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SHORT COMMUNICATION

Inhibition of Calcium Accumulation by the Sarcoplasmic Reticulum: A Putative Mechanism for the Cardiotoxicity of Adriamycin

Irit Halili-Rutman, Chaim Hershko,† Gavriela Link,‡ Avram J. Rutman* and Asher Shainberg*§*

*The Otto Meyerhoff Drug Receptor Center, Department of Life Sciences, Bar-Ilan University, Ramat Gan 52900 Israel; †Department of Medicine and ‡Department of Human Nutrition and Metabolism, Shaare Zedek Medical Center, Jerusalem, Israel

ABSTRACT. The aim of this study was to examine the effect of adriamycin (ADR) on calcium accumulation by the sarcoplasmic reticulum (SR). Chemical skinning of cultured rat myocardial cells compromised the barrier function of the cell membrane and thus permitted direct exposure of mitochondrial and non-mitochondrial sites to ADR. In the presence of ATP, and sodium azide, mitochondrial calcium accumulation was negligible. Furthermore, it has previously been shown that non-mitochondrial calcium accumulation is mediated mainly by the SR under these conditions. Incubation with 10 μM ADR for 2 hr reduced the level of calcium accumulation by the SR by 50%. A similar effect was obtained after 24 hr incubation with 1 μM ADR. The addition of ferric iron to the culture medium further reduced the level of calcium accumulation. Neither vitamin E nor β-carotene affected calcium accumulation by the SR. These results suggest that ADR interferes with the calcium accumulation activity of the SR and that ferric iron potentiates this effect. BIOCHEM PHARMACOL **54**;1:211–214, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. adriamycin; cardiotoxicity; sarcoplasmic reticulum; calcium accumulation; iron

Adriamycin (ADR) is a potent chemotherapeutic agent with a broad spectrum of activity against a variety of human malignancies [1]. Unfortunately, the therapeutic effects of ADR are accompanied by acute and chronic cardiotoxicity, thus limiting its usefulness. ADR-induced cardiotoxicity is characterized by impairment of intracellular calcium regulation in myocytes [2], and by dilation of the specific regions of sarcoplasmic reticulum (SR) [3]. This latter phenomenon appears to coinicide with decreased diastolic function [4].

The mechanism of ADR-induced cardiotoxicity is still unclear, although free radicals are believed to play an important pathogenic role [5]. According to this hypothesis, ADR generates free radicals which cause oxidation of cellular lipids and proteins, thus compromising cardiomyocyte structure and function. It has been further suggested that the ADR-induced generation of free radicals is mediated by both enzymatic and nonenzymatic pathways [4] and that ferric iron plays an important role in the latter route. Several different free radical scavengers, antioxidants and iron chelators have been used in order to prevent or reduce ADR-cardiotoxicity. In a study performed on mice, it was reported that a single injection of vitamin E given 24 hr prior to ADR administration prevented cardiotoxicity [6]. In other studies performed in cultured cardiac cells [7] and in heart microsomes [8], it was found that ADR-induced cardiotoxicity was inhibited by deferoxamine [7 and by di-*N,N'*-Dicarboxamidomethyl-*N,N'*-dicarboxymethyl-1,2-diaminopropane (ICRF-198) and *d*-1,2-Bis (3,5-dioxopiperazine-1-yl) propane (ICRF-187) [8].

The aim of this study was to examine the effect of ADR on the calcium accumulation activity of the SR of rat myocardiocytes using "skinned" cultured cells. The mechanisms underlying the effect of ADR on calcium accumulation were investigated by pretreating the cells with ferric iron or antioxidants prior to ADR administration.

MATERIALS AND METHODS

Chemical

ADR was purchased from Farmitalia Carloerba (Milano, Italy). Vitamin E, β-carotene and ferric ammonium citrate were purchased from Sigma (Missouri, U.S.A).

Preparation of Rat Heart Cell Cultures

The hearts of 1–2 day old rats were removed under sterile conditions and washed three times in PBS to remove excess blood cells. The hearts were minced into small fragments

§Corresponding author: Prof. Asher Shainberg, Department of Life Sciences, Bar-Ilan University, Ramat Gan 52900, Israel, Tel: 972-3-5318265; FAX 972-3-5351824, E-mail: shaina@ashur.cc.biu.ac.il.

Abbreviations: ADR, adriamycin; SR, sarcoplasmic reticulum.

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and gently agitated in a solution containing the protease mixture, RDB (Ness-Ziona, Israel), as described previously [9]. Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY, U.S.A.), containing 10% horse serum (Biolab, Jerusalem, Israel), was added to the supernatant suspensions containing dissociated cells, which were then centrifuged at 500 *g* for 5 min. The cell pellet was resuspended in high glucose (5 mg/mL) DMEM supplemented with 10% heat-inactivated horse serum and 2% chick embryo extract. The cell suspension was diluted to 10^6 cells/ml and plated on 35 mm collagen-gelatin-coated plastic culture dishes (1.5 mL per dish). Cultures were incubated in a humidified 5% CO_2 and 95% air atmosphere at 37°C. Twenty-four hr after plating, the incubated cells were washed extensively, in order to remove unattached cells. Confluent monolayers exhibiting spontaneous contractions developed in culture within 2 days. The growth medium was replaced every 3–4 days.

Skinning Procedure

A modification of the skinning method described by Weinstein *et al.* [10], in which cultured cells were incubated in 1 mL of skinning solution (140 mM KCl, 20 mM HEPES, 10 mM EGTA, 50 μg/mL saponin, 5 mM NaN_3, 5 mM potassium oxalate, 1 μM ruthenium red, pH 7.4) at 25°C for 30 min, was used.

Assay of SR-Mediated Ca^{2+} Accumulation

Accumulation of Ca^{2+} by the SR was measured as previously described [10]. The skinned heart cells attached to the dish were washed twice with Ca^{2+}-uptake solution containing 140 mM KCl, 20 mM HEPES, 0.5 mM EGTA, 0.5 mM $CaCl_2$, 5 mM NaN_3, 5 mM potassium oxalate, 5 mM $MgCl_2$, pH 7.0 at 37°C. One ml of radioactive uptake solution (^{45}Ca 0.2 μCi/mL) was then added to the cells which were incubated at 37°C for 10 min, either in the presence or absence of 5 mM ATP. The uptake solution was removed, and the cells were rinsed five times with ice-cold fresh uptake solution. Cells were then lysed with 0.3 mL of 1% Triton X-100 and collected in counting vials containing 4 ml scintillation liquid (Hydrofluor, National Diagnostics). Radioactivity was determined using a scintillation counter (Kontron). Values for specific calcium uptake were derived by substraction of dpm counts obtained from calcium accumulation experiments performed in the absence of ATP. These values were converted to units of nmole calcium/min/mg protein by calibration with uptake solutions containing known amounts of calcium.

Protein Content

Protein determination was performed according to the method of Lowry *et al.* [11], using bovine serum albumin as the standard.

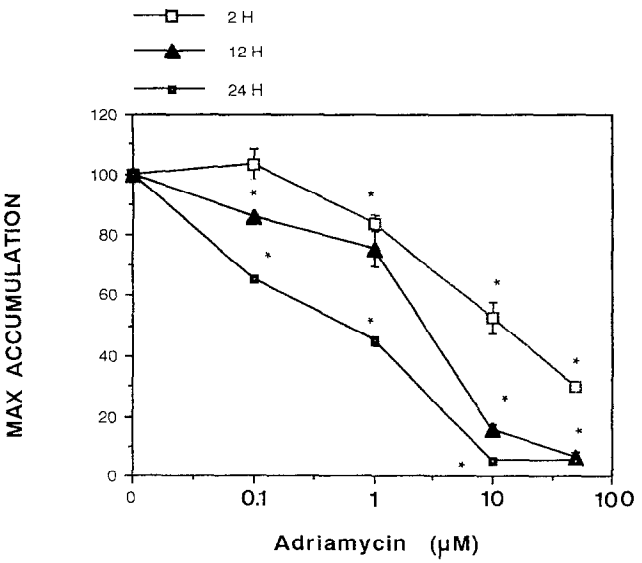

FIG. 1. Effect of ADR on calcium accumulation by the SR. 6-Day-old cultured cardiomyocytes were incubated for 2, 12 and 24 hr with the indicated concentrations of ADR. The cells were then skinned and calcium accumulation was measured. The data shown are the means of triplicate experiments performed on three separate occasions (±SD). *$P < 0.01$ compared with control; df = 16.

Data Analysis

Each experiment was performed on triplicate culture dishes, on three seperate occasions. Results are presented as mean ± standard deviation (SD). The significance of differences between the various treatments and the control was analyzed using Student's *t*-test. A probability level of $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of ADR on Calcium Accumulation by the SR

Cardiomyocytes were incubated for 2, 12 and 24 hr with different concentrations of ADR. After incubation, the cells were skinned, and calcium accumulation was measured both in the presence and the absence of ATP as described above. It was found that ADR inhibited calcium accumulation by the SR. The log dose response curve of this effect is shown in Fig. 1. The IC_{50} (dose causing half maximal inhibition) for the 2 hr experiment was calculated to be 10 μM, while the corresponding values for 12 hr and 24 hr were 3 μM and 1 μM, respectively. All treatments gave results which were significantly different from control except for 0.1 μM ADR at 2 hr. ADR did not affect the level of calcium uptake in the absence of ATP (data not shown).

Effect of Iron on Calcium Accumulation

In order to further elucidate the mechanisms of action of ADR on heart cells, the effect of ferric iron was investigated. As ferric iron is assumed to play an important role in

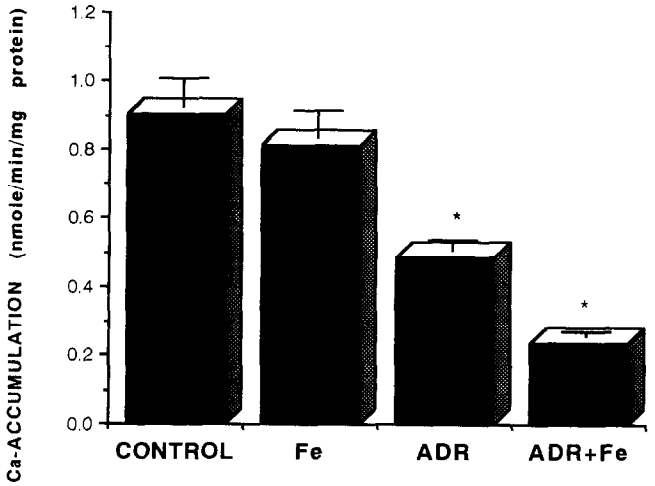

FIG. 2. Effect of iron loading on calcium accumulation by the SR of ADR-treated cardiomyocytes. 6-Day-old cultured cardiomyocytes were incubated with 0.25 mM ferric ammonium citrate (Fe) for 24 hr. ADR (10 μM) was added to the growth medium and the cells were incubated for 2 hr. After incubation, the cells were skinned and calcium accumulation was measured. The data shown are the means of triplicate experiments performed on three separate occasions (±SD). *$P < 0.01$ compared with control; df = 16.

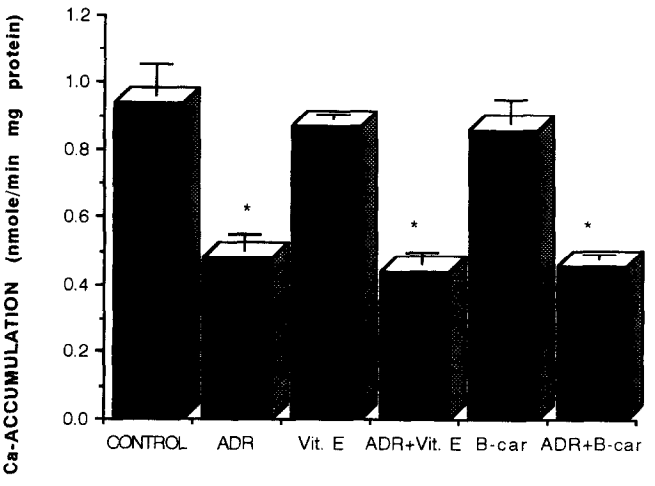

FIG. 3. Effect of vitamin E or β-carotene on calcium accumulation by the SR of ADR-treated cardiomyocytes. 6-Day-old cultured cardiomyocytes were incubated with 40 μM Vitamin E (Vit E) or 15 μM β-carotene (B-car) for 1 hr. ADR (10 μM) was then added to the growth medium and the incubation continued for a further 2 hr. After incubation, the cells were skinned and calcium accumulation was measured. The data shown are the means of triplicate experiments performed on three separate occasions (±SD). *$P < 0.01$ compared with control; df = 16.

the generation of free radicals by ADR [4], it was anticipated that ferric iron loading would potentiate the effect of ADR on calcium accumulation. Cells were incubated with ferric ammonium citrate 0.25 mM for 24 hr. ADR (10 μM, final concentration) was added to the growth medium and incubation was continued for a further 2 hr. After incubation, the cells were skinned, and calcium accumulation was measured both in the presence and absence of ATP. In the absence of ADR, ferric iron treatment had no significant effect on calcium accumulation. In cells treated with both ferric iron and ADR, accumulation was approximately 50% of that in cells treated with ADR alone (Fig. 2). Calcium uptake in the absence of ATP was not affected by ferric iron, either in the presence and absence of ADR (data not shown).

Effect of Antioxidants on Calcium Accumulation

In order to study the effect of antioxidants on ADR-induced inhibition of calcium accumulation by the SR, the cells were incubated with either vitamin E (40 μM) or β-carotene (15 μM) for 1 hr prior to ADR treatment. The cells were then skinned, and calcium accumulation was measured both in the presence and absence of ATP, as described above. Addition of antioxidants did not affect either the amount of specific calcium accumulation by the SR (Fig. 3) or the uptake of calcium in the absence of ATP (data not shown).

DISCUSSION

Several studies suggest an important role for the SR in the development of ADR cardiotoxicity. In a study performed on mice, microscopic SR lesions were reported 14 hr after a single injection of ADR [4]. In a different study using cardiac SR membranes fused with planar bilayers, it was shown that ADR increases the probability of single channel opening, and induces abnormal function of the SR [3]. The aim of the present study was to investigate the effect of ADR on the calcium accumulation property of the SR in skinned cultured cardiomyocytes. This was achieved by measuring calcium accumulation in "skinned" cardiomyocytes exposed to ADR. All solutions contained sodium azide, which has been shown to inhibit mitochondrial calcium uptake [10]. Furthermore, it was previously determined that calcium uptake under these conditions is mediated mainly by the SR [10]. The results of the present study demonstrate that ADR inhibits calcium accumulation by the SR in a dose- and time-dependent manner. This effect is consistent with the recent results described above. Further investigation is needed to determine whether the drug inhibits Ca-uptake by the SR, or enhances the permeability of the SR membrane, thus allowing the passive leakage of calcium.

In order to further elucidate the mechanism of the cardiotoxic effect of ADR, we investigated the effect of antioxidants and ferric iron. Our results show that the addition of ferric iron to the culture medium further inhibits the calcium accumulation. This observation lends further support to an oxygen-free radical-mediated mechanism of cell damage, and is in agreement with studies which propose that ferric iron is an important mediator of this type of cellular damage in several different tissues: in the heart following ADR treatment [6], in the CNS following

ischemia or trauma [12], and in a variety of pathophysiological conditions in the vascular system [13].

Antioxidants, which are known to inhibit lipid oxidation, did not inhibit the effect of ADR. This suggests that the biochemical mechanisms underlying the effect of ADR on calcium accumulation by the SR are not likely to involve lipid oxidation; further investigation is needed to determine if they involve protein oxidation.

In conclusion, our results demonstrate that ADR inhibits calcium accumulation by the SR of cultured cardiomyocytes. We therefore propose that SR might be one of the targets of ADR-induced cardiotoxicity. In addition, ferric iron potentiates this effect, implicating a role for oxidative mechanisms in the pathogenesis of the cardiotoxic effects of ADR.

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