Letter

HSP70-Ran-RCC1 transport system during cadmium-induced apoptosis in porcine kidney LLC-PK_1 cells

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ABSTRACT — Cadmium alters the temporal dynamics of various cellular proteins, including heat shock proteins and protooncogenes. To reveal the molecular dynamics during cadmium cytotoxicity, we investigated the conventional nucleocytoplasmic transport pathways. Western blot analyses revealed that exposure of porcine kidney LLC-PK_1 cells to cadmium (10 μM) temporally induced HSP 70 and Ran in the cytoplasm and Ran and RCC1 in the nucleus. RCC1 could be detected in the nucleus as early as 1 hr after exposure of the cells to the metal. Thus, HSP70-Ran-RCC1 transport system appears to be involved in early phase cadmium cytotoxicity.

Key words: Cadmium, HSP70 chaperone system, LLC-PK_1 cells

INTRODUCTION

Exposure of cells to cadmium evokes a number of responses that involve not only death-signaling reactions, but also reactions that protect the cells against toxicity (Ishido and Kunimoto, 2001). Accordingly, it has proven difficult to identify the pathways activated by the metal during cell death. Glutathione is the first line of defense against cadmium cytotoxicity (Singhal *et al.,* 1987). Intracellular cadmium ions, which escape the sulfhydryl reaction with glutathione, activate numerous cellular responses, including the induction of proto-oncogenes (Jin and Ringertz, 1990), heat shock proteins (Levinson *et al.,* 1980), and the Bcl-2 family (Ishido *et al*., 2002). Ultimately, free cadmium ions are sequestered by a chemical reaction with metallothionein, which is inducible at a later phase of cadmium exposure (Ishido *et al*., 2002). Cell fate depends on which reaction dominates.

Protooncogenes such as c-myc might not be involved in cadmium-induced apoptosis of porcine kidney LLC-PK_1 cells (Ishido *et al.*, 1998), and it has been reported that HSP70 would protect against cadmium cytotoxicity (Levinson *et al.,* 1980; Mahmood *et al*., 2014). Here, we investigated the effect of cadmium on the HSP70-Ran-RCC1 transport system in porcine kidney LLC-PK_1 cells.

MATERIALS AND METHODS

Materials

Porcine renal LLC-PK_1 cells were obtained from the American Type Culture Collection (CRL1392; Rockville, MD, USA). Cell culture media was from Sigma Chemical Corp. (St. Louis, MO, USA). Antibodies for HSP70, Ran, and RCC1 were from Transduction Laboratories (Lexington, KY, USA). The Enhanced Chemiluminescence (ECL) Western Blotting Detection Kit was from Amersham Pharmacia Biotech (Uppsala, Sweden). Protease inhibitors were from Peptide Institute Inc. (Osaka, Japan).

Cell culture

LLC-PK_1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The cells were subcultured (1:4) 2 to 3 times per week.

Cell lysates and Western Blot Analysis

Cells were collected by centrifugation at 600 x *g* for 5 min and homogenized in 20 mM Hepes, pH 7.8, containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and protease inhibitors (0.5 mM phenylmethyl- sulfonyl fluoride (Wako Co., Osaka, Japan), 5 μg/mL pepstatin

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(Peptide Institute Inc., Osaka, Japan), and 5 µg/mL leupeptin (Peptide Institute Inc.). The homogenates were kept on ice for 10 min and used as total cell lysates. For cell fractionation, cytoplasmic and nuclear factions were prepared as follows. Total cell lysates were centrifuged at 2,000 x *g* for 10 min and the supernatants were used as cytoplasmic fractions. The pellets were resuspended in 20 mM Hepes, pH 7.8, containing 0.42 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM dithiothreitol, 25% glycerol, and the above-mentioned protease inhibitors at 4°C for 30 min. Then, the samples were centrifuged at 25,000 x *g* for 20 min at 4°C and the clear supernatants were used as nuclear fractions. Protein concentrations were measured with a bicinchoninic acid kit (Pierce Chemical Co., Rockford, IL, USA) using bovine serum albumin as a standard. Proteins (20 µg) were subjected to 7.5-15% polyacrylamide gels containing 0.1% SDS under reducing conditions. Proteins in an SDS gel were electrophoretically transferred at 2 mA/cm^2 for 20 min onto Immobilon membranes (Millipore Co., Osaka, Japan) in 25 mM Tris, 192 mM glycine, and 20% methanol with an Atto semidry horizontal electrophoretic transfer unit (Atto, Tokyo, Japan). The membrane was blocked with 8% casein in PBS containing 0.1% Tween 20 at room temperature for 3 hr. The transferred membrane was then incubated with antibodies against HSP70, Ran, and RCC1 at a concentration of a 1-2 µg/mL in PBS containing 0.1% Tween 20 and 5% bovine serum albumin overnight at 4°C. After incubation with primary antibodies, the sheets were washed three times for 5 min each with PBS containing 0.1% Tween 20, and the antibodies were detected with horseradish peroxidase-conjugated secondary IgG using an ECL Western Blotting Detection Kit according to the manufacturer's instructions (Amersham Pharmacia Biotech). Gels were calibrated with prestained molecular markers (Bio-Rad, Hercules, CA, USA).

RESULTS AND DISCUSSION

We previously demonstrated that DNA fragmentation, a biochemical characteristic of apoptosis, was clearly detectable with ethidium bromide staining of agarose gel, as early as 7 hr after exposure of $LLC\text{-}PK_1$ cells to 10 µM cadmium (Ishido *et al*., 1995).

To examine the effects of cadmium on the HSP70-Ran-RCC1 transport system 10 µM cadmium was added to cultured $LLC\text{-}PK_1$ cells for 0, 1, 3, 7, or 24 hr; the treated cells were harvested and homogenized. The total cell lysates were further fractionated into the nuclear and cytoplasmic fractions. Twenty micrograms of proteins/

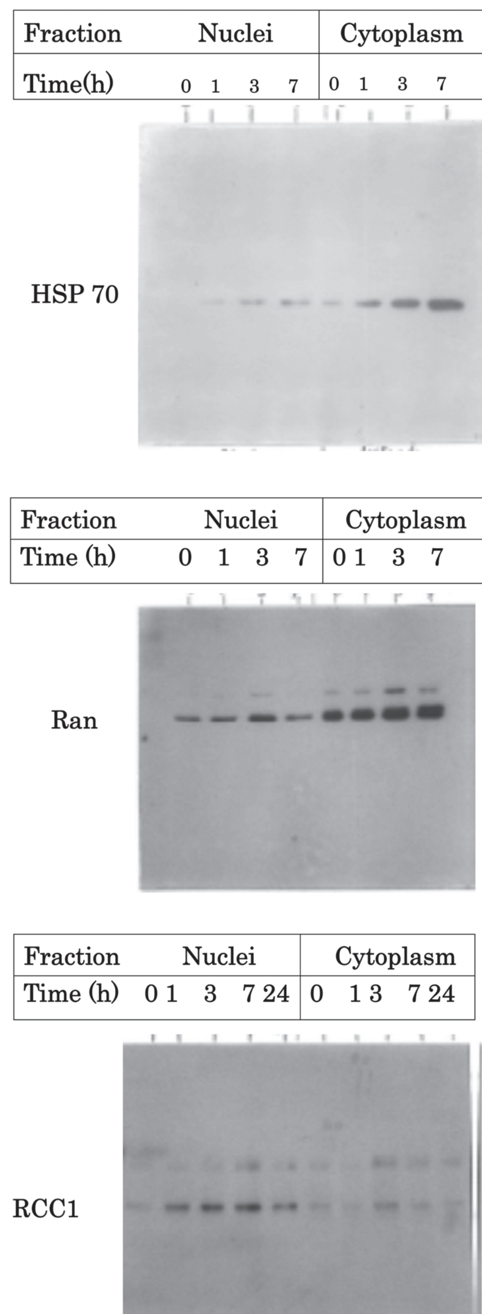

Fig. 1. Western blot analysis of HSP70, Ran, and RCC1 induction by cadmium. Porcine kidney $LLC\text{-}PK_1$ cells were incubated with 10 µM cadmium for the indicated periods. The treated cells were then harvested, homogenized and fractionated, as described in the MATERIALS AND METHODS section. Twenty micrograms of proteins/fraction was subjected to western blot analyses, using anti-HSP70 antibody (*top*), anti-Ran antibody (*middle*), and anti-RCC1 antibody (*bottom*), followed by ECL western blot analyses.

fraction was analyzed by western blotting for HSP70, Ran and RCC1 proteins. Figure 1 shows the time course of the induction of the HSP70-Ran-RCC1 transport system in both the nucleus and cytoplasmic fractions. Prior to exposure to the metal, HSP70 was mainly found in the cytoplasm and RCC1 in the nucleus; Ran was found in both the cytoplasm and nucleus. Exposure of porcine kidney LLC-PK_1 cells to cadmium (10 μM) temporally induced HSP 70 and Ran in the cytoplasm and Ran and RCC1 in the nucleus. RCC1 was detectable in the nucleus as early as 1 hr after exposure of cells to the metal.

A variety of cellular stresses induce the nucleocytoplasmic redistribution of various functional proteins and perturbation of conventional nucleocytoplasmic transport pathways (Kose and Imamoto, 2014). HSP70 plays a protective role in cadmium cytotoxicity (Levinson *et al*., 1980; Mahmood *et al*., 2014). In this study, HSP70-Ran-RCC1 transport system was induced by cadmium exposure. The driving force of this transport system is the concentration gradient of Ran across the nuclear envelope (Blackinton and Keene, 2014), whose activation is initiated by induction of cytoplasmic HSP70. One of the roles of this transport system is to facilitate DNA repair; if the cellular damage is irreparable, apoptosis occurs. Therefore, further study is needed to reveal the role of this transport system in cadmium-induced apoptosis.

Conflict of interest---- The authors declare that there is no conflict of interest.

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