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Biochemical and Biophysical Research Communications 339 (2006) 463–468
BBRC
www.elsevier.com/locate/ybbrc

Doxorubicin prevents endoplasmic reticulum stress-induced apoptosis

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Received 2 November 2005
Available online 15 November 2005

Abstract

Several cellular stress signaling pathways initiate apoptosis in eukaryotic cells, but the interactions and coordination between the pathways have not been elucidated. In this study, apoptosis was triggered in MCF7 human breast carcinoma cells using doxorubicin, a topoisomerase inhibitor, and an endoplasmic reticulum (ER) stress inducer, thapsigargin, the latter causing the unfolded protein response (UPR). Interestingly, compared to treatment with doxorubicin or thapsigargin alone, cell death was reduced by treatment with both stress inducers. In contrast to another topoisomerase inhibitor, etoposide, doxorubicin markedly decreased apoptosis induced by thapsigargin; this doxorubicin effect was accompanied by reduced expression of the UPR-specific proapoptotic protein, C/EBP-homologous protein, and its upstream transcription factor, ATF4. We further found that doxorubicin downregulates the expression of ATF4 mRNA, indicating that doxorubicin interferes with the UPR at the level of ATF4 transcription. Taken together, the data suggest that ER stress-initiated cell death might be regulated by doxorubicin.

Keywords: Stress signaling; Doxorubicin; Thapsigargin; Apoptosis; Unfolded protein response

In order to maintain homeostasis, cells must sense and respond to stress, including viral infection, genetic mutations, chemical insult, and nutrient depletion. Specific response programs are activated to circumvent each type of stress. For instance, the unfolded protein response (UPR) is activated upon disruption of the endoplasmic reticulum (ER) environment by such events as a decrease in calcium concentration or a change in the oxidative environment, which result in the accumulation of unfolded or misfolded proteins in the ER [1]. The UPR is activated through ER stress sensors, namely the PKR-like ER kinase, PERK, the ER-bound transcription factor, ATF6, and the kinase/transcription factor, IRE1 [2].

In cases where cellular damage is so great that homeostasis cannot be recovered, survival of the organism is intimately linked to pathways that trigger apoptosis. As an adaptive mechanism, PERK phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α) to attenuate protein translation [3,4]. However, phosphorylation of eIF2α also promotes the translation of ATF4 [5], which upregulates the expression of the proapoptotic protein, CHOP (C/EBP-homologous protein-10, also known as GADD153) [4,6]. CHOP was originally identified based on its induction by DNA-damaging agents, especially methyl methanesulfonate (MMS) [7], but it was subsequently discovered that CHOP is most responsive to perturbations in the ER [8,9] and plays a critical role in facilitating ER stress-induced apoptosis by downregulating the antiapoptotic protein, Bcl2 [10,11].

Similar to the UPR, DNA damage induced by topoisomerase inhibitors such as doxorubicin and etoposide is sensed by enzymes, including ataxia telangiectasia mutated (ATM) and ataxia telangiectasia Rad3-related (ATR). Then, the kinases Chk1 and Chk2 activate the tumor suppressor gene, p53, to induce proapoptotic proteins, including Bax [12]. While both doxorubicin and etoposide treatments induce a common initial pathway to active p53, the downstream mechanism for the induction of apoptosis differs. Doxorubicin is a quinone-containing anthracycline that is converted to the corresponding

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.11.040

semiquinone by NADH dehydrogenase in mitochondria and subsequently generates superoxide and H_2O_2 [13,14]. These reactive oxygen species are considered to play a key role in apoptosis induced by doxorubicin [15–17]. In contrast, apoptosis triggered by etoposide does not involve reactive oxygen species, but etoposide elicits the FADD/TRADD death receptor-mediated pathway that activates caspase-8 [18].

In the present study, we have identified a functional interaction between doxorubicin-mediated signaling and the UPR. Moreover, our studies reveal for the first time that doxorubicin disrupts the UPR at the level of ATF4 transcription and suggest that doxorubicin-induced signaling might utilize this step to control ER stress-induced apoptosis.

Materials and methods

Cell culture conditions. MCF7 human breast carcinoma cells were maintained at 37 °C in a humidified incubator containing 5% CO_2, in RPMI supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin (JBI, Korea). DNA damage was induced using doxorubicin or etoposide (Sigma, St. Louis, MO), and ER stress was induced with thapsigargin (Calbiochem, San Diego, CA) for the indicated periods.

Western blotting. Cells were lysed in 100 μl (per well of a six-well plate) 1% SDS, 100 mM Tris, pH 8.0, and immediately heated at 100 °C. Proteins were resolved on 12% Tris/tricine gels and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) that were incubated with primary antibodies. Anti-Bip and anti-caspase-7 were purchased from BD (San Diego, CA), and anti-eIF2α and anti-phosphorylated-eIF2α (anti-p-eIF2α) were obtained from Cell Signaling (Beverly, MA). Anti-eIF2α detects both unphosphorylated and phosphorylated eIF2α. Anti-actin and anti-CHOP were purchased from Sigma, and anti-ATF4 and the secondary antibodies, anti-rabbit IgG-HRP and anti-mouse IgG-HRP, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Immunopositive bands were visualized using ECL solutions (Pierce, Rockford, IL).

DNA fragmentation assay. MCF7 cells were grown in six-well plates and were incubated with various stress inducers for different periods of time. Cells were harvested to assess DNA fragmentation using the Cell Death Detection ELISA (Roche, Mannheim, Germany) according to the manufacturer's recommendations. The cytosolic fraction (20,000*g* for 10 min) from approximately 1×10^4 cells was incubated in a microtiter plate precoated with a primary antibody against histones, after which an HRP-conjugated secondary antibody against DNA was added. The relative level of DNA fragmentation was compared with that of the control that did not receive stress inducers.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR. Total RNA was prepared using the RNeasy mini kit (Qiagen, Valencia, CA) from MCF7 cells treated with either doxorubicin or thapsigargin or both reagents. Reverse transcriptase (MBI, Germany) was used to generate cDNA from total RNA. The primers used to amplify ATF4 cDNA were forward: 5′-CCACTAGGTACCGCCAGAAG-3′ and reverse: 5′-GCCTTGCGGACCTCTTCTAT-3′. The primers used to amplify actin were forward: 5′-CTGGAGAAGAGCTACGAGCTGC-3′, and reverse: 5′-CTAGAAGCATTTGCGGTGGACG-3′. The amplified cDNA for ATF4 was 156 bp. Identical primer sets were used for both RT-PCR and real-time PCR. After RT-PCR, ATF4 and actin were separated by electrophoresis on a 1.2% agarose gel. Real-time PCR was carried out using an Exicycler Quantitative Thermal Block (Bioneer, Korea) and SYBR Green dye (Takara Bio, Japan) for detection. The PCR conditions were 95 °C for 30 s, followed by 45 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s, and finally 72 °C for 10 min.

Results and discussion

Doxorubicin reduces thapsigargin-induced apoptosis

In order to study the interaction between DNA damage-induced apoptosis signaling and the apoptosis induced by ER stress, MCF7 cells were treated with the ER stress inducer, thapsigargin; the DNA-damaging drug, doxorubicin; or both reagents, and apoptosis was estimated by the level of DNA fragmentation (ELISA; Fig. 1A, upper panel). In cells treated with 0.2 μM thapsigargin, DNA fragmentation was more extensive after the 24-h treatment compared with the 16-h treatment. Cells treated with 5 μM doxorubicin showed DNA fragmentation at both 16 and 24 h. Interestingly, cells treated with both 0.2 μM thapsigargin and 5 μM doxorubicin had a reduced level of DNA fragmentation (and thus apoptosis) compared with treatment using either individual reagent.

To substantiate our findings, after treating cells with the same concentrations of reagents described above, cells were harvested and analyzed by Western blotting to determine the level of one of the executioner caspases, caspase-7 (Fig. 1A, lower panel). In the presence of thapsigargin, the cleavage of caspase-7 to the ~20 kDa activated form was negligible after a 16-h incubation, but was enhanced at the 24-h time point. Treatment with doxorubicin for either 16 or 24 h also induced caspase-7 cleavage. However, treatment with both thapsigargin and doxorubicin led to a decline in caspase-7 cleavage, especially at the 24-h time point. In agreement with the results in Fig. 1A (upper panel), these data indicate that apoptosis decreases in cells treated with both thapsigargin and doxorubicin when compared with cells treated with only one of these reagents.

We next examined DNA fragmentation in MCF7 cells treated with varying concentrations of thapsigargin (0.1, 0.5, and 1 μM) in the absence or presence of 5 μM doxorubicin. Compared with cells treated with either doxorubicin or thapsigargin, cells treated with both reagents showed a minimal level of DNA fragmentation (apoptosis), similar to that measured in the non-treated control (Fig. 1B, upper panel). In this experiment, it appears that doxorubicin-induced apoptosis is reduced by treatment with thapsigargin. However, an even greater reduction of thapsigargin-induced apoptosis by treatment with doxorubicin was observed at all three concentrations tested. Western blotting using anti-caspase-7 clearly confirmed this observation, in that at each concentration of thapsigargin, doxorubicin treatment substantially reduced the level of thapsigargin-induced activation of caspase-7.

In Fig. 1B, activation of caspase-7 as well as DNA fragmentation were not enhanced with increasing thapsigargin concentration, indicating that 0.1 μM thapsigargin is sufficient to cause the maximal level of DNA fragmentation and caspase-7 activation in MCF7 cells. Thus, we next used reduced concentrations of thapsigargin to verify the effect