Electrical stimulation induced Hsp70 response in C_2C_{12} cells

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ABSTRACT

Electrical stimulation (ES) is widely used in experimental and clinical settings and shows effects on cellular response to stress; however, mechanisms underlying ES-induced effects are not thoroughly understood. We investigated the Hsp70 response in mouse myoblast derived C_2C_{12} cells to ES at 13V in different groups (A: 12 Hz, 11 min; B: 12 Hz, 90 min; C: 100 Hz, 11 min) and harvested before ES and at 0h, 1h, 4h, 8h and 12h after ES, respectively. Control cells without ES were parallel treated to each stimulated group. Hsp70 expression was determined at protein level by quantitative Western-blot and at mRNA level by real-time PCR, respectively. ES in group A caused a modest biphasic Hsp70 response at mRNA level with a slight increase at protein level. In group B Hsp70 increased significantly ($P<0.01$) at mRNA (559%) and protein level (413%), and remained elevated 12h after ES. In group C the highest Hsp70 mRNA level (14-fold increase, $P<0.01$) was observed at 4h after ES with only a moderate increase at protein level (147%, $P<0.05$) at 8h after ES. Thus, ES induced distinct Hsp70 responses at both protein and mRNA level, and the characteristics of ES determined the pattern and time course of Hsp70 response in the cultured cells. ES induced Hsp70 response may serve as a common mechanism underlying diverse effects of ES and plays an important role in cellular adaptive response to ES.

Key words: heat shock protein; cellular stress; cellular response; gene expression

INTRODUCTION

Electrical stimulation (ES) has been widely applied in clinical and experimental settings in order to achieve beneficial effects on the clinical care process or to facilitate cellular adaptation to an experimental intervention (9; 14). ES has been

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shown to have a beneficial effect on the wound healing process (37) and a fundamental impact on regeneration of injured tissues (8) and cellular maturation (14). There is evidence that ES can distinctly affect the immunologic response (2; 12; 45). It was shown in an animal study that transcutaneous needle stimulation causes a reversal of stress-induced suppression of antibody production (31). Thus, ES can bring about a variety of functional and structural changes in the cells (15; 41; 43). However, the mechanisms underlying ES-induced cellular changes are not thoroughly investigated. Based on the fact that ES exerts diverse effects, it is likely that treatments with different kinds of ES on different tissues share a common molecular mechanism.

Among complex and diverse mechanisms responsible for cellular adaptation to stresses is the response of the stress protein or heat shock protein (Hsp). The induction of Hsps, especially Hsp with 70 kD (Hsp70), serves as an essential and universal cellular mechanism in response to stress (19). A wide spectrum of stressors including physical, chemical and physiological stimuli has been proven to induce Hsp70 (19), and furthermore, the Hsp70 response has been shown to depend upon the intensity of stimuli, for instance, exercise-induced Hsp70 response in the skeletal muscle (19; 28). However, not every stimulus will induce a Hsp70 response. For example, low frequency ultrasound (continuous or pulsed) was shown not to increase Hsp70 content in the skeletal muscle in an animal experiment (25). Hsp70 is known to be widely involved in different cellular processes including protein degradation and synthesis as well as energy metabolism (16; 19). With its "sensing" function to initiate the cellular response to stress and its "molecular chaperone" role Hsp70 has profound impact on cellular processes in adaptation to stress (19; 23). Studies have shown that Hsp70 has distinct effects on cell growth and apoptosis (29; 38). Since ES can induce a series of cellular changes that have been reported to be able to induce a Hsp70 response (23), we hypothesize that ES can induce a Hsp70 response in cultured C_2C_{12} cells, and that the Hsp70 response may vary with different kinds of ES. In this study we applied ES to C_2C_{12} cells with varying ES duration and intensity to investigate the Hsp70 response at protein as well as at mRNA level.

MATERIAL AND METHODS

Cell Culture and Sampling

C_2C_{12} derived from the mouse myoblasts (Amer. Type Culture Collection) were cultured in DMEM medium (Gibco-BRL, Berlin, Germany) containing 10% fetal bovine serum and penicillin/streptomycin (5000 U/5000 μg/ml) and maintained at 37°C in a 5% CO_2 humidified atmosphere. After about 3 day cultivation by reaching a confluence at 75-80%, cells were divided into three groups (group A, B and C) and stimulated with different kinds of ES. To each ES group, a control group of cells without ES was parallel treated in the same way.

Experimental Protocol

Original C_2C_{12} cells were cultured to amplify the total cell numbers for about 3 days and then distributed to each dish ($1x10^6$ cells). By a confluence at 75%, cells were

stimulated with different ES impulses according to the experimental protocol (Fig. 1). These cells were electrically stimulated at 13V in different groups (A: 12 Hz, 11 min; B: 12 Hz, 90 min; C: 100 Hz, 11 min) and harvested before ES and at 0h, 1h, 4h, 8h and 12h after ES, respectively. The harvested cells were re-suspended, and shock frozen immediately and then kept at -80 °C till subsequent analysis.

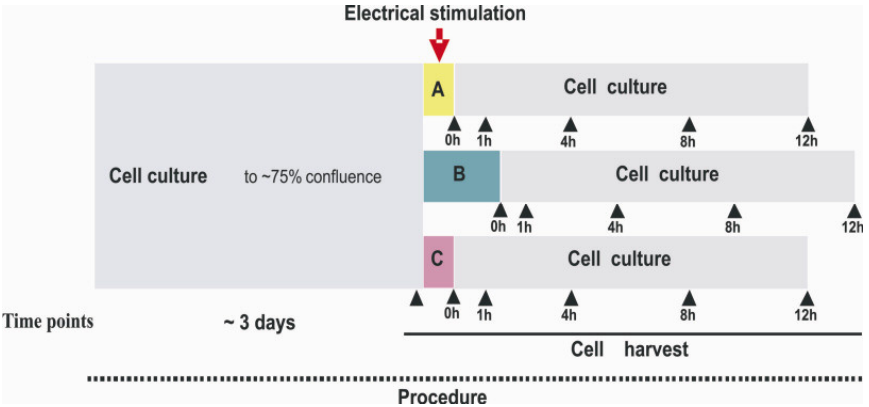


Fig. 1. Study protocol. During the cell (C_2C_{12}) culture process electrical stimulation was applied at 13 V with different characteristics, and the cells were harvested at the time points indicated. For details see text.

Electrical Stimulation

ES was applied to the cells using the device described previously (27). We have modified this device by integrating the platinum electrodes into the lid of a rectangular dish (80 x 120 mm, Nunc Omny Tray) in order to cover the whole area of the cultured cells and to get an even electrical field in the medium. The dishes were put in the incubator when ES was applied by the stimulator (Grass S-48, New York, USA).

Determination of Hsp70 Expression at Protein and mRNA Level

One part of the harvested cells ($1.2x10^6$) was used to extract the total protein using protein extraction method described previously (18; 22). The cells were at first homogenized in 100 µl extraction buffer (100 mM $Na_4P_2O_7 \cdot 10H_2O$, 5 mM

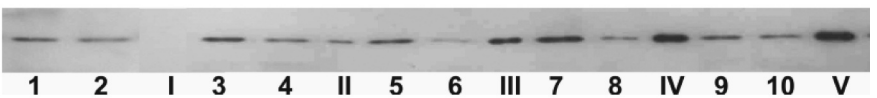


Fig. 2. Quantitative Western-blot for Hsp70 expression at protein level. Lane I-V: Standard Hsp70 with known amount of 0, 10, 15, 20, 25 ηg, respectively. Lanes 1, 3, 5, 7, 9 indicate Hsp70 obtained from ES treated group at 0h, 1h, 4h, 8h, and 12h after ES (40 µg total protein loaded), and lanes 2, 4, 6, 8, 10 indicate Hsp70 obtained from control group.

EGTA, 5 mM $MgCl_2 \cdot 6H_2O$, 0.3 mM KCl, and 1 mM dithiothreitol) with an ultrasonic homogenizer (Bandelin Sonoplus Homogensator HD2070, Berlin, Germany). The total protein concentration of each sample was determined according to the Lowry´s method (26). For determination of Hsp70 content in a sample, 40 μg total protein were loaded and separated by SDS-PAGE, and Western-blot with specific monoclonal antibody (SPA-810, Stressgen, Canada) was performed (Fig. 2), the details were described elsewhere (22).

Another part of the harvested cells ($1.2x10^6$) was taken for total RNA extraction using QIA Pure Extraction Kit (Qiagen, Germany) according to the standard protocol of the provider. The first-stand cDNA was reverse-transcribed from the RNA extract using Sensiscript reverse transcription kit (Qiagen, Germany) with random oligo-dT primer according to the standard protocol of the provider. The cDNA samples were taken for quantitative real-time PCR. The reagents used for real-time PCR were purchased from Quanti Master Sybr Green PCR kit (Qiagen, Germany). In general, in 20 μl total volume of each reaction were 10 μl of SYBR PCR Master Mix, 0.5 μl forward primer (10μM/μl), 0.5 μl reverse primer (10μM/μl), and 2.0 μl cDNA sample. The concentration of MgCl2 as well as RNAase-free water volume was adjusted for PCR reaction volume according to the prior experiments. The forward and reverse primer sequences are AGCCTTCCAGAAGCAGAGC and GGTCGTTGGCGATGATCT, respectively. The expected length of the PCR product is 124 bp.

Real-time PCR was run by LightCycler 2.1 (Roche Diagnostics, Switzerland). The PCR program was: Hot-start at 94°C for 15 min, 50 cycles with denaturation (94°C for 15 sec), annealing (60°C for 15 sec) and DNA elongation (72°C for 10 sec), respectively. To control the quality of PCR reactions, no-cDNA template control and no-reverse transcription control (DNAase-treated total RNA not subjected to cDNA synthesis) reactions were performed prior to real-time PCR, so that any contamination of DNA including genomic DNA could be excluded. To estimate the changes of Hsp70 gene expression at mRNA level, the efficiency of the real-time PCR was determined prior to the study. The purity of the PCR products was analyzed by a melting curve analysis. Additionally, PCR products were verified by running an electrophoresis on agarose gel based on their DNA size and purity. To assure the comparable amount of harvested cells used for real-time PCR, the gene expression of an internal reference gene (beta-2 microglobulin) was parallel determined using the real-time PCR.

Data Analysis

The content of Hsp70 was quantitatively determined by the densitometric integral of the protein bands on Western-blot films with refer to the known standard Hsp70 amounts (18; 22) (Fig. 2). The relative changes of Hsp70 content in ES-treated samples were related to those of the corresponding controls, and the baseline relation (before ES) was set at 100%.

From real-time PCR, the cycle number at the so-called crossing point where fluorescence signal (reflecting the production of double-stranded DNA) begins to increase exponentially, can be calculated. Based on the PCR efficiency, the ES-

Figure 3a

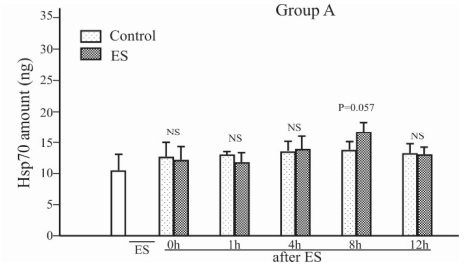


Figure 3b

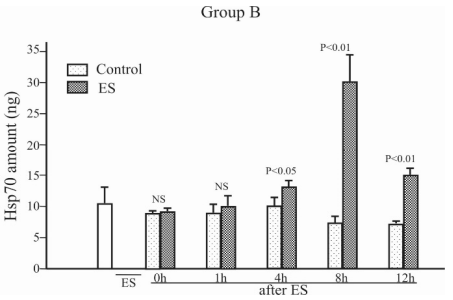
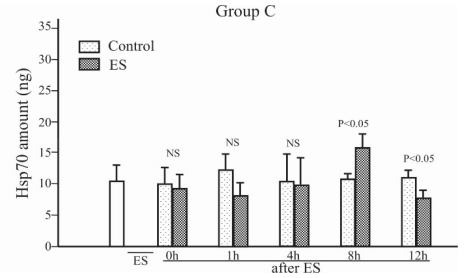


Figure 3c



induced changes of the gene expression were related to those of the internal reference gene, and the ratios were then related to that of the control samples, the baseline (before ES) was set at 100%. The data presented here are the mean of triplicate experiments. The one-way ANOVA with post hoc Scheffe test was performed, and statistic significance was assumed when $P<0.05$.

RESULTS

In the control cells, Hsp70 at both protein and mRNA level did not change clearly in comparison with that at baseline, indicating a relatively stable Hsp70 expression during the cell culture procedures.

In group A, Hsp70 at protein level kept relatively constant except for that at 8h after the stimulation (Fig. 3a) where it increased by 18% ($P=0.057$) in comparison with control cells. No statistically significant difference was found in this group between the stimulated and control cells at other time

Figure 3d

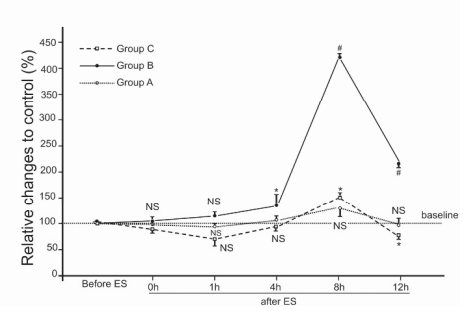


Fig. 3. Results of the Hsp70 response at protein level to different kinds of electrical stimulation (ES). Figure 3a-c represent results of group A, B and C, respectively; 3d shows ES-induced relative changes of Hsp70 expression at protein level in comparison with control (baseline). NS: no significance; * $P<0.05$; # $P<0.01$.

points (Fig. 3d). At mRNA level Hsp70 response to ES showed a biphasic increase at 1h (189%, P<0.01) and 8h (202%, P<0.01) after ES, and returned at 12h after ES to the level slightly below the baseline (Fig. 4).

In group B, Hsp70 protein level increased continuously after ES (Fig. 3b). This increase was statistically significant at 4h after ES (P<0.05) and peaked at 8h after ES (P<0.01), and remained clearly elevated at 12h after ES (P<0.01). The relative change in comparison with the control cells showed significant increase at 4h and 8h after ES (131% and 413%, respectively; Fig. 3d) and maintained still elevated at 12h after ES (212%, P<0.01). ES-induced Hsp70 mRNA expression in group B is illustrated in Fig. 4. It showed a significant increase at 1h after ES (165%, P<0.01), peaked at 4h after ES by 559% (P<0.01) and remained distinctly augmented at 12h after ES (319%, P<0.01).

In group C, Hsp70 protein level showed a tendency for a slight decrease after ES till 4h (n.s.), a significant increase at 8h after ES, but was then depressed at 12h after ES (Fig. 3c). The relative changes compared with the control cells showed an increase at 8h after ES (147%, P<0.05) and a depression at 12h after ES (67%, P<0.05) (Fig. 3d). At mRNA level, Hsp70 expression showed dramatic changes: a slight increase at 1h after ES (145%, P<0.01), a peak at 4h after ES (14-folds, P<0.01) and 182% at 12h after ES (P<0.01) (Fig. 4).

Figure 4

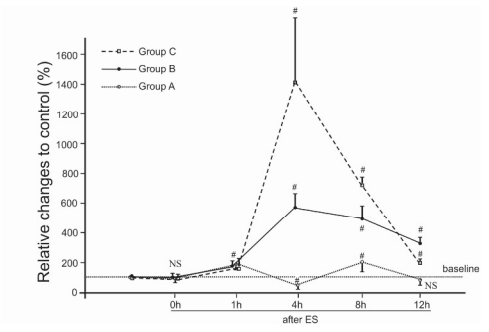


Fig. 4. Relative changes of the Hsp70 response at mRNA level to different kinds of electrical stimulation (ES) in comparison to control (baseline, set at 100%): NS: no significance; # P<0.01.

DISCUSSION

Although electrical stimulation has been widely applied in experimental and clinical settings to elucidate cellular responses, and ES-induced effects are well-documented, the mechanisms underlying ES-induced effects at the cellular as well as the molecular level remain to a large extent unknown. It is known that in response to cellular stress Hsp70 induction plays an important role and the Hsp70 response serves as an important mechanism responsible for cellular adaptation to stress (16; 19). Therefore, in the present study we investigated the Hsp70 response to different kinds of electrical stimulation. The results show that ES induces a distinct Hsp70 response depending upon the characteristics of ES.

It is well-known that a variety of stresses can induce a Hsp70 response; however, studies on the Hsp70 response to electrical stimulation seem not to have been reported. In the present study it is shown that an ES at relatively low intensity (at

low frequency for a short time) caused a biphasic Hsp70 response at mRNA level (Fig. 4) and a slight increase at protein level (Fig. 3a). With prolongation of the electrical stimulation (group B), the Hsp70 response was clearly higher and maintained for longer at both mRNA and protein level (Fig. 3b and 4). In contrast, ES at high intensity (high frequency) caused a distinctly higher Hsp70 response at mRNA level, but this response was followed by only a moderate increase at protein level (Fig. 3c and 3d), indicating that a high frequency ES induced Hsp70 response was higher at mRNA level but lower at protein level in comparison with that induced by ES at low frequency for longer duration. All these results may suggest that 1) Electrical stimulation can induce a Hsp70 response; 2) The ES-induced Hsp70 response depends upon the characteristics of ES, and 3) High intensity ES represents a high cellular stress but does not bring about a higher Hsp70 response at protein level. Though a variety of documented stresses can cause a Hsp70 response, some kinds of stress - like ultrasound at low frequency - seem not to be able to induce Hsp70 response (25), and to the best of our knowledge, this is the first report on the Hsp70 response to electrical stimulation. Previous studies have shown that the Hsp70 response depends on stress intensity, for instance, an exercise-induced Hsp70 response depended on exercise intensity (23; 28). These are quite in accordance with our present study. The biphasic response of Hsp70 at mRNA level might be attributable to different cellular processes: The earlier response may represent a stress-sensing process while the later response might be involved in the cellular response. It is known that Hsp70 has two basic biological functions: Stress sensing and molecular chaperone (19). Thus, it seems that the early response may trigger the stress-sensing process which can further induce a signaling cascade, and the later response may serve a molecular chaperone role that is indispensable for protein synthesis and degradation during cellular adaptation. The high frequency ES induced high response at mRNA level with only moderate change at protein level may suggest a specific response manner of Hsp70 when the stress is “too high” for the cells. Such a phenomenon was also observed in our previous study on the Hsp70 response in skeletal muscle of patients with peripheral arterial occlusive disease (PAOD) (21). In that study we found that the most severe ischaemic situation in PAOD at stage IV (according to Fontaine Classification) did not bring about as high a Hsp70 response as that at stage III. In our present study we did not include a further group treated with high frequency ES for longer duration because our prior experiment showed that such treatment caused dramatic cell damage so that not enough viable cells could be harvested for the subsequent analysis. These results suggest that the frequency of an electrical stimulation may be determinant for an ES-induced Hsp70 response, and to induce higher Hsp70 response, ES at low frequency for a longer time seems to be more reasonable. This is supported by the study of Goldman and Pollack in which an ES-induced increase in total DNA in human dermal fibroblasts occurred at 10 Hz but not 100 Hz (7), suggesting the determinant effect of ES frequency.

The mechanisms underlying ES-induced Hsp70 response are still to be elucidated. Because ES can lead to a variety of physiological changes in the stimulated cells (14; 30) that are shown to be able to induce Hsp70 response (24), it can be speculated that Hsp70 can be induced in response to ES-induced challenges in

energy metabolism. Different to the adult skeletal muscle, ES applied to C_2C_{12} cells (myoblasts) in the current study did not cause mechanical contraction, but probably affected more the activity of ion channels, for instance the ATP-sensitive potassium channel. It has been reported that the subunit of this channel called $K_{IR}6.2$ can serve as a metabolic sensor and plays an important role in metabolic regulation (47). Indeed, we have observed that through electrical stimulation, glucose turnover in the C_2C_{12} cells was accelerated (more glucose consumption and lactate production) in comparison with that in the unstimulated control cells (unpublished data). Certainly, it remains unknown whether this ES-induced metabolic effect might be attributable to $K_{IR}6.2$, and whether ES might have impact on glucose uptake through improving insulin function in the cells studied. To clarify this point, a study on effect of electrical stimulation on the expression and functional status of $K_{IR}6.2$ is needed. Another possible pathway for ES-induced Hsp70 response might be through protein turnover. It is known that denatured proteins can be captured by Hsp70 that can trigger Hsp70 induction (42). It has been reported that ES can affect cellular proliferation as well as cellular apoptosis in which some protein turnover is involved (32; 34). In this way Hsp70 may take on its chaperone role to help synthesize new protein and degrade abnormal or aged protein (16). It is also likely that the ES-induced Hsp70 response is associated with reactive oxygen species (ROS) because it was shown in a very recent study that ES has an impact on ROS generation (40).

The investigation of the Hsp70 response to electrical stimulation is of significance since this kind of stimulation has been widely used in clinical as well as experimental settings. According to characteristics and the stimulated target cells, ES can be divided into two kinds: one stimulating motor neuron or skeletal muscle to induce muscle contraction (39), and the other stimulating cells to facilitate response in cellular regeneration, adaptation and maturation without mechanical contraction (8; 14; 46). The former ES form seems not suitable for the purpose of the present study because it is known that muscle contraction per se can cause a Hsp70 response (19). Hence, we have chosen the latter form of ES to elucidate the direct effect of ES on the Hsp70 response, and this kind of ES has a wide spectrum of application. Recently, a study on nerve regeneration demonstrated that ES accelerates axon outgrowth in association with elevated neuronal neurotrophic factor and receptors (8). In the field of cardiology, ES has been shown to induce cardiomyocyte predifferentiation of fibroblasts *in vitro* (6), and another recent study implies that ES plays a role in cardiac differentiation of human embryonic stem cells, through mechanisms associated with the intracellular generation of ROS (40). In skeletal muscle, activation of satellite cells which triggers myogenesis plays a pivotal role in muscle regeneration and hypertrophy (10; 17; 36). It is evident for human skeletal muscle that strength training can induce response of growth and myogenic factors (20; 36). In a previous study, ES was shown to have a distinct impact on myoblast maturation and to accelerates myotube formation and to set contractile function to `on´ (14). Recently, Flaibani et al. have shown on muscle precursor cells that ES influences cell orientation and induces myocyte alignment along with down-regulation of the myogenin gene and upregulation of the desmin gene (4). In the field of immunology and cancer therapy, effects of ES have been investigated (2). It is reported that ES applied to

rat brain can modulate the immune response (11; 13). Using transcutaneous needle stimulation the stress induced suppression of antibody production can be reversed (31). In cancer therapy, ES has been used in experiments to improve the effect of chemotherapy (35), and ES seems to facilitate delivery of drugs or gene transfer to the tumour cells (3; 5; 33; 44). It is experimentally evident that through electrical gene transfer an enhancement of humoral and cellular immunity can be achieved (48). There is evidence that released extracellular Hsp70 interacts with cells of the immune system and exerts immunoregulatory effects – known as the chaperokine activity of Hsp70 (1). Therefore, ES has diverse effects on the immune response to stress, which might imply that different effects brought about by ES might share a common mechanism and that the Hsp70 response might serve as such a mechanism. Certainly, further studies have to be done to clarify this point.

From the present study it can be concluded that electrical stimulation can bring about a distinct Hsp70 response, depending on the characteristics of the ES applied. Low frequency ES for a short time induced a modest Hsp70 response. With prolongation of ES, the Hsp70 response becomes more distinct and lasts longer whereas high frequency ES leads to a higher Hsp70 response at mRNA level but only a moderate response at protein level. Therefore, our data provide strong support for the clinical and experimental application of electrical stimulation to facilitate cellular responses and also help us to understand the mechanism(s) underlying ES-induced cellular effects.

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