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ER stress inducer, thapsigargin, decreases extracellular-superoxide dismutase through MEK/ERK signalling cascades in COS7 cells

TETSURO KAMIYA[1,2], AYA OBARA[1], HIROKAZU HARA[1], NAOKI INAGAKI[2] & TETSUO ADACHI [1]

[1]*Department of Biomedical Pharmaceutics, Laboratory of Clinical Pharmaceutics, Gifu Pharmaceutical University, 1-25-4 Daigaku-nishi, Gifu 501-1196, Japan, and* [2] *Bioresponses and Regulation, Field of Biofunctional Control, Medical Information Sciences Division, United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan*

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Abstract

It has been reported that tubular cells suffer an endoplasmic reticulum (ER) stress during the development of chronic kidney disease, which is a potent risk factor of cardiovascular disease. Moreover, under these conditions, reactive oxygen species are generated and induce cell injury. Extracellular-superoxide dismutase (EC-SOD) is a member of SODs and protects the cells from oxidative stress. Here, it is demonstrated that thapsigargin, an ER stress inducer, decreased EC-SOD expression, whereas the expression of Cu,Zn-SOD and Mn-SOD was not changed. On the other hand, another ER stress inducer, tunicamycin, did not affect the expression of EC-SOD. Further, it was shown that thapsigargin has the ability to activate extracellular-signal regulated kinase (ERK), but tunicamycin does not. Moreover, pre-treatment with U0126, an inhibitor of mitogen-activated protein kinase kinase (MEK)/ERK, suppressed thapsigargin-triggered EC-SOD reduction, suggesting that MEK/ERK signalling should play an important role in the regulation of EC-SOD in COS7 cells under ER stress conditions.

Keywords: *Extracellular-superoxide dismutase, endoplasmic reticulum stress, thapsigargin, chronic kidney disease, extracellular-signal regulated kinase*

Introduction

The excess production of reactive oxygen species (ROS) caused by an oxidant and anti-oxidant imbalance can result in oxidative stress, leading to altered metabolism by dysregulation of signal transduction events and biomolecular injury, all of which contribute to pathological changes in cell and tissue function [1,2]. To protect the cells from oxidative stress, organisms have antioxidant enzymes, such as superoxide dismutase (SOD), catalase and glutathione peroxidase [3,4]. SOD is a major anti-oxidant enzyme that protects cells from oxidative stress by accelerating the dismutation reaction of superoxide. In mammalians, three types of SOD isozymes have been cloned and characterized in different locations: cytosolic copper and zinc-containing SOD (Cu,Zn-SOD), mitochondrial manganese-containing SOD (Mn-SOD) and extracellular-SOD (EC-SOD) [5]. EC-SOD is the only isozyme of SOD that is expressed extracellularly, binding to tissues via its heparin-binding domain to give affinity to heparan sulphate proteoglycans on the cell surface, in basal membranes and in the extracellular matrix [6,7].

Chronic kidney disease (CKD), defined as kidney injury lasting more than 3 months, is a global public health problem [8]. The significant correlation between the degree of tubular-interstitial injury and renal function indicates that tubular-interstitial injury plays a

Correspondence: Tetsuro Kamiya, Department of Biomedical Pharmaceutics, Laboratory of Clinical Pharmaceutics, Gifu Pharmaceutical University, 1-25-4 Daigaku-nishi, Gifu 501-1196, Japan. Tel: +81 58 230 8100. Fax: +81 58 230 8105. Email: tekamiya@gifu-pu.ac.jp

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pivotal role in CKD progression [9]. While the development of CKD, proteinuria or renal toxic drugs directly injure tubular cells; further, these factors are also known to induce hypoxia. In the presence of hypoxia, cells must respond to pathological conditions by the co-ordinated expression of numerous genes to adaptation through the activation of hypoxia inducible factor-1α (HIF-1α) [10–12]. On the other hand, it is well recognized that hypoxia is associated with endoplasmic reticulum (ER) stress, which induces the apoptotic process [13]. To cope with ER stress, cells trigger a set of pathways known as the unfolded protein response (UPR), which is mediated by three types of ER-transmembrane proteins: inositol-requiring protein-1 (IRE-1), RNA-dependent protein kinase-like ER eukaryotic translation initiation factor 2α kinase (PERK) and activating transcriptional factor 6 (ATF6) [14].

Recently, we have reported that hypoxia and cobalt chloride ($CoCl_2$), a hypoxia mimetic, decrease the expression of EC-SOD, but not Cu,Zn-SOD and Mn-SOD through intracellular ROS generation and mitogen-activated protein kinase (MAPK) activation in green monkey kidney COS7 cells [15]; however, the regulation of EC-SOD under ER stress remains unclear. In order to address these issues, we investigated the effects of ER stress inducers, thapsigargin and tunicamycin, on the expression of SODs in COS7 cells. We found that thapsigargin, but not tunicamycin, decreased the expression of EC-SOD mRNA in a dose- and time-dependent manner. Moreover, we determined the involvement of MAPK kinase (MEK) and ERK in the reduction of EC-SOD. From our observations, we considered that MEK/ERK play a critical role in the regulation of EC-SOD in COS7 cells under ER stress conditions.

Materials and methods

Cell culture

COS7 cells were cultured as described in our previous report [15]. After the cells had grown to confluence, the medium was replaced with Dulbecco's modified Eagle's medium supplemented with 0.5% calf serum and antibiotics 12 h before exposure to the test reagents. The induction of hypoxia (1% O_2) was carried out in a culture chamber which controls O_2 concentration by supplying N_2 gas together with 5% CO_2. The conditioned medium was collected at the time indicated for the assay of EC-SOD concentration and the cells were washed with ice-cold phosphate-buffered saline (PBS) and then used for the mRNA assay, measurement of cellular protein and Western blot analysis.

RT-PCR analysis

The preparation of cDNA and RT-PCR were performed by the methods described in our previous report [15] with the primers indicated below. The pairs of sequence-specific primers used are as follows: EC-SOD, F 5′-AGA AAG CTC TCT TGG AGG AG-3′, R 5′-ACC GCG AAG TTG CCG AAG TC-3′ (496 base pair (bp)); Cu,Zn-SOD, F 5′-GCG ACG AAG GCC GTG TGC GTG-3′, R 5′-TGT GCG GCC AAT GAT GCA ATG-3′ (351 bp); Mn-SOD, F 5′-CGA CCT GCC CTA CGA CTA CGG-3', R 5′-CAA GCC AAC CCC AAC CTG AGC-3′ (365 bp); GRP78, F 5′-TTT CTG CCA TGG TTC TCA CT-3′, R 5′-CCC AGA TGA GTA TCT CCA TT-3′ (328 bp); β-actin, F 5′-CAA GAG ATG GCC ACG GCT GCT-3′, R 5′-TCC TTC TGC ATC CTG TCG GCA-3′ (275 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), F 5′-GAA GGT GAA GGT CGG AGT C-3′, R 5′-CAA AGT TGT CAT GGA TGA CC-3′ (452 bp). Densitometric analysis of the PCR products was performed with Multi Gauge V3.0 (Fuji film, Tokyo, Japan).

Measurement of cellular protein

We measured the cellular protein as an index of cell injury. After the cells were treated, they were washed twice with ice-cold PBS and then scraped in 1 mL PBS. The cell suspension was homogenized using an ultrasonic homogenizer. The total protein in the supernatant was assayed using a Bio-Rad protein assay reagent (Bio-Lad Lab., Hercules, CA).

Measurement of cell viability

The cytotoxicity of ER stress inducers was determined by 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described in our previous report [15]. Following treatment of the COS7 cells in a 96-well microplate with ER stress inducers for 24 h, the culture medium was aspirated and the cells were added to 110 μL of 10% FCS-DMEM containing MTT (CHEMICON Int., Inc., Temecula, CA) and were then incubated for 3 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. After incubation, the cells were added to 100 μL of isopropanol containing 0.04 N HCl and were then mixed thoroughly to dissolve the MTT formazan. Finally, the MTT formazan formed was measured at 595 nm with a reference wavelength of 655 nm.

ELISA of EC-SOD

The EC-SOD concentration in the conditioned medium and cells was determined by enzyme-linked immunosorbent assay (ELISA), as described in our previous report [16], with minor modifications. We confirmed previously using another cell line that

EC-SOD concentration is closely correlated with SOD activity [17].

Western blotting

Whole cell extracts were prepared in lysis buffer as described previously [15]. For phosphorylated protein detection, the cells were scraped and lysed in 200 μL lysis buffer (20 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM Na_3VO_4, 20 mM β–glycerophosphate, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 2 μg/mL leupeptin and 1% Triton X-100). After centrifugation, the protein concentration of extracts was estimated with protein assay reagent. Extracts containing 20 μg protein were boiled with sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 2% sodium dodecylsulphate (SDS), 10% glycerol, 50 mM DTT and 0.01% bromophenol blue) for 5 min and separated by SDS-PAGE on 10 or 12% (w/v) polyacrylamide gels. After being transferred electrophoretically onto PVDF membranes, non-specific binding sites were blocked with PBS containing 1% bovine serum albumin. Subsequently, the membranes were incubated with the respective specific primary antibodies (1:1,000). After the membranes had been washed three times with PBST (PBS containing 0.1% Tween 20), the blots were incubated with biotin-conjugated goat anti-rabbit or -mouse antibody (1:1,000). After the membranes had been washed three times with PBST, the blots were incubated with ABC reagents (Vector Laboratories, Inc., Burlingame, CA) (1:5000). After the membranes had been washed with PBST three times, the bands were detected using SuperSignal® West Pico (Thermo Scientific, Rockford, IL) and imaged using an LAS-3000 UV mini (Fuji Film).

Statistical analysis

Data are expressed as the mean ± SD of three independent experiments. Statistical evaluation of the data was performed using ANOVA followed by post-hoc Bonferroni tests. A p-value less than 0.05 was considered significant.

Results

Induction of ER stress during hypoxia and by thapsigargin

To assess ER stress induction during hypoxia, COS7 cells were incubated under hypoxia (1% O_2) for 24 h and the induction of GRP78, which is a good marker of ER stress, was assessed by RT-PCR (Figure 1A). COS7 cells constitutively expressed GRP78 mRNA at a very low level, while culturing under normoxia did not affect the expression of GRP78. On the other hand, GRP78 expression was significantly induced when these cells were incubated under hypoxia, suggesting that hypoxia induces ER stress in COS7 cells. It is well known that thapsigargin induces ER stress by blocking sarco-endoplasmic reticulum Ca^2+-ATPase. To determine whether thapsigargin induces ER stress in COS7 cells, we first investigated the effect of thapsigargin on the protein content and the cell viability. As shown in Figures 1B and C, the protein content and the cell viabilities were not affected by thapsigargin treatment below 1 μM and so from this we determined the concentrations of thapsigargin (0–1 μM) to use in this

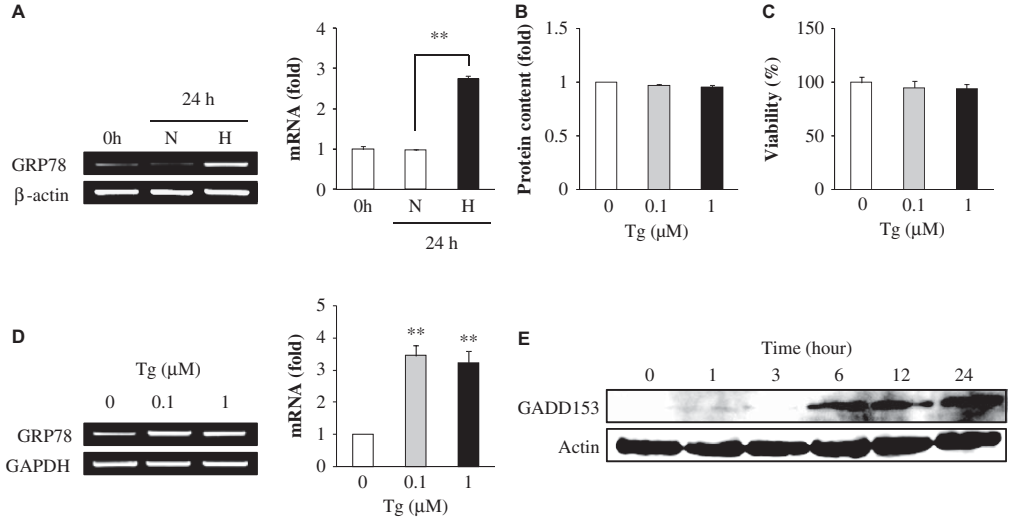

Figure 1. Induction of ER stress during hypoxia and by thapsigargin. (A) COS7 cells were cultured under normoxia (N; 20% O_2) or hypoxia (H; 1% O_2) for 24 h. After the cells were treated, RT-PCR was carried out. PCR data were normalized using β-actin. ($^{**}p < 0.01$ vs normoxia cells). The cells were treated with the indicated concentrations of thapsigargin (Tg) for 24 h (B, C, D) or 1 μM Tg for the indicated hours (E). After the cells were treated, measurement of protein content (B), cell viabilities (C), RT-PCR (D) and Western blotting (E) were carried out. PCR data was normalized using GAPDH levels ($^{**}p < 0.01$ vs untreated cells).

study. We next investigated the expression patterns of several molecular indicators of ER stress in these cells. Treatment with thapsigargin increased the GRP78 mRNA expression (Figure 1D). Moreover, we confirmed that the expression of growth arrest- and DNA damage-inducible gene 153 (GADD153) protein expression was also increased at 6 h and the expression was maintained until 24 h (Figure 1E), suggesting that the addition of thapsigargin induced ER stress under our experimental conditions.

Expression of SODs under ER stress induced by thapsigargin

The effect of ER stress on SOD expression in the kidney has not been clearly defined. Treatment with thapsigargin decreased the expression of EC-SOD compared to untreated cells in a dose-dependent manner, whereas the expressions of Cu,Zn-SOD and Mn-SOD were not changed (Figure 2A). Further, thapsigargin significantly decreased EC-SOD protein levels similar to mRNA expression (Figure 2B). In the time-course experiments, the mRNA expression of EC-SOD was not changed up to 12 h; however, the EC-SOD expression was significantly decreased at 24 h compared with the untreated cells (Figure 2C). On the other hand, the protein expression of EC-SOD was significantly decreased in a short time (6 h) after thapsigargin treatment.

Effect of tunicamycin on the expression of SODs

We next investigated the effect of another ER stress inducer, tunicamycin, on the expression of SODs. Treatment with tunicamycin did not induce cell cytotoxicity (Figures 3A and B), but increased the expression of GRP78 (Figure 3C) and GADD153 (data not shown) similar to thapsigargin treatment. These observations indicated that tunicamycin also induced ER stress in COS7 cells; however, interestingly, tunicamycin did not have the capacity to decrease the expression of EC-SOD, indicating that ER stress did not directly decrease EC-SOD in COS7 cells.

Involvement of MAPK in the reduction of EC-SOD by thapsigargin

It is well recognized that MAPK is activated under ER stress conditions in many kinds of cells [18–20]. Moreover, our previous reports showed that the expression of EC-SOD was regulated by several kinds of MAPK in COS7 [15], 3T3-L1 [21] and U937 cells [22]. To clarify the involvement of MAPK in the reduction of EC-SOD, we first investigated the effects of MAPK inhibitors, SP600125 for JNK, U0126 for MEK/ERK and SB203580 for p38-MAPK. Pre-treatment with MAPK inhibitors did not affect the basal expression of EC-SOD (data not shown) and U0126 significantly blocked thapsigargin-induced reduction of EC-SOD, but SP600125 and SB203580 had no effect on the reduction (Figure 4A). Indeed, treatment with thapsigargin induced MEK and ERK activation (Figures 4B and C). Moreover, pre-treatment with U0126 completely blocked thapsigargin-triggered ERK activation (Figure 4D); however, the activation of ERK was not determined after tunicamycin exposure (Figure 4C), suggesting that MEK/ERK signalling plays an important role in the reduction of EC-SOD in COS7 cells under ER stress conditions.

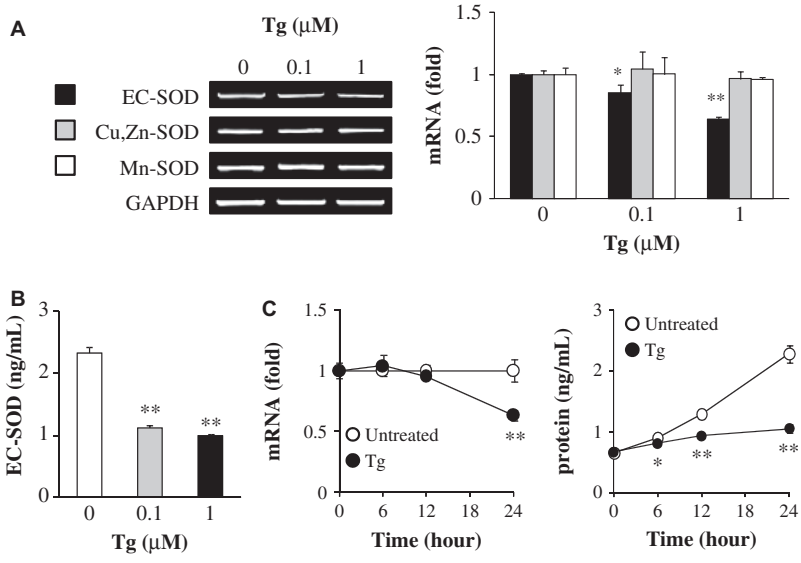

Figure 2. Expression of SODs under ER stress induced by thapsigargin. COS7 cells were treated with the indicated concentrations of thapsigargin (Tg) for 24 h (A, B) or 1 μM Tg for the indicated hours (C). After the cells were treated, RT-PCR and ELISA were carried out. All PCR data were normalized using GAPDH levels ($^{*}p < 0.05$, $^{**}p < 0.01$ vs untreated cells).

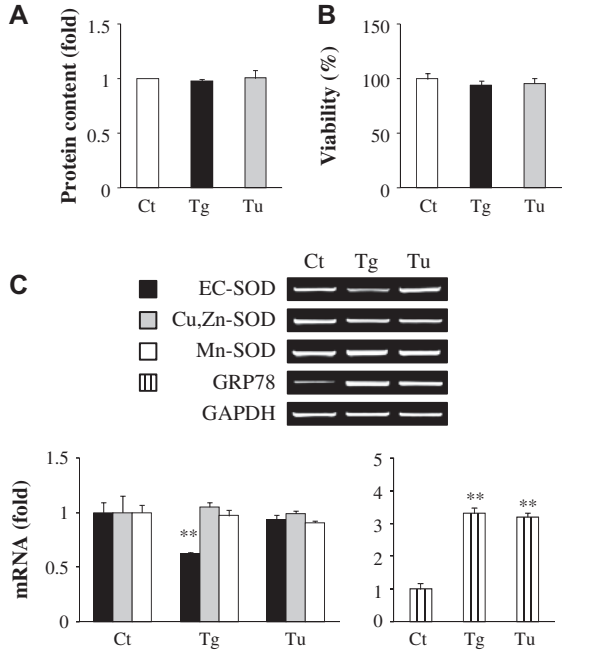

Figure 3. Effect of tunicamycin on the expression of SODs. COS7 cells were treated with 1 μM thapsigargin (Tg) or 1 μg/mL tunicamycin (Tu) for 24 h. After the cells were treated, measurement of protein content (A), cell viabilities (B) and RT-PCR (C) were carried out. All PCR data were normalized using GAPDH levels ($^{**}p < 0.01$ vs untreated cells).

Discussion

It has been considered that the excess production of ROS and the induction of ER stress induces many kinds of diseases such as atherosclerosis [23,24], retinopathy [25] and metabolic disorders [26]. Recently, it has been well recognized that CKD is one of the potent risk factors of cardiovascular disease and atherosclerosis. During the development of CKD, tubular cells suffer chronic hypoxia by fibrillization of the kidney tissue. Under hypoxic conditions, the cells were exposed to excess production of ROS. To recover the cells from oxidative stress and decreased O_2 consumption, numerous genes, such as heme oxygenase-1 (HO-1), vascular endothelial growth factor and erythropoietin, were up-regulated by HIF-1α [10–12]. Indeed, in our previous report, we detected ROS generation and the induction of HO-1 mRNA in COS7 cells under hypoxia [15]. On the other hand, it is well known that hypoxia induces ER stress via HIF-1α-independent mechanisms and these stresses lead to cell injury. To cope with ER stress, cells trigger a set of pathways known as UPR and increase the expression of GRP78, which repairs misfolding proteins. In this study, incubation of cells under hypoxia significantly induced the expression of GRP78 (Figure 1A), suggesting that hypoxia induced ER stress similar to previous reports.

EC-SOD is a major SOD isozyme in the vascular system and the presence of a high level of EC-SOD throughout the vessel walls might have an important protective role as an anti-inflammatory and anti-arteriosclerosis factor against superoxide and inflammatory cytokines in the vascular system [27–29]. In our previous report [15] we investigated the effect of $CoCl_2$ on the expression of EC-SOD, but the regulation of EC-SOD under ER stress conditions is unclear. We therefore investigated the effect of thapsigargin, an ER stress inducer, on the expression of EC-SOD. Treatment of cells with thapsigargin significantly induced the expression of GRP78 and GADD153 (Figures 1D and E). Moreover, thapsigargin significantly decreased the expression of EC-SOD in both mRNA and protein levels (Figure 2); however, this reagent did not affect the expression of Cu,Zn-SOD and Mn-SOD (Figure 2A). These results were similar to the effect of $CoCl_2$ on the expression of SODs in COS7 cells [15]. Surprisingly, treatment of cells with tunicamycin did not affect the expression of EC-SOD mRNA in spite of the induction of GRP78 (Figure 3C) and GADD153 (data not shown), indicating that the regulation of EC-SOD was not directly mediated by ER stress, but by other mechanisms.

MAPK has been shown to play a pivotal role in biological responses, including cell growth and death. Moreover, our previous reports showed the involvement of MAPK in the regulation of EC-SOD in several cell types [15,21,22,30]. We therefore next investigated the involvement of MAPK in the reduction of EC-SOD using MAPK inhibitors. As shown in Figure 4A, pre-treatment with U0126 exhibited a significant inhibitory effect on EC-SOD reduction, but not other inhibitors. We determined the activation of the MEK/ERK pathway in thapsigargin-treated COS7 cells (Figures 4B and C), but did not detect

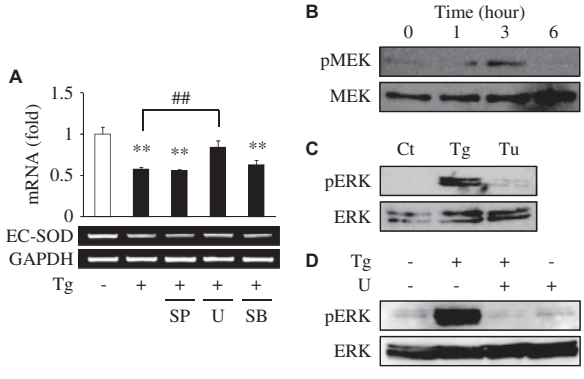

Figure 4. Involvement of MAPK in the reduction of EC-SOD by thapsigargin. (A) COS7 cells were pre-treated with or without 1 μM SP600125 (SP), U0126 (U) or SB203580 (SB) for 30 min and then the cells were treated with or without 1 μM thapsigargin (Tg) for 24 h. After the cells were treated, RT-PCR was carried out. All PCR data were normalized using GAPDH levels ($^{**}p < 0.01$ vs untreated cells, $\#\#p < 0.01$ vs Tg-treated cells). (B) The cells were treated with 1 μM Tg for the indicated hours. (C) The cells were treated with 1 μM Tg or 1 μg/mL tunicamycin (Tu) for 20 h. (D) The cells were pre-treated with or without 1 μM U0126 (U) for 30 min and then with or without 1 μM Tg for 20 h. After the cells were treated, Western blotting was carried out.

the activation of ERK by the administration of tunicamycin (Figure 4C), indicating that MEK/ERK plays a pivotal role in the regulation of EC-SOD under ER stress conditions. In our previous report, we showed the involvement of ROS and p38-MAPK in the regulation of EC-SOD in COS7 cells [15]; however, in this study, we did not observe the involvement of intracellular ROS and p38-MAPK in the reduction of EC-SOD by thapsigargin. From these observations, we speculated that the difference of signal molecules leads to the activation of different MAPK involved in the regulation of EC-SOD.

Recently, it has been reported that the difference in biological consequence between thapsigargin and tunicamycin are explained by calcium disturbance [31]. Further, we reported the differences between thapsigargin and tunicamycin responded to an ER stress-regulated factor, eukaryotic initiation factor 2α (eIF2α) [32]. We therefore investigated the involvement of calcium disturbance and eIF2α in the thapsigargin-triggered reduction of EC-SOD using a cell-permeable calcium chelator, BAPTA-AM and an inhibitor of the dephosphorylation of eIF2α, salubrinal, respectively; however, we did not detect the involvement of calcium disturbance and eIF2α in that reduction (data not shown). Therefore, the reduction of EC-SOD by thapsigargin may be not regulated by calcium disturbance, but by other mechanisms.

Conclusions

Our results suggest that the expression of EC-SOD was decreased by the addition of thapsigargin and it was speculated that the activation of MEK/ERK signalling was necessary for the reduction of EC-SOD in this model. From our results, it is speculated that the reduction of EC-SOD leads to decreased resistance to oxidative stress and accelerates ROS-derived diseases and prevents the reduction of EC-SOD, contributing to the control of redox homeostasis under ER stress conditions.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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