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Hsp70 Response to Electrical Stimulation in C2C12 cells

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Content

Abbreviations

Ab	Antibody
ABF	Albumin Bovine Fraction
APS	Ammoniumperoxodisulfate
AIF	Apoptotic Inducing Factor
AMP	Adenosine Monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine Triphosphate
Apaf-1	Apoptotic Protease-activating Factor-1
Bcl-2	B-cell leukemia/lymphoma
Bax	Bcl-2-associated X Protein
Bp	Base Pair
BSA	Bovine Serum Albumin
Casp	Caspase
cDNA	Complementary Deoxyribonucleic Acid
CK	Creatine Kinase
COX	Cytochrome Oxidase
Cyt.C	Cytochrome C
Cp	Crossing Point
dATP	Deoxyadenosine Triphosphate
ddH_2O	Double Distilled Water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DNP	2,4-dinitrophenol
dNTP	Deoxy-ribonucleoside Triphosphate
DTT	Dithiothreitol

EDTA	Ethylenediaminetetraacetic Acid
ECL	Enhanced Chemiluminescence
ES	Electrical Stimulation
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
G	Gram
HSE	Heat Shock Element
HSF	Heat Shock Factor
Hsp	Heat Shock Protein
Hz	Hertz
IL-6	Interleukin 6
IL-8	Interleukin 8
kDa	Kilodalton
mAb	Monoclonal Antibody
IGF-1	Insulin-like Growth Factor-1
$LFES_{11}$	Low Frequency Electrical Stimulation for 11 min
$LFES_{90}$	Low Frequency Electrical Stimulation for 90 min
$HFES_{11}$	High Frequency Electrical Stimulation for 11 min
Ml	Milliliter
mM	Milli Molar
mRNA	Messenger Ribonucleic Acid
MAPK	Mitogen-activated Protein Kinase
NaOH	Sodium Hydroxide
Ng	Nanogram
NRF-1	Nuclear Respiratory Factors
NF-AT	Nuclear Factor of Activated T-cells
P21	Cyclin-dependent Kinase Inhibitor
PCR	Polymerase Chain Reaction
PAGE	polyacrylamide gel electrophroresis

PAOD	peripheral Arterial Occlusive Disease
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Containing Tween-20
PKC	Protein Kinase C
PI-3K	Phosphatidylinositol-3-kinase
PVDF	Polyvinylidene Difluoride
RLT	RNeasy Lysis Buffer
RNA	Ribonucleic Acid
RNase	Ribonuclease
RT-PCR	Real-time Quantitative PCR
SDS	Sodium Dodecyl Sulphate
TBST	Tris-buffered Saline Containing Tween-20
TEMED	N,N,N,N-Tetramethylenediamine
TNF-α	Tumor Necrosis Factor α
Tris	Tris(hydroxymethyl) Aminomethane
µg	Microgram
µl	Microliter
µM	Micro molar
UV	Ultraviolet Light
V	Voltage
VEGF	Vascular Endothelial Growth Factor
β2-M	β2 -microglobulin

1 Introduction

1.1 Concept of Hsp70

Cells from virtually all organisms response to stress with rapid synthesis of highly conserved proteins termed heat shock proteins (Hsps). This was first observed in 1962 in cells exposed to heat shock (Ritossa F, 1962), and it is known that the response with Hsps production can also be induced by a variety of stresses (Lindquist, 1986). Up to date, numerous Hsps have been identified, which can be divided into five families according to their molecular mass: Hsp104/110, Hsp90, Hsp70, Hsp60 and small Hsp family (Villar and Mendez-Alvarez, 2003). It is evident that Hsps plays an essential and universal role in maintaining cellular homeostasis and vitality.

Of the Hsps studied to date, the best characterized Hsps are those with apparent molecular mass 70 kilodalton (kDa). There are at least four distinct proteins in the Hsp70 family: Hsp72 (Hsp70), Hsp73 (Hsc70), Hsp75 (mHsp70), and Hsp78 (Glucose-regulated protein 78) (Snoeckx et al., 2001b). Hsp70 is highly inducible and considered as one of the most prominent Hsp70s. Hsp70 has been extensively studied with regard to its cellular location, regulation, and function. The molecular structure of Hsp70 comprises three parts, i.e. N-terminal ATPase domain, peptide-binding site and c'-terminus. Different genes located on different chromosomes encodes Hsp70, for instance, a 2440 base pair (bp) gene containing 212 bp leader sequence and a 242 bp downstream or 3'-untranslated region (Wu et al., 1986). Hsp70 is found in a variety of cells, and can be clearly induced by diverse cellular stresses. Hsp70 is indispensable for facilitating cellular response to stress and is essential for cellular homeostasis.

1.2 Regulation of cellular Hsp70 response in skeletal muscle

In unstressed cells Hsp70 is expressed at very low level. The induction of Hsp70 is usually a stress-mediated process. Hsp70 can be induced very rapidly in a great amount with exposure to stress (Donati et al., 1990). The regulation of Hsp70 response is highly complex and has not been completely understood. Hsp70 response varies with kinds and intensities of stresses, is regulated at different levels including gene level, transcriptional and post-transcriptional level, and

represents a self-regulated manner.

1.2.1 Inducing factors of Hsp70 response

Although Hsp70 response is recognized as a highly conserved and universal characteristic of cells, Hsp70 induction can be distinctly affected by the inducing factors. It is evident that factors leading to cellular stress can induce Hsp70, which include physic, chemical as well as physiological or pathophysiological factors (Lindquist, 1986).

Physical exercise is considered as an important and established physiological stress in terms of Hsp70 induction in human. Numerous studies have demonstrated that physical exercise induces Hsp70 production in blood cells (Fehrenbach et al., 2001), liver, heart and skeletal muscle (Salo et al., 1991). The induction of Hsp70 by exercise is associated with temperature variation, contraction related stress, energy metabolism, hormones and cytokines, and perfusion-related stress (J.M.Steinacker and Yuefei Liu, 2002). Similarly, the up-regulation of Hsp70 expression in skeletal muscle has been observed during pathophysiological process, such as ischemia/reperfusion (Liu et al., 2002). Lepore and Morrion found a modest induction of Hsp70 in skeletal muscle undergoing 10 minute ischemia followed by 15 minute reperfusion (Lepore and Morrison, 2000).

Additionally, it is well known that heat stress and chemical factors can cause Hsp70 response in various cell types or tissues. Heat shock was the first factor observed in inducing Hsp70 response and it has been extensively studied in diverse cells and tissues over the last 50 years. It was also demonstrated that chemical stress (such as sodium arsenite) induced Hsp70 response at both mRNA and protein level in cultured cells (Kim et al., 2001). Moreover, evidence from studies showed that some kinds of physical stresses (such as: mechanical stretch stress) could also induce Hsp70 response in cells and tissues. Luo et al. reported that various forms of stretch stimulation increased expression of Hsps 25 and 70, but not that of Hsp 90 in endothelial cells (Luo et al., 2007). It is well accepted that electrical stimulation (ES) can activate skeletal muscle cells and facilitate contraction; therefore, ES is deemed as one kind of physic stress. Previous study on the expression of stress proteins in skeletal muscle after chronic electrical stimulation has showed that Hsp60 was significantly increased after stimulation, along with

increased mitochondrial enzyme activity. However, up to date, there is no evidence whether there is an Hsp70 response in the ES-induced cellular process.

There are different inducing factors which can induce Hsp70 production. The regulation of Hsp70 response may represent a complex system, in which many influencing factors are involved. The inducers themselves can regulate Hsp70 response. The degree of Hsp70 induction is closely related to the intensity and duration of stress exposure. For example, Liu et al. reported that in human skeletal muscle Hsp70 response to physical exercise training depended on exercise intensity (2004). Furthermore, there was a different Hsp70 response to high-intensity strength training and low-intensity endurance training (Liu et al., 2000). Similar results can be obtained from previous study on Hsp70 response to ischemia/reperfusion (I/R). It has been shown that Hsp70 expression level in the skeletal muscle depends not only on the severity of I/R but also on the ischemic duration (Liu et al., 2002; Gampert L et al., 2004). The dependence of Hsp70 response on stress intensity can also be elucidated by heat stress. A hyperthermia induced Hsp70 expression level is related to the changes of temperature and the duration of the hyperthermic exposure in cell culture as well as in animals and human beings (Widelitz et al., 1987; Mitchell et al., 2002). Up to date, it is generally accepted that stress-induced Hsp70 response may be determined by the intensity, duration, and patterns of stress, with variation among species, organs, tissues, and cells (Blake et al., 1990; Mathur et al., 1994).

1.2.2 Influencing factors of Hsp70 response

In general, Hsps can be rapidly produced in response to cellular stress. It is evident that in many tissues Hsps can be induced within short time by stress. For instance, in cell culture and rodent animal experiments, it has been demonstrated that Hsp70 response can be activated within minutes or hours (Maglara et al., 2003; Zhang et al., 2003). In human beings, Fehrenbach et al. have reported that in leukocytes Hsp27 and Hsp70 transcripts increased significantly immediately after exercise (Fehrenbach et al., 2001). However, Hsp70 response to stress in human skeletal muscle seems to be much slower with a time delay, especially at protein level. In addition, there is a discrepancy of the time course of Hsp70 expression between mRNA and protein level. In human beings, Hsp70 mRNA usually increases

immediately after stress and remains elevated for a few hours, whereas an increase in Hsp70 protein has been observed days after stress (J.M.Steinacker and Yuefei Liu, 2002). Collectively, Hsp70 mRNA usually increases immediately (in minutes/hours) after stress, remains elevated for a few hours and then decreases rapidly, especially in cell culture; whereas an increase of Hsp70 protein occurs hours/days after stress and maintains for longer time. Furthermore, the change in Hsp70 mRNA content seems to be more dramatic than that of Hsp70 protein.

Among the diverse influencing factors, the experimental setting itself may affect Hsp70 response. For instance, the cell culture is a kinetic process in which cultured cells should be in different stages at different time points during cell culture and the expression of Hsp70 in different cellular stage may be different. In other words, cellular status is also a factor which may have effect on the Hsp70 response. There are still some chemical agents (i.e. glucose, insulin) and cell signal transducers (such as cyclic AMP, growth factors, Ca^{2+}) can influence the expression of Hsp70.

1.2.3 Regulation of Hsp70 at different levels

Hsp70 expression is regulated at gene transcriptional, translational and posttranslational level. A number of previous studies have shown that Hsp70 mRNA is rapidly increased in response to stress, which indicates that transcription of Hsp70 gene is transiently induced by environmental stress. In cell culture, Hsp70 mRNA response can be activated within several minutes or hours (Liu and Steinacker, 2001). Puntschart et al. reported a significant Hsp70 mRNA up-regulation within 4 minutes in response to acute exercise in skeletal muscle (Puntschart et al., 1996). Previous studies in our lab demonstrated that exercise training led to an increase of Hsp70 mRNA in well-trained human skeletal muscle (Liu et al., 1999). Furthermore, Moseley et al. investigated the role of post-transcriptional regulation of human Hsp70 gene through its 3'-untranslated region and reported that this region itself is heat responsive (Moseley et al., 1993).

Like most of other proteins, Hsp70 expression is also regulated at protein level. Previous studies have shown that there is a discrepancy in Hsp70 between mRNA level and protein level in response to stress (Puntschart et al., 1996; Bruce et al., 1993). In cell culture, Hsp70 mRNA can increase in response to a challenge, although there is little Hsp70 protein produced, which suggests HSP promoter

activity and protein accumulation may be uncoupled (Bruce et al., 1993). The discrepancy of the expression of Hsp70 mRNA and protein could also be shown in human skeletal muscle after exercise (Puntschart et al., 1996). These results are also supported by ischemia-induced Hsp70 response. It has been documented that the effect of ischemia alone on Hsp70 response was smaller than that of the ischemia followed by reperfusion (Lepore and Morrison, 2000), suggesting that Hsp70 response to ischemia may be influenced by protein synthesis which is limited under ischemia condition and improved by reperfusion. All the evidences may suggest that Hsp70 response is regulated at protein level and transcriptional activation of Hsp70 gene can be independent of protein synthesis. In addition, Theodorakis and Morimoto examined the translational regulation of Hsp70 expression in human cells, and showed that heat shock, inhibition of protein synthesis and mRNA stability might be involved in the post-transcriptional events (Theodorakis and Morimoto, 1987). Therefore, the difference in Hsp70 response is related to events subsequent to promoter activation and may include important post-transcriptional regulatory mechanism.

The meanings of Hsp70 expression at mRNA and protein level might be different during the cellular response and adaptation to stress. The response of Hsp70 at mRNA level indicates that the signal transduction pathway involved in the activation of the transcription of Hsp70-genes has been initiated. In eukaryotic cells this transcription is effectuated via a transcriptional factor known as the heat shock factor (HSF). Generally, the activation of Hsp70-gene transcription leads to the protein synthesis. The expression of Hsp70 mRNA can serve as an indicator for the cellular stress (stress sensor). The denatured proteins induced by stress can be first detected by Hsp70, which subsequently transduces the stress signals (Liu et al., 2006). Therefore, it has been demonstrated that Hsp70 mRNA can be viewed as a molecular marker of thermal injury (Flanagan et al., 1995), neurotoxicity (Pratt and Toft, 2003) or environmental metal strain (Lepock et al., 1990). Furthermore, Hsp70 mRNA may be probably involved in other signal pathways via interaction with other key transcriptional factors in response to stress. These signal pathways control cell homeostasis, proliferation, differentiation and apoptosis.

It is evident that Hsp70 protein has effects on cellular protection against the environmental stress or challenges. Proposed mechanisms of cellular protection for

Hsp70 protein include their functioning as molecular chaperones to assist in the assembly and translocation of newly synthesized proteins within the cell and the repair and refolding of damaged. Evidence from studies showed that Hsp70 protein could prevent protein denaturation or process leading to protein denaturation. Additionally, Hsp70 protein can capture antigenic peptides and trigger their cross-presentation through immune effector cells, which suggests an immune-modulatory role of Hsp70 (Fabian et al., 2007; Theriault et al., 2005).

1.2.4 Mechanisms underlying regulation of Hsp70

There is evidence that intracellular signaling cascade ultimately leading to the activation of Hsp70-gene transcription and subsequent protein synthesis. Hest shock factor (HSF) is considered as a transcription factor and plays an important role in the regulation of Hsp70 response. Heat shock factors (HSFs), present in the cytosol, are bound by Hsp70 and maintained in an inactive state. HSF activation is first important step for initiating Hsp70 gene transcription. The central process in HSF activation is the equilibrium between the binding of free Hsp70 molecules to HSF and to stress-mediated unfolding proteins. Under stressful condition, the free Hsp70 captures the denatured proteins, causing the decrease of free Hsp70 in cytosol and this can cause a dissociation of Hsp-HSF complex and allow a formation of HSF trimer. These HSF trimer complexes enter the nucleus and bind to heat shock elements (HSE) in the promoter region of the Hsp70 gene (trimer-HSE). Hsp70 mRNA is then transcribed and leaves the nucleus for the cytosol, where new Hsp70 is synthesized (Figure 1).

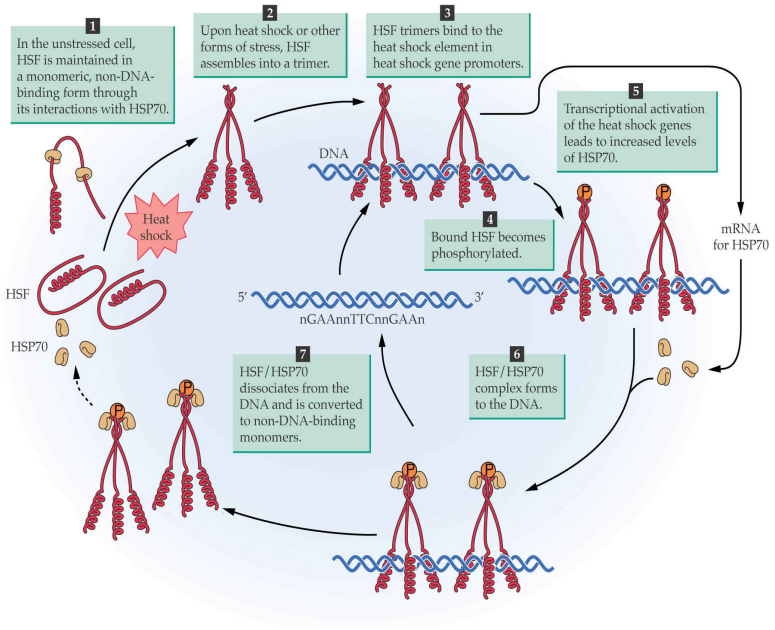

Figure 1: Regulation of Heat shock protein 70 (Hsp70) induction and possible mechanism (Kregel, 2002). **HSF**: Heat shock factor; **HSE**: Heat shock element; **DNA**: Deoxyribonucleic acid; **mRNA**: Messenger ribonucleic acid

Furthermore, when Hsp70 is accumulated to a high level, the pool of unbound Hsp70 increases, which thereafter increases the possibility of complex formation with activated HSF1 (Snoeckx et al., 2001a). The binding of Hsp70 to HSF1 will facilitate the deactivation of HSF1 in a negative feedback loop, leading to the attenuation of the stress response. Recently, it was documented that over-expression of Hsp70 inhibited the phosphorylation of HSF1 by activating protein phosphatase and inhibiting protein kinase C (PKC) activity (Ding et al., 1998).

It is also known that HSF can be activated indirectly by some stressors that activate phospholipase C, thereby activating of PKC, which plays a role in the activation of Hsp70-gene transcription (Snoeckx et al., 2001a). Additionally, changes in intracellular calcium concentration may be involved in the activation of Hsp-gene transcription. It has been reported that heat shock, stretch, and α-adrenoreceptor lead to increase of intracellular free calcium in cardiac tissue along with enhanced Hsp-gene transcription (Calderwood et al., 1988; Vidair et al., 1990). Furthermore, evidence from studies also indicated that a long-term incubation with high does of calcium antagonists may have a blocking effect on Hsp70 synthesis. Low-Friedrich and Schoeppe demonstrated that calcium channel blockers could reduce Hsp70

synthesis in isolated cardiomyocytes (Low-Friedrich and Schoeppe, 1991). This suggests that intracellular calcium concentration may play a role in the regulation of Hsp70 response.

1.3 Functions of Hsp70

It has been proven that Hsp70 exerts different functions including cellular protection against stress, protein metabolism (i.e. protein degradation, folding and synthesis), facilitating cellular adaptation to stress and development as well as effects on cellular energy metabolism. All these functions of Hsp70 can be attributed to two basic functions: molecular chaperone and stress sensing.

1.3.1 Molecular chaperone

Molecular chaperone is a major function of Hsp70 and a permanent cellular event during both non-stressed and stressed condition. First, Hsp70 passes the newly synthesized, unfolded proteins to members of the HSP60 leading to folded proteins. Second, Hsp70 carries proteins for translocation into different cellular compartments. Finally, Hsp70 may serve as cohort proteins to other proteins such as glial-axon transfer proteins (Kiang and Tsokos, 1998).

It is shown that an immediate translocation of newly synthesied Hsp70 into cell nucleus and nucleolus occurs in various cell types and tissues after heat stress (Snoeckx et al., 2001a). Hsp70 is reportedly involved in the process of protein turnover including protein stabilization and protein degradation. On the contrary, inhibition of Hsp70 production and translocation led to loss of cellular protection. These results suggest that Hsp70 is an intracellular molecular chaperone involved in nascent and damaged protein refolding.

Another role of Hsp70 as molecular chaperone is described in innate/adaptive immune and inflammatory response (Moseley, 2000). Elevations in intracellular Hsp70 level have been shown to improve cell tolerance to inflammatory cytokines such as tumor necrosis factor a (TNF-α) and interleukin-1 (IL-1) (Muller et al., 1993). Additionally, Hsp70 is also involved in antigen presentation in cells such as macrophages and dendrites (Todryk et al., 1999).

These functions of Hsp70 appear to be based on its property to interact with

hydrophobic peptide segments of proteins in an ATP-controlled fashion. Substrate binding is localized to a 15-kDa region near the C-terminal end of Hsp70 (Feige and Polla, 1994). The substrate binding and release cycle are regulated by the switching of Hsp70 between the low-affinity ATP bound state and the high-affinity ADP bound state (Mayer and Bukau, 2005). The ATP cycle is regulated by co-chaperones of the family of J-domain proteins, which target Hsp70 to their substrates, and by nucleotide exchange factors, which determine the lifetime of the Hsp70-substrate complex (Mayer and Bukau, 2005).

1.3.2 Stress Sensing

The significance of the stress sensing role of Hsp70 is that Hsp70 can initiate and transduce signals involved in cellular response to stress. Hsp70 may serve as a sensor to detect the accumulation of the abnormal proteins induced by various stresses and transduce the stress signal (Liu et al., 2006). Studies have shown that Hsp70 can be considered as a molecular marker of thermal injury (Flanagan et al., 1995), neurotoxicity (Pratt and Toft, 2003) or environmental metal strain (Lepock et al., 1990). With the stress sensing function Hsp70 is involved in a variety of cellular processes as immune reaction, energy metabolism and stress response.

Hsp70 interacts with key regulators of many signal transduction pathways, controlling cell homeostasis, proliferation, differentiation and cell death. Feder et al. have suggested that Hsp70 might specifically interact with cell cycle regulators (Feder and Hofmann, 1999). For instance, over-expression of Hsp70 in HL60 cells reduced proliferation and promoted their differentiation (Kwak et al., 1998). However, in some situations elevated expression of Hsp70 has been shown to increase proliferation, especially in some cancer cell lines (Barnes et al., 2001). Further research is needed to clarify this issue.

Cellular stress can trigger a process of self-destruction known as apoptosis. With the protective molecular mechanism, Hsp70 is probably capable of inhibiting the apoptotic pathway. It has been demonstrated that Hsp70 can affect the apoptotic pathway in both cytochrome C release and initiator caspase activation (Li et al., 2000). Beere et al. have shown that Hsp70 had a negative influence on apoptotic signaling by preventing activation of caspase-9 via direct binding to apoptotic protease-activating factor-1 (Apaf-1), which precludes the eventual recruitment of

procaspase-9 to the apoptosome (Beere et al., 2000). In addition, Hsp70 has been shown to inhibit apoptotic inducing factor (AIF)-induced chromatin condensation of purified nuclei (Ravagnan et al., 2001).

With stress-sensing role, Hsp70 is also involved in immune and inflammatory response. It is evident that exogenous Hsp70 stimulated TNFα, interleukin-1β (IL-1β) or IL-8 in human monocytes via CD14 and calcium dependent pathways (Asea et al., 2000). Observations from studies demonstrated that Hsp70 is important modulator of antigen presentation, which can activate pro-inflammatory cytokine (e.g. IL-6, TNFα) production or other innate immune factors (i.e. nuclear factor of activated T-cells (NF-κB)) and specifically bind with high affinity to the plasma membrane of antigen presenting cells (Moseley, 2000). Furthermore extracellular Hsp70 has been shown to stimulate the complement pathway, to increase phagocytosis and to function as a powerful adjuvant for eliciting antigen-specific immune responses and anti-tumour immunity (Liu et al., 2006).

1.4 Effects of electrical stimulation on cells

1.4.1 Introduction of ES

Electrical signals provide important functions in diverse tissues and cells of the mammal body, ranging from skeletal muscle contraction to conduction of nerve impulses. Electrical signals in the body occur by cell membrane depolarization, which allows influx of ions creating a potential difference. These electrical signals occur naturally in the body, but artificial stimulation can be applied to the body to induce a physiological response.

It is well known that electrical stimulation (ES) can activate skeletal muscle cells and facilitate contraction. Significant enhancement of angiogenesis produced by ES has been reportedly used in ischemic and non-ischemic rat limbs (Kanno et al., 1999). ES is commonly used in clinical setting to mimic voluntary contractions and enhance the rehabilitation of human skeletal muscles. The ability of ES in terms of improving skeletal muscle performance in healthy as well as dysfunctional muscle is widely accepted and demonstrated in lab research as well as clinical practice. Although ES is a commonly accepted modality in the management of muscle dysfunction, the mechanisms associated with its effects are poorly understood.

1.4.2 Cellular response and adaptation to ES

It is well established that chronic low-frequency electrical stimulation to skeletal muscle can induce functional, structural and metabolic changes (Pette and Vrbova, 1999). A large body of studies has demonstrated that after long-term of ES two main functional changes in skeletal muscle were observed: slowing of the time-courses of contraction and relaxation; and increased fatigue resistance. These functional changes reflect profound alterations in gene expression leading to a transformation of the muscle fiber phenotype. Numerous in vivo studies have demonstrated that ES of skeletal muscle can induce a fast - to -slow phenotype changes, which occurs in an orderly sequence and involves most cellular elements. However, the mechanisms associated with this process are still unclear. Additionally, metabolic changes among the adaptive response were also observed in animal experiments, such as increase in mitochondrial volume (Eisenberg and Salmons, 1981), changes in enzyme activities (Henriksson et al., 1986) and isozyme patterns. These metabolic changes result in alteration in metabolic profile of the muscle fiber with a shift from preferentially glycogenolytic-glycolytic to predominantly aerobic-oxidative (Pette and Vrbova, 1999).

Although effects of ES on the skeletal muscle have been extensively studied, few data derived from cellular level are available. Studies have shown that in vitro ES can cause fast-to-slow phenotype changes in primary muscle culture as well as in C2C12 cell line (Naumann and Pette, 1994; Stern-Straeter et al., 2005). A study on satellite cells showed an up-regulation in myosin heavy chain type I in the cultured satellite cell myotubes that underwent a chronic ES (Wehrle et al., 1994). A further study on maturation and differentiation of rat aneural myotubes revealed that ES could promote these processes (Xia et al., 1998). It seems that ES may have different effects on proliferation and differentiation in cell culture. Pedrotty et al. reported that ES stimulated proliferation of skeletal muscle blasts whereas it did not stimulate differentiation (Pedrotty et al., 2005). This result seems to be supported by a further study, in which a negative impact on the differentiation process was suggested by the myogenic gene expression in ES 3-D myoblast-fibrin cultures undergoing ES process (Stern-Straeter et al., 2005). However, there is also a study reporting that ES applied to L6 myoblasts accelerated the mature process from myotubes to muscle fibers (Kawahara et al., 2006). ES may have impact on the

cellular apoptosis. It was reported that ES applied to cardiomyocytes induced an activation of stress-activated kinase, leading to apoptosis (Kuramochi et al., 2006).

Although limited data are available with regard to effects of ES on cellular response and adaptations, especially in the cell culture settings, there is evident that ES may have profound impact on the cellular stress response, cell growth process as well as apoptosis.

1.4.3 Mechanisms underlying ES-induced cellular changes

Although ES seems to have profound effects on many kinds of cells, especially on myotube and myoblast, the cellular and molecular mechanisms underlying ES-induced cellular changes are not completely understood and are needed to be clarified further.

The regulation of cellular growth factor by ES can be considered as one of the basical mechanisms involved in the cellular response. Previous studies have demonstrated that ES could induce up-regulation of growth factors (i.e. insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF)) in muscle cells and endothelial cells (Zhao et al., 2004; Bayol et al., 2005). Both IGF and VEGF are thought to be principal angiogenic factors and stimulate cellular proliferation. Recently, it is demonstrated that IGF can induce changes in muscle phenotype through a calcineurin/nuclear factor of activated T-cells (NF-AT) pathway, which is activated by ES of myotubes in culture (Semsarian et al., 1999). It has been indicated that ES improves insulin response in human skeletal muscle cell model, and has effect on the glucose metabolism in cells (Aas et al., 2002). Other studies suggested that treatment of C2C12 cells with IGF-1 or insulin and dexamethasone results in hypertrophy of differentiated myotubes and a switch to glycolytic metabolism (Semsarian et al., 1999).

It is evident that ion channels of surface and intracellular membranes are crucially involved in the control of muscle cell excitability and consequently of muscle contraction. Thus changes in the expression and/or function of ion channels during cellular response and adaptation may have major consequences in the physiology of muscle cells. It has been reported that ES appears to stimulate myoblast proliferation via an L-type calcium channel-dependent pathway (Pedrotty et al.,

2005). Furthermore, calcium serves as a second messenger that plays a role in cell survival, proliferation, motility, apoptosis, and differentiation. It is known that there are different types of K^+ channels presenting the sarcolemma of skeletal muscle (Lin-Shiau et al., 1991). K^+ channels were considered to not only modulate the contraction of skeletal muscle but also play a role during cellular response to ES as well.

1.5 The aim of the study

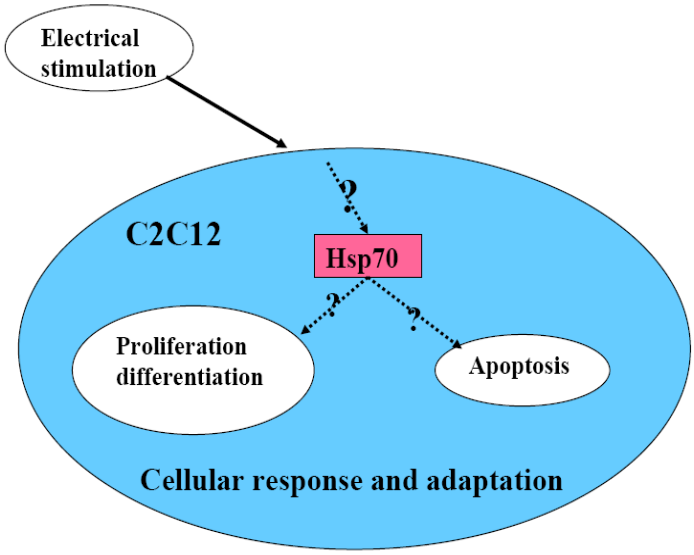

Figure 2: The aim of the study. **Hsp70**: Heat shock protein 70.

Though ES is wildly applied in clinical as well as experimental settings, and it is shown that ES can induce a variety of functional, structural, and cellular changes. The available data and knowledge with regard to the mechanisms underlying ES-induced cellular response are very limited, and the mechanisms are to a great extend not completely understood. Among the mechanisms responsible for cellular response to stress, Hsp70 plays an essential and universal role, and thus, it is likely that ES might induce Hsp70 response in cell culture settings and Hsp70 response might be involved in the cellular response. It is known that in cell culture experiment stress induces cellular response with regulation of cell growth process as well as apoptotic activity. We hypothesize, therefore, that ES can induce Hsp70 response in the cultured C2C12 along with changes in cellular growth process and apoptotic activity (Figure 2). In the present study the following questions are addressed.

1. Is there any Hsp70 response in the ES-induced cellular process?

2. Do different kinds of ES induce different Hsp70 responses in C2C12 cells?

3. Do different kinds of ES have effects on cell growth process?

4. Do different kinds of ES have effects on cell apoptotic activity?

5. Is an Hsp70 response to ES associated with ES-induced response in cell growth regulation and apoptotic activity?

2 Material and Methods

2.1 Cell culture

C2C12 mouse muscle cell line is a subclone isolated from parental C2 myoblasts, which were originally derived from the generating thigh muscle of an adult mouse (Yaffe and Saxel, 1977). These cells have capacity to proliferate and differentiate.

Frozen C2C12 cell line, purchased from the Amer. Type Culture Collection, was taken from liquid nitrogen tank to water bath (37°C). Thaw vial of cells rapidly in water bath with constant agitation. The cells were then transferred to T25 flask filled with 5ml growth medium (Gibco, U.K.). The growth medium consists of DMEM with 4.5 g/l glucose, 4mM L-Glutamine, 10% FBS (fetal bovine serum), and 1% penicillin. C2C12 cells were cultured at 37°C in a 5% CO_2 humidified atmosphere. On the following day, cells were observed using microscope. The medium was aspirate and replaced with fresh pre-warmed medium. Medium was changed the day after seeding and every second day or every third day thereafter. By reaching 50%-60% confluence, cells were sub-cultured by trypsinization using a 5% trypsin solution containing 2% EDTA and 0.9% NaCl. Before sub-culture, cells were resuspended in a known volume of culture medium and a viable-cell count was performed with trypanblau exclusion. C2C12 cells divide rapidly when maintained under conditions favoring cell division (low density and growth stimulation by fetal bovine serum) (Figure 3A and 3B).

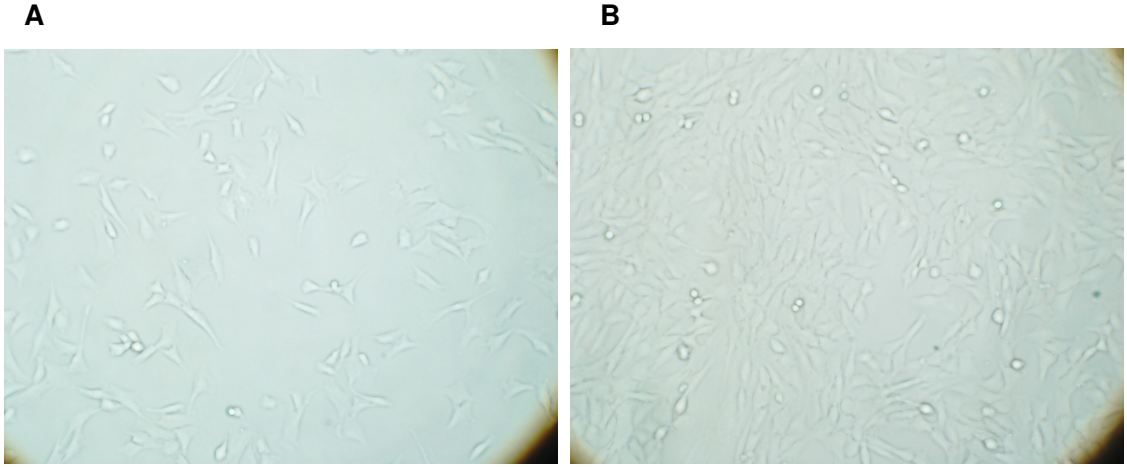

Figure 3: C2C12 cells were cultured in growth medium after recovery (100X). **A)** 24h culture; **B)** 48 h culture.

Cells were harvested according to experimental protocol (see below). Cell monolayers were washed three times with ice-cold phosphate buffered solution

(PBS) before cell harvest and then detached from culture dish by cell scraper. The suspension was collected to 15 ml falcon and centrifuged at 1200 rpm for 5 minutes. Cell pellet from one dish was resuspended with 1.5 ml PBS which was aliquoted into two 1.5 ml tubes. The resuspended cells were centrifuged at 9300 rpm for 60 seconds and then PBS was removed. Cell pellets were shock-frozen in liquid nitrogen and then stored at -80℃.

2.2 Experimental protocol

2.2.1 Cell sampling

C2C12 cells were seeded in rectangular dishes at a density of ca. 1.2 x 10^6 per dish and freshly growth medium was added to the culture. By 75% confluence, cells were divided into three groups (Group A, B and C) and stimulated with three different kinds of ES. To each of these groups, a corresponding group of cells was parallel treated in the same way except for ES. Each cell group was divided to different dishes from which the cultured cells were harvested at different time points (see below). Thus, from each cell group, six cell samples were obtained, making all together 36 cell samples with about 3x10^6 cells each. This experiment setting was repeated two times.

2.2.2 Experimental protocol

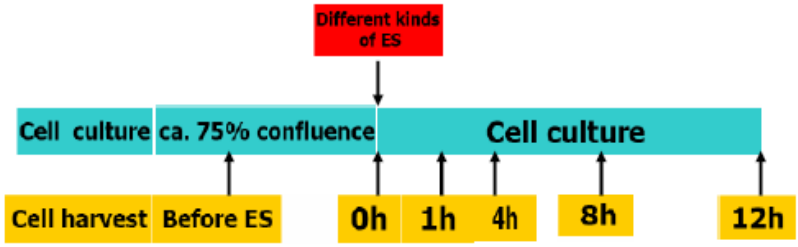

Figure 4: Illustration of the experimental protocol (for details see text). **ES**: Electrical stimulation.

As shown in the figure 4, cells were harvested before ES, and 0h, 1h, 4h, 8h, 12h after ES respectively. Meanwhile, C2C12 cells without ES were also harvested at each time point and served as control group.

2.3 Electrical stimulation

2.3.1 Electrical stimulation apparatus

Although ES is widely applied in clinical as well as experimental settings, and continuous ES of isolated cells in culture provides an excellent model for the study of the ensuing biochemical and molecular changes in vitro, there is no well-designed device which allows simultaneously and uniformly stimulating a large number of independent cultured muscle cells. Therefore, the first important thing in the present study was to develop a device of continuous ES for isolated cells in culture. An earlier attempt to develop such an in vitro model of ES was reported by Brevet et al. (Brevet et al., 1976) and modified by others (McDonough et al., 1994). Recently, Marotta et al. described an original ES of cultured cells suitable for continuous uniform stimulation of a large number of wells in standard multiwell culture plates (Marotta et al., 2004). However, there are still some limitations in these previously reported systems. First, they required specially designed flasks for each sample of the cells, making it difficult to generate a high number of replicates. Second, the number of wells in standard 6- or 24-well culture plates adopted in other studies was not suitable for simultaneously stimulating a large number of cells.

In the present study, the principles for the design of the ES unit were as following: 1) the design and fabrication of an electrical stimulation unit matched a suitable controlled and sterile environment for cell growth and allowed to be autoclaved, and avoided reactions with chemicals and proteins in media; 2) it produced a uniform electric field throughout the area of interest; 3) Possibility for simultaneously stimulating a large number of cells. In the current study, some modifications were made to optimize the system of ES on C2C12 cells based on the previous ES device and protocols.

First, the rectangular dishes (Nunc Omny Tray) were selected for cell culture in our experiment. The suitable new lids were built by polyethylene which was fitted to the dishes. Two holes were drilled in the new lid to allow the wire to enter. Two platinnum (99% pure) electrodes (0.5mm) were integrated into the new lid by glued with silicone (Figure 5). The electrodes were adjacent to the short axis of the lid and the ends of electrodes were connected to a commercial stimulator (Grass S-48)

(Figure 6) which generated variable electrical impulses. The portion of electrodes was immersed in the culture medium and touched the bottom of culture dish to obtain a maximal area of stimulation. This new device allows simultaneously stimulating a large number of cultured cells over a long period in standard incubation conditions. Furthermore, it can produce a range of outputs based on the stimulation parameters delivered from the Grass S-48 stimulator.

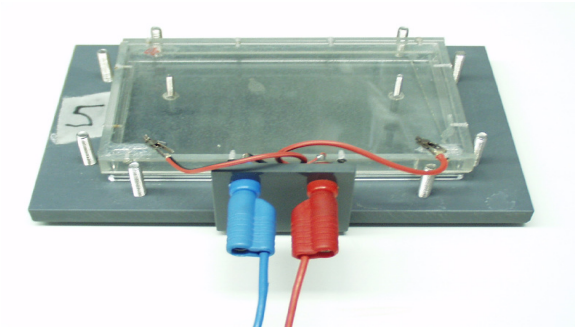

Figure 5: Electrical stimulator unit

Figure 6: Grass S-48 electrical stimulator

2.3.2 Electrical stimulation parameters

Previous studies have reported different ES conditions used to stimulate the cells according to different study purposes. To determine the stimulation conditions is critical for the experiment because an electrical stimulation varying in intensity (voltage or frequency) may have performed impact on cellular response. Therefore, a series of experiments had been performed to set ES parameters with regard to electrical impulse and stimulation duration. ES of 13 Voltage (V) on C2C12 cells was an empirical value which has been proven to be the highest possible voltage causing myotubes contraction without killing cells through our pre-experiments. In addition, it was also suggested that different frequency and duration of ES would have different effects on cellular response and adaptation according to previous studies. Therefore, in our experiment, cells were divided into three groups (A, B

and C) for different frequencies and duration of ES. Specifically, Group A with 13 voltages, 12 hertz (Hz), 11 min; Group B with 13 voltages, 12 Hz, 90 min; and Group C: 13 voltages, 100 Hz, 11 min.

2.4 Determination of the study parameters

To investigate cellular response to ES, a series of parameters were taken from the experiment that covers Hsp70 response, cell growth process and apoptosis. These parameters were generally analyzed at mRNA and /or protein level.

2.4.1 Analysis of gene expression at mRNA level

To analyze the gene expression at mRNA level for the study parameters, the following procedure including total RNA extraction, complementary deoxyribonucleic acid (cDNA) synthesis, real-time PCR was undertaken, which is described below.

2.4.1.1 Extraction of total RNA

The commercially available Kit for extraction of total RNA from cultured cells was used (Product No. 74904, Qiagen, Germany). The frozen harvested cells were thawed at room temperature and suspended with phosphate buffered saline (PBS) (1M, PH 7.5). 600ml RNeasy lysis buffer (RLT buffer) (provided by the Kit) was added to lyse and homogenize the cells. The lysate was transferred to GIAshredder spin column and centrifuged for 2 min at 12,000 rpm. Subsequently, 70% ethanol was added into the homogenized lysate and well mixed by pipetting. After lysates being transferred into high pure filter tubes and centrifuged, samples were incubated with DNAse I working solution for 15 minutes at room temperature, subsequently they were washed with provided different washing buffers according to the standard protocol of the provider. Finally, RNA was eluted with RNase-free water (60-100 µl). The concentration and purity of RNA were determined by spectrometry with absorbance at 260 nm and 280 nm.

2.4.1.2 First Strand cDNA Synthesis

To estimate mRNA level by PCR, it is necessary to reverse-transcribe mRNA to complementary DNA (cDNA). An oligo-dT primer first-stand cDNA was produced

using the Sensiscript reverse transcription Kit (Product No. 205213, Qiagen, Germany). The regents added to the tube for reverse transcription PCR were shown in table 1. Reverse transcription PCR reaction was performed at 37 ℃ for 60 minutes. A total reaction volume 200 µl was obtained from each cell sample. All cDNA samples were stored at -20℃ for subsequent PCR analysis.

Table 1: Reverse transcription polymerase chain reaction (RT-PCR) reaction system in the present study

Component	Volume per reaction (µl)
RT-buffer (10x)	20
dNTP Mix (5 mM)	20
oligo (dT) primer (0.5 µg/µl)	2
RNase inhibitor (10 units/µl)	2.5
sensiscript reverse transcriptase	10
RNase-free water	125.5
total cellular RNA	20
Total per Reaction	200

RT: reverse transcription; **dNTP**: Deoxy-ribonucleoside triphosphate; **RNase**: ribonuclease; **RNA**: ribonucleic acid.

2.4.2 Real-time quantitative PCR

2.4.2.1 Principle of real-time PCR

A real-time PCR combines the chemical principle of a conventional PCR and a technique for real-time quantitating colour signal that specifically indicates PCR production. Since the conventional PCR is well-known to all, only the technique to detect the PCR production signal in real-time modules is here described. The primers used for PCR in this study are summarised in table 2.

In the present study, β2-Microglobulin was employed as an internal reference gene in C2C12 cells, which was generally used in mouse muscle cells and the stable expression of β2-Microglobulin following stress has been proven. First, Hsp70 mRNA was examined in C2C12, which is the most important parameter representing cellular response to ES. Second, several parameters involved in cellular proliferation and differentiation (e.g. P21, Cyclin D1 and IGF-1) were investigated at mRNA level. P21 is an inhibitor of cellular proliferation, whereas,

both Cycin D1 and IGF-1 can be served as promoters of cell growth. Finally, apoptotic parameters, such as Bax, Bcl-2, Caspase8, 9, Apaf-1, AIF, were also been examined at mRNA level, in which Bcl-2 is negative regulator of cellular apoptosis and the others are promoters involved in the process of apoptosis.

Table 2: The primers used for polymerase chain reaction in the present study

Gene name	Accession number	Forward primer	Reverse primer	Product length(bp)
β2-Micro globulin	NM_009735	gagactgatacatacgcctgcaga	tcacatgtctcgatcccagtaga	81
Hsp70	NM_010479	agccttccagaagcagagc	ggtcgttggcgatgatct	124
IGF-1	Y18062	gctatggctccagcattcg	gctccggaagcaacactca	65
P21	AF035683	gaaaacggaggcagaccagc	cacagcagagggcggg	108
CyclinD1	NM_007631	tctttccagagtcatcaagtgtg	gactccagaagggcttcaatc	75
Bcl-2	NM_009741	catgccgtccttagaaaataca	ctgctttttatttcatgaggtacatt	132
Bax	NM_007527	cggcgaattggagatgaa	gtgtccacgtcagcaatcat	65
AIF	NM_012019	atcatgctgttgtgagtggaa	caggacccaaatcactcca	99
Apaf-1	NM_009684	cttgacattaaagctcatgaagatg	cagtcgcagaatcccaaatc	115
Caspase8	NM_009812	cagagagaagaatgagccttgaa	gtcacacagttccgccattt	89
Caspase9	NM_015733	gtacatcgagaccttggatgg	tcgcagaaacagcattgg	95

Hsp70: Heat shock protein 70; **Bcl-2**: B-cell leukemia/lymphoma 2; **Bax**: BCL2-associated X protein; **AIF**: Apoptosis-inducing factor; **Apaf-1**: Apoptotic peptidase activating factor 1; **IGF-1**: Insulin-like growth factor 1; **P21**: Cyclin-dependent kinase inhibitor 1A; product length is according to the order of results.

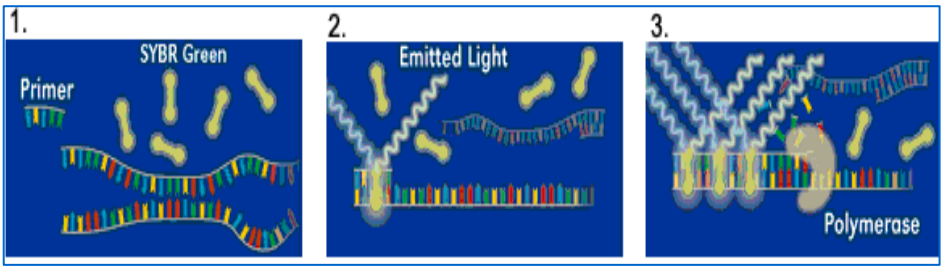

Figure 7: SYBR Green I technique. (1) At the beginning of amplification, the unbound dye molecules weakly fluoresce, producing a minimal background fluorescence signal which is subtracted during computer analysis. **(2)** After annealing of the primers, DNA binding results in a dramatic increase of the SYBR Green I molecules to emit light upon excitation. **(3)** During elongation, more and more dye molecules bind to the newly synthesized DNA. An increase in fluorescence is viewed in real-time. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls.

On a real-time PCR, a color indicator to reflect PCR product is adopted. The most commonly used color indicator is Sybr Green I (Figure 7). Sybr Green I can bind specifically to the synthesized double-stranded DNA, and therefore given information about changes in PCR product amount.

2.4.2.2 Real-Time PCR quantitation

As stated above, real-time PCR quantitation follows changes of the color indicator for PCR product. Figure 8 shows a typical real-time PCR run curve that represents a sigmoid shape. At the beginning of PCR, the amount of the double stranded DNA is low or blank. After one cycle of PCR, this amount is theoretically doubled. This makes, however, at the early phage only trivial change in the total amount of PCR product, leading to a fluctuation in the color indicator signal (baseline). When PCR product is accelerated enough, an additional PCR cycle can result in a distinct increase in color indicator signal, which is followed by a rapid change phage in an exponential manner. The synthesized PCR product reaches a so-called threshold when the color signal begins to increase exponentially, and the corresponding cycle number of PCR is termed cross point (Cp).

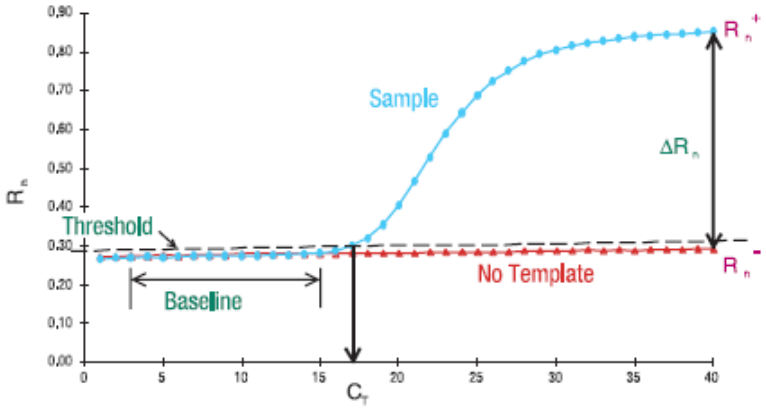

Figure 8: Model of a single amplification plot, showing terms commonly used in real-time quantitative polymerase chain reaction. The threshold line is the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The cycle at which the sample reaches this level is called the Cycle Threshold (C_T) or crossing point (Cp). **Rn** is normalized Reporter signal, level of fluorescence detected during PCR; **Rn^+** is the Rn value of a reaction containing all components (the sample of interest); **Rn^-** is the Rn value detected in no template control (baseline value); **ΔRn** is the difference between Rn^+ and Rn^-

It is evident that Cp is related the original copy number of the tested samples (Pfaffl,

2001). Thus, Cp serves as the important parameter to estimate mRNA level. According to the Cp, the relation of mRNA level between two samples can be estimated as follow:

$$\frac{A_{\text{original}}}{B_{\text{original}}} \longrightarrow \frac{Cp_B}{Cp_A}$$

Given an efficiency factor = 2, (PCR product doubled after each cycle), the relation can be expressed:

$$\frac{A_{\text{original}}}{B_{\text{original}}} = 2^{Cp_B - Cp_A}$$

Hence, to estimate gene expression level with real-time PCR, the crossing points of the samples and the efficiency of PCR must be determined. Furthermore, to preclude potential influence in real-time PCR quantitation resulted from variability in workflow (e.g. adding samples), a so-called internal reference gene that should express universally and stably in the cells is usually taken to make samples comparable.

2.4.2.3 Real time quantitative PCR with LightCycler in the present study

In the present study the real-time PCR was run with Lightcycler system (Version 1.2, Software 3.51, Roche Diagnostics, Switzerland). The agents added to Lightcycler for PCR are stated in table 3. Sybr green PCR master-mix contains PCR buffer, Tag polymerase, deoxy-ribonucleoside triphosphate (dNTP) and $MgCl_2$.

Table 3: Regents for real-time PCR with Lightcycler in the present study

Component	Volume per reaction (µl)
SYBR PCR master mix	10
Forward primer(10 mM)	0.5
Reverse primer(10 mM)	0.5
RNase-free water	7
cDNA sample	2
Total per Reaction	20

PCR: polymerase chain reaction; **RNase:** ribonuclease; **cDNA:** complementary deoxyribonucleic acid.

Prior to quantitative analysis, several titration experiments, for $MgCl_2$ and efficiency test were performed to determine optimum amplifications conditions. The real-time

PCR program run with the Lightcycler Sybr Green was described in table 4

Table 4: Real-time polymerase chain reaction program with Lightcycler SYBR Green in the present study

Program		Hotstart		Cycles: 1
Segment Number	Temperature Target ℃	Hold time (sec)	Slope (℃/sec)	Acquisition Mode
1	94	900	20	None

Program		Amplification		Cycles: 60
Segment Number	Temperature Target ℃	Hold time (sec)	Slope (℃/sec)	Acquisition Mode
1	94	15	20	None
2	60*	20	20	None
3	72	15	20	Single

*Annealing temperature for each primer is different (specifically, Hsp70, Apaf-1, Caspase9, IGF-1, P21, Cyclin D1 at 60 ℃, whereas, Bcl-2, Bax, AIF, Caspase8 at 63℃)

Melting curve analysis: To confirm the unique amplified products, melting curve analysis was performed at the end of the amplification by cooling the samples at 20℃/seconds down to 60 ℃, and then increasing the temperature to 95 ℃, at 0.1 ℃/seconds with continuous fluorescence measurement.

Table 5: Real-time polymerase chain reaction melting curve analysis program

Program		Melting curve		Cycles: 1
Segment Number	Temperature Target ℃	Hold time (sec)	Slope (℃/sec)	Acquisition Mode
1	95	5	20	None
2	60*	15	20	None
3	95	0	0.1	Continuous
4	40	10	20	None

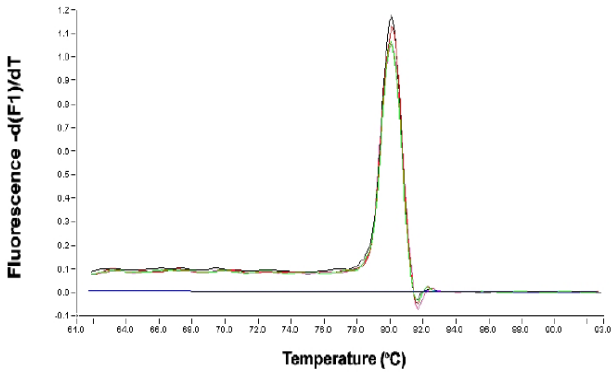

Figure 9: Lightcycler meting curve of β2 microglobulin. The unique amplified product was confirmed by melting temperature =80 ℃.

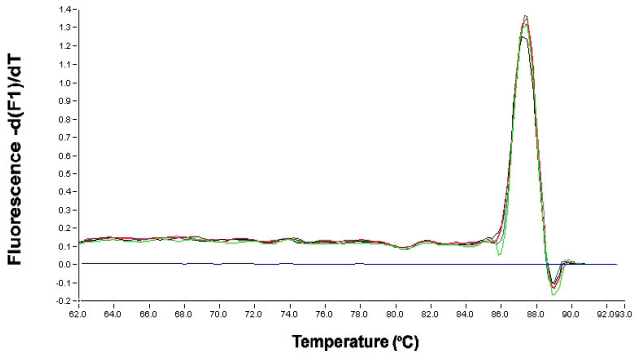

Figure 10: Lightcycler meting curve of Heat shock protein 70 (Hsp70). The unique amplified product was confirmed by melting temperature= 87 °C.

To verify an expected PCR product, the PCR amplicons were electrophoresed on 3% agarose gel stained with ethidium bromide (Figure 11). Based on the positions of DNA size in the gel, the size of a PCR product could be estimated.

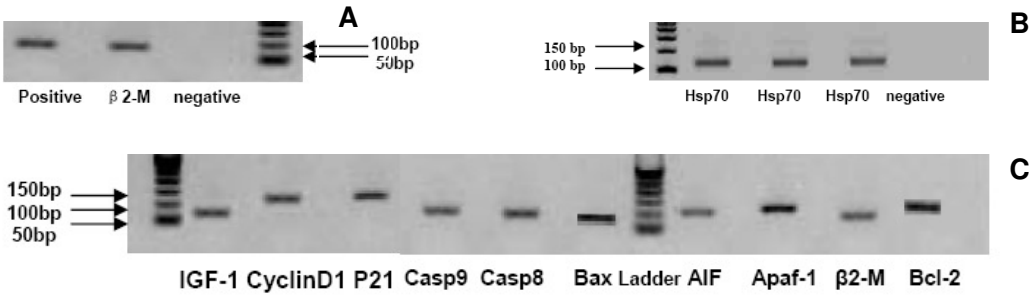

Figure 11: Agarose gel electrophoresis. A) β2 microglobulin Real-time PCR product size: 81bp; **B)** Heat shock protein 70 (Hsp70) Real-time PCR product size: 124bp; **C)** Real-time PCR product size: IGF-1: 65bp; Cyclin D1: 75bp; P21: 108bp; AIF:99bp; Apaf-1: 115bp; Bcl-2: 132bp; Bax: 65bp; Caspase8: 89bp; Caspase9: 95bp.

2.4.2.4 Calculation of real-time PCR results

As stated above, Cp and amplification efficiency (*E*) serve as the important parameter to estimate mRNA level. According to the Cp and *E*, the relation of mRNA level between two samples can be estimated. Generally two quantification types in Real-time PCR are possible: absolute quantification method and relative quantification method. Relative quantification method is employed in our experiment, which means that target concentration is expressed in relation to the concentration of a housekeeping gene. To investigate the physiological changes in gene expression, the expression ratio is adequate for the purpose of the present study. As the relative quantification calculation is based on the assumption that amplification of individual genes occurs with similar reaction efficiencies,

quantitative PCR assays of 10-step 10X serial dilutions of real-time PCR product were performed with each gene primer set used. Then the curve for each gene was got and the reaction efficiencies were calculated. Real-time PCR amplification efficiencies were calculated using the given slopes in lightCycler software. The corresponding real-time PCR efficiency factor (E_f) of one cycle in the exponential phase was calculated using the equation: $E_f = (10^{-1/slope})$. Generally, for a 100% efficient reaction 2^n = fold dilution: where n = cycle number difference, therefore: 10-fold dilution series: n ≅ 3.3. The efficiency of the PCR should be 90-100% and the ideal slope of efficiency curve should be 3.3.

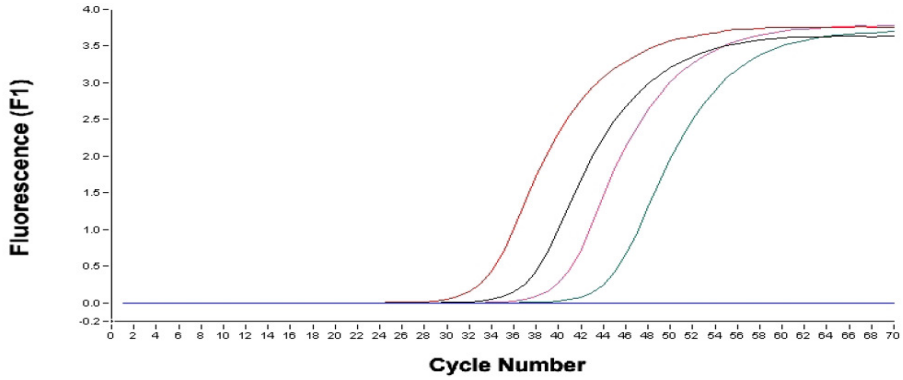

Figure 12: Real-time PCR of Heat shock protein 70 (Hsp70) efficiency test. Hsp70 amplified from 10X serial dilutions of real-time PCR Hsp70 product (10^{-6},10^{-7},10^{-8},10^{-9}).

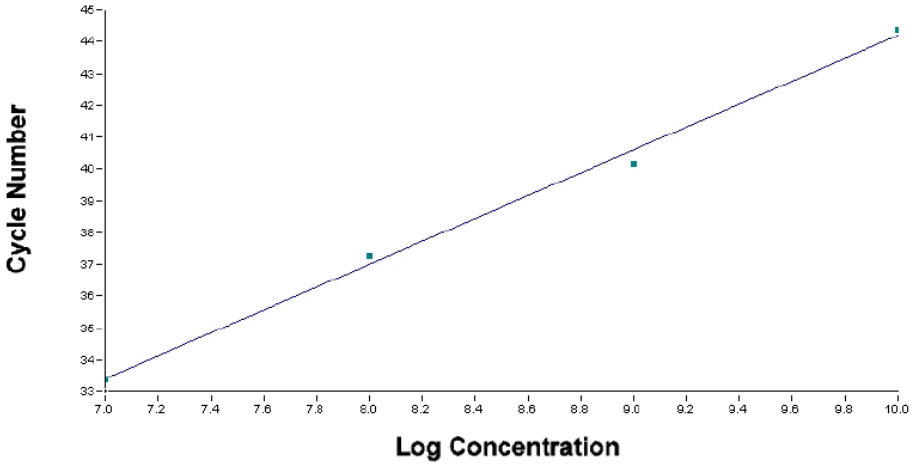

Figure 13: When plotted against the log of Heat shock protein 70 (Hsp70) product concentration, the crossing point (Cp) can be used to estimate efficiency of amplification. Slope=3.407, Intercept=9.667.

The ratio of a target gene is expressed in a sample versus a control in comparison to a reference gene. E_{target} is the real-time PCR efficiency of target gene transcript; E_{ref} is the real-time PCR efficiency of a reference gene (β2-Microglobulin) transcript;

ΔCp_{target} is the Cp deviation of control - sample of the target gene transcript; ΔCp_{ref} =Cp deviation of control - sample of the reference gene transcript. For the calculation of R, the individual real-time PCR efficiencies and the ΔCp of the investigated transcripts must be known.

For example (Figure 14), the change of Hsp70 mRNA expression of C2C12 in the ES group was analyzed with β2-Mikroglobulin as the reference gene, and the relation to the expression of Hsp70 mRNA in the control group.

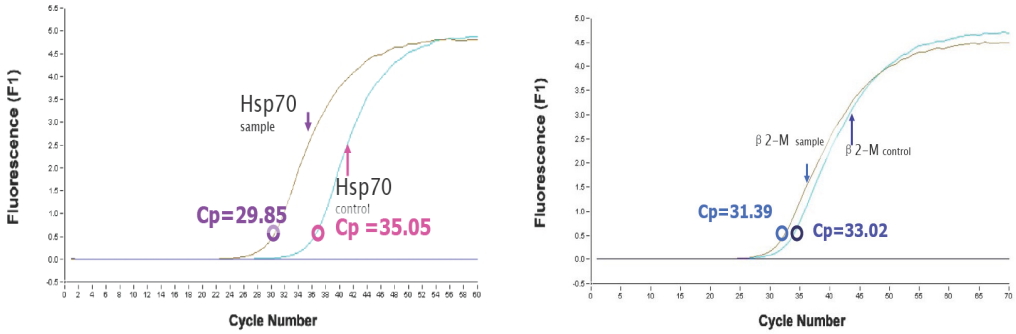

Figure 14: The formula for the calculation of the ratio. $Ratio_1 = (E_{Hsp70})^{\Delta Cp\ Hsp70\ (control\text{-}sample)} = (1.967)^{35.05-29.85} = 1.967^{5.2} = 33.711$; $Ratio_2 = (E_{\beta2\text{-}M})^{\Delta Cp\ \beta2\text{-}M\ (control\text{-}sample)} = (1.952)^{33.02-31.39} = 1.952^{1.63} = 2.975$; $Ratio = Ratio_1 / Ratio_2 = (E_{Hsp70})^{\Delta Cp\ Hsp70\ (control\text{-}sample)} / (E_{\beta2\text{-}M})^{\Delta Cp\ \beta2\text{-}M\ (control\text{-}sample)} = 33.711 / 2.975 = 11.33$. **Hsp70**: Heat shock protein 70; **Cp**: Crossing point; **β2-M**: β2 microglobulin.

2.5 Determination of Hsp70 at protein level

2.5.1 Total protein extraction

The C2C12 cell pellet was homogenized in 200 µl extraction buffer containing 100 mmol/l $Na_4P_2O_7$ $10H_2O$, 5 mmol/l EGTA, 5 mmol/l $MgCl_2$ $.6H_2O$, 0.3 mMol/l KCl, and 1 mMol/l dithiothreitol with an ultrasonic homogenizer. The cell homogenates were stirred on ice for 20 min and then centrifuged at 4℃ and 16,000 g for 10 min (Liu et al., 1999). The supernatant was collected and the total protein concentration was determined according to the method of Lowry (Lowry et al., 1977).

2.5.2 Protein concentration measurement by Lowry's method

The principle of Lowry's method is quite simple. Briefly, the phenolic group of tyrosine and trytophan residues (amino acid) in a protein will produce a blue purple color complex, with Folin- Ciocalteau reagent which consists of sodium tungstate

molybdate and phosphate. The intensity of color depends on the amount of these aromatic amino acids present and will thus vary with different proteins.

Standard curve should be made with various concentration of the standard protein (Albumin bovine fraction, ABF). In order to calculate the protein concentration for the samples based on their absorption values at 750nm wave length. This standard curve will linearly correlate the protein concentration and the Abs 750nm. The forum of the standard curve is Y=aX+b. Y is absorption of the protein; X is the concentration of the protein. Then the concentration of the unknown sample is determined by comparison to this curve. The formula is shown: X=(Y-b)/a. As shown in table 6:

Table 6: Standard protein (ABF) concentration

Standard concentration (mg/ml)	0,0	0,4	0,8	1,2	1,6	2,0	2,4	2,8	3,2	3,6	4,0
Absorption 750 nm	0.000	0.079	0.149	0.218	0.263	0.345	0.372	0.413	0.467	0.473	0.517

Standard curve: Y= 0.0445+ 0.1276X

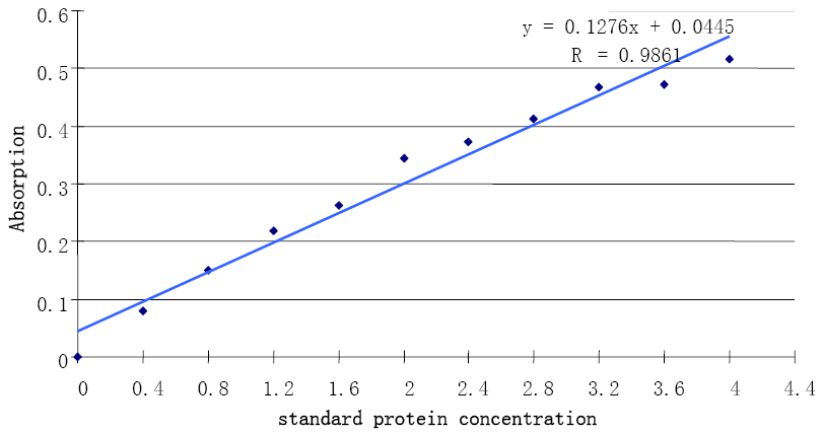

Figure 15: Standard protein curve. Standard curve: Y= 0.0445+ 0.1276X

Table 7: Calculation of the protein concentration (Figure 15).

Unknown protein Absorption 750 nm	0.355	0.463	0.300	0.487	0.369	0.562	0.350	0.470	0.451	0.603	0.586
Protein concentration (mg/ml)	2.434	3.28	2.002	3.468	2.543	4.056	2.395	3.335	3.186	4.377	4.244

2.5.3 Hsp70 quntification by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blot

The protein samples were prepared for electrophoresis by adjusting total protein concentration with Laemmli sample buffer to 0.5 µg / µl, then heated at 95 ℃, for 5 minutes. 40 µg protein were loaded to SDS-PAGE with a 4% stacking and 7.2% seperation gels using the Hoefer SE600 electrophoresis unit equipped with a cooling system developed in our lab to keep the temperature of running buffer constant. Standard Hsp70 protein (10 ng, 15 ng, 20 ng, 25 ng, respectively) was also loaded to indicate the protein molecular size and used to calculate the amount of Hsp70 in each sample. SDS-PAGE was run at 100 V, 50 A for about 20 hours.
After electrophoretic separation, proteins were transferred to a polyvinylidene difluoride (PVDF, pore size 0.45 µm) sheet using the LKB 2117 multiphot II electrophoresis system at 200 V and 0.8 mA / cm^2 for 60 minutes. The membranes were then blocked in PBS-T containing 5% nonfat dried milk at 4 ℃ for overnight. After three times of washing (15 min each) with PBS-T, membranes were incubated with anti-Hsp70 mouse monoclonal IgG1 diluted 1:1000 in 5% milk for 1h. After washing with PBS-T three times (15 min each), membranes were incubated for 1h with goat anti-mouse IgG Fc diluted 1:10000 in 5% milk. This second antibody was conjugated with horse-radish-peroxidase (HRP). After five times of washing with PBS-T, the membranes were incubated in ECL-regent solution (Kodak, New York, USA) for one minute, and then expose to ECL film (Eastman Kodak Company, New York, USA). The ECL films were then developed according to the standard protocol by Kodak Company (Figure 16). The protein bands were densitometrically digitalized by using a digital camera. The densitometric integral derived from each band, i.e. the integral of a mean density over a measured area, was taken to calculate the amount of Hsp70 in each sample. The procedure was done using the software Mars (Mecam, Labor fur Bildverarbeitung, Fachhochschule ULM, ULM, Germany). A linear regression could be obtained from the series of different known amount of standard Hsp70 and their corresponding optical density integrals (Figure 17). According to this standard curve Hsp70 amount of each sample was calculated.

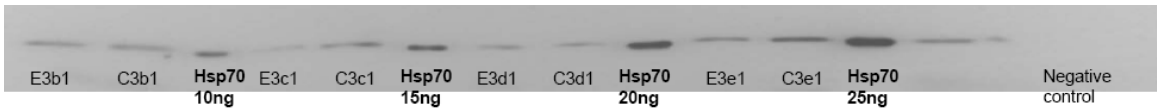

Figure 16: Heat shock protein 70 (Hsp70) blot depicting standard Hsp70 and Hsp70 obtained from samples.

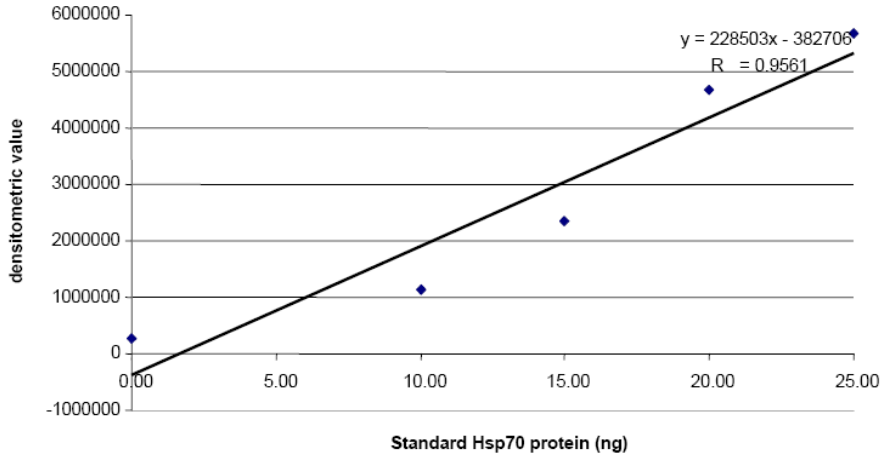

Figure 17: Relationship of standard Heat shock protein 70 (Hsp70) protein to density. Densitometric value = 228503 Hsp70- 382706.

A linear relationship was found between the known Hsp70 and the densitometric values: densitometric value = 228503 Hsp70- 382706 (r^2 =0.91, Figure 16 and 17). On the basis of this linear relationship, Hsp70 of each sample was calculated. For example, Hsp70 from each sample (40 µg of the total protein) was calculated to be E3b1:9.19 ng, C3b1: 9.83 ng; E3C1:8.11 ng; C3b1:11.82 ng; E3d1: 9.71 ng; C3d1:10.34 ng; E3e1:13.23 ng; C3e1: 18.49 ng.

2.6 Statistic analysis

The experimental setting was repeated three times. The gene expression of the ES-treated cells was estimated by the ratio to their corresponding control cells, where the baseline level (before ES) was set to 100%. The protein expression of Hsp70 was determined by western-blot and Hsp70 protein amount of each sample was calculated according to the standard curve between densitometric integral and the known standard Hsp70 amounts. Hsp70 protein of ES-treated samples was also expressed in relation to the corresponding control sample and baseline (before ES) was set to 100%. All samples were run with real-time PCR or western-blot in replicate. The final results were expressed as mean value.

3 Results

3.1 Performance of the experiment

Experiments were accomplished following the study design. During the experiments the parameters of ES were monitored, and they kept constant in the stimulation period. The temperature of the cell culture media also maintained at 37 ℃ constantly. Potential influence of ES on morphology and cell growth was observed through microscope. For determination of Hsp70 content in samples with quantitative western-blot, repeated measurements were done to examine the reproducibility and stability. In addition to verification of the PCR products, the amplification efficiency in real-time PCR was tested for each parameter, the respective efficiencies are stated in table 8.

Table 8: Amplification efficiency of the parameters studied in present study

Parameter	Amplification efficiency
β2-microglobulin	1.952
Hsp70	1.967
Bcl-2	1.945
Bax	1.965
AIF	1.908
Apaf-1	1.921
Caspase8	1.834
Caspase9	1.823
IGF-1	1.912
P21	1.893
CyclinD1	1.868

Hsp70: Heat shock protein 70; **Bcl-2:** B-cell leukemia/lymphoma 2; **Bax:** BCL2-associated X protein; **AIF:** Apoptosis-inducing factor; **Apaf-1:** Apoptotic peptidase activating factor 1; **IGF-1:** Insulin-like growth factor 1; **P21:** Cyclin-dependent kinase inhibitor 1A

3.2 Influence of ES on morphology and cell growth

C2C12 cells were cultured in growth medium (DMEM with 10% FBS) after recovery. In this condition, cells were myoblastic and divided rapidly. Usually, the number of cells will be doubled in about 20 to 24 hours. To avoid fusion and differentiation, C2C12 cells were sub-cultured by 50%-60% confluence. When they are growing,

C2C12 myoblasts are flat, not confluent and closely adherent to the substrate. They are fusiform or star-shaped, rigorously showing one central nucleus. However, a number of cells were observed at different mitotic phases.

By 70%-80% confluence, cells were stimulated. In Group A and B, there were no obvious morphologic changes observed in C2C12 cells after LFES under microscope (Figure 18). However, in Group C, it could be observed that cells near the electrode were died (Figure 19).

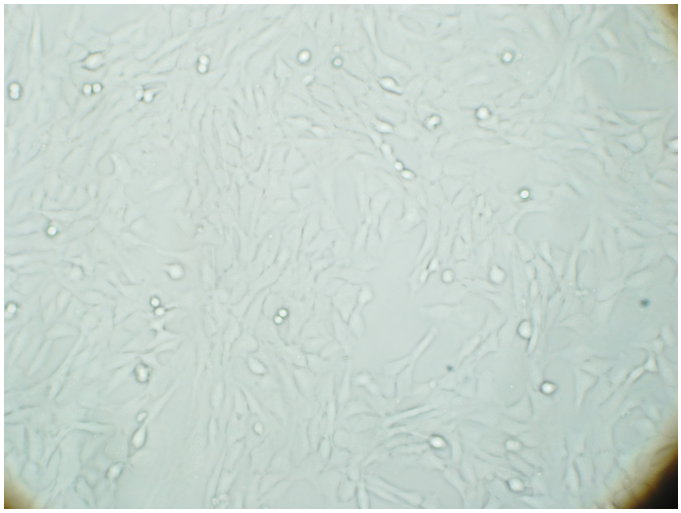

Figure 18: Effects of low frequency electrical stimulatiom (LFES) on C2C12 cells. No obvious morphological changes of C2C12 were observed after LFES. (100X)

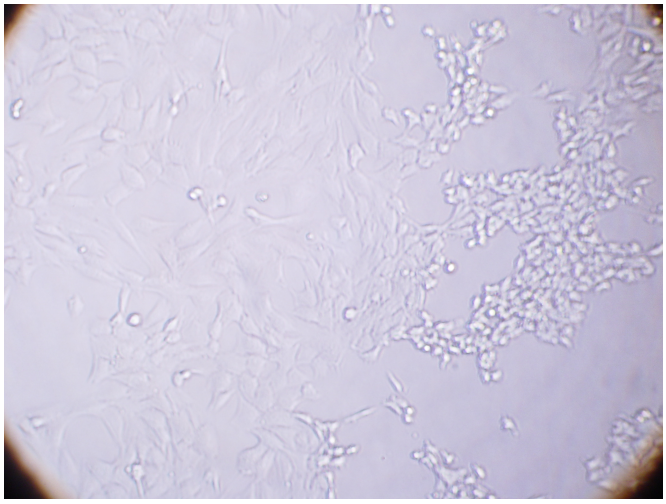

Figure 19: Effects of high frequency electrical stimulatiom (HFES) on C2C12 cells. C2C12 cells near the electrode were dead after HFES. (100X)

3.3 Hsp70 response to ES

3.3.1 Hsp70 response to ES at mRNA level

As stated in methods, gene expression at mRNA level was derived from relative quantitative real-time PCR. It means that the comparison among different samples is done by calculating ratios between the samples (see data analysis). These results are expressed here in percentage.

3.3.1.1 Hsp70 response at mRNA level to ES with low frequency for short duration ($LFES_{11}$)

The expression of Hsp70 at mRNA level is depicted in figure 20A. In the control group, expression of Hsp70 mRNA showed a slight tendency to decline during the 12h cell culture. In the stimulated group, expression of Hsp70 mRNA increased distinctly at 1h and 8h after ES, revealing a biphasic response following the ES. Specifically, Hsp70 mRNA was 89% (1h after ES) and 102% (8h after ES) higher compared to baseline, respectively. Then it returned below baseline after 12h recovery from ES.

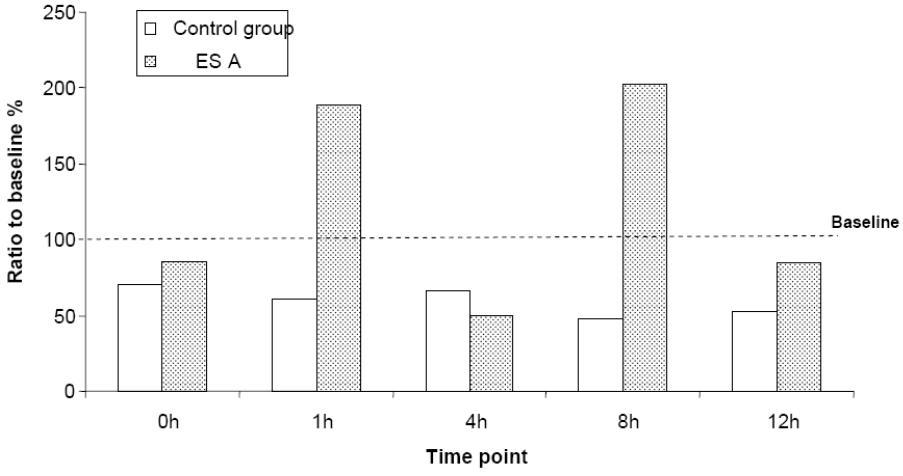

Figure 20 (A): Heat shock protein 70 (Hsp70) mRNA expression. Hsp70 response at mRNA level to electrical stimulation (ES) at 13V with 12Hz for 11min (group A). 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively. Value before ES was set at 100% as baseline. Hsp70 expression level is expressed as ratio to baseline at the corresponding time points. **ES**: electrical stimulation.

3.3.1.2 Hsp70 response at mRNA level to ES with low frequency for long duration ($LFES_{90}$)

The expression of Hsp70 at mRNA level is depicted in Figure 20B. In the control group, there was little change in Hsp70 mRNA along with cell culture. However, compared with baseline in ES group, Hsp70 mRNA level increased by 65% 1h after ES, peaked by 459% 4h after ES and kept elevated at by 219% 12h after ES.

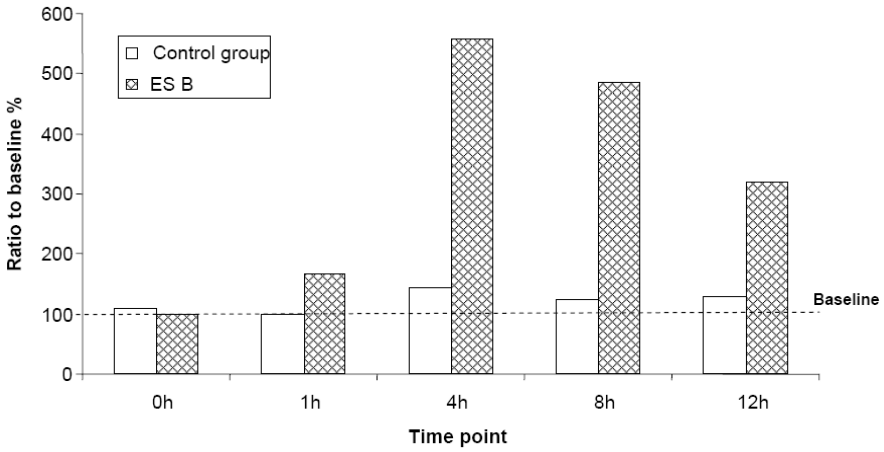

Figure 20 (B): Heat shock protein 70 (Hsp70) mRNA expression. Hsp70 response at mRNA level to electrical stimulation (ES) at 13V with 12Hz for 90 min (group B). 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively. Value before ES was set at 100% as baseline. Hsp70 expression level is expressed as ratio to baseline at the corresponding time points.

3.3.1.4 Hsp70 response at mRNA level to ES with high frequency for short duration ($HFES_{11}$)

The expression of Hsp70 at mRNA level is depicted in figure 20C. The expression of Hsp70 mRNA in control was quite stable along cell culture and decreased slightly at 12h. In the ES group, Hsp70 mRNA significantly increased at by 45% 1h after ES, peaked by 1310% 4h after ES and kept elevated at by 82% 12h after ES.

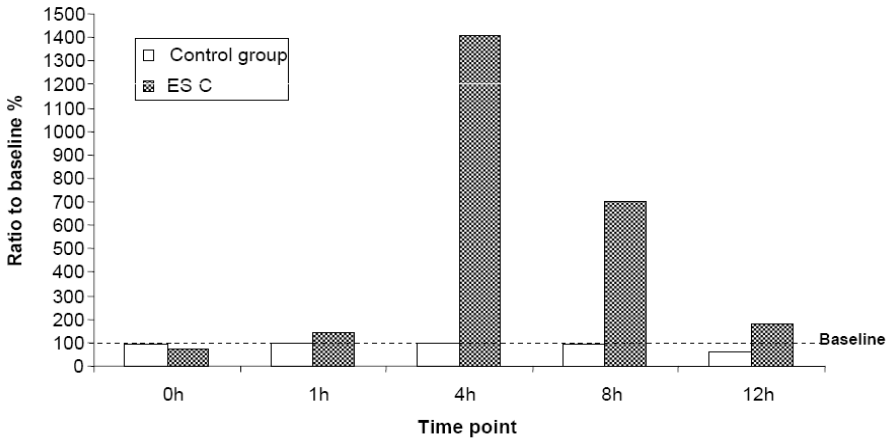

Figure 20 (C): Heat shock protein 70 (Hsp70) mRNA expression. Hsp70 response at mRNA level to electrical stimulation (ES) at 13V with 100 Hz for 11 min (group C). 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively. Value before ES was set at 100% as baseline. Hsp70 expression level is expressed as ratio to baseline at the corresponding time points.

3.3.1.4 Effects of different kinds of ES on Hsp70 mRNA expression in C2C12

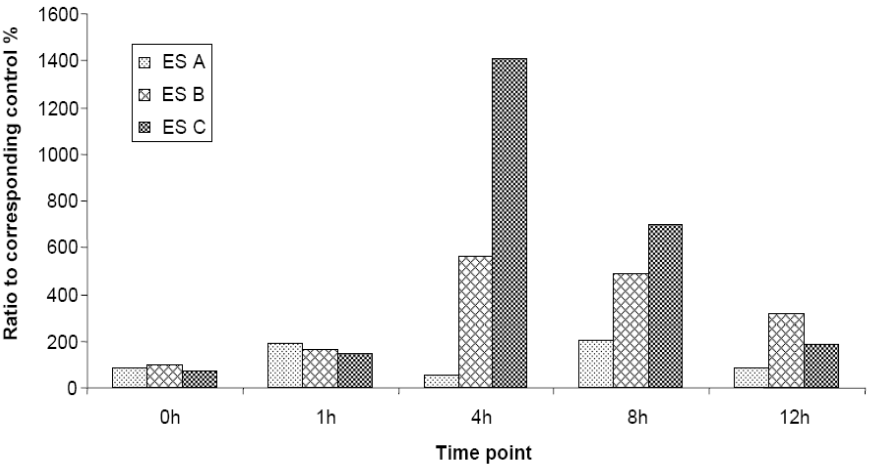

Figure 20 (D): Heat shock protein 70 (Hsp70) mRNA expression. Hsp70 response at mRNA level to electrical stimulation (ES) at 13V, with 12Hz for 11min (group A), 12Hz for 90min (B), and 100 Hz for 11 min (C), respectively. 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively. Hsp70 mRNA expression of ES treated cells was estimated by the ratio to their corresponding control cells, which was set to 100%.

In figure 20D, different response of Hsp70 mRNA were compared among the three groups at each time point. Besides a biphasic response in group A, peak Hsp70 mRNA level was clearly lower and an elevated expression duration was shorter than that in group B and C, showing an order in amplitude of Hsp70 response:

C>B>A, and in duration: B>C>A.

3.3.2 Hsp70 response to ES at protein level

3.3.2.1 Hsp70 response at protein level to ES with $LFES_{11}$

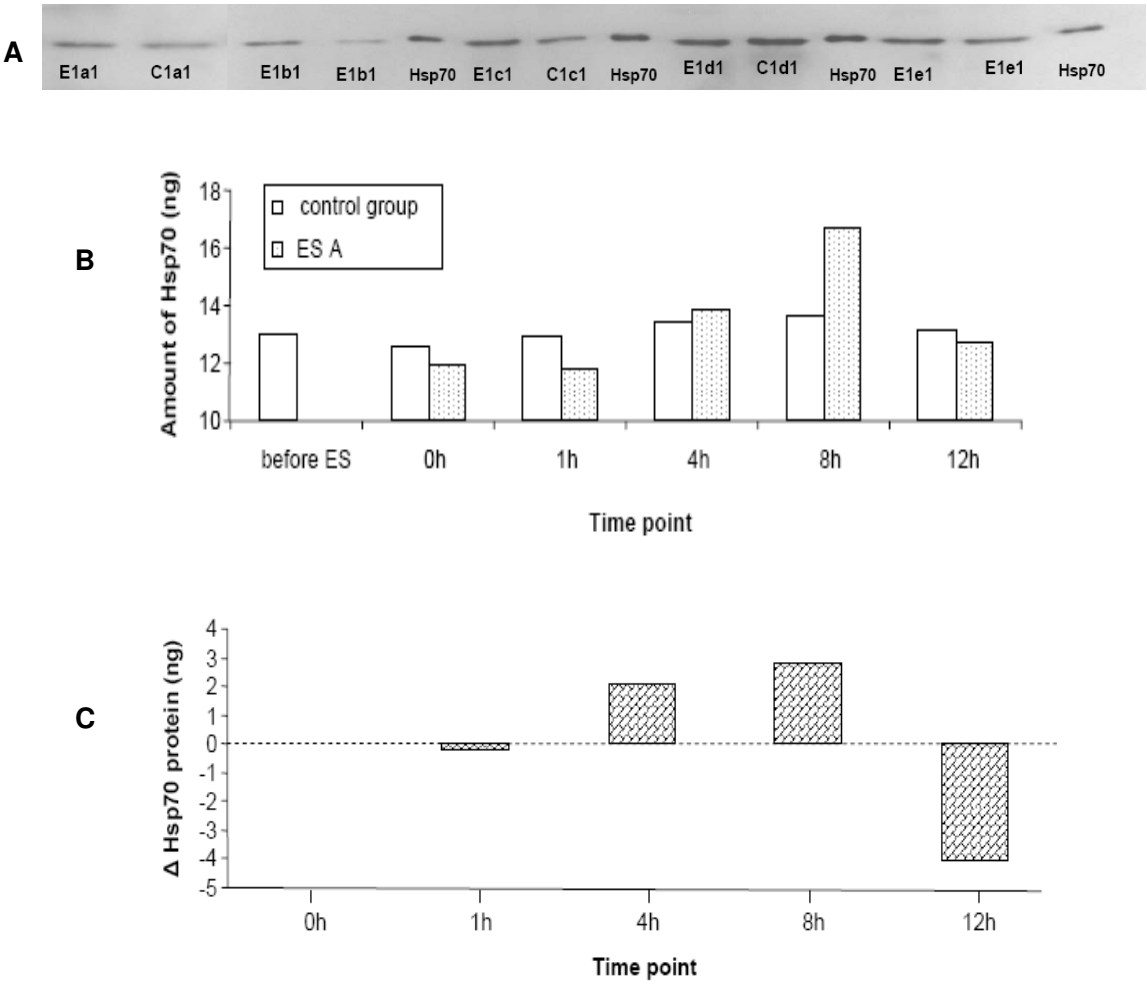

Figure 21: Heat shock protein 70 (Hsp70) response at protein level to electrical stimulation (ES) at 13V with 12Hz and 11min (group A). A: Determined by western-blot. Hsp70 protein immunoblot depicting standard Hsp70 (10, 15, 20 and 25ng, respectively) and Hsp70 obtained from samples (40ug total protein loaded). “E” and “C” indicate ES treated group and control group. “a, b, c, d and e” indicate each time point (0h, 1h, 4h, 8h, and 12h after ES), respectively; **B:** Hsp70 protein level of each sample (40ug total protein loaded); **C:** Hsp70 protein changes in group A. Difference (Δ) of Hsp70 protein between consecutive time points serves as an indicator of Hsp70 protein production. 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately, 1h, 4h, 8h and 12h after ES, respectively.

Hsp70 protein level at each time point as depicted in figure 21. In the absence of ES, Hsp70 protein expression in the control group was fairly constant at each time point. Hsp70 protein began to increase by 3.02% 4h after ES and peaked by

22.13% at 8h after ES. Thereafter, Hsp70 protein decreased below control 12h after ES. Furthermore, difference of Hsp70 protein between consecutive time points, serving as an indicator of Hsp70 protein production, showed that the maximum Hsp70 increase happened at 8h after ES.

3.3.2.2 Hsp70 response at protein level to $LFES_{90}$

Hsp70 protein level at each time point is depicted in Figure 22. In the control group, Hsp70 protein showed less change over the observation period. In the stimulated cells, Hsp70 protein level began to increase at 4h after ES by 30.94%, reached a peak at 8h after ES (increase by 313.12%) and kept elevated at 12h after ES.

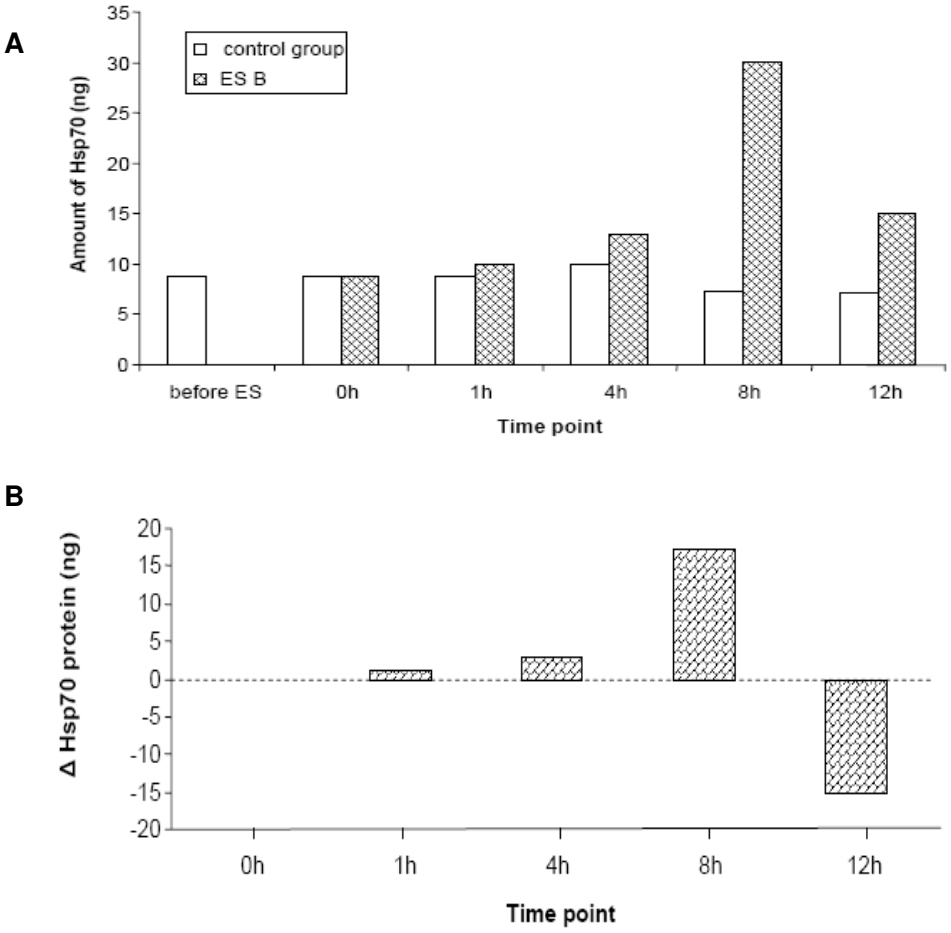

Figure 22: Heat shock protein 70 (Hsp70) response at protein level to electrical stimulation (ES) at 13V with 12Hz and 90min (group B). 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively. **A**: Hsp70 protein level of each sample (40ug total protein loaded). **B**: Hsp70 protein changes in group B. Difference (Δ) of Hsp70 protein between consecutive time points serves as an indicator of Hsp70 protein production.

3.3.2.3 Hsp70 response at protein level to $HFES_{11}$

Hsp70 protein level at each time point is depicted in Figure 23. In the absence of ES, Hsp70 protein showed less change over the observation period. There was a significant up-regulation of Hsp70 protein only at 8h by 46.29% after ES. However, the expression of Hsp70 protein was depressed compared to the control at other time points after ES. Furthermore, difference of Hsp70 protein between consecutive time points, serving as an indicator of Hsp70 protein production, showed that the maximum Hsp70 increase happened at 8h after ES.

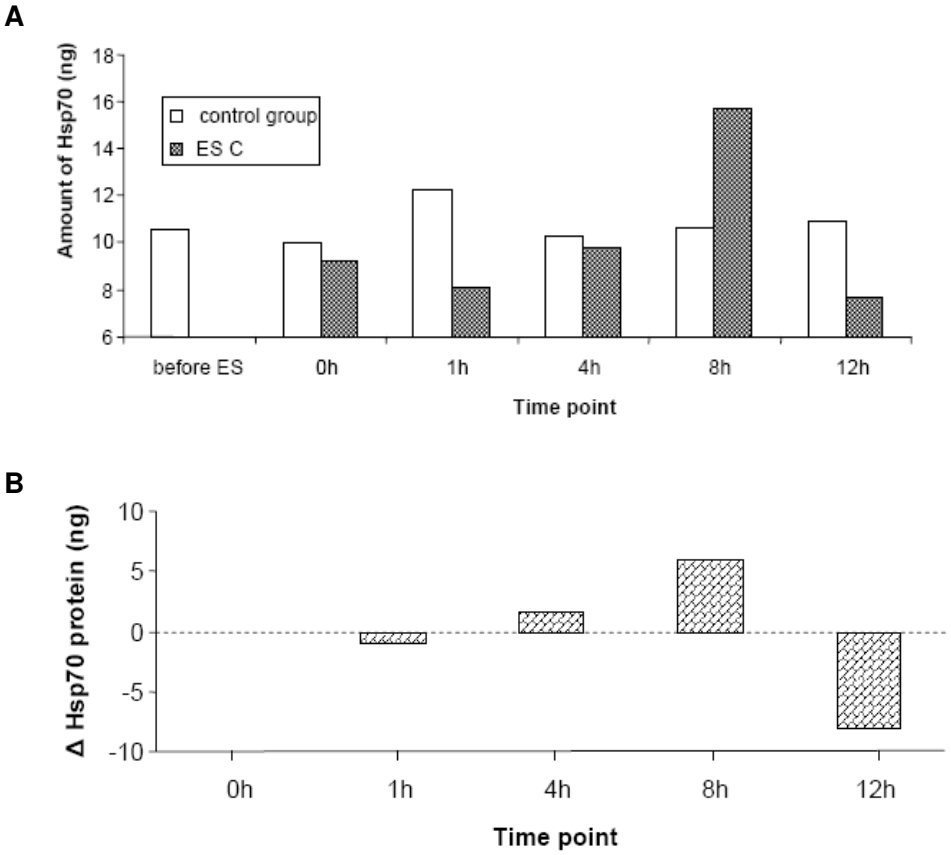

Figure 23: Heat shock protein 70 (Hsp70) response at protein level to electrical stimulation (ES) at 13V with 100Hz and 11min (group C). 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively. **A:** Hsp70 protein level of each sample (40ug total protein loaded). **B:** Hsp70 protein changes in group C. Difference (Δ) of Hsp70 protein between consecutive time points serves as an indicator of Hsp70 protein production.

3.3.2.4 Effects of different kinds of ES on Hsp70 protein expression in C2C12

As shown in figure 24, it is obvious that different kinds of ES had different effects on the expression of Hsp70 protein in C2C12 cells. In comparison among group A, B

and C, 1) the amplitude of up-regulated Hsp70 protein was in order of B more than C more than A; 2) the duration of up-regulated Hsp70 mRNA was in order of B more than A more than C.

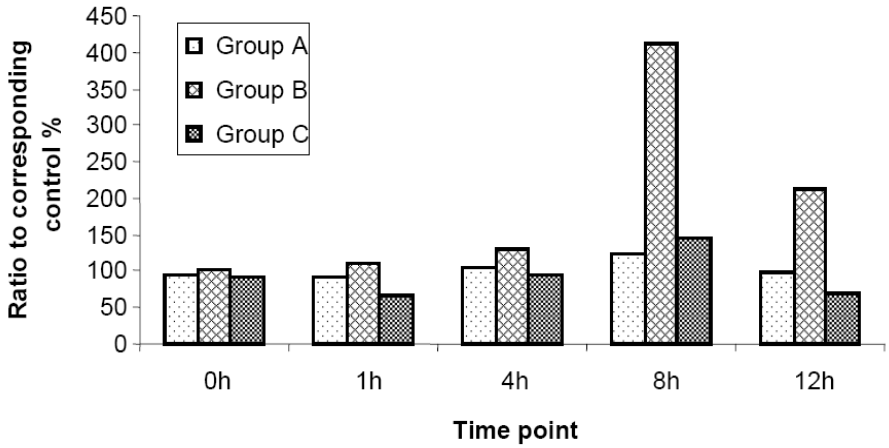

Figure 24: Heat shock protein 70 (Hsp70) response at protein level to electrical stimulation (ES) at 13V, with 12Hz and 11min (group A), 12Hz and 90min (group B), and 100 Hz and 11 min (group C), respectively. 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively. Hsp70 protein expression of ES treated cells was estimated by the ratio to their corresponding control cells, which was set to 100%.

3.4 IGF-1, Cyclin D1 and P21 response to ES at mRNA level

The results of these parameters are expressed here as ratios between ES and corresponding controls. The ration for each parameter was set to 100% before ES as baseline, and the changes of these ratios at different time points were compared to the baseline level and expressed in percentages.

3.4.1 IGF-1 response to ES at mRNA level

In the cells of group A (low frequency, short duration), IGF-1 expression showed a slight increase immediately after ES and a further distinct increase at 1h after ES, and kept elevated over 8h after ES.

In group B (low frequency, longer duration), ES caused an early augmentation in IGF-1 expression at mRNA level immediately after ES with peak level, and maintained clearly elevated over 12h after ES.

In comparison with that of group A and B, electrical stimulated cells (high

frequency, short duration) did not reveal any clear increase in IGF-1 expression within 1h after ES, and showed a depressed expression at 4h and afterwards after ES.

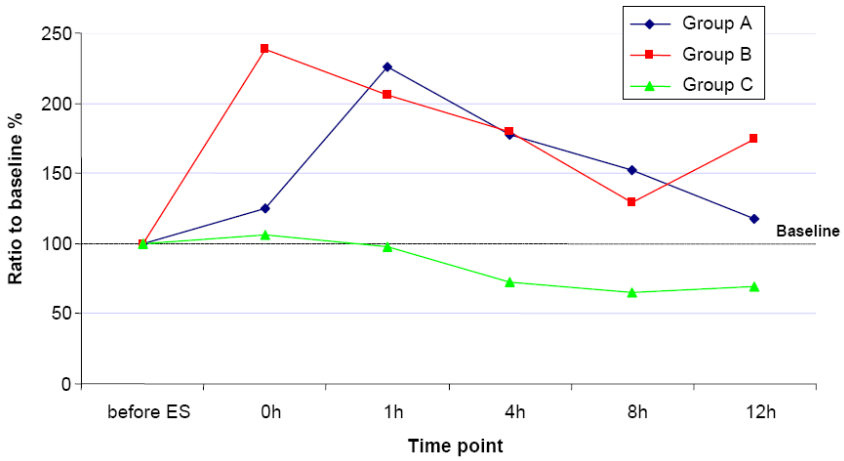

Figure 25: Insulin-like growth factor-1 (IGF-1) response at mRNA level to electrical stimulation (ES) at 13V, with 12Hz and 11min (group A), 12Hz and 90min (group B), and 100 Hz and 11 min (group C), respectively. 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively.

3.4.2 Cyclin D1 response to ES at mRNA level

In figure 26, the expression of cyclin D1 at mRNA level is illustrated. In the cells of group A, cyclin D1 expression showed a slight increase at 1h after Es and a further distinct increase at 4h after ES, and then returned back to baseline at 8h after ES.

In group B, cyclin D1 expression at mRNA level immediately increased after ES and went up further at 1h after ES with the peak level, and maintained elevated over 8h after ES.

In comparison with those of group A and B, cyclin D1 mRNA did not real any clear increase within 1h after ES, and showed a depression at 4h and afterward after ES.

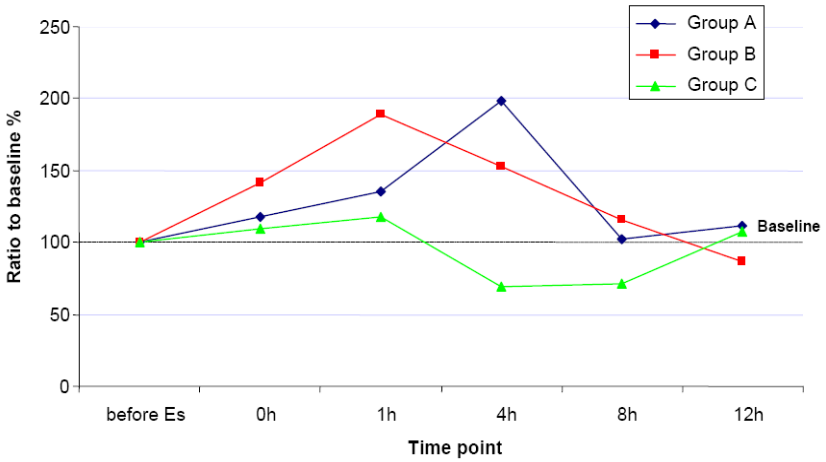

Figure 26: Cyclin D1 response at mRNA level to electrical stimulation (ES) at 13V, with 12Hz and 11min (group A), 12Hz and 90min (group B), and 100 Hz and 11 min (group C), respectively. 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively.

3.4.3 P21 response to ES at mRNA level

In figure 27, the expression of cyclin D1 at mRNA level is illustrated. No distinct changes were shown in P21 mRNA after ES in group A. In group B, P21 mRNA increased immediately after ES and reached a peak level at 4h after ES, and kept clearly elevated over 12h after ES. In group C, ES caused an early augmentation in P21 expression at mRNA level immediately after ES and peaked at 1h after ES, and kept clearly elevated over 8h after ES.

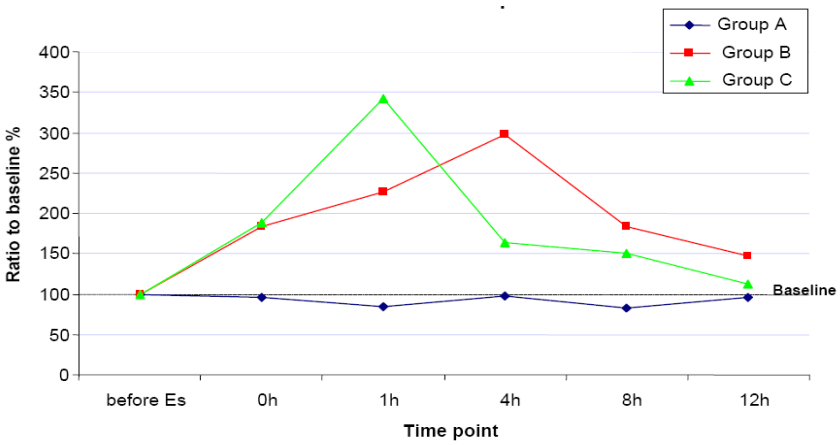

Figure 27: Cyclin-dependent Kinase Inhibitor (P21) response at mRNA level to electrical stimulation (ES) at 13V, with 12Hz and 11min (group A), 12Hz and 90min (group B), and 100 Hz and 11 min (group C), respectively. 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively.

3.4.4 Comparison between IGF-1, Cyclin D1 and P21 response to ES in a certain group

IGF-1 is known as a potent stimulator for cell growth, and cyclin D1 and P21 are established parameters indicating cell cycle phases in the cell growth process. Therefore, these parameters are associated each other during the cell culture, and in the following figures, the relationship among these parameters is shown as response to a certain kind of ES.

In figure 28A, the expression of IGF-1, cyclin D1 and P21 at mRNA level is depicted along with different time points in group A. In response to ES at low frequency for short duration, IGF-1 and cyclin D1 increased simultaneously with different time course for their peak. The expression of P21 did not show clear change over 12h after ES.

In figure 28B, the expression of IGF-1, cyclin D1 and P21 at mRNA level is depicted along with different time points in group B. In response to ES at low frequency for long duration, the expression of IGF-1 and cyclin D1 showed a similar tendency as that in group A. Unlike that in group A, P21 mRNA increased immediately after ES and reached a peak level at 4h after ES, and kept elevated over 12h after ES.

In figure 28C, the expression of IGF-1, cyclin D1 and P21 at mRNA level is depicted along with different time points in group C. In response to ES at high frequency for short duration, IGF-1 and cyclin D1 showed a slight increase after ES and a depressed expression at 4h after ES and afterward after ES. P21 mRNA increased immediately after ES and peaked at 1h after ES, and kept clearly elevated over 8h after ES.

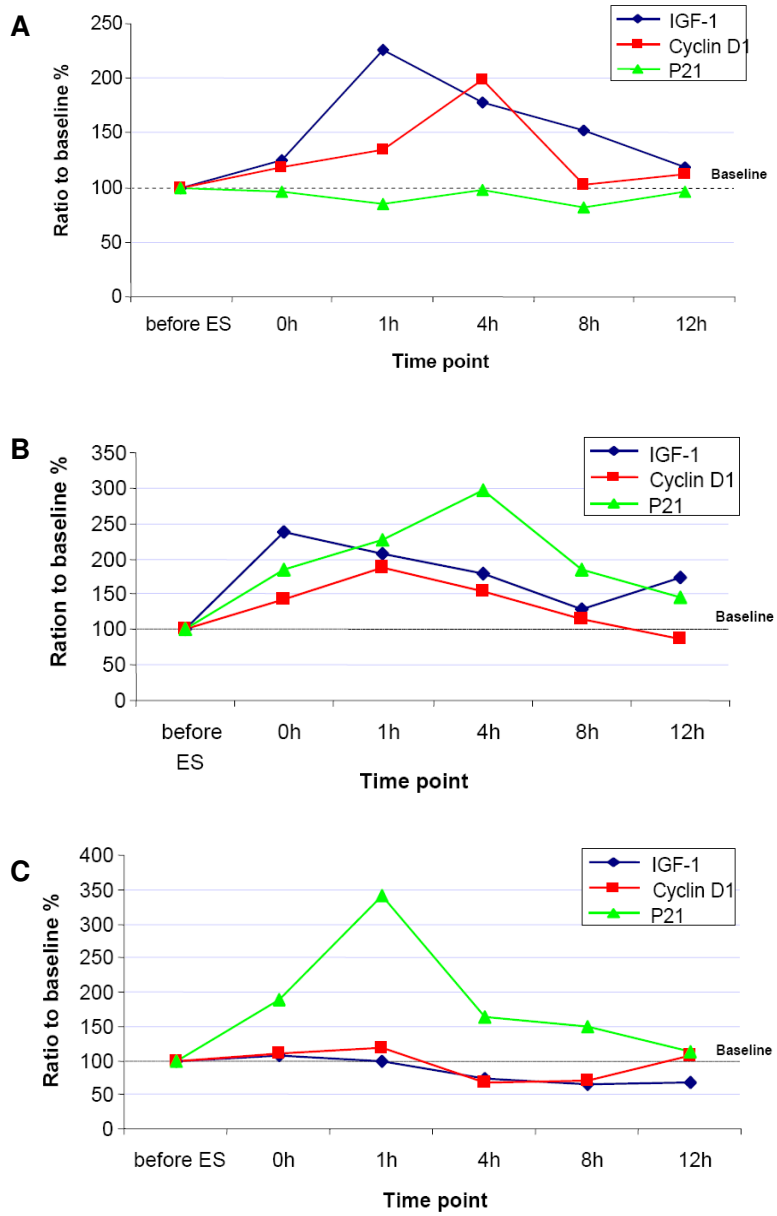

Figure 28: Insulin-like growth factor-1 (IGF-1), Cyclin D1 and Cyclin-dependent Kinase Inhibitor (P21) response at mRNA level to electrical stimulation (ES) at 13V, with 12Hz and 11min (group A), 12Hz and 90min (group B), and 100 Hz and 11 min (group C), respectively. 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively.

3.5 Apoptotic gene response to ES

To examine whether ES has effects on cellular apoptosis, we investigated expression of several genes which have been demonstrated to be involved in the apoptotic process.

3.5.1 Bax response to ES at mRNA level

Bax response to ES at mRNA level is shown in figure 29. In group A, the expression of Bax mRNA showed a slight increase immediately after ES and a further distinct increase at 1h after ES, and then decreased rapidly. In group B, ES caused an early augmentation in Bax mRNA after ES and peaked at 1h after ES, and kept elevated over 8h after ES. In group C, Bax mRNA showed a slight decrease immediately after ES and a dramatic increase at 1h after ES with its peak level, and kept elevated over 8h after ES.

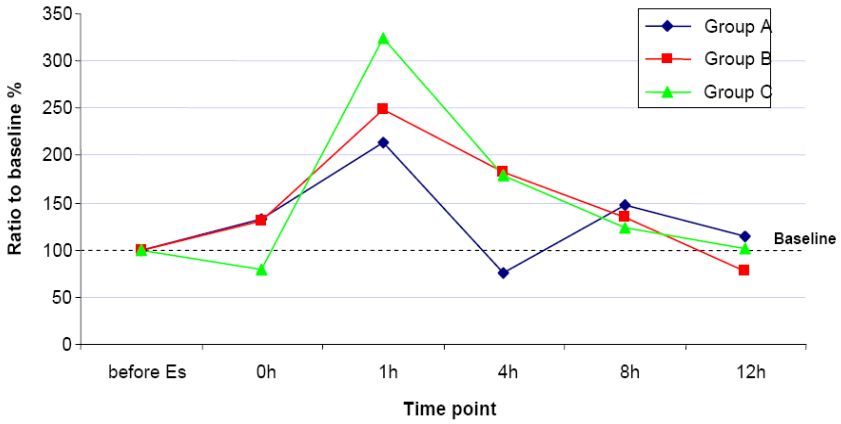

Figure 29: Bcl-2-associated X protein (Bax) response at mRNA level to electrical stimulation (ES) at 13V, with 12Hz and 11min (group A), 12Hz and 90min (group B), and 100 Hz and 11 min (group C), respectively. 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively.

3.5.2 Bcl-2 response to ES at mRNA level

Bcl-2 response at mRNA level to ES was shown in figure 30. In group A, the expression of Bcl-2 mRNA showed a slight increase immediately after ES and a further distinct increase at 1h after ES, and kept elevated over 8h after ES. In group B and C, Bcl-2 mRNA did not change distinctly till 4h after ES, and thereafter depressed.

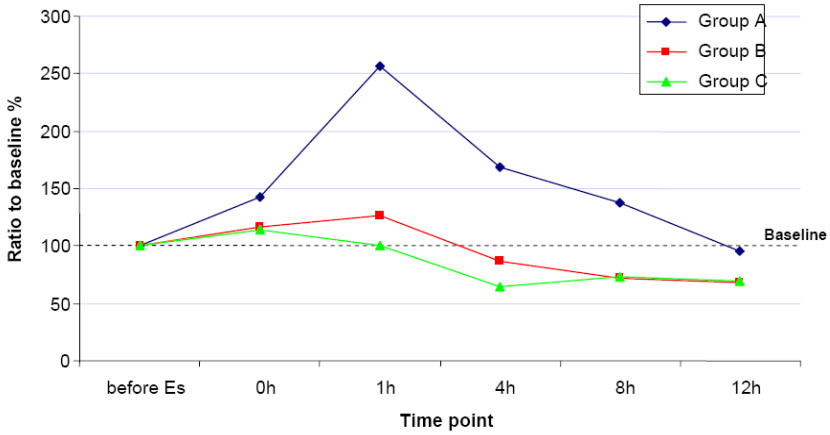

Figure 30: B-cell leukemia/lymphoma (Bcl-2) response at mRNA level to electrical stimulation (ES) at 13V, with 12Hz and 11min (group A), 12Hz and 90min (group B), and 100 Hz and 11 min (group C), respectively. 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively.

3.5.3 Apoptotic index (Bax/Bcl-2) changes to ES

Effect of ES on the apoptotic index (Bax/Bcl-2) was also investigated in group A, B and C. In group A, the apoptotic index was clearly depressed till 4h after ES and then increased slightly 8h and 12h after ES. In comparison with group A, the apoptotic index was clearly augmented in group B and C and kept elevated over 12h after ES.

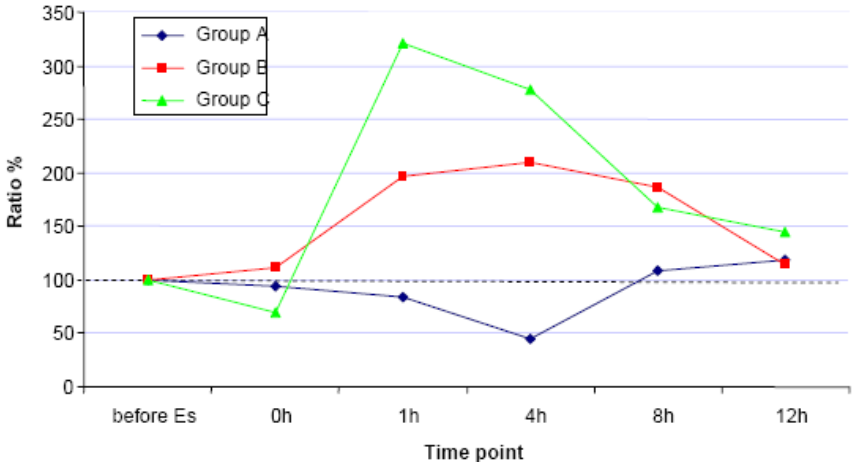

Figure 31: Bcl-2-associated X protein / B-cell leukemia/lymphoma (Bax/Bcl-2) changes to electrical stimulation (ES) at 13V, with 12Hz and 11min (group A), 12Hz and 90min (group B), and 100 Hz and 11 min (group C), respectively. 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively.

3.5.4 AIF response to ES at mRNA level

AIF response at mRNA level to ES is shown in figure 32. In group A, AIF mRNA expression was up-regulated at 1h after ES and then decreased steadily. However, there was no distinct increase of AIF mRNA in group B. In group C, AIF mRNA increased and peaked at 1h after ES and then returned to baseline at 12h after ES.

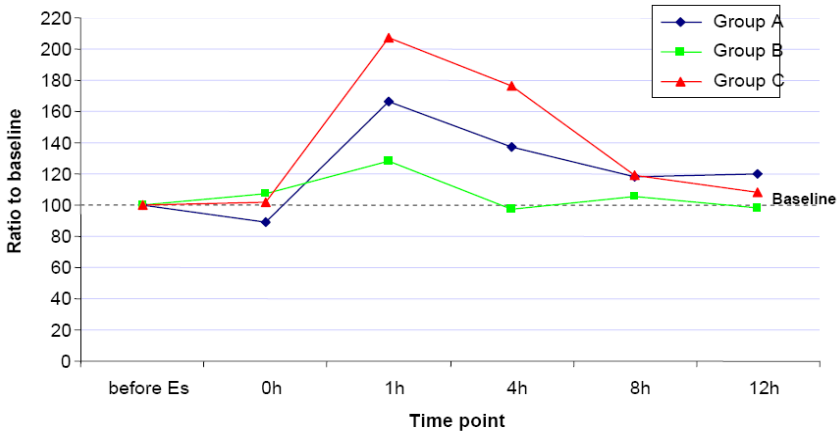

Figure 32: Apoptotic inducing factor (AIF) response at mRNA level to electrical stimulation (ES) at 13V, with 12Hz and 11min (group A), 12Hz and 90min (group B), and 100 Hz and 11 min (group C), respectively. 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively.

3.5.5 Apaf-1 response to ES

Apaf-1 response at mRNA level to ES is shown in figure 33. In group A, Apaf-1 mRNA began to increase at 1h (128%) after ES and kept elevated 12h after ES. In group B, there was a continuous down-regulation of Apaf-1 mRNA after ES. In group C, Apaf-1 mRNA increased immediately after ES, peaked at 4h after ES and returned to baseline at 8h after ES.

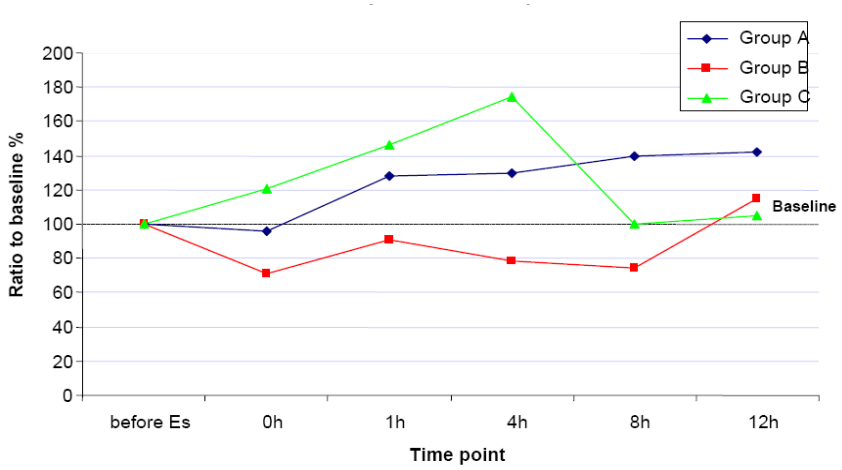

Figure 33: Apoptotic protease-activating factor-1 (Apaf-1) response at mRNA level to electrical stimulation (ES) at 13V, with 12Hz and 11min (group A), 12Hz and 90min (group B), and 100 Hz and 11 min (group C), respectively. 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively.

3.5.6 Caspase9 response to ES at mRNA level

Caspase9 response at mRNA level to ES is shown in figure 34. The expression of Caspase9 in group A and C showed similar change within 1h after ES, however, it depressed rapidly afterwards in group C and kept depressed thru 12h after ES, whereas it in group A showed a further increase at 4h after ES and returned to baseline at 8h after ES. In group B, the expression of Caspase9 did not change clearly within 1h after ES and increased slightly afterwards.

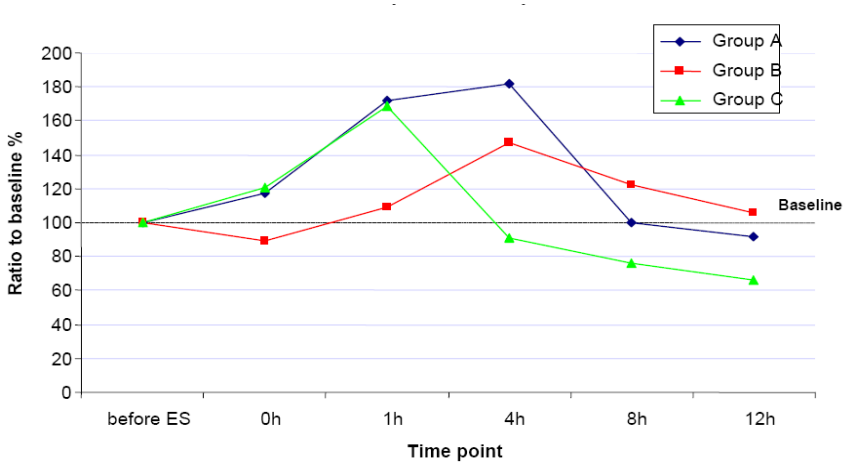

Figure 34: Caspase 9 response at mRNA level to electrical stimulation (ES) at 13V, with 12Hz and 11min (group A), 12Hz and 90min (group B), and 100 Hz and 11 min (group C), respectively. 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively.

3.5.7 Caspase8 response to ES at mRNA level

Caspase8 response at mRNA level to ES is shown in figure 35. In group A, Caspase8 mRNA began to increase after ES, peaked at 4h after ES and then dropped back to baseline. There was no distinct change of Caspase8 mRNA in group B. In group C, the up-regulation of Caspase8 mRNA occurred at 1h after ES and then decreased to baseline.

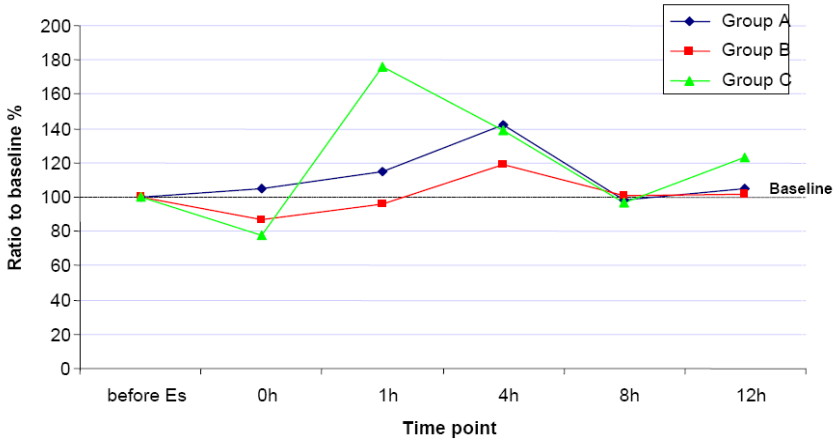

Figure 35: Caspase 8 response at mRNA level to electrical stimulation (ES) at 13V, with 12Hz and 11min (group A), 12Hz and 90min (group B), and 100 Hz and 11 min (group C), respectively. 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively.

3.5.8 AIF, Apaf-1, Caspase9 and Caspase8 response to ES

In order to examine the relationship among AIF, Apaf-1, Caspase9 and Caspase8, the expression of these parameters is parallel illustrated in the same group along with different time points.

In group A, all these parameters showed an increase at 1h after ES, and this increase was more dramatic for AIF and Caspase9 while only modest for Caspase8, For Apaf-1, the expression increase was lower but kept longer elevated in comparison with that of AIF and Caspase9.

In group B, AIF showed a slight increase 1h after ES, followed by an increase in Caspase9 at 4h after ES, and returned to baseline afterwards. There was no clear change in Caspase8 after ES. Apaf-1 mRNA showed a depressed expression over 8h after ES.

In group C, all the parameters showed an increase at 1h after ES and decreased steadily, while Apaf-1 showed a further increase at 4h after ES and returned to baseline.

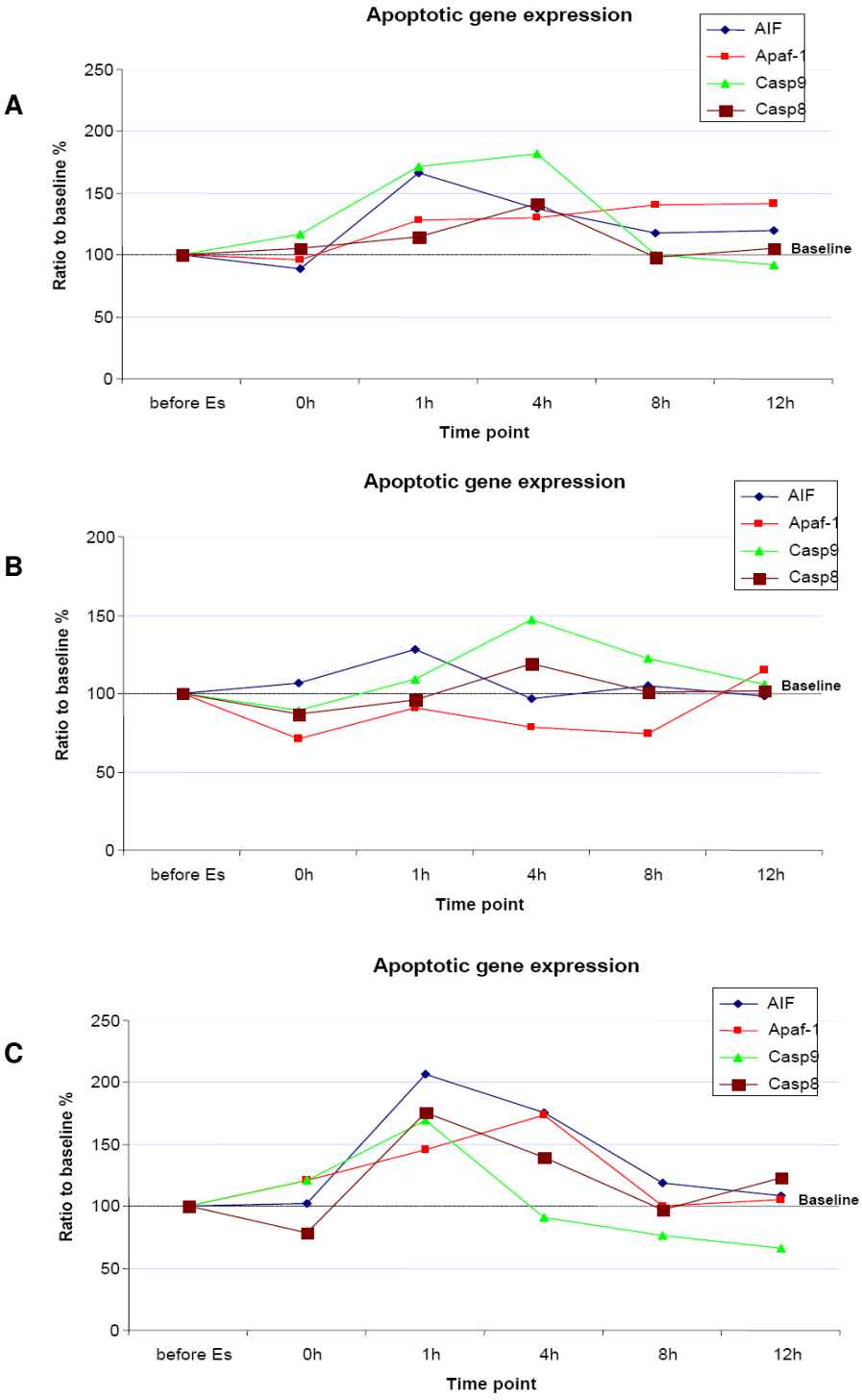

Figure 36: Apoptotic inducing factor (AIF), Apoptotic protease-qctivating factor-1 (Apaf-1), Caspase 9 and Caspase 8 response at mRNA level to electrical stimulation (ES) at 13V, with 12Hz and 11min (group A), 12Hz and 90min (group B), and 100 Hz and 11 min (group C), respectively. 0h, 1h, 4h, 8h and 12h indicate the time points when cell harvested immediately as well as 1h, 4h, 8h and 12h after ES, respectively.

4 Discussion

Electrical stimulation has been used in clinical and experimental settings and regarded as an established cellular stress which can cause diverse cellular processes. Among the mechanisms responsible for cellular response to stress, Hsp70 response is known to play an important role. Furthermore, in cell culture settings, cells response to stress with regulation of cell growth process and apoptosis activity. However, whether these mechanisms are involved in the cellular response to ES, has not been investigated. We hypothesize that ES applied to cell culture would induce Hsp70 response, and Hsp70 response may be attributed to regulation of cell growth and apoptosis. We have thus investigated Hsp70 response induced by different kinds of ES along with changes in myogenic as well as apoptosis markers in C2C12 cells.

4.1 C2C12 cell line and Electrical stimulation system

Skeletal muscle is formed by myofibers, whose development is called myogenesis. During mammalian skeletal muscle myogenesis, totipotencial mesenchymal cells provide a population of mononucleated fusiform cells, named myoblasts, which are known to be the precursors of contractile muscle cells (Lawson and Purslow, 2000). Myoblasts progressively fuse to form myotubes, which further differentiate to myofibers. Cultured myoblasts can serve as an in vitro model to investigate response and adaptation to cellular stress.

C2C12 mouse muscle cell line is a subclone isolated from parental C2 myoblasts, which were established from the thigh muscle of adult mice (Yaffe and Saxel, 1977). C2C12 cells are thought to be derived from satellite cells, which lie along the muscle fibers and exist throughout the adult life as a potential source of new myoblasts. These cells have capacity of proliferation and differentiation. Under conditions favouring cell division (low density and growth stimulation by fetal bovine serum), these cells divide rapidly. After induction of differentiation by withdrawal of growth factors, C2C12 cells cease dividing and fuse into myotubes (Liu et al., 1996).

ES has been widely used in studies on skeletal muscle plasticity, and ES-induced

structural and functional alterations in skeletal muscle have been proven (Pette and Vrbova, 1999). In previous studies, ES apparatus has been reported, which can stimulate cells by giving electrical impulses and imitating muscle contractions in vitro. In the present study, we have developed our own “ES unit” in order to get optimized results based on the previous experience and our study purposes. Furthermore, a series of experiments had been performed to set reasonable ES parameters with regard to electrical impulse and stimulation duration (as described in methods).

4.2 ES-induced Hsp70 response

4.2.1 ES-induced Hsp70 response at mRNA level

In the present study, quantitative real-time PCR was used to determine the level of Hsp70 mRNA after different kinds of ES, and β2-Microglobulin was taken as internal reference, which was generally used in mice muscle cells, and a stable expression of β2-Microglobulin following stresses has been shown. Therefore, based on the expression level of β2-Microglobulin, Hsp70 expression at mRNA level can be compared among the samples.

It is known that expression of Hsp70 is under complex regulatory control in cells. In addition to physiological stress such as heat shock or transition metals, Hsp70 gene is induced by serum stimulation and polyoma large tumor antigen genes. Milarski and Morimoto showed that expression of the human Hsp70 gene is regulated during the cell cycle (Milarski and Morimoto, 1986). Based on its biological characteristic, Hsp70 is expected to vary with C2C12 cell culture procedure, and it is true in this study that Hsp70 expression at mRNA level changed during the study period. Therefore, not the absolute Hsp70 mRNA level, but the change is of interest, and the effect of ES on Hsp70 mRNA was examined in comparison with the baseline (value before ES was set at 100% as baseline) or with the corresponding controls without ES.

The results from the present study showed a distinct Hsp70 response at mRNA level to ES, though the response of Hsp70 at mRNA level showed different magnitudes as well as different time courses. Previous studies have shown that diverse cellular stresses can induce Hsp70 response at mRNA level (Lindquist,

1986). It was demonstrated that physical or chemical stress (such as heat shock, sodium arsenite or $CdCl_2$) induced Hsp70 response at mRNA level in cell culture (Kim et al., 2001). Cultured myotubes have demonstrated increased Hsp70 mRNA production in response to hypoxia (Benjamin et al., 1990) and heat shock (Luo et al., 2001). Furthermore, previous study on the expression of stress proteins in skeletal muscle after chronic ES showed that Hsp60 mRNA was significantly up-regulated after ES (Ornatsky et al., 1995). All these previous studies seem in accordance with the present study, and therefore, support our findings that ES brings about Hsp70 response at mRNA level in cultured cells.

In the present study, three different kinds of ES were applied to stimulate the cells, which induced different response of Hsp70 at mRNA level. The low frequency ES with short duration (group A) caused only a modest up-regulation of Hsp70 at mRNA level. In comparison with group A, Hsp70 mRNA increased clearly higher and longer in group B with ES prolongation at the same frequency. ES at high frequency (group C) induced a dramatically increase in Hsp70 gene expression that was even higher compared with that in group B, and the increase kept above baseline over 12h after ES. Therefore, different kinds of ES may lead to different Hsp70 response at mRNA level, and the frequency of ES seems to have more impact on Hsp70 response at mRNA level. It was evident that many factors may have effects on the degree of Hsp70 response to cellular stress, including the intensity and duration of stress exposure. For example, a hyperthermia induced Hsp70 mRNA response was closely related to the changes of temperature and the duration of the hyperthermic exposure in cell culture (Widelitz et al., 1987). It has also been shown that Hsp70 expression level in skeletal muscle depends not only on the severity of ischemia but also on the ischemic duration (Liu et al., 2002). In the present study, three kinds of ES were different with regard to intensity and duration of stimulation, which definitely induced different Hsp70 response at mRNA level in C2C12 cells.

Although Hsp70 mRNA response to variety of stresses (such as, thermal stress, ischemia/ reperfusion, physical exercise etc.) in various cells or tissues (Kregel, 2002) has been extensively studied, it is the first report, to our best knowledge, that the changes of Hsp70 expression in C2C12 cells following different kinds of ES. Thus, the kinetics of Hsp70 response to ES was not known prior to this study. It is

true that in many tissues Hsps can be induced within a short time after cells exposed to the stress (Lindquist, 1986). In human endothelial cell culture, for instance, Hsp70 mRNA level began to increase 1h after heat shock, reached a maximum after 2h and then decreased (Wagner et al., 1999). Similar results were obtained in another study which showed heat shock and hydrogen peroxide induced a rapid and marked increase in Hsp70 mRNA level in cell culture (Jornot et al., 1991). Furthermore, Bardella and Comolli showed that ischemia caused an almost immediate increase of Hsp70 mRNA in renal tubular cells, that reached a maximum at 1h of reperfusion (Bardella and Comolli, 1994). With the study on skeletal muscle, Liu et al. demonstrated that Hsp70 mRNA significantly increased 2h after ischemia in skeletal muscle and peaked 4h after ischemia (Gampert L et al., 2004). In the present study, there was a modest elevation in Hsp70 mRNA at 1h after ES in group A. In the group B and C, Hsp70 mRNA began to increase at 1h, reached the peak at 4h after ES and then decreased. Therefore, the up-regulation of Hsp70 mRNA to ES in C2C12 cells was rapid and could keep a few hours after ES, which is consistent with the previous studies.

It is evident that the amplitude of Hsp70 response to stresses at mRNA level is quite different. In cell culture, for instance, Luo et al. showed that Hsp70 mRNA level increased more than 10 times compared to the control in L6 myotubes 3h after heat shock (43℃, 1h) (Luo et al., 2001). Walsh et al. reported a significant elevation of Hsp70 mRNA (6.5 fold) in serum at the 2h after a single bout of high intensity exercise in humans (Walsh et al., 2001). It was also shown that Hsp70 mRNA level increased about 8 folds after acute ischemia in skeletal muscle (Gampert L et al., 2004). In the present study, increase of Hsp70 mRNA induced by ES reached about 6 folds and 14 folds in group B and C. In this respect, the results of the current study are quite in accordance with these of the previous studies.

4.2.2 Hsp70 response to ES at protein level

Hsp70 response to different kinds of ES at protein level was determined by quantitative SDS-PAGE and Western-blot. Hsp70 protein level of each sample was calculated according to the linear regression between the densitometric values and a series of different known amount of standard Hsp70 (Liu et al., 1999). The expression of Hsp70 at protein level showed different response with regard to

magnitude and time course.

In this study, Hsp70 protein expression began to increase 4h after ES, peaked 8h after ES and then decreased in group A and C. In group B, the expression of Hsp70 protein increased continuously after ES, reached peak 8h after ES and kept elevated 12h after ES. These results clearly demonstrated that ES induced Hsp70 response at protein level in C2C12 cells. Hsp70 response at protein level to a variety of stressful conditions has been reported. It was shown that Hsp70 was up-regulated at protein level after heat stress in cultured L6 myotubes (Luo et al., 2001) and C2C12 cells (Maglara et al., 2003). Wagner et al. reported that heavy metal ions (such as Zn^{2+}, Cd^{2+}) and heat shock caused Hsp70 protein up-regulation in human endothelial cells (Wagner et al., 1999). Numerous studies have demonstrated that physical exercise induced Hsp70 production at protein level in liver, heart and skeletal muscle (Salo et al., 1991). It has been proven that Hsp70 protein production after physical exercise training in human skeletal muscle (Liu et al., 2004). Furthermore, ischemia/reperfusion-induced Hsp70 protein response in skeletal muscle has also been demonstrated (Liu et al., 2002). Hence, Hsp70 response at protein level to ES also sounds reasonable.

In this study, ES-induced Hsp70 response at protein level was quite different, varying with the characters of ES. In comparison with group A, the increase of Hsp70 protein in group B was higher at each time point after ES and kept longer. The results indicated that low frequency ES could induce a modest up-regulation of Hsp70 protein in C2C12 cells, and the increase maintained higher and longer with prolongation of ES. Like Hsp70 mRNA response, the amount of Hsp70 response to ES at protein level was also determined by the duration and intensity of stress exposed. It was documented that Hsp70 protein significantly increased in well-trained rowers during a 4-week training period (Liu et al., 1999). In previous studies, it was demonstrated that Hsp70 protein response to training/exercise was related to the training volume (Liu et al., 1999) and exercise intensity (Liu et al., 2000). The does-dependent response of Hsp70 protein can also be elucidated by ischemia in skeletal muscle. The examination of Hsp70 protein expression in ischemia muscle in patients with PAOD showed that the expression of Hsp70 protein was linked to the severity of the disease (Liu et al., 2002). These results of previous studies seem to support the results of the present study that Hsp70

response to ES at protein level was different among different kinds of ES in terms of stress intensity and duration.

Results from this study showed that there was only a little increase in Hsp70 protein at 8h after ES in group C and a down-regulation at other time points. Interestingly, Hsp70 response at protein level did not increase further along with the increase of the frequency of ES. This seems not to be in accordance with intensity-dependent Hsp70 response. Several factors might be responsible for the unexpected result. First, extremely strong stress may kill the cells directly, as observed in group C (the frequency of ES was too high); second, Hsp70 response may not serve as mechanism in response to extremely strong stress; third, the transcriptional activation of Hsp70 mRNA could be independent of protein synthesis. Similar result was also documented in the study on Hsp70 protein expression in patients with PAOD (Liu et al., 2002), which showed that the highest level of Hsp70 protein in patients with PAOD was observed in stage III, but not in stage IV.

4.2.3 Discrepancy of the Hsp70 expression between mRNA level and protein level

It is clearly shown in this study that the changes of Hsp70 content between mRNA level and protein level were different. The changes of Hsp70 mRNA content were more dramatic than that of Hsp70 protein in this study, especially in group C where the Hsp70 mRNA level increased 14 times related to control, whereas the maximum increase of Hsp70 protein was about 0.5 times related to control. This discrepancy of Hsp70 expression between mRNA level and protein level was also observed in previous studies. Puntschart et al. showed that 30 minute treadmill exercise could induce Hsp70 mRNA up-regulation within 4 minutes after exercise, while no significant Hsp70 protein increase within three hours after exercise (Puntschart et al., 1996). Actually, there have been studies demonstrating that the changes of Hsp70 mRNA are not accompanied by the corresponding changes of Hsp70 at protein level (Liu et al., 2006). The discrepancy may suggest that Hsp70 response is regulated at different levels and posttranscriptional mechanisms play important role in regulating Hsp70 expression level.

4.2.4 Mechanisms involved in ES-induced Hsp70 response

The mechanisms of Hsp70 induction by ES are largely unknown and may be complicated and multi-factorial. In eukaryotic cells HSF is a transcription factor and plays an important role in the regulation of Hsp70 gene transcription. Generally, HSF is inactive under non-stress conditions, and HSF activation induced by stress leads to the activation of Hsp-gene transcription and subsequent protein synthesis. It is known that many factors can cause HSF activation. The central process in HSF activation is the equilibrium between the binding of free Hsp70 to the HSF and to stress-mediated unfolding proteins. Any increase in the presence of unfolding proteins shifts this equilibrium to the Hsp-unfolded proteins side, thereby activating HSF. It is known that hyperthermia is a well-established stress which can induce an accumulation of unfolding proteins and subsequently lead to Hsp70 production. However, in this study, the temperature of cell culture medium maintained at 37 ℃ constantly. Therefore, hyperthermia was probably not response for Hsp70 response in this study.

It is evident that challenges to energy metabolism are known to be able to induce Hsp70 response. Glycogen and ATP deletion can induce Hsp70 response (Liu and Steinacker, 2001; Steinacker J.M and Y Liu, 2002). The decrease of glucose in culture medium after ES has been observed in other study in our lab. Therefore, the ES-induced energy metabolic changes in the cells might at least partly be responsible for Hsp70 response. Furthermore, cellular physiological stresses occurred in this study, such as cell division, differentiation and growth factors, may also induce Hsp70 production (Kiang and Tsokos, 1998).

It has been reported that changes of intracellular or extracellular pH value can influence Hsp70 expression (Mosser et al., 1990a). It is demonstrated that exercise-induced decrease in pH could also induce Hsp70 response (Whelan and Hightower, 1985). The other study's results in our lab showed that the concentration of lactate in culture medium significantly increased after ES which decreased the PH value of culture medium. Therefore, lactate accumulation and pH decrease in culture medium may be attributed to Hsp70 induction by ES.

Reactive oxygen species (ROS) induced by stresses are also thought as important mechanism responsible for Hsp70 response. It has been demonstrated that

extracellular ROS (O_2-, OH, H_2O_2) can activate phospholipase C, thereby enhance intracellular concentration of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, leading to subsequent activation of protein kinase C (PKC). PKC plays tan important role role in the Hsp70 gene transcription. Multiple studies have shown that oxidative stress can induce Hso70 expression in cells and muscles (Liu et al., 2006). Although ROS was not investigated in present study, oxidative stress might be attributed to the mechanisms of ES-induced Hsp70 response.

It has been shown that HSF can be activated by Ca^{2+}, Mn^{2+} and La^{2+} (Mosser et al., 1990b) and increase in Ca^{2+} concentration induced by ionomycin (a Ca^{2+} ionophore) promotes Hsp70 production in human epidermoid A-431 cells (Ding et al., 1996), MDCK cells (Yamamoto et al., 1994) and rat luteal cells (Khanna et al., 1994). Evidence from studies also shows that inhibition of increase in Ca^{2+} concentration by removal of external Ca^{2+} or treatment with Ca^{2+} chelator greatly attenuated Hsp70 mRNA and protein synthesis (Kiang et al., 1994). In the present study, we speculate that ES to C2C12 cells might affect the concentration of intracellular Ca^{2+}, and probably serve as one of the mechanisms of ES-induced Hsp70 response.

Cytokines induced by variety stresses may also be associated with Hsp70 production. It is proven that IL-6 activated Hsp70 gene expression in human skeletal muscle (Febbraio et al., 2002). More recent studies have shown that exercise-induced Hsp70 response in skeletal muscle is related to IL-6, IL4 and IL13 (Febbraio et al., 2002; Prokopchuk et al., 2007). In the present study, IL-4 and IL-13 receptor were also examined (data not shown), and gene expression of IL-4 and IL-13 receptor significantly increased after ES. These results may indicate that cytokines induced by ES might have impact on Hsp70 production in C2C12 cells.

4.3 Effects of ES on cell growth process

Cellular response and adaptation to stresses have been reported in previous studies, suggesting that stress-induced cellular response involves regulation of cell growth process. In the present study, we examined the changes of several parameters at mRNA level including IGF-1, Cyclin D1 and P21, which have been demonstrated to be involved in the process of cellular proliferation and differentiation. A large body of studies has demonstrated that IGF-1 is a stimulator of cell growth in many kinds of cells (including muscle cells), one of the most potent

natural activators of the AKT signaling pathway, and a potent inhibitor of apoptosis. Cyclin D1 promotes progression through the G1-S phase of the cell cycle and amplification or over-expression of cyclin D1 plays pivotal roles in the cell proliferation especially in cancer cells (Fu et al., 2004). The cyclin-dependent kinase inhibitor P21 is a major player in cell cycle control and it is regulated at the transcriptional level. It mainly inhibits the activity of cyclin/cdk2 complexes and negatively modulates cell cycle progression, as well as blocks DNA synthesis (Gartel and Radhakrishnan, 2005). P21 can withdraw cells from cell cycle and promote cell differentiation, and thus promote cell differentiation.

In group A the expression of IGF-1 mRNA was increased clearly at 1h and kept above base line till 8h after ES, and this was followed by an increase in the expression of Cyclin D1 mRNA. However, in group A, there was no distinct change in P21. It seemed that low frequency ES with short duration to C2C12 cells had more impact on cell proliferation rather than on differentiation. In group B, similar results could be derived from IGF-1 and cyclin D1 expect for P21 that increased gradually up to 4h after ES. These results suggested that this kind of ES has effects on both cell proliferation and differentiation. Contrastly in group C, there was a lack of an increase in the expression of IGF-1 and cyclin D1, but an early distinct response in P21 expression. This suggests that ES at high frequency had effect on cell differentiation rather than proliferation.

Up to date few studies dealing with effect of ES on cell growth regulation have been reported. A previous study showed an accelerated proliferation of P6 Hela cells by ES at 1.0v for 20 min (Kumagai et al., 2004). Pedrotty et al. have observed effect of ES on proliferation of muscle blasts in culture, but not on differentiation (Pedrotty et al., 2005). Similar results were also obtained in bone cells and endothelial cell culture (Hartig et al., 2000; Zhao et al., 2004). Furthermore, there is another study demonstrating that ES on the L6 rat myoblasts accelerated the appearance of myotubes, and subsequently produced muscle fibers (Kawahara et al., 2006). This result indicates that ES can affect muscle cell differentiation.

Up to date, available data and knowledge with regard to the mechanisms underlying ES-induced cellular response are very limited. In consideration of the basic biological role of Hsp70 and the regulation of cell growth process as an

essential mechanism responsible for cellular response to stress, Hsp70 response to ES along with response of IGF-1, cyclin D1 and P21 to ES was investigated in the present study. It is likely that Hsp70 induced by ES interacts with some key factors involved in the process of cellular proliferation and differentiation.

4.4 Effects of ES on apoptotic response in C2C12 cells

Apoptosis is a programmed cell death and a kind of “cell suicide”. This program is targeted to eliminate cells which are no longer needed or are potentially endangering for the whole organism. The activation of the apoptosis program can be brought about through either exogenous or endogenous factors. It is known that cellular stress can trigger apoptosis in variety of cells. However, it is still unclear whether ES can induce cellular apoptosis in C2C12 cells. Therefore, we examined several parameters at mRNA level including Bax, Bcl-2, Caspase8/9, Apaf-1, AIF. These parameters except for Bcl-2 are promoters for the process of apoptosis.

The results in the present study showed that ES can induce the changes of apoptotic gene expression. The up-regulation of Bax was observed after ES, especially in group B and C. However, Bcl-2 expression only significantly increased at 1h after ES in group A and was depressed after ES in group B and C. Furthermore, the ratio of Bax/Bcl-2 was depressed in group A, whereas it was significant increased in groups B and C, especially in group C. These results indicate that ES at high frequency or low frequency for longer duration can induce up-regulation of apoptotic genes in C2C12 cells. Previous study has demonstrated that ES in cardiomycytes can induce activation of stress-related kinases, leading to apoptosis in myocytes (Kuramochi et al., 2006). There is evidence that cellular apoptosis could be induced by variety of stresses, which included heat stress, oxidative stress, calcium overload, pH changes, etc. It has been shown that the different kinds of stresses varying in pattern, intensity and duration might have different effects on cellular apoptosis (Pchejetski et al., 2007; Salinas et al., 2006). In the present study, the up-regulation of apoptotic genes may suggest an activation of cellular apoptosis.

Interestingly, the expression of Apaf-1, AIF, Caspase8, 9 mRNA in group B was lower compared to that in group A and C. These results seemed inconsistent with the expression of Bax and Bax/Bcl-2 in group B, since Apaf-1, AIF and Caspase8/9

are up-stream regulators for Bax. A possible explanation is that a higher upregulation of Hsp70 was simultaneously observed. It has been reported that Hsp70 in regulating apoptosis may be at the level of signal transduction. For instance, Hsp70 up-regulation, as a consequence of either cellular stress or transfection, prevents the induction of apoptosis by several insults (Kwak et al., 1998). In contrast, Hsp70 down- regulation is to facilitate the induction of apoptosis (Ravagnan et al., 2001). The protective molecular mechanism of Hsp70 may result from down-stream of cytochrome c release, and upstream of caspase3 activation (Li et al., 2000). It was demonstrated that Hsp70 interacted directly with Apaf-1 with subsequently inhibition of cytochrome c release and processing of caspase9 (Beere et al., 2000). Hsp70 has also been shown to inhibit AIF-induced apoptosis, but the mechanism related to this process is unknown (Ravagnan et al., 2001). Therefore, the highest Hsp70 response at protein level induced by ES in group B seems to be associated with ES-induced apoptotic activity in the present study.

4.5 Meanings of Hsp70 production after ES

It is well known that Hsp70 response serves as a very important mechanism underlying cellular adaptation to various stresses. As stated above, there are two basic functions of Hsp70: molecular chaperone and stress sensing. Based on these functions, Hsp70 response can have different effects on cellular protection, protein metabolism (i.e. protein degradation, folding and synthesis), and facilitating cellular adaptation to stress as well as effects on cellular energy metabolism.

It is evident that Hsp70 response can serve as an indicator for cellular stress (stress sensor). Upon stress, protein damage and denaturation may occur and Hsp70 can serve as a sensor to detect the accumulation of abnormal proteins and transduce the stress signals. In the present study, ES induced Hsp70 response at both mRNA and protein level, suggesting a presence of cellular stress initiated by ES. Furthermore, does-dependent response of Hsp70 to ES as was observed in this study, implies that Hsp70 response can reflect the extent of cellular stress induced by ES.

Previous studies have shown that cellular response and adaptation to ES are associated with cell growth process (Pedrotty et al., 2005). It was documented that ES could stimulate the proliferation of skeletal muscle cells (Pedrotty et al., 2005;

Stern-Straeter et al., 2005), and similar results were also obtained in the study of other cell types, such as bone cells (Hartig et al., 2000), Hela cells (Kumagai et al., 2004) and endothelial cells (Zhao et al., 2004). In the present study, IGF-1 and cyclin D1 mRNA significantly increased after low frequency ES, indicating that low frequency ES may promote cell proliferation. Additionally, the highest Hsp70 response at protein level induced by ES at low frequency for longer duration seems to be associated with long standing elevated IGF-1 and cyclin D1 mRNA. Therefore, ES-induced Hsp70 response may be associated with cell growth process. It is evident that the protein synthesis and protein folding occur during the cell growth process, in which Hsp70 may play a role (Beckman R.P et al., 1990).

It was also documented that ES to L6 myoblasts could promote cell differentiation and accelerate cell mature process (Kawahara et al., 2006). In the present study, low frequency ES with longer duration led to higher up-regulation of P21 along with the up-regulation of Hsp70. Therefore, it seems that ES-induced Hsp70 may be involved in the regulation of cell cycle. Feder and Hofmann have suggested that Hsp70 might specifically interact with cell cycle regulators (Feder and Hofmann, 1999). It is evident that Hsp70 had impact on the cell proliferation and/or the differentiation (Kwak et al., 1998; Barnes et al., 2001). For instance, over-expression of Hsp70 in HL60 cells reduced proliferation and promoted their differentiation (Kwak et al., 1998). However, the exact role of Hsp70 related to cell proliferation and/or differentiation is still uncertain and should be clarified further.

Study on effect of ES on apoptosis is very limited. There is only one study showing that ES caused apoptosis in cardiomyocytes (Kuramochi et al., 2006). Results from the present study also showed that some apoptotic parameters clearly increased after ES accompanied by the augmentation of Hsp70, indicating that Hsp70 may be attributed to cell apoptotic process. Previous studies demonstrated that Hsp70 could affect the apoptotic pathway in both cytochrome C release and initiater caspase activation (Li et al., 2000). In the present study, ES did not induce significant increase in AIF, Caspase8, Caspase9 and Apaf-1 in group B (compared with that in group A) though a higher Hsp70 response. Therefore, Hsp70 may exert a negative influence on apoptotic signaling pathway.

Previous study in our lab examined the direct effect of Hsp70 on energy

metabolism. In that study, over-expressing Hsp70 in the cells distinctly increased the rate of glucose consumption and lactate production, and the cellular ATP level in the over-expressing Hsp70 cells was higher compared to the control, suggesting that Hsp70 enhanced cellular ATP level by stimulating glycolytic activity (Steinacker J.M et al., 2005). Another study in our lab examined the glucose and lactate in the culture medium after ES, showing a significant increase of lactate concentration and a clear decrease of glucose concentration. These results indicated that the energy turnover increased after ES. It is known that energy metabolism is crucial for cellular function and Hsp70 plays a pivotal role in facilitating cellular adaptation to stress. Therefore, Hsp70 is closely related to energy metabolism: 1) any changes in energy metabolic status significantly induce Hsp70 response; which has been demonstrated in a number of studies; 2) Hsp70 may exert direct or indirect effects on energy metabolism (Liu et al., 2006). However, the molecular mechanisms associated with effects of Hsp70 on cellular energy metabolism are still unclear and should be studied further.

It was demonstrated that Hsp70 could play a role in the process of cellular protection against stress, in which Hsp70 could prevent protein denaturation and /or processing denatured proteins and protein fragments (Kiang and Tsokos, 1998). It is reported that preconditioned cells with Hsp70 induction significantly improve their thermal stability or thermal tolerance (Selvakumar and Geraldine, 2005; Xi et al., 2001). It was shown that induction of Hsp70 was associated with the development of tolerance to a variety of stresses, including hypoxia, ischemia, energy depletion, cytokines such as TNF-a, and ultraviolet radiation (Snoeckx et al., 2001b). In the present study, it is possible that ES-induced Hsp70 has influence on protecting cellular vital function and preventing cellular damage.

4.6 Shortcomings of this study

Although all the experiments in the present study were accomplished following the study design, there are still some shortcomings in this study. First, cell culture is kinetic process in which C2C12 cells are in different stages of cell cycle. The genes studied in this project may be probably regulated during the cell cycle, such as Hsp70. Therefore, it is difficult to avoid the influence of cell culture when analysing

the results. Therefore, a set of non-stimulated cells were treated in same way and taken as control. Second, it seems that there is a defect in the latter part of this study, where some parameters involved in cell growth and apoptotic process were only investigated at mRNA level. Though we were aware of this fact, to determine these parameters at protein level, especially for the active form, was quite cumbersome since the lab method was not yet established for most of the parameters. However, we have conserved the samples at protein level, so that the corresponding parameters at protein level may be determined in the further no sooner than the method is established.

4.7 Conclusion

1) ES leads to distinct Hsp70 response at both mRNA level and protein level in C2C12 cells.
2) ES-induced Hsp70 response is associated with the frequency and duration of ES and differed at mRNA and protein level.
3) It seems that ES at low frequency for short time had effect principally on cell proliferation while ES at high frequency on differentiation, and ES at low frequency for a longer time on both of them. Therefore, ES had profound impacts on cell growth regulation, which varied with different kinds of ES as well.
4) Apoptotic activity reflected by apoptosis index (Bax/Bcl-2) was depressed by ES at low frequency for short time, and ES at high frequency or low frequency for a longer time led to an increase in this index. Therefore, the regulation of apoptosis was obviously involved in the ES-induced cellular response.
5) ES applied in the present study can bring about distinct response with Hsp70 induction along with changes in the regulation of cell growth and apoptosis. It is likely that in response to ES, Hsp70 interacts with the regulation of cell growth and apoptosis.

5. Summary

Background: Electrical stimulation (ES) is widely applied in clinical as well as experimental settings, and it is evident that ES can induce a series of functional, structural, and cellular changes. However, up to date, available data and knowledge with regard to the mechanisms underlying ES-induced cellular response are very limited. It is known that among the mechanisms responsible for cellular response to stress, Heat shock protein with 70 kilodalton (Hsp70) plays an essential and universal role. Furthermore, in cell culture experiments, stress induces cellular response with regulation of cell growth process as well as apoptotic activity. However, it is still unclear whether ES would induce Hsp70 response in cultured C2C12 cells, and especially whether ES-induced Hsp70 response may be related to other cellular response with regard to regulation of cell growth and apoptosis. Since ES is thought as an established cellular stressor, we hypothesize ES can induce Hsp70 response in cultured cells, which are vary with characters of ES. Furthermore, we hypothesize that in the ES-induced cellular response changes in the regulation of cell growth process as well as apoptotic activity would be involved. We speculate that Hsp70 response is related to cell growth and apoptosis in ES-induced cellular response.

Purpose: To investigate 1) Hsp70 response to ES in C2C12 cells and its association with characters of ES; 2) Response with cell growth process and apoptotic activity to ES, and 3) Possible association of Hsp70 expression with cell growth process and apoptosis in ES-induced cellular response.

Methods: Cultured C2C12 cells were divided into different groups with regard to ES characters (at 13 voltages): A (ES: 12 Hz, 11 min), B (12 Hz, 90 min) and C (100 Hz, 11 min). By a confluence at ca. 75%, the cells were stimulated. Cells were harvested before ES, and 0, 1, 4, 8, 12h after ES. Cells taken as control were parallel treated as the corresponding group. From the harvested cells total protein and ribonucleic acid (RNA) were extracted. A quantitative Western-blot was used to determine Hsp70 at protein level. For estimation of expression of at messenger ribonucleic acid (mRNA) level of assigned genes involved in cell growth, such as insulin-like growth factor (IGF-1), cyclin D1 and cyclin-dependent kinase inhibitor

(P21), as well as in apoptosis, such as B-cell leukemia (Bcl-2), Bcl-2-associated X protein (Bax), a reverse- transcription quantitative real-time polymerase chain reaction (PCR) was performed, referring to the expression level of an internal reference gene (β2-microglobulin). The changes in gene expression level of the ES-treated cells were compared with those of the controls. Experiment for each treatment was triplicate.

Results: 1) Hsp70 response to ES. In group A, Hsp70 mRNA showed a biphasic increase at 1h and 8h after ES. In group B and C, Hsp70 mRNA level increased at 1h, peaked at 4h and kept elevated at 12h after ES, where the peak level in group C was higher than in group B. At protein level, Hsp70 in group B began to increase at 4h after ES and maintained elevated afterwards with its peak at 8h. Hsp70 protein in group C showed depressed at 1h and augmented at 8h after ES. 2) Response in cell growth parameters to ES: In group A, IGF-1 increased clearly 1h after ES, followed by an increase in Cyclin D1 4h after ES, and no distinct change was shown in P21. In group B, IGF-1 increased and peaked immediately after ES accompanied by elevated P21 expression with peak level 4h after ES, while Cyclin D1 did not change significantly. In group C, P21 increased dramatically 1h after ES, and then decreased steadily with depressed expression of IGF-1 and Cyclin D1. 3) Effects of ES on apoptotic parameters: In comparison with group A, where both Bax and Bcl-2 were upregulated, Bcl-2 showed no change 1h after ES and decreased at 4h after ES in group B and C, along with significant increase in Bax. Therefore, the apoptotic index (Bax/Bcl-2) was depressed in group A whereas clearly augmented in group B and C

Discussion and conclusion: ES led to distinct Hsp70 response at both mRNA and protein level in C2C12 cells, and ES-induced Hsp70 response was associated with characteristics of ES. It was also shown that ES had profound impacts on cell growth regulation, which varied with different kinds of ES as well. Furthermore, the regulation of apoptosis was obviously involved in the ES-induced cellular response. Therefore, ES applied in the present study can bring about distinct response with Hsp70 induction along with changes in the regulation of cell growth and apoptosis. It is likely that in response to ES, Hsp70 interacts with the regulation of cell growth and apoptosis.

6. Reference

Aas V, Torbla S, Andersen MH, Jensen J, Rustan AC: Electrical stimulation improves insulin responses in a human skeletal muscle cell model of hyperglycemia. Ann N Y Acad Sci 967: 506-515 (2002)

Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, Calderwood SK: HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nat Med 6: 435-442 (2000)

Bardella L, Comolli R: Differential expression of c-jun, c-fos and Hsp 70 mRNAs after folic acid and ischemia-reperfusion injury: effect of antioxidant treatment. Exp Nephrol 2: 158-165 (1994)

Barnes JA, Dix DJ, Collins BW, Luft C, Allen JW: Expression of inducible Hsp70 enhances the proliferation of MCF-7 breast cancer cells and protects against the cytotoxic effects of hyperthermia. Cell Stress Chaperon 6: 316-325 (2001)

Bayol S, Brownson C, Loughna PT: Electrical stimulation modulates IGF binding protein transcript levels in C2C12 myotubes. Cell Biochem Funct 23: 361-365 (2005)

Beckman RP, Mizzen LA, Welch WJ: Interaction of HSP70 with newly synthesized proteins implications for perotein folding and assembly. Science 248: 850-954 (1990)

Beere HM, Wolf BB, Cain K, Mosser DD, Mahboubi A, Kuwana T, Tailor P, Morimoto RI, Cohen GM, Green DR: Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nat Cell Biol 2: 469-475 (2000)

Benjamin IJ, Kroger B, Williams RS: Activation of the heat shock transcription factor by hypoxia in mammalian cells. Proc Natl Acad Sci U.S.A. 87: 6263-6267 (1990)

Blake MJ, Gershon D, Fargnoli J, Holbrook NJ: Discordant expression of heat shock protein mRNAs in tissues of heat-stressed rats. J Biol Chem 265: 15275-15279 (1990)

Brevet A, Pinto E, Peacock J, Stockdale FE: Myosin synthesis increased by electrical stimulation of skeletal muscle cell cultures. Science 193:1152-1154 (1976)

Bruce JL, Price BD, Coleman CN, Calderwood SK: Oxidative injury rapidly activates the heat shock transcription factor but fails to increase levels of heat shock proteins. Cancer Res 53: 12-15 (1993)

Calderwood SK, Stevenson MA, Hahn GM: Effects of heat on cell calcium and inositol lipid metabolism. Radiat Res 113: 414-425 (1988)

Ding XZ, Smallridge RC, Galloway RJ, Kiang JG: Increases in HSF1 translocation and synthesis in human epidermoid A-431 cells: role of protein kinase C and $[Ca^{2+}]i$.

J Investig Med 44: 144-153 (1996)

Ding XZ, Tsokos GC, Kiang JG: Overexpression of HSP-70 inhibits the phosphorylation of HSF1 by activating protein phosphatase and inhibiting protein kinase C activity. FASEB J 12: 451-459 (1998)

Donati YR, Slosman DO, Polla BS: Oxidative injury and the heat shock response. Biochem Pharmacol 40: 2571-2577 (1990)

Eisenberg BR, Salmons S: The reorganization of subcellular structure in muscle undergoing fast-to-slow type transformation. A stereological study. Cell Tissue Res 220: 449-471 (1981)

Fabian TK, Fejerdy P, Nguyen MT, Soti C, Csermely P: Potential immunological functions of salivary Hsp70 in mucosal and periodontal defense mechanisms. Arch Immunol Ther Exp (Warsz) 55: 91-98 (2007)

Febbraio MA, Steensberg A, Fischer CP, Keller C, Hiscock N, Pedersen BK: IL-6 activates HSP72 gene expression in human skeletal muscle. Biochem Biophys Res Commun 296: 1264-1266 (2002)

Feder ME, Hofmann GE: Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61: 243-282 (1999)

Fehrenbach E, Niess AM, Veith R, Dickhuth HH, Northoff H: Changes of HSP72-expression in leukocytes are associated with adaptation to exercise under conditions of high environmental temperature. J Leukoc Biol 69: 747-754 (2001)

Feige U, Polla BS: Hsp70--a multi-gene, multi-structure, multi-function family with potential clinical applications. Experientia 50: 979-986 (1994)

Flanagan SW, Ryan AJ, Gisolfi CV, Moseley PL: Tissue-specific HSP70 response in animals undergoing heat stress. Am J Physiol 268: R28-R32 (1995)

Fu M, Wang C, Li Z, Sakamaki T, Pestell RG: Minireview: Cyclin D1: normal and abnormal functions. Endocrinology 145: 5439-5447 (2004)

Gampert L, Baur AC, Steinacker JM: Ischemia induces HSP70 expression in porcine skeletal muscle. Med Sci Sports Exerc 36: S318-S319 (2004)

Gartel AL, Radhakrishnan SK: Lost in transcription: p21 repression, mechanisms, and consequences. Cancer Res 65: 3980-3985 (2005)

Hartig M, Joos U, Wiesmann HP: Capacitively coupled electric fields accelerate proliferation of osteoblast-like primary cells and increase bone extracellular matrix formation in vitro. Eur Biophys J 29: 499-506 (2000)

Henriksson J, Chi MM, Hintz CS, Young DA, Kaiser KK, Salmons S, Lowry OH: Chronic stimulation of mammalian muscle: changes in enzymes of six metabolic pathways. Am J Physiol 251: C614-C632 (1986)

Steinacker JM, Liu YF: Exercise and Stress Response: the role of stress protein.

CRC 2:197-215 (2002)

Jornot L, Mirault ME, Junod AF: Differential expression of Hsp70 stress proteins in human endothelial cells exposed to heat shock and hydrogen peroxide. Am J Respir Cell Mol Biol 5: 265-275 (1991)

Kanno S, Oda N, Abe M, Saito S, Hori K, Handa Y, Tabayashi K, Sato Y: Establishment of a simple and practical procedure applicable to therapeutic angiogenesis. Circulation 99: 2682-2687 (1999)

Kawahara Y, Yamaoka K, Iwata M, Fujimura M, Kajiume T, Magaki T, Takeda M, Ide T, Kataoka K, Asashima M, Yuge L: Novel electrical stimulation sets the cultured myoblast contractile function to 'on'. Pathobiology 73: 288-294 (2006)

Khanna A, Aten RF, Behrman HR: Heat shock protein induction blocks hormone-sensitive steroidogenesis in rat luteal cells. Steroids 59: 4-9 (1994)

Kiang JG, Carr FE, Burns MR, McClain DE: HSP-72 synthesis is promoted by increase in [Ca2+]i or activation of G proteins but not pHi or cAMP. Am J Physiol 267: C104-C114 (1994)

Kiang JG, Tsokos GC: Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. Pharmacol Ther 80: 183-201 (1998)

Kim D, Somji S, Garrett SH, Sens MA, Shukla D, Sens DA: Expression of Hsp 27, Hsp 60, hsc 70, and Hsp 70 by immortalized human proximal tubule cells (HK-2) following exposure to heat shock, sodium arsenite, or cadmium chloride. J Toxicol Environ Health A 63: 475-493 (2001)

Kregel KC: Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J Appl Physiol 92: 2177-2186 (2002)

Kumagai E, Tominaga M, Harada S: Sensitivity of chronically HIV-1 infected HeLa cells to electrical stimulation. Appl Microbiol Biotechnol 63: 754-758 (2004)

Kuramochi Y, Guo X, Sawyer DB, Lim CC: Rapid electrical stimulation induces early activation of kinase signal transduction pathways and apoptosis in adult rat ventricular myocytes. Exp Physiol 91: 773-780 (2006)

Kwak HJ, Jun CD, Pae HO, Yoo JC, Park YC, Choi BM, Na YG, Park RK, Chung HT, Chung HY, Park WY, Seo JS: The role of inducible 70-kDa heat shock protein in cell cycle control, differentiation, and apoptotic cell death of the human myeloid leukemic HL-60 cells. Cell Immunol 187: 1-12 (1998)

Lawson MA, Purslow PP: Differentiation of myoblasts in serum-free media: effects of modified media are cell line-specific. Cells Tissues Organs 167: 130-137 (2000)

Lepock JR, Frey HE, Inniss WE: Thermal analysis of bacteria by differential scanning calorimetry: relationship of protein denaturation in situ to maximum growth temperature. Biochim Biophys Acta 1055: 19-26 (1990)

Lepore DA, Morrison WA: Ischemic preconditioning: lack of delayed protection against skeletal muscle ischemia-reperfusion. Microsurgery 20: 350-355 (2000)

Li CY, Lee JS, Ko YG, Kim JI, Seo JS: Heat shock protein 70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation. J Biol Chem 275: 25665-25671 (2000)

Lin-Shiau SY, Day SY, Fu WM: Use of ion channel blockers in studying the regulation of skeletal muscle contractions. Naunyn Schmiedebergs Arch Pharmacol 344: 691-697 (1991)

Lindquist S: The heat-shock response. Annu Rev Biochem 55: 1151-1191 (1986)

Liu Y, Gampert L, Nething K, Steinacker JM: Response and function of skeletal muscle heat shock protein 70. Front Biosci 11: 2802-2827 (2006)

Liu Y, Gao WD, O'Rourke B, Marban E: Cell-type specificity of preconditioning in an in vitro model. Basic Res Cardiol 91: 450-457 (1996)

Liu Y, Lehmann M, Baur C, Storck M, Sunder-Plassmann L, Steinacker JM: HSP70 expression in skeletal muscle of patients with peripheral arterial occlusive disease. Eur J Vasc Endovasc Surg 24: 269-273 (2002).

Liu Y, Lormes W, Baur C, Opitz-Gress A, Altenburg D, Lehmann M, Steinacker JM: Human skeletal muscle HSP70 response to physical training depends on exercise intensity. Int J Sports Med 21: 351-355 (2000)

Liu Y, Lormes W, Wang L, Reissnecker S, Steinacker JM: Different skeletal muscle HSP70 responses to high-intensity strength training and low-intensity endurance training. Eur J Appl Physiol 91: 330-335 (2004)

Liu Y, Mayr S, Opitz-Gress A, Zeller C, Lormes W, Baur S, Lehmann M, Steinacker JM: Human skeletal muscle HSP70 response to training in highly trained rowers. J Appl Physiol 86: 101-104 (1999)

Liu Y, Steinacker JM: Changes in skeletal muscle heat shock proteins: pathological significance. Front Biosci 6: D12-D25 (2001)

Low-Friedrich I, Schoeppe W: Effects of calcium channel blockers on stress protein synthesis in cardiac myocytes. J Cardiovasc Pharmacol 17: 800-806 (1991)

Lowry SF, Goodgame JT, Jr, Norton JA, Jones DC, Brennan MF: Effect of protein malnutrition on host-tumor composition and growth. Surg Forum 28: 143-145 (1977)

Luo G, Sun X, Hungness E, Hasselgren PO: Heat shock protects L6 myotubes from catabolic effects of dexamethasone and prevents downregulation of NF-kappaB. Am J Physiol Regul Integr Comp Physiol 281: R1193-R1200 (2001)

Luo SS, Sugimoto K, Fujii S, Takemasa T, Fu SB, Yamashita K: Role of heat shock protein 70 in induction of stress fiber formation in rat arterial endothelial cells in response to stretch stress. Acta Histochem Cytochem 40: 9-17 (2007)

Maglara AA, Vasilaki A, Jackson MJ, McArdle A: Damage to developing mouse skeletal muscle myotubes in culture: protective effect of heat shock proteins. J Physiol 548: 837-846 (2003)

Marotta M, Bragos R, Gomez-Foix AM: Design and performance of an electrical stimulator for long-term contraction of cultured muscle cells. Biotechniques 36: 68-73 (2004)

Mathur SK, Sistonen L, Brown IR, Murphy SP, Sarge KD, Morimoto RI: Deficient induction of human Hsp70 heat shock gene transcription in Y79 retinoblastoma cells despite activation of heat shock factor 1. Proc Natl Acad Sci U.S.A. 91: 8695-8699 (1994)

Mayer MP, Bukau B: Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci 62: 670-684 (2005)

McDonough PM, Stella SL, Glembotski CC: Involvement of cytoplasmic calcium and protein kinases in the regulation of atrial natriuretic factor secretion by contraction rate and endothelin. J Biol Chem 269: 9466-9472 (1994)

Milarski KL, Morimoto RI: Expression of human HSP70 during the synthetic phase of the cell cycle. Proc Natl Acad Sci U.S.A. 83: 9517-9521 (1986)

Mitchell CR, Harris MB, Cordaro AR, Starnes JW: Effect of body temperature during exercise on skeletal muscle cytochrome c oxidase content. J Appl Physiol 93: 526-530 (2002)

Moseley PL: Exercise, stress, and the immune conversation. Exerc Sport Sci Rev 28: 128-132 (2000)

Moseley PL, Wallen ES, McCafferty JD, Flanagan S, Kern JA: Heat stress regulates the human 70-kDa heat-shock gene through the 3'-untranslated region. Am J Physiol 264: L533-L537 (1993)

Mosser DD, Kotzbauer PT, Sarge KD, Morimoto R.I: In vitro activation of heat shock transcription factor DNA-binding by calcium and biochemical conditions that affect protein conformation. Proc Natl Acad Sci U.S.A. 87: 3748-3752 (1990)

Muller E, Munker R, Issels R, Wilmanns W: Interaction between tumor necrosis factor-alpha and HSP 70 in human leukemia cells. Leuk Res 17: 523-526 (1993)

Naumann K, Pette D: Effects of chronic stimulation with different impulse patterns on the expression of myosin isoforms in rat myotube cultures. Differentiation 55: 203-211 (1994)

Ornatsky OI, Connor MK, Hood DA: Expression of stress proteins and mitochondrial chaperonins in chronically stimulated skeletal muscle. Biochem J 311 (Pt 1): 119-123 (1995)

Pchejetski D, Kunduzova O, Dayon A, Calise D, Seguelas MH, Leducq N, Seif I, Parini A, Cuvillier O: Oxidative stress-dependent sphingosine kinase-1 inhibition mediates monoamine oxidase A-associated cardiac cell apoptosis. Circ Res 100: 41-49 (2007)

Pedrotty DM, Koh J, Davis BH, Taylor DA, Wolf P, Niklason LE: Engineering skeletal myoblasts: roles of three-dimensional culture and electrical stimulation. Am J Physiol Heart Circ Physiol 288: H1620-H1626 (2005)

Pette D, Vrbova,G: What does chronic electrical stimulation teach us about muscle plasticity? Muscle Nerve 22: 666-677 (1999)

Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45 (2001)

Pratt WB, Toft DO: Regulation of signaling protein function and trafficking by the Hsp90/Hsp70-based chaperone machinery. Exp Biol Med (Maywood) 228: 111-133 (2003)

Prokopchuk O, Liu Y, Wang L, Wirth K, Schmidtbleicher D, Steinacker JM: Skeletal muscle IL-4, IL-4Ralpha, IL-13 and IL-13Ralpha1 expression and response to strength training. Exerc Immunol Rev 13: 67-75 (2007)

Puntschart A, Vogt M, Widmer HR, Hoppeler H, Billeter R: Hsp70 expression in human skeletal muscle after exercise. Acta Physiol Scand 157: 411-417 (1996)

Ravagnan L, Gurbuxani S, Susin SA, Maisse C, Daugas E, Zamzami N, Mak T, Jaattela M, Penninger JM, Garrido C, Kroemer G: Heat-shock protein 70 antagonizes apoptosis-inducing factor. Nat Cell Biol 3: 839-843 (2001)

Ritossa F: A new puffing pattern induced by temperature shock and DNP in Drosophila. Experentia 18: 571-573 (1962)

Salinas LS, Maldonado E, Navarro RE: Stress-induced germ cell apoptosis by a p53 independent pathway in Caenorhabditis elegans. Cell Death Differ 13: 2129-2139 (2006)

Salo DC, Donovan CM, Davies KJ: HSP70 and other possible heat shock or oxidative stress proteins are induced in skeletal muscle, heart, and liver during exercise. Free Radic Biol Med 11: 239-246 (1991)

Selvakumar S, Geraldine P: Heat shock protein induction in the freshwater prawn Macrobrachium malcolmsonii: acclimation-influenced variations in the induction temperatures for Hsp70. Comp Biochem. Physiol A Mol Integr Physiol 140: 209-215 (2005)

Semsarian C, Wu MJ, Ju YK, Marciniec T, Yeoh T, Allen DG, Harvey RP, Graham RM: Skeletal muscle hypertrophy is mediated by a Ca^{2+}-dependent calcineurin signalling pathway. Nature 400: 576-581 (1999)

Snoeckx LH, Cornelussen RN, van Nieuwenhoven FA, Reneman RS, Van D: Heat shock proteins and cardiovascular pathophysiology. Physiol Rev 81: 1461-1497 (2001)

Steinacker JM, Wang L, Gampert L: Effects of overexpression of heat shoch protein 70 on energy metabolism. Med Sci Sports Exerc 37: S455-S461 (2005)

Steinacker JM, Liu Y: stress proteins and applied exercise physiology. Front Biosci 211-230 (2002)

Stern-Straeter J, Bach AD, Stangenberg L, Foerster VT, Horch RE, Stark GB, Beier JP: Impact of electrical stimulation on three-dimensional myoblast cultures - a

real-time RT-PCR study. J Cell Mol Med 9: 883-892 (2005)

Theodorakis NG, Morimoto RI: Posttranscriptional regulation of Hsp70 expression in human cells: effects of heat shock, inhibition of protein synthesis, and adenovirus infection on translation and mRNA stability. Mol Cell Biol 7: 4357-4368 (1987)

Theriault JR, Mambula SS, Sawamura T, Stevenson MA, Calderwood SK: Extracellular HSP70 binding to surface receptors present on antigen presenting cells and endothelial/epithelial cells. FEBS Lett 579: 1951-1960 (2005)

Todryk S, Melcher AA, Hardwick N, Linardakis E, Bateman A, Colombo MP, Stoppacciaro A, Vile RG: Heat shock protein 70 induced during tumor cell killing induces Th1 cytokines and targets immature dendritic cell precursors to enhance antigen uptake. J Immunol 163: 1398-1408 (1999)

Vidair CA, Wang ZH, Dewey WC: Noninvolvement of the heat-induced increase in the concentration of intracellular free Ca^{2+} in killing by heat and induction of thermotolerance. Radiat Res 124: 156-164 (1990)

Villar J, Mendez-Alvarez S: Heat shock proteins and ventilator-induced lung injury. Curr Opin Crit Care 9: 9-14 (2003)

Wagner M, Hermanns I, Bittinger F, Kirkpatrick CJ: Induction of stress proteins in human endothelial cells by heavy metal ions and heat shock. Am J Physiol 277: L1026-L1033 (1999)

Walsh RC, Koukoulas I, Garnham A, Moseley PL, Hargreaves M, Febbraio MA: Exercise increases serum Hsp72 in humans. Cell Stress Chaperon 6: 386-393 (2001)

Wehrle U, Dusterhoft S, Pette D: Effects of chronic electrical stimulation on myosin heavy chain expression in satellite cell cultures derived from rat muscles of different fiber-type composition. Differentiation 58: 37-46 (1994)

Whelan SA, Hightower LE: Differential induction of glucose-regulated and heat shock proteins: effects of pH and sulfhydryl-reducing agents on chicken embryo cells. J Cell Physiol 125: 251-258 (1985)

Widelitz RB, Duffy JJ, Gerner EW: Accumulation of heat shock protein 70 RNA and its relationship to protein synthesis after heat shock in mammalian cells. Exp Cell Res 168: 539-545 (1987)

Wu BJ, Kingston RE, Morimoto RI: Human HSP70 promoter contains at least two distinct regulatory domains. Proc Natl Acad Sci U.S.A. 83: 629-633 (1986)

Xi L, Tekin D, Bhargava P, Kukreja RC: Whole body hyperthermia and preconditioning of the heart: basic concepts, complexity, and potential mechanisms. Int J Hyperther 17: 439-455 (2001)

Xia Y, Buja LM, McMillin JB: Activation of the cytochrome c gene by electrical stimulation in neonatal rat cardiac myocytes. Role of NRF-1 and c-Jun. J Biol Chem 273: 12593-12598 (1998)

Yaffe D, Saxel O: Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature 270: 725-727 (1977)

Yamamoto N, Smith MW, Maki A, Berezesky IK, Trump BF: Role of cytosolic Ca2+ and protein kinases in the induction of the Hsp70 gene. Kidney Int 45: 1093-1104 (1994)

Zhang Z, Ferraris JD, Brooks HL, Brisc I, Burg MB: Expression of osmotic stress-related genes in tissues of normal and hyposmotic rats. Am J Physiol Renal Physiol 285: F688-F693 (2003)

Zhao M, Bai H, Wang E, Forrester JV, McCaig CD: Electrical stimulation directly induces pre-angiogenic responses in vascular endothelial cells by signaling through VEGF receptors. J Cell Sci 117: 397-405 (2004)

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