Deferoxamine Induces Endoplasmic Reticulum Stress in PC12 Cells

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Deferoxamine (DFA, *N*′-[5-(acetyl-hydroxy-amino)-pentyl]-*N*-[5-[3-(5-aminopentyl-hydroxy-carbamoyl) propanoylamino]pentyl]-*N*-hydroxy-butane diamide) is a chelating agent used to remove excess iron from the body and to reduce organ and tissue damage. DFA enhances both iron regulatory protein 1 (IRP1) expression and its endoplasmic reticulum (ER) membrane-binding activity, as occurs in hypoxia, an ER stress, in cultured cells. Here, we show that DFA promotes ER stress via an ER signal pathway.

Key words: Deferoxamine (DFA), Endoplasmic Reticulum (ER) Stress

Introduction

Deferoxamine (DFA, *N*′-[5-(acetyl-hydroxy-amino)pentyl]-*N*-[5-[3-(5-aminopentyl-hydroxy-carbamoyl) propanoylamino]pentyl]-*N*-hydroxy-butane diamide) is a chelating agent used to remove excess iron from the body, which can occur in anemic patients that receive multiple blood transfusions. DFA is also used to treat acute iron poisoning, especially in small children. DFA combines with iron in the bloodstream and enhances its elimination in the urine to reduce organ and tissue damage, such as to the liver. Iron regulatory protein 1 (IRP1) regulates iron uptake and storage (Palmer *et al*., 1994). Hypoxia, an endoplasmic reticulum (ER) stress, enhances IRP1 expression and ER membrane-binding activity (Xin *et al*., 2004). We hypothesized that DFA could induce ER stress in PC12 cells.

ER membrane kinases (inositol-requiring enzyme 1, IRE1; protein kinase-like ER kinase, PERK; and activating transcription factor 6, ATF6) directly or indirectly participate in the unfolded protein response (UPR) of mammalian cells. The ER stress response in mammalian cells is triggered by the dissociation of Bip from stress transducers such as PERK, ATF6, and IRE1. Bip binds to ER lumenal un-/misfolded proteins activating of the ER stress response. Activation (autophosphorylation and dimerization) of IRE1 activates the endonuclease domains that cleave X-box DNA-binding protein (XBP) mRNA and generates an activated form of XBP1 by removing 23 nucleotides of the Pst1 restriction enzyme site, activation of PERK results in phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2-α) and inhibits translation initiation. ATF6 is cleaved at the cytosolic face of the membrane in response to ER stress, causing nuclear translocation of the *N*-terminal cytoplasmic domain, which contains the DNA-binding, dimerization, and transactivation domains, and subsequent binding to both ER stress-response element (ERSE) and ATF6 sites to enhance ER molecular chaperone genes. UPR is rapidly sensitive to environmental or physical changes associated with apoptosis (Marciniak and Ron, 2006; Ron and Walter, 2007). Here, we show that DFA promotes ER stress at the cellular level against neuronal PC12 cells through the ER signaling pathway.

Materials and Methods

Rat pheochromocytoma PC12 cells were routinely cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% horse serum and 5% fetal calf serum on collagen-coated dishes in a humidified 5% CO_2 atmosphere at 37 °C (De León *et al*., 1994). Total RNA was extracted using an SV Total RNA isolation system (Promega, Madison, USA). RT-PCR was performed using the forward primer (F) (5′-ACCACCAGTCCATCGCCATT-3′) and reverse primer (R) (5′-CCACCCTGGACGGAAGTTTG-3′) for IRE1; F (5′-AGTGGTGGCCACTAATGGAG-3′) and R (5′-TCTTTTGTCAGG GGTCGTTC-3′) for Bip; F (5′-CTAGGCCTGGAGGCCAG-

GTT-3′) and R (5′-ACCCTGGAGTATGCGG-GTTT-3′) for ATF6; F (5′-ATCGAGTTCACC-GAGCAGAC-3′) and R (5′-TCACAGCTTTCT-GGTCATCG-3′) for PDI; F (5′-GGTCTGGTT-CCTTGGTTTCA-3′) and R (5′-TTCGCTGGCT-GTGTAACTTG-3′) for PERK; F (5′-ACATC-AAATGGGGTGATGCT-3′) and R (5′-AGGA-GACAACCTGGTCCTCA-3′) for GAPDH; and F (5′-AAACAGAGTAGCAGCTCAGACTGC-3′) and R (5′-TCCTTCTGGGTAGACCTCTGGG-AG-3′) for XBP1. RT-PCR primers were supplied by Bioneer Co. (Taejon, Korea). Unless otherwise noted, all other chemicals were purchased from Sigma (St. Louis, Mo., USA). RT-PCR conditions for 30 cycles were: 94 °C for 30 s; 58 °C for 30 s; and 72 °C for 1 min (but 10 min in the final cycle) using both primers mentioned above with *Taq* DNA polymerase. Immunoblotting analysis was performed according to the standard procedure. PC12 cells were scraped, lysed by the addition of SDS sample buffer [62.5 mM Tris (tris(hydroxymethyl)aminomethane)-HCl, pH 6.8, 6% (w/v) SDS, 30% glycerol, 125 mM DTT, 0.03% (w/v) bromophenol blue] and separated by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane, and the membrane was incubated with the primary antibodies overnight at 4 °C. The blots were developed using an enhanced chemiluminescence Western blotting detection system kit (Amersham, Uppsala, Sweden). Rabbit anti-eIF2 antibody, eIF2-P antibody and goat anti-actin antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-ATF6 antibody was obtained from Imgenex (San Diego, CA, USA).

Results and Discussion

Accumulation of un-/misfolded proteins in the ER lumen triggers an ER stress signal pathway through ER membrane stress transducers (IRE1, PERK and ATF6) and an ER lumen regulator (Bip). Releasing Bip from the ER luminal stress-sensing domain of IRE1 induces autophosphorylation and dimerization of IRE1, which activates endonuclease domains that can cleave XBP1 mRNA and generate an activated form of XBP1 protein, finally binding to ERSE in the nucleus. PERK is also autophosphorylated and dimerized by releasing Bip, and then blocks total translation by phosphorylation of eIF2-α. ATF6 consists of a *C*-terminal ER luminal stress-sensing domain and a cytosolic *N*-terminal basic leucine zipper (b-ZIP) domain, which is cleaved via S1P and S2P proteases translocated into the nucleus to bind to both ERSE and ATF6. We tested whether DFA stimulates ER stress signaling via IRE1, PERK, ATF6 and ER chaperones or not (Marciniak and Ron, 2006; Ron and Walter, 2007).

DFA (0.4 mM) time-dependently increased the mRNA levels of Bip, IRE1, and ATF6 (Fig. 1A), slightly increased the mRNA levels of PERK, but not of PDI (Fig. 1A). Other ER chaperones, including Bip, have higher ER chaperone activity than PDI has whose main function is enzyme activity of disulfide isomerase. In unstressed cells, Bip binds to the lumenal domains of ER membrane stress transducers and acts as an ER chaperone to help protein folding.

ER-inducible drugs such as tunicamycin, DTT, and the calcium ionophore A23187 activate IRE1 kinase activity and trigger the attached RNase activity to produce spliced XBP1 mRNA (Yoshida, 2007). To confirm the induction of IRE1 gene expression by DFA, XBP1 mRNA splicing (the proximal step of IRE1) was tested by an RT-PCR assay (Fig. 1B). DFA treatment stimulated the XBP1 mRNA-splicing activity. ER-inducible drugs enhance phosphorylation of eIF2α protein through PERK dimerization. The expression of both IRE1 and ATF6 increased after DFA treatment (Fig. 1A), but that of PERK did not. DFA also dose-dependently increased the expression and phosphorylation of eIF2α (phosphorylation is the proximal step of PERK activation) (Fig. 1C). AFT6 is constitutively expressed as a 90-kDa protein. ATF6 is cleaved at the cytosolic face of the membrane in response to ER stress, leading to nuclear translocation of the *N*-terminal cytoplasmic domain, which contains the DNA-binding, dimerization, and transactivation domains (Liu and Kaufman, 2003). There the *N*-terminus binds to both ERSE and ATF6 to enhance ER molecular chaperone genes. DFA also enhanced ATF6 protein expression (Fig. 1D).

In summary, we showed that DFA increased the expression of an ER chaperone and ER membrane stress transducers (IRE1, PERK, and ATF6) via typical UPR in PC12 cells. Thus, chemical hypoxia induced by DFA can trigger ER stress. Our findings provide new insight into the possible role of DFA in ER stress, and may help in developing novel drugs for ER stress-associated diseases.

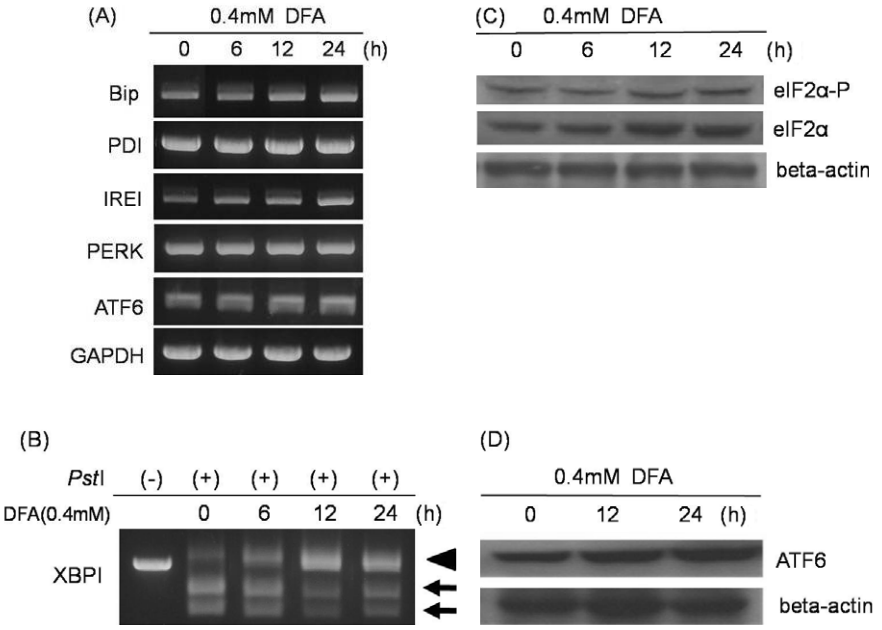

Fig. 1. Results of DFA treatment in the ER signal pathway. (A) Induction of ER chaperones and ER membrane stress transducer gene expression in PC12 cells. PC12 cells were treated with or without 0.4 mM DFA for indicated times. mRNA levels were measured by RT-PCR. (B) XBP1 cDNA after *Pst*I digestion. PC12 cells were treated with 0.4 mM DFA for indicated times and RT-PCR was performed. This fragment was further digested by *Pst*I to reveal a restriction site that is lost upon splicing of XBP1 by ER stress. The resulting XBP1 cDNA products were visualized on a 2% agarose gel. By *Pst*1 restriction digestion, unspliced XBP1 mRNA produces the two lower bands indicated by arrows (290 bp and 183 bp), whereas spliced XBP1 mRNA gives one 450 bp band (an arrow head). (C) Effects of DFA on phosphorylation of eIF2α protein. PC12 cells were cultured in 60-mm dishes until 80% confluence were incubated in the presence of 0.4 mM DFA for the indicated periods. Cells were washed with PBS, scraped with a rubber policeman, and lysed in 100 μl of SDS sample buffer. After boiling for 5 min, 5 μl aliquots of each sample were subjected to SDS-PAGE (10% gel) and analyzed by immunoblotting with anti-phosphorylated-eIF2α antibody (eIF2α-P) and anti-eIF2α antibody (eIF2α). (D) Immunoblotting analysis of ATF6. Cells were treated with 0.4 mM DFA for indicated times. Cell lysates were subjected to Western blotting with mouse anti-ATF6 monoclonal antibody. Although the experiments were performed in triplicate, only a representative blot is shown.

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De León M., Nahin R. L., Mendoza M. E., and Ruda M. A. (1994), SR13/PMP-22 expression in rat nervous system, in PC12 cells, and C6 glial cell lines. J. Neurosci. Res. **38**, 167–181.

Liu C. Y. and Kaufman R. J. (2003), The unfolded protein response. J. Cell Sci. **116**, 1861–1862.

Marciniak S. J. and Ron D. (2006), Endoplasmic reticulum stress signaling in disease. Physiol. Rev. **86**, 1133–1149.

Palmer C., Roberts R. L., and Bero C. (1994), Deferoxamine posttreatment reduces ischemic brain injury in neonatal rats. Stroke **25**, 1039–1045.

Ron D. and Walter P. (2007), Signal integration in the endoplasmic reticulum unfolded protein response. Nat. Rev. Mol. Cell Biol. **8**, 519–529.

Xin Q. i., Okuma Y., Hosoi T., and Nomura Y. (2004), Edaravone protects against hypoxia/ischemia-induced endoplasmic reticulum dysfunction. J. Pharmacol. Exp. Ther. **311**, 388–393.

Yoshida H. (2007), Unconventional splicing of XBP-1 mRNA in the unfolded protein response. Antioxid. Redox. Signal **9**, 2323–2333.