*Short Communication***Mild Electrical Stimulation Increases Ubiquitinated Proteins and Hsp72 in A549 Cells via Attenuation of Proteasomal Degradation**

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Abstract. To explore the cellular effects of mild electrical stimulation (MES), we treated A549 cells with low-intensity direct current at 5 V applied for 10 min. MES did not induce cell cytotoxicity or the unfolded protein response (UPR). Interestingly, the expression of ubiquitinated proteins and heat shock protein (Hsp) 72 was increased but not that of other Hsps. MES attenuated the degradation of Hsp72, which is a substrate of the proteasome-ubiquitin system. These results, along with the observed increase in expression of ubiquitinated proteins, imply that MES may affect the proteasome system, which regulates the fate of many proteins.

Keywords: mild electrical stimulation, proteasomal degradation, ubiquitinated protein

It has been established that direct-current electrical fields impact on cellular functions (1). Positive medical effects of applied low electric current, such as decreased inflammation, bone fracture healing, and alleviation of pain, have been reported (2, 3). It is hypothesized that the therapeutic effects of applied low electrical field strength are due to enhanced signal transduction (4), a hypothesis that was partly validated by a study demonstrating that electrical signals activate the phosphatidylinositol-3-OH kinase (PI-3 kinase) and promote wound healing (5). However, aside from this study, the physiological processes that are influenced by mild electrical current are still largely unexplored.

To determine the physiological processes affected by applied low-intensity current, we studied the cellular effects of mild electrical stimulation (MES) in a lung adenocarcinoma cell line, A549. A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., Carlsbad, CA, USA) containing 10% fetal bovine serum and antibiotics in a 37°C incubator. Cells were plated on 60-mm culture dishes, and at 70% confluency, they were treated with MES.

During treatment, the culture plate cover was exchanged with a plate cover with slits at the sides designed to accommodate insulated wires bearing a pair of flat rubber electrodes, which were fitted at the walls of the culture plate. The electrodes were connected to a Bio·Metronome™ (Tsuchiya Gum Co., Ltd., Kumamoto). Electrical stimulation of cells was delivered using 5 V [55 pulses per second (pps)] of low-intensity (3.85 μ A) direct current with individual pulse duration of 0.1 ms (Fig. 1A). After treatment, cells were re-incubated at 37°C for the indicated time before the assays.

First, to determine the effect of MES on cell viability, we performed lactate dehydrogenase (LDH) assay in A549 cells treated with MES for 10 min using a cytotoxicity detection kit (Roche, Mannheim, Germany) following the manufacturer's protocol. MES did not increase LDH release at 2 to 10 V, suggesting that this condition did not cause cell death in A549 cells, unlike that of the ROS inducer, menadione (50 μ M), which served as the positive control for LDH release (Fig. 1B). Consistently, MES did not induce a change in the cell morphology of A549 cells (Fig. 1C).

Despite the lack of cytotoxic effect of MES on the cells, MES could be considered a form of cellular stress and thus might up-regulate a number of stress response. To investigate this possibility, we analyzed the expres-

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Fig. 1. MES did not exhibit cytotoxic effect in A549 cells. A: The parameters for mild electrical stimulation. B: Lactate dehydrogenase activity was determined in A549 cells treated with MES or 50 μM menadione. %LDH release was computed as follows: LDH in medium/total LDH. Menadione was used as a positive control for LDH release. Data are each presented as the mean $\pm$ S.E.M. ($n = 3$). * $P < 0.05$ assessed by one-way ANOVA. C: Cells were treated with MES at 5 V for 10 min and were then examined with phase contrast microscopy.

sion of heat shock proteins (Hsps), ubiquitinated proteins, Grp94, and BiP in A549 cells treated with MES at 5 V for 10 min. Cell lysates were extracted using radioimmunoprecipitation (RIPA) buffer and subjected to Western blotting according to the protocol described previously (6) using the indicated antibodies. Interestingly, we observed a notable and significant increase in the expression of ubiquitinated proteins (Fig. 2: A and B) and Hsp72 (Fig. 2: A and C) in MES-treated cells compared with the control, but the expressions of Hsp90 and Hsp40 were not remarkably altered (Fig. 2A). MES did not stimulate the expression of unfolded protein response (UPR) marker proteins, Grp94 and BiP, which are recognized by anti-KDEL antibody, indicating that MES did not induce UPR (Fig. 2A, KDEL).

To determine the mechanism of how MES increases the expression of Hsp72, we first examined the effect of MES on the transcription of Hsp72. As positive control, we performed heat shock experiment. Cellular response to heat shock involves the synthesis of Hsp72 mRNA, through the binding of heat shock factor-1 (HSF-1) to the Hsp72 promoter (7). For heat shock treatment, the culture plate was sealed and immersed in a water bath at a temperature of 42°C. MES or heat shock treatment was carried out for 10 min. Total RNA was collected from A549 cells, using Isogen (Nippongene, Tokyo). RT-PCR experiments were performed with an RT-PCR kit (Takara, Ohtsu) according to the manufacturer's instructions. The primers used for human Hsp72 and GAPDH were described previously (6). As expected, heat shock at 42°C for 10 min increased the mRNA (Fig. 2: D and E, HS) as well as the protein levels of

Hsp72 (Fig. 2: F and G, HS). Interestingly, MES did not affect the Hsp72 mRNA (Fig. 2: D and E, MES) but it significantly increased the protein expression of Hsp72 (Fig. 2: F and G, MES). Furthermore, we assayed the promoter activity of Hsp72 by luciferase assay as previously described (6), using the promoter of Hsp72 cloned in pGL2 basic vector. Heat shock but not MES increased the transcriptional activity of Hsp72 (Fig. 2H). Taken together, these results indicated that, unlike heat shock, the effect of MES was not at the transcriptional level but at the protein level of Hsp72.

Because Hsp72 protein is regulated via the ubiquitin/proteasome pathway (8), we assessed the effect of MES on the proteasomal degradation of Hsp72. A549 cells were pretreated with cycloheximide (5 μM) for 1 h and then were treated with MES at 5 V for 10 min. Cells were re-incubated at 37°C in media containing cycloheximide and then harvested at the indicated time. Cell lysates were subjected to Western blotting analysis. Treatment of A549 cells with MES significantly attenuated the degradation of Hsp72 compared with the control (Fig. 2I), an effect that is similar to that of treatment with MG132, a proteasome inhibitor (data not shown). These findings are consistent with a previous study showing that proteasome inhibition by ALLN (*N*-acetyl-leucyl-leucyl-norleucinal), a proteasome inhibitor, elevated the expressions of ubiquitinated proteins and Hsp72, but not those of Hsp40, Hsp90, and GRP 94 (9).

To confirm our hypothesis that MES affects the proteasome, we examined the effect of MES on the expression of cystic fibrosis transmembrane conductance regulator (CFTR) protein, a well-known substrate

Fig. 2. MES increased the expression of ubiquitinated proteins and Hsp72 in A549 cells. **A:** A549 cells were treated with MES at 5 V for 10 min. After 8 h of re-incubation at 37°C, cell lysates were extracted and analyzed by Western blotting with the indicated antibodies. CNX was used as an internal control. **B** and **C:** Quantification of ubiquitinated proteins (**B**) and Hsp72 protein (**C**) expression using Image Gauge software (ver. 4.23; Fujifilm, Tokyo). Values are each presented as the mean $\pm$ S.E.M. ($n = 3$) and expressed as relative expression to untreated samples. * $P < 0.05$ vs untreated cells, assessed by Student's t test. **D** and **F:** A549 cells were untreated or treated with MES at 5 V or heat shock at 42°C for 10 min. **D:** After 1 h of re-incubation at 37°C, total RNA was extracted for RT-PCR analysis. **F:** Cell lysates were extracted from treated samples after 5 h of re-incubation at 37°C and analyzed by Western blotting. **E** and **G:** Quantification of Hsp72 mRNA expression (**E**) and Hsp72 protein expression (**G**) using Image Gauge software. Values are each presented as the mean $\pm$ S.E.M. ($n = 3$) and expressed as relative expression to untreated samples. * $P < 0.05$ vs untreated samples, assessed by one-way ANOVA. **H:** A549 cells transfected with Hsp72 promoter in pGL2 basic vector were untreated or treated with MES or heat shock for 10 min. Luciferase activity of the lysates was determined 5 h after treatment and is expressed as fold activation over the untreated sample. The data shown are representative of 3

independent experiments. Data are each presented as the mean $\pm$ S.E.M. ($n = 3$). *** $P < 0.001$ vs untreated cells, assessed by one-way ANOVA. **I:** A549 cells were pretreated with 5 μ M cycloheximide for 1 h and then were treated with MES at 5 V for 10 min. Cell lysates were extracted and analyzed by Western blotting at the indicated time point. Relative amount of Hsp72 was normalized to Hsc70, and blots were quantified using Image Gauge software. Data are each presented as the mean $\pm$ S.E.M. ($n = 3$). * $P < 0.05$ assessed by Student's t test. Filled circles, control; open circles, MES. **J:** CHO cells stably expressing CFTR were subjected to MES at 5 V for 10 min. After 5 h of re-incubation at 37°C, lysates were isolated and analyzed by Western blotting with the indicated antibodies. CNX was used as an internal control.

of the proteasome (10). It has been reported that inhibition of proteasomal degradation leads to the accumulation of misfolded CFTR and the appearance of high molecular weight (HMW)-complex caused by poly-ubiquitinated proteins (11). Chinese hamster ovary (CHO-K1) cells stably expressing CFTR were treated with MES for 10 min at 5 V. Protein lysates were analyzed by Western blotting with anti-CFTR antibody. As shown in Fig. 2J, although the expression of mature and immature CFTR (band C and B, respectively) was not changed by MES, the expression of HMW-complex

was dramatically induced by MES treatment, demonstrating that MES attenuated the proteasomal degradation of CFTR. In addition, consistent with the results in A549 cells, the expression levels of ubiquitinated proteins and Hsp72 were increased by MES treatment in CHO-K1 cells (Fig. 2J).

A previous study showed that as a protective cellular response to ionic stress, Hsp72 expression is induced by high extracellular potassium concentration ($[K^+]_e$) due to rapid Ca^{2+} influx (12). We next determined whether MES had a similar membrane depolarizing effect as

Fig. 3. MES did not induce calcium influx in A549 cells. **A:** A549 cells were placed in medium containing KCl for 10 min. After 5 h of re-incubation at 37°C, cell lysates were extracted and analyzed by Western blotting. **B:** A549 cells were loaded with Fura-2 AM and were stimulated with the indicated concentrations of KCl. Representative traces are shown. **C and D:** A549 cells were loaded with Fura-2 AM and were treated with MES at 5 V (**C**) or 10 V (**D**) for 10 min. Representative traces are shown. Ionomycin was used as positive control for calcium influx.

$[K^+]_e$. Cells grown on cover glasses were rinsed with Hank's buffered salt solution (HBSS). They were then loaded with 5 μ M Fura-2 AM (Dojindo Molecular Technologies, Kumamoto), incubated at 37°C for 45 min and subsequently rinsed with HBSS. The average fluorescence of the whole population of Fura-2 AM-loaded cells was measured by using continuous rapid alternating excitation from monochromators (340 and 380 nm) and emission at 510 nm in a fluorescence spectrophotometer. The fluorescence ratio was recorded every 5.0 s using a digitized image processor (Argus-50; Hamamatsu Photonics, Hamamatsu). We were able to confirm in A549 cells that Hsp72 expression was up-regulated in the presence of high $[K^+]_e$ (50–100 mM) with an accompanying increase of Ca^{2+} influx (Fig. 3: A and B). However, although Hsp72 expression level was increased in MES-treated cells (Fig. 2: A, F, and J), there was no difference in Ca^{2+} influx with MES treatment at 5 or 10 V (Fig. 3: C and D), indicating that the effect of MES on Hsp72 expression was not due to membrane depolarization. Collectively, these data suggest that MES increases the expression of Hsp72 and ubiquitinated proteins via attenuation of proteasomal degradation.

We have firstly shown here that low-intensity current affects the degradation of proteins, which are substrates of the ubiquitin-proteasome system, such as Hsp72 and CFTR. However, whether other client proteins of the proteasome are likewise affected still has to be clarified. The proteasome is the main intracellular protein-degradation machinery in higher eukaryotes and the proteasome substrates are diverse, hence the proteasomal

system impacts on a variety of cellular processes such as the cell cycle, differentiation, apoptosis, adaptation to stress, and integration of environmental signals. In addition, the ubiquitin-proteasome pathway has recently gained much attention due to the fact that the modulation of its activity can impinge on pathways utilized by malignant cells (reviewed in ref. 13).

Because there are many substrates of the proteasome, MES may have the potential to affect various physiological and/or pathological pathways. Electrical stimulation has been the focus of attention in recent years, and studies have revealed the effect of electrical stimulation on life processes (14, 15). Although the mechanism of how MES affects the proteasome system has yet to be elucidated, our finding that MES attenuates proteasomal degradation provides useful information for evaluating the therapeutic applications of low-intensity current.

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