

The Endoplasmic Reticulum Chaperone GRP94 Is Induced in the Thyrocytes by Cadmium

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We established a relationship between the toxic effects of cadmium on the expression of the endoplasmic reticulum (ER) chaperone GRP94 (glucose regulated protein 94) and cell survival in cultured rat-thyrocytes of FRTL5 cells. There are no data reporting that the enhanced expression of GRP94 by Cd stimulation is detectable in thyrocytes. Western blot analysis revealed higher levels of GRP94 expression in those cells post-treated with low concentrations of Cd, following a step-down treatment method, than in Cd pre-treated cells or cells not treated with any Cd, due to changes in cellular sensitivity after pre-treatment with Cd and the possible induction of GRP94 expression after removal of a low concentration of Cd. Elevated GRP94 expression in thyrocytes post-treated with Cd confers a survival advantage by rendering them resistant to cytotoxic stress, and the existence in the thyrocytes of a Cd-specific pathway regulates the expression of stress proteins by Cd.

Introduction

Cadmium (Cd) is a poisonous heavy metal responsible for acute and chronic toxicities. In animals, including humans, it can cause damage to a variety of organs such as the liver, kidneys and testes (Kwon *et al.*, 1997; Morselt, 1991). Various aspects of Cd cytotoxicity have already been described in several comprehensive studies (Goering and Fisher, 1995; Piscator, 1988; Waalkes *et al.*, 1992). One of the main toxic effects of Cd on cells is the induction of abnormal and denatured proteins by its reaction with thiol groups or its substitution of zinc, causing the accumulation of dysfunctional proteins in the cells (Vallee and Ulmer, 1972). It is known that the generation of abnormal proteins is recognized as a stress signal and induces cellular stress responses, which result in enhanced expression of stress proteins to protect intracellular proteins and organelles against various stimuli (Lindquist, 1986; Nover, 1991; Parsell and Lindquist, 1994). In general and under normal conditions, stress proteins have roles as intracellular housekeepers or molecular chaperones, and are

synthesized in response to various physiochemical or environmental stimuli to maintain cell homeostasis (Harboe and Quayle, 1991; Lindquist, 1986; Morimoto 1993). However, it has been unclear how this stress response is related to cell protection or survival. It is also unknown whether the endoplasmic reticulum (ER) chaperones are expressed from or protect cells when abnormal proteins are synthesized and accumulated due to the presence of Cd. We examined whether a GRP94 could be overexpressed in the thyrocytes for resistance or adaptation against Cd, since some biochemical studies have demonstrated that GRP94 associates with a variety of cellular proteins including tyrosine kinases, actin, tubulin as well as steroid hormones or dioxin receptors depending on xenobiotic (Barque *et al.*, 1996). In the field of thyroidology, the expression of hsp72 in thyrocytes from patients with Grave's disease and Hashimoto's thyroiditis (Heufelder *et al.*, 1992) and its continuous expression in cultured thyrocytes have been reported (Misaki *et al.*, 1994). Our previous results have demonstrated that several types of ER-resident stress proteins, GRP94, Bip, ERp72,

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calreticulin, ER60 and PDI, were highly expressed under conditions of misfolded protein accumulation in the ER, in which the expression of GRP94 was about four-fold higher than in normal cells and it actually recognized misfolded protein (Kim *et al.*, 1996). Here, using the thyroid cell line FRTL5 from well established rat thyrocytes (Kohn and Valente, 1989), we examined the expression of the ER resident chaperone, GRP94, using Cd exposure to understand its role in encountering a heavy metal stressor. When the cells were immediately incubated at the concentrations of 0.1 μM Cd at 37 °C for a further hour after removal from the medium containing 30 μM Cd, GRP94 expression is higher than its in the control cells and 1.7 times greater than normal cell colony formation.

Materials and Methods

Cell culture and cadmium treatments

FRTL5 cells [American Type Culture Collection, Rockville, USA, (CRL #8305)] were cultured in Coon's medium containing 5% calf serum and 4 hormones mixture [bovine TSH (1^{-10} milliunits/ml), bovine insulin (1 $\mu\text{g}/\text{ml}$), hydrocortisone (10 nm/ml), and human transferrin (5 $\mu\text{g}/\text{ml}$)] under 5% CO_2 at 37 °C. All cell culture reagents were obtained from Sigma Chemicals, St. Louis, USA. Confluent monolayer FRTL5 cells were exposed to concentrations of 0, 10, 30, 50 and 100 μM Cd for 1 h. After washing three times with cold PBS (phosphate-buffered saline, pH 7.4) with 10% $\text{Ca}^{2+}/\text{Mg}^{2+}$, cells were trypsinized with 0.05% trypsin and 0.02% EDTA in PBS. Some of the single cells were transferred onto a new culture plate and reincubated in a Cd-free medium for 24 h at 37 °C to allow the growth and proliferation of the cells. Cell colonies were rinsed twice with PBS, fixed in 70% ethanol and stained using the Giemsa method (Bancroft and Cook, 1984) and then counted under a microscope. Results shown are the averages of duplicate samples of each and the experiment was repeated five times. The next set of experiments for the post-treatment effects of cadmium were designed such that cells were cultured under normal culture conditions, as outlined above, at 30 μM Cd for 1 h and then sequentially post-treated with low concentrations of 0.05, 0.1 and 0.5 μM Cd for 1 h.

Immunoblotting of GRP94 expression

The cells treated with Cd were washed three times in ice-cold PBS and then lysed in 0.5 ml of a solution containing 0.1 M NaCl, 25 mM Tris (hydroxymethyl) aminomethane-HCl, pH 6.8, 5 mM EDTA, 0.1% Triton X-100 and a cocktail of protease inhibitors (0.1 mM leupeptin, 10 mM pepstatin, 1 mM diisopropylfluorophosphate, 1 mU/ml aprotinin). After the removal of cell debris by centrifugation at 8,000 $\times g$ for 10 min at 4 °C, soluble proteins were fractionated by denatured discontinuous 4–8% SDS-PAGE and the proteins were transferred to nitrocellulose membranes. Blots were blocked in TBST [25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% (v/v) Tween 20] containing 5% (w/v) nonfat milk, washed several times with TBST, and incubated with the GRP94 antibody (1:1000) (StressGen, Victoria, Canada) at room temperature for 1 h. Blots were then washed three times in TBST and incubated with HRP-conjugated secondary antibody (1:1000) for 2 h. Signals recognized by the antibody were exposed and developed on the X-ray film.

Results and Discussion

The results of the cell survival test against various concentrations of Cd are shown graphically in Fig. 1A. Confluent FRTL5 cells on the culture plate were washed three times with cold PBS either by agitating in a box or by hand. Different concentrations of Cd (10, 30, 50 and 100 μM) were added to the medium and the cells were incubated at 37 °C for 1 h. The resulting cells were split and transferred to a new culture plate and cultured under normal culture conditions for 24 h and counted using the Giemsa staining method (Bancroft and Cook, 1984). No significant decrease in cell survival rate was observed at Cd concentrations up to 30 μM . However, cell death markedly increased when cells were exposed to Cd concentrations in excess of 30 μM . At 100 μM concentration, most cells died and no surviving cells were observed at 0.5 mM Cd concentration (data not shown). Our results also show that cell division is slightly higher at low Cd concentrations than in the control cells at 30 μM Cd. One possible reason for this may be that although no effects were observed at low Cd concentrations on the expression of GRP94 (as shown by the results of Western blot

Fig. 1A

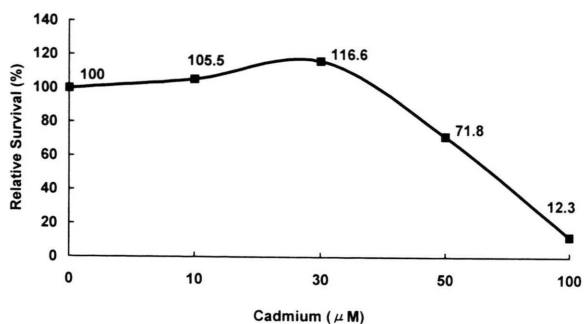


Fig. 1B

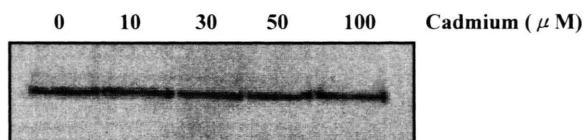


Fig. 1. Effect of cadmium on the proliferation of FRTL5 cells.

(A) Confluent monolayer FRTL5 cells were incubated with 0, 10, 30, 50 and 100 μM of cadmium for 1 h. The number of cadmium-treated cells is given as a percentage against the number of without cadmium treated cells is 100%. The experiments above were performed with duplicate samples; in particular the colony-forming results were the averages of five repeated experiments. (B) Equal amounts of protein from each sample was separated by the denatured discontinuous 4–8% SDS-PAGE and GRP94 on the nitrocellulose membrane was recognized by anti-GRP94 1st-antibody derived from a rabbit and HRP-conjugated 2nd-antibody for the Western blotting.

analysis in Fig. 1B), the activation of intracellular stressors by relatively low concentrations of Cd stimulated cell division somewhat thus enhancing the colony-forming ability. Similar phenomena have already been reported in the other cell lines – low concentrations of cytotoxic matter induce cell division, while excess concentrations lead to cell death.

Previous studies have shown that a stress protein was induced when cells were exposed to a single heavy metal treatment or to heat shock. However, in this study, using FRTL5 cells, no induced stress protein, such as GRP94, was detected after a single treatment of Cd (Fig. 1A) although enhanced colony formation was observed (Fig. 1B). Two aspects of the Cd-induced stress response have received little attention so far. However, with regard to the enhanced induction of GRP94, this study tested: (1) the change in cellular sensitivity after exposure to Cd and (2) the possible induction of GRP94 expression after the removal of a low concentration of same stressor (a step-down treatment), i.e. Cd.

The following experiment estimates whether the Cd-pretreated cells can induce GRP94 following the subsequent application of low concentrations of Cd. The concentration of 30 μM Cd for 1 h is considered to be the critical point for cell division and proliferation based on the result of the cell survival test (Fig. 1A). Confluent FRTL5 cells were washed three times with cold PBS and incu-

bated with 30 μM Cd at 37 °C for 1 h. The cells were then immediately incubated at lower concentrations of 0.05, 0.1, 0.5 and 60 μM Cd at 37 °C for a further hour after removal from the medium containing 30 μM Cd. The resulting cells were transferred onto a new culture plate, reincubated under normal culture conditions for 24 h and counted using the Giemsa staining method.

As shown in Fig. 2A, a smaller increase in colony formation was observed at 0.05 μM Cd concentration, while the largest increase in colony size was observed at 0.1 μM Cd, which is about 1.7 times greater than normal cell colony formation. At concentrations higher than 0.1 μM , colony formation rapidly decreased and all cells died at 60 μM Cd. However, non-Cd-pretreated cells did not appear to show enhanced cell division following the subsequent application of low concentrations of Cd (dotted line in Fig. 2A). Enhanced colony forming efficiency by the post-treatment of cells with low doses of Cd has already been reported in other cell lines (Ovelgonne *et al.*, 1995a; Ovelgonne *et al.*, 1995b). The explanation for this phenomenon may be that the application of low concentrations of post-treatment Cd over a relatively short time stimulates the already slightly enhanced intracellular stressor, GRP94, which when expressed increases cell proliferation (Laszlo and Bissell, 1983; Li, 1983). Recently some researchers have demonstrated that GRP94 is one of the factors involved in cell differentiation and prolifer-

Fig. 2A

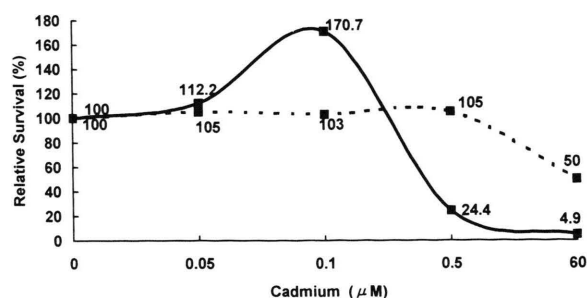


Fig. 2B

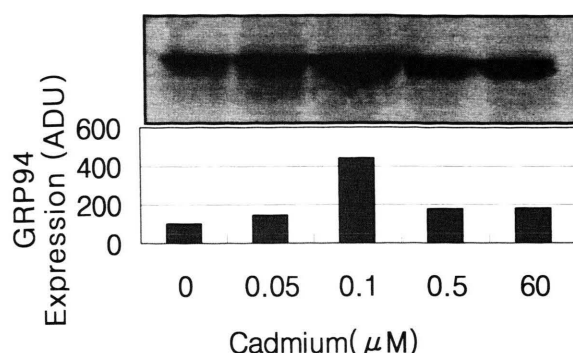


Fig. 2. The subsequent exposure of low concentrations of cadmium.

(A) Confluent monolayer FRTL5 cells were pre-treated with both 0 and 30 μM Cd for 1 h, the resulting cells were then post-treated with 0.05, 0.1, 0.5, and 60 μM Cd, (indicated by a continuous line) and compared with non-pretreated cells (indicated by a dotted line). The number of Cd-treated cells is given as a percentage against the number of without cadmium treated cells is 100%. The experiments above were performed with duplicate samples and the results of colony-forming experiments were the averages of five repeated experiments. (B) Equal amounts of protein from each sample was subjected to the denatured discontinuous 4–8% SDS-PAGE. Western blotting was performed under the same conditions as in Fig. 1B. The quantitation of band intensities of GRP94 was measured by the ImageQuant software package and indicated in arbitrary densitometry units (ADU).

ation in other cell lines (Ananthan *et al.*, 1986; Sato and Torigoe, 1998). The results of Western blotting of a Cd step-down treatment are presented in Fig. 2B. Post-treated cells with low concentrations of Cd show changes in their expression of GRP94 in contrast to non-pretreated cells. At 0.1 μM Cd concentration, GRP94 is higher than its expression in the control cells, non-pretreated cells and other cells exposed to different concentrations of Cd. Quantification of the Western blotting bands using the ImageQuant software package showed that the expression of GRP94 in the 0.1 μM Cd is about 4.4-fold higher than its expression from normal cells pretreated but not post-treated with Cd (Fig. 2B). Stimulation of GRP94 synthesis is considered to be due to the stress-induced accumulation of abnormal or denatured proteins in the ER by Cd. In this paper we present evidence that pre-treatments with Cd induce a bi-phasic change in sensitivity; an initial sensitization is followed by the development of a tolerance towards a second application of Cd. During this phase of increased

sensitivity, the induction of the stress response can be further stimulated by low doses of the initial stressor, which under control conditions are without any effect. We can conclude from our above-mentioned data that the degree of modulation of the response depends on the post-treatment conditions, which provide cytoprotection from Cd up to a reasonable Cd concentration. This may represent an important cellular defence mechanism in the thyrocytes against environmental heavy metal pollution. In addition the finding that a rapid increase in cellular sensitivity due to low concentrations of post-treatment Cd, seems to represent a general principle since this phenomena has been observed in the case of heat shock and arsenite post-treatment (Delpino *et al.*, 1992; Ovelgonne *et al.*, 1995b).

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