Manganese Superoxide Dismutase Is Induced by Endoplasmic Reticulum Stress through IRE1-Mediated Nuclear Factor (NF)-κB and AP-1 Activation

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Manganese superoxide dismutase (MnSOD) is an antioxidative enzyme that scavenges superoxide radicals and is localized in the mitochondrial matrix. MnSOD is induced by a variety of stimuli through nuclear factor (NF)-κB and AP-1 activation. We investigated the expression of MnSOD in HeLa cells exposed to various agents interfering with endoplasmic reticulum (ER) functions. All agents caused an increase in the mRNA and protein levels of MnSOD. Although ER stress-responsive genes often are up-regulated by ATF6, IRE1 and XBP1, which are ER stress-related transcription factors/transducers, the overexpression of neither molecule affected the levels of MnSOD mRNA and protein. Furthermore, we showed that ER stress reagents induced NF-κB and AP-1 activation that were inhibited by a dominant-negative IRE1 mutant. We finally demonstrated that ER stress-induced MnSOD expression was reduced by the IRE1 mutant. These results suggest that the MnSOD expression is controlled by ER stress through IRE1-mediated NF-κB and AP-1 activation.

Key words manganese superoxide dismutase (MnSOD); endoplasmic reticulum (ER) stress; IRE1; nuclear factor (NF)-κB; AP-1

Superoxide dismutase (SOD) is an antioxidative enzyme that specifically catalyzes the disproportionate reaction of superoxide radical anion ($O\cdot^{2-}$) to hydrogen peroxide. SOD, distinguished by subcellular localization and enzymatic properties, consists of three isozymes, cytosolic copper/zinc SOD (Cu/ZnSOD), mitochondrial manganese SOD (MnSOD) and an extracellular SOD.[1] Reactive oxygen species (ROS) have been implicated in a wide range of degenerative processes including amyotrophic lateral sclerosis,[2] ischemic disease,[3] Alzheimer's disease,[4] Parkinson's disease,[5] and aging.[6] ROS are generated by mitochondria as the toxic by-products of oxidative phosphorylation in their energy-generating pathway. Thus, MnSOD plays a critical role in scavenging intramitochondrial free radicals as a defense against the injurious effect of mitochondrial DNA and proteins.

The endoplasmic reticulum (ER) is an organelle in which secretory and membrane proteins are correctly folded and assembled by ER-resident molecular chaperones. Exposure to various agents interfering with the ER functions, such as tunicamycin (inhibition of N-linked glycosylation), thapsigargin (inhibition of sarco/endoplasmic reticulum Ca^{2+}-ATPases) and DTT (reduction of disulfide bonds), leads to the accumulation of unfolded protein in the ER lumen followed by induction of the ER chaperone. This signal pathway from the ER to the nucleus is termed the unfolded protein response (UPR).[7] When unfolded proteins accumulate and aggregate in the ER, two major molecules are activated in the ER membrane and transduce the signal to the nucleus. One is IRE1, whose cytosolic domain contains a Ser/Thr protein kinase domain and endoribonuclease domain.[8,9] IRE1 self-associates in the ER membrane by autophosphorylation, then cleaves an intron of the precursor mRNA for a transcription factor, XBP1, which induces the transcription of ER stress-responsive genes, through the unfolded protein response element (UPRE).[10] The other is ATF6, a transcription factor, whose N-terminal facing cytoplasm is liberated from the ER membrane by ER stress-induced proteolysis[11] and translocated into the nucleus, and then binds to the ER stress response element (ERSE).[12] It is well known that activation of the UPR leads to the enhanced expression of ER-resident chaperone proteins, such as GRP78 and GRP94.[13]

ER stress triggers the activation of other well-known molecules, nuclear factor-κB (NF-κB) and JNK.[14—18] The signaling is transduced by IRE1 that binds to tumor necrosis factor-α (TNF-α) receptor-associated factor 2 (TRAF2).[16,18] A number of studies have demonstrated that MnSOD expression is induced by a variety of stimuli, including proinflammatory cytokines,[19,20] bacterial lipopolysaccharide,[21] UV irradiation,[22] and phorbol esters.[23] Most of these treatments activate transcription factors, mainly NF-κB and AP-1, and induce a gene expression related to the stress response. We here show that MnSOD was induced by ER stress through IRE-mediated NF-κB and AP-1 activation.

MATERIALS AND METHODS

Materials and Reagents Thapsigargin, tunicamycin, brefeldin A and dithiothreitol were purchased from WAKO; A23187 was purchased from Calbiochem; tetracycline for T-REx system was purchased from Invitrogen. Anti-MnSOD (SOD-110) and anti-KDEL (SPA-827) antibodies were purchased from StressGen; anti-GRP78 (N-20) antibody was purchased from Santa Cruz.

Cell Culture and Stable Cell Line Construction HeLa and human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified 5% CO_2/95% air atmosphere. HEK293 cell lines stably expressing human IRE1α (K599A) dominant-negative mutants were selected in medium containing 80 μg/ml of GENETICIN (Invitrogen). Overexpressions of IRE1α, ATF6 (N-terminal side in the cytosol acting as a transcription factor; 1—373 amino acid residues), or XBP1

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(spliced form) were performed using the T-REx™ system. The pcDNA4/TO (Invitrogen) plasmids carrying those genes with the tetracycline-controlled cytomegalovirus (CMV) promoter were transfected in a 293 cell line (T-REx-293; Invitrogen) that expresses the tetracycline repressor protein Tet-R. The T-REx-293 cell lines were selected in DMEM containing 10% FCS and 4 μg/ml blasticidin (Invitrogen) and Zeocin (Invitrogen) was added to 300 μg/ml.

Immunoblotting Proteins (20 μg/lane) prepared as whole cell lysates were subjected to SDS-PAGE and transferred at 4 °C to nitrocellulose membranes. The transferred membranes were blocked in TBST (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk, and then incubated with anti-MnSOD polyclonal (1 : 10000 dilution), anti-KDEL monoclonal (1 : 5000 dilution) or anti-GRP78 polyclonal (1 : 1000 dilution) antibodies in TBST. Horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG HRP-linked antibody (Amersham, 1 : 5000 dilution) was used as the secondary antibody. The bands were detected using the ECL system (Amersham).

RNA Isolation and Real-Time PCR Total RNA was prepared from cells using TRI-REAGENT (SIGMA) according to the manufacturer's protocol. cDNA was synthesized from 2 μg of total RNA by reverse transcription using 100 U of SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen) and 0.25 μg of oligo $(dT)_{12-18}$ primer (Invitrogen). The expression of mRNA was measured by TaqMan-base real-time PCR in duplicate. The assay was performed in a 96-well optical tray with a final reaction volume of 25 μl containing 2× TaqMan Universal Master Mix (Applied Biosystems), forward and reverse primers (300 nM), the probes (125 nM), and cDNA, which was produced in the same manner as above. Gene-specific PCR products were measured continuously by an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) during 40 cycles. Primer and probe sequences were as follows: human GRP78/BiP, ⟨forward-primer⟩ 5′CTACTCCTGCGTCGGCGT-3′, ⟨reverse-primer⟩ 5′TGATGCGGTTGCCCTGAT-3′, ⟨probe⟩ 5′ (FAM) CCGCGTGGAGATCATCGCCA (TAMRA)-3′; human GAPDH, ⟨forward-primer⟩ 5′CGACAGTCAGCCGCATCTT-3′, ⟨reverse-primer⟩ 5′CCCCATGGTGTCTGAGCG-3′ ⟨probe⟩ 5′ (FAM) CGTCGCCAGCCGAGCCCACA (MGB)-3′. Primers and probes were designed with Primer Express software (Applied Biosystems). Human MnSOD was detected with an Assays-on-demand™ primer and probe set, Hs00167309_m1 (Applied Biosystems). The quantity of specific mRNA was normalized as a ratio to the amount of GAPDH mRNA.

Electrophoretic Mobility Shift Assays (EMSA) Nuclear extracts were prepared using previously described methods.[24,25] Oligonucleotide probes for NF-κB and AP-1 containing a NF-κB-binding site (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) and AP-1-binding site (5′-CGC TTG ATG ACT CAG CCG GAA-3′) were purchased from Santa Cruz (the core recognition sequence of this oligonucleotide is underlined). For the supershift assay, the nuclear extracts were incubated with 1 μg of specific antibodies (anti-NF-κB p65, A, Santa Cruz; anti-NF-κB p50, NL5, Santa Cruz) against each NF-κB component and (anti-c-Jun, Ab-3, Oncogene; anti-c-Fos, 4, Santa Cruz) against each AP-1 component for 2 h at 4 °C before the addition of the oligonucleotide probe.

RESULTS

We initially examined whether the expression of MnSOD is induced by ER stress. HeLa cells were treated with thapsigargin (Tg; sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor), tunicamycin (Tm; *N*-glycosylation inhibitor), brefeldin A (BFA; ER-Golgi transport inhibitor), A23187 (calcium ionophore) and dithiothreitol (DTT; inhibitor of disulfide formation). Quantitative PCR revealed that the expression of MnSOD mRNA was increased by treatment with Tg and Tm (Fig. 1A). Western blotting analysis showed the increased expression of MnSOD in cells treated with ER stress-inducing regents (Fig. 1B). The expression of GRP78, a well-known ER stress responsive gene, in mRNA and protein, is concomitantly, but faster than MnSOD, induced by the reagents, peaking at 12 h (Figs. 1A, B).

In mammals, ER stress signaling, including the UPR, is mediated by molecules in the ER membrane, such as IRE1 and ATF6.[8—13] To determine whether the ER stress-induced MnSOD expression is mediated by the UPR, we next examined the induction of MnSOD expression by the overexpression of ER stress transducers/transcription factors, ATF6,

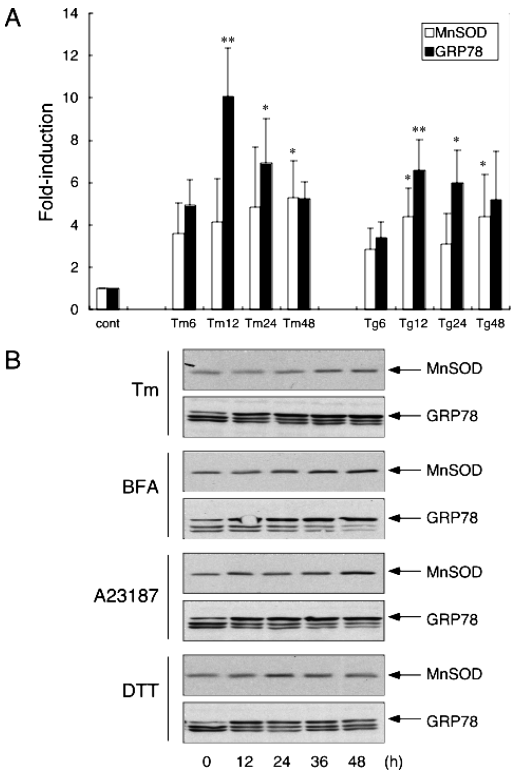

Fig. 1. Induction of MnSOD by ER Stress

(A) Expression of MnSOD mRNA. HeLa cells were treated with 2 μg/ml of tunicamycin (Tm) or 0.1 μM of thapsigargin (Tg) for the periods indicated, and total RNA was prepared and reverse-transcripted, then subjected to real-time PCR using TaqMan probes and primers (MnSOD, GRP78 and GAPDH). The expression levels of mRNA were normalized for those of GAPDH and expressed as a fold increase compared with unstressed cells (results from four independent experiments in duplicate). (B) Expression of MnSOD protein. HeLa cells were treated with 2 μg/ml of Tm, 1 μg/ml of brefeldin A (BFA), 2 mM of A23187 and 1 mM of dithiothreitol (DTT) for the periods indicated. The whole cell lysates were subjected to Western blot analysis using anti-MnSOD or anti-GRP78 antibodies. Results were expressed as the means±S.D. (three independent experiments). Statistical analysis was performed with Dunnett's test for multiple comparison after one-way analysis of variance (*, $p<0.05$; **, $p<0.01$).

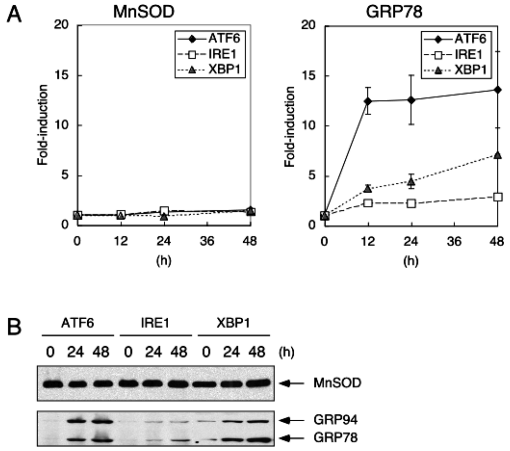

Fig. 2. The Effect of ATF6, IRE1 and XBP1 Overexpression on the Expression of MnSOD

(A) Expression of MnSOD mRNA. 293 cells were overexpressed with IRE1α, ATF6 (N-terminal side in the cytosol acting as a transcription factor; 1—373 amino acid residues), or XBP1 (spliced form) using the T-REx^{TM} system. The cells were treated with 1 μg/ml tetracycline for the periods indicated, and total RNA was prepared and subjected to reverse transcription. cDNA was amplified using TaqMan probes and primers (MnSOD, GRP78 and GAPDH). The expression levels of mRNA were normalized for those of GAPDH and expressed as a fold increase compared with unstressed cells (results from three independent experiments in duplicate). (B) Expression of MnSOD protein. 293 cells were overexpressed with IRE1α, ATF6 (1—373), or XBP1 (spliced form) using the T-REx^{TM} system. The whole cell lysates were subjected to Western blot analysis using anti-MnSOD or anti-KDEL (to detect both GRP78 and GRP94) antibodies.

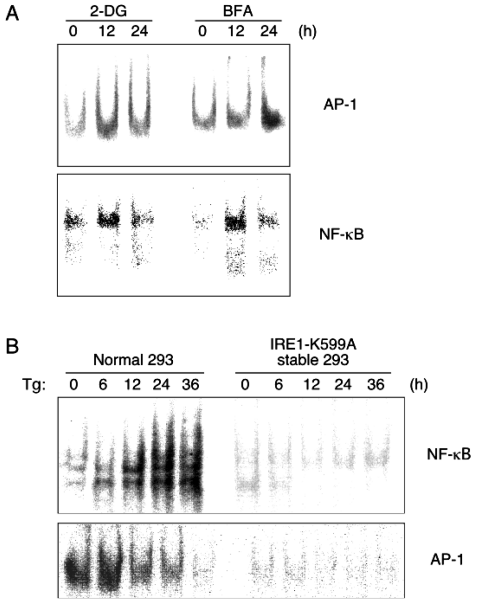

Fig. 3. Effect of Dominant-Negative IRE1 Mutant on ER Stress-Induced NF-κB and AP-1 Activation

(A) Activation of NF-κB and AP-1 by ER stress. 293 cells were treated for the periods indicated with 10 mM of 2-deoxyglucose (2-DG) and 2 μg/ml of BFA. Nuclear extracts were prepared and subjected to EMSA using ^{32}P-labeled oligonucleotides including NF-κB and AP-1 sites. (B) Effect of dominant-negative IRE1 mutant on NF-κB and AP-1 activation in response to ER stress. 293 cells and those stably expressing K599A-IRE1 were treated with 2 mM of Tg for the periods indicated. Nuclear extracts were prepared and subjected to EMSA.

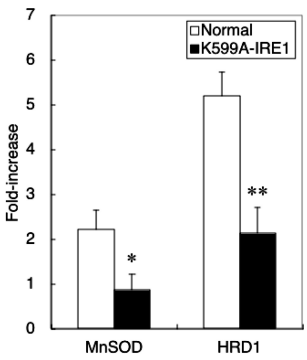

Fig. 4. Effect of Dominant-Negative IRE1 Mutant on ER Stress-Induced MnSOD mRNA Expression

Normal 293 cells and those stably expressing IRE1α-K599A were treated with 2 μg/ml Tm for 6 h. Purified total RNA was reverse-transcribed and then subjected to real-time PCR. The expression levels of mRNA were normalized for those of GAPDH and expressed as a fold increase compared with unstressed cells. Results were expressed as the means$\pm$S.D. (three independent experiments). Statistical analysis was performed with Student's *t*-test (*, $p<0.05$; **, $p<0.01$).

IRE1 and XBP1. The overexpression of ER stress transducers was performed using the T-REx system in HEK293 cells. Unexpectedly, none of the transducers affected the expression of MnSOD mRNA, whereas the expression of GRP78 was markedly induced by ATF6 and XBP1 (Fig. 2A). In addition, the MnSOD protein expression was not altered by overexpression of the transducers, consistent with the mRNA expression (Fig. 2B). These results indicated that the well-known mediators are not involved in the induction of MnSOD expression by ER stress.

Transcriptional activation in mammalian UPR is mediated by the binding of ATF6 and XBP1 to *cis*-elements including ERSE and UPRE.[10] The promoter region of MnSOD, however, contains two NF-κB sites and AP-1 sites, but neither an ERSE nor UPRE site.[27] It has been reported that MnSOD expression is induced by NF-κB and AP-1 activated by treatment with TPA and cytokines.[27—29] Furthermore, we and other researchers have demonstrated that NF-κB and JNK are activated by ER stress, and that the signaling is mediated by the IRE1-TRAF2 pathway.[16,18] We confirmed the ER stress-induced activation of NF-κB and AP-1 using EMSA. Each of the ER stress reagents (2-deoxyglucose, 2-DG; inhibitor of glycosylation) showed the activation of NF-κB and AP-1 (Fig. 3A). Furthermore, a competition assay was performed with a 1000-fold molar excess of unlabelled probes (data not shown); for the supershift assay, the nuclear extracts were incubated with 1 μg of specific antibodies against each NF-κB and AP-1 component, p50 and p65, and c-Jun and c-Fos, respectively (data not shown). To further investigate the involvement of IRE1 in ER stress-induced NF-κB and AP-1 activation, we performed EMSA with 293 cells stably expressing a dominant negative IRE1-K599A mutant, defective in kinase activity. In 293 cells with the dominant-negative mutant of IRE1, both activations of NF-κB and AP-1 by ER stress were completely suppressed, compared with those in normal cells (Fig. 3B). These results indicate that IRE1 is required for the activation of NF-κB and AP-1 in response to ER stress.

To investigate the involvement of IRE1 in the ER stress-induced MnSOD expression, we performed RT-PCR analysis using the dominant negative IRE1-K599A mutant. MnSOD expression induced by tunicamycin was significantly attenuated in 293 cells stably expressing the IRE1 mutant, compared with normal 293 cells (Fig. 4). On the other hand, the ER stress-induced HRD1 expression, depending on the IRE1-mediated signaling pathway,[30] was also significantly reduced by the dominant negative mutant (Fig. 4), suggesting

that the induction of MnSOD expression by ER stress is mediated through the pathway from IRE1 to NF-κB and/or AP-1.

DISCUSSION

In this study, we demonstrated that MnSOD mRNA and protein levels were up-regulated by a variety of ER stress-inducing reagents, such as thapsigargin, tunicamycin, brefeldin A, dithiothreitol and A23187, concomitantly with the induction of a typical ER stress-responsive gene, GRP78/Bip (Figs. 1A, B). To further elucidate the mechanism by which ER stress induces MnSOD expression, we examined the effects of transducers and transcription factors, ATF6, IRE1 and XBP1, associated with ER stress. However, the overexpression of these molecules had no effect on mRNA and protein levels of MnSOD, while that of GRP78 was elevated by ATF6 and XBP1 (Figs. 2A, B), indicating that the induction of MnSOD is mediated by unconventional signaling pathways. On the other hand, the induction of ER stress-responsive genes has hitherto been reportedly induced by ER stress-specific transcription factors, ATF6 and XBP1, through ER stress-responsive *cis*-elements, such as ERSE and UPRE.[10] However, neither ERSE nor UPRE was found in the promoter region of MnSOD, whereas NF-κB and AP-1 sites exist in the promoter.[26—29]

MnSOD is up-regulated by a variety of stimuli that activate a number of transcription factors, including NF-κB and AP-1.[19—23] On the other hand, although it has reported that ER stress evokes NF-κB and JNK activation,[14—18] the types of genes induced by the pathways is unknown. JNK activation leading to the cleavage of Bid is associated with apoptosis,[30] while c-Jun, which constitutes AP-1, is phosphorylated and activated by JNK.[31,32] JNK activation is mediated by ER stress-activated IRE1 that interacts with TRAF2, whose signal leads to JNK.[16] As it has yet to be determined whether ER stress induces AP-1 activation *via* the signaling pathway, we demonstrated AP-1 activation by ER stress and its inhibition by an IRE1 dominant-negative mutant (Figs. 3A, B). We have previously shown that ER stress-induced NF-κB activation was also mediated by IRE1 and TRAF2, similar to JNK activation,[18] although the ER stress-induced NF-κB activation pathway is known as the ER overload response (EOR), which is transduced by the release of calcium from the ER by overloading the ER with accumulated proteins and the subsequent production of reactive oxygen species (ROS).[14,15] Therefore, we hypothesized that the induction of MnSOD by ER stress depends on IRE1-TRAF2-mediated NF-κB and/or AP-1 activation. As expected, the response was effectively inhibited by the dominant negative IRE1 mutant (Fig. 4). Although it is still unknown whether other IRE1-mediated transcription factors and those that bind to other *cis*-elements are involved in the MnSOD induction in response to ER stress, at least in part, ER stress-induced NF-κB and AP-1 activation likely affects the induction.

ER stress-responsive gene induction is extended to a wide variety of genes involved in the ER and its surrounding function, such as translocation, glycosylation/modification, protein folding, protein degradation, vesicle trafficking/transport and lipid/inositol metabolism.[32] Furthermore, CHOP, a transcription factor, is induced by ER stress and causes apoptosis,[33] indicating that genes unrelated to ER participate in the ER stress response. MnSOD is primarily localized in mitochondria in a steady state, although the subcellular localization remains to be determined under ER stress conditions. In addition, the roles of MnSOD under ER stress conditions and the effects of ER stress on mitochondria are unclear. MnSOD scavenges ROS in mitochondria under various stress conditions as well as unstressed conditions. The efflux of calcium from ER stores induces an increase in cytoplasmic calcium concentration and its uptake to mitochondria.[34] The calcium influx into mitochondria leads to the collapse of mitochondria inner-membrane potential and the subsequent release of cytochrome c from mitochondria to the cytoplasm, resulting in the activation of caspase-9 that induces apoptosis.[35] It has been reported that MnSOD serves to protect mitochondria from damage caused by increased calcium-induced ROS generation.[36] Therefore, ER stress-induced MnSOD also prevents ROS increase in such circumstances. Although some mechanisms by which ER stress induces apoptosis have been proposed,[33,37,38] the protection of mitochondria from ER stress appears to be important. It is presumed that ER stress-induced MnSOD expression plays a critical role in protection cells against increased mitochondrial ROS production, indicating cross talk between ER and mitochondria under ER stress.

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