Am J Physiol Cell Physiol 312: C418–C427, 2017.
First published January 18, 2017; doi:10.1152/ajpcell.00356.2016.

RESEARCH ARTICLE

Doxorubicin-induced nitrosative stress is mitigated by vitamin C via the modulation of nitric oxide synthases

Gauri Akolkar, Ashim K. Bagchi, Prathapan Ayyappan, Davinder S. Jassal, and Pawan K. Singal

Institute of Cardiovascular Sciences, St. Boniface Hospital Albrechtsen Research Centre, Department of Physiology and Pathophysiology, Max Rady College of Medicine, Rady Faculty of Health Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

Submitted 15 December 2016; accepted in final form 12 January 2017

Akolkar G, Bagchi AK, Ayyappan P, Jassal DS, Singal PK. Doxorubicin-induced nitrosative stress is mitigated by vitamin C via the modulation of nitric oxide synthases. *Am J Physiol Cell Physiol* 312: C418–C427, 2017. First published January 18, 2017; doi: 10.1152/ajpcell.00356.2016.—An increase in oxidative stress is suggested to be the main cause in Doxorubicin (Dox)–induced cardiotoxicity. However, there is now evidence that activation of inducible nitric oxide synthase (iNOS) and nitrosative stress are also involved. The role of vitamin C (Vit C) in the regulation of nitric oxide synthase (NOS) and reduction of nitrosative stress in Dox-induced cardiotoxicity is unknown. The present study investigated the effects of Vit C in the mitigation of Dox-induced changes in the levels of nitric oxide (NO), NOS activity, protein expression of NOS isoforms, and nitrosative stress as well as cytokines TNF-α and IL-10 in isolated cardiomyocytes. Cardiomyocytes isolated from adult Sprague-Dawley rats were segregated into four groups: *1*) control, *2*) Vit C (25 μM), *3*) Dox (10 μM), and *4*) Vit C + Dox. Dox caused a significant increase in the generation of superoxide radical ($O_2^{\cdot-}$), peroxynitrite, and NO, and these effects of Dox were blunted by Vit C. Dox increased the expression of iNOS and altered protein expression as well as activation of endothelial NOS (eNOS). These changes were prevented by Vit C. Dox induced an increase in the ratio of monomeric/dimeric eNOS, promoting the production of $O_2^{\cdot-}$, which was prevented by Vit C by increasing the stability of the dimeric form of eNOS. Vit C protected against the Dox-induced increase in TNFα as well as a reduction in IL-10. These results suggest that Vit C provides cardioprotection by reducing oxidative/nitrosative stress and inflammation via a modulation of Dox-induced increase in the NO levels and NOS activity.

oxidative stress; nitrosative stress; nitric oxide synthase; doxorubicin; vitamin C; ascorbic acid

EVEN THOUGH ADVANCES in early diagnosis and improved treatment modalities have significantly increased the survival of cancer patients (39, 44, 48), cardiovascular complications and heart failure in cancer survivors still accounts for ~33% of deaths (22, 52). Doxorubicin (Dox) is one of the most potent and widely used chemotherapeutic agents for the treatment of a variety of cancers (22, 36, 44). However, the secondary and latent cardiotoxic side effects of the chemotherapy drugs are a serious concern (22, 29, 36, 44). One of the major mechanisms associated with Dox-induced cardiotoxicity is through an increased generation of reactive oxygen species (ROS) (24, 29, 36, 41, 44). In addition to oxidative stress, there is also evidence for the involvement of nitrosative stress in Dox-induced cardiotoxicity by an increase in the generation of reactive nitrogen species such as peroxynitrite (3, 8, 35). Peroxynitrite, a potent, reactive and cytotoxic free radical, is formed by near diffusion limited reaction of a high concentration of nitric oxide (NO) with superoxide ($O_2^{\cdot-}$) and is involved in various pathological conditions (35, 38, 47).

Endogenously, NO is a product of an enzymatic reaction of nitric oxide synthase (NOS) to convert arginine to citrulline. NOS exists in three different isoforms, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Both nNOS and eNOS are constitutively active and produce NO in the physiological range (15, 25). On the other hand, iNOS is activated in response to cytokines or stress signal and results in the generation of NO in the high micromolar range (15, 25). A reduction in peroxynitrite-induced cytotoxicity by a strategic use of antioxidant(s) can be a viable strategy for the mitigation of pathological conditions associated with a number of diseases, including Dox-induced cardiotoxicity. Therefore, in this study, the effect of Vit C on $O_2^{\cdot-}$, NO, peroxynitrite, NOS isoforms, protein modifications, and the release of TNF-α as well as IL-10 in relation to cell viability were evaluated in Dox-challenged cardiomyocytes.

MATERIALS AND METHODS

The study was approved by the University of Manitoba Animal Care Committee and conforms to the guidelines of the Canadian Council of Animal Care.

Reagents

Vit C (sodium ascorbate) was purchased from Sigma-Aldrich (Oakville, ON, Canada), and Dox was obtained from Pfizer (New York). All the reagents for cardiomyocyte isolation and cell culture except collagenase (Worthington-Biochem, Lakewood, NJ) were purchased from Sigma-Aldrich. Hydroxyphenyl fluorescein (HPF) and diaminofluorescein-2-diacetate (DAF-2DA) were obtained from Cell Technology (Mountain view, CA), and dihydoxyethidium (DHE) was obtained from Sigma-Aldrich. NO (cat. no. NB98) and NOS detection kits (cat. no. NB78) were obtained from Oxford Biomedical Research (Oxford, MI). *S*-nitrosylated protein detection assay kit (cat. no. 10006518) was purchased from Cayman Chemicals (Ann Arbor, MI). ELISA kits for detection of TNFα (cat. no. 900-M73) and IL-10 (cat. no. 900-M53) were obtained from Peprotech (Rocky Hill, NJ). Antibodies (iNOS, eNOS, phospho-eNOS Ser^{1177}, phospho-eNOS Thr^{495}, and GAPDH) were purchased from Cell Signaling (Mississauga, ON,

Address for reprint requests and other correspondence: P. K. Singal, Institute of Cardiovascular Sciences, St. Boniface Hospital Albrechtsen Research Centre, R3021, 351 Tache Ave., Winnipeg, Canada R2H 2A6 (e-mail: psingal@sbrc.ca).

Canada) except nitrotyrosine (NT), which was purchased from Oxford Biomedical Research.

Cardiomyocyte Isolation and Treatment

Cardiomyocytes were isolated from male Sprague-Dawley rats (250–300 g, ~8–10 wk of age) as previously described (30) and were plated (10^6 per dish) in laminin-coated (20 μg/ml) polystyrene tissue culture dishes. Cells were incubated in culture medium (M199) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA) and antibiotics (streptomycin/penicillin, 100 mg/ml) at 37°C under a 5% CO_2-95% O_2 atmosphere. This culture medium does not support growth as well as survival of fibroblasts. Unattached dead cells were removed after 2 h, and the viable cardiomyocytes were incubated overnight in M199 with 0.5% FBS under the same incubation conditions.

Viable cardiomyocytes (95%) were divided into four groups and treated as follows: control (cardiomyocytes cultured in M199 only), Vit C (25 μM sodium ascorbate), Dox (10 μM of doxorubicin hydrochloride), and Vit C (25 μM sodium ascorbate) + Dox (10 μM doxorubicin hydrochloride) for 24 h. For the combination group of Vit C + Dox, cells were treated with Vit C 1 h before the addition of Dox. The concentration and the time of treatments were based on our previous studies (28).

Cell viability. MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], a tetrazole, was used to determine viability of cardiomyocytes from different treatment groups. Cardiomyocytes (2×10^4 cells/well) plated on 96-well plates were incubated with 5 mg/ml MTT and further incubated at 37°C for 2 h. All the remaining supernatant was removed carefully and 150 μl of dimethyl sulfoxide was added to each well and mixed thoroughly to dissolve the formed crystal formazan. The absorbance of each well was recorded at 570 nm using a plate reader (Dynex Technologies, Chantilly, VA).

Superoxide anion. Isolated cardiomyocytes (10^3 cells/well) were seeded in 24-well plates. After 24 h of treatment, cells were incubated with 5 μM DHE for 30 min at 37°C. Cells were visualized by fluorescence microscopy using Olympus microscope (1X81) (Olympus America, Melville, NY) at excitation and emission wavelengths of 515/605 nm, respectively. Fluorescence intensity was determined using ImageJ Software ((National Institutes of Health, Bethesda, MD; https://imagej.nih.gov/ij/) and expressed relative fluorescence intensity per myocyte area; 100 ± 10 myocytes were used in each group for the quantification of fluorescence intensity.

NO levels. In aqueous solution, NO rapidly degrades to nitrate and nitrite. Nitrate is further enzymatically converted to nitrite by nitrate reductase. Spectrophotometric quantification of nitrite was done using Greiss reagent. Levels of NO released into the media as well as in the cardiomyocytes were assessed using a commercially available kit. Absorbance was recorded at 540 nm using a plate reader, and the concentration of samples was determined using a standard curve. Intracellular NO levels were also measured using a fluorescent dye, DAF-2DA. After 24 h of treatment, cardiomyocytes were washed with phosphate-buffered saline (PBS) and then incubated with DAF-2DA (5 μM) at 37°C for 1 h in the dark. Fluorescent images were obtained using a microscope (Olympus 1X81) at excitation and emission wavelengths of 488 and 515 nm, respectively. Images were taken randomly from 10 different fields for each treatment group and were analyzed using Image Pro Software (Media Cybernetics, Rockville, MD). Fluorescence intensity was determined using ImageJ Software and expressed as relative fluorescence intensity per myocyte area; 100 ± 10 myocytes were used in each group for the quantification of fluorescence intensity.

Peroxynitrite detection. Peroxynitrite levels in cardiomyocytes were detected using peroxynitrite specific dye, HPF. Isolated cardiomyocytes (2×10^3 cells/well) were seeded in black 96-well plates. Twenty-four hours after treatment, cells were washed with PBS and loaded with 10 μM HPF and incubated at 37°C for 1 h in the dark. The fluorescence was measured using a fluorescence plate reader (Glomax Multidetection System, Promega, Madison, WI) at excitation and emission wavelengths of 488 and 515 nm, respectively. Peroxynitrite levels were expressed as fold change compared with control. Cellular peroxynitrite levels were also monitored using a fluorescence microscope (Olympus 1X81). Images were acquired at excitation and emission wavelengths of 488 and 515 nm, respectively at 400× magnification. Images were taken randomly from 10 different fields and analyzed using Image Pro Software. Fluorescence intensity was determined using ImageJ Software and expressed as relative fluorescence intensity per myocyte area; 100 ± 10 myocytes were used in each group for the quantification of fluorescence intensity.

NOS activities and protein expression. Assessment of total NOS (iNOS and eNOS) activities in cardiomyocytes was done using a commercially available kit, and the absorbance was recorded at 540 nm using a microplate reader (Dynex Technologies). Concentration of samples was determined by interpolation from the standard curve and was expressed as micromoles of NO produced per microgram protein per minute.

iNOS and eNOS protein expression. The effect of different treatments on protein expression of iNOS as well as phosphorylated eNOS (p-eNOS Ser^{1177} and p-eNOS Thr^{495}) and total eNOS was determined in cardiomyocyte lysate by Western blotting. Protein samples (30 μg protein) were subjected to one dimensional 8-15% SDS polyacrylamide gel electrophoresis (PAGE). Separated proteins were transferred to 0.45-μm polyvinylidene difluoride (PVDF) membranes (Thermo-Fisher Scientific, Waltham, MA). Nonspecific sites on the membrane were blocked by incubating membranes with 10% skim milk. Membranes were incubated overnight at 4°C with primary antibodies. Unbound primary antibody was washed with Tris-buffered saline with Tween-20 (TBS-T). Membranes were placed in a solution containing secondary antibody goat anti-rabbit immunoglobulin G horseradish peroxidase (Bio-Rad) (1:5,000) for 1 h at room temperature (RT). Membrane-bound proteins were visualized by incubating the membranes with enhanced chemiluminiscence (ECL) (Thermo-Fisher Scientific) reagent and developed on X-ray film. The bands were quantified by image analysis software Quantity One (Bio-Rad). Equal protein loading for all the samples was confirmed using rabbit anti-GAPDH antibody (1:2,000) as an internal standard. Chemiluminiscence intensity of each band in each lane was normalized to the intensity of GAPDH band in respective lane.

Monomeric/dimeric eNOS. Low temperature electrophoresis was performed to determine the monomeric and dimeric forms of eNOS as described previously (51). Briefly, protein samples were incubated with Laemmli buffer without β-mercaptoethanol for 5 min at 37°C. Samples were loaded on 6% SDS-PAGE at 4°C. Transfer of proteins on PVDF membrane and probing of membrane with antibody was done as routine Western blot analysis as described earlier. eNOS and β-actin were detected by ECL and analyzed using Quantity One software. Protein expression of eNOS was expressed as the ratio of monomeric/dimeric form.

S-nitrosylation of protein. S-nitrosylation of proteins was determined using a commercially available kit. Cardiomyocyte lysate (30 μg protein) from different treatment groups was processed as per the supplier's manual. The processed samples were run on SDS-PAGE and then transferred onto PVDF membranes. The membranes were blocked using 2% bovine serum albumin (BSA) and then incubated with *S*-nitrosylation detection reagent (HRP) for 1 h. The membranes were developed using ECL reagent.

Protein estimation. Cardiomyocytes from different treatment groups were washed with PBS and scraped. The pellet was resuspended in ice-cold cell lysis buffer containing protease inhibitor cocktail (Roche Diagnostics, Mississauga, ON, Canada), phosphatase inhibitor (Pierce-Thermo-Fisher Scientific), 1 mM phenylmethylsulfonyl fluoride, and DTT. Cell lysate was incubated on ice for 30 min and then centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected in separate tubes and stored at −80°C for further

analysis. Total protein concentration was determined using BSA as standard according to modified Bio-Rad procedure.

Cytokine analysis. Levels of TNF α and IL-10 released from cardiomyocytes into the medium were detected in different treatment groups using a commercially available sandwich ELISA development kit as per the manufacturer's instructions, and the color development was monitored using ELISA plate reader (Dynex Technologies) at 405 nm. Concentration of cytokines in treated samples was extrapolated from color development of known standard concentration.

Statistical Analysis

All experiments were performed in duplicate for each treatment group and repeated three times. Data are expressed as means ± SE. Treatment groups were compared by one-way analysis of variance (ANOVA), and Tukey-Kramer's test was performed to identify differences between the group. $P \leq 0.05$ was considered to be significant.

RESULTS

Viability of Cardiomyocytes

The viability of control and treated cardiomyocytes was determined using MTT assay where viable cardiomyocytes can convert the yellow colored tetrazole dye into purple formazan dye. Viability of the control cardiomyocytes was considered as 100% and Vit C treatment did not cause any significant difference. Dox treatment caused a significant reduction in the viability to 63% ($P < 0.001$) compared with the control and Vit C groups. Pretreatment of cardiomyocytes with Vit C significantly increased the viability to 87% ($P < 0.001$) in the Vit C + Dox group (Fig. 1).

Generation of Reactive Oxygen and Nitrogen Species

The effect of Vit C and Dox on the generation of reactive oxygen and nitrogen species was determined in isolated cardiomyocytes. Oxidative and nitrosative stresses were determined by measurement of cellular levels of $O_2^{\cdot -}$ and peroxynitrite, respectively. There were very low levels of $O_2^{\cdot -}$ and peroxynitrite in control and Vit C-treated cells. Treatment of cardiomyocytes with Dox caused a significant increase ($P < 0.001$) in the generation of both $O_2^{\cdot -}$ (Fig. 2, *A* and *B*) and peroxynitrite (Fig. 2, *C–E*) compared with control and Vit C-treated cells. Pretreatment with Vit C caused a significant reduction ($P < 0.001$) in the generation of these reactive species compared with the Dox-alone group.

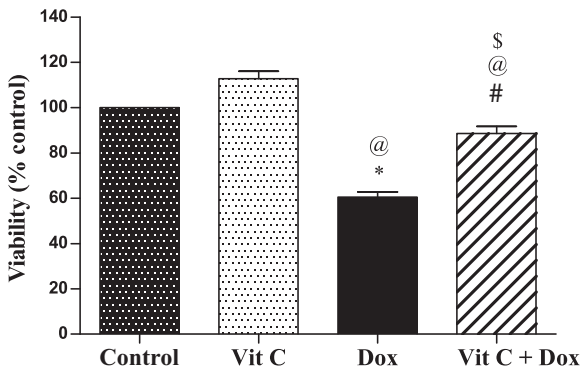

Fig. 1. Effects of different treatments on cardiomyocyte viability. Cardiomyocytes were treated with Vitamin C (Vit C) (25 μM), doxorubicin (Dox) (10 μM), or Vitamin C + doxorubicin (Vit C + Dox) for 24 h, and viability was analyzed using MTT assay. Data represented as % viability compared with control. Data are means ± SE from 3 individual experiments done in duplicate. *$P < 0.001$ compared with control; $$P < 0.05$ compared with control; @$P < 0.001$ compared with Vit C; #$P < 0.001$ compared with Dox.

Levels of Nitric Oxide and NOS Activity

Since peroxynitrite is formed by the reaction of $O_2^{\cdot -}$ with NO, we determined the effect of Dox and Vit C on the levels of NO in the cardiomyocytes as well as in the media. Control and Vit C-treated cardiomyocytes showed very minimal levels of NO. There was higher fluorescence intensity in Dox-treated cardiomyocytes compared with control and Vit C-treated groups, indicating higher production of NO by Dox. Vit C pretreatment attenuated NO production in Dox-treated cardiomyocytes (Fig. 3, *A* and *B*). As NO rapidly dissociates into nitrate and nitrite, we measured the levels of nitrate as an indicator of total NO concentration. Dox treatment significantly increased the levels of nitrate (Fig. 3*C*) compared with control and Vit C. Pretreatment of cardiomyocytes with Vit C was able to reduce the generation of NO by Dox. NO, a gaseous signaling molecule, can easily diffuse through the membrane to exert its paracrine effects. Therefore, we measured the levels of NO released from cardiomyocytes into the media. Dox-treated cardiomyocytes showed an ~3-fold increase in the levels of released NO compared with control (Fig. 3*D*). Vit C in the Vit C + Dox group significantly ($P < 0.05$) prevented Dox-mediated release of NO into the media (2.2-fold decrease) (Fig. 3*D*).

NOS Isoforms

Since NO is enzymatically produced by the action of NOS enzymes, we determined the effect of Dox and Vit C on total NOS activity (Fig. 4). Dox-treated cardiomyocytes showed a significant increase (almost threefold compared with control) in the total NOS activity. In comparison to Dox alone, Vit C pretreatment reduced the NOS activity to 1.5-fold in the Vit C + Dox group.

iNOS protein expression showed an upregulation in Dox-treated cardiomyocytes compared with control (Fig. 5*A*). Vit C protected against Dox-induced upregulation of iNOS (Fig. 5*A*). Since the phosphorylation site for eNOS determines its functional activity, we determined protein expression of the phosphorylated form of eNOS at its activating (Ser^{1177}) as well as inhibitory site (Thr^{495}) in the cardiomyocytes from different treatment groups. The regulation of eNOS activity occurs through differential modulation of its phosphorylation state at Ser^{1177} or Thr^{495} residue (Fig. 5, *B* and *C*). Treatment with Dox caused a reduction of protein expression of phosphorylated eNOS at Ser^{1177}, but upregulation in the protein expression of phospho-eNOS at Thr^{495}. Vit C treatment prevented these Dox-mediated changes (Fig. 5, *B* and *C*) in the differential expression of the eNOS.

The dimerized form of eNOS is critical in the function of the enzyme for the synthesis of NO. The ratio of eNOS monomer/dimer was observed to be higher in Dox-treated cardiomyocytes compared with control (Fig. 5*D*). Vit C protected against Dox-induced uncoupling of eNOS by reducing the ratio of monomeric to dimeric form (Fig. 5*D*).

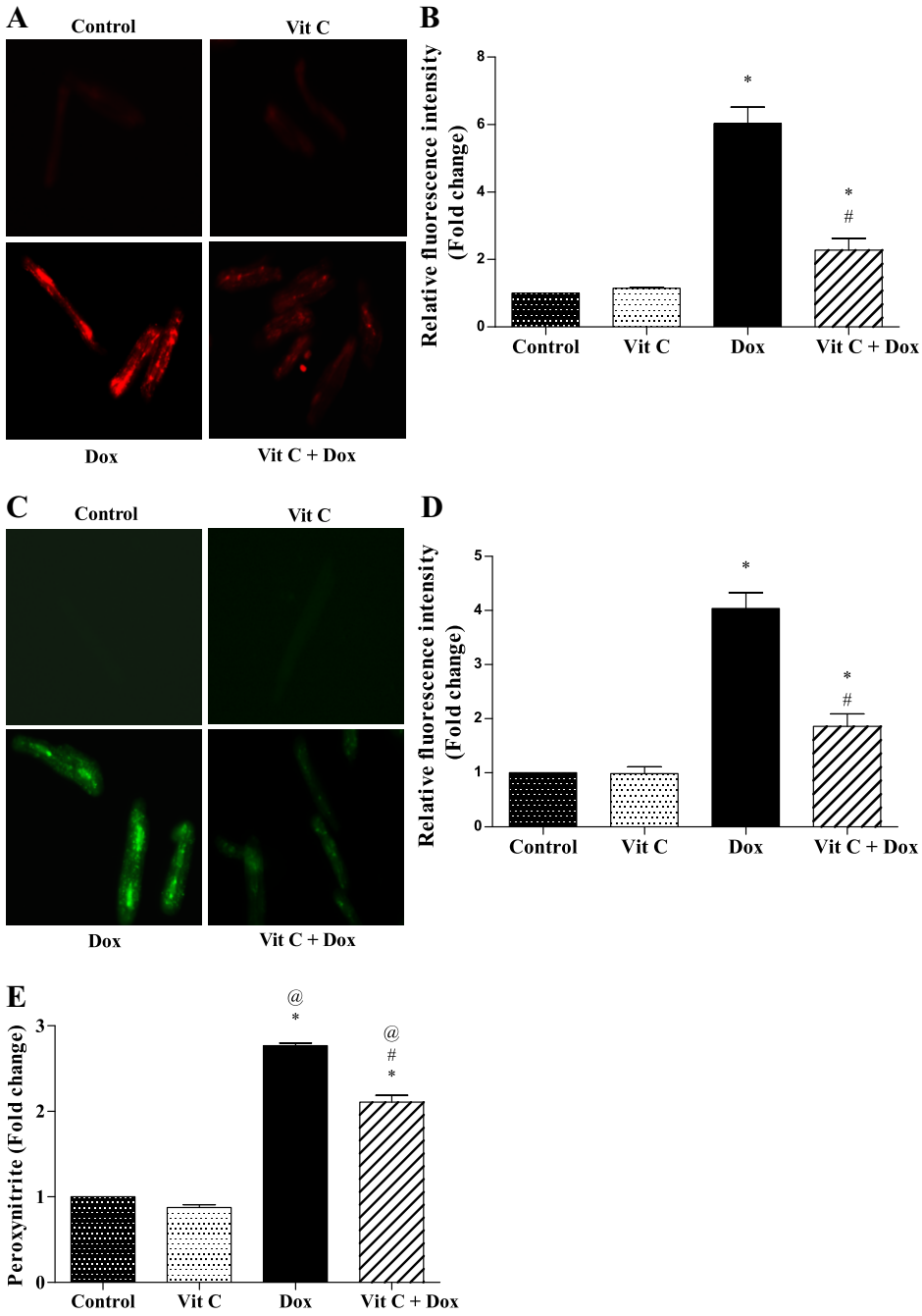

Fig. 2. Effects of different treatments on production of superoxide and peroxynitrite radicals in cardiomyocytes. *A*: DHE fluorescence images for superoxide detection in control, Vit C, Dox, and Vit C+ Dox (400×). *B*: semiquantitative image analysis of superoxide fluorescence intensity. *C*: HPF fluorescence images for peroxynitrite in control, Vit C, Dox, and Vit C+ Dox (400×). *D*: semiquantitative image analysis of peroxynitrite fluorescence intensity. *E*: peroxynitrite levels as fold change in arbitrary units compared with control. Data are expressed as relative fluorescence units and are mean ± SE from 3 individual experiments done in duplicate. $^{*}P < 0.001$ compared with control; $^{@}P < 0.001$ compared with Vit C; $^{\#}P < 0.001$ compared with Dox.

Protein Modifications

NO exerts its biological action via modification of proteins by forming NO adducts at its cysteine residue (nitrosylation) or tyrosine residue (nitration). Therefore, we determined the effect of Dox and Vit C on protein modifications caused by protein *S*-nitrosylation or protein nitrotyrosine (NT) formation. There was a significant ($P < 0.001$) increase in total nitrosylated proteins by Dox treatment. Vit C pretreatment showed a reduction in the levels of protein nitrosylation induced by Dox (Fig. 6*A*). Dox treatment increased the levels of protein NT by ~1.6 times compared with control. Vit C pretreatment in Dox-treated cardiomyocytes significantly reduced the protein NT levels upregulated by Dox (Fig. 6*B*). Vit C alone had no change in the levels of protein nitration or nitrosylation.

Cytokine Analysis

After 24 h of treatment, Dox caused a significant increase in the levels of TNF-α. Vit C pretreatment prevented Dox-induced upregulation of TNF-α. In fact the levels of TNF-α in the Vit C + Dox group were significantly lower even from the control levels. Vit C alone did not cause any difference in the levels of TNF-α compared with control (Fig. 7*A*). On the other hand, Dox treatment significantly reduced the levels of anti-inflammatory cytokine IL-10 compared with control. Vit C

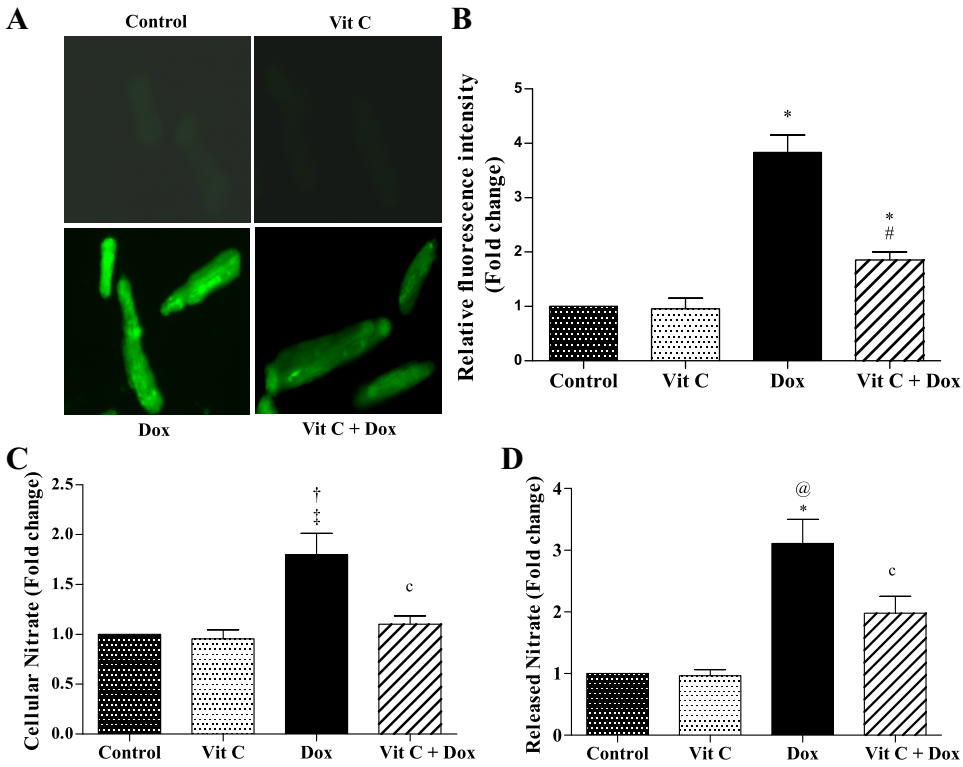

Fig. 3. Changes in nitric oxide levels in cardiomyocytes. *A*: DCF-2DA fluorescence images for nitric oxide radicals. *B*: semiquantitative image analysis of nitric oxide fluorescence intensity represented as relative fluorescence. Cardiomyocyte (*C*) as well as released (*D*) nitrate levels. Data are mean ± SE from 3 individual experiments in duplicate. $^{*}P < 0.001$ compared with control; $^{\dagger}P < 0.01$ compared with control; $^{@}P < 0.001$ compared with Vit C; $^{\ddagger}P < 0.01$ compared with Vit C; $^{c}P < 0.05$ compared with Dox; $^{\#}P < 0.001$ compared with Dox.

pretreatment upregulated the levels of IL-10 in Dox-treated cardiomyocytes (Fig. 7*B*). Dox treatment caused an almost threefold increase in the ratio of TNF-α to IL-10 compared with control. Vit C treatment significantly lowered this ratio in the Dox + Vit C group (Fig. 7*C*).

DISCUSSION

It is known that plasma levels of Vit C are decreased in cancer patients and are also lowered by chemotherapy drugs (32, 49). A reduction in oxidative stress and improved antioxidant enzyme activities was observed in breast cancer patients under chemotherapy with the use of Vit C (34, 45). A randomized trial is under way for adding antioxidants to chemotherapy regime in newly diagnosed ovarian cancer patients (9). However, clinical trials using Vit C or other antioxidants have provided inconclusive results. This inconsistency may be due to variations in the dose and duration of the treatment, end points evaluated, and the patient population involved. In this regard, the time of administration of Vit C is critical (11). Moreover, most of the studies have investigated these effects in already sick or at-risk populations, where extensive damage may have already occurred. Therefore, it is crucial to understand if prophylactic treatment with Vit C can be cardioprotective in reducing Dox-mediated cardiac insults, which has been the objective of our study. The recommended dietary allowance of 60 mg/day results in the plasma concentration of 24 μM of Vit C. Furthermore, a dose of up to 2 g/day did not show any deleterious effects (6). Thus the concentration of 25 μM used in our study can be easily reached in patients.

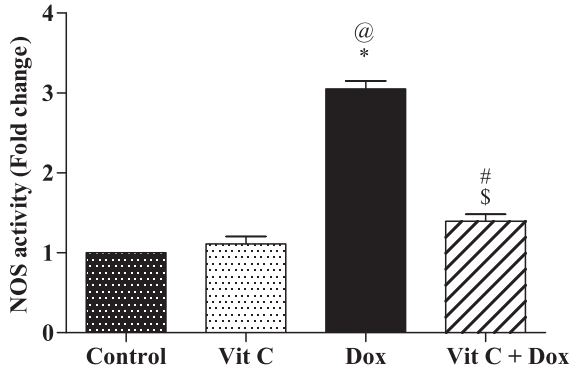

Fig. 4. Total nitric oxide synthase (NOS) activity in cardiomyocytes from different treatment groups. Data are means ± SE from 3 individual experiments done in duplicate. $^{*}P < 0.001$ compared with control; $\$P < 0.05$ compared with control; $^{@}P < 0.001$ compared with Vit C; $^{\#}P < 0.001$ compared with Dox.

In our previous study, we evaluated the effect of Vit C on Dox-induced changes in oxidative stress and signaling proteins (p38, p53, and ASK-1). It was reported that Dox-mediated upregulation of oxidative stress as well as signaling proteins was reduced by Vit C (28). The present study demonstrates the cardioprotective role of Vit C in attenuating nitrosative stress, modulation of various NOS isoforms, as well as release of cytokines in an in vitro model of Dox-induced cardiotoxicity using adult rat cardiomyocytes. Dox increased the levels of peroxynitrite as a result of enhanced NO levels as well as specific alterations in iNOS and eNOS activities. These effects of Dox were accompanied by increased protein nitration and nitrosylation as well as TNF-α generation. Pretreatment with Vit C reduced nitrosative stress mainly as a result of downregulation of TNF-α and iNOS activation. Additionally, Vit C prevented eNOS uncoupling, thereby reducing its monomeric form and thus impeding the production of peroxynitrite.

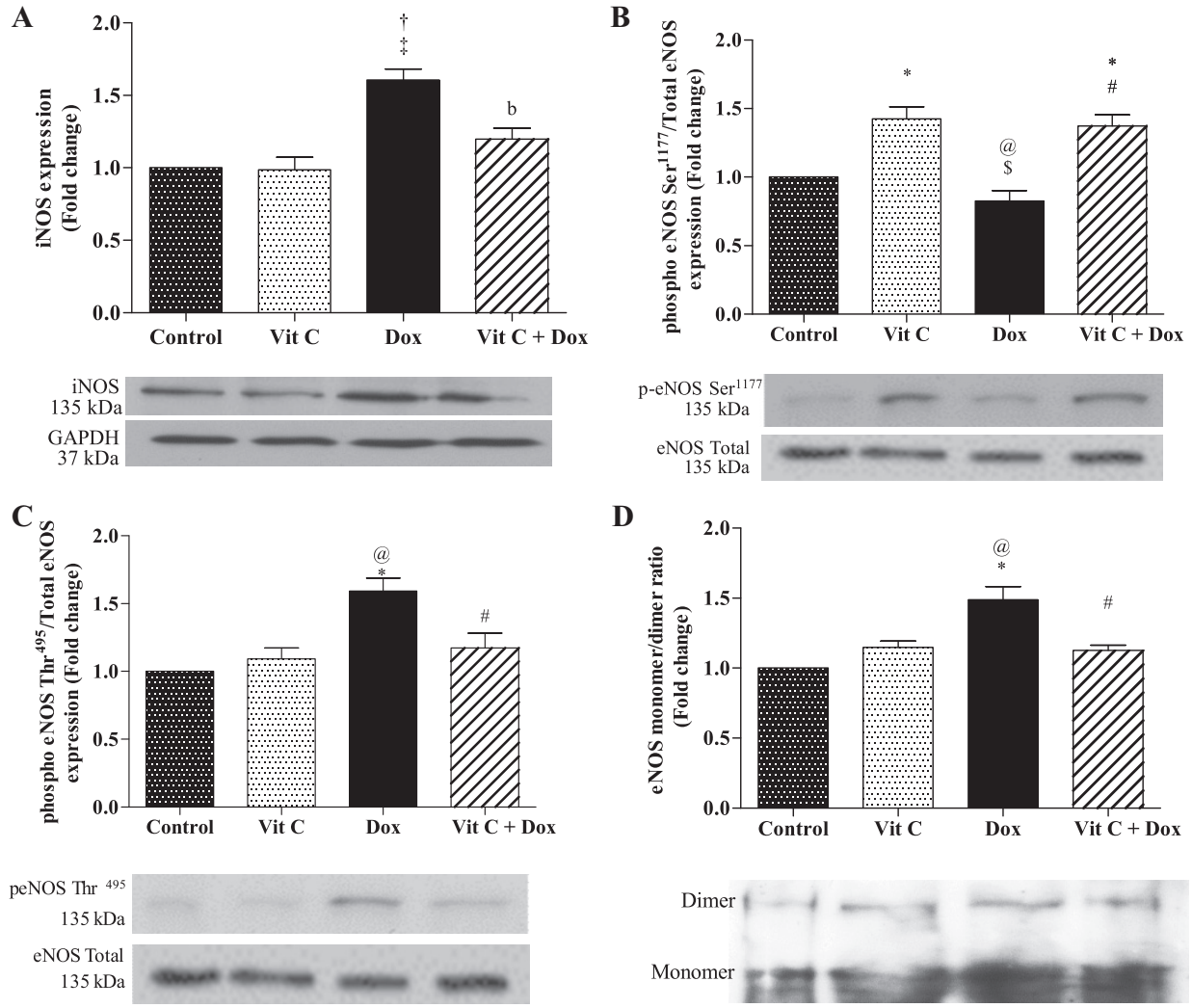

Fig. 5. Protein expression of nitric oxide synthase isoforms in different treatment groups. Densitometric analysis of protein expression of inducible nitric oxide synthase (iNOS) (*A*); phosphorylated forms of endothelial nitric oxide synthase (eNOS) at serine (Ser) 1177 (*B*) and threonine (Thr) 495 (*C*); and monomeric/dimeric forms of eNOS (*D*) in cardiomyocyte lysate using low temperature SDS-PAGE. iNOS was normalized by using GAPDH as the loading control while total eNOS protein expression was used to normalize phosphorylated eNOS. *Bottom* panels: Western blot for respective proteins. Data are means ± SE from 3 individual experiments done in duplicate. $^{*}P < 0.001$ compared with control; †$P < 0.01$ compared with control; \$$P < 0.05$ compared with control; $^{@}P < 0.001$ compared with Vit C; ‡$P < 0.01$ compared with Vit C; #$P < 0.001$ compared with Dox; $^{b}P < 0.01$ compared with Dox.

Vit C Improves Survival of Cardiomyocytes via Reduction in Dox-Induced Nitrosative Stress

We observed diminution of cardiomyocyte viability in Dox-treated group as has also been previously reported (2, 27, 28). Dox is associated with the activation of various cell death pathways such as apoptosis and necrosis. Increased levels of peroxynitrite and $O_2^{\cdot -}$ act as a trigger for initiation of apoptosis via changes in mitochondrial membrane permeability and opening of mitochondrial permeability transition pore (35) as well as the activation of caspase-3 and PARP (18, 23). In addition to apoptosis, peroxynitrite also triggers necrotic cell death (18). The involvement of peroxynitrite in cell death is evident through studies with peroxynitrite scavengers resulting in enhanced cardiomyocyte viability (35). We observed that Vit C pretreatment increased the viability of Dox-treated cardiomyocytes, and we propose that this improved viability is due to abatement of peroxynitrite as well as $O_2^{\cdot -}$ levels and further cellular insults. Recently it has been reported that Tempol, another water-soluble antioxidant, reduces Dox-induced oxidative stress as well as apoptosis (4).

In the present study, Dox caused an increased generation of peroxynitrite as a result of enhanced $O_2^{\cdot -}$ and NO production. Although the initial trigger for the generation of peroxynitrite is through increased $O_2^{\cdot -}$ generation via upregulation of NADPH oxidase (13, 38), further stimulation is triggered through TNFα and IL-1β mediated upregulation of iNOS (13) as well as eNOS uncoupling (3, 7). Peroxynitrite not only exacerbates the toxicity by direct oxidation of DNA, proteins, and lipids, but also inhibits the activity of antioxidant enzymes (17) and accelerates the production of ROS such as $O_2^{\cdot -}$ (7). Additionally, high levels of peroxynitrite lead to nitration and nitrosylation of a variety of proteins, thereby altering its function and downstream signal transduction (33, 50). Our results indicated extensive nitration and nitrosylation of a large number of proteins in Dox-treated cardiomyocytes. Although

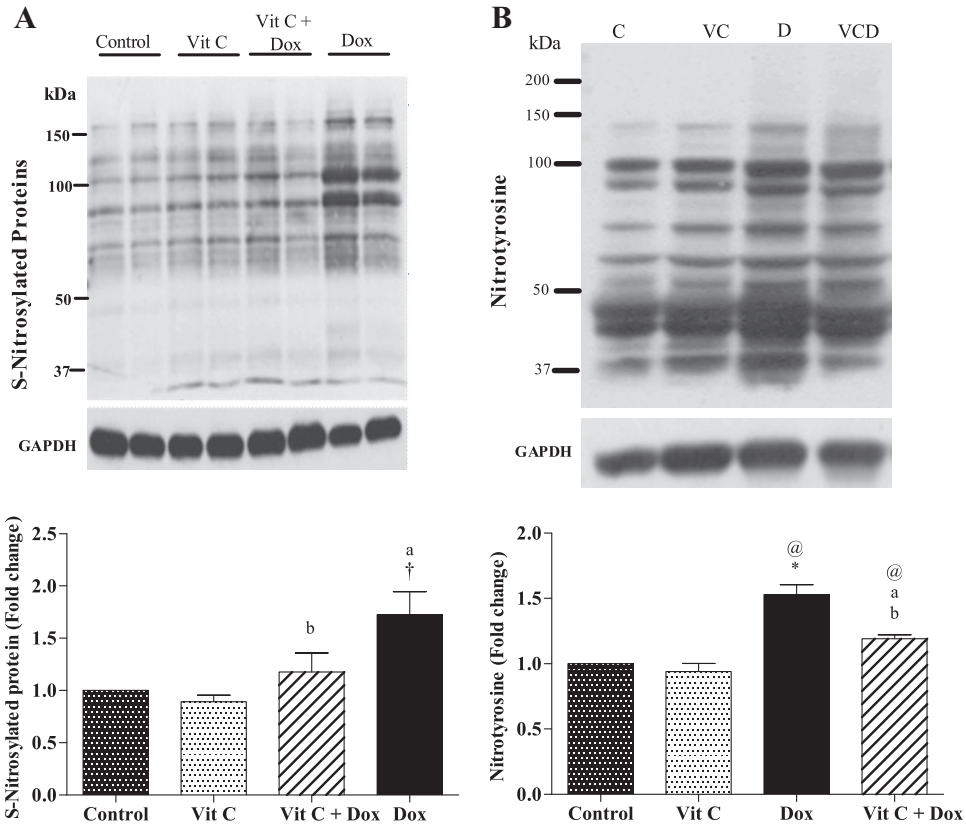

Fig. 6. Protein modifications in terms of nitrosylation and nitration in cardiomyocytes in different treatment groups. Western blots: *S*-nitrosylation (*A*) and nitration (*B*) of proteins in control and treated cardiomyocytes. GAPDH was used as loading control. *Bottom* panels: densitometric analysis. Data are expressed as means ± SE from 3 individual experiments done in duplicate. $^{*}P < 0.001$ compared with control; $^{\dagger}P < 0.01$ compared with control; $^{@}P < 0.001$ compared with Vit C; $^{a}P < 0.05$ compared with Vit C; $^{b}P < 0.01$ compared with Dox.

we have not identified the involvement of specific proteins, various studies have shown that nitration/nitrosylation of cardiac myofibrils, apoptosis, inflammation as well as calcium handling proteins resulted in ventricular dysfunction (19, 33, 50). However, the protein modifications are dependent on redox state of the cell and can be prevented or reversed in the presence of antioxidants (5, 40). Here we observed that Vit C pretreatment reduced Dox-mediated protein nitration and nitrosylation. Our data suggest that Vit C-mediated reduction in nitrosative stress is manifold: one through a reduction in generation of $O_2^{\cdot -}$ through a decrease in monomeric eNOS; and the second is via simultaneous reduction in generation of NO via downregulation of iNOS activation.

Vit C Reduced Activation of Dox-Mediated iNOS Activation and Inflammatory Cytokines

Excess generation of NO in the high micromolar range through an activation of iNOS is implicated in various pathological conditions, including heart failure (1). iNOS-mediated enhanced NO levels for prolonged period of time are responsible for enhanced peroxynitrite formation as well as protein modification by *S*-nitrosylation and nitration. Studies with genetic and pharmacological modulation of iNOS have highlighted the role of iNOS in the pathogenesis of heart failure (25). In the present study mitigation of the Dox-induced increase in iNOS by Vit C treatment lends support to these

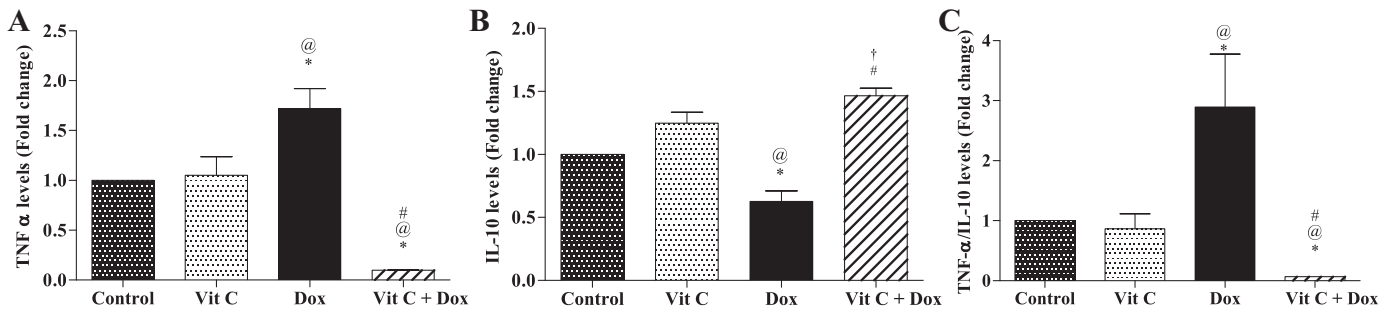

Fig. 7. Levels of TNF α and IL-10 released from cardiomyocytes in different treatment groups. Change in the release of proinflammatory cytokine TNF-α (*A*); anti-inflammatory cytokine, IL-10 (*B*); and ratio of TNFα/IL-10 released from control and treated cardiomyocytes. Data are means ± SE from 3 individual experiments done in duplicate. $^{*}P < 0.001$ compared with control; $^{\dagger}P < 0.01$ compared with control; $^{@}P < 0.001$ compared with Vit C; $^{\#}P < 0.001$ compared with Dox.

observations. Disruption of iNOS has been shown to reduce protein nitrosylation (42) as well as improve cardiac function (12) via reduction in cardiac NO and thereby peroxynitrite formation. Upregulation of iNOS has been previously reported in the progression of Dox-induced cardiotoxicity (2, 35, 37). We observed an upregulation of iNOS in Dox-treated group. The increase in NO levels and total NOS activity observed in Dox-treated cardiomyocytes in this study can be pinned down to upregulation of iNOS protein expression and not via eNOS which is rendered inactive by the interplay of phosphorylation at the Thr495 and Ser1177 sites. This upregulation and activation of iNOS has been reported to be triggered as a result of increased levels of TNF-α and IL-1 (46). We did observe a Dox-induced increase in TNF-α in this study. Despite the conflicting evidence for the levels of different cytokines in Dox-treated hearts (2, 26, 53), the need to maintain a balance between TNF-α and IL-10 in pathological condition is crucial (20). Our results demonstrated differential changes in the levels of TNF-α and IL-10 in Dox-treated cardiomyocytes as evident by an increase in TNF-α and reduction in IL-10. We do not have a good explanation for the enhanced Vit C effect on the TNF- α levels induced by Dox. One possibility could be that Dox-induced stress in Vit C + Dox may result in overshooting the mark.

Vit C Enhances eNOS Stabilization in Its Dimeric Form

As NOS are stable in their dimeric state, maintaining the dimeric form of eNOS is crucial for its enzymatic activity. Nevertheless, dissociation of the dimeric form into monomeric form under the conditions of oxidative stress or cofactor oxidation can lead to uncoupling of enzyme, resulting in production of $O_2^{\cdot -}$ instead of NO. Despite iNOS being a more potent source of NO and $O_2^{\cdot -}$ than eNOS, uncoupling of eNOS is implicated in many pathological conditions as a result of its inability to produce physiological levels of eNOS-derived NO required for cardiovascular functions (14). Uncoupling of eNOS is associated with the progression of many pathological conditions such as endothelial dysfunction, diabetes, obesity, and aging (10, 16, 51). Although the mechanism for uncoupling of eNOS is not clearly understood, it can be a consequence of monomerization of eNOS as a result of oxidation of the cofactor of NOS. Using low-temperature nonreducing Western blot, we observed an enhanced disruption of the dimeric form of eNOS into monomeric subunits upon Dox treatment. Compromised dimer binding can be as a result of increased oxidation of cofactor involved in binding of two monomers (10) or enhanced generation of NO adduct at cysteine residue of eNOS, resulting in disruption of dimer stability (40). Moreover, maintaining the redox potential through upregulation of thioredoxin reductase resulted in protection of eNOS dimer and the restoration of enzyme activity (40). In our study, Vit C pretreatment in Dox-treated cardiomyocytes stabilized eNOS, leading to reduced dissociation of the dimer and thus preventing eNOS uncoupling. Our results highlight that protecting eNOS uncoupling impedes further amplification of generation of $O_2^{\cdot -}$ and thus peroxynitrite in Dox-treated cardiomyocytes. Thus reduction of oxidative/nitrosative stress by Vit C can be one of the key mechanisms involved in Vit C-mediated protection against eNOS uncoupling.

Our data further inform the differential regulation of eNOS in Dox-treated cardiomyocytes. Since eNOS is compartmentalized in plasmalemma caveoli (31) in proximity to the sarcoplasmic reticulum, its target for effective signaling, eNOS-derived NO in physiological levels plays an important role in cardiomyocyte contraction (15, 54). Dox treatment caused an increase in phosphorylation at Thr^{495} but reduced the expression of Ser^{1177} phosphorylation. Since eNOS is constitutively expressed, the activity of the enzyme is regulated by differential phosphorylation of its activating or inhibitory site. Phosphorylation of eNOS at Ser^{1177} enhances the enzyme activity, whereas phosphorylation at Thr^{495} inactivates the enzyme. This phosphorylation of eNOS is regulated through various kinases and phosphatases such as Akt, AMP activated protein kinase (AMPK), protein kinase C (PKC), and protein phosphatase 2A (PP2A) (21). Under nonstimulated conditions, Thr^{495} is phosphorylated, whereas stimulation by estrogen, VEGF, or bradykinin activates eNOS through phosphorylation at Ser^{1177} through Akt/AMPK (15). Downregulation of Akt and AMPK has been reported in Dox-treated cardiomyocytes (27, 43). These Dox-mediated changes in eNOS phosphorylation were reversed by Vit C. Vit C pretreatment increased the phosphorylation of eNOS at Ser^{1177} while it reduced Thr^{495} phosphorylation in Dox-treated cardiomyocytes. Similarly, phosphorylation of eNOS at Ser^{1177} is observed to be upregulated by oleuropein (2). We suggest that the Vit C-mediated increase in phosphorylation of Ser^{1177} may be the result of Akt or AMPK upregulation (2, 28) or reduced dephosphorylation through an inhibition of PP2A (21).

In conclusion, our findings demonstrate for the first time that Vit C provides a protective effect in reducing nitrosative stress in Dox-induced cardiotoxicity via reduction in NO, $O_2^{\cdot -}$, iNOS activation, and decrease in proinflammatory cytokine. We also documented that Vit C stabilized eNOS and modulated the expression of NOS isoforms altered by Dox. These results highlight the beneficial effects of Vit C at the molecular level in the mitigation of Dox-induced cardiotoxicity.

GRANTS

This study was supported by operating grants from the Canadian Institutes of Health Research and Research Manitoba. G. Akolkar is supported by Bank of Montreal studentship. P. K. Singal is the holder of the Naranjan Dhalla Chair in Cardiovascular Research supported by St. Boniface Hospital Research Foundation.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

G.A. and P.K.S., conception and design of study; G.A., A.K.B., and P.A. performed experiments; G.A., A.K.B., P.A., D.S.J., and P.K.S. analyzed and interpreted results; G.A. and P.K.S. drafted manuscript; G.A., A.K.B., P.A., D.S.J., and P.K.S. edited and revised manuscript; G.A., A.K.B., P.A., D.S.J., and P.K.S. approved the final version of the manuscript.

REFERENCES

1. **Aldieri E, Bergandi L, Riganti C, Costamagna C, Bosia A, Ghigo D.** Doxorubicin induces an increase of nitric oxide synthesis in rat cardiac cells that is inhibited by iron supplementation. *Toxicol Appl Pharmacol* 185: 85–90, 2002. doi:10.1006/taap.2002.9527.
2. **Andreadou I, Mikros E, Ioannidis K, Sigala F, Naka K, Kostidis S, Farmakis D, Tenta R, Kavantzas N, Bibli SI, Gikas E, Skaltsounis L, Kremastinos DT, Iliodromitis EK.** Oleuropein prevents doxorubicin-induced cardiomyopathy interfering with signaling molecules and cardiomyocyte metabolism. *J Mol Cell Cardiol* 69: 4–16, 2014. doi:10.1016/j.yjmcc.2014.01.007.

3. **Angsutararux P, Luanpitpong S, Issaragrisil S.** Chemotherapy-induced cardiotoxicity: overview of the roles of oxidative stress. *Oxid Med Cell Longev* 2015: 795602, 2015. doi:10.1155/2015/795602.
4. **Ayaz S, Bhandari U, Pillai KK.** Tempol ameliorates oxidative stress, apoptosis in Doxorubicin induced cardiotoxicity. *Adv Appl Pharm Sci J* 1: 30–38, 2016.
5. **Beltrán B, Orsi A, Clementi E, Moncada S.** Oxidative stress and S-nitrosylation of proteins in cells. *Br J Pharmacol* 129: 953–960, 2000. doi:10.1038/sj.bjp.0703147.
6. **Carr AC, Vissers MC.** Synthetic or food-derived vitamin C–are they equally bioavailable? *Nutrients* 5: 4284–4304, 2013. doi:10.3390/nu5114284.
7. **Cassuto J, Dou H, Czikora I, Szabo A, Patel VS, Kamath V, Belin de Chantemele E, Feher A, Romero MJ, Bagi Z.** Peroxynitrite disrupts endothelial caveolae leading to eNOS uncoupling and diminished flow-mediated dilation in coronary arterioles of diabetic patients. *Diabetes* 63: 1381–1393, 2014. doi:10.2337/db13-0577.
8. **Chaiswing L, Cole MP, Ittarat W, Szweda LI, St Clair DK, Oberley TD.** Manganese superoxide dismutase and inducible nitric oxide synthase modify early oxidative events in acute adriamycin-induced mitochondrial toxicity. *Mol Cancer Ther* 4: 1056–1064, 2005. doi:10.1158/1535-7163.MCT-04-0322.
9. **Drisko JA, Chapman J, Hunter VJ.** The use of antioxidants with first-line chemotherapy in two cases of ovarian cancer. *J Am Coll Nutr* 22: 118–123, 2003. doi:10.1080/07315724.2003.10719284.
10. **d'Uscio LV, Milstien S, Richardson D, Smith L, Katusic ZS.** Long-term vitamin C treatment increases vascular tetrahydrobiopterin levels and nitric oxide synthase activity. *Circ Res* 92: 88–95, 2003. doi:10.1161/01.RES.0000049166.33035.62.
11. **Farbstein D, Kozak-Blickstein A, Levy AP.** Antioxidant vitamins and their use in preventing cardiovascular disease. *Molecules* 15: 8098–8110, 2010. doi:10.3390/molecules15118098.
12. **Feng Q, Lu X, Jones DL, Shen J, Arnold JM.** Increased inducible nitric oxide synthase expression contributes to myocardial dysfunction and higher mortality after myocardial infarction in mice. *Circulation* 104: 700–704, 2001. doi:10.1161/hc3201.092284.
13. **Ferdinandy P, Danial H, Ambrus I, Rothery RA, Schulz R.** Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. *Circ Res* 87: 241–247, 2000. doi:10.1161/01.RES.87.3.241.
14. **Förstermann U, Münzel T.** Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation* 113: 1708–1714, 2006. doi:10.1161/CIRCULATIONAHA.105.602532.
15. **Förstermann U, Sessa WC.** Nitric oxide synthases: regulation and function. *Eur Heart J* 33: 829–837, 2012. doi:10.1093/eurheartj/ehr304.
16. **Gamez-Mendez AM, Vargas-Robles H, Ríos A, Escalante B.** Oxidative Stress-Dependent Coronary Endothelial Dysfunction in Obese Mice. *PLoS One* 10: e0138609, 2015. doi:10.1371/journal.pone.0138609.
17. **Han YJ, Kwon YG, Chung HT, Lee SK, Simmons RL, Billiar TR, Kim YM.** Antioxidant enzymes suppress nitric oxide production through the inhibition of NF-kappa B activation: role of H(2)O(2) and nitric oxide in inducible nitric oxide synthase expression in macrophages. *Nitric Oxide* 5: 504–513, 2001. doi:10.1006/niox.2001.0367.
18. **Islam BU, Habib S, Ahmad P, Allarakha S, Moinuddin, Ali A.** Pathophysiological Role of Peroxynitrite Induced DNA Damage in Human Diseases: A Special Focus on Poly(ADP-ribose) Polymerase (PARP). *Indian J Clin Biochem* 30: 368–385, 2015. doi:10.1007/s12291-014-0475-8.
19. **Iwakiri Y.** S-nitrosylation of proteins: a new insight into endothelial cell function regulated by eNOS-derived NO. *Nitric Oxide* 25: 95–101, 2011. doi:10.1016/j.niox.2011.04.014.
20. **Khaper N, Bryan S, Dhingra S, Singal R, Bajaj A, Pathak CM, Singal PK.** Targeting the vicious inflammation-oxidative stress cycle for the management of heart failure. *Antioxid Redox Signal* 13: 1033–1049, 2010. doi:10.1089/ars.2009.2930.
21. **Ladurner A, Schmitt CA, Schachner D, Atanasov AG, Werner ER, Dirsch VM, Heiss EH.** Ascorbate stimulates endothelial nitric oxide synthase enzyme activity by rapid modulation of its phosphorylation status. *Free Radic Biol Med* 52: 2082–2090, 2012. doi:10.1016/j.freeradbiomed.2012.03.022.
22. **Lefrak EA, Pitha J, Rosenheim S, Gottlieb JA.** A clinicopathologic analysis of adriamycin cardiotoxicity. *Cancer* 32: 302–314, 1973. doi:10.1002/1097-0142(197308)32:2<302::AID-CNCR2820320205>3.0.CO;2-2.
23. **Levrand S, Vannay-Bouchiche C, Pesse B, Pacher P, Feihl F, Waeber B, Liaudet L.** Peroxynitrite is a major trigger of cardiomyocyte apoptosis in vitro and in vivo. *Free Radic Biol Med* 41: 886–895, 2006. doi:10.1016/j.freeradbiomed.2006.04.034.
24. **Li T, Singal PK.** Adriamycin-induced early changes in myocardial antioxidant enzymes and their modulation by probucol. *Circulation* 102: 2105–2110, 2000. doi:10.1161/01.CIR.102.17.2105.
25. **Liu VW, Huang PL.** Cardiovascular roles of nitric oxide: a review of insights from nitric oxide synthase gene disrupted mice. *Cardiovasc Res* 77: 19–29, 2008. doi:10.1016/j.cardiores.2007.06.024.
26. **Lou H, Danelisen I, Singal PK.** Cytokines are not upregulated in adriamycin-induced cardiomyopathy and heart failure. *J Mol Cell Cardiol* 36: 683–690, 2004. doi:10.1016/j.yjmcc.2004.03.004.
27. **Lou Y, Wang Z, Xu Y, Zhou P, Cao J, Li Y, Chen Y, Sun J, Fu L.** Resveratrol prevents doxorubicin-induced cardiotoxicity in H9c2 cells through the inhibition of endoplasmic reticulum stress and the activation of the Sirt1 pathway. *Int J Mol Med* 36: 873–880, 2015. doi:10.3892/ijmm.2015.2291.
28. **Ludke A, Sharma AK, Bagchi AK, Singal PK.** Subcellular basis of vitamin C protection against doxorubicin-induced changes in rat cardiomyocytes. *Mol Cell Biochem* 360: 215–224, 2012. doi:10.1007/s11010-011-1059-z.
29. **Ludke AR, Al-Shudiefat AA, Dhingra S, Jassal DS, Singal PK.** A concise description of cardioprotective strategies in doxorubicin-induced cardiotoxicity. *Can J Physiol Pharmacol* 87: 756–763, 2009. doi:10.1139/Y09-059.
30. **Ludke AR, Sharma AK, Akolkar G, Bajpai G, Singal PK.** Downregulation of vitamin C transporter SVCT-2 in doxorubicin-induced cardiomyocyte injury. *Am J Physiol Cell Physiol* 303: C645–C653, 2012. doi:10.1152/ajpcell.00186.2012.
31. **Massion PB, Feron O, Dessy C, Balligand JL.** Nitric oxide and cardiac function: ten years after, and continuing. *Circ Res* 93: 388–398, 2003. doi:10.1161/01.RES.0000088351.58510.21.
32. **Mayland CR, Bennett MI, Allan K.** Vitamin C deficiency in cancer patients. *Palliat Med* 19: 17–20, 2005. doi:10.1191/0269216305pm970oa.
33. **Mihm MJ, Yu F, Weinstein DM, Reiser PJ, Bauer JA.** Intracellular distribution of peroxynitrite during doxorubicin cardiomyopathy: evidence for selective impairment of myofibrillar creatine kinase. *Br J Pharmacol* 135: 581–588, 2002. doi:10.1038/sj.bjp.0704495.
34. **Mikirova N, Casciari J, Riordan N, Hunninghake R.** Clinical experience with intravenous administration of ascorbic acid: achievable levels in blood for different states of inflammation and disease in cancer patients. *J Transl Med* 11: 191, 2013. doi:10.1186/1479-5876-11-191.
35. **Mukhopadhyay P, Rajesh M, Bátkai S, Kashiwaya Y, Haskó G, Liaudet L, Szabó C, Pacher P.** Role of superoxide, nitric oxide, and peroxynitrite in doxorubicin-induced cell death in vivo and in vitro. *Am J Physiol Heart Circ Physiol* 296: H1466–H1483, 2009. doi:10.1152/ajpheart.00795.2008.
36. **Octavia Y, Tocchetti CG, Gabrielson KL, Janssens S, Crijns HJ, Moens AL.** Doxorubicin-induced cardiomyopathy: from molecular mechanisms to therapeutic strategies. *J Mol Cell Cardiol* 52: 1213–1225, 2012. doi:10.1016/j.yjmcc.2012.03.006.
37. **Oktem G, Uysal A, Oral O, Sezer ED, Olukman M, Erol A, Akgur SA, Bilir A.** Resveratrol attenuates doxorubicin-induced cellular damage by modulating nitric oxide and apoptosis. *Exp Toxicol Pathol* 64: 471–479, 2012. doi:10.1016/j.etp.2010.11.001.
38. **Pacher P, Obrosova IG, Mabley JG, Szabó C.** Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies. *Curr Med Chem* 12: 267–275, 2005. doi:10.2174/0929867053363207.
39. **Raj S, Franco VI, Lipshultz SE.** Anthracycline-induced cardiotoxicity: a review of pathophysiology, diagnosis, and treatment. *Curr Treat Options Cardiovasc Med* 16: 315, 2014. doi:10.1007/s11936-014-0315-4.
40. **Ravi K, Brennan LA, Levic S, Ross PA, Black SM.** S-nitrosylation of endothelial nitric oxide synthase is associated with monomerization and decreased enzyme activity. *Proc Natl Acad Sci USA* 101: 2619–2624, 2004. doi:10.1073/pnas.0300464101.
41. **Rochette L, Guenancia C, Gudjoncik A, Hachet O, Zeller M, Cottin Y, Vergely C.** Anthracyclines/trastuzumab: new aspects of cardiotoxicity and molecular mechanisms. *Trends Pharmacol Sci* 36: 326–348, 2015. doi:10.1016/j.tips.2015.03.005.
42. **Ropelle ER, Pauli JR, Cintra DE, da Silva AS, De Souza CT, Guadagnini D, Carvalho BM, Caricilli AM, Katashima CK, Carvalho-Filho MA, Hirabara S, Curi R, Velloso LA, Saad MJ, Carvalheira JB.** Targeted disruption of inducible nitric oxide synthase protects against

aging, S-nitrosation, and insulin resistance in muscle of male mice. *Diabetes* 62: 466–470, 2013. doi:10.2337/db12-0339.

43. **Sharma AK, Ludke AR, Bagchi AK, Al-Shudiefat AA, Akolkar G, Jassal DS, Singal PK.** Phosphorylation of AktSer473/Ask1Ser83 in Trolox-induced suppression of Doxorubicin-induced changes in rat cardiomyocytes. *Curr Res Cardiol* 1: 7–12, 2014. doi:10.4172/2368-0512.1000003.
44. **Singal PK, Iliskovic N.** Doxorubicin-induced cardiomyopathy. *N Engl J Med* 339: 900–905, 1998. doi:10.1056/NEJM199809243391307.
45. **Suhail N, Bilal N, Khan HY, Hasan S, Sharma S, Khan F, Mansoor T, Banu N.** Effect of vitamins C and E on antioxidant status of breast-cancer patients undergoing chemotherapy. *J Clin Pharm Ther* 37: 22–26, 2012. doi:10.1111/j.1365-2710.2010.01237.x.
46. **Treuer AV, Gonzalez DR.** Nitric oxide synthases, S-nitrosylation and cardiovascular health: from molecular mechanisms to therapeutic opportunities (review). *Mol Med Rep* 11: 1555–1565, 2015. doi:10.3892/mmr.2014.2968.
47. **Turko IV, Murad F.** Protein nitration in cardiovascular diseases. *Pharmacol Rev* 54: 619–634, 2002. doi:10.1124/pr.54.4.619.
48. **Vejpongsa P, Yeh ET.** Prevention of anthracycline-induced cardiotoxicity: challenges and opportunities. *J Am Coll Cardiol* 64: 938–945, 2014. doi:10.1016/j.jacc.2014.06.1167.
49. **Weijl NI, Hopman GD, Wipkink-Bakker A, Lentjes EG, Berger HM, Cleton FJ, Osanto S.** Cisplatin combination chemotherapy induces a fall in plasma antioxidants of cancer patients. *Ann Oncol* 9: 1331–1337, 1998. doi:10.1023/A:1008407014084.
50. **Weinstein DM, Mihm MJ, Bauer JA.** Cardiac peroxynitrite formation and left ventricular dysfunction following doxorubicin treatment in mice. *J Pharmacol Exp Ther* 294: 396–401, 2000.
51. **Yang YM, Huang A, Kaley G, Sun D.** eNOS uncoupling and endothelial dysfunction in aged vessels. *Am J Physiol Heart Circ Physiol* 297: H1829–H1836, 2009. doi:10.1152/ajpheart.00230.2009.
52. **Zhang J, Cui X, Yan Y, Li M, Yang Y, Wang J, Zhang J.** Research progress of cardioprotective agents for prevention of anthracycline cardiotoxicity. *Am J Transl Res* 8: 2862–2875, 2016.
53. **Zhu J, Zhang J, Zhang L, Du R, Xiang D, Wu M, Zhang R, Han W.** Interleukin-1 signaling mediates acute doxorubicin-induced cardiotoxicity. *Biomed Pharmacother* 65: 481–485, 2011. doi:10.1016/j.biopha.2011.06.005.
54. **Ziolo MT, Bers DM.** The real estate of NOS signaling: location, location, location. *Circ Res* 92: 1279–1281, 2003. doi:10.1161/01.RES.0000080783.34092.AF.