Antioxidants protect against reactive oxygen species associated with adriamycin-treated cardiomyocytes

S. Michael DeAtley, Michael Y. Aksenov, Marina V. Aksenova, Bart Harris, Robert Hadley, Pamela Cole Harper, John M. Carney, D. Allan Butterfield

Abstract

Adriamycin (ADM) is a broad-spectrum antineoplastic antibiotic used to treat cancer patients. However, the usefulness of this drug is presently limited by the development of a dose-dependent cardiotoxicity. A current hypothesis for the ADM-induced cardiotoxicity involves the production of free radicals [4–7]. For example, we showed that ADM treatment of erythrocytes leads to protein oxidation [8], a key marker of oxidative stress [9].

1. Introduction

Adriamycin (ADM) is an anthracycline antibiotic that is one of the most frequently used antineoplastic agents in the treatment of human malignancies [1]. However, the clinical effectiveness of ADM treatment is diminished due to the dose-limiting side effect of cardiotoxicity [2,3]. A predominant hypothesis for the mechanism of ADM-induced cardiotoxicity involves the production of free radicals [4–7]. For example, we showed that ADM treatment of erythrocytes leads to protein oxidation [8], a key marker of oxidative stress [9].

Studies of the ability of antioxidants to protect against the ADM-induced cardiotoxicity in animal models have not shown a consistent pattern. Reports of vitamin E being cardioprotective [10,11] have been disputed by other investigators who have shown no changes in the mortality of animals during ADM administration [12–14]. N-Acetylcysteine has also been shown to be ineffective in the prevention of the
ADM-induced cardiotoxicity in a rat model [15]. However, other antioxidants have demonstrated a protective effect. Paracchini et al. established that the spin trap agent alpha-phenyl-tert-butyl nitrone (PBN) significantly reduced the myocardial impairment in rats during ADM treatment [16]. Recently, Siveski-Iliskovic et al. showed that the antioxidant probucol was effective in preventing ADM-induced cardiomyopathy in a rat model [17].

A highly sensitive method of assessing oxidative stress in cultured cardiomyocytes involves use of an oxidant-sensitive fluorescence probe combined with high spatial and temporal resolution laser-scanning confocal microscopy. Sarvazyan [18] utilized this method to confirm an oxidative mechanism of ADM toxicity in cardiomyocytes. If ADM were to induce ROS oxidative stress, then one could predict that appropriate antioxidants should modulate the stress. The present study utilizing 2',7'-dichlorodihydrofluorescein fluorescence demonstrates the ability of antioxidants to protect against ADM-generated reactive oxygen radicals in cardiomyocyte cultures.

2. Materials and methods

2.1. Chemicals

Adriamycin HCl was purchased from the University of Kentucky Hospital pharmacy. 5-Aminosalicylic acid (5A-SA), alpha-phenyl-tert-butyl nitrone (PBN), and cell culture materials were purchased from Sigma Chemical Co. (St. Louis, MO.). Trolox was purchased from Fisher Scientific (Pittsburgh, PA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH/DA) was obtained from Molecular Probes (Eugene, OR).

2.2. Cardiomyocytes

Rat heart myoblasts H9c2 (2-1) were purchased from American Type Culture Collection (Rockville, MD) and incubated with Dulbecco’s modified Eagle’s medium with 4 mM l-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 1.0 mM sodium pyruvate, fetal bovine serum (10%), with 50 units penicillin/ml, 100 mg streptomycin/ml at 37°C in 5% CO2 and the media changed every 2–3 days. The cultured myocytes were allowed to reach confluency before being used experimentally. Confluent myocytes were defined in accordance with the observations of Kimes and Brandt, who described the dividing myocytes as large, flat, spindle-shaped cells, while confluent myocytes form multinucleated tubular structures [19].

2.3. Adriamycin incubation

Upon reaching confluency, myocytes were incubated with either 50 μM Adriamycin, vehicle, 50 μM adriamycin + trolox 100 μM, 50 μM adriamycin + 100 μM PBN, 50 μM Adriamycin + 100 μM 5-aminosalicylic acid, for 24 and 48 h at standard culture conditions. The antioxidants were loaded into the culture dishes 1 h prior to the addition of 50 μM ADM. Following incubation, the media was removed and the myocytes were used for DCFH/DA confocal analysis.

2.4. Dichlorodihydrofluorescein assay

For measurement of ROS, cardiomyocytes were loaded with DCFH/DA by incubating for 50 min in the presence of 100 mM of the dye. The culture plates were washed three times with Hank’s balanced saline solution containing 10 mM HEPES buffer and 10 mM glucose. The culture plates were placed on the stage of an inverted microscope and were imaged using a laser-scanning confocal microscope (Nikon RCM 8000, Melville, NY). DCF fluorescence was excited using the 488 nm wavelength output of an argon laser. Emitted fluorescence was collected at 510 nm using a Nikon 40× water-immersion objective (N.A. 1.15). Six cells for each treatment group were chosen for image analysis on a random basis. Images were immediately stored on an optical disk recorder as 8-bit, 512×483 pixel TIFF file. The files were later transferred to an 80486 microcomputer for off-line analysis using the Metamorph program (Universal Imaging, West Chester, PA).

2.5. Statistical analysis

Statistical comparisons were made using ANOVA followed by the Student Newman–Keuls post hoc test for multiple comparisons. Significance was set at P < 0.01.
3. Results and discussion

DCFH/DA is a sensitive fluorimetric probe of the production of oxidative stress in living cells [20]. DCFH/DA enters the cell and is deacetylated by esterases to DCFH which, because of the negative charge, remains enclosed within the cell. When the DCFH is oxidized by ROS, it is transformed into the highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF).

Fig. 1 is a representative picture of the DCF fluorescence in myocytes during the various treatments: (A) control, (B) 50 μM ADM for 24 h, (C) 100 μM PBN and 50 μM ADM for 24 h, (D) 100 μM 5-ASA and 50 μM ADM for 24 h, (E) 100 μM trolox and 50 μM ADM for 24 h.

Fig. 1. Representative pictures of the DCF fluorescence in myocytes during the various treatments: (A) control, (B) 50 μM ADM for 24 h, (C) 100 μM PBN and 50 μM ADM for 24 h, (D) 100 μM 5-ASA and 50 μM ADM for 24 h, (E) 100 μM trolox and 50 μM ADM for 24 h.

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Fig. 1 is a representative picture of the DCF fluorescence in myocytes during the various treatments. It is obvious that ADM leads to ROS production and that the antioxidants studied provide various degrees of protection. These images were digitized and analyzed for ROS-induced DCF fluorescence (Fig. 2). All three of the water-soluble antioxidants used in this study modulated the oxidative stress induced by ADM (Fig. 2A–C). Relative to control, the mean percent increase of DCF fluorescence was: ADM, 273%, P < 0.01, n = 6; PBN, 147%, n = 6; 5-ASA, 129%, n = 6; Trolox, 106%, n = 6. Trolox provided the greatest degree of protection while PBN provided the least, but effective reduction of ADM-induced DCF fluorescence.

This is the first study using DCF to demonstrate the ability of antioxidants to quench reactive oxygen species generated during ADM treatment in cardiomyocyte cultures. The capacity of the DCF assay to evaluate the protection by antioxidants to oxidative stress generated by different prooxidants has previously been demonstrated in several studies [21–25].

A partial overlap of the ADM and DCF emission spectra could suggest a possibility of some interaction between the two molecules. However, Ubezio and Civoli, using cancer cells, were able to demonstrate...
that the fluorescence intensity observed during ADM treatment was not due to technical artifacts, but is attributable to the reactive oxygen radicals produced in the cells by the drug [26].

The increase of DCF fluorescence in ADM-treated cultured cardiomyocytes, observed in this study, is consistent with the suggested oxidative mechanism of ADM cardiotoxicity. Several reports have demonstrated the production of oxygen generated free radicals due to ADM in intact cardiac tissues and organelle preparations [5,7,27–29]. The heart is highly susceptible to oxygen radical injury because it contains considerably less protective mechanisms, such as glutathione, superoxide dismutase or catalase, than other metabolic organs like the liver or kidney [18,30].

Oxidative injury to the cardiomyocyte mitochondria by ADM, has been the subject of several studies [27–29,31,32]. Taken together, these reports suggest that the ADM-induced oxidative injury at the mitochondrial level precedes alterations in Ca$^{2+}$ homeostasis, which could result in an increase of the permeability of the inner mitochondrial membrane and the release of superoxide anion to the mitochondria cytosolic face. Oxidative-induced dysfunction of the cardiomyocyte mitochondria has been suggested to be a possible primary event in the development of cardiotoxicity due to ADM administration.

The supplement of antioxidant agents is one possible means of augmenting the cardiomyocyte endogenous oxidative defenses and may be cardioprotective in patients receiving ADM therapy. In our study, we observed that all three antioxidants tested were protective against the ADM-generated oxygen radicals. Trolox and 5-ASA were demonstrated to be the most effective in abrogating the increased oxidative stress in our myocyte cultures. One possible explanation for this result is the effect these two agents have on cellular membranes. ADM is a well-known inducer of lipid peroxidation in cellular membranes [10,33], and lipid peroxidation products such as HNE can diffuse into cells causing oxidative damage to cellular components [34,35]. Trolox is a scavenger of lipid peroxyl radicals, thus preventing the lipid peroxidation cascade from damaging cellular and possibly mitochondrial membranes. Similarly, 5-ASA has not only been shown to be a powerful scavenger of the hydroxyl radical [35], but it has also recently been demonstrated to associate with the membrane surface, allowing chain-breaking antioxidant activity when peroxidation is initiated within the membrane [36]. The spin trap agent PBN has been used to show protective effects against oxidative stress associated with stroke [37], hyperoxia [38] and accelerated aging [39]. In the current study, PBN also was effective in decreasing the ADM-generated reactive oxygen radical, but not to the same extent as trolox or 5-ASA. This result is consistent with the work of Paracchini et al. who demonstrated that PBN was cardioprotective against the ADM-induced cardiotoxicity in a rat model [16].

In conclusion, we provide the first evidence of protection by antioxidants against ADM-induced oxidative stress in cardiomyocytes using the DCF fluorescence assay. These results are consistent with the notion that the use of antioxidants may be beneficial in modulating or preventing the cardiotoxicity associated with ADM chemotherapy.

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References
