THE EFFECTS OF LONG-TERM EXERCISE TRAINING ON SP72 AND MHC-I EXPRESSION IN RATS

by

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Abstract

The present study was designed to determine if the increase in 72-kD stress protein expression observed following acute exercise and short-term training would persist after 24 weeks of endurance training. It was also of interest to examine if the relationship between SP72 expression and type I myosin heavy chain demonstrated under non-stress conditions, yet lost after short-term training, would be restored subsequent to long-term training. Female rats were either maintained at rest (n=6-9) or exercised on a treadmill (n=7-8). The training protocol consisted of an 8-week build-up followed by 16 weeks at 70-100% VO_2max. Results indicate that after 24 weeks of endurance training, the exercise-induced increase in SP72 associated with acute exercise and short-term training persists in the muscles and fibre types most likely to be recruited during the exercise. Also following long-term endurance training, the constitutive SP72-MHC-I positive correlation demonstrated under non-stress conditions does not exist.

Keywords

stress proteins; type I myosin heavy chain; rat; long-term endurance training; skeletal muscle; liver

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List of Abbreviations

ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
BSA	Bovine Serum Albumin
°C	degrees Celcius
ddH_2O	double distilled water
DMF	Dimethylformamide
DNA	Diribonucleic Acid
DTT	Dithiothreitol
E. coli	Escherichia coli
ER	Endoplasmic Reticulum
g	gram
GRP	Glucose Regulated Protein
HST	Heat Shock Testicular
HSE	Heat Shock Element
HSF	Heat Shock Transcription Factor
HSP	Heat Shock Protein
HSR	Heat Shock Response
ip	intra-peritoneal
kD	kilo Dalton
kg	kilogram
L	Litre
m	metre
M	Molar
mg	milligram
µg	microgram
MHC	Myosin Heavy Chain
mm	millimetre
mM	milli Molar
mRNA	messenger Ribonucleic Acid
MW	Molecular Weight
PAGE	Polyacrylamide Gel Electrophoresis
pI	Isoelectric point
PMSF	Phenylmethylsulfonyl Fluoride
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
SP	Stress Protein
SR	Sarcoplasmic Reticulum
TBS	Tris-Buffered Saline
TTBS	Tween Tris-Buffered Saline
VO_2max	maximal oxygen uptake
vol	volume
wt	weight

1. INTRODUCTION

Various stressors may lead to a disruption of cellular homeostasis. During episodes of stress, the disruption in homeostasis may compromise the survival of the entire organism. At the cellular level, specific stress proteins (SPs) may play an important role in maintaining cellular homeostasis. Cells from all organisms respond to increases in temperature of 3 to 5°C above normal by rapid gene transcription and subsequent mRNA translation to yield a class of highly conserved proteins known as the heat shock proteins (HSPs), or more generally referred to as the stress proteins (SPs) (Locke, 1997). This induction, termed the stress response (SR), provides the cell with a mechanism to re-establish homeostasis.

Although heat shock has been the most extensively characterized stressor (Cairo et al., 1985; Currie and White, 1983, Locke et al., 1990), many other stressors are capable of inducing the synthesis of some or all of the SPs. These include ischemia (Emami et al., 1991; Knowlton et al., 1991), accumulation of reactive oxygen species (Salo et al., 1991), alterations in pH (Jurivich et al., 1992; Petronini et al., 1995), alterations in calcium (Lee, 1987), and glucose deprivation Pouyssegur et al., 1977), to name a few. In animals, exercise (Kelly et al., 1996; Locke et al., 1990; Locke and Tanguay, 1996; Skidmore et al., 1995) is capable of inducing the stress response because the conditions experienced by the exercising animal are often physiologically and/or biochemically similar to those mentioned above.

Exercised induced increases in certain SPs may help to preserve cell function under stressed conditions in a manner analogous to that following heat stress (Welch, 1990). While the protective role of SPs has been most extensively examined in cardiac tissue (Currie et al., 1993; Donnelly et al., 1992; Plumier et al., 1995), the involvement of SPs in skeletal muscle protection is strongly implicated (Garramone et al., 1994). For instance, a single bout of

eccentric exercise can provide protection against future injury (Schwane and Armstrong, 1983), and exercise of a similar duration and intensity has been shown to increase SP synthesis in skeletal muscle (Locke et al., 1990; Salo et al., 1991).

Little is known regarding SP expression in tissues following long-term exercise training. Previous studies suggest that certain SPs may be elevated with exercise training (Brickman et al., 1996; Kelly et al., 1996; Samelman and Alway, 1996; Sim et al., 1991), yet in all cases, exercise training was of a short duration or there was a progressive increase in speed or duration of exercise. Any increase in SP content may, therefore, reflect the relative change in exercising intensity or duration from the previous exercise level. In addition, exercise training results in minimized homeostatic disruption during exercise bouts by diminishing the acute exercise responses, such as high intracellular temperature and increased cellular lactate concentration, known to induce the stress response. Therefore, the adaptation to exercise training may have an effect on SP expression in subsequent exercise bouts.

SPs may also be involved in protecting specific fibre types. SP72, although generally considered to be an inducible protein, is constitutively expressed in rat skeletal muscle in proportion to the number of type I fibres (Locke et al., 1991; Locke and Tanguay, 1996). The reason for the differences in SP72 content in various muscles and muscle fibres remains to be determined, though it could be related to the relative stress imposed on the muscle or to a specific function for SP72 in fibres expressing MHC-I (reviewed in Locke, 1997). Such a specific function might include a role in the assembly and/or turnover of certain constituents of type I muscle fibres, or a response to the increased use of these fibres (Rice et al., 1988) and a subsequent need for protection. Therefore, type I fibres may be less

vulnerable to certain stresses and exhibit a reduced SP content (reviewed in Booth and Baldwin, 1996).

1.1 Purpose and Rationale

While the expression of SP72 following acute exercise (Locke et al., 1990; Salo et al., 1991, Skidmore et al., 1995) and short-term training (Kelly et al., 1996) has been well studied, the effect of long-term endurance training on SP72 expression in rat skeletal muscle has received little previous attention. Given the increased SP72 expression demonstrated following acute exercise and short-term training, it was of interest to determine if this response would persist following long-term endurance training and offer a continued cellular protection, or if the response would diminish as a result of an adaptive response to training.

In addition, studies in the endurance-trained rodent reveal that training induces transformations in MHC phenotype from the faster to the slower isoforms (Green et al., 1984). It has been demonstrated that for most locomotory movement there is an orderly pattern of recruitment from type I to type IIA to type IIX/IIB (Rice et al., 1988). This may account for the observation that the constitutive SP70 expression and MHC-I content in skeletal muscle appear to be related at rest (Locke et al., 1991). While this relationship is lost with electrical stimulation (Ornatsky et al., 1995) and short-term exercise training (Kelly et al., 1996), it may be regained after a longer period of adaptation to exercise. It was of interest, therefore, to examine if the SP72-MHC-I relationship would be restored following long-term endurance training where there might be a change in fibre type composition towards the slower type I fibres.

1.2 Hypotheses

The experiments described in the Methods chapter were designed to test the following three hypotheses:

1) The previously demonstrated exercise-induced increase in SP72 associated with acute exercise and short-term training, will not persist following 24 weeks of endurance training due to the adaptive response of muscle to training.
2) 24 weeks of endurance training will not cause a shift in the myosin heavy chain expression of trained muscle toward type I MHC.
3) The positive correlation between SP72 and MHC-I expression observed under control conditions will not exist following 24 weeks of endurance training.

2. LITERATURE REVIEW

2.1 The Heat Shock Proteins

All organisms, from the simplest prokaryotes to the most complex eukaryotes, respond to blunt changes in environmental circumstances by the rapid and preferred transcription, and subsequent translation of a set of highly conserved proteins known as heat shock proteins (HSPs) (Lis and Wu, 1993; Wu, 1995). The high conservation of the HSPs through evolution suggests that these proteins must serve a vital, universal function within the cell (Donnelly et al., 1992; Hunt and Morimoto, 1985).

Although HSPs are also referred to as stress proteins (SPs) (Welch, 1992), most of these proteins are constitutively expressed in the unstressed cell. They are essential for cell function under normal conditions of growth, and represent essential gene products involved in a number of important biological pathways (Georgopoulos et al., 1990; Gething and

Sambrook, 1992; reviewed in Morimoto et al., 1990). A select few of the SPs are expressed only in times of stress and their appearance is often an indication that the cell has experienced some type of trauma. The degree of expression is dependent upon the metabolic activities of the cell and induction is accomplished with remarkable speed (Welch, 1992).

Tissières and colleagues (1974) discovered the major SPs by analyzing newly synthesized proteins in *Drosophilia melanogaster* larvae. Since that time, SPs have been classified into groups according to the primary apparent mode of regulation. The two major groups arising from this differentiation are the HSPs and the glucose regulated proteins (GRPs). SPs are then further classified according to the protein's molecular weight and isoelectric point. In mammalian cells many SPs have been identified and grouped as follows: 20-30 kD-, 50-60 kD-, 70 kD-, 90 kD-, and 110 kD-family (Burrel et al., 1992; Knowlton, 1991; Morimoto et al., 1994; Welch and Suhan, 1986). The information regarding the role and expression of many of these SPs in most cells or tissues is limited.

2.1.1 The SP70 Family

The most strongly induced forms of stress proteins in mammalian cells have molecular masses of approximately 70 kD and isoelectric points between pH 5.2-6.3 (Pelham, 1986). The majority of exercise-related investigations, to date, have focused on the expression of these particular SPs. In rodent cells, 5 distinct isoforms of the SP70 family have been identified: HST70, HSP72, HSC73, GRP75 and GRP78 (Beckmann et al., 1990; Hunt et al., 1993; Wisniewski et al., 1990). Although separate, the genes coding for the various members of the SP70 family have a high degree of sequence similarity (Longo et al., 1993). The N-terminal domain of the SP70 protein contains an ATP binding site (Milarski

and Morimoto, 1989), whereas regions in the C-terminal domain are responsible for polypeptide binding (Hightower et al., 1994) and nuclear localization (Milarski and Morimoto, 1989). All of the related SP70 proteins share the common property of binding nucleotides, especially ADP and ATP, but different members of the SP70 family are localized to particular cellular compartments, and in response to physiological stress are redistributed to different intracellular locales (Beckmann et al., 1992a; Brown et al., 1993).

In mammalian cells, there are two major members of the SP70 family: the constitutive 73 kD protein and the inducible 72 kD protein, referred to in this paper as SP73 and SP72 respectively (Blake et al., 1990; Tanguay et al., 1993). These proteins exhibit sequence homology of approximately 95%, and similar biochemical properties (Guidon and Hightower, 1986; Li and Laszlo, 1985; Welch, 1992). One distinguishing feature between SP73 and SP72 is the presence of intervening sequences in the SP73 gene and the lack thereof in the SP72 gene (Gunther and Walter, 1994; Mestril et al., 1994). SP73 is expressed constitutively in all cells and is slightly increased in expression by heat shock or other oxidative stresses (Sorger and Pelham, 1987). While SP72 is present in the non-stressed cell, it is further induced following stress to the cell. During stress, both SP70 isoforms migrate from the cytoplasm to the nucleus and become associated with the preribosomal-containing granular region of nucleoli. During recovery from stress, SP72 and SP73 return to the cytoplasm and become associated with proteins and polyribosomes (Welch and Mizzen, 1988).

The glucose regulated proteins (GRPs), also members of the SP70 family, are not heat inducible. However, GRPs are induced by a diversity of stresses which perturb the N-linked glycosylation of nascent proteins, including but not limited to glucose deprivation,

calcium influx, and prolonged hypoxia (Lee, 1987; reviewed in Mestril and Dillmann, 1995; Mizzen et al., 1989). Although the regulation of GRPs is distinct from HSPs, they share considerable homology (Gunther and Walter, 1994). GRP75, found within the mitochondria, facilitates the translocation of precursor proteins across the mitochondrial membrane and is involved with the subsequent stabilization and folding of proteins once they are in the mitochondria (Mizzen et al., 1991). GRP78 resides in the sarcoplasmic reticulum (SR) and endoplasmic reticulum (ER) of cells (Mizzen et al., 1989; Munro and Pelham, 1986; Volpe et al., 1992). Under normal growth conditions, GRP78 is synthesized constitutively and abundantly and comprises about 5% of the lumenal content of the ER of mammalian cells (Bole et al., 1986; reviewed in Welch, 1992). Its synthesis can be further induced by the accumulation of secretory precursors or mutant proteins in the ER, or by a number of different stress conditions that lead to the accumulation of unfolded polypeptides (Gething and Sambrook, 1992).

2.1.2 The Heat Shock Response

The heat shock response was first observed in *Drosophilia* larvae, where altered puffing patterns of salivary gland chromosomes were noted in response to elevations in temperature (Ritossa, 1962). In virtually all organisms studied to date, SPs become the dominant product of protein synthesis within 15 minutes following exposure to high temperatures (ranging between 41°C and 42°C in rats) (Hutter et al., 1994; Lindquist, 1986; Lindquist and Peterson, 1990).

In addition to heat (Cairo et al., 1985; Currie and White, 1983; Donnelly et al., 1992; Locke et al., 1990), a variety of conditions have been observed to induce the heat shock

response (HSR) in mammalian cells. Therefore, the HSR is now more commonly referred to as the stress response. Changes in pH (Jurivich et al., 1992; Petronini et al., 1995), oxidative stress (Essig and Nosek, 1997; Salo et al., 1991; Sortz et al., 1979), glucose deprivation (Pouyssegur et al., 1977), increased intracellular calcium ion concentration (Jurivich et al., 1992; Lee, 1987), exposure to glucose or amino acid analogues (Beckmann et al., 1992a), hypoxia (Dwyer et al., 1989; Iwaki et al., 1993), ischemia (Emami et al., 1991; Iwaki et al., 1993; Knowlton et al., 1991), and exercise (Kelly et al., 1996; Locke et al., 1990; Locke et al., 1995b; Locke and Tanguay, 1996; Salo et al., 1991; Skidmore et al., 1995), all activate the response.

The activation of the stress response is mediated by the oligomerization (Westwood et al., 1991), nuclear translocation and binding of a transcriptional activator, the heat shock transcription factor (HSF), to a highly conserved DNA sequence known as the heat shock element (HSE) (Abravaya et al., 1992; Bornstein and Craig, 1990; Larson et al., 1988; Sarge et al., 1993). The HSE is located in the 5'-flanking sequence of all genes coding for SPs (Bienz and Pelham, 1986, 1987; Sorger, 1991). Phosphorylation of the HSF follows its binding to the HSE on the promoter of the SP gene to create a complex with high transcriptional activity (Bienz and Pelham, 1986; Mosser et al., 1990; Sarge et al., 1993; Sorger, 1991).

In yeast and *Drosophilia*, only one heat shock transcription factor has been cloned (Clos et al., 1990). However in larger eukaryotes, there exists a family of HSFs (Nakai and Morimoto, 1993). HSF1 is the primary activator of heat shock gene transcription (Sarge et al., 1993; Shi et al., 1998), while HSF2 is activated by hemin and may function to activate heat shock gene transcription during differentiation or other cellular processes (Morimoto,

1993). Although HSF3 has been identified, its activators remain to be determined (Nakai and Morimoto, 1993).

The HSF exists in the cytoplasm of unstressed cells as a non-DNA-binding monomer. Following exposure to protein-damaging stresses HSF is converted to a trimeric state and acquires DNA-binding activity (Baler et al., 1993). The DNA-binding activity of the HSF is negatively regulated through a feedback system, specifically by interfering with the function of the HSF (Abravaya et al., 1991; Baler et al., 1992; Morimoto et al., 1994; Mosser et al., 1993). The binding of the HSF to the HSE is termed HSF activation (Locke and Tanguay, 1996). HSF activation is one of the early steps in the stress response necessary for the stress-induced transcription/translation of SPs (Jurivich et al., 1992; reviewed in Locke and Noble, 1995), and has been shown to occur within minutes after exposure to stresses. During a heat shock treatment, SP70 transcription rates increase rapidly and then decline within 60 minutes (Findly and Pederson, 1981). By two hours HSF activation is undetectable (Locke and Tanguay, 1996). In *vivo*, a threshold between 41°C and 42°C must be reached for HSF activation to occur (Locke and Tanguay, 1996).

Heat shock gene expression seems to be autoregulated by SP70 (Abravaya et al., 1992; Beckmann et al., 1992b). In unstressed vertebrate cells, HSF exists in the cytoplasm complexed with free SP70 (Baler et al., 1992; Locke and Tanguay, 1996; Morimoto, 1993; Morimoto et al., 1994). Inducers of the HSR are proteotoxic or protein damaging, therefore during episodes of stress, the accumulation of damaged and/or malfolded proteins causes SP70 and HSF to separate in an ATP-dependent process, permitting SP70 to bind to the target proteins (Beckmann et al., 1990; Lewis and Pelham, 1985). The non-DNA-binding HSF monomer is rapidly converted to a DNA-binding trimer, at which point it binds to the

HSE, resulting in enhanced SP70 gene expression (Abravaya et al., 1992; Clos et al., 1990; Mosser et al., 1990; Morimoto, 1993; Shi et al., 1998). When the free pool of SP70 is restored to some critical concentration, activation of HSF is inhibited and the rate of SP70 protein synthesis is halted (Morimoto et al., 1994). Thus, the competition for SP70 by newly damaged proteins may alter the equilibrium between free and substrate-bound forms of SP70. Altering this equilibrium has been suggested as the signal leading to HSF activation and the stress response (Baler et al., 1992).

Although transcriptional regulation is the primary form of control for heat shock gene expression, the effects of heat shock on post-transcriptional events include the rapid and preferential translation of SPs, seemingly in part due to features within the 5' noncoding region of the mRNA (Lindquist, 1981), and a rapid reduction in the constitutive expression of non-heat shock genes. These events can be accounted for by the following 3 factors. First, an interesting and remarkable feature of SP72 genes, one common to all organisms, is an absence of intervening sequences or introns (Lindquist, 1986), as previously mentioned. This unique feature allows transcripts to bypass the general stress induced block in RNA processing (Wu et al., 1985; Yost and Lindquist, 1986), which prevents the splicing of transcripts produced at 25°C for at least 2 hours (Su et al., 1996). Pre-treatments at temperatures inducing SPs protect RNA splicing from disruption by subsequent severe heat shock, and the level of protection varies with the concentration of SPs in the cell. Second, within minutes of heat exposure, there is a general reduction in the expression of the non-heat shock genes typically expressed in the unstressed cell (Lindquist, 1986). This reduced expression seems to be regulated by decreased transcription of the non-heat shock genes due to faulty ribonucleoprotein assembly (Mayrand and Pederson, 1983), and results in less

efficient processing of the nascent transcripts. A rapid accumulation of SPs following stress is favored over other cellular proteins that require further processing. Finally, SP70 mRNA appears very unstable when expressed at normal temperatures (Petersen and Lindquist, 1989). During SP70 mRNA translation, pre-existing control mRNAs are under-translated due to the reduced stability of the mRNA (Theodorakis and Morimoto, 1987). A regulatory element at the 3' end of the SP70 mRNA has the ability to stabilize the mRNA at increased temperatures and destabilize the message once the temperature is returned to normal (Moseley et al., 1993). This feature targets SP70 mRNAs for rapid turnover at elevated temperatures thereby enhancing the selective translation of SPs. After heat shock, the SP70 mRNA can be detected immediately, remains elevated for at least two hours, and returns to control levels approximately six to eight hours post heat shock (Kiang et al., 1994; Locke et al., 1995a).

2.1.3 Protective Function of SPs

Welch (1990) demonstrated that a number of morphological and biochemical lesions, which occur as a result of heat shock treatment, are repaired more rapidly if the cells are first made thermotolerant. Thermotolerance is thought to be acquired through the functions of SPs, particularly SP72, since cells expressing elevated levels of the inducible SP72 protein show protection against high temperatures (Angelidis et al., 1991; Heads et al., 1994; Mizzen and Welch, 1988). Thermotolerance is typically acquired within 4 to 8 hours of the initial heat shock and is dependent on the kinetics of SP synthesis (Li and Laszlo, 1985; Welch, 1992). Cell culture studies have demonstrated that cells expressing a high SP72 content become capable of surviving normally lethal temperatures (Li et al., 1991). In addition,

when SPs are induced by stressors other than heat, thermotolerance is still displayed (Li, 1983).

Evidence for the protective role of SPs can also be obtained from investigations using whole animals. The term 'cross-tolerance' is most often used in these studies and describes the concept which suggests that the production of SPs by one stressor protects against a subsequent, yet different form of assault (Locke et al., 1995a; Marber et al., 1993). In cardiac muscle, the increased SP70 content after heat shock has been associated with myocardial protection and a reduction in infarct size (Currie et al., 1993; Karmazyn et al., 1990). For instance, rabbits subjected to hyperthermia with subsequent accumulation of SP72, demonstrate a significantly improved myocardial salvage following coronary occlusion and reperfusion (Currie et al., 1993; Donnelly et al., 1992). Plumier and colleagues (1995) demonstrated an enhanced post-ischemic recovery in the hearts of SP72 transgenic mice subjected to global ischemia. These studies, as well as others (Bradford et al., 1996; Liu et al., 1992), strongly suggest that heat shock and the accompanying accumulation of SP70 and/or other SPs provides protection to the myocardium during episodes of stress.

While the exact mechanism(s) by which SPs protect cells from stress remains unknown, it has been proposed that SP70 may prevent protein aggregation and denaturation or restore the function of damaged proteins (Marber et al., 1993; Sarge et al., 1994). A protective role for skeletal muscle has not been consistently shown, unlike that found in cardiac tissue. However, Garramone and co-workers (1994) found that prior heat shock treatment resulted in significant biochemical protection against ischemic injury to rat muscle.

The notion of cross-tolerance could have implications for the induction of SPs via exercise. Since induction of SPs by heat shock provides protection to cardiac tissue from

ischemic stress, it follows that induction of SPs by exercise might also provide protection to cells and tissues. Schwane and Armstrong (1983) have shown that a single bout of exercise can provide protection against injury from future exercise bouts. Therefore, it is offered that the ability of the pre-conditioning stress to protect against a subsequent more severe stress correlates with the ability to induce enhanced synthesis of SPs (Amin et al., 1995,1996; Li et al., 1992; Marber et al., 1993).

2.1.4 Additional SP Functions

In addition to protection from heat shock and other stresses (Bienz and Pelham, 1987; Li et al., 1991; Pelham, 1984; Riabowol et al., 1988), SPs have many functions such as translocation of nascent proteins (Brown et al., 1993; Gething and Sambrook, 1992; Langer et al., 1992), dissociation of certain protein complexes (Georgopoulos et al., 1990), protection from phenotype alterations, removal of abnormal proteins from the cell (Gething and Sambrook, 1992; Sherman and Goldberg, 1992), and participation in protein folding and stabilization (Beckmann et al., 1990).

SPs which perform any of these various functions have been grouped under the heading of 'molecular chaperones' (Ellis, 1987). Originally used to describe the SP60 family (Ellis and Van Der Veis, 1991), molecular chaperones refer to proteins directly implicated in protein degradation, as well as the stabilization, import and refolding of nascent polypeptides from the time of initiation of synthesis to the release of the native protein at its active site (Craig, 1992; Frydman et al., 1994; Hayer-Hartl et al., 1995; Morimoto et al., 1994). The interaction of the SP70 family member with its target protein is transient and non-covalent (Craig et al., 1992), and occurs in the presence of ADP (Hightower et al.,

1994). Release of the target is enhanced by ATP hydrolysis (Beckmann et al., 1990; Welch, 1992), although it is not required (Schmid et al., 1994).

During heat shock, SP72 is rapidly synthesized in the cytoplasm and migrates to the heat sensitive nucleus (Welch and Feramisco, 1984). Following heat shock, SP72 returns to the cytoplasm interacting transiently with newly synthesized and maturing polypeptides (Welch and Mizzen, 1988). Cytoplasmic SP70 stabilizes these unfolded proteins until they achieve final conformation or stabilizes the proteins in a translocation-competent form facilitating posttranslational translocation across the ER or mitochondrial membranes (Beckmann et al., 1990; Welch, 1992). The restoration of damaged proteins crucial for normal cell functioning could contribute to the restoration of cellular homeostasis, thus resulting in protection to the cell.

GRP75 is involved in translocation of precursor proteins through the lipid bilayer at mitochondrial contact sites (Black and Subject, 1991), and in folding of imported polypeptides in the mitochondrial matrix (Welch, 1992). As the precursor bound to SP70 enters the mitochondrion, it interacts with GRP75, which binds to unfolded segments appearing on the matrix side. GRP75 then releases the precursor, which then binds to the SP60 complex, acting as a scaffold for protein refolding and oligomer assembly (Ostermann et al., 1989). In the ER, GRP78 facilitates assembly of monomeric proteins into their final multimeric structure (Beckmann et al., 1990).

2.2 SPs and Exercise

2.2.1 Metabolic Effects of Exercise and Training

Skeletal muscle can undergo multiple phenotypic adaptations in response to physiological demands. A function of exercise adaptation is to minimize disruption of homeostasis during an exercise bout. Less disruption in homeostasis permits the animal to undergo physical work for longer durations at the same power output before fatigue (Holloszy and Coyle, 1984).

Physiological and biochemical adaptations that occur with exercise training in animals fall into two categories based on the duration of the change in environment. Alterations that occur on the same scale as a single bout of exercise are said to be acute exercise responses, whereas changes that persist for appreciable periods or as a consequence of physical training are said to be chronic physical adaptations (Booth and Thomason, 1991). Training adaptations can result from aerobic, anaerobic, and resistance training. However only adaptations to aerobic type training will be discussed within this paper.

In response to a single bout of exercise, decreased ATP, increased Ca^{2+} concentration, and decreased pH occur within the cell (Metzger and Fitts, 1987; Thompson et al., 1992). In the exercising muscle, glycogen depletion, elevated temperature, and increased lactate also occur (Newsholme and Leech, 1983). More precisely, there is a shift in glucose uptake to exercising muscles, a decrease in function of the SR and a decrease in malonyl-CoA concentration in exercising muscles. Malonyl-CoA serves as a regulatory molecule to inhibit fatty acid oxidation. Therefore, a decrease in malonyl-CoA enhances fatty acid oxidation, which in turn conserves the limited stores of carbohydrate in the body (Winder et al., 1989).

Oxyradical formation appears to be a certain outcome of mitochondrial metabolism. Aerobic exercise may increase the production of oxygen species as the result of mitochondrial uncoupling associated with elevated temperatures during exercise (Ji, 1995). Oxygen species damage functional components involved in the production of force, by attacking cellular membranes, affecting permeability characteristics and ionic gradients, and inducing cellular injury by modifying protein conformation (Davies and Goldberg, 1987). Following exhaustive exercise, Davies and colleagues (1982) reported increased production of oxyradicals in skeletal muscle. If the degree of membrane disruption is sufficient to result in cell damage, initiation of the stress response might occur (Amelink et al., 1990). It has been suggested that oxidative stress may be a direct initiator of the stress response (Salo et al., 1991).

The changes related to endurance training are associated with increased mitochondrial density and increased capacity of the trained skeletal muscle to generate ATP aerobically by oxidative phosphorylation (Holloszy, 1967; Holloszy and Booth, 1976). Associated with the increased capacity for mitochondrial oxygen uptake is an increase in both the size and number of mitochondria and a potential twofold increase in the level of aerobic system enzymes (Annex et al., 1991; Galbo, 1983). Endurance training also increases the potential for maximal blood flow per unit of trained skeletal muscle during aerobic exercise due to an increase in capillary density per unit volume of muscle (Rowell, 1993; Saltin and Gollnick, 1985). Skeletal muscle myoglobin content is increased resulting in an increase in the quantity of oxygen within the cell at any one time (Katch et al., 1991). Following endurance training, exercise at a given submaximal VO_2 elicits smaller increases in muscle and blood lactate concentrations, a slower utilization of carbohydrate, and an

increased reliance on fat oxidization as an energy source (Abdelmalki et al., 1996; Green et al., 1984; reviewed in Holloszy and Booth, 1976). Acute exercise responses such as high intracellular calcium, elevated body temperature, muscle damage, and change in cellular pH have been shown to be diminished with training, permitting the muscle to regain homeostasis during exercise.

Exercise-induced muscle damage is reflected biochemically by elevations of serum enzymes and myoglobin (Noakes, 1987). Interestingly, factors influencing the release of these enzymes are the intensity and duration of the physical exercise and the type of exercise, but also individual variables such as training status and sex (Ebbeling and Clarkson, 1989; Noakes, 1987). Oestrogen is a hormone found in much higher concentrations in females than in males (Shangold, 1984), and has been shown to reduce post-exercise muscle damage. Oestrogen has been shown to stimulate the transcription and translation of HSF1 and HSF2, which makes oestrogen the first identified cellular factor involved in the regulation of HSF gene expression (Yang et al., 1995). Furthermore, it has been demonstrated (Amelink and Bär, 1986) that oestradiol is a mediator of the sex-linked difference in the susceptibility to muscle damage, possibly by modifying intracellular calcium homeostasis (Amelink et al., 1990).

2.2.2 SPs and Protein Turnover

Exercise is a physiological stress in which the synthesis and degradation of proteins directly involved in muscle contraction and energy provision are stimulated (Booth and Thomason, 1991; Pette and Vrbova, 1992). The regulation of protein turnover in skeletal muscle not only determines protein homeostasis in this tissue, but also influences energy

metabolism and the physiological status of the whole body (Zhou and Thompson, 1997). The continuous turnover of proteins permits muscle plasticity (Goldberg and Rice, 1974), and the time required to alter muscle plasticity is a function of protein half-life (Schimke, 1970). The amino acid glutamine has been reported to regulate protein turnover in skeletal muscle, and it has been shown that increased glutamine concentrations increase the levels of SP70 in stressed *Drosophilia* cells (reviewed in Hughes et al., 1997). These findings suggest that SP70 may play a role in the mechanism by which glutamine enhances the rate of protein synthesis.

Protein turnover has been shown to increase with the muscle remodeling associated with exercise (Booth and Thomason, 1991). Noble and associates (1984) demonstrated an increased protein turnover following compensatory hypertrophy in the rat. During adaptation to exercise, proteins, which turn over rapidly, reach a new steady state level faster than proteins that turn over slowly. Goldberg and Rice (1974) suggest that proteins with short half-lives may have evolved to permit crucial enzymes to fluctuate rapidly with changing physiological conditions. This could provide enhanced survival, or a more rapid adaptability to exercise training (reviewed in Booth and Baldwin, 1996).

2.2.3 SP Expression and Exercise

As with heat shock (Locke et al., 1995a), the physiological stress created by exercise causes alterations to the cellular homeostasis resulting in a very rapid and substantial stress response (Hernando and Manso, 1997; Locke et al., 1990; Ryan et al., 1991; Salo et al., 1991; Su et al., 1996). Hammond and associates (1982) were the first to use exercise as a stimulus for the induction of SPs. It has since been shown that the physiological stress

created by treadmill running is sufficient to induce SPs in a variety of tissues (Locke et al., 1990; Salo et al., 1991, Skidmore et al., 1995). Following 20 minutes of treadmill running, exercising rats exhibit HSF activation, and SP72 and mRNA activation follows thereafter (Locke et al., 1990). In a study where rats were run to exhaustion (Salo et al., 1991), significant increases in SPs were found in the liver, heart, and hindlimb muscles following exercise. Interestingly, with 8 weeks of exercise training (Kelly et al., 1996) or with continuous electrical stimulation (Neufer et al., 1996), the increase in SP70 response is not diminished as the muscle adapts to the exercise load. Kelly and co-workers (1996) suggest that the increased synthesis of SP72 observed in skeletal muscle after a single bout of exercise may not represent simply an acute stress response but may be associated ultimately with some more long-term, adaptive process. However, since the exercise training periods used in the above-mentioned studies are relatively short, it is possible that the increase in SP72 demonstrated following exercise might still only represent an acute response.

Although the exact component of exercise responsible for the induction of SPs remains unknown, exercise-induced hyperthermia might be expected to induce or enhance SP expression. Skidmore and associates (1995) employed exercise in a cool environment as a means of separating other potential SP-inducing effects of exercise from that of elevated temperatures, and found an accumulation of SP72 in several muscles of treadmill-running rats despite their having a core temperature that was not different from baseline. Therefore, factors other than elevated temperature must be involved in the initiation of the stress response to exercise.

At present, little is known regarding the expression of SPs in tissues following long-term exercise training. Preliminary studies suggest that certain SPs may be elevated with

exercise training (Brickman et al., 1996; Samelman and Alway, 1996; Sim et al., 1991). However, in all cases, the speed or duration of exercise was progressively increased. Therefore any increase in SP content may simply reflect the relative change in exercising intensity or duration from the previous exercise level. A large amount of evidence proposes that cells of an exercising organism with elevated SP70 content become more resistant to protein damage by permitting the cells to more easily withstand the biochemical and physiological stresses that often accompany exercise. However, because the HSR is observed as a transient response required by cells to allow them to cope or adapt to a new level of stress, it remains unknown what happens to SP content following exercise at the same intensity and duration for several weeks (Locke, 1997). The present study may offer an explanation.

2.3 Skeletal Muscle Myosin

Myosin, a protein consisting of two heavy (approximately 200 kD each) and four light chains (approximately 20 kD each), makes up the largest part of the contractile apparatus in skeletal muscle fibres (reviewed in Barton and Buckingham, 1985). The ability of different light and heavy chains to associate in many various combinations makes for a large variety of myosin isoforms (reviewed in Pette and Staron, 1990). However, the contractile characteristics of muscle are determined essentially by the pattern of myosin heavy chain (MHC) expression (Maughan et al., 1997). Myosin light chain (MLC) composition, although not the primary determining factor, can modify the contractile characteristics of the muscle (Bottinelli et al., 1994).

2.3.1 Fibre Type Classification

The organization of fibre types with different characteristics is a primary determinant of the various expressions of muscle function (Lamb et al., 1991; Maughan et al., 1997). Different fibre classification schemes have been developed based on contractile properties, metabolic markers, and histochemical and biochemical parameters (Gollnick and Hodgson, 1986; Gordon and Pattullo, 1993; Pette and Staron, 1990).

Mammalian skeletal muscles are composed of a heterogeneous population of muscle fibres, individually adapted and differing in a number of ways. Slow oxidative, or type I, fibres are responsible for sustained contractions of muscle and contain slow MHC, which has a low actin-activated ATPase activity. Type I fibres predominate in motor units used extensively for antigravity function (Booth and Baldwin, 1996). In addition, type I skeletal muscle is known to have a higher intrinsic rate of protein turnover than other muscle types (Obinata et al., 1981). In contrast, there are at least three fast, or type II, fibre types with distinct contraction speeds that are sequentially recruited for progressively more forceful movements. Fast oxidative IIA, fast intermediate IIX and fast glycolytic IIB fibres express IIA, IIX, and IIB MHC genes, respectively, and the corresponding MHC proteins hydrolyze ATP successively faster so that the fibre types contract with progressively faster kinetics (Schiaffino and Reggiani, 1996; Maughan et al., 1997). Motor units that are used almost exclusively for short-duration, high-power output activity express chiefly the IIB and IIX MHCs (Adams et al., 1993), whereas, fast motor units used for more endurance types of activity appear to abundantly express the IIA MHC isoform (Green et al., 1984).

Muscles frequently contain a mosaic of different fibre types to give them a wide range of contractile responses in normal movement (Burke et al., 1981; reviewed in Hughes

et al., 1997; Pette and Staron, 1990), and several general patterns of fibre type distribution have been described in the rat. The deepest portion of limb muscles of rodents are typically composed of high proportions of type I and type IIA fibres and the most superficial regions of type IIB fibres (Armstrong and Phelps, 1984).

It appears that under most circumstances there is an orderly recruitment pattern of different motor unit types for most locomotory movement (Rice et al., 1988). In general, smaller motor units posses axons with the lowest firing threshold. These axons usually innervate type I fibres and as a result, when recruited, forces are small but can be sustained for long periods of time. At the other spectrum, large motor units possessing large high-threshold myelinated axons innervating type IIB fibres are recruited for the production of great force and fire in brief high frequency bursts for a limited period of time (McComas, 1996). These fibre types are progressively recruited in the exercising muscle with increasing levels of force requirement in the following order, type I → IIA→ II X → IIB (Rice et al., 1988).

2.3.2 MHC Gene Expression

Adaptations in skeletal muscle, in response to training, are specific to the muscles used in the activity. Changes in the activity patterns of a particular muscle can cause alterations in gene expression. Thus, it seems that muscle genes are regulated largely by mechanical and/or metabolic stimuli (Maughan et al., 1997).

The genes coding for myofibrillar protein isoforms belonging to the same family share a similar basic structure, which reflects a common evolutionary origin. Myofibrillar

protein genes are regulated by a variety of transcription factors, in particular MyoD and myogenin, which bind to DNA sequences located in 5'- or 3'-flanking regions of the genes (Hughes et al., 1993, 1997; Schiaffino and Reggiani, 1996). Individual fibres within a muscle can differ from one another in the members of the MHC gene family that each expresses (Stockdale, 1997). For example, MyoD mRNA is expressed primarily in fast muscle of adult rats (Hughes et al., 1997). This expression changes in response to manipulations that alter muscle fibre type, which suggests that MyoD may be involved in the regulation of fibre type-specific gene expression in adult muscle fibres (Hughes et al., 1993). Hughes and colleagues (1997) propose that the presence of MyoD in specific rodent tissue types may reflect continued growth or the ongoing turnover or repair of lesions caused by normal exercise in freely moving animals.

Myosin genes can alter contractile properties by rebuilding myofibrils using a different type of MHC (Maughan, 1997). A fast-twitch muscle fibre could become a slow fibre by switching off the gene for the fast-MHC and switching on the gene for the slow isoform. Most genes in the cells of the body are switched on and off by the incident actions of signaling molecules such as hormones or growth factors. Therefore, MyoD may regulate transcription of different genes in distinct fibre types by acting in combination with other transcription factors that differ between fibres.

2.3.3 MHC Expression and Exercise

In spite of a high degree of specialization, mammalian skeletal muscle fibres represent versatile entities, which adapt to altered functional demands, hormonal signals, and neural input. In the process, muscle fibres acquire physiological and biochemical

characteristics which appear better suited to the new functional requirements. An increase in demand can be generated by the central nervous system, for example, in exercise (Locke et al., 1994), or by electrical stimulation (Neufer et al., 1996). Endurance exercise and chronic stimulation differ in degree of change. The properties which change in response to exercise are those that are altered at an early stage of stimulation. Properties resistant to change under exercise conditions alter only after prolonged stimulation (Salmons and Henricksson, 1981). Thus, exercise differs from electrical stimulation in the more intermittent pattern of activation imposed on the muscle, the more selective recruitment of motor units, and the potential provided for the operation of hormonal and other systemic factors.

Training can have a strong influence on the functional and metabolic properties of muscle and its component fibres. Studies in the endurance trained rodent reveal that low force, high frequency activity patterns are capable of inducing transformations in MHC phenotype to the slower isoforms seen in endurance trained musculature (Abdelmalki et al., 1996; Fitzsimons et al., 1990; Green et al., 1984; Hernando and Manso, 1997). An increase in type I and type IIA fibres and a decrease in type IIB have been demonstrated to occur in rat hindlimb muscle following 15 weeks of extreme training (Green et al., 1984). Conversely, following 8 weeks of treadmill training, Kelly and co-workers (1996) found that the rat hindlimb muscles examined failed to show significant shifts in MHC-I content between sedentary and trained groups. This difference could be due in part to the differing duration and intensity of training. In the former study (Green et al., 1984), rats were run on a treadmill twice a day and the intensity and duration of the run were progressively increased. In the latter study (Kelly et al., 1996), the subjects were run only once a day, and

the speed, grade and duration, were not altered. The more aggressive and longer duration study by Green and asscciates may have caused fatigue to occur in type I muscle fibres forcing the recruitment of type II fibres to sustain the endurance-type exercise load, therefore increasing the demand for fibres with type I characteristics. In addition, the less aggressive training program in the 8-week study may not have permitted fibre type changes to occur.

A non-physiological stress commonly used to study adaptability of muscle is chronic electrical stimulation, which can dramatically alter biochemical and functional properties (Ausoni et al., 1990; Booth and Thomason, 1991; Neufer et al., 1996). Unlike exercise, the activity is restricted to the stimulated muscle, and the muscle is less influenced by other changes that can occur in the body during training, such as hormonal changes. Artificial stimulation bypasses the central nervous system and activates all motor units equally, whereas during exercise individual motor units are activated in a graded and hierarchical manner (Pette and Vrbova, 1992). Chronic electrical stimulation evokes changes in nearly every aspect of the structural and biochemical characteristics of the innervated muscle (e.g. induction of mitochondrial biogenesis) (Ornatsky et al., 1995).

Changes in muscle load by factors other than exercise and electrical stimulation, have also been shown to affect the composition of muscle fibre types, myofibrillar protein isoform patterns, and gene expression (Essig et al., 1991; reviewed in Pette and Vrbova, 1992). Exposure of rodents to hindlimb suspension or limb immobilization induces alterations in both fibre mass and MHC phenotype in the unloaded skeletal muscle (Baldwin et al., 1990; Diffee et al., 1991; Haddad et al., 1993). Tsika and co-workers (1987) used hindlimb suspension to unload the predominantly type I muscles causing the myosin profile to shift from type I to the faster, less oxidative type IIX and IIB isoforms. Other studies (Locke et

al., 1994; McCormick et al., 1994) have used compensatory overload, by surgical removal of synergists, in fast muscle and found that the muscles expressed the slower, more oxidative type I myosin isoform.

2.4 SP-MHC Relationship

There is increasing evidence of a muscle phenotype specific pattern of stress protein expression (Riley et al., 1993; Neufer and Benjamin, 1996). Although primarily considered a stress inducible protein, it has been demonstrated that the constitutive expression of SP72 is roughly proportional to the type I muscle fibre composition (Kelly et al., 1996; Locke et al., 1991; Locke and Tanguay, 1996; Ornatsky et al., 1995). Resting levels of SP73, however, are relatively constant among different muscle types and other tissues (Hernando and Manso, 1997; Kelly et al., 1996). This may suggest that the expression of both stress proteins, SP72 and SP73, is not coordinated and points towards a possible specific function for SP72 in skeletal muscle that is only prevalent in slow-twitch fibres (Hernando and Manso, 1997). Due to the higher oxidative capacity of these fibres, one of the suggested functions of SP72 involves protection and repair of proteins from oxidative stress (Locke et al., 1991). However, this explanation does not account for the very low SP72 expression in highly oxidative tissues such as the liver and myocardium (Currie and White, 1983; Locke et al., 1991). Myocardium is known to express a large amount of β-MHC, which is identical to type I MHC (Lompré et al., 1984). The β-MHC gene is under the control of separate regulatory systems in cardiac and skeletal muscle (Morkin, 1993; Rindt et al., 1993) which suggests that regulatory factors may account for this apparent inconsistency.

Since type I muscle fibres tend to be recruited more frequently for ambulation and postural maintenance, the stress of muscle loading could account for the elevated constitutive expression of SP72 (Noble and Aubrey, 1994). Similarly, the higher rate of protein turnover demonstrated in frequently recruited type I muscle fibres could represent a stimulus for the enhanced SP72 expression (Ornatsky et al., 1995). Myogenic regulatory factors seem to be responsible for the distinctive pattern of protein expression in various muscle fibre types (Hughes et al., 1993). Specifically it has been demonstrated that MyoD, a muscle-specific transcription factor, may reflect continued growth or the ongoing turnover or repair of lesions caused by normal exercise in freely moving animals (Hughes et al., 1997). Hence, regulation by specific myogenic factors could also provide an explanation for the constitutive SP72 expression in type I MHC- rich muscle.

Alpha B-crystallin is a major structural protein of the ocular lens (Wistow and Piatigorsky, 1988) as well as a genuine heat shock protein in non-lens tissue (Jakob, 1993). This protein is abundantly expressed in tissues with high oxidative capacity, including the heart and type I skeletal muscle fibres (Piatigorsky, 1992), and is regulated by MyoD transcription factor during myogenesis (Edmonson and Olson, 1993). Alpha B-crystallin and SP27 share considerable sequence and structural similarity and are co-induced in response to heat and oxidative stresses (Head et al., 1994). Interestingly, Neufer and Benjamin (1996) demonstrated that αB-crystallin expression is found in type I skeletal muscles and suggested that this protein may be regulated by shifts in the demand for oxidative metabolism. This raises the possibility that the expression of specific SPs such as SP70 may identify those fibres that have initiated an adaptive response to exercise, thereby implying that recruitment

during the remodeling process may proceed sequentially from type I and type IIA to type IIB fibres (Neufer et al., 1996).

A number of studies have documented the expression of SPs in specific skeletal muscles in conjunction with acute exercise (Kilgore et al., 1994; Locke et al., 1990; Locke et al., 1991; Salo et al., 1991; Skidmore et al., 1995). Neufer and colleagues (1996) demonstrated that the induction of SP70 during the adaptive response to chronic motor nerve stimulation proceeds from type I/IIA to type IIX/B fibres. However, Ornatsky and co-workers (1995) indicate that this relationship is not maintained during the physiological stress imposed by chronic contractile activity. Following 10 days of chronic stimulation, trace amounts of type I MHC in the tibialis anterior muscle remained unchanged, whereas the amount of type IIB/IIX MHC decreased. Despite this, a 9-fold increase in the level of SP70 occurred, which suggests that MHC and SP72 gene expression are independently regulated in skeletal muscle.

Locke and Tanguay (1996) demonstrated that HSF activation and inactivation are more rapid in muscles predominantly comprised of type I fibres. In addition, the lack of HSF activation in type I muscle during unstressed conditions may suggest that the increased SP70 expression observed in these muscles during unstressed conditions is not the result of HSF-HSE interaction. Therefore, it is proposed (Locke and Tanguay, 1996) that some other region of the SP70 promoter may be responsible for the high level of SP70 expression observed in type I muscles during non-stressed conditions. As well, the tissue distribution of the HSF (Nakai and Morimoto, 1993) could account for the tissue-specific expression of SP72.

The increased amount of constitutive SP72 in slow muscles might permit these fibres to respond more rapidly to stress. Since type I muscle fibres are recruited more frequently than type II fibres, type I fibres may have to respond to intracellular changes more frequently and more rapidly than type II muscle fibres. Locke and Tanguay (1996) suggest that the stress of the cellular environments of the fast and slow muscles and their respective fibres may be very different and might necessitate the need for high levels of specific SPs to provide a more precise control of the stress response.

3. METHODS

All procedures involving animals were approved by the University of Western Ontario Committee for Animal Care, and were performed in accordance with the guidelines of the Canadian Council on Animal Care (Olfert et al., 1993).

3.1 Animal Care and Training Paradigm

Untrained female Sprague-Dawley rats (Charles River Laboratories), 3-4 months old at the beginning of the study, were employed. The animals were maintained on a 12:12h dark-light cycle, individually housed at 20 ± 1°C with 50% relative humidity, and fed and watered ad libitum.

Animals chosen randomly were either maintained at rest (C, n = 6-9) or run on a motor driven treadmill (T, n = 8). The training paradigm consisted of an 8-week build up, starting with 10 min/day at 23 m/min, 10 % grade. The first 4 weeks of the 8-week build-up involved a time increase, and the second 4 weeks involved an intensity increase. The 8-week

build up was followed by 16 weeks at full intensity, which consisted of 15 min at 25 m/min, followed by 30 min at 30 m/min, and 15 min at 40m/min, 18% grade.

3.2 Tissue Removal and Collection

Animals were anaesthetized with pentobarbital sodium (60 mg/kg i.p.). The skeletal muscles of interest [soleus (Sol), plantaris (Pla), diaphragm (Dia), red (RV) and white (WV) portions of the vastus, red (RG) and white (WG) portions of the gastrocnemius, frontalis (Fro)], and the liver (Liv) were carefully dissected prior to sacrifice, immediately frozen in liquid nitrogen, and stored at -80°C for subsequent analysis. In certain animals, particular muscles were unavailable for dissection due to damage, which occurred to the tissue sample. Animals were sacrificed 48 hours after the last exercise bout of the training program.

The liver, an organ subjected to increased abdominal heat but inactive, and the frontalis (type IIB), an inactive skeletal muscle also subjected to the circulating hormonal milieu, provide control models for SP induction due to heat, catecholamines and blood hormones generated in the body as a result of exercise. The diaphragm (type I/IIA) provides a model for chronically active muscle exposed to transient increases in activity. The plantaris (mixed), soleus (predominantly type I), red gastrocnemius (type IIA), white gastrocnemius (type IIB), red vastus (type IIA/IIB), and white vastus (type IIB), muscles highly active during exercise, provide models for skeletal muscles with different average daily activity levels, recruitment patterns and fibre types (Armstrong and Phelps, 1984; Gosselin et al., 1996).

3.3 SP72 and MHC-I Protein Analysis

3.3.1 Polyacrylamide Gel Electrophoresis

Frozen muscle samples weighing 40-80 mg were homogenized in 20 vol. of 600 mM NaCl and 15 mM tris (hydroxymethyl) amino-methane (Tris), pH 7.5, containing 0.1 mM EDTA, 1.0 mM dithiothreitol (DTT), 0.5 mM PMSF, 0.5 μ/ml aprotinin, 1.0 mM benzamidine, 1.0 mM leupeptine, and 1.0 mM pepstatin in ddH_2O as protease inhibitors. Samples were homogenized with a Tekmar TR-10, set at 70 on rheostat scale. During homogenization, samples were kept on ice. Protein concentrations were determined using the technique described by Lowry and colleagues (1951) using known amounts of purified bovine serum albumin (BSA) to generate a standard curve. To determine the individual muscle sample loads for use in gel electrophoresis, a standard curve was used and values were taken within the linear range.

Whole muscle homogenates were processed using a BioRad mini protein II one-dimensional sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) system to separate the component proteins. A modification of the method of Laemmli (1970) was used, as described previously (Locke et al., 1994), except that the separating gel consisted of either a 10% in acrylamide for SP72, or a 15% polyacrylamide gradient for type I MHC immunoblotting. As a positive control, samples were run against a standard containing a constant concentration of SP72 (50μg load) and MHC-I (25μg) (Sprague-Dawley Sol muscle homogenates). For SP72 analysis, gels were loaded with 40 μg Sol, 75 μg RG, 80 μg Dia, 125 μg Pla, 120 μg RV, 200 μg WV, 200 μg WG, 250 μg Fro, or 150 μg Liv of protein homogenates. For MHC-I protein analysis, gels were loaded with 25 μg

Sol, 40 μg RG, 30 μg Dia, 30 μg Pla, 20 μg RV, 50 μg WV, 100 μg WG, or 100 μg Fro protein homogenates.

3.3.2 Protein Transfer and Immunoblotting

After electrophoretic separation, protein transfer was performed (Towbin et al., 1979) in a BioRad Mini-Protean trans-blot apparatus as described previously by Locke and associates (1994). Two sandwiches consisting of 2 pieces of filter paper, a gel, a piece of 0.2 mm pore size nitrocellulose paper and two more pieces of filter paper were placed in a blotting folder between two scouring pads. The apparatus was placed at 4°C and run for a total of 180 volt·hours, while maintaining the transfer buffer temperature below 15°C.

Following protein transfer, the nitrocellulose membrane was blocked with 5% non-fat dry milk powder (DMP) in Tris-buffered saline (TBS; 500 mM NaCl, 20 mM Tris·HCL, pH 7.5) for 6 hours at room temperature. To check for any residual protein, gels were stained with 0.5% Coomassie Brilliant Blue G in 50% methanol and 10% glacial acetic acid, and de-stained in 50% methanol containing 10% glacial acetic acid. Blots were washed twice for 5 minutes in TTBS (TBS plus 0.05% Tween 20) and incubated in a primary antibody, which consisted of SP72 (1:1000 dilution) or type I MHC (1: 100 dilution) in TTBS with 2% DMP and 0.02% sodium azide as a preservative. Blots were flipped several times over the first two hours of the incubation and then left in the solution for a total of 16 hours at 4°C on a rotary shaker. Following the primary antibody incubation, blots were washed twice in TTBS for 5 minutes each with shaking and transferred to a secondary antibody solution for 3 hours. This solution consisted of goat anti-mouse immunoglobulin G conjugated to alkaline

phosphatase (Bio Rad) in a 1: 3000 dilution with 2% DMP in TTBS. The membranes were washed twice in TTBS and once in TBS, with shaking, and developed immersed in a carbonate buffer (100 mM Na_2CO_3, 1 mM $MgCl_2$, pH 9.8), which contained 3% (wt/vol.) *p*-nitro blue tetrazolium chloride *p*-toluidine salt in 70% *N,N*-dimethylformamide (DMF) and 15% (wt/vol.) 5-bromo-4-chloro-3-indolyl phosphate in 100% DMF. After development, membranes were washed in H_2O and quantification of SP72 and MHC-I from immunoblots relative to a coelectrophoresed standard was performed by scanning the blots using a laser densitometer and recording integrator. The anti-heat-shock 72-kD protein monoclonal antibody was purchased commercially (StressGen #SP-810), while the type I MHC specific monoclonal antibody was generously donated by Dr. Peter Merrifield, Department of Anatomy, The University of Western Ontario, London, Ontario.

3.4 Statistical Analysis

Levels of SP72 and MHC-I concentrations within each muscle and tissue examined from the long-term endurance trained animals and the control animals were reported as means ± SEM and compared by using individual t-tests. The level of significance was set at $P < 0.05$. Utilizing a Pearson Product Moment Correlation, all SP72 data from the trained and control groups were compared to the MHC-I data. A correlation was detected with the level of significance set at $P < 0.05$.

4. RESULTS

4.1 SP72 Protein Data

Figure 1a shows a typical Western blot sample (diaphragm) indicating inducible SP72 content from three control animals (C) and three animals subjected to 24 weeks of endurance exercise training (T). Similar gels were loaded with Sol, RG, Dia, Pla, RV, WV, WG, Fro or Liv protein homogenates, separated by SDS-PAGE, transferred to nitrocellulose and reacted with antibody for SP72, as described in the Methods section.

SP72 levels were measured and quantified as a percentage of a standard (Sprague-Dawley) soleus sample with a constant SP72 content. The data from control and trained animals presented in Figure 2 and Appendix 1 show the SP72 content of Sol, RG, Dia, Pla, RV, WV, WG, Fro, and Liv as determined by Western blotting with anti-SP72. Data are percentages (means ± SEM) of standard soleus sample. Differences between control and trained groups are significant at $P < 0.05$.

T-tests were used to compare the heat shock response among the control and trained groups within each tissue (Appendix 2). Compared to control groups, the exercise trained Sol, RV, RG, and WV muscle demonstrated significantly higher SP72 content, while all other muscles examined demonstrated no significant differences. Elevation of body temperature, as evidenced by the lack of increase in Liv, or hormonal milieu, as evidenced by the lack of response in the non-exercised Fro muscle, do not appear to account entirely for the observed training effects.

To some degree, SP72 content following long-term training appeared to be inversely proportional to the level of SP72 present in the untrained muscle. Accordingly, Pla muscle

Std T1 C1 T2 C2 T3 C3

Figure 1a Sample Western blot indicating SP72 content of diaphragm (Dia; load 80ug) from three control animals (C) and three animals subjected to 24 weeks of endurance training (T). 50ug of soleus standard were loaded for quatification and measurement. Similar gels were loaded with 40ug Sol, 75ug RG, 125ug Pla, 200ug WV, 200ug WG, 250ug Fro, or 150ug Liv, transferred to nitrocellulose and reacted with anti-SP72 as described in METHODS.

Std T6 C6 T7 C7 T8 C8

Figure 1b Sample Western blot indicating MHC-I content of diaphragm (Dia; load 30ug) from three control animals (C) and three animals subjected to 24 weeks of endurance training (T). 25ug of soleus standard were loaded for quatification and measurement. Similar gels were loaded with 25ug Sol, 40ug RG, 30ug Pla, 50ug WV, 100ug WG, or 100ug Fro, transferred to nitrocellulose and reacted with anti-MHC-I as described in METHODS.

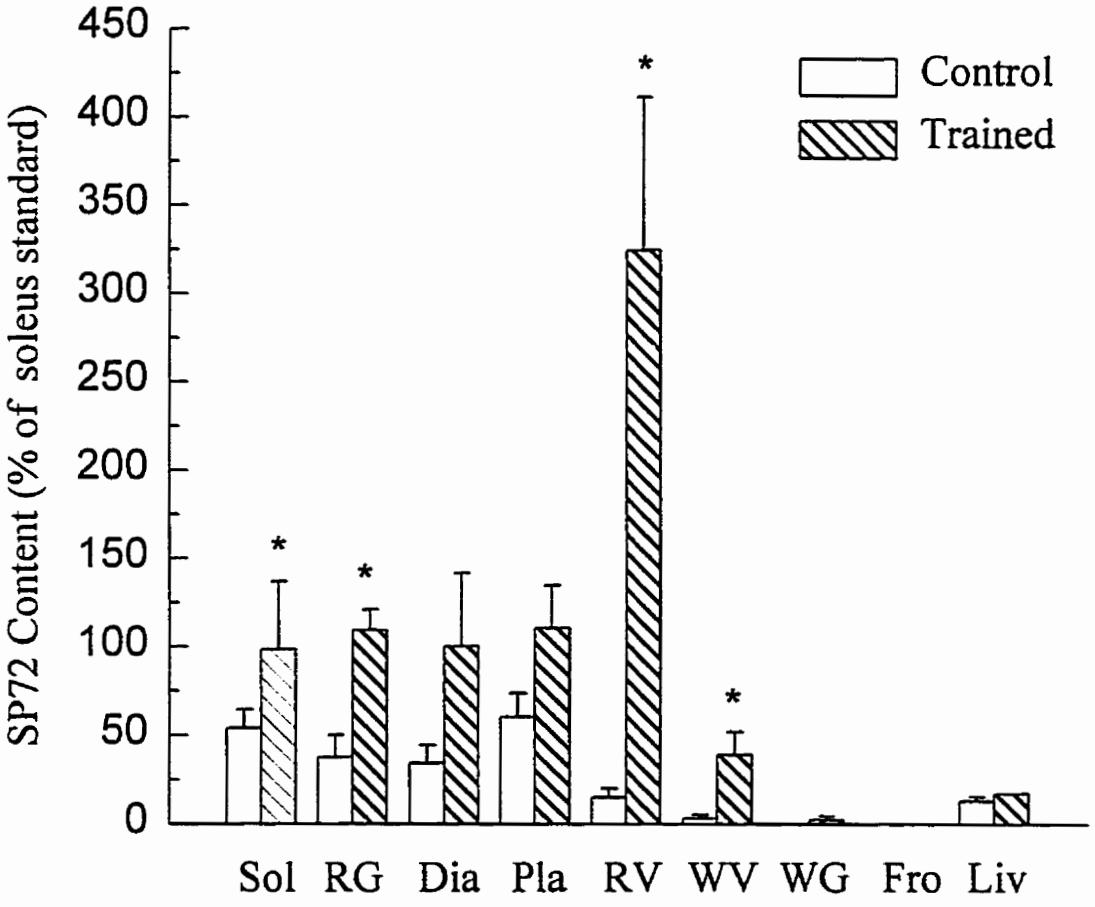

Figure 2 SP72 content of soleus (Sol), red gastrocnemius (RG), diaphragm (Dia), plantaris (Pla), red vastus (RV), white vastus (WV), white gastrocnemius (WG), frontalis (Fro), and liver (Liv) as determined by Western blotting with anti-SP72. Data are percentages (means $\pm$ SEM) of standard (Sprague-Dawley) Sol sample. C, control animals (n = 6-9); T, 24 weeks endurance trained animals (n = 6-8). Differences between control and trained animals are significant (*) at $P < 0.05$.

expressed the highest constitutive SP72 levels but exhibited less than a twofold increase in SP72 levels following training. In contrast, RV had low SP72 sedentary control levels, yet SP72 levels rose dramatically following training exhibiting a greater than 23-fold increase (Figure 2, and Appendix 2). Other factors were involved, however, as low control SP72 levels in WG were not significantly elevated by exercise training.

4.2 MHC-I Protein Data

Figure 1b shows a typical Western blot sample (diaphragm) indicating type I MHC content from three control animals (C) and three animals subjected to 24 weeks of endurance exercise training (T). Similar gels were loaded with Sol, RG, Dia, Pla, RV, WV, WG, or Fro protein homogenates, separated by SDS-PAGE, transferred to nitrocellulose and reacted with antibody for MHC-I, as described in the Methods section.

MHC-I levels were measured and quantified as a percentage of a standard (Sprague-Dawley) soleus sample with a constant MHC-I concentration. The data from control and trained subjects presented in Figure 3 and Appendix 3 show the MHC-I content of Sol, RG, Dia, Pla, RV, WV, WG, and Fro as determined by Western blotting with anti-MHC-I. Data are percentages (means ± SEM) of standard soleus sample. Differences between control and trained groups are significant at $P < 0.05$.

T-tests were used to compare the main effects among the control and trained groups within each muscle (Appendix 4). Compared to control groups, RG and RV demonstrated significantly higher MHC-I levels following 24 weeks of long-term endurance training, while all other muscles examined failed to show a significant difference in MHC-I content.

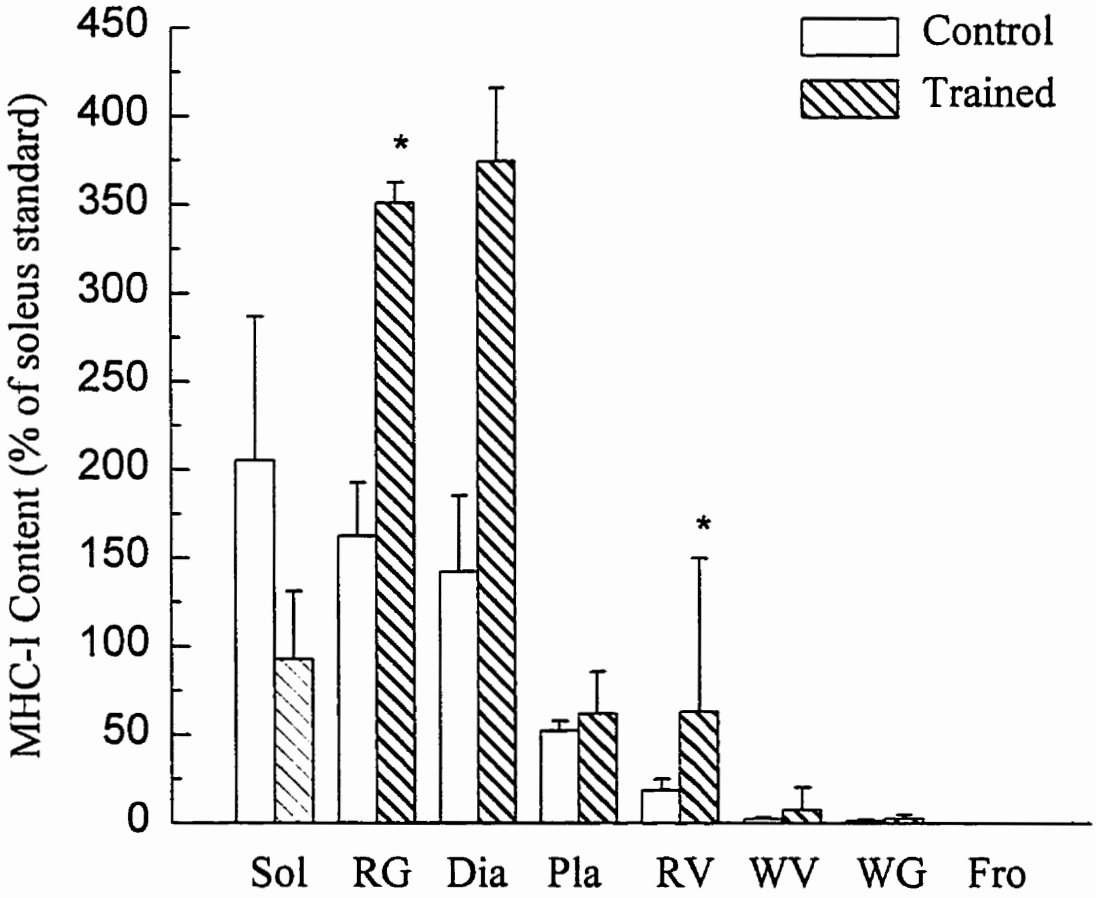

Figure 3 Type I myosin heavy chain (MHC-I) content of soleus (Sol), red gastrocnemius (RG), diaphragm (Dia), plantaris (Pla), red vastus (RV), white vastus (WV), and frontalis (Fro) as determined by Western blotting with anti-MHC-I. Data are percentages (means $\pm$ SEM) of standard (Sprague-Dawley) Sol sample. C, control animals (n = 6-9); T, 24 weeks endurance-trained (n = 7-8). Differences between control and trained animals are significant (*) at $P < 0.05$.

4.3 SP72-MHC-I Relationship Data

To determine whether a relationship existed between SP72 and type I MHC expression following 24 weeks of endurance training, data from muscles were combined and compared within the control and trained groups respectively. Using the Pearson Product Moment Correlation, a positive correlation was detected between the control levels of SP72 and MHC-I ($r = 0.391$, $P = 0.00169$). Figure 4 shows the correlation between SP72 and MHC-I content of rat skeletal muscles in control subjects. Although statistically significant, the coefficient of determination (r^2) indicates that only about 15% of the variation in SP72 expression may be accounted for by MHC-I expression. This suggests that many factors are involved in the expression of SP72 in skeletal muscle. Protein content data (as percentages of standard Sol sample) from Sol, RG, Dia, Pla, RV, WV, WG, and Fro were combined. No significant correlation was found between the SP72 and MHC-I content in the trained group ($r = 0.242$, $P = 0.0564$).

5. DISCUSSION

The potential role of SPs in preserving cells from stress and preparing them to survive various types of environmental challenge suggests that these proteins could also play a relevant role in mediating the adaptive response of skeletal muscle to physical exercise. Both chronic and acute physical exercise are stressors which ultimately necessitate an adaptive response in order for cellular homeostasis to be maintained (Booth and Thomason, 1991). Adaptive responses to muscle loading are not confined to whole organisms, but are also evident in the differential capacities of muscle fibres that are recruited to greater or lesser degrees (Pette and Staron, 1990).

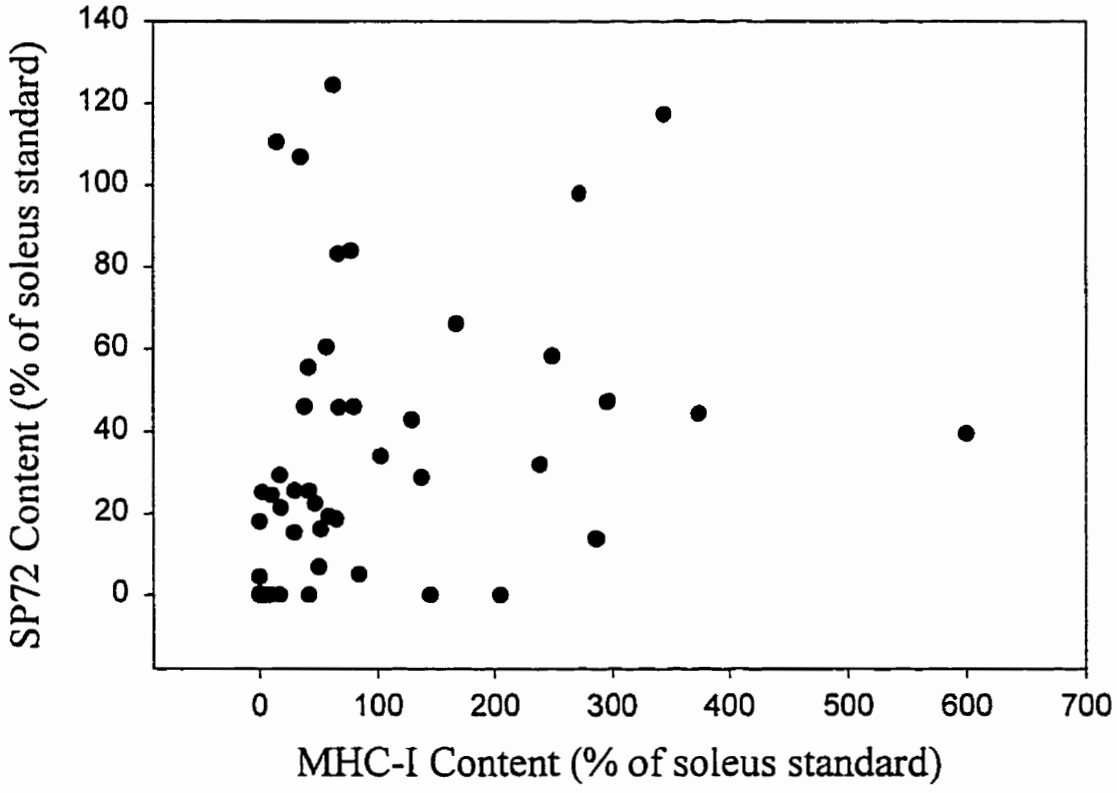

Figure 4 Correlation (r = 0.391, P = 0.00169) between SP72 and MHC-I content within individual tissue samples (excluding Liv) taken from control animals (n = 62). Protein content data (as percetnages of standard soleus sample) from soleus (Sol), red gastrocnemius (RG), diaphragm (Dia), plantaris (Pla), red vastus (RV), white vastus (WV), white gastrocnemius (WG), and frontalis (Fro) were combined in this correlation.

With this in mind, two distinct possibilities were considered to answer the question of whether the exercise-induced SP72 expression demonstrated following acute exercise and short-term training would persist or not be induced subsequent to long-term endurance training. First, it was postulated that a training program of longer duration than those utilized in the studies to date would result in a decreased SP72 expression due to the adaptation to training. Hence, factors responsible for inducing the stress response following acute exercise and short-term training might be stabilized following long-term training, thereby decreasing the need for SP expression. Second and in contrast, it was theorized that after 24 weeks of treadmill training, SP72 would continue to be expressed in muscles due to an increase in type I MHC content. The shift towards an increase in type I MHC content demonstrated following continuous long-term treadmill training might result in a greater SP72 expression. Therefore, the present study was designed to investigate the expression of both SP72 and MHC-I in selected rat tissue following 24 weeks of endurance treadmill training. It was also of interest to investigate whether the SP72-MHC-I relationship, observed under non-stress conditions, but lost during acute exercise and short-term training, would exist following 24 weeks of endurance training.

5.1 SP72 Protein Expression

Exercise is a well-characterized model of increased metabolic demand and illustrates the remarkable plasticity of skeletal muscle, which can undergo multiple phenotypic changes in response to disturbances to cellular homeostasis. Exercise adaptation minimizes the disruption in cellular homeostasis during an exercise bout (Holloszy and Coyle, 1984). This

may contribute to increased ability of the whole organism to withstand various forms of stress.

The induction of SPs, particularly SP72, confers protection to cells and tissues from subsequent stress (Welch et al., 1990). Previous studies have demonstrated the induction of various SPs, including SP72, following both acute exercise and short-term training (Kelly et al., 1996; Locke et al., 1990; Locke et al., 1995a; Salo et al., 1991; Su et al., 1996). A single bout of exhaustive exercise results in decreased glycogen stores, elevated temperature, and increased lactate (Newsholme and Leech, 1983), and also causes decreased ATP, increased Ca^{2+} concentration, decreased pH, increased production of oxygen radicals (Davies and Goldberg, 1987), and increased cellular protein turnover (Booth and Thomason, 1991; Noble et al., 1984) within the cells of the exercising muscle (Metzger and Fitts, 1987; Thompson et al., 1992). As discussed in the Literature Review, the stress response can be readily activated by these factors.

Acute exercise responses such as high intracellular calcium, elevated body temperature, muscle damage, and change in cellular pH have been shown to diminish with training due to the adaptation of various systems within the exercising muscle (reviewed in Booth and Thomason, 1991). These adaptations which permit the muscle to regain homeostasis during exercise, could possibly decrease the induction of SP72. For example, in response to aerobic training, there is an adaptive increase in mitochondrial density resulting in increased oxidative capacity of the trained muscle (Saltin and Gollnick, 1983), and less of a disturbance of energy metabolism. Compared to acute exercise conditions, adaptation to training causes less muscle damage, which results in a decreased protein turnover occurring within the trained muscle (Booth and Thomason, 1991). Therefore with

training there is not as great a need for protein degradation which may result in a decreased response in SP72 expression as per Hypothesis 1.

Surprisingly, following 24 weeks of endurance training, all muscle and tissue samples studied demonstrated a tendency towards an increase in SP72 content, with Sol, RV, RG, and WV muscles demonstrating significantly higher SP72 levels. The adaptation to training, which occurs in frequently recruited muscle fibre types following long-term training, could provide an additional explanation for these observations. A progressive recruitment pattern of muscle fibres from type I → type IIA → type IIX → type IIB is observed with exercise (Rice et al., 1988). The work by Neufer and associates (1996) using electrical stimulation suggests that the recruitment of muscle fibres may increase SP72 content in these specific fibres and that such increases may diminish as the fibres adapt to the exercise load. The increased recruitment accompanying daily exercise training appears to result in a further increase in SP72 expression in the type I fibre-rich soleus muscle. The WV, although containing a low percentage of type I fibres and a high percentage of type IIB fibres, demonstrates increased SP72 expression following training. This response is unexplained, however it may be due to the location of the muscle and its activity during exercise. With low intensity exercise, other motor units such as those composed of predominantly type IIA fibres, for instance the red portions of the gastrocnemius and vastus muscles, may also be recruited. As the most frequently recruited type I muscle fibres fail to meet the duration and force requirements of the activity, a greater demand is placed on the type IIA fibres to sustain the exercise load. Only in extreme cases, such as very intense or long-duration exercise, are the activity requirements so great as to recruit type IIB fibres. In

the present study, it is suggested that muscles predominantly composed of type IIB fibres will not sufficiently recruited, during exercise so as to alter SP72. In addition, the higher intrinsic rate of protein turnover, seen in type I skeletal muscle (Obinata et al., 1981), could be responsible for the increased protein turnover rate demonstrated in muscle cells.

It should be noted that post-pubescent female rats were used in the present study. Oestradiol has been shown to play an important role in the reduction of muscle damage in the intact animal (Amelink and Bär, 1986), and therefore the higher levels of circulating oestrogen seen in female rats may yield enhanced membrane stability during stress such as exercise (Amelink et al., 1990). Paroo and co-workers (unpublished) demonstrated a decrease in post-exercise SP72 levels of male rats injected with oestradiol. Oestrogen also limits post-exercise skeletal muscle inflammation and consequential tissue damage (Dallegri and Ottonello, 1997). Therefore the increase in SP72 expression normally seen following a single bout of exercise training may be lessened due to the protective effect of circulating oestradiol in the subjects, thereby decreasing the intensity of the HSR. Male subjects may thus display a different response to long-term exercise training.

The induction of SP synthesis in skeletal muscle fibres after exercise is a local process, which may occur only in active muscles or, on the contrary, may also occur in passive or less active muscles. This question is relevant to understand the nature of the exercise-induced signal responsible for initiating the muscle stress response. According to Hernando and Manso (1997), the existence of a generalized response of skeletal muscle to physical stress is consistent with the biological role actually assigned to stress proteins. The stress response appears to have evolved to protect cells from damage during stress and to prepare them to withstand environmental challenges. It is conceivable, therefore, that the

perception by muscle of a sustained signal potentially associated with the recruitment of fibres could be enough to activate transcription of stress genes (Morimoto, 1993), whether or not these fibres were actually recruited. However, due to the relatively high biosynthetic cost of anticipated induction of stress protein synthesis, it is possible that only muscle directly affected by the stress of exercise induces SP72 expression. The frontalis is a skeletal muscle that is inactive during exercise training but one which is subjected to the circulating hormonal milieu resulting from exercise. The liver is an organ subjected to increased abdominal heat during exercise. The lack of SP72 expression demonstrated in both tissues suggests that factors other than heat, catecholamines and blood hormones generated in the body as a result of exercise, are responsible for SP induction. Skidmore and associates (1995), using acute exercise stress, also found that factors other than temperature must be involved in the initiation of the HSR. This study supports these conclusions.

A finding, which warrants further examination, is the observation that those muscles with the highest control levels of SP72 tended to respond with the lowest increase after exercise training. Using short-term exercise training, Kelly and associates (1996) observed similar findings to those of this study. The soleus and plantaris contain the highest initial SP72 levels and exhibited only a twofold increase following training, while the red and white portions of the vastus lateralis muscle, initially demonstrating very low SP72 levels, exhibited greater than tenfold increases in most subjects. It has been observed using cell culture that cells, which over-express SP72, achieve reduced peak levels of SP72 when stressed. It is believed that this occurs because of the increased constitutive SP72 content (Mosser et al., 1993). The diaphragm has an important role in sustaining ventilation, and unlike most skeletal muscle, is chronically active and exposed to transient increases in

activity during control and exercise conditions. The diaphragm contains relatively equal percentages of fibre types, 22% type I, 32% type II, 32% type IIX, and 14% type IIB (Gosselin et al., 1996). Therefore, the increased amount of SP72 in slow muscles from the constitutive expression might permit these fibres to respond more rapidly to stress, and hence reduce the ultimate response to exercise training. In contrast, the less frequently recruited muscles will lower SP levels.

5.2 MHC-I Protein Expression

Increased muscular activity, such as endurance training, brings about a variety of metabolic adaptations (Holloszy and Booth, 1976). Controversy continues to exist as to whether or not endurance training is capable of inducing more extensive changes, such as transforming the fibre type within a muscle. Green and colleagues (1984) suggest that this incertitude may be due to the failure to provide training programs that increase muscle activity to a level approaching that common to chronic nerve stimulation. Continuous motor nerve stimulation has been used extensively to study the adaptive response of fast-twitch skeletal muscle to increased contractile activity (Pette and Vrbova, 1992). With this model, it has been demonstrated that a previously fast-twitch glycolytic muscle is transformed to a slow-twitch oxidative muscle. In addition, high intensity endurance training is capable of transforming specific characteristics of muscle fibres and has been demonstrated to alter type I fibre proportion within some rat hindlimb muscles (Green et al., 1984). The time courses of the various changes, similar to those in chronic nerve stimulation experiments, indicate that various functional systems of the muscle fibre do not change simultaneously.

In the present study, muscles examined failed to demonstrate a shift towards an

increased MHC-I content following long-term endurance training, with the exception of the red portions of the gastrocnemius and vastus muscles, thereby supporting Hypothesis 2. Interestingly, the type I fibre-rich soleus muscle demonstrated a decrease in MHC-I content, however not significant, while most type II fibre-rich muscles demonstrated a tendency towards increased MHC-I content. Although the MHC-I response to training demonstrated in the soleus cannot be explained at this time, it may be of interest to note that Kelly and associates (1996) demonstrated a similar finding. Again, the issue of fibre type recruitment pattern may come into play. Pette (1990) proposed that muscle fibre recruitment leads to a progressive switch from type IIB → IIX → IIA → I fibres. With exercise, only those motor units required to complete the activity are recruited. With endurance training, this includes muscles or portions of muscles rich in type I or type IIA motor units. In the present investigation, the RG and RV, two muscles rich in type IIA fibres, are recruited often enough during the endurance-training program to demonstrate an increase in MHC-I content. However, muscles composed of predominantly type IIB fibres, such as the WV, WG, and Fro, are not recruited enough, if at all, to demonstrate a shift towards increased MHC-I content. The seemingly high, yet non significant, increase in MHC-I content in the Dia may be explained by the amount of variability illustrated in the MHC content of the diaphragm (Appendix 3).

5.3 SP72-MHC-I Relationship

Locke and Tanguay (1996) suggest that the higher constitutive amount of SP70 in type I muscle might allow these fibres to respond more rapidly to stress. Since, type I fibres

are recruited more frequently than type II fibres, type I fibres may have to respond to intracellular changes more frequently and more rapidly than type II fibres. Short-term acute treadmill training results in an increased SP72 expression (Hernando and Manso, 1997; Kelly et al., 1996), whereas shifts in MHC-I content do not occur following 8 weeks of treadmill training (Kelly et al., 1996). A similar uncoupling of the relationship between SP72 and MHC-I existing in the skeletal muscle of sedentary animals has been reported after 10 days of chronic electrical stimulation (Ornatsky et al., 1995). In the present study, it was of interest to determine whether this relationship changed with long-term endurance type training when further adaptations in either SP72 or MHC-I might have occurred. Given the potential protective role of SPs, however, we hypothesized that SP72 would remain elevated in exercise-trained skeletal muscle compared to MHC-I. The results supported our hypothesis but were equivocal.

Supporting previous observations (Hernando and Manso, 1997; Kelly et al., 1996; Locke et al., 1994; 1991), a relationship between muscle fibre composition and SP72 content appears to be present under non-stressed or control conditions ($r = 0.391$, $P = 0.00169$; Figure 4). This suggests that SP72 plays a specific role in muscles rich in type I fibres (Kelly et al., 1996). Several factors could be proposed to explain a potential relationship. For example, muscle specific transcription factors such as myogenin are responsible for the expression of MHC-I. Myogenin content may be altered in response to a variety of conditions that are ultimately associated with changes in MHC-I expression (Hughes et al., 1997). As previous investigations have demonstrated a relationship between the expression of muscle specific transcription factors and stress proteins (Neufer et al., 1996), it is possible that the expression of both SP72 and MHC-I is linked. When examined in comparison to all

muscles, while a positive correlation is detected, the relationship is weak. Only about 15% of the MHC-I expression is determined to be accountable for the induction of SP72, which suggests that other factors may be involved in the expression of SP72 in skeletal muscle.

Because of the higher oxidative capacity of type I fibres, one of the suggested functions for SP72 involves protection or repair of proteins from oxidative stress (Locke et al., 1991). This could also contribute to explaining differences in the expression levels of SP72 among skeletal muscle types, but it seems inadequate to explain the very low constitutive expression levels of this protein in other highly oxidative tissues like liver. Further, the decrease in MHC-I content accompanied by the increased SP72 expression seen in the soleus muscle after 24 weeks of training suggests that factors other than solely those involved in inducing MHC-I may be responsible for the induction of SP72.

Although a correlation was not present between SP72 and MHC-I levels following 24 weeks of endurance training ($r = 0.242$, $P = 0.0564$), it was nearly significant. With short exercise bursts, the SP72-MHC-I relationship is lost due to dramatic increases in SP72 content with no corresponding shift in MHC-I content. However, it is possible that a return of such a relationship may be anticipated with increased intensity and duration of training given the results presented above.

6. CONCLUSIONS

Although SP cellular function is not completely understood, SPs have been widely proposed to serve central roles in the adaptation to stress (Morimoto et al., 1994). The findings from this study indicate that after 24 weeks of endurance treadmill training, the exercise-induced increase in SP72 associated with acute and short-term exercise persists in

the muscles and fibre types most likely to be recruited during the exercise. The mechanisms responsible for the maintenance of elevated SP72 expression with long-term training, cannot be wholly resolved by the present study. Hypothesis 1 proposes that, with long-term endurance training, the exercise-induced increase in SP72 associated with acute and short-term exercise will not persist. Given that a significant increase in SP72 content was demonstrated following 24 weeks of endurance training in four out of six exercising muscles, Hypothesis 1 must be rejected. Hypothesis 2, which proposes that long-term training will not cause a shift in the myosin heavy chain expression of trained muscle toward type I MHC, is accepted given that a shift towards increased type I MHC expression in trained muscle was not demonstrated across a majority of muscles examined. Lastly, the findings of the present study demonstrate that a positive relationship between SP72 and MHC-I expression is observed under non-exercise control conditions. However this relationship does not persist following long-term endurance training. Hypothesis 3, which proposes that the control-condition relationship between SP72 and MHC-I will not be re-established following 24 weeks of endurance training, is also accepted. Although a constitutive SP72-type I MHC relationship is found, the factors responsible for the activation of the stress response, which occur for a number of reasons, are independent of fibre type.

6.1 Limitations and Future Research

Given that the majority of the research on the protective role of SPs has involved cardiac tissue, it may have been useful to include, in the present study, heart tissue samples from animals for SP72 and MHC-I analysis. Also, additional evidence, which would support a training effect, could have been provided by the measurement of citrate synthase activity

within the exercising muscle cell. Citrate synthase is a common marker for aerobic capacity in animals (Holloszy and Booth, 1976).

It is clear that in animals, exercise is a stimulus capable of inducing the stress response and increasing the expression of certain SPs in certain cells and tissues. The exercise-induced SP expression has been associated with providing protection to tissues during episodes of stress. One can only speculate regarding the transfer of the findings of this study to investigations in humans. In the future, it would be valuable to directly assess the protective role of increased SP expression induced by exercise by measuring contractile function following a known stressor such as ischemia reperfusion. It would also be helpful to attempt to assess the mechanism by which exercise induces SP72 expression, which may explain how long-term exercise training may protect vital organs and tissues from stress and disease.

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Appendix 1 Muscle content of SP72, expressed as a percentage of a standard (Sprague-Dawley) soleus sample, in control (C) and 24-week endurance trained (T) rats.

N	Sol C	Sol T	RG C	RG T	Dia C	Dia T	Pla C	Pla T
n1	18.59	129.40	55.51	107.28	42.79	108.16	45.99	134.46
n2	60.48	76.75	66.11	115.84	97.90	246.55	106.83	62.13
n3	45.94	139.93	34.01	136.84	47.27	317.72	124.46	171.38
n4	110.48	71.15	117.34	139.09	13.84	19.39	22.37	31.22
n5	58.25	84.01	0	106.67	21.39	28.17	15.38	59.97
n6	39.48	84.24	0	147.96	19.19	25.06	83.14	137.92
n7	44.29	104.52	31.97	60.19	25.58	43.66	83.87	229.19
n8	n/a	n/a	5.12	62.35	6.94	13.78	45.82	58.99
n9	n/a	n/a	28.81	n/a	16.14	n/a	n/a	n/a

N	RV C	RV T	WV C	WV T	WG C	WG T	Fro C	Fro T	Liv C	Liv T
n1	0	739.48	0	55.80	0	0	0	0	16.33	17.01
n2	0	34.87	0	0	0	0	0	0	8.14	12.64
n3	25.19	133.80	0	20.81	0	0	0	0	11.06	17.90
n4	0	130.47	0	4.50	0	0	0	0	0	3.13
n5	25.40	515.22	18.05	34.02	0	0	0	0	21.29	25.77
n6	29.33	500.73	0	110.63	0	0	0	0	18.15	26.56
n7	24.50	370.56	0.37	64.83	0	17.67	n/a	n/a	15.49	19.28
n8	n/a	172.01	4.48	22.63	0	0	n/a	n/a	14.37	15.60
n9	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Appendix 2 A comparison of SP72 content of control and 24-week endurance trained rats: Summary table of t-tests. Significant difference detected with $P < 0.05$.

Muscle	*N*	(C) Mean	Std Dev	SEM	*N*	(T) Mean	Std Dev	SEM	P	Sig. Diff
Sol	7	53.930	28.501	10.772	7	98.571	26.901	10.167	0.011	yes
RG	9	37.652	37.908	12.636	8	109.528	33.402	11.809	<0.001	yes
Dia	8	34.362	29.090	10.285	8	100.311	117.611	41.582	0.146	no
Pla	9	60.444	40.589	13.530	8	110.658	68.610	24.257	0.082	no
RV	7	14.917	14.039	5.306	8	324.643	245.797	86.902	0.006	yes
WV	8	2.863	6.330	2.238	8	39.152	36.674	12.966	0.015	yes
WG	8	0	0	0	8	2.209	6.247	2.209	0.334	no
Fro	6	0	0	0	8	0	0	0	1.000	no
Liv	8	13.104	6.662	2.356	8	17.236	7.438	2.630	0.261	no

Appendix 3 MHC-I content, expressed as a percentage of a standard (Sprague-Dawley) soleus sample, in control (C) and 24-week endurance trained (T) rats.

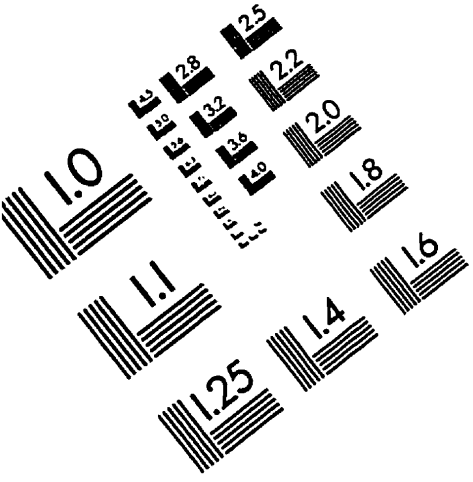
N	Sol C	Sol T	RG C	RG T	Dia C	Dia T	Pla C	Pla T
n1	64.67	15.67	41.01	560.11	129.00	607.98	37.86	88.12
n2	56.26	3.76	166.54	325.80	271.90	602.24	34.53	53.19
n3	79.74	0.62	102.46	181.40	296.00	1017.09	62.11	77.34
n4	14.21	32.71	344.06	492.45	286.46	524.77	46.79	87.39
n5	248.74	241.69	204.78	578.69	17.69	4.47	28.88	11.81
n6	599.88	172.14	144.49	240.91	58.27	109.62	66.09	30.14
n7	373.86	182.81	238.76	241.30	29.65	63.23	77.16	60.72
n8	n/a	n/a	83.85	188.38	49.96	68.77	66.91	86.73
n9	n/a	n/a	137.34	n/a	n/a	n/a	51.36	n/a

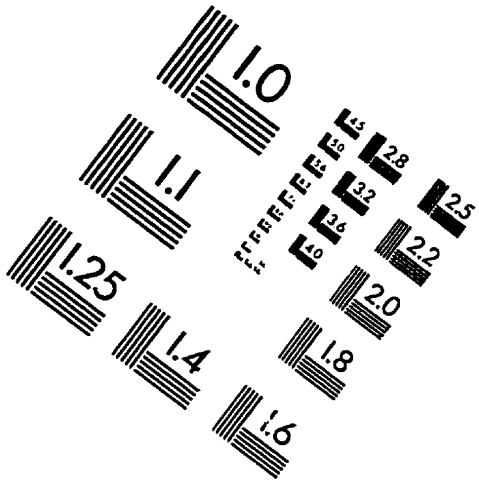
N	RV C	RV T	WV C	WV T	WG C	WG T	Fro C	Fro T
n1	16.68	118.53	3.51	0	7.59	10.80	0	0
n2	41.60	103.56	9.26	12.55	0	4.26	0	0
n3	2.24	68.58	4.69	34.56	0	2.68	0	0
n4	1.34	74.85	0	8.65	3.14	0	0	0
n5	41.88	41.81	0	0	0	0	0	0
n6	17.00	53.92	0	0	0	0.33	0	0
n7	10.06	29.51	0	4.46	0	0	n/a	0
n8	n/a	15.35	0	0	0	3.12	n/a	0
n9	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

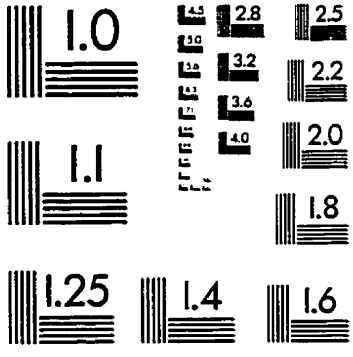
Appendix 4 A comparison of MHC-I content of control and 24-week endurance trained rats: Summary table of t-tests. Significant difference detected with $P < 0.05$.

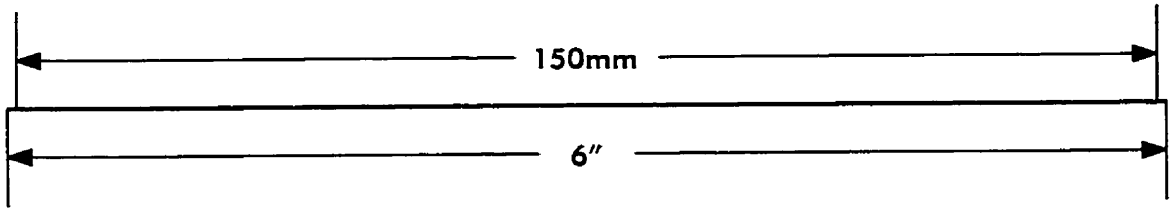
Muscle	*N*	(C) Mean	Std Dev	SEM	*N*	(T) Mean	Std Dev	SEM	P	Sig. Diff
Sol	7	205.337	216.126	81.688	7	92.771	102.103	38.591	0.237	no
RG	9	162.588	90.892	30.297	8	351.130	167.151	59.097	0.010	yes
Dia	8	142.366	122.582	43.339	8	374.771	366.318	129.513	0.111	no
Pla	9	52.410	16.665	5.555	8	61.930	28.753	10.166	0.410	no
RV	7	18.686	16.910	6.391	8	63.264	35.503	12.552	0.010	yes
WV	8	2.183	3.422	1.210	8	7.527	11.912	4.212	0.243	no
WG	8	1.341	2.754	0.974	8	2.649	3.697	1.307	0.436	no
Fro	6	0	0	0	8	0	0	0	1.000	no
Liv	8	13.104	6.662	2.356	8	17.236	7.438	2.630	0.261	no

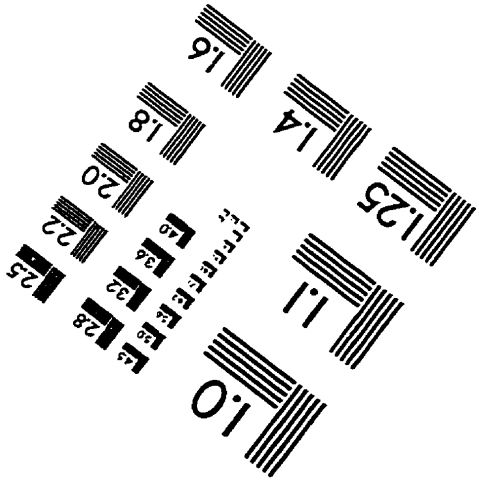
IMAGE EVALUATION
TEST TARGET (QA-3)

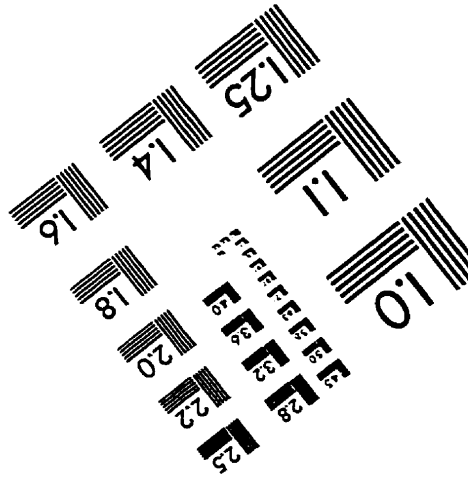












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