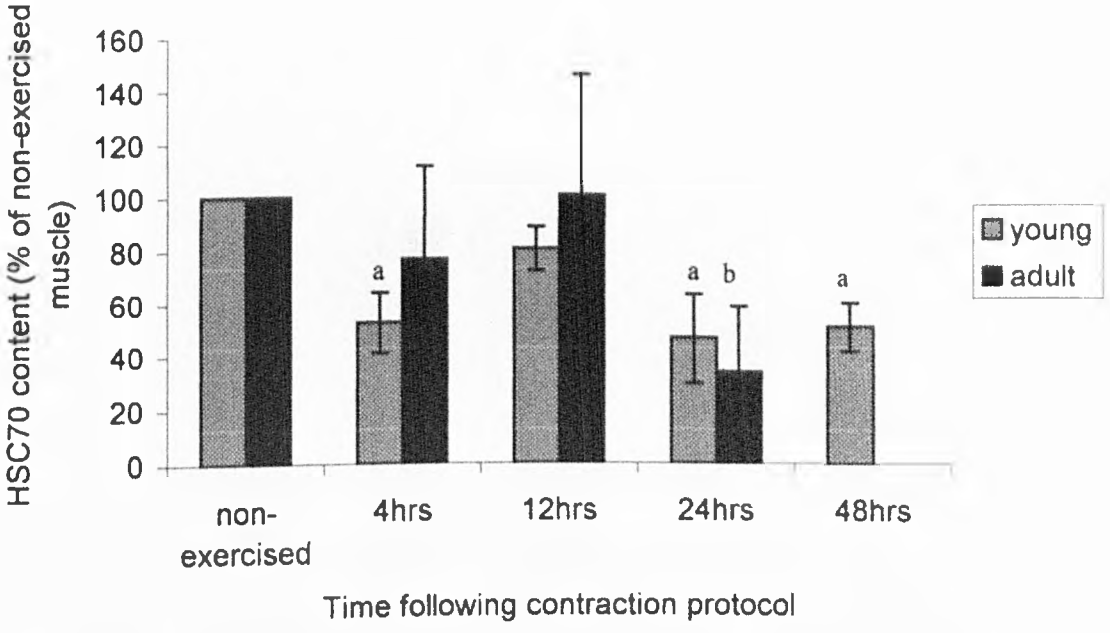


Figure 5.11a. HSC70 content of AT muscle from non-exercised young and adult female mice and AT muscles at 4, 12, 24 and 48 hours following a period of isometric contractions. [a]P<0.05 in comparison with the HSC70 content of muscles from non-exercised young female mice, [b]P<0.05 in comparison with the HSC70 content of muscles from non-exercised adult female mice (n=4-6).

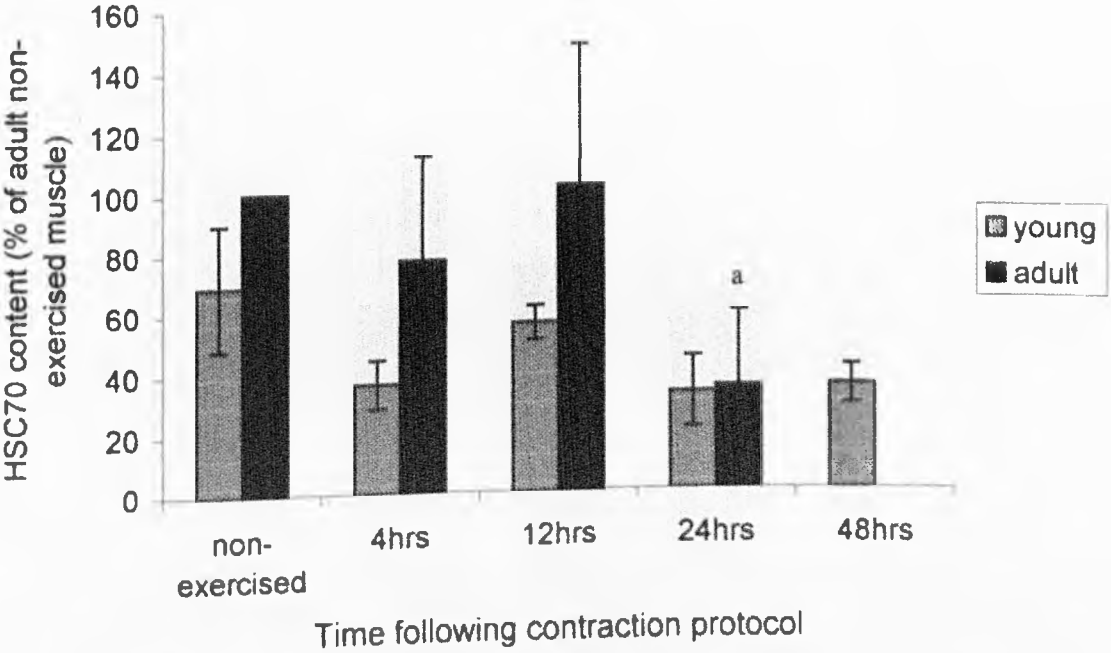


Figure 5.11b. HSC70 content of AT muscle from non-exercised young and adult female mice and AT muscles at 4, 12, 24 and 48 hours following a period of isometric contractions. [a]P<0.05 in comparison with the HSC70 content of muscles from non-exercised adult female mice (n=4-6).

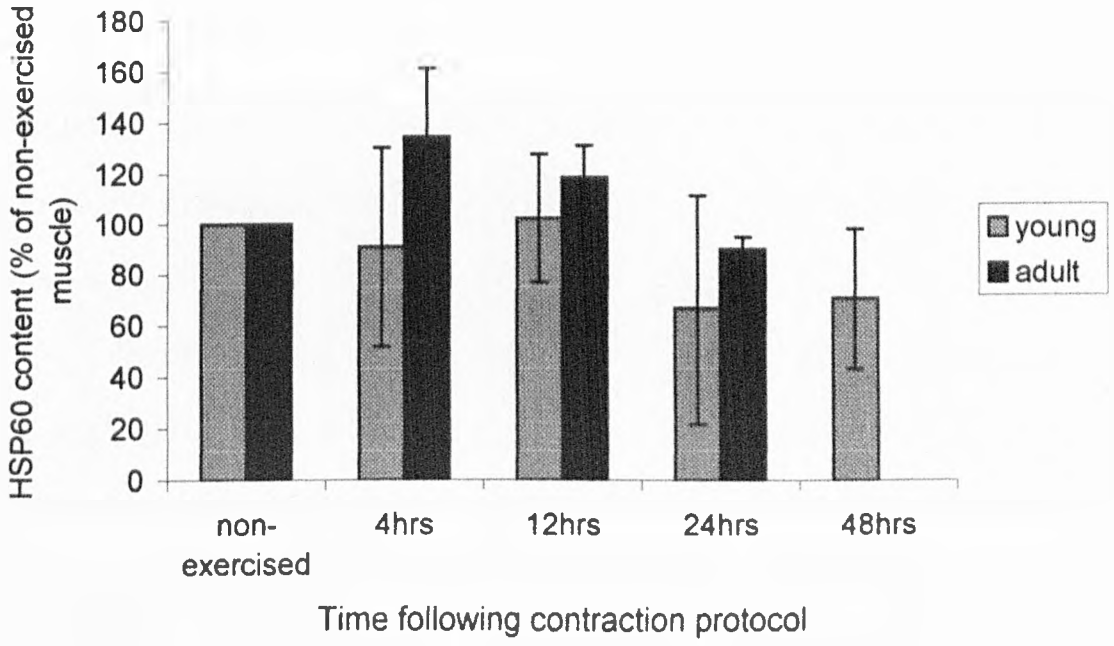


Figure 5.12a. **HSP60 content of AT muscle from non-exercised young and adult female mice and AT muscles at 4, 12, 24 and 48 hours following a period of isometric contractions (n=4-6).**

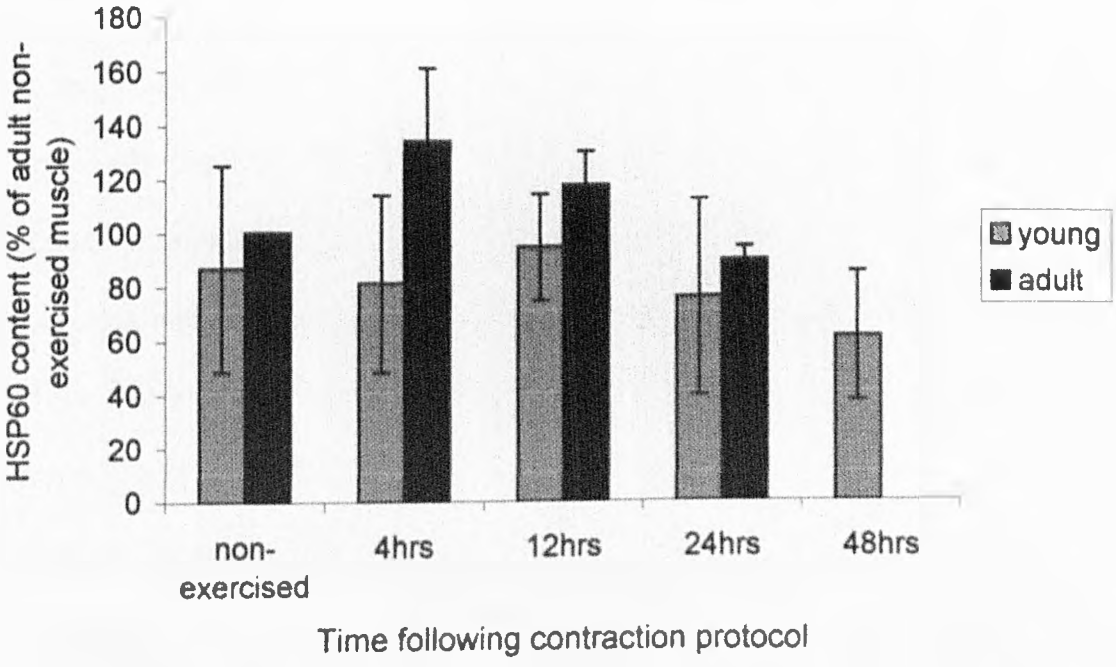


Figure 5.12b. **HSP60 content of AT muscle from non-exercised young and adult female mice and AT muscles at 4, 12, 24 and 48 hours following a period of isometric contractions (n=4-6).**

5.4. DISCUSSION

The aim of the work described in this chapter was to examine the influence of ageing and isometric contractile activity on the HSP content of AT muscles from male and female mice. The differences between the stress response in male and female mice were striking. The response in muscles of male mice will be discussed first.

There was a significant increase in the HSP70 and HSC70 contents of muscles of non-exercised aged male mice compared with adult mice. In contrast, the HSP60 and HSP25 contents from non-exercised aged male mice remained unchanged compared with those from non-exercised adult male mice.

Both HSP70 and HSC70 act as molecular chaperones in non-stressed tissue, facilitating the early steps of the protein maturation, preventing protein aggregation and restoring the function of damaged proteins following stress (Welch, 1992). In the unstressed cell HSP70 is present in low or undetectable quantities and is highly induced during stress. HSC70 is constitutively expressed, however the HSC70 content is also increased following stress. As discussed previously (Section 1.5.1), one of the characteristics of the ageing process is a decline in muscle mass and strength due to a large decrease in the total number of individual muscle fibres within the muscle bulk and an atrophy of the remaining fibres, particularly type II fibres (Lexell *et al*, 1988). The reduction in the proportion of type II fibres results in the muscle moving toward the characteristics of type I muscle fibres. Type I fibres at rest have been shown to contain significantly more HSPs than muscles composed of primarily type II fibres (Locke *et al*, 1991), which may explain why the HSP70 and HSC70 content is

higher in skeletal muscles of non-exercised male aged mice compared with that from skeletal muscles of non-exercised adult male mice.

The increased content of HSP70 and HSC70 in skeletal muscles of non-exercised aged male mice is similar to the up-regulation in catalase and SOD activities demonstrated in the previous chapter. It has been suggested that this up-regulation of the antioxidant defence in tissues is due to the increased production of ROS with ageing. Since ROS can damage a variety of cellular macromolecules, including proteins, increased content of HSPs and antioxidant defence enzymes is a necessary adaptation. Thus, the increased content of HSC70 and HSP70 in skeletal muscles from non-exercised aged mice suggests that the cellular oxidative environment in muscles of aged male mice has been altered and therefore an up-regulation of HSPs is important in order to adapt to these stressful conditions.

The lack of change in HSP25 and HSP60 content with age may reflect the specialised nature of these proteins. HSP25 is generally expressed in low levels in unstressed cells, in which it has been shown to be involved in signal transduction, differentiation, growth, as well as the formation of oligomeric complexes (Locke, 1997). HSP60 is primarily (~95%) located within the mitochondria were it functions with HSP10. Data from HSP60 is difficult to interpret in this situation since changes in absolute numbers of mitochondria per unit muscle will affect the HSP60 content of muscle. Since type I muscle fibres contain relatively more mitochondria, it may be expected that the HSP60 content should also increase with age. However, as discussed previously, mitochondria are a major site of ROS generation (see Section 1.2.1) as well as a primary target of ROS and are thought to be particularly important during aging. It has been shown that several mitochondrial functions decline with age due to oxidative damage to

mitochondrial macromolecules such as mitochondrial DNA, proteins and lipids (for a review see Shigenaga *et al*, 1994). Loss of mitochondrial function can have a major impact on the fidelity of cellular defence mechanisms and repair processes. This may result in an increased mutational load and an increased accumulation of dysfunctional macromolecules. Thus, there are several levels at which mitochondria may have changed during ageing. This makes it difficult to interpret this data and may be the reason for the lack of detection of change in the HSP60 content with age, unlike the other HSPs measured.

Data also demonstrate that skeletal muscles from adult male mice respond to the contraction protocol by a general increase in the HSP content. A transient but significant increase in the HSC70 and HSP60 content of muscle was observed at 4 hours following contraction. In addition, HSP70 content was increased at 12 and 24 hours following the contraction protocol and HSP25 content was also increased at 4, 12 and 24 hours following contractions. Other workers have also shown similar increases in the HSP content following acute periods of exercise. Skidmore *et al* (1995), have reported that moderate intensity exercise significantly increased the concentration of HSP70 in skeletal muscles of male rats. Smolka *et al* (2000) have also demonstrated that a single period of exercise results in an increased HSP70 expression in soleus muscles of male rats.

In contrast, the content of HSPs in skeletal muscle from aged male mice following contractions was generally reduced or remained unchanged. The exception to this was HSP60, which demonstrated a variable but significant increase at 12 hours following the contraction protocol. This was similar to the pattern in muscles of adult male mice following contractions and may reflect the complexity of interpretation of changes in HSP60 in relation to changes in

mitochondrial number and status. Other studies have shown that the ability of some cells to induce HSPs following various stresses is reduced in aged individuals. For example, a reduced accumulation of HSP70 following heat stress has been shown in heart (Kregel *et al*, 1995) and liver (Hall *et al*, 2000) of male rats. In skeletal muscle, we have demonstrated that the production of HSP70 in response to a period of contractile activity was severely blunted at 24 hours following the contraction protocol in comparison with muscles of young rats (Vasilaki *et al*, 2002). This lack of adaptation in the HSP content in tissues of aged animals may play a major role in the more general failure of adaptation to stress.

The HSP content of skeletal muscles from non-exercised aged female mice was variable. In general, the HSP content from non-exercised aged female mice was decreased (HSP60 and HSC70 contents) or remained unchanged (HSP70 content). The exception to this was HSP25, which was increased in comparison with the HSP25 content of skeletal muscles from adult female mice.

The variability observed in the HSP content of non-exercised skeletal muscles from aged female mice may be due to several possibilities. For example, protein synthesis is known to fall in cells with age. Therefore, the need of HSPs such as HSP70 and HSC70 may be reduced in skeletal muscles of female mice. Moreover, as a muscle ages, fibres are lost. Another role of HSPs is as antigen presenting molecules and it has been suggested that HSPs may be involved in this process in skeletal muscle. In addition, the decline in HSP production associated with ageing has been correlated with a reduced activation of the HSF1 transcription factor (Liu *et al*, 1996). This decrease may result in a decrease of HSP content in skeletal muscle of aged female mice. Likewise, the redox state of

cells from aged animals is known to be more oxidative (Ames *et al*, 1993). This change in redox state may result in damage to HSF1 at the gene. There is increasing evidence that damage to DNA is an important factor in the ageing process. Several studies have demonstrated an accumulation of somatic mutations, at various loci, with age (Akiyama *et al*, 1995; Cole *et al*, 1988). This observation has led to the proposal that oxidative damage to DNA may result in mutations in genes important for cellular defence mechanisms such as HSF1. The resultant reduced repair mechanisms could then exacerbate the problem leading to an increased accumulation of mutations in important genes. Such defect in gene encoding HSF1 would lead to the production of defective HSF1 protein. Furthermore, somatic mutations in the DNA binding element (HSE) of the HSP genes or the gene for HSF1 itself may result in a reduced binding affinity of HSF1 to the HSE of the HSPs. However, the current study did not examine this in detail.

In contrast to the data from adult and aged male mice, no significant increases were observed in the HSP content in muscles of either adult or aged female mice following the contraction protocol. There are various possible reasons for this lack of adaptation in muscles of adult female mice. Most likely, it appears that the signal for adaptation is reduced /absent in these mice for multiple reasons. Alternatively, it may be that the muscles from female mice are "aged" more prematurely than those of male mice and the response to stress is already altered. Finally, it may be that the response to stress is delayed and so is not evident at 24 hours following the contraction protocol. To address these latter two possibilities, an extra group of AT muscles from young female B6XSJL mice (4-6 months old) was subjected to the same contraction protocol and analysed for HSP content. In addition, in order to detect whether there is a delay in the stress

response in skeletal muscles of female mice, an extra group of AT muscles from young female mice was studied at 48hrs following the contraction protocol.

Data demonstrated no significant differences in the HSP content between skeletal muscles of non-exercised young and adult female mice. Furthermore, no significant increases in the HSP content of skeletal muscles of young mice were observed at any time point studied. Finally, no significant increase in the HSP content of skeletal muscles of young female mice were shown at 48hrs following the contraction protocol.

It has been proposed that the lack of adaptation in skeletal muscles of female mice compared with skeletal muscles of male mice may be due to sex hormone effects. Recent studies have concentrated on the effects of sex hormones in the regulation of HSP expression with exercise. Paroo *et al* (1999, 2002) have demonstrated that oestrogen, a steroid hormone that is found at much higher concentrations in females than males, attenuates post-exercise HSP70 expression in skeletal muscle. This group have shown that the HSP70 content from skeletal muscles of male rats was greater than that from female rats following an acute period of treadmill running. In addition, oestrogen treatment of male rats resulted in a two- to four fold reduction in post-exercise HSP70 content compared to the HSP70 content of non-treated male rats and that the HSP response from oestrogen-treated male rats was similar to that of females following exercise. This pattern of HSP70 expression was also found in other tissues such as liver, lung and heart, implicating a common mechanism of hormone action. Thus, these findings suggest that the effects exerted by sex hormones extend beyond their role in reproductive function and that sex hormones play a crucial role the gender-specific differences in response to stress.

In summary, the HSP70 and HSC70 content of skeletal muscles from non-exercised aged male mice were significantly higher than those from skeletal muscles of adult male mice. Data from muscles of adult male mice demonstrated a clear increase in the HSP content following non-damaging isometric contractions. This was not evident in muscles of aged male mice or muscles from young, adult or aged female mice. Data suggest that the differences observed in the stress response between male and female mice may be due to the biological differences between the two sexes and that other factors such as sex hormones may play an important role in the increased attenuation in the stress response of skeletal muscles from female mice.

CHAPTER 6

DNA BINDING ACTIVITY OF NF-κB, AP-1 AND HSF1 IN ANTERIOR TIBIALIS MUSCLES OF ADULT AND AGED MALE MICE FOLLOWING THE CONTRACTION PROTOCOL

6.1. INTRODUCTION

Gene expression in eukaryotes is controlled primarily at the level of transcription. Most eukaryotic genes are not expressed unless they are specifically turned on in response to environmental stimuli. The on-and-off switch of gene expression is precisely controlled by a group of proteins known as transcription factors. Transcription factors are regulatory proteins that recognize specific DNA sequences, bind to them and activate transcription by recruiting the correct RNA polymerase to carry out RNA synthesis (Sun and Oberley, 1996; Figure 6.1).

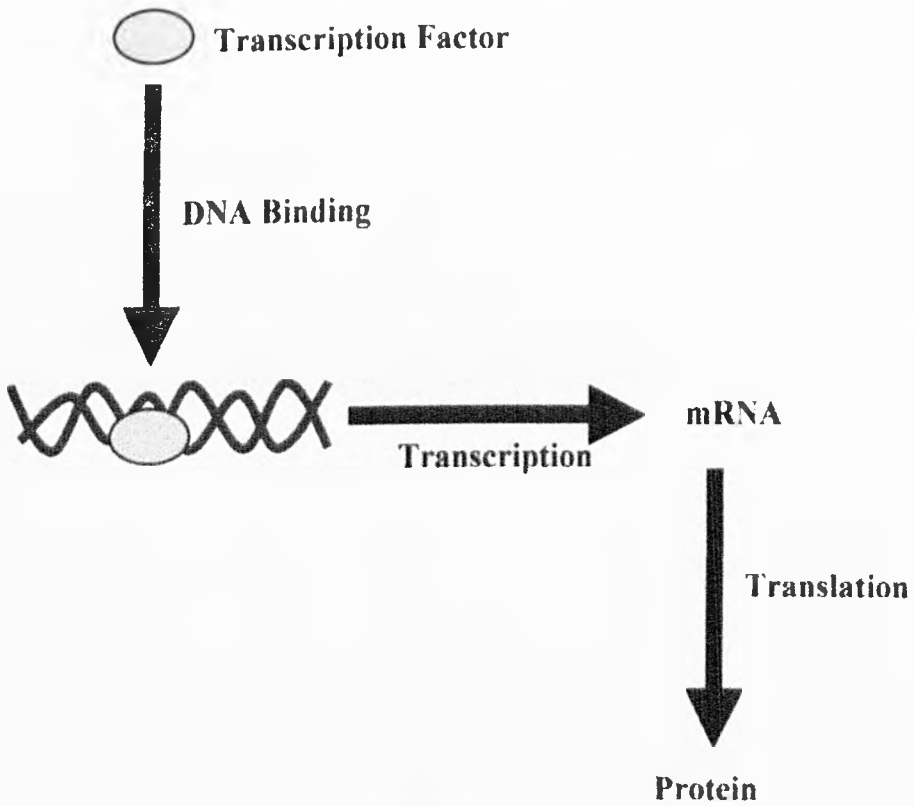


Figure 6.1. Basic model of activation of transcription by transcription factors.

Transcription factors can be generally divided into two categories: the general transcription factors and the sequence specific transcription factors (also known as transcriptional activators). General transcription factors are part of the RNA polymerase transcription machinery and direct the basal transcription through the minimal promoter elements consisting of a TATA box and the initiation region. In contrast, transcriptional activators contain two essential regions: a DNA binding domain that recognises and binds to a particular DNA sequence and a transactivation domain that facilitates the stimulation of transcription in co-operation with the basic transcription machinery (Sun and Oberley, 1996). This part of the thesis will focus on three of the most extensively characterised sequence specific transcription factors, NF-κB, AP-1 and HSF1.

NF-κB is an inducible transcription factor that has been found to be activated in many cell types in response to a broad range of stimuli such as viral and bacterial products, tumour promoters, physical stress such as UV light, chemical agents, as well as oxidants such as H_2O_2 (Sen *et al*, 1996; Flohe *et al*, 1997).

The classical form of NF-κB is a heterodimer containing a 50- and 65-kDa subunits (termed p50 and p65 respectively). Both p50 and p65 contain a highly conserved N-terminal region responsible for DNA binding. The p65 subunit also contains a C-terminal region as the transactivation domain, allowing NF-κB to act as transcriptional activator (Sun and Oberley, 1996). Under basal conditions, the inactive NF-κB is found in the cytosol where is bound to a specific inhibitor, IκB (See section 1.4.1). NF-κB activation is initiated by the phosphorylation of IκB, which acts a trigger for IκB dissociation and subsequent

degradation. Dissociation of IκB allows NF-κB to migrate to the nucleus where it binds to a decameric DNA sequence, 5'-GGGACTTTCC-3' and activates target genes (Sun and Oberley, 1996; Jackson *et al*, 2002).

AP-1 is a transcription factor that regulates the expression of genes associated with growth, neuronal excitation and stress (see Jackson *et al*, 2002 for a review). AP-1 is a complex of oncogene proteins of the Jun and Fos families. The Jun family consists of three proteins (c-Jun, JunB and JunD), whereas the Fos family consists of four proteins (c-Fos, Fra-1, Fra-2 and FosB; Sun and Oberley, 1996). The classical form of AP-1 consists of c-Fos/c-Jun dimers (see Section 1.4.2). Induction of AP-1 by exogenous stimuli relies predominantly on the *de novo* synthesis of its DNA binding subunits, which is regulated by pre-existing transcription factors (Zhou *et al*, 2001). Increased levels of c-Jun and c-Fos proteins result in the formation of AP-1 dimer. Both c-Jun and c-Fos subunits are then phosphorylated and bind to an octameric DNA sequence, 5'-TGAGTCAG-3' in order to activate target genes (Jackson *et al*, 2002; see Section 1.4.2).

Inducible HSP expression in eukaryotes is regulated by the heat shock transcription factors (HSFs). Four different HSFs have been identified: HSF1, HSF2, HSF3, and HSF4 (see Section 1.4.3). HSF1 has been identified as the transcription factor that mediates stress-induced heat shock gene expression in eukaryotes. HSF1 is comprised of a conserved amino terminal localised DNA binding domain, multiple arrays of hydrophobic heptad repeats essential for trimer formation and a carboxyl terminal transactivation domain (Morimoto, 1998). Activation of HSF1 occurs following a variety of stresses such as heat shock, oxidative stress and amino acid analogues (Morimoto *et al*, 1996; Morimoto, 1998). The mechanism by which the stress results in the activation of HSF1 is

relatively well characterised. It is proposed that HSF1 is present in the cytoplasm of the unstressed cell as a monomer, associated with HSP70 or HSP90 (see Section 1.4.4). Upon stress, cellular proteins are destabilised and HSP70 has a greater binding affinity for these destabilised proteins, releasing HSF1 and allowing it to migrate to the nucleus. In the nucleus, HSF1 forms a homotrimer, binds to the Heat Shock Element (HSE) that contains multiple adjacent and inverse iterations of the pentanucleotide motif 5'-nGAAn-3' and becomes inducibly hyperphosphorylated (Morimoto, 1998). This results in increased transcription of the heat shock genes and subsequently, an increase in cellular content of HSPs. As the stress is removed, cellular proteins are refolded. HSP70 is released from these cellular proteins and the cellular content of HSP70 is increased due to increased transcription. HSP70 is then free to re-associate with HSF1 in the nucleus. This negative feedback results in the inactivity of HSF1 (Morimoto, 1998).

The activation of NF-κB, AP-1 and HSF1 in response to oxidative stress has been studied extensively in several tissues and numerous reports indicate that intracellular ROS levels can play a role in the activation mechanisms for these transcription factors. Both NF-κB and AP-1 are activated in response to oxidative stress in a variety of cells (Sun and Oberley, 1996). It is thought that ROS signalling though activation of protein kinases can specifically phosphorylate IκB and due to the lower binding affinity of the phosphorylated IκB it is released from NF-κB, which is then free to translocate to the nucleus and activate transcription, whereas ROS-mediated activation of AP-1 seems to occur through activation of the MAP kinase pathways as well as its subsequent phosphorylation (Roy *et al*, 2002).

In addition, binding sites for both NF-κB and AP-1 have been found in genes encoding a number antioxidant defence enzymes such as glutathione peroxidase, catalase and Mn-SOD (Hollander *et al*, 2000; Zhou *et al*, 2001) suggesting that NF-κB and AP-1 are not only activated following oxidative stress but they may also be involved in the regulation of antioxidant enzyme induction in response to oxidative stress.

HSF1 is also activated in response to oxidative stress (Locke *et al*, 1995; Nishizawa *et al*, 1999). It has been suggested that one pathway through which ROS activate HSF-1 is via oxidation of protein thiols (McDuffee *et al*, 1997; see Section 1.4.5). In skeletal muscle, a study in our laboratory has demonstrated an increase in the HSP content following non-damaging isometric contractions, which is associated with the generation of superoxide anion radicals and a transient, reversible oxidation of protein thiols (McArdle *et al*, 2001). Although the study did not examine the activation of HSF1 following the contraction protocol, the data suggested an involvement of mild oxidative stress in the activation of HSF1, which resulted in an increased content of HSPs.

The aim of this part of the study was to examine the NF-κB, AP-1 and HSF1 binding activity in skeletal muscles of male adult and aged mice prior to and following the contraction protocol.

6.2. EXPERIMENTAL METHODS

6.2.1. Contraction protocol

Skeletal muscles from anaesthetised adult and aged male B6XSJL mice were subjected to a 15-minute period of non-damaging isometric contractions (Section 2.1). Mice were killed immediately following the contraction protocol and anterior tibialis muscles were removed and frozen in liquid nitrogen. Anterior tibialis muscles from non-exercised adult and aged male B6XSJL mice were also obtained and rapidly frozen in liquid nitrogen. Muscles were stored at -70°C until analysis.

6.2.2. Examination of transcription factor DNA binding activity of skeletal muscles by electrophoresis mobility shift assay (EMSA)

Anterior tibialis muscle from non-exercised and exercised adult and aged male mice were ground under liquid nitrogen and the ground powder was homogenised in extraction buffer and centrifuged at 10,000g for 10mins at 4°C (Section 2.5.1). The supernatants was retained and analysed for protein content using the bicinconinic acid protein determination kit (Section 2.3.1; Sigma Immunochemicals, Dorset, UK). Supernatants were used for examination of transcription factor binding activity using gel mobility shift assay (Section 2.5). In addition, competition and supershift experiments were performed (Section 2.5.4).

6.2.3. Analysis of transcription factor content of skeletal muscles by SDS-PAGE and western blotting

Anterior tibialis from non-exercised and exercised adult and aged mice were ground under liquid nitrogen, and portions of ground powder were

homogenised in 100µl of 1% SDS and protease inhibitors and centrifuged at 10,000g for 10mins at 4°C (Section 2.2). The supernatants was retained and analysed for protein content using the bicinconinic acid protein determination kit (Section 2.3.1; Sigma Immunochemicals, Dorset, UK). Supernatants were used for determination of HSF1, IκBα, p50, p65, c-Fos and c-Jun protein levels by SDS-PAGE and western blotting using various antibodies (Section 2.4).

6.3. RESULTS

6.3.1. NF-κB binding activity in AT muscles of adult and aged male mice following the contraction protocol

The NF-κB binding activity of AT muscles of non-exercised, adult and aged male mice is shown in Figure 6.2. The NF-κB binding activity of muscles of non-exercised aged male mice was significantly increased compared with the NF-κB binding activity of muscles of non-exercised adult male mice (adult male: 694.25 ± 34.57 arbitrary units; aged male: 1348.24 ± 53.07 arbitrary units; $P<0.05$).

The NF-κB binding activity of AT muscles from adult and aged male mice immediately following a period of isometric contractions is shown in Figure 6.2. A significant rise in the NF-κB binding activity was seen in muscles of adult male mice following the contraction protocol, whereas a decrease in the NF-κB binding activity was observed in muscles of aged male mice at this time point (adult male: 2404.825 ± 202.19 arbitrary units; aged male: 952.25 ± 98.68 arbitrary units; $P<0.05$).

In order to determine the specificity of the binding protein, competition and supershift experiments were also performed (See Section 2.5.4). Addition of the unlabelled specific competitor resulted in a decrease in the intensity of the NF-κB band, demonstrating the specificity of the binding protein (Figure 6.3a). Addition of p50 and p65 antibodies (Figure 6.3b) resulted in the production of supershift bands for both the antibodies.

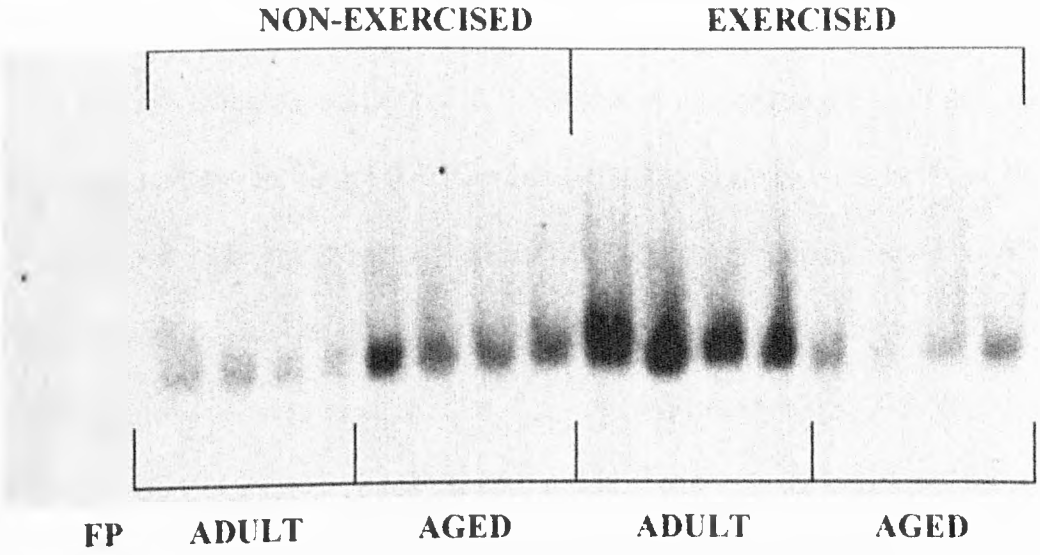


Figure 6.2. NF-kB binding activity of non-exercised AT muscle from adult and aged male mice and AT muscles immediately following a period of isometric contractions (n=4); (FP) free probe.

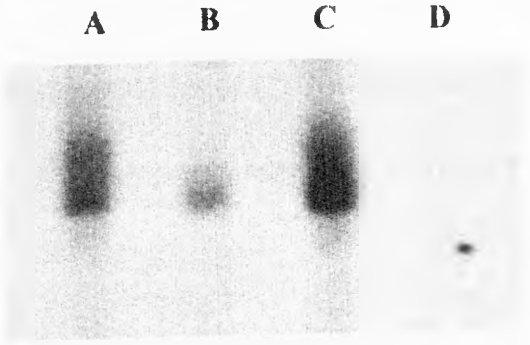


Figure 6.3a. Competition experiment of NF-κB binding activity. (A) control (B) specific competitor (C) non-specific competitor (D) free probe.

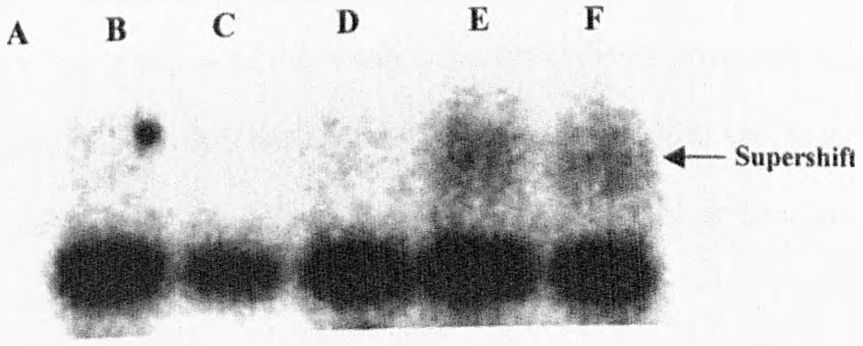


Figure 6.3b. Supershift experiment of NF-κB binding activity. (A) free probe (B) control (C) specific competitor (D) non-specific competitor (E) control + p50 antibody (F) control + p65 antibody.

6.3.2. AP-1 binding activity in AT muscles of adult and aged male mice following the contraction protocol

The AP-1 binding activity of AT muscles of non-exercised adult and aged male mice is shown in Figure 6.4. The AP-1 binding activity of muscles of non-exercised aged male mice was significantly increased compared with the AP-1 binding activity of muscles of non-exercised adult male mice (adult male: 1027.08 ± 72.92 arbitrary units; aged male: 2146.2 ± 210.39 arbitrary units; $P<0.05$).

The AP-1 binding activity of AT muscles from adult and aged male mice immediately following a period of isometric contractions is shown in Figure 6.4. A significant rise in the AP-1 binding activity was seen in muscles of adult male mice following the contraction protocol, whereas a decrease in the AP-1 binding activity was observed in muscles of aged male mice at this time point (adult male: 4043.95 ± 302.39 arbitrary units; aged male: 1252.19 ± 213.4 arbitrary units; $P<0.05$).

In order to determine the specificity of the binding protein, competition and supershift experiments were also performed. As with NF-κB, addition of the unlabeled specific competitor resulted in a decrease in the intensity of the AP-1 band (Figure 6.5a). Addition of c-Fos and c-Jun antibodies (Figure 6.5b) resulted in the production of supershift bands for both the antibodies, however the intensity of the supershift band produced by the c-Jun antibody was greater than that produced by the c-Fos antibody.

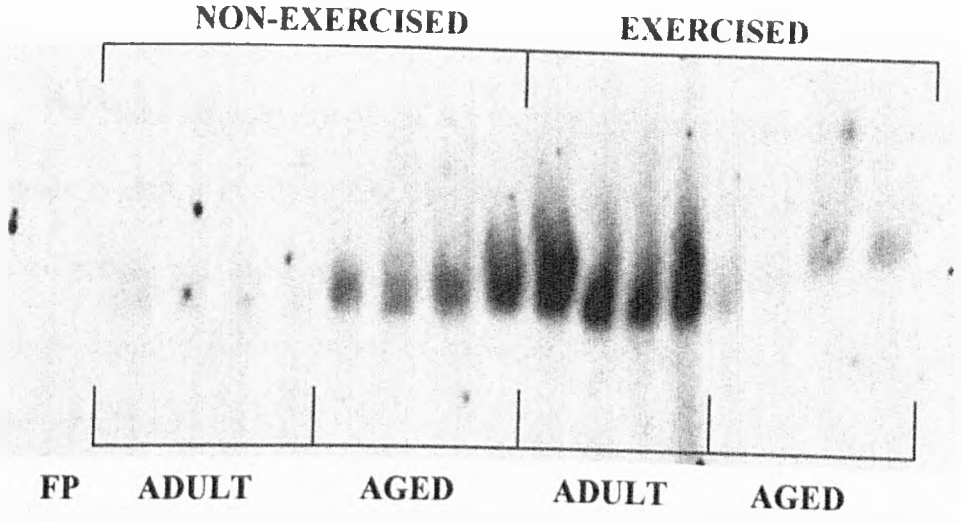


Figure 6.4. AP-1 binding activity of non-exercised AT muscle from adult and aged male mice and AT muscles immediately following a period of isometric contractions (n=4); (FP) free probe.

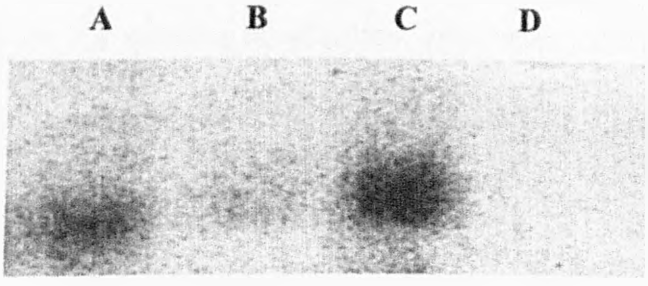


Figure 6.5a. Competition experiment of AP-1 binding activity. (A) control (B) specific competitor (C) non-specific competitor (D) free probe.

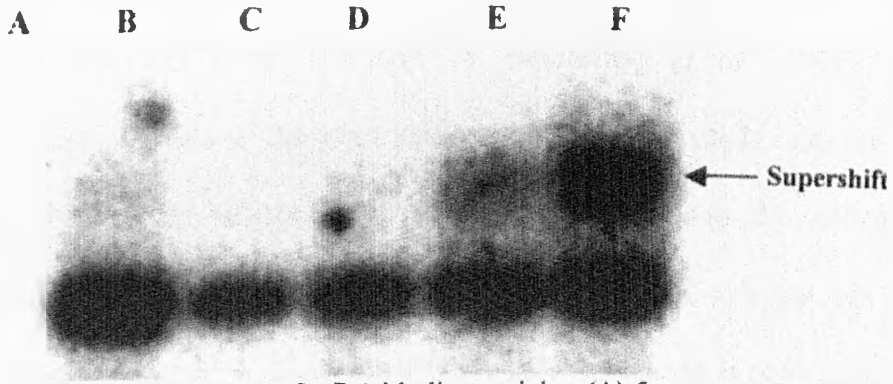


Figure 6.5b. Supershift experiment of AP-1 binding activity. (A) free probe (B) control (C) specific competitor (D) non-specific competitor (E) control + c-Fos antibody (F) control + c-Jun antibody.

6.3.3. HSF-1 binding activity in AT muscles of adult and aged male mice following the contraction protocol

The HSF1 binding activity of AT muscles of non-exercised adult and aged male mice is shown in Figure 6.6. The HSF1 binding activity of muscles of non-exercised adult and aged male mice was generally not detectable, although occasional samples from muscles of either adult or aged mice gave a faint but positive signal (e.g. lane 1).

The HSF1 binding activity of AT muscles from adult and aged male mice immediately following a period of isometric contractions is shown in Figure 6.6. The HSF1 binding activity was increased in both adult and aged mice following contractions. No significant differences in the HSF1 binding activity were observed between AT muscles of adult and aged mice at this time point (adult male: 695.48 ± 18.5 arbitrary units; aged male: 602.6 ± 56.7 arbitrary units).

In order to determine the specificity of the binding protein, competition and supershift experiments were also performed (Figure 6.7). The competition experiment demonstrated the specificity of the HSF1 band and addition of the HSF1 antibody (kindly provided by R. Morimoto, North western University, Evanston, USA) resulted in a decrease in the density of the HSF1 band (Figure 6.7). Similar data has been produced by Bharadwaj *et al* (1999), who demonstrated that addition of the same antibody used in this study resulted in a decrease in the density of the HSF1 band. This is unlike the additional supershifted bands produced by addition of antibodies to the NF-κB and AP-1 gel shift experiments (Figure 6.3b, Figure 6.5b), but in this instance it is thought that addition of an antibody may prevent protein-DNA interactions by blocking regions of the protein that bind DNA and, as a result, it decreases the ability of the

transcription factor to induce a mobility shift (Mueller and Pahl, 2000; Promega Corporation technical bulletin, Madison, USA).

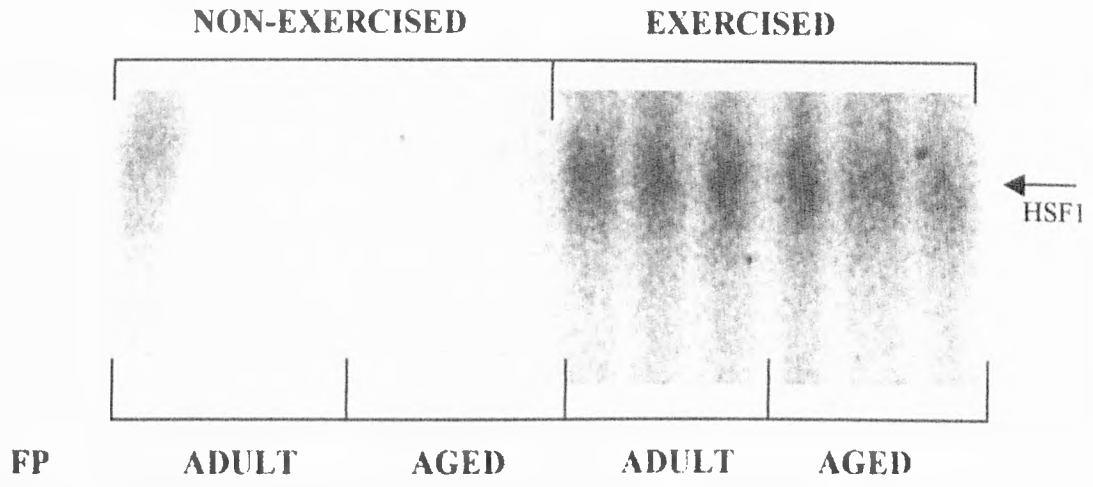


Figure 6.6. HSF1 binding activity of non-exercised AT muscle from adult and aged male mice and AT muscles immediately following a period of isometric contractions (n=3); (NS) non-specific binding, (FP) free probe.

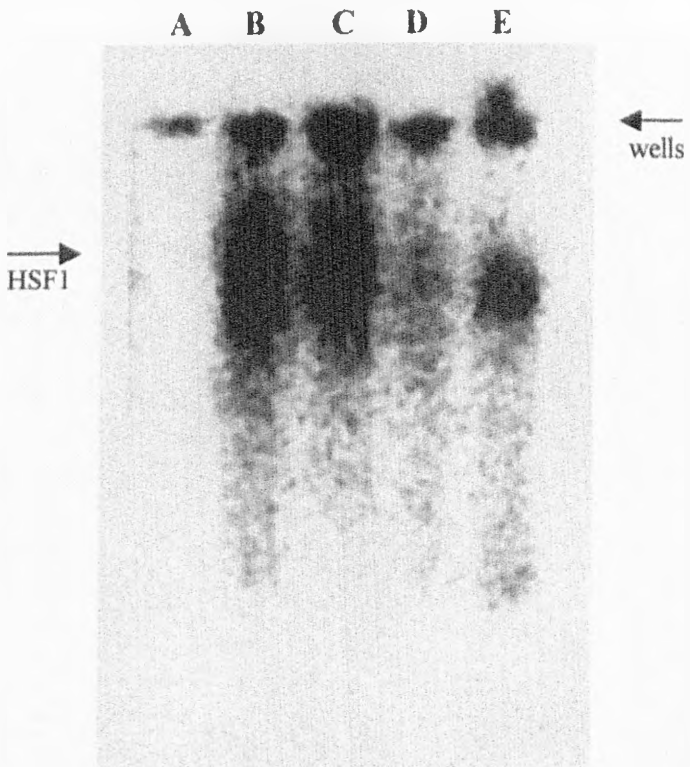


Figure 6.7. Competition and supershift experiment of HSF1 binding activity. (A) free probe (B) control (C) non-specific competitor (D) specific competitor (E) control + HSF1 antibody.

6.3.4. Protein levels of I-κBα and NF-κB components in AT muscles of non-exercised adult and aged male mice

A representative western blot of the protein levels of I-κBα and NF-κB components (p50 and p65) of AT muscles of non-exercised adult and aged male mice is shown in Figure 6.8. No obvious differences in the I-κBα content of muscles from non-exercised adult and aged male mice were observed. In addition, no age-related changes in the p50 and p65 protein levels were observed in skeletal muscles of non-exercised mice.

6.3.5. Protein levels of AP-1 components in AT muscles of non-exercised adult and aged male mice

The protein levels of AP-1 components (c-Fos and c-Jun) of AT muscles of non-exercised adult and aged male mice were undetectable.

6.3.6. Protein levels of HSF1 in AT muscles of non-exercised adult and aged male mice

A representative western blot of the protein levels of HSF1 in AT muscles of non-exercised adult and aged male mice are shown in Figure 6.9. No age-related changes in the HSF1 protein levels were observed in skeletal muscles of non-exercised mice.

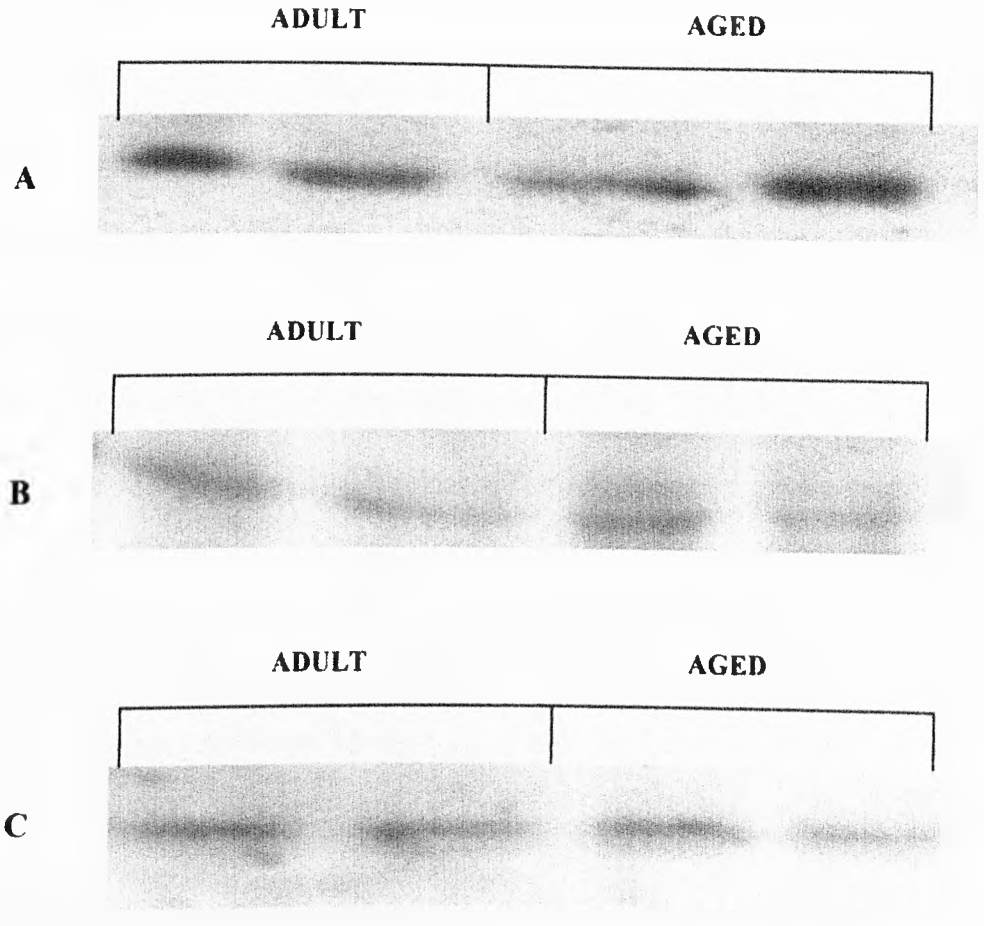


Figure 6.8. Representative western blots of (A) IκBα (B) p50 and (C) p65 protein levels in skeletal muscles of non-exercised male adult and aged mice.

Figure 6.9. Representative western blot of HSF1 protein levels in skeletal muscles of non-exercised male adult and aged mice.

6.4. DISCUSSION

Data presented in Chapters 4 and 5 demonstrated a reduced production of antioxidant defence enzymes and HSPs in muscles of aged mice following the contraction protocol. The aim of this section of the study was to examine the influence of ageing and isometric contractile activity on the DNA binding activity of the three primary transcription factors, NF-κB, AP-1 and HSF1, responsible for stress-induced production of these proteins in AT muscles from male mice.

Data demonstrated a significant increase in both NF-κB and AP-1 binding activities in muscles of non-exercised aged male mice compared with the NF-κB and AP-1 binding activities in muscles of non-exercised adult male mice. Other workers have previously reported an increase in the binding activity of NF-κB with ageing in other tissues. For example, Helenius *et al* (1996a) reported an up-regulation of NF-κB binding activity during ageing in the mouse heart. In a different study, Helenius *et al* (1996b) have also demonstrated an age-associated induction of NF-κB in the liver, heart, kidney and brain from aged rats and mice.

In order to assess whether the increase in the NF-κB and AP-1 binding activities were due to changes in the protein levels of NF-κB and AP-1 components, the protein levels of p50, p65, c-Fos and c-Jun, as well as the IκBα levels were also analysed by western blotting. Data demonstrated that there are no differences in the p50, p65 or IκBα levels between skeletal muscles of adult and aged mice. Similar data have been produced by Helenius *et al* (1996b) who demonstrated an increase in the NF-κB binding activity with ageing in different tissues (e.g. liver), even though the p50, p65 and IκBα levels remained unaffected by ageing. Data also demonstrated that, although an increase in the AP-1 binding

activity was observed during ageing (Figure 6.4), the protein levels of AP-1 components (c-Fos and c-Jun) remained undetectable in muscles of aged mice.

The increased DNA binding activity of NF-κB and AP-1 in non-exercised skeletal muscles from aged male mice may be due to several possible reasons. Several studies have provided evidence of an age-related increase in the production of ROS (see Section 1.5.5). Both NF-κB and AP-1 are redox-sensitive transcription factors and therefore, the redox status of the cells, and especially a pathological increase in the production of ROS with ageing, can interfere with the activation of the NF-κB and AP-1 signalling pathways (Roy *et al*, 2002).

In addition, binding sites for both NF-κB and AP-1 have been found in genes encoding a number of antioxidant defence enzymes such as glutathione peroxidase, catalase, Cu/Zn-SOD and Mn-SOD (Hollander *et al*, 2000; Zhou *et al*, 2001). Thus, the increased DNA binding activity of NF-κB and AP-1 in non-exercised skeletal muscles of aged male mice may be related to the up-regulation of the activities of the antioxidant defence enzymes in skeletal muscles of non-exercised aged male mice observed in Chapter 4 (Figures 4.2 and 4.4).

In contrast to the NF-κB and AP-1 data, the HSF1 binding activity in both non-exercised adult and aged mice was generally undetectable and was not affected by ageing. Similar data has been produced by Locke (2000), who also could not detect HSF1 binding activity in extracts from soleus and white gastrocnemius muscles of adult and aged rats.

There were no differences in HSF1 protein levels between skeletal muscles of adult and aged mice (Figure 6.9). This is in agreement with a study by Heydari *et al* (2000), who demonstrated that the cellular level of the HSF1 protein

remains unaffected by ageing in rat hepatocytes, as well as with a study by Locke and Tanguay (1996a) which demonstrated that myocardial HSF1 protein content was similar between hearts from young and old rats.

Skeletal muscles from adult male mice responded to the contraction protocol by a general increase in the NF-κB and AP-1 binding activities. Both NF-κB and AP-1 are redox-sensitive transcription factors and studies have shown that an increase in the intracellular ROS levels can play an important role in their activation mechanisms (For reviews see Sun and Oberley, 1996; Jackson *et al*, 2002a). In skeletal muscle, Zhou *et al*, (2001) demonstrated that chemically induced oxidative stress results in an increase in the DNA binding activity of both NF-κB and AP-1 in differentiated cells and that this increase results in an up-regulation of antioxidant enzymes, such as catalase and glutathione peroxidase, in response to oxidative stress. Thus, since NF-κB and AP-1 are actively involved in the induction of antioxidant gene expression, the increased NF-κB and AP-1 binding activities observed following the contraction protocol may be responsible for the up-regulation of the catalase and SOD activities in skeletal muscles of adult male mice observed in Chapter 4.

In contrast, NF-κB and AP-1 binding activities in skeletal muscle from aged mice following the contraction protocol were generally reduced or remained unchanged. Decrease in the NF-κB binding activity has also been reported by Pahlavani and Harris (1998) who demonstrated that ROS generated by xanthine-xanthine oxidase treatment resulted in a 48% decrease in the DNA binding activity of NF-κB in T cells from aged rats. The present data suggest that the lack of increased production of antioxidant defence enzymes in muscles of aged mice

following the contraction protocol observed in Chapter 4 may be, at least in part, due to inability of NF-κB and AP-1 to bind to DNA.

In contrast, the binding activity of HSF1 was increased in skeletal muscles of both adult and aged mice following the contraction protocol. Other workers have previously demonstrated an increase in the HSF1 binding activity in tissues from young/adult animals following oxidative stress. For example, Locke *et al* (1995) have reported that a single bout of treadmill running is a sufficient stimulus to activate HSF1 in rat heart. Furthermore, Nishizawa *et al* (1999) demonstrated a significant increase in the HSF1 DNA binding activity with H_2O_2 treatment or xanthine/xanthine oxidase perfusion in isolated hearts of rats. Therefore, the present data suggest that the contraction protocol used results in an increase in the HSF1 DNA binding activity in skeletal muscles of adult male mice and that this increase may be responsible for the increased production of HSPs in skeletal muscles of adult mice demonstrated in Chapter 5.

Several studies have demonstrated an age-dependent decline in the DNA binding activity of HSF1. For example, Liu *et al* (1996) demonstrated a decrease in the HSF1 DNA binding activity in aged human diploid lung fibroblasts following heat shock. Locke and Tanguay (1996a) have also demonstrated a significant reduction in the HSF1 binding activity following heat shock in hearts from aged rats compared with hearts from young rats. In addition, a study by Heydari *et al* (2000) have demonstrated an age-related decrease in the DNA binding activity of HSF1 from rat hepatocytes following heat shock. However, this was not apparent in this study. Skeletal muscles of aged male mice respond to the contraction protocol by increasing the HSF1 binding activity. A study by Locke (2000) also showed an increase in the HSF1 binding activity in skeletal

muscles of aged rats following heat shock. However, the effect of exercise and ageing on the HSF1 activation in skeletal muscle has not been previously examined.

Although the current work has shown an increase in the binding activity of HSF1 from skeletal muscles of aged male mice following the contraction protocol in vitro, the content of HSPs in muscles of aged male mice following contractions was generally reduced or remained unchanged (see Chapter 5).

There are several possible reasons for the lack of adaptation in muscles of aged male mice:

a) Although HSF1 from aged mice is capable of binding to the idealised DNA sequence used in EMSA experiments *in vitro*, this may not be the case *in vivo*. Any deletions or mutations in the Heat Shock Element (HSE) of the HSP genes will result in a decrease in the HSF1 DNA binding activity as well as in a decrease in transcriptional activation and synthesis of HSPs.

b) HSF1 phosphorylation plays an important role in transcriptional activation and in mammalian cells, HSF1 is phosphorylated both constitutively and inducibly at serine residues (Cotto and Morimoto, 1999). However, inducible HSF1 phosphorylation (also known as hyperphosphorylation) has been found to be very important for transcriptional activation. It has been demonstrated that in human cells, treatment with anti-inflammatory drugs induces HSF1 binding activity to levels comparable with those observed during heat shock, however the drug-induced form of HSF1 is transcriptionally inert (Jurivich *et al*, 1992). Comparison of the heat shock and drug induced forms of HSF1 revealed that the drug induced HSF1 is

constitutively but not inducibly phosphorylated, whereas heat shock induced HSF1 is both constitutively and inducibly phosphorylated and that the transcriptionally inert drug induced HSF1 can be converted to the transcriptionally active state by subsequent exposure to heat shock (Cotto *et al*, 1996). Results from these studies suggest that inducible phosphorylation correlates with transcriptional activation and that acquisition of DNA binding activity by HSF1 is necessary but insufficient for transcriptional activation which may be the case in skeletal muscles of aged mice.

c) Finally, the attenuated adaptive response in skeletal muscles of aged mice following the contraction protocol may be due to alterations during gene transcription or translation. Both gene transcription and translation are complex and extremely specific procedures and thus changes in these two cellular mechanisms may result in a decreased production of HSPs.

In summary, there is an age-related increase in the NF-κB and AP-1 binding activities in skeletal muscles of non-exercised mice, whereas the HSF1 binding activity was not affected by ageing. Following the contraction protocol, NF-κB and AP-1 binding activities were increased only in skeletal muscles of adult mice, whereas the HSF1 binding activity was increased in both adult and aged mice. Data from HSF1, NF-κB and AP-1 binding activities following the contraction protocol suggest that the inability of muscles of aged mice to produce HSPs following contractile activity may not be due to an altered binding of HSF1 to the binding domain but due to incomplete HSF1 phosphorylation or alterations during gene transcription or during translation. In contrast, the inability of skeletal muscles from aged mice to increase the antioxidant defence enzyme activity

following the contraction protocol appears be due to the limited ability of NF-κB and AP-1 to bind to DNA in order to activate antioxidant defence enzyme gene transcription.

The aim of the work in the next section of this thesis was to examine the mRNA level of HSP25, one of the HSPs that was most affected by age, and to put the antioxidant defence enzyme and HSP data in context with a more global assessment of activation of genes in muscles of adult and aged mice.

CHAPTER 7

mRNA EXPRESSION IN SKELETAL MUSCLES OF ADULT AND AGED MALE MICE FOLLOWING THE CONTRACTION PROTOCOL

7.1. INTRODUCTION

The current study has demonstrated that skeletal muscles of aged male mice fail to increase their antioxidant defence enzyme activities (see Chapter 4) and HSP content (see Chapter 5) following contractile activity. Examination of NF-κB and AP-1 transcription factor binding activities has demonstrated that the inability of skeletal muscles from aged mice to increase their antioxidant defence enzyme activity following the contraction protocol appears, at least in part, be due to the limited ability of NF-κB and AP-1 from muscles of aged mice to bind to DNA (see Chapter 6). In contrast, there was no gross difference in the ability of HSF1 to bind to HSE to produce HSPs in muscles of adult or aged mice since HSF1 binding was increased in muscles of both adult and aged mice following the contraction protocol. Thus, the attenuated HSP response in skeletal muscles of aged mice following the contraction protocol may be due to other reasons, including alterations during gene transcription.

Gene expression is a combination of many procedures including transcription, transport of mRNA and translation. There is now significant evidence that alterations during gene expression occur during differentiation, development as well as during ageing. Age-related alterations in gene expression are usually reflected at the level of transcription (e.g. transcription factors), as well as both mRNA and protein levels (Brewer, 2002).

The use of DNA microarray analysis has provided a revolutionary tool for evaluation of age-related changes in gene expression because they allow the simultaneous detection and analysis of multiple genes. DNA microarrays can be broadly divided into two classes, cDNA based arrays and oligonucleotide arrays (Weindruch *et al*, 2002).

A cDNA microarray includes hundreds of cDNAs spotted on positively charged nylon membrane. The membrane can be used to quantify RNA abundance following hybridisation with tissue-specific cDNA pools obtained through reverse transcription. The hybridization pattern can then be analysed and quantified by phosphoimaging. The relative expression levels of a given cDNA from two different RNA sources can be assessed by comparing the signal obtained from one RNA source to that obtained from another source.

Oligonucleotide arrays contain thousands of gene-specific oligonucleotides synthesised *in situ* (Weindruch *et al*, 2002).

DNA microarrays have been successfully used in a range of ageing-related studies, including examination of ageing of dwarf mouse liver (Dozmorov *et al*, 2001); the molecular events associated with brain ageing and its retardation by calorie restriction in male mice (Prolla, 2002) and age-related changes in cardiac muscle of mice (Weindruch *et al*, 2002). In addition, DNA microarrays have been used for the examination of stress-related gene expression following cadmium exposure (an environmental toxic heavy metal) in liver tissue of mice (Liu *et al*, 2002).

In skeletal muscle, DNA microarrays have been used for the examination of the effect of caloric restriction during ageing in mouse gastrocnemius muscles (Lee *et al*, 1999); the influences of ageing and caloric restriction on the transcriptional profile of vastus lateralis muscles from rhesus monkeys (Kayo *et al*, 2001); the differential gene expression in red soleus and white quadriceps muscles (Campbell *et al*, 2001); differential gene expression in rat soleus muscles during work overload-induced hypertrophy (Carson *et al*, 2002) and the effect of

high-fat diet and antioxidant supplementation on rat skeletal muscle (Sreekumar *et al*, 2002).

The aim of this part of the study was to examine the mRNA levels of HSP25, the increased production of which was most affected by age, using northern blotting. This was then put into context with changes in the overall activation of stress-related genes in muscles of aged male mice following contractile activity compared with muscles of adult male mice using Atlas cDNA mouse stress arrays.

7.2. EXPERIMENTAL METHODS

7.2.1. Contraction protocol

Skeletal muscles of adult and aged male and female B6XSJL mice were subjected to the 15-minute period of non-damaging isometric contractions *in vivo* as described previously (Section 2.1). Mice were killed immediately, 4 hours and 12 hours following the contraction protocol and gastrocnemius muscles were removed and frozen in liquid nitrogen. Gastrocnemius muscles from non-exercised adult and aged male B6XSJL mice were also removed and frozen in liquid nitrogen. Muscles were stored at -70°C until analysis.

7.2.2. Analysis of RNA from gastrocnemius muscles by northern hybridisation

The RNA isolation from mouse gastrocnemius muscles was performed as described in Section 2.6.1 using TRI REAGENT™ (~50mg of ground muscle in 0.5ml of TRI REAGENT™; Sigma Immunochemicals, Dorset, UK). Following isolation, the RNA content of the samples was measured using the RiboGreen RNA quantitation assay kit (Section 2.6.3; Molecular probes, Leiden, The Netherlands).

The quality of the total RNA from gastrocnemius muscles was examined by electrophoresis of 1μg of RNA on a denaturing formaldehyde/ agarose/ ethidium bromide gel (Section 2.6.4). Preliminary studies demonstrated that the total RNA extracted from individual muscles was insufficient to produce a signal. Thus, equal amounts of RNA from each sample were therefore pooled and 10μg of total RNA was loaded onto a 1% agarose gel (see Section 2.7.2). Following

electrophoresis, the RNA was transferred to a nylon membrane (Section 2.7.3). The nylon membrane was then hybridized overnight with continuous agitation using a [^{32}P]-labeled oligonucleotide for HSP25 (Section 2.7.4). The membrane was exposed to a phosphor screen (Amersham International, UK) for 24 hours and the phosphor screen was scanned using a Biorad Personal Molecular Imager FX (Biorad, Hercules, USA) and analysed using Quantity one Software (Biorad, Hercules, USA).

7.2.3. Analysis of mRNA from gastrocnemius muscles using cDNA mouse stress arrays

The analysis of mRNA from gastrocnemius muscles of adult and aged male mice prior to and following the contraction protocol was performed by using Atlas Mouse Stress arrays (Cat #7749-1; Clontech, Palo Alto, USA) as described in Section 2.6.

The RNA isolation from mouse gastrocnemius muscles was performed as described in Section 2.6.1 using TRI REAGENT (~100mg of ground muscle in 1ml of TRI REAGENT; Sigma Immunochemicals, Dorset, UK), the RNA content of the samples was measured using the RiboGreen RNA quantitation assay kit (Section 2.6.3; Molecular probes, Leiden, The Netherlands) and the samples were then pooled. The quality of the total RNA was examined by electrophoresis of 1-2μg of RNA on a denaturing formaldehyde/agarose/ethidium bromide gel. RNA probes were synthesized as described in Section 2.6.5.

7.3. RESULTS

7.3.1. HSP25 mRNA levels in gastrocnemius muscles of adult and aged male mice prior to and following non-damaging isometric contractions

Prior to northern blotting, the quality of the total RNA from samples from gastrocnemius muscles of adult and aged male mice was examined by electrophoresis of 1μg of RNA on a denaturing formaldehyde/ agarose/ ethidium bromide gel (Figure 7.1).

Figure 7.2A and Figure 7.2B are northern blots of the HSP25 and 18S mRNA levels respectively of gastrocnemius muscles from non-exercised adult and aged male mice and gastrocnemius muscles immediately following the contraction protocol. The densities of the individual bands are shown in Table 7.1.

No significant differences in the HSP25 mRNA levels were observed between gastrocnemius muscles of non-exercised adult and aged mice (non-exercised aged /non-exercised adult density ratio following standardisation to 18S: 0.997).

The contraction protocol resulted in an increase in the HSP25 mRNA levels in gastrocnemius muscles of adult mice compared with the HSP25 mRNA levels in gastrocnemius muscles of non-exercised adult mice (exercised adult /non-exercised adult density ratio following standardisation to 18S: 1.21).

In contrast, the HSP25 mRNA levels in gastrocnemius muscles of aged mice demonstrated no evidence of an increase immediately following the contraction protocol compared with the HSP25 mRNA levels in gastrocnemius muscles of non-exercised aged mice (exercised aged /non-exercised aged density ratio following standardisation with 18S: 0.903).

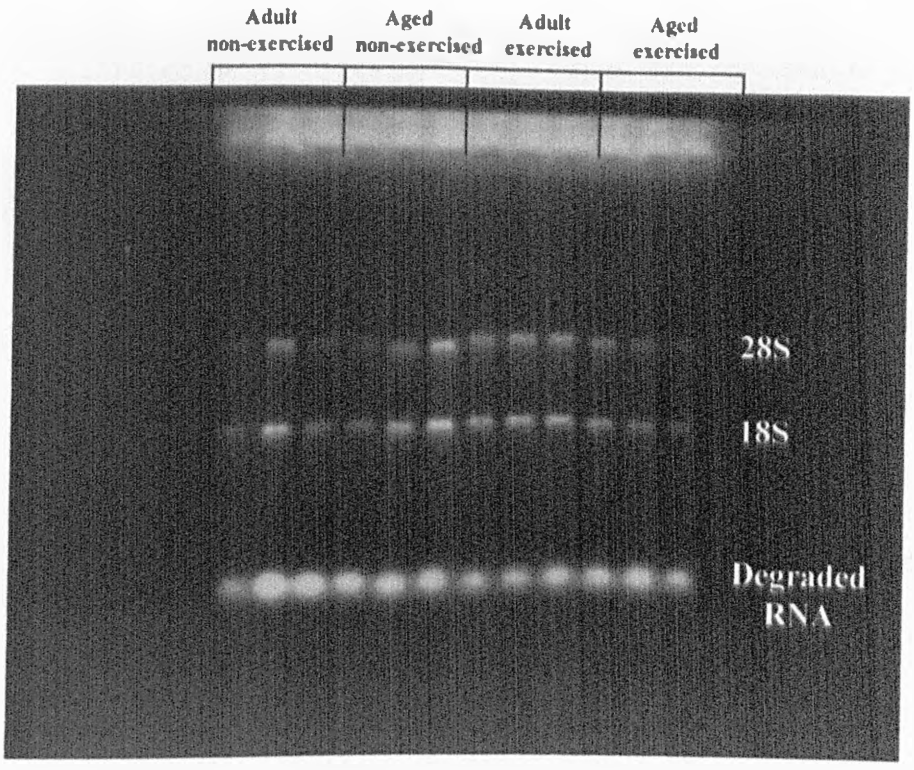


Figure 7.1. Formaldehyde/ agarose/ ethidium bromide gel of the total RNA of samples from gastrocnemius muscles from non-exercised adult and aged male mice and muscles from adult and aged male mice immediately following the contraction protocol.

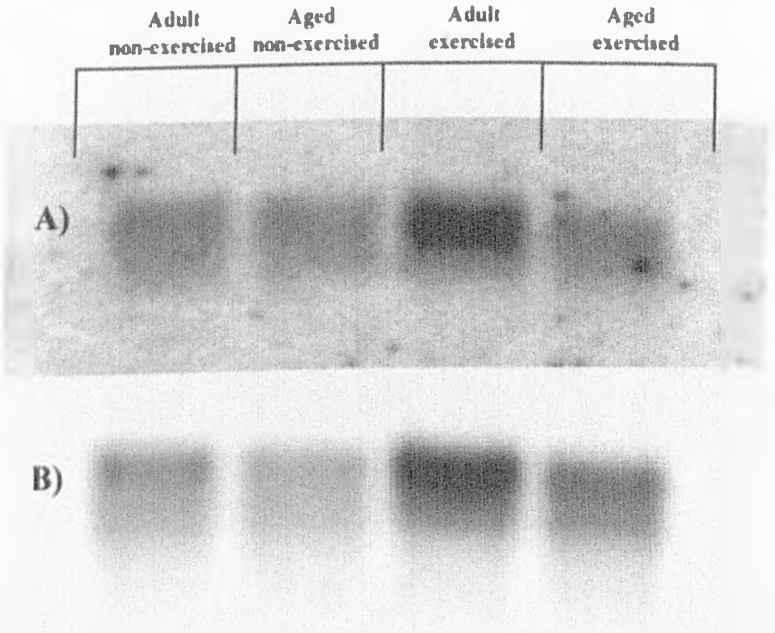


Figure 7.2. Northern blots showing A) the HSP25 mRNA levels and B) the 18S mRNA levels in gastrocnemius muscles from non-exercised adult and aged male mice and muscles immediately following the contraction protocol.

Table 7.1. **18S and HSP25 RNA levels of gastrocnemius muscles from non-exercised adult and aged male mice and gastrocnemius muscles of adult and aged male mice immediately following the contraction protocol.**

	18S	HSP25
ADULT NON-EXERCISED	37,768	17,870
AGED NON-EXERCISED	32,373	15,320
ADULT EXERCISED	58,967	33,740
AGED EXERCISED	48,069	20,430

Data are presented as arbitrary units.

7.3.2. Analysis of stress protein mRNA in gastrocnemius muscles from non-exercised adult and aged male mice by cDNA microarrays

The quality of the total RNA of pooled samples from gastrocnemius muscles of adult and aged male mice prior to, immediately, 4 hours and 12 hours following the contraction protocol was examined by electrophoresis of 1-2 μg of RNA on a denaturing formaldehyde/ agarose/ ethidium bromide gel (Figure 7.3).

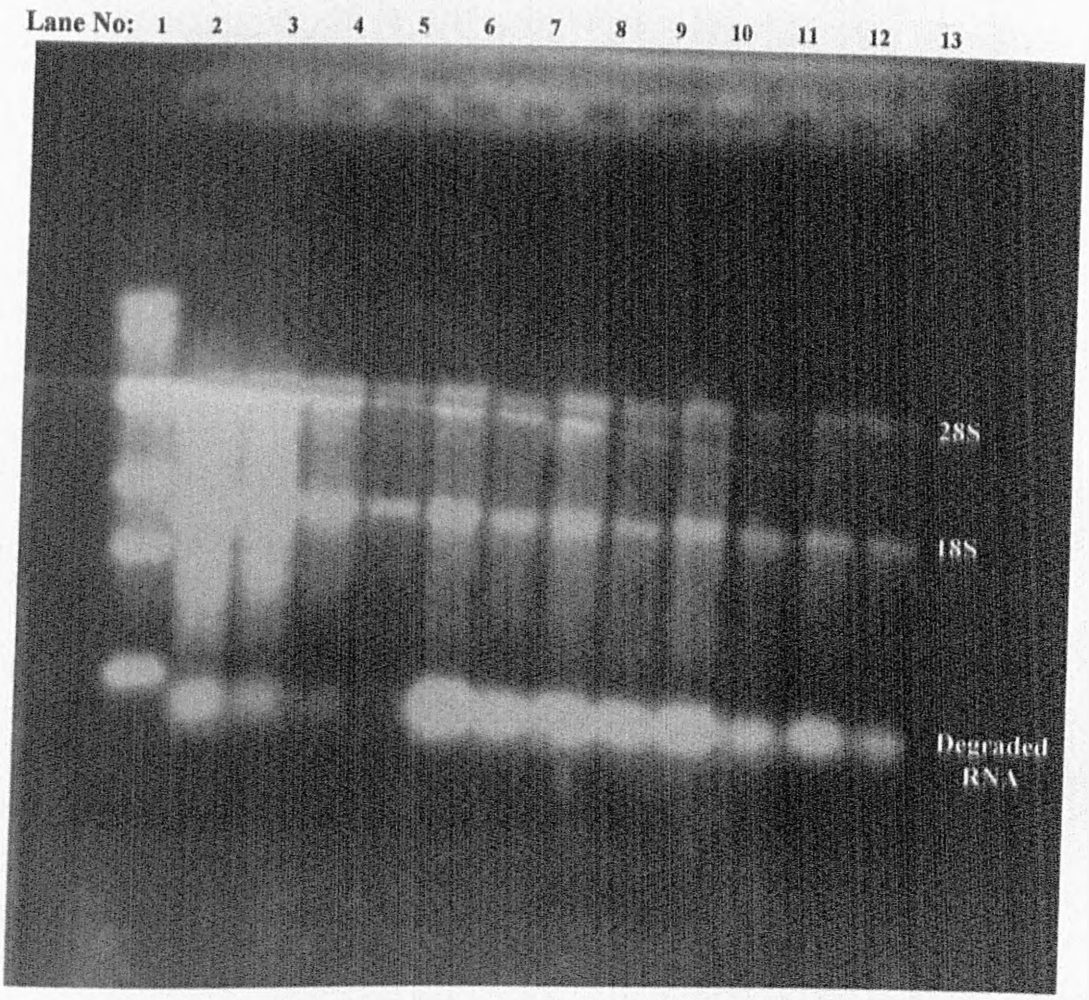


Figure 7.3. Formaldehyde/ agarose/ ethidium bromide gel of the total RNA of samples from gastrocnemius muscles from non-exercised adult and aged male mice and muscles immediately, 4 and 12 hours following the contraction protocol. Lane 1) RNA ladder; lane 2) 3μg mouse heart total RNA (Ambion Inc, Huntingdon, UK); lane 3) 2μg of mouse heart total RNA (Ambion Inc, Huntingdon, UK); lane 4) 1μg of mouse heart total RNA (Ambion Inc, Huntingdon, UK); lane 5) 0.5μg mouse heart total RNA (Ambion Inc, Huntingdon, UK); lane 6) adult non-exercised; lane 7) aged non-exercised; lane 8) adult immediately following contractions; lane 9) aged immediately following contractions; lane 10) adult at 4 hours following contractions; lane 11) aged at 4 hours following contractions; lane 12) adult at 12 hours following contractions; lane 13) aged at 12 hours following contractions.

To define gene expression in skeletal muscles from non-exercised adult and aged male mice, isolated RNA from the muscles was subjected to cDNA microarray analysis. Microarray images from non-exercised adult and aged male mice were shown in Figure 7.4. Microarray images were normalized by global normalization and compared in order to identify differentially expressed genes. Table 7.2 summarises the genes that are differentially expressed in gastrocnemius muscles from non-exercised aged mice compared with non-exercised adult mice. Comparison of the cDNA microarray images demonstrated a decrease in the expression of two genes in skeletal muscles of non-exercised aged mice compared with skeletal muscles of non-exercised adult mice (Table 7.2).

Table 7.2. **Differential mRNA expression between skeletal muscles of non-exercised adult and aged male mice.**

Descriptor	Identifier	Fold change
Growth arrest and DNA-damage-inducible, alpha (Gadd45)	L28177	**-2.88**
Oxidative stress-induced (OSI)	U40930	**-52.7**

Fold changes in the mRNA levels of gastrocnemius muscles of non-exercised aged mice compared with the mRNA levels of gastrocnemius muscles of non-exercised adult mice; (-) down regulation.

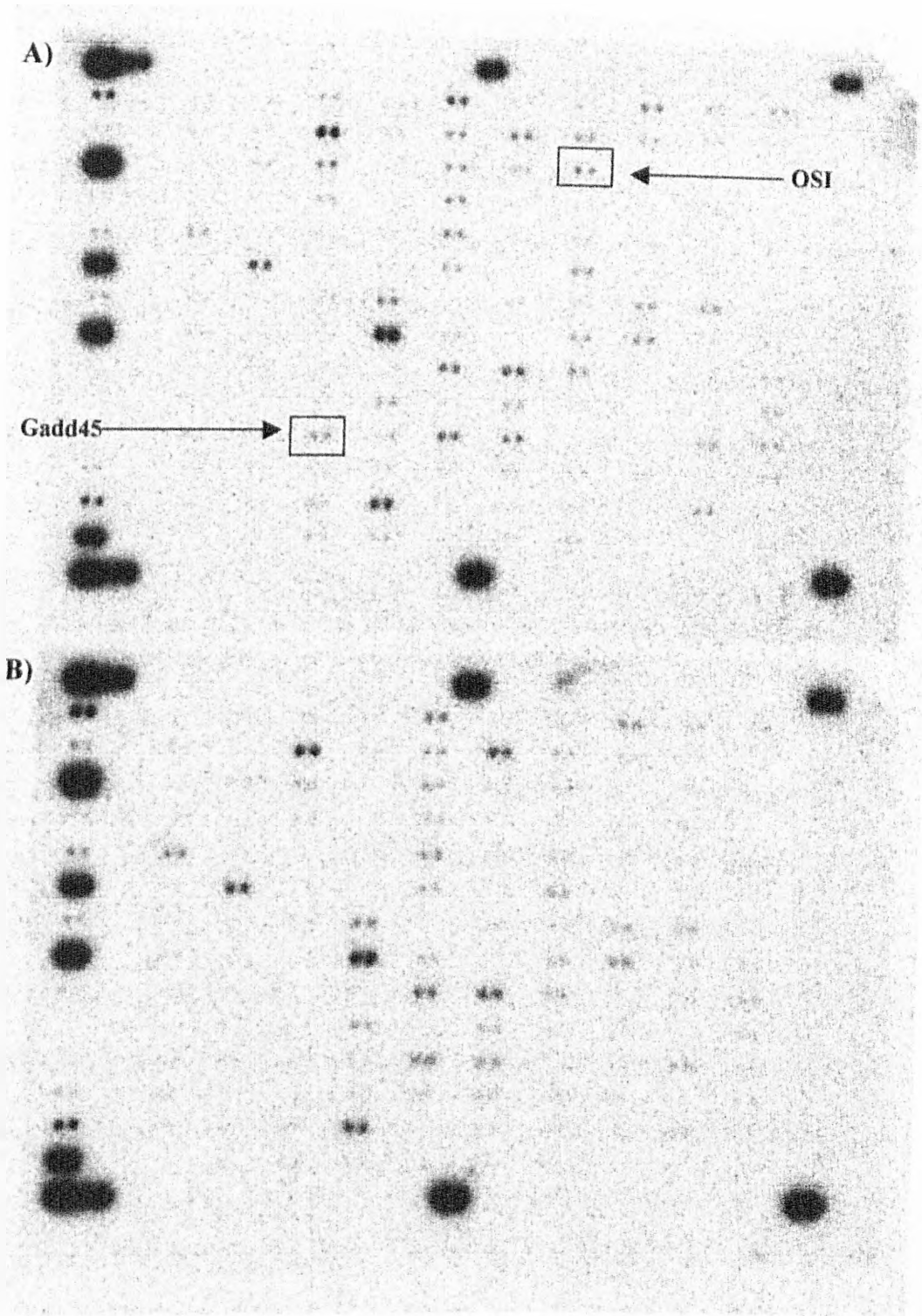


Figure 7.4. Representative Clontech Mouse Stress microarray images. A) gastrocnemius muscles from non-exercised adult male mice B) gastrocnemius muscles from non-exercised aged male mice.

7.3.3. Gene expression in gastrocnemius muscles of adult male mice at various time points following the contraction protocol

cDNA microarray images of gastrocnemius muscles of adult male mice immediately, 4 and 12 hours following the contraction protocol are shown in Figure 7.5A, Figure 7.6A and Figure 7.7A respectively. Microarray images were normalized by global normalization using Atlas Image v2.01 (Clontech, Palo Alto, USA) and compared with the microarray image from non-exercised adult mice in order to identify differentially expressed genes. Table 7.3 summarises the genes that are differentially expressed in gastrocnemius muscles from adult mice immediately, 4 and 12 hours following the contraction protocol compared with non-exercised adult mice.

Comparison of the microarray images demonstrated an increase in the expression of 3 genes in skeletal muscles of adult mice following contractions (heme oxygenase 1 at 4 hours following the contraction protocol; topoisomerase I and crystallin beta A1 at 12 hours following the contraction protocol) and a decrease in 13 genes (e.g. chaperonin subunit 4, subunit 5, subunit 7 and subunit 8, oxidative stress induced and ferredoxin 1 immediately following the contraction protocol; growth arrest and DNA-damage-inducible, cytochrome P450-2f2 and cytochrome P450-2e1 at 4 hours following the contraction protocol) compared with skeletal muscles of non-exercised adult mice (Table 7.3).

Table 7.3. **Differential mRNA expression between skeletal muscles of adult male mice immediately, 4 hours and 12 hours following the contraction protocol.**

GENE NAME	GENBANK	Fold Change	Fold change	Fold change
		Immediately	4 hours	12 hours
Growth arrest and DNA-damage-inducible, alpha (Gadd45)	L28177	NC	-2.75	-3.4
Topoisomerase (DNA) I / (Top I)	D10061	ND	ND	2.45
Cyclophilin 40 (CYP40; CYPD)	AA407024	NC	NC	-2.03
Chaperonin subunit 4 (delta)	Z31554	-2.95	NC	-5.36
Chaperonin subunit 5 (epsilon)	Z31555	-3.35	NC	-8.28
Chaperonin subunit 7 (eta)	Z31399	-3.32	NC	-3.53
Chaperonin subunit 8 (theta)	Z37164	-2.6	NC	NC
Heme oxygenase (decycling) 1 (HO-1)	M33203	ND	3.23	ND
Crystallin, beta A1	V00728	ND	ND	14.2
Endoplasmic reticulum protein	M73329	-2.7	NC	-2.14
Oxidative stress induced (OSI)	U40930	-2.22	NC	NC
P glycoprotein 2	J03398	ND	-3.28	ND
Nucleophosmin 1	M33212	-2.11	NC	-3.3
Ferredoxin 1	L29123	-2.83	NC	-3.22
Cytochrome P450, 2f2	M77497	NC	-3.47	-2.68
Cytochrome P450, 2e1, ethanol inducible	L11650	NC	-3.04	NC

Fold changes in the mRNA expression in gastrocnemius muscles of adult mice immediately, 4 hours and 12 hours following the contraction protocol compared with mRNA expression in gastrocnemius muscles of non-exercised adult mice. (ND) non-detectable, (NC) no change.

7.3.4. Gene expression in gastrocnemius muscles of aged male mice at various time points following the contraction protocol

Microarray images of mRNA expression from aged male mice immediately, 4 and 12 hours following the contraction protocol are shown in Figure 7.5B, Figure 7.6B and Figure 7.7B respectively. Microarray images were normalized by global normalization using Atlas Image v2.01 (Clontech, Palo Alto, USA) and compared with the microarray image from non-exercised aged mice in

order to identify differentially expressed genes. Table 7.4 summarises the genes that are differentially expressed in gastrocnemius muscles from aged mice immediately, 4 and 12 hours following the contraction protocol compared with non-exercised adult mice.

Comparison of the microarray images demonstrated an increase in the mRNA expression of 8 genes in skeletal muscles of aged mice following contractions (e.g. mitogen activated protein kinase kinase 4 immediately following the contraction protocol; oxidative stress induced and growth arrest and DNA-damage-inducible at 4 hours following the contraction protocol) and a decrease in the mRNA expression of 16 genes (e.g. chaperonin subunit 3, subunit 4, subunit 5 and subunit 8, calnexin, ferredoxin and RAD23b homologue immediately and 12 hours following the contraction protocol) compared with skeletal muscles of non-exercised aged mice (Table 7.4).

A comparison of the 16 genes that were modified by the contraction protocol in skeletal muscles of adult mice with the 24 genes that were modified by the contraction protocol in skeletal muscles of aged mice revealed that only 9 of the genes that were up/down regulated in skeletal muscles of adult mice were also up/down regulated in skeletal muscles of aged mice (Table 7.5b), whereas the remaining 7 genes modified by the contraction protocol in skeletal muscles of adult mice were not modified in skeletal muscles of aged mice (Table 7.5a).

Table 7.4. **Differential mRNA expression between skeletal muscles aged male mice immediately, 4 hours and 12 hours following the contraction protocol.**

GENE NAME	GENBANK	Fold change	Fold change	Fold change
		Immediately	4 hours	12 hours
Growth arrest and DNA-damage-inducible, alpha (Gadd45)	L28177	NC	2.2	NC
Ornithine decarboxylase structural	M10624	-2.11	NC	-2.92
Uracil-DNA glycosylase	X99018	ND	ND	-5.05
Chaperonin subunit 4 (delta)	Z31554	-6.14	NC	-3.36
Chaperonin subunit 5 (epsilon)	Z31555	-6.86	NC	NC
Chaperonin subunit 3 (gamma)	Z31556	-3.19	NC	-3.43
Chaperonin subunit 8 (theta)	Z37164	-3.64	NC	NC
Tumor rejection antigen gp96	J03297	-2.64	NC	-2.3
Calnexin (CANX)	L188888	-3.3	NC	-2.28
Endoplasmic reticulum protein	M73329	-18.5	-2.24	-15.5
Oxidative stress induced (OSI)	U40930	NC	34.5	NC
Cyclophilin 40 (CYP40; CYPD)	AA407024	-2.9	NC	-4.94
t-complex protein 1	D90344	-2.26	NC	-2.34
Ferredoxin 1	L29123	-5.82	NC	-18.75
Cytochrome P450, 4a12	X71479	3.68	ND	ND
Cytochrome P450, 2f2	M77497	-4.75	NC	ND
Kinesin family member 5B	U86090	-4.33	NC	ND
Peroxisome proliferator activator receptor delta (PPARδ)	L28116	3.98	ND	ND
Mitogen activated protein (MAP) kinase kinase 4	U18310	2.04	ND	ND
Aplysia ras-related homolog B (RhoB)	X99963	4.19	ND	ND
Thymine glucol DNA glycosylase/AP lyase	Y09688	-2.9	ND	ND
Terminal deoxynucleotidyl transferase	X04123	ND	2.14	ND
RAD23a homolog (S. cerevisiae)	X92410	ND	2.31	ND
RAD23b homolog (S. cerevisiae)	X92411	-2.27	NC	-2.31

Fold changes in the mRNA expression in gastrocnemius muscles of aged mice immediately, 4 hours and 12 hours following isometric contractions compared with gastrocnemius muscles of non-exercised aged mice. (ND) non-detectable, (NC) no change.

Table 7.5. **Comparison of mRNA expression changes between skeletal muscles of adult and aged male mice immediately, 4 hours and 12 hours following the contraction protocol.**

GENE NAME	GENBANK	Immediately		4 hours		12 hours	
		Adult	Aged	Adult	Aged	Adult	Aged
Topoisomerase (DNA) I / (Top 1)	D10061	–	–	–	–	Up (+2.45)	–
Chaperonin subunit 7 (eta)	Z31399	Down (-3.32)	–	–	–	Down (-3.53)	–
Heme oxygenase (decycling) 1 (HO-1)	M33203	–	–	Up (+3.23)	–	–	–
Crystallin, beta A1	V00728	–	–	–	–	Up (+14.2)	–
P glycoprotein 2	J03398	–	–	Down (-3.28)	–	–	–
Nucleophosmin 1	M33212	Down (-2.11)	–	–	–	Down (-3.3)	–
Cytochrome P450, 2e1, ethanol inducible	L11650	–	–	Down (-3.04)	–	–	–

a. Genes up or down regulated only in skeletal muscles of adult mice following the contraction protocol

GENE NAME	GENBANK	Immediately		4 hours		12 hours	
		Adult	Aged	Adult	Aged	Adult	Aged
Growth arrest and DNA-damage-inducible, alpha	L28177	–	–	Down (-2.75)	Up (2.2)	Down (-3.4)	–
Cyclophilin 40 (CYP40; CYPD)	AA407024	–	Down (-2.9)	–	–	Down (-2.03)	Down (-4.94)
Chaperonin subunit 4 (delta)	Z31554	Down (-2.95)	Down (-6.14)	–	–	Down (-5.36)	Down (-3.36)
Chaperonin subunit 5 (epsilon)	Z31555	Down (-3.35)	Down (-6.86)	–	–	Down (-8.28)	–
Chaperonin subunit 8 (theta)	Z37164	Down (-2.6)	Down (-3.64)	–	–	–	–
Endoplasmic reticulum protein	M73329	Down (-2.7)	Down (-18.5)	–	Down (-2.2)	Down (-2.14)	Down (-15.5)
Oxidative stress induced (OSI)	U40930	Down (-2.22)	–	–	Up (34.5)	–	–
Ferredoxin 1	L29123	Down (-2.83)	Down (-5.82)	–	–	Down (-3.22)	Down (-18.8)
Cytochrome P450, 2f2	M77497	–	Down (-4.75)	Down (-3.47)	–	Down (-2.68)	–

b. Genes up or down regulated in skeletal muscles of adult and aged mice following the contraction protocol

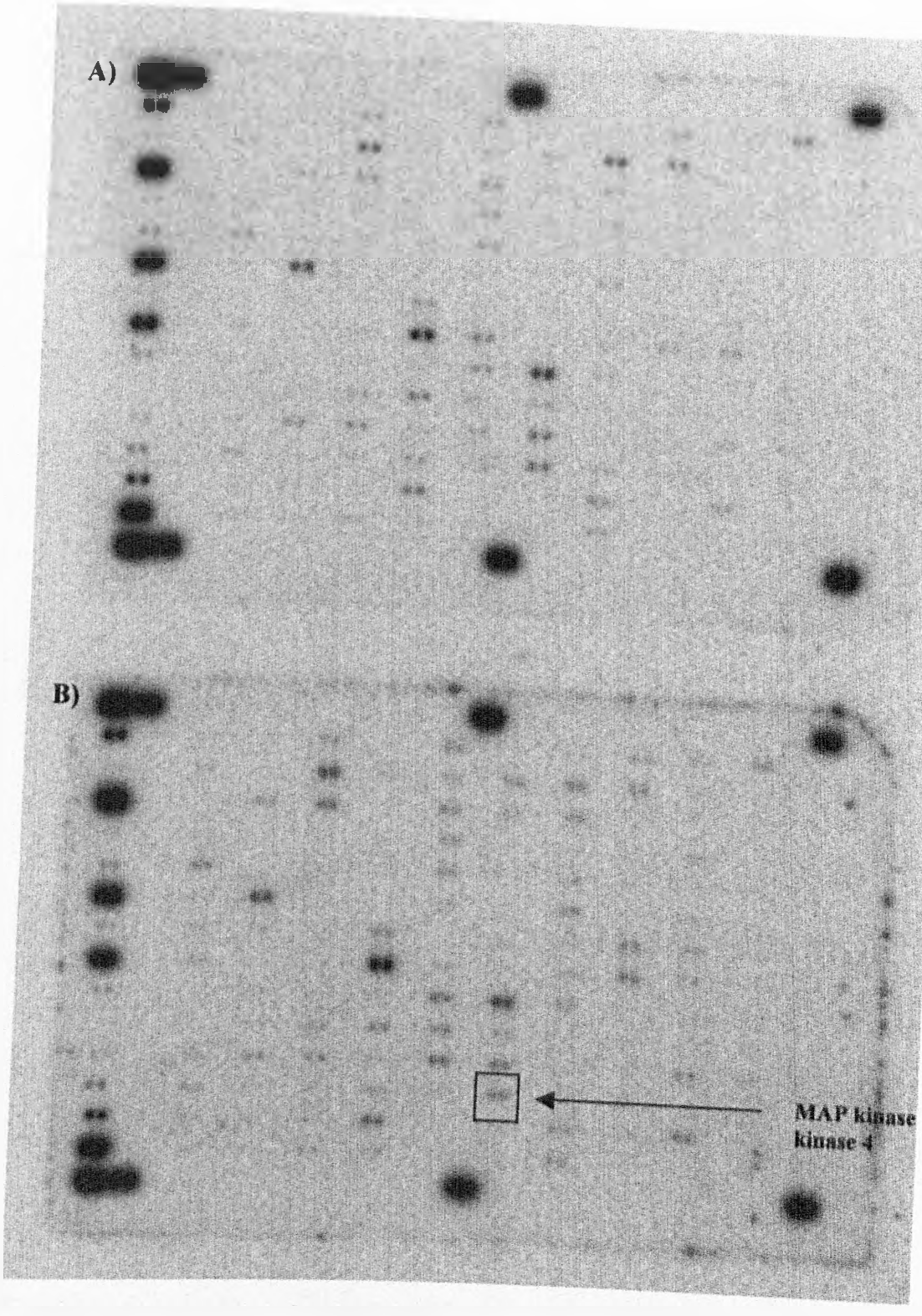


Figure 7.5. Representative Clontech Mouse Stress microarray images. A) RNA from gastrocnemius muscles of adult male mice immediately following the contraction protocol B) RNA from gastrocnemius muscles of aged male mice immediately following the contraction protocol.

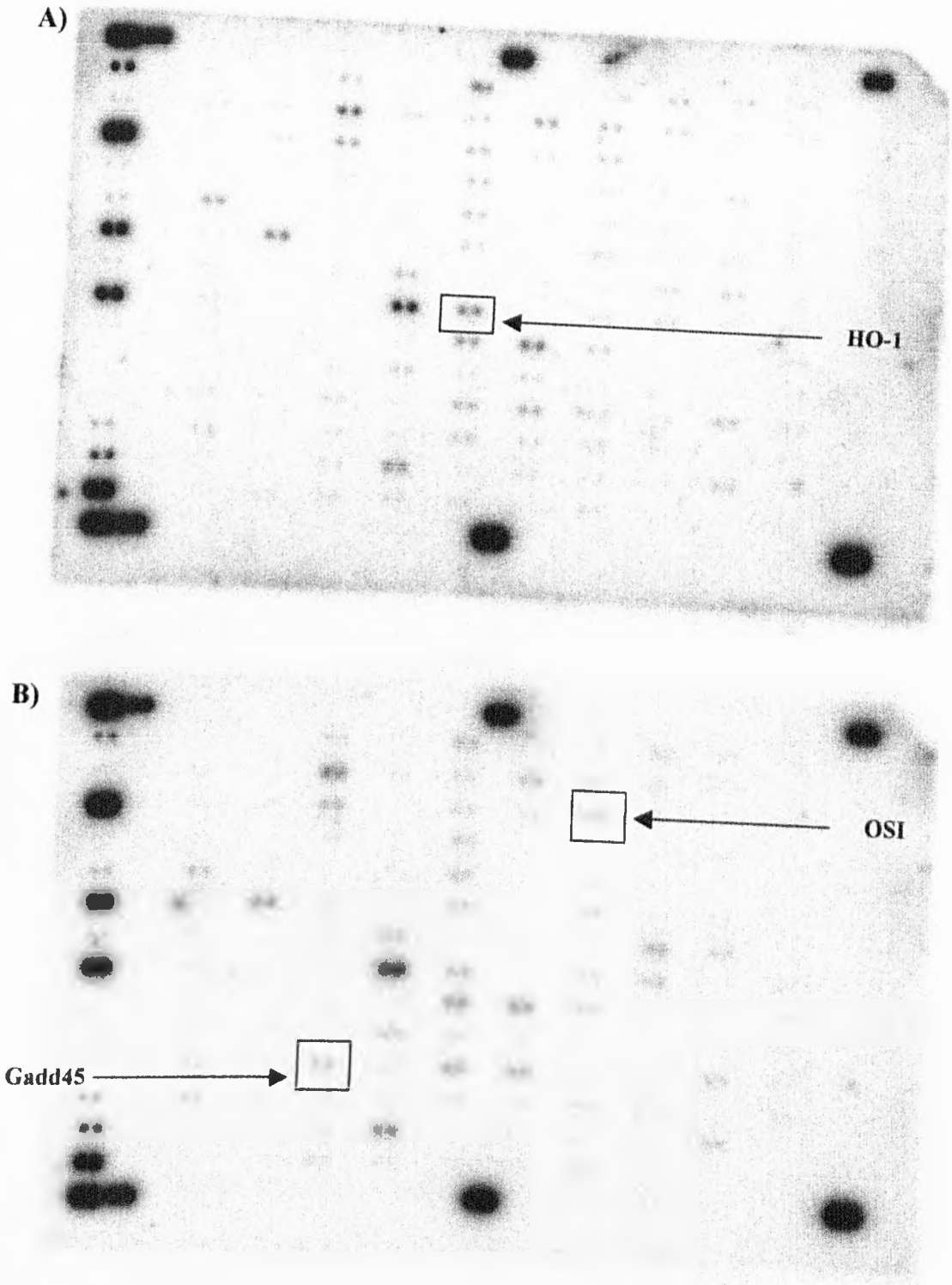


Figure 7.6. Representative Clontech Mouse Stress microarray images. A) RNA from gastrocnemius muscles of adult male mice at 4 hours following the contraction protocol B) RNA from gastrocnemius muscles of aged male mice at 4 hours following the contraction protocol.

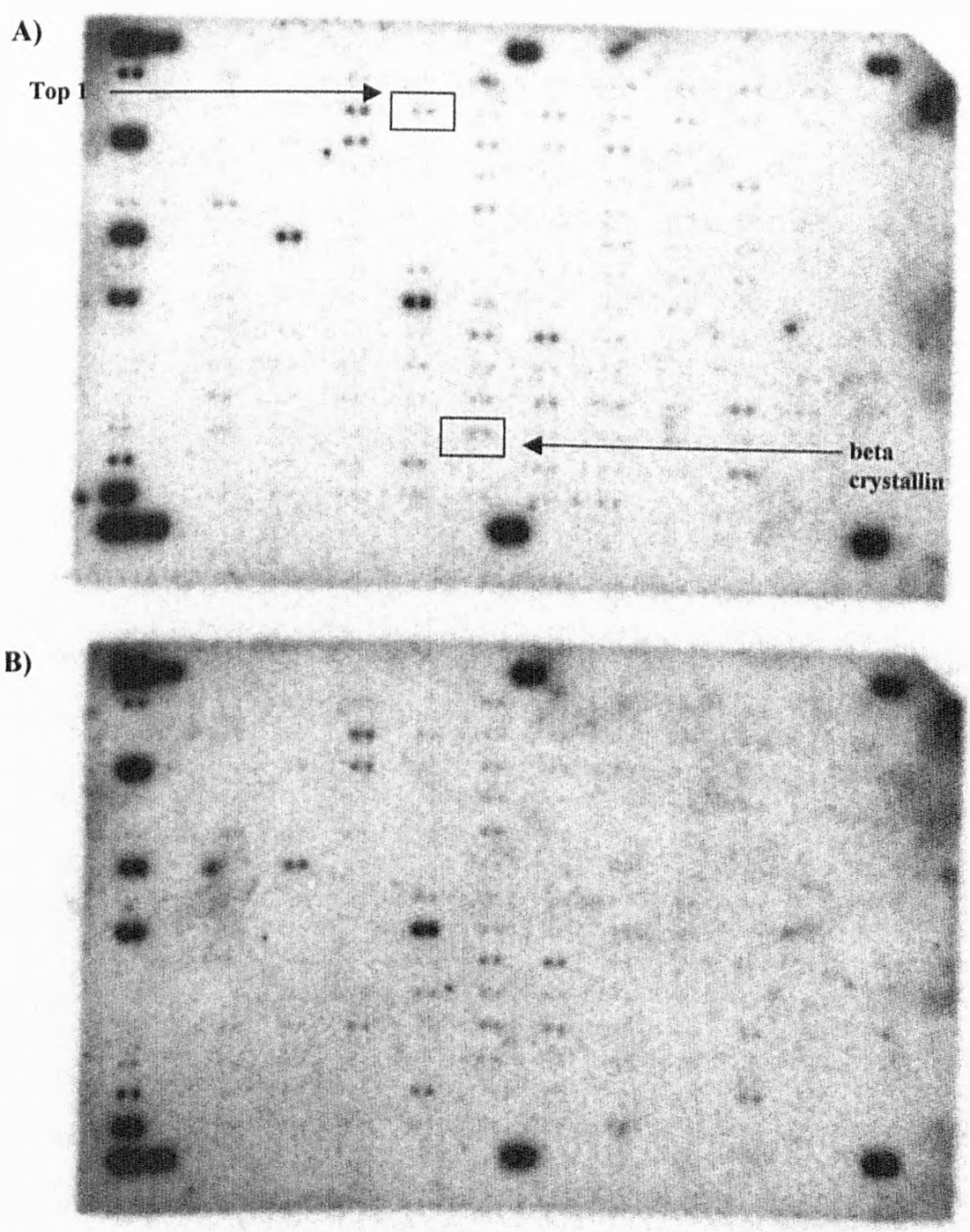


Figure 7.7. Representative Clontech Mouse Stress microarray images. A) RNA from gastrocnemius muscles of adult male mice at 12 hours following the contraction protocol B) RNA from gastrocnemius muscles of aged male mice at 12 hours following the contraction protocol.

7.4. DISCUSSION

Data presented in Chapter 5 demonstrated that although skeletal muscles from adult male mice respond to the contraction protocol by a general increase in the HSP content, skeletal muscles of aged mice fail to adapt following contractile activity. In addition, data presented in Chapter 6 demonstrated that the inability of muscles of aged mice to produce HSPs following contractions is not due to a gross alteration binding of HSF1 to the HSE DNA binding domain.

To further characterise the mechanism responsible for the alternated production of HSPs the HSP25 mRNA levels were examined by northern blotting. HSP25 was chosen since its production following the contraction protocol was significantly increased in skeletal muscles of adult mice, whereas the HSP25 production in skeletal muscles of aged mice following the contraction protocol remained unchanged (See Chapter 5). The northern blot of total RNA from non-exercised and exercised adult and aged mice was also hybridised with an 18S rRNA probe to equalise density measurements. Data demonstrated no difference in the HSP25 mRNA levels between gastrocnemius muscles of non-exercised adult and aged male mice. Following the contraction protocol, the HSP25 mRNA levels were increased by 20% in skeletal muscles of adult mice. The HSP25 mRNA levels were not increased in skeletal muscles of aged male mice. Therefore, these data suggest that the attenuation in the HSP response in skeletal muscles of aged mice following the contraction protocol, and especially that of HSP25, appears to be, at least in part, due to alterations occurring during transcription of the HSP25 gene.

To define overall changes in stress-related protein expression in muscles of adult and aged mice following contractile activity, mRNA from skeletal

muscles of adult and aged male mice prior to and following the contraction protocol was subjected to microarray analysis. Microarray images were analysed and compared in pairs using Atlas Image v2.01 (Clontech, Palo Alto, USA). Initially, Atlas Image provides a rough orientation by identifying two gene spots to serve as anchors. Then the Auto-Alignment function precisely aligns the array with the Grid Template and automatically finds the precise location of all hybridization signals. Once this step is performed for both arrays and the background calculation is adjusted, a pseudo-coloured image is generated that clearly identifies up- and down-regulated genes. Clicking any gene box in this image immediately provides the gene identity and signal intensity data. Prior to analysis, microarray images were also normalised by global normalisation i.e. the signal values of all the genes present in the array are used for normalisation (this method is suited for the comparison of similar tissues). There has been considerable discussion about the variability in mRNA data from arrays including strategies to eliminate false positive and negative apparent changes in expression (Dozmorov *et al*, 2001). In order to try to minimise the occurrence of false positives and negatives only data showing at least a two-fold change in expression is reported.

Comparison of microarray images from mRNA of non-exercised adult and aged mice demonstrated a down regulation in the mRNA levels of two genes in skeletal muscles of non-exercised aged mice; growth arrest and DNA-damage-inducible gene (Gadd45) and oxidative stress induced gene (also known as A170). Gadd45 is believed to play a role in growth arrest and possibly cell death (Shaulian and Karin, 1999). It has been shown that the expression of Gadd45 protein is induced following DNA damage (Zhan *et al*, 1994) and following stress

such as treatment with inorganic arsenicals (Liu *et al*, 2001). Oxidative stress induced gene encodes A170 protein which is induced by pro-oxidants such as diethyl maleate and paraquat (Ishii *et al*, 1996; Nakaso *et al*, 1999). Although A170 protein is specifically induced by mild oxidative stress, it is not an antioxidant itself, but is thought to be a modulator of signal transduction to induce cellular responses under oxidative stress (Ishii *et al*, 1996).

Contraction of skeletal muscles from adult male mice demonstrated an increase in the mRNA levels of three proteins; topoisomerase I, heme oxygenase 1 (also known as HO-1 or HSP32) and beta crystallin A1. HO-1 is the inducible form of heme oxygenase. Induction of HO-1 has been proposed as a general response to oxidative stress including exercise (Essig *et al*, 1997) and although HO-1 has a heat shock element in the promoter region, it is more often up-regulated following oxidative stresses that may not operate through the heat shock factor 1 activation pathway but may involve activation of NF-κB or other transcription factors (Tacchini et al, 1995; Noble, 2002). In addition, HO-1 does not appear to involve typical chaperone functions e.g. transportation or stabilisation of cellular proteins but its induction appears to play a role in cellular protection against injury caused by increased production of ROS (Noble, 2002). β crystallins are major structural proteins in the lens of the eye, but they are expressed in other tissues such as skeletal muscle (Neufer and Benjamin, 1996) and their function involves prevention of aggregation of denatured proteins and protein refolding upon removal of stress (Jacob *et al*, 1993; Neufer and Benjamin, 1996). The increased expression of HO-1 and β crystallin genes in skeletal muscles of adult male mice following the contraction protocol, in addition with the increased activity of antioxidant defences enzymes and increased production

of HSPs discussed in Chapters 4 and 5, provides evidence of adaptation in skeletal muscles of adult mice following non-damaging contractile activity.

Contraction of skeletal muscles from adult male mice also resulted in a decrease in the mRNA levels of 13 proteins. These included: DNA-damage-inducible gene (Gadd45), oxidative stress induced gene (A170). Interestingly, the levels of these mRNAs were also decreased in muscles of aged mice prior to the contraction protocol in comparison with muscles of non-exercised adult mice. In addition, genes encoding cytochrome P450 enzymes and chaperonin subunits delta, epsilon, eta and theta encoding subunits of the cytosolic chaperonin-containing t-complex polypeptide 1 (CCT; a molecular chaperone that plays an important role in the folding of proteins in the eukaryotic cytosol such as actin; Kubota, 2002) were also down regulated.

Contraction of skeletal muscles from aged male mice resulted in more dramatic alterations in gene expression. In contrast to skeletal muscles from adult mice, the contraction protocol resulted in the enhanced expression of DNA-damage-inducible gene (Gadd45) and oxidative stress induced gene (A170) as well as other genes such as RAD23a that is involved in nucleotide excision repair, peroxisome proliferator activator receptor delta that encodes transcription factor PPARδ involved in the inflammatory response and mitogen activated protein kinase kinase 4 (MKK4), a member of the stress-activated protein kinase signaling cascade.

The contraction protocol also resulted in a reduction in the expression of 16 genes in skeletal muscles of aged mice. These included: chaperonin subunits gamma, delta, epsilon, and theta, t-complex protein 1 gene that encodes protein TCP-1 (t-complex polypeptide 1), which is a subunit of the hetero-oligomeric

complex CCT (chaperonin containing TCP-1), uracil-DNA glucosylase involved in base excision repair, as well as calnexin gene which encodes calnexin, an integral membrane protein of the ER that binds to Ca^{2+} and may function as a chaperone involved in the transition of proteins from ER to the outer cellular membrane (Tjoelker *et al*, 1994).

Comparison of the 16 genes which their expression changed following the contraction protocol in skeletal muscles of adult mice with those from skeletal muscles of aged mice revealed that only 9 of the genes that were up/down regulated in skeletal muscles of adult mice were also up/down regulated in skeletal muscles of aged mice. The remaining 7 genes modified by the contraction protocol in skeletal muscles of adult mice were not modified in skeletal muscles of aged mice. These 7 genes include genes encoding proteins such as heme oxygenase 1, beta crystallin and topoisomerase I which suggests that the response of these 3 genes, as well as the response of the remaining 4 genes, is attenuated in skeletal muscles of aged male mice following the contraction protocol.

Although Clontech Atlas Mouse Stress microarrays contain genes encoding antioxidant defence enzymes such as catalase, Mn-SOD and Cu/Zn-SOD, as well as HSP60 no differences in the expression of these genes were observed between skeletal muscles of non-exercised adult and aged mice or between skeletal muscles of adult and aged mice subjected to the contraction protocol at any time point studied. However, it is possible that an increase or a decrease in the expression of these genes following the contraction protocol may have occurred at an earlier or a later time point than the ones studied (e.g. 1 hour following the contraction protocol). In addition, Clontech Atlas Mouse Stress microarrays contain a limited amount of genes and do not contain genes encoding

other HSPs such as HSP70, HSC70 and HSP25 thus, examination of the expression of these genes was not possible.

In summary, data demonstrated an increase in the HSP25 mRNA in skeletal muscles of adult male mice following the contraction protocol, whereas the HSP25 mRNA in skeletal muscles of aged male mice was not increased following contractions. These data suggest that the attenuation in the HSP25 response in skeletal muscles of aged mice following the contraction protocol is due, at least in part, to alterations occurring during transcription of the HSP25 gene. The use of DNA microarrays has provided a tool for the global assessment of the genes whose expression levels change in response to the contraction protocol. Data demonstrated that a number of genes showed an attenuated response in skeletal muscles of aged male mice following the contraction protocol compared with their expression in skeletal muscles of adult mice following contractions. Further analysis of the proteins whose mRNA levels showed up/down regulation in response to the contraction protocol will define the molecular mechanisms underling the biological adaptation of skeletal muscle in response to contractile activity.

CHAPTER 8

DISCUSSION

8.1. SUMMARY OF THE MAIN FINDINGS

The initial aims of the work reported in this thesis were to examine:

1. The oxidative status of skeletal muscles from adult and aged B6XSJL mice.
2. The relationship of this to the ability of the muscle to adapt following a period of non-damaging contractile activity.
3. The extent and time course of attenuation in the stress response in skeletal muscles of aged mice following contractile activity.
4. The mechanisms that may be responsible for any attenuation detected.
5. Whether there are any gender-specific differences in the stress response of adult and aged mice following contractile activity.

The major findings of the work were:

1. Data have demonstrated that the cellular oxidative environment in muscles of non-exercised aged male mice may be altered. The muscle appears to have attempted to adapt to an increased stress by an increased content of some of the antioxidant defence enzymes, such as catalase and SOD.
2. Data from muscles of adult male mice demonstrated a clear increase in the production of HSPs and antioxidant defence enzymes following the non-damaging isometric contraction protocol. This was not evident in muscles of aged male mice. In addition, data demonstrated an increase in the HSP25 mRNA in skeletal muscles of adult male mice following the contraction protocol, whereas the HSP25 mRNA levels in skeletal muscles of aged male mice demonstrated no evidence of an increase following contractions.

3. Data from studies examining HSF1, NF-κB and AP-1 binding activity suggested that the inability of muscles of aged mice to produce HSPs following contractile activity is not due to an altered binding of HSF1 to the binding domain. In contrast, this may be the mechanism by which production of antioxidant enzymes is altered in aged mice following the contraction protocol since binding of NF-κB and AP-1 was attenuated.
4. No significant differences were observed in the antioxidant enzyme activity and HSP content in skeletal muscles of adult and aged female mice prior to and following the contraction protocol. Data suggests that there are major differences in the adaptive response in muscles of male and female mice. The reasons for this are unknown but there is now considerable evidence suggesting that the sex hormone differences play a major role.
5. Microarray analysis demonstrated that the expression of more genes was down regulated than up regulated in skeletal muscles of both adult and aged male mice following the contraction protocol. Furthermore, more genes appeared to show up or down regulation following the contraction protocol in skeletal muscles of aged male mice than in skeletal muscles of adult male mice. Skeletal muscles of adult mice showed a change in expression of 16 genes, of which 7 were attenuated in muscles of aged mice, confirming the widespread nature of this attenuation in adaptive capacity in aged muscle.

8.2. GENERAL DISCUSSION OF THE DATA PRESENTED

A number of general areas for discussion are highlighted by these data:

8.2.1. Effect of ageing on antioxidant defence enzyme content of skeletal muscles from mice

Skeletal muscle generates a number of ROS during contraction, including superoxide anion radical, hydroxyl radical and nitric oxide. Increased production of ROS cause a wide spectrum of cell damage including lipid peroxidation, inactivation of enzymes, alteration of intracellular oxidation-reduction state, and damage of DNA (Ji *et al*, 1990). However, muscle cells have developed mechanisms to protect against an increased production of ROS. This response of muscle cells to oxidative stress includes a highly ordered set of events that is often represented by rapid changes in gene expression, followed by the synthesis of proteins involved in adaptation to stress.

Ageing is hypothesised to be caused by the deleterious effects of ROS accumulating throughout the lifespan (Leeuwenburgh *et al*, 1994). It is proposed that, as an organism ages, the production of reactive oxygen species is increased as a result of the functional deterioration of mitochondria. There is strong evidence that increased free radical generation may be the underlying reason for several age-related pathogeneses. Thus, cellular antioxidant defences play a more crucial role at advanced age (Leeuwenburgh *et al*, 1994). However, it has been demonstrated that during ageing, the cellular response to oxidative stress becomes defective.

The overall purpose of this study was to examine the effect of age and gender on the ability of muscles from mice to adapt following a period of non-damaging isometric contractions.

An increase was observed in catalase and SOD activities in skeletal muscles of non-exercised aged male mice (Figure 4.2; Figure 4.4). It has been suggested that mitochondria of aged tissues have a higher rate of superoxide anion radical leakage, which would result in increased need for SOD, especially Mn-SOD. In addition, subsequent increased generation of hydrogen peroxide would result in increased need for catalase. It has been demonstrated that transcription factors such as Nuclear Factor κB (NF-κB) and activator protein-1 (AP-1) are important mediators of redox-responsive gene expression in skeletal muscle and that both NF-κB and AP-1 are actively involved in the upregulation of antioxidant enzymes such as SOD and catalase in response to oxidative stress. Examination of the DNA binding activity of these two transcription factors revealed an increase in the DNA binding of NF-κB and AP-1 in skeletal muscles of non-exercised aged male mice compared with that of non-exercised adult male mice (Figure 6.2; Figure 6.4). Therefore, the present data suggests that muscles from non-exercised male mice may have been exposed to a more oxidative environment. Whether this adaptation is sufficient to return the cell to the oxidative status of muscles of young/adult mice is unclear.

Physical exercise increases the production of ROS (Lawler *et al*, 1993; McArdle *et al*, 2001). Thus, an increase in antioxidant defences of skeletal muscles from adult mice following the contraction protocol was predicted. This study has demonstrated that skeletal muscle of adult male mice responds to non-damaging isometric contractions by increasing the levels of catalase and SOD activities (Figure 4.2; Figure 4.4) whereas, the activities of catalase and SOD in skeletal muscles of aged male mice were unchanged. Examination of the DNA binding activity of NF-κB and AP-1 revealed a significant increase in the DNA

binding of NF-κB and AP-1 in skeletal muscles of adult male mice, whereas the NF-κB and AP-1 binding activities in skeletal muscles of aged male mice were significantly decreased (Figure 6.2; Figure 6.4). Increased production of ROS can cause changes in the biochemical structure and function of nucleic acids and proteins and damage several cellular mechanisms. NF-κB and AP-1 contain several redox sensitive sites and so it is possible that such alterations may have occurred in the structure or function of NF-κB and AP-1, potentially leading to a functional inhibition of these two transcription factors in skeletal muscles of aged male mice was observed. Therefore, the present data suggests that the inability of skeletal muscles from aged mice to increase the antioxidant defence enzyme activity following the contraction protocol may be due to the inability of NF-κB and AP-1 to bind to DNA in order to activate antioxidant defence enzyme gene transcription.

8.2.2. Effect of ageing on HSP content of skeletal muscles from mice

The content of HSP70 and HSC70 in skeletal muscles from non-exercised aged male mice was increased (Table 5.1; Figure 5.2b). This increase in the HSP70 and HSC70 content of skeletal muscles of non-exercised aged male mice was similar to the up-regulation in catalase and SOD activities discussed in section 8.2.1. Both HSP70 and HSC70 are necessary for chaperone function in non-stressed tissue. It has been suggested that this up-regulation of the cellular defence mechanisms in tissues of aged rodents is possibly due to the increased production of ROS with ageing. Since ROS can damage cellular macromolecules, including proteins, increased content of HSPs, such as HSP70 is a necessary adaptation.

In contrast, the HSP60 and HSP25 contents from non-exercised aged male mice remained unchanged compared with those from non-exercised adult male mice (Table 5.1). The lack of change in HSP25 and HSP60 content with age may reflect the specialised nature of these proteins. Under normal conditions, HSP25 is involved with general cell mechanisms such as signal transduction, differentiation and growth (Locke, 1997), whereas HSP60 is expressed constitutively in mitochondria and plays an important role in facilitating protein transport across intracellular membranes, such as the mitochondrial membrane (Welch, 1992) and folding of mitochondrial proteins. There seems to be a discrepancy between the elevated levels of HSP70 / HSC70 in muscles of non-exercised aged male mice and a lack of change in other HSPs with age. This may be due to the general function of HSP70 / HSC70 in cells compared with the highly specialised function of other HSPs.

In order to examine whether the increase in the HSP70 and HSC70 content of non-exercised skeletal muscles from aged male mice was due to activation of HSF1 transcription factor, the HSF1 binding activity of non-exercised adult and aged mice was also examined. Data demonstrated no significant differences in the DNA binding activity of HSF1 between skeletal muscles of non-exercised adult and aged male mice (Figure 6.6). In addition, data demonstrated that that there are no differences in HSF1 protein levels between skeletal muscles of adult and aged mice (Figure 6.9). Thus, the data suggests that the variability in the HSP content observed in skeletal muscles of non-exercised aged male mice is not due to the increased binding activity of HSF1 but probably due to other reasons such as chronic changes in the cellular redox state which may result in an increased content of HSP70 and HSC70 as an adaptive response.

This study has also demonstrated that skeletal muscles from adult male mice respond to the contraction protocol by a general increase in the content of all HSPs studied. This was not apparent in muscles of aged mice following the contraction protocol. The most dramatic increase observed was HSP25 content (Figure 5.1a; Figure 5.1b).

In order to examine whether the attenuated adaptive response in skeletal muscles of aged mice following the contraction protocol is due to alterations during transcription, the HSP25 mRNA levels were examined. Data revealed that the HSP25 mRNA levels from skeletal muscles of adult mice were not only significantly higher than those from skeletal muscles of non-exercised adult mice but there were also significantly higher than the HSP25 mRNA levels from skeletal muscles of aged mice following the contraction protocol (Figure 7.2A).

In order to assess whether the lack of adaptation in the HSP content in tissues of aged mice was due to an attenuation of the HSF1 activation mechanism, the HSF1 binding activity in skeletal muscles of aged mice following the contraction protocol was compared to that of skeletal muscles of adult mice following the same protocol. Data demonstrated that in muscles of aged mice, binding of HSF1 to the Heat Shock Element (HSE) binding domain was not grossly altered following the contraction protocol compared with muscles of adult mice. However, the attenuation of HSP production observed in muscles of aged mice may be due to several other possible reasons. Although HSF1 from aged mice is capable of binding to the consensus DNA sequence, this may not be the case *in vivo*. Somatic mutations in the DNA binding element (HSE) of the HSP genes may result in a reduced binding affinity of HSF1 to the HSE of the HSPs, and so lead to an attenuated stress response in aged mice. In addition, it has been

shown that although DNA binding activity by HSF1 is necessary, this alone is insufficient for transcriptional activation and hyperphosphorylation of the transcription factor is necessary for transcriptional activation (Cotto *et al*, 1996). However, the current study did not examine this in detail.

8.2.3. Sex differences in the adaptive response of skeletal muscles following the contraction protocol

In contrast to the major differences observed between skeletal muscles of non-exercised and exercised adult and aged male mice, no significant changes were observed in the antioxidant defence enzyme activities and HSP content in muscles of adult and aged female mice prior to or following the contraction protocol (Chapter 4; Chapter 5). In order to assess whether the muscles from female mice are "aged" more prematurely than those of male mice and the response to stress is already altered, an extra group of AT muscles from young female B6XSJL mice (4-6 months old) was subjected to the same contraction protocol and analysed for antioxidant defence enzyme activities and HSP content. In addition, in order to detect whether there is a delay in the response in skeletal muscles of female mice and so is not evident at 24 hours following the contraction protocol, an extra group of AT muscles from young female mice was studied at 48hrs following the contraction protocol.

Data demonstrated no significant differences in the antioxidant defence enzyme activities and HSP content between skeletal muscles of non-exercised young and adult female mice. Furthermore, no significant increases in the antioxidant defence enzyme activities and HSP content of skeletal muscles of young mice were observed at any time point studied. Finally, no significant increases in the antioxidant defence enzyme activities and HSP content of skeletal

muscles of young female mice were shown at 48hrs following the contraction protocol.

Data suggest that the signal for adaptation in skeletal muscles of female mice is reduced or is absent. The reasons for this is unknown but there is considerable evidence suggesting that the biological differences between the two sexes play an important role. Recent studies that have concentrated on the effects of sex hormones in the regulation of HSP expression with exercise have demonstrated that oestrogen attenuates post-exercise HSP expression in skeletal muscle and other tissues such as liver, lung and heart (Paroo *et al*, 1999; Paroo *et al*, 2002). These findings suggest that sex hormones play a crucial role the gender-specific differences in response to stress, although, the current study did not examine this in detail.

8.2.4. Global changes in gene expression with age

To define overall changes in the mRNA levels of stress-related proteins in skeletal muscle, RNA from skeletal muscles of adult and aged mice prior to and following the contraction protocol was subjected to microarray analysis. In general, the contraction protocol resulted in the up regulation of 3 genes and the down regulation of 13 genes in skeletal muscles of adult mice, and the up regulation of 8 genes and the down regulation of 16 genes in skeletal muscles of aged male mice.

In skeletal muscles of adult mice, the contraction protocol resulted in the enhanced expression of heme oxygenase 1 and beta crystallin, the induction of which plays a role in cellular protection against injury (Table 7.3) whereas no changes in the mRNA levels of these two proteins were seen in skeletal muscles

of aged mice following contractions (Table 7.4; Table 7.5a). The contraction protocol resulted in the down regulation of genes encoding proteins such as Gadd45 and oxidative stress induced protein A170 whereas, in skeletal muscles of aged mice, the contraction protocol resulted in the increased expression of genes encoding Gadd45 and oxidative stress induced protein A170. Genes encoding subunits of the chaperonin-containing t-complex polypeptide 1 (CCT) were down regulated in skeletal muscles of both adult and aged mice following the contraction protocol.

In conclusion, data demonstrated that more genes are down regulated than up regulated in skeletal muscles of adult and aged male mice following the contraction protocol with more dramatic attenuation in the gene expression in skeletal muscles of aged male mice.

8.3. POTENTIAL FUTURE STUDIES

8.3.1. Detection of ROS release from muscles of adult and aged mice by microdialysis

Since skeletal muscles generate a number of ROS during contraction (See Chapter 1) and since ageing is hypothesised to be caused by the deleterious effects of ROS accumulating throughout the lifespan, the detection of ROS release, especially following physical activity, is important. Microdialysis in a novel technique, which enables continuous monitoring of the extracellular environment of the tissue under study. This technique was developed for study of the kinetics of pharmacological agents in the brain and was initially used by our laboratory to study potential mediators of inflammation (McArdle *et al*, 1997). The technique was modified to monitor superoxide (McArdle *et al*, 2001) and hydroxyl radical (Pattwell *et al*, 2001) release from skeletal muscle *in vivo* during a period of contractile activity or ischemia and reperfusion. The use of the microdialysis technique would allow investigation of the production of ROS prior to and following the contraction protocol in skeletal muscles of adult and aged mice and will provide evidence of the role that oxidants play in the adaptive responses is skeletal muscle.

8.3.2. Detection of mutations in the Heat shock element (HSE) of the HSP genes

This study has demonstrated that the HSP production in skeletal muscles of male mice following the contraction protocol is attenuated. Although HSF1 from aged mice is capable of binding to the idealised DNA sequence used in EMSA experiments *in vitro* (See Chapter 6), this may not be the case *in vivo*. Any deletions or mutations in the Heat Shock Element (HSE) of the HSP genes will

result in a decrease in the HSF1 DNA binding activity as well as in a decrease in transcriptional activation and synthesis of HSPs. Denaturing gradient gel electrophoresis (DGGE) analysis of PCR amplified DNA can be employed to detect mutations and deletions in HSE of the HSP genes. This technique has been successfully used to identify genetic variation in virus populations. The use of this technique will allow examining whether the reduced transcription of HSPs in skeletal muscles of aged mice is due to genetic changes on the HSE of the HSP genes.

8.3.3. Intervention studies to examine the effect of stress proteins on skeletal muscle function in aged mice

Muscle dysfunction is currently a consequence of ageing but previous data from our laboratory has shown that overexpression of heat shock protein 70 (HSP70) in muscle of transgenic mice throughout life significantly reversed some aspects of this age-related decline in muscle function. Examination of the site(s) at which the HSP70 content provides protection to muscle cells and whether the elevated HSP content of the cell facilitated improved protein folding and also increased resistance of proteins to aggregation may provide further insights into the potential mechanisms of protection of a maintenance of the ability of muscles to produce HSP70 during the ageing process.

Although HSP70 has the capacity to refold a wide array of proteins, other HSPs have more specific roles within the cell and it would be interesting to examine the potential protection afforded by other HSPs, including HSP32 (heme oxygenase 1) and beta crystallin since their gene expression was significantly increased in skeletal muscles of adult mice following contractions (See Chapter 7).

A physiological intervention study could also be used to examine the possibility that non-damaging exercise can chronically elevate muscle HSP70 and other HSPs. A treadmill running protocol in mice throughout life could be used to assess the ability of muscle from these mice to produce HSPs in response to stress and the susceptibility of muscles to exercise-induced damage and recovery from damage. If this approach proved beneficial the effect of a much shorter period of exercise conditioning in aged animals could be used to determine whether exercise intervention in aged individuals could be an appropriate therapeutic approach.

There is much to be learned about the stress response of aged cells in cell biology. Future targeted studies will impact significantly on our understanding of the pathophysiology of ageing.

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