Downregulation of vitamin C transporter SVCT-2 in doxorubicin-induced cardiomyocyte injury

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Submitted 4 June 2012; accepted in final form 28 June 2012

Ludke AR, Sharma AK, Akolkar G, Bajpai G, Singal PK. Down-regulation of vitamin C transporter SVCT-2 in doxorubicin-induced cardiomyocyte injury. Am J Physiol Cell Physiol 303: C645–C653, 2012. First published July 6, 2012; doi:10.1152/ajpcell.00186.2012.—Vitamin C (Vit C) has been shown to be protective against doxorubicin (Dox)-induced cardiotoxicity. However, Vit C uptake into cardiomyocytes is poorly understood. Furthermore, whether the antioxidant enzyme reserve is enhanced by Vit C is also not known. The present study investigated an influence of Dox on Vit C transporters, expression of endogenous antioxidant reserve as well as enzymes, oxidative stress, and apoptosis in isolated cardiomyocytes. Cardiomyocytes isolated from adult Sprague-Dawley rats were exposed to control (culture medium 199 alone), Dox (10 μM), Vit C (25 μM), and Vit C + Dox for 24 h. Vit C transporter expression and localization, oxidative stress, antioxidant enzymes, and apoptosis were studied. Expression and localization of sodium-dependent vitamin C transporter-2 (SVCT-2) in the sarcolemma was reduced by Dox, but Vit C supplementation was able to blunt this change. There was a decrease in the expression of antioxidant enzymes glutathione peroxidase (GPX), catalase, and CuZn superoxide dismutase (SOD) due to Dox, but only GPX expression was completely prevented and CuZn SOD was partially rescued by Vit C. Dox-induced decrease in antioxidant reserve and increase in oxidative stress were partially mitigated by Vit C. Dox-induced apoptosis was ameliorated by Vit C. It is suggested that cardioprotection offered by Vit C in Dox-induced cardiomyopathy may involve an upregulation of SVCT-2 transporter followed by a reduction in oxidative stress as well as blunting of cardiomyocyte injury.

VITAMIN C (Vit C), also known as ascorbate, is a strong antioxidant. It readily reacts with reactive oxygen species (ROS) by donating electrons and in the process ascorbate is oxidized first to monodehydroascorbate and then to dehydroascorbate (DHA) (23). These oxidized forms of Vit C are relatively stable and do not cause cellular damage. DHA is structurally similar to glucose, and it has been proposed to enter into the cell via glucose transporters (GLUT) (46, 47). Furthermore, its entry into cells can be competitively inhibited by glucose (47). Since under physiological conditions ascorbate predominates (95% in human plasma), it is unlikely that DHA uptake by glucose transporters is sufficient to meet the needs of most cells (6, 33).

More recently, another transport system specific to Vit C, the sodium-dependent Vit C transporter (SVCT), has been identified (44) and two subtypes of SVCT (SVCT-1 and SVCT-2) have also been cloned (5, 32). The expression of SVCT-1 is detected mainly in the intestinal epithelium and in the liver, whereas SVCT-2 is ubiquitously expressed in various organs, including the myocardium (11). Furthermore, SVCT-2 has also been reported to be crucial for Vit C uptake in metabolically active and specialized tissues, thus offering protection against oxidative stress (29, 39). Considering the fact that Vit C levels vary in different tissues, data are lacking on how Vit C transport is affected by various drug treatments in different cell types including cardiomyocytes.

In addition to its fundamental properties as a ROS scavenger, Vit C has been recognized to modulate endogenous antioxidants as well as influence redox-sensitive molecular pathways and other proteins (19). Antioxidant supplements are well-recognized beneficial therapeutic strategies to treat oxidative stress-mediated disease pathologies (13, 14, 24, 38). In this regard, there is cumulative evidence showing that oxidative stress and higher susceptibility of cardiomyocytes to apoptosis and cell injury are important in doxorubicin (Dox)-induced cardiomyopathy and failure (4, 27, 30).

We have recently reported that Vit C protects against Dox-induced cardiomyocyte damage by decreasing oxidative stress, phosphorylation of proapoptotic signaling molecules such as mitogen-activated kinases (MAPK) and p53 as well as restoring cell viability (27). In the present study, we investigated the effects of Dox on the expression and localization of Vit C transporters in cardiomyocytes, antioxidant reserve, and endogenous antioxidant enzymes to better explain changes in oxidative stress and cell viability with and without Vit C.

MATERIALS AND METHODS

The investigation was approved by the University of Manitoba Animal Care Committee and conformed with the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.

Cardiomyocyte Isolation and Treatments

Cardiomyocytes were isolated from normal adult male Sprague-Dawley rats (250–300 g) using a previously described procedure (26). Cardiomyocytes (10⁶ per dish) were plated in laminin-coated (20 μg/ml) polystyrene tissue culture dishes. Plated cells were incubated in serum-free culture medium 199 (M199) supplemented with antibiotics (streptomycin-penicillin, 100 mg/ml) at 37°C under a 5% CO₂ and 95% O₂ atmosphere. Two hours after plating, the culture medium was changed to remove unattached dead cells, and the viable cardiomyocytes were incubated overnight.

Viable cardiomyocytes (95%) were divided into four groups and treated for 24 h as follows: Control (cardiomyocytes cultured in M199 only); Vit C (25 μM); Dox (10 μM); Vit C + Dox. For the treatment with Vit C groups, a pretreatment period of 1 h was allowed before the addition of Dox.

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Assessment of Cardiomyocyte Viability

Viability of the cultured cardiomyocytes was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole] assay (27). For this technique, cells were seeded in 96-well microplates each having 2 x 10^4 cells/well. After 24 h of different treatments, 5 mg/ml MTT was added to each well and cells were further incubated at 37°C for 2 h. The supernatant was removed carefully and 150 μl of dimethyl sulfoxide was added to each well and mixed thoroughly to dissolve the formed crystal formazan. After 10 min of incubation to ensure that all crystal formazan was dissolved, the cytotoxicity of each treatment was quantified by recording the absorbance in each well at 570 nm using an ELISA plate reader.

Western Blot Analysis

Whole cell protein extracts were prepared from control and treated cardiomyocytes cultured in 60-mm dishes. After different treatments, cells were washed in PBS and then placed on ice, and RIPA lysis buffer, pH 7.6, containing 150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, phosphatase inhibitors (10 mM NaF, 1 mM Na3VO4, and 1 mM EGTA), and protease inhibitors (4 μM leupeptin, 1 μM pepstatin A, and 0.3 μM aprotinin) was used to lyse the cells and extract protein. The protein samples obtained (45 μg) were then subjected to electrophoresis, immunoblotting, and protein detection (18) using specific antibodies for the study of transporters, antioxidant enzymes, and apoptotic marker described later in this section. Detection of membrane-bound proteins was visualized using the luminol/iodophenol substrate for horseradish peroxidase-conjugated antibodies (Roche) and developed on X-ray film. The bands were quantified by image analysis software (Quantity One, Bio-Rad). Rabbit GAPDH antibody and rabbit cyclophilin D antibody were used as general and mitochondrial loading control, respectively.

Study of Transporters

SVCT-2 and GLUT4 transporters using rabbit SVCT-2 antibody and rabbit GLUT4 antibody were analyzed by immunofluorescence and Western blotting. Cardiomyocytes from different treatment groups were grown on glass coverslips coated with laminin. Cells were washed with PBS, fixed in 4% paraformaldehyde, quenched in 100 mM glycine, permeabilized in 0.1% (vol/vol) Triton X-100, and blocked in 3% BSA. The cells were then incubated with rabbit SVCT-2 or GLUT4 antibodies followed by incubation with FITC-goat anti-rabbit antibody. Cells were then washed in PBS and mounted for microscopy using a Floursave reagent. Images were obtained using fluorescent microscope (Olympus BX 51) with excitation and emission at the wavelength of 488 nm and 525 nm, respectively. Controls were set using untreated cells and autofluorescence was examined by tagging the treated cells with secondary antibody only. Fluorescence intensity was quantified using Image Pro-Plus software (version 5.1.2).
For GLUT4 detection, treated cardiomyocytes were incubated in the presence or absence of 1 μM insulin (Sigma-Aldrich) in K-H buffer (4% bovine serum albumin, pH 7.4), for 1 h at 37°C before immunofluorescent study.

**Lipid Hydroperoxides**

Lipid hydroperoxides were assayed using a commercially available kit. Briefly, treated and untreated cardiomyocytes were washed with PBS, and an aliquot of 45 μl (containing 30 μg of protein) of the homogenate was used to quantify hydroperoxides using an ELISA plate reader at 675 nm. Mean absorbance for all the groups was quantified.

**Antioxidant Reserve and Enzymes**

Total antioxidant capacity of cardiomyocytes was measured using Antioxidant Assay Kit. An aliquot of 20 μl of cell lysates (containing 30 μg of protein) from different treatment groups was used to assay the ability of antioxidants in the sample to inhibit the oxidation of ABTS [2,2'-azino-di-(3-ethylbenzthiazoline sulfonate)] to ABTS+ by metmyoglobin. The mean absorbance from each sample was read at 405 nm (ELISA reader) and calculated as molar Trolox equivalents obtained from a standard Trolox curve.

Protein expression of antioxidant enzymes was studied using Western blot analysis as described earlier in this section using rabbit: GSHPx, Cu/Zn-SOD, catalase, and heme oxygenase-1 (HO-1) antibodies.

**Apoptosis**

Annexin-propidium iodide assay. Occurrence of apoptosis in isolated cardiomyocytes was detected using a commercially available Annexin-V-FLUOS assay kit (45). After the treatments, cardiomyocytes were washed with PBS and incubated for 30 min with 20 μl of Annexin-V-FLUOS staining solution and 20 μl of propidium iodide (PI) in a total volume of 250 μl of PBS per dish. After the incubation, the cells were examined using a fluorescent microscope (Olympus BX 51). The rod-shaped myocytes exhibiting green fluorescence only (Annexin-V-FLUOS) were counted as cells in early apoptosis. The cells exhibiting no fluorescence at all were counted as normal living cells. Rounded cardiomyocytes showing red nuclei (positive for PI) stained with green were counted as dead cells. The cells were counted by examining 10 random microscopy fields. Data are expressed as a count of apoptotic cells/100 cells.

**Proapoptotic markers.** Mitochondrial and cytosolic fractions were isolated using Mitochondria Isolation Kit for cells. Cytochrome c and Bax protein expressions were analyzed by Western blot analysis (described earlier) using rabbit cytochrome c and Bax antibodies in both of the fractions.

**Reagents**

Vitamin C was purchased from Sigma-Aldrich Canada (Oakville, ON, Canada) and doxorubicin (Adriamycin) was obtained from Pfizer. Cardiomyocyte isolation and culture reagents were purchased from Sigma-Aldrich Canada. Antibodies were purchased from Cell Signaling (New England Biolabs, Mississauga, ON, Canada) with the exception of transporter antibodies and GAPDH, which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Annexin-V-FLUOS assay kit and polyvinylidene difluoride membranes were purchased from Roche Diagnostics (Roche, Indianapolis, IN). Antioxidant assay kit was purchased from Cayman Chemical (Ann Arbor, MI). Lipid hydroperoxides LPO-CC assay kit was purchased from Kamiya Biomed (Tukwila, WA). Mitochondria Isolation Kit for cells and Western blot reagents were purchased from Thermo Scientific (Ottawa, ON, Canada) unless otherwise specified.

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**Fig. 2. Glucose transporter GLUT4 immunofluorescence in the presence or absence of insulin.**

A: representative GLUT4 fluorescent microscopic images of adult rat cardiomyocytes. B: fluorescence intensity (percentage of insulin control). Data are expressed as means ± SE of five different experiments. *Significantly different (P < 0.05) from control. Groups are the same as in Fig. 1.
Statistical Analysis

All experiments were done in duplicate for each treatment group and repeated for five times (n = 5). For the study of fluorescence techniques and Annexin-V-FLUOS, a total of 10 dishes (35 mm) were prepared for each treatment group and 10 different fields per dish were counted for the quantitative analysis. Data are expressed as means ± SE. Groups were compared by one-way analysis of variance (ANOVA), and Bonferroni’s test was performed to identify differences between groups. P < 0.05 was considered significant.

RESULTS

Cardiomyocyte Viability

The concentrations for Dox and Vit C used in the present study were based on our previous study (27). Briefly, cardiomyocytes were exposed to Dox (10 μM) for 24 h in the presence of different concentrations of the vitamin (5, 10, 25, 50, and 100 μM) and analyzed in terms of viability of cells and ROS production (27). There was no significant protection at 5 and 10 μM of Vit C. The most optimal effect was seen at 25 μM, and further increase to 50 and 100 μM of Vit C did not show any additional benefit (27). For this reason, 25 μM was chosen as the concentration of Vit C for this study. In this study, using MTT assay for testing the viability of cells, it was noted that Dox caused a 23% decrease in MTT conversion, which was significant (P ≤ 0.05). This change was mitigated in the Vit C + Dox group with only a 11% decrease in MTT. Control values were set as 100% and no differences were seen in the control and Vit C groups. These results are in agreement with previously published data (27) and confirmed the suitability of the concentrations used in the present study.

Localization and Expression of Vit C Transporters

To analyze Vit C transport, we studied SVCT-2 due to its presence in the myocardium and its property of being the stereoselective high-affinity transport for Vit C. GLUT4 was chosen to understand whether Vit C could be entering the cell through its oxidized form, DHA; besides, this GLUT isoform is expressed exclusively in cardiac, skeletal, and adipose tissue (10). SVCT-2 was prominently labeled on the cell surface as well as in the vesicle compartments (Fig. 1A). Quantification of fluorescence intensity showed a significant decrease (20%) of SVCT-2 immunoreactivity after Dox treatment, which was partially reversed by cotreatment with Vit C (Fig. 1B). In the

Fig. 3. A and B: effects of different treatments on SVCT2 (A) and GLUT4 (B) transporter expression. In A and B, the top panel is Western blots and the bottom panel shows densitometric analysis. Data are expressed as means ± SE of five different experiments. *Significantly different (P < 0.05) from control; #significantly different (P < 0.05) from the Dox group. Groups are the same as in Fig. 1.

Fig. 4. Effects of different treatments on oxidative stress. A: lipid peroxidation in cell lysates (percentage of control). B: total antioxidant capacity measured in cell lysates (percentage of control). Data are expressed as means ± SE of five different experiments. *Significantly different (P < 0.05) from control; #significantly different (P < 0.05) from the Dox group. Groups are the same as in Fig. 1.
absence of insulin, GLUT4 was located in the intracellular compartments, and the intensity of immunofluorescence was low in all groups and the reaction was no different from its control, as shown in Fig. 2A. However, in the presence of insulin, GLUT4 was translocated to the cardiomyocyte surface and there was very prominent GLUT4-specific cell surface labeling. Cardiomyocytes treated with Dox expressed small but significantly higher GLUT4 immunofluorescence intensity in the presence of insulin than in all other groups (Fig. 2B).

To assess SVCT-2 and GLUT4 protein expression after respective treatments, Western blotting of cell lysates was performed (Fig. 3, A and B). In the Dox-treated group, SVCT-2 was downregulated by 22%, but Vit C was able to blunt the change caused by Dox. There was no difference between control and Vit C alone groups (Fig. 3A). In accordance with immunofluorescence results, GLUT4 protein expression was slightly increased in the Dox group compared with the other groups (Fig. 3B).

Oxidative Stress and Antioxidant Enzymes

Oxidative stress was monitored in terms of lipid hydroperoxides along with the study of total antioxidant capacity in cell lysates (Fig. 4, A and B). The production of lipid hydroperoxides was increased by ~50% in Dox-exposed cardiomyocytes, which was only partially reduced ($P \leq 0.05$) in the Vit C + Dox group. Lipid hydroperoxide production was not different between control and Vit C-treated cells (Fig. 4A).

Dox treatment led to a 25% decrease ($P \leq 0.05$) in the total antioxidant capacity of cells. Vit C alone did show some increase, but the change was not significant. Dox-induced decrease was significantly ($P \leq 0.05$) mitigated in the presence of Vit C (Fig. 4B).

Effects of Vit C on Dox-induced changes in the endogenous antioxidant enzyme protein expression were studied using Western blot analysis (Fig. 5, A–D). Dox significantly decreased protein expression of antioxidant enzymes glutathione peroxidase (GPx) by 15%, cytosolic Cu/Zn superoxide dismutase (SOD) by 50%, and catalase by 40%. Vit C pretreatment was able to rescue Dox-induced decrease in GPx, and it partially prevented the decrease seen in Cu/Zn SOD but had no effect on catalase levels. There was no difference found in heme oxygenase levels among all the studied groups.

Apoptosis

Dox-induced apoptosis and its modulation by Vit C was studied by staining the cardiomyocytes with Annexin-PI (Fig. 6, A and B) as well as using proapoptotic markers (Fig. 7, A and B). In the control group, the majority of cells were rod-shaped and binucle-
ated, and the nuclei had a normal appearance. Exposure to Dox caused a six-fold increase (P ≤ 0.05) in Annexin staining in cells that excluded PI dye (Fig. 6A). Treatment with Vit C completely prevented this change caused by Dox (Fig. 6B).

Proapoptotic protein Bax and cytochrome c release were also analyzed (Fig. 7, A and B). Mitochondrial permeability change results in cytochrome c release from mitochondria to the cytosol and activates downstream apoptotic signaling events. Dox caused a significant increase in cytosolic cytochrome c and decrease in its mitochondrial content. This shift in cytochrome c distribution was prevented by Vit C (Fig. 7A). Dox-induced overexpression of Bax was seen both in the mitochondria and in the cytosol, which was mitigated by Vit C treatment (Fig. 7B).

**DISCUSSION**

Cardiovascular disease and cancer, the two leading causes of death worldwide, have been shown to be closely related (8, 16). Cancer survivors are at higher risk to develop heart failure due to the chemotherapeutic regimen used (42). For this reason, Dox-induced cardiomyopathy still remains an important clinical problem, and an understanding of the molecular mechanisms involved is crucial to find ways to protect the heart against such an insult. The present study provides evidence for the first time that Dox-induced treatment downregulates sodium-dependent Vit C transporter-2 (SVCT-2) protein expression on the cardiomyocyte cell surface. Dox also resulted in an increase in lipid hydroperoxides and a decrease in total antioxidant capacity of the cells, culminating in apoptosis. The down-regulation of Vit C transporter may also have accentuated these Dox-induced changes.

Since the functional role of SVCT-2 in mammalian cells is to induce the transport of Vit C which is sodium-dependent and specific for ascorbate (32), a specific reduction in SVCT-2 in cardiomyocytes implies that the entry of Vit C may be affected in vivo with Dox exposure. In addition, we have noticed that SVCT-2 is present in the sarcolemma as well as vesicle compartments within the cardiomyocyte. In this regard, previous studies in different cell types have also suggested that, in addition to its membrane localization, SVCTs can also be found in intracellular compartments (3, 41). The reduction in SVCT-2 seen in the present study may suggest that Dox could potentially influence the flux of other transporter-dependent exogenous defense molecules as well as influence their intracellular distribution. Thus, the importance of antioxidant co-treatment should be highlighted since Vit C was able to blunt the Dox-induced decrease in SVCT-2 as well as partially rescue the cardiomyocytes from Dox-induced injury.

There is enough evidence that production of reactive oxygen species, as a by-product of Dox metabolism, and promotion of oxidative stress play a role in Dox-induced cardiotoxicity due to the unique vulnerability of the heart (28, 37). Cardiomyocytes depend on oxidative phosphorylation to execute their contractile function; thus antioxidant reserve may already be tightly balanced under physiological circumstances (7, 28). Upon an insult such as Dox-induced oxidative stress in the heart, the cardiomyocyte becomes overwhelmed and is more prone to oxidative damage. The present study confirmed the
prooxidant effect of Dox in cardiomyocytes with the summation of events such as increased lipid peroxidation, decreased total antioxidant capacity, and diminution observed in the protein expression of the well-known antioxidant enzymes catalase, Cu/Zn SOD, and GPx, though no change was found in HO-1 protein expression. Dox-induced decrease in the overall antioxidant capacity of the cells demonstrated that not only enzymatic antioxidant systems are affected, but the nonenzymatic defense may also be blunted. In agreement with our findings, Dox has already been shown to alter a variety of cellular proteins (1, 22, 43) as well as decrease antioxidant activity (2, 21, 49) in the heart.

Pretreating the cells with Vit C, specifically preserved GPx decrease caused by Dox and blunted the decrease in SOD, but had no effect on rescuing catalase. These data suggest some selectivity in the beneficial effects of Vit C on antioxidant enzymes. Some other antioxidants have already been shown to increase or preserve the endogenous antioxidant enzymes which are decreased by Dox (2, 17, 21, 25, 49) suggesting that antioxidant therapy may be a reliable cardioprotective strategy.

Our in vitro model of Dox-induced cardiotoxicity confirmed a diminution of cell viability after Dox treatment with the activation of apoptosis analyzed by Annexin-positive staining and proapoptotic markers. Annexin detects cell membrane exposure of phosphatidylserine, one of the earliest features of apoptosis (45); this change was seen only in Dox-treated cells. These data suggest some selectivity in the beneficial effects of Vit C on antioxidant enzymes. Some other antioxidants have already been shown to increase or preserve the endogenous antioxidant enzymes which are decreased by Dox (2, 17, 21, 25, 49) suggesting that antioxidant therapy may be a reliable cardioprotective strategy.

In summary, our findings show that Dox induces cardiomyocyte oxidative stress via decrease in the Vit C transporter which was associated with a decrease in endogenous antioxidant enzymes, generation of lipid peroxidation culminating in a decrease in cell viability and apoptosis. This study supports
beneficial effects of Vit C supplementation in the mitigation of Dox-induced adverse cardiac effects.

ACKNOWLEDGMENTS

Support from the Canadian Institute of Circulatory and Respiratory Health (to P. K. Singal) is greatly acknowledged. A. R. Laude is supported by a studentship from Manitoba Health Research Council. P. K. Singal is holder of the Naranjan Dhalia Chair in Cardiovascular Research supported by the St. Boniface Hospital and Research Foundation.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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