Transferrin Receptor-dependent Iron Uptake Is Responsible for Doxorubicin-mediated Apoptosis in Endothelial Cells

ROLE OF OXIDANT-INDUCED IRON SIGNALING IN APOPTOSIS

Received for publication, December 5, 2001, and in revised form, February 6, 2002 Published, JBC Papers in Press, February 20, 2002, DOI 10.1074/jbc.M111604200

Srigiridhar Kotamraju‡, Christopher R. Chitambar§, Shasi V. Kalivendi‡, Joy Joseph‡, and B. Kalyanaraman‡††

From the ¶Biophysics Research Institute and Free Radical Research Center and the §Division of Neoplastic Diseases, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

In the past, investigators have successfully used iron chelators to mitigate the cardiotoxicity of doxorubicin (DOX), a widely used anticancer drug that induces reactive oxygen species (ROS), oxidative damage, and apoptosis. Although intracellular iron plays a critical role in initiating DOX-induced apoptosis, the molecular mechanism(s) that link iron, ROS, and apoptosis are still unknown. In this study, we demonstrate that apoptosis results from the exposure of bovine aortic endothelial cells to DOX and that the apoptotic cell death is accompanied by a significant increase in cellular iron (55Fe) uptake and activation of iron regulatory protein-1. Furthermore, DOX-induced iron uptake was shown to be mediated by the transferrin receptor (TfR)-dependent mechanism. Treatment with the anti-TfR antibody (IgA class) dramatically inhibited DOX-induced apoptosis, iron uptake, and intracellular oxidant formation as measured by fluorescence using dichlorodihydrofluorescein. Treatment with cell-permeable iron chelators and ROS scavengers inhibited DOX-induced cellular 55Fe uptake, ROS formation, and apoptosis. Based on these findings, we conclude that DOX-induced iron signaling is regulated by the cell surface TfR expression, intracellular oxidant levels, and iron regulatory proteins. The implications of TfR-dependent iron transport in oxidant-induced apoptosis in endothelial cells are discussed.

Doxorubicin (DOX) or adriamycin is a popular antitumor drug that has been used to treat a variety of cancers including breast cancer and prostate cancer (1, 2). A major long term toxic effect of this drug is the development of cardiac damage (e.g. cardiomyopathy and heart failure) in cancer patients treated with DOX (3, 4). Endothelial dysfunction is an acute toxic side effect of DOX. One of the proposed mechanisms responsible for the acute and chronic toxicity is the formation of ROS formed from the redox activation of DOX (5–8). DOX, a quinone-containing drug, undergoes a one-electron reduction to a semiquinone intermediate that generates superoxide anion and hydrogen peroxide (9, 10). Several flavoprotein reductases, including endothelial nitric-oxide synthase, are capable of initiating the redox activation of DOX (11–13).

The possible involvement of iron in DOX-induced cardiotoxicity became evident from studies in which iron chelators (ICRF-187 or dexrazoxane) were shown to be cardioprotective (14, 15). It was postulated that iron needed to catalyze intracellular radical reactions originated from mitochondrial aconitase or ferritin, the intracellular iron storage protein (16–18). Both superoxide and DOX semiquinone were shown to release iron from aconitate or ferritin (16, 17). Recently, it was reported that apoptosis in endothelial cells and myocytes was induced by submicromolar concentrations of DOX (19, 20). Treatment with intracellular iron chelator inhibited DOX-induced apoptosis in neonatal myocytes (19). Thus, the intracellular iron was thought to play a major role in initiating DOX-induced apoptosis (19).

Previously it has been shown that cellular iron is regulated by the cell surface transferrin receptor (TfR)-mediated uptake of iron as transferrin iron (21, 22). Recent reports indicate that the cellular oxidative damage caused by ROS is critically controlled by cellular iron homeostasis (23–25). Exposure of murine fibroblasts to hydrogen peroxide decreased ferritin synthesis and enhanced the expression of TfR mRNA (24), implicating a possible link between oxidant generation and TfR-mediated iron regulation.

In this study, we tested the hypothesis that the TfR-dependent uptake of iron is responsible for DOX-induced apoptosis in endothelial cells. The results from this study show that an anti-TfR monoclonal antibody (42/6) dramatically blocked apoptosis in endothelial cells, suggesting that the TfR-dependent influx of extracellular iron is responsible for mediating DOX-induced apoptosis. DOX-induced iron uptake was inhibited by the pretreatment of cells with cell-permeable antioxidants and iron chelators. The biological implications of oxidant-induced iron signaling in endothelial cells are discussed.

EXPERIMENTAL PROCEDURES

Materials—α-Pheny1-tert-butyl nitro (PB), 2-phenyl-1,2-benziselenazol-3-zH1-one (ebselen), doxorubicin (DOX), and deferral (or also deferoxamine) were obtained from Sigma. HBED and dexrazoxane (ICRF-187) were gifts from Dr. Cherakuri Muralikrishna (National...
Cancer Institute, National Institutes of Health). Dihydroethiodium and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Molecular Probes Inc. Fe(III) tetrakis (4-benzoic acid) porphyrin (FeTBAP) and Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) were synthesized according to published methods (26). Monoclonal antibody against human TfR was a gift from Dr. Ian Trowbridge (Salk Institute, San Diego, CA).

**Endothelial Cell Culture**—BAEC were obtained from Clonetics. The cells were obtained at the third passage, transferred to 75-cm² filter vent flasks (Costar, Cambridge, MA), grown to confluence (5.2 × 10⁶ cells/75 cm²) in Dulbeco’s modified Eagle’s medium containing 10% fetal calf serum, L-glutamine (4 mmol/liter), penicillin (100 units/ml), and streptomycin (100 µg/ml), and incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. On the day of the treatment, the medium was replaced with Dulbecco’s modified Eagle’s medium containing 2% FBS, which contains 25–30 µg transferrin/ml. The above condition was applied to all of the experiments performed in this study. The cells were passaged as described by Balla et al. (27) and used between passages 4 and 12.

**Measurement of ⁵⁵Fe Uptake in Endothelial Cells**—Bovine aortic endothelial cells were grown in Dulbecco’s modified Eagle’s medium containing 10% FBS until confluence. On the day of the treatment, the medium was replaced with Dulbecco’s modified Eagle’s medium containing 2% FBS, and the cells were allowed to adjust to the medium conditions 2–3 h. Then, 0.1 Ci of ⁵⁵Fe (ferric chloride) was added to the medium for 0–16 h. An aliquot of medium was taken to measure the label in the medium. The cells were washed twice with Dulbecco’s phosphate-buffered saline (DPBS) and lysed with PBS (containing 0.1% Triton), an aliquot was taken for the protein estimation (Lowry method), and the remaining lyse was used for counting in a β counter (28).

**Measurement of Apoptosis by TUNEL Assay**—The terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL) assay was used for microscopic detection of apoptosis (19, 20). This assay is based on labeling of 3′-free hydroxyl ends of the fragmented DNA with fluorescein-dUTP catalyzed by terminal deoxynucleotidyl transferase. Procedures were followed according to the commercially available kit (ApoAlert) from CLONTECH. Apoptotic cells exhibit a strong nuclear green fluorescence that can be detected using a standard fluorescein (ApoAlert) from CLONTECH. Apoptotic cells exhibit a strong nuclear green fluorescence at 620 nm. The areas of apoptotic cells were homogenized by passing the suspension through a 25-gauge needle. The following agents were added: 1 mM sodium vanadate, 10 µM aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin inhibitors. The cells were homogenized by passing the suspension through a 25-gauge needle (10 strokes). The lysate was centrifuged at 750 × g for 10 min at 4 °C to remove the cellular debris. The caspase-3 activity in the supernatant was measured in a spectrophotometer using acetyl-Asp-Glu-Val-Asp-7-amido-4-methyl coumarin (Ac-DEVD-AMC) as a substrate according to the manufacturer’s instructions provided with the assay kit (20).

**Determination of Tfr Levels**—BAEC were washed with ice-cold PBS and resuspended in 100 µl of RIPA buffer (20 µg Trit-HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 100 µg/ml NaCl, 100 mM sodium fluoride). To a 10-ml solution of the above, the following agents were added: 1 mM sodium vanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin inhibitors. The cells were homogenized by passing the suspension through a 25-gauge needle (10 strokes). The lysate was centrifuged at 750 × g for 10 min at 4 °C to pellet out the nuclei. The remaining supernatant was centrifuged for 30 min at 12,000 × g. Protein was determined by the Lowry method, and 20 µg was used for the Western blot analysis. The proteins were resolved on 8% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were washed with Tris-buffered saline (140 mM NaCl, 50 mM Trit-HCl, pH 7.2) containing 0.1% Tween 20 and 5% skim milk to block the nonspecific protein binding. Membranes were incubated with mouse anti-human transferrin receptor monoclonal antibody (Zymed Laboratories Inc., San Francisco, CA; 1 µg/ml) in Tris-buffered saline containing 0.1% Tween 20 for 2 h at room temperature), washed, and incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:5000) for 1.5 h at room temperature. The TfR band was detected using the ECL method (Amersham Biosciences) (32, 33).

**Measurement of Acidity**—TfR in endothelial cells were grown in Dulbecco’s modified Eagle’s medium containing 10% FBS, and the cells were allowed to adjust to the medium conditions 2–3 h. Then, 0.1 Ci of ⁵⁵Fe (ferric chloride) was added to the medium for 0–16 h. An aliquot of medium was taken to measure the label in the medium. The cells were washed twice with Dulbecco’s phosphate-buffered saline (DPBS) and lysed with PBS (containing 0.1% Triton), an aliquot was taken for the protein estimation (Lowry method), and the remaining lysate was used for counting in a β counter (28).

**Measurement of Caspase-3 Activity**—Caspase-3-like activity is increased through a protease cascade during apoptosis in the early stage (29–31). Following treatment with DOX and other antioxidants, the cells were washed with ice-cold PBS and lysed with cell lysis buffer (caspase-3 assay kit; CLONTECH). The samples were incubated on ice for 10 min and then centrifuged in a microcentrifuge at 12,000 × g for 3 min at 4 °C to remove the cellular debris. The caspase-3 activity in the supernatant was measured in a spectrophotometer using acetyl-Asp-Glu-Val-Asp-7-amido-4-methyl coumarin (Ac-DEVD-AMC) as a substrate according to the manufacturer’s instructions provided with the assay kit (20).

**Measurement of Oxidative Stress**—The determination of intracellular oxidant production was based on the oxidation of DCFH to a fluorescent 2′,7′-dichlorofluorescein (DCF) (36, 37). Following pretreatment of BAEC with DOX and antiapoptotic antioxidants, the medium was aspirated, and cells were washed twice with DPBS and incubated in 1 ml of medium without FBS. DCFH diacetate was added at a final concentration of 10 µM and incubated for 20 min. The cells were then washed once with DPBS and maintained in 1 ml of culture medium. Fluorescence was monitored using a Nikon fluorescence microscope equipped with an fluorescein isothiocyanate filter. The intensity values were calculated using the Metamorph software.

**Measurement of Oxidative Stress**—The determination of intracellular oxidant production was based on the oxidation of DCFH to a fluorescent 2′,7′-dichlorofluorescein (DCF) (36, 37). Following pretreatment of BAEC with DOX and antiapoptotic antioxidants, the medium was aspirated, and cells were washed twice with DPBS and incubated in 1 ml of medium without FBS. DCFH diacetate was added at a final concentration of 10 µM and incubated for 20 min. The cells were then washed once with DPBS and maintained in 1 ml of culture medium. Fluorescence was monitored using a Nikon fluorescence microscope equipped with an fluorescein isothiocyanate filter. The intensity values were calculated using the Metamorph software.

**Hydroethidine (Dihydroethiodium) Staining**—The redox-sensitive fluorophore hydroethidine (dihydrothiodium) has been used to monitor intracellular oxidative stress (38). Following pretreatment of BAEC with antiapoptotic antioxidants and DOX, culture medium was aspirated, and the cells were washed once with DPBS and incubated in fresh culture medium without FBS. Hydroethidine (10 µmol/liter) was added to the cells, and the incubation was continued for 20 min, during which hydroethidine was oxidized to fluorophore ethidium. Fluorescence images were obtained using a Nikon fluorescence microscope equipped with a rhodamine filter. The fluorescence intensity values from three different fields of view were calculated using the Metamorph software, and the average values are represented.
FIG. 2. The effect of anti-transferrin receptor antibody on DOX-induced $^{55}$Fe uptake in BAEC. A. BAEC were treated with 0.5 μM DOX for different time periods as indicated, and TfR levels were determined by Western analysis using the anti-TfR antibody. Note that following a 2-h incubation with DOX, TfR levels were increased and remained elevated for 16 h. B. Represents the densitometric analysis of TfR levels as shown in A and C. DOX-induced $^{55}$Fe uptake was measured in BAEC after a 2-h incubation with DOX in control cells and in cells treated with the specific anti-TfR antibody, 42/6 (12 μg/ml). Note that anti-TfR antibody treatment drastically lowered DOX-induced $^{55}$Fe uptake. The data represent the means ± S.D. of three independent experiments.

FIG. 3. The effect of antioxidants on DOX-induced $^{55}$Fe uptake (A) and TfR expression (B) in BAEC. BAEC were pretreated independently with either MnTBAP (100 μM), FeTBAP (20 μM), PBN (100 μM), or ebselen (50 μM) for 2 h prior to treating cells with 0.5 μM DOX and $^{55}$Fe. Following a 4-h incubation with DOX, $^{55}$Fe uptake was determined as described under “Experimental Procedures.” B, BAEC were treated with 0.5 μM DOX in the presence of either 20 μM FeTBAP, 100 μM MnTBAP, or 100 μM PBN for 4 h, and transferrin receptor levels were measured by Western blotting. The data shown are representative of three separate experiments. C, the densitometric analysis of TfR levels as shown in B.
RESULTS

Transferrin Receptor-mediated $^{55}$Fe Uptake by Endothelial Cells—Incubation of BAEC with 0.5 μM DOX caused an increase in the cellular uptake of $^{55}$Fe (Fig. 1A). As shown in Fig. 1, DOX treatment induced a decrease in $^{55}$Fe content in the medium and an increase in $^{55}$Fe uptake by cells. Fig. 1B shows the time-dependent increase in $^{55}$Fe uptake by cells following DOX treatment. DOX-induced iron uptake reached a maximum within a period of 2–3 h. To examine the involvement of the transferrin receptor, we monitored the effect of DOX on the expression of cellular TfR levels by Western blotting analysis. Fig. 2 (expression of cellular TfR levels by Western blotting analysis.

- cells were treated with 0.5 μM DOX. C, cells were pretreated (2 h) with anti-TfR antibody prior to the addition of DOX. D, the same as above, except that DOX and anti-TfR antibody were added at the same time. E, cells were treated with anti-TfR antibody alone. F, cells were pretreated with ICRF-187 iron chelator for 2 h prior to the addition of 0.5 μM DOX. G, cells were treated with 0.5 μM DOX and ICRF-187 without pretreatment. H, cells were treated with 100 μM PBN for 2 h prior to the addition of 0.5 μM DOX. I, cells were treated with 50 μM ebselen for 2 h prior to the addition of 0.5 μM DOX. J, cells were treated with 20 μM FeTBAP for 2 h prior to the addition of 0.5 μM DOX. K, percentage of apoptosis in A–J calculated using Metamorph image analysis software. The data shown are representative of three separate experiments.

Effect of Anti-TfR Antibody, Antioxidants, and Iron Chelators on DOX-induced Apoptosis—Treatment of BAEC with 0.5 μM DOX enhanced the fraction of TUNEL-positive BAEC from 2 to 65% (Fig. 4, A and B). Cells treated with anti-TfR monoclonal antibody 42/6 (12 μg/ml) dramatically decreased the fraction of TUNEL-positive nuclei (Fig. 4C). Pretreatment with nitrone spin trap, PBN, or ebselen (data not shown) along with DOX inhibited TfR expression by 75 and 60%, respectively, as measured by densitometry scanning (Fig. 3C). These findings suggest that antioxidants inhibit DOX-mediated $^{55}$Fe uptake through the down-regulation of TfR.

Effect of Anti-TfR Antibody, Antioxidants, and Iron Chelators on DOX-induced Apoptosis—We examined the effects of small molecular weight, cell-permeable superoxide dismutase mimetics (MnTBAP and FeTBAP) (39, 40), the lipophilic glutathione peroxidase mimetic (ebselen) (41), and an open chain nitrite-free radical trap (PBN) (42) on DOX-induced $^{55}$Fe uptake and TfR levels. BAEC were treated with each compound for 2 h prior to the addition of 0.5 μM DOX. In the presence of FeTBAP (20 μM), ebselen (50 μM), and PBN (100 μM), DOX-induced iron uptake was inhibited (Fig. 3A). Concomitantly, TfR levels were measured. Fig. 3B shows that antioxidants that inhibit DOX-induced $^{55}$Fe uptake caused a marked decrease in the expression of TfR. For example, treatment of BAEC with either FeTBAP, PBN, or ebselen (data not shown) along with DOX inhibited TfR expression by 75 and 60%, respectively, as measured by densitometry scanning (Fig. 3C). These findings suggest that antioxidants inhibit DOX-mediated $^{55}$Fe uptake through the down-regulation of TfR.
Effect of Anti-TfR Antibody and Cell-permeable Iron Chelators on DOX-induced Caspase-3 Activation—Previously we reported that exposure of BAEC or cardiomyocytes to submicromolar levels of DOX induced caspase-3 activation (20). Caspase-3 activity was presumably increased through a proteolytic activation.

Fig. 5. The effect of anti-transferrin receptor antibody, cell-permeable iron chelators, and antioxidants on DOX-induced caspase-3 proteolytic activation. A, BAEC were treated with 0.5 μM DOX in the presence and absence of anti-TfR antibody (12 μg/ml), and caspase-3 activity was measured as a function of time. Note that anti-TfR antibody alone caused caspase-3 activation after 16 h treatment. B, BAEC were treated with 0.5 μM DOX and 10 μM iron chelators (with or without pretreatment for 2 h), 100 μM PBN, 50 μM ebselen, and 20 μM FeTBAP (all of the antioxidants were preincubated for 2 h prior to the treatment with DOX) for 8 h, and caspase-3 activity was measured by monitoring the release of p-nitroaniline as described under “Experimental Procedures.” The values are the means ± S.D. of three separate experiments.

Effect of Anti-TfR Antibody and Cell-permeable Iron Chelators on DOX-induced Reactive Oxygen Species—The oxidation of DCFH, a nonfluorescent probe, to a fluorescent DCF has been used to measure intracellular H2O2 (36, 37) and redox-active iron as described under “Experimental Procedures.”

The oxidation of DCFH, a nonfluorescent probe, to a fluorescent DCF was mediated by TfR-dependent transport exacerbates DOX-induced aconitase inactivation. Earlier studies (24, 25) have shown that IRP-1, a central cytoplasmic regulator of cellular iron metabolism, is oxidatively activated to bind to mRNAIRE. Treatment of cells with 0.5 μM DOX for 0–8 h caused a dose-dependent increase in IRP-1 activity. This activity was significantly increased within 2 h of DOX treatment (Fig. 6D, right panel) with respect to total IRP-1 (active and inactive). To determine whether the increase in IRP-1 activity with DOX treatment was due to an increase in total IRP-1, lysates were treated with 1% 2-mercaptoethanol, which activates IRP-1 to the high affinity RNA-binding form (46). Under these conditions, major differences in IRP binding to IRE were not observed between control and DOX-treated cells (Fig. 6D, left panel). This suggests that DOX treatment clearly induces activation of IRP-1. The present findings are in agreement with a recent study reporting that DOX at low concentrations (∼1 μM) activates IRP-1 in cardiomyocytes (47). However, at higher concentrations (>5 μM), DOX irreversibly inactivates IRP-1 (47).

Effect of Anti-TfR Antibody and Iron Chelators on DOX-induced Inactivation of Aconitase and IRP-1 Activity—Inactivation of aconitase has been used as a physiologically relevant indicator of the intracellular iron oxidation (43–45). As shown in Fig. 6A, DOX (0.5 μM) treatment inhibited aconitase activity (40%) within 2–3 h. Pretreatment with antioxidants (FeTBAP, PBN, and ebselen) almost fully restored aconitase activity (Fig. 6B). Fig. 6C shows that anti-TfR antibody and pretreatment with cell-permeable iron chelators restored the activity of aconitase, indicating that TfR-mediated cellular iron transport exacerbates DOX-induced aconitase inactivation.

The present findings strongly suggest that preincubation with cell-permeable chelators of iron is extremely critical to ameliorating DOX-induced apoptosis.

Effect of Antioxidants and Anti-TfR Antibody on DOX-induced Inactivation of Aconitase and IRP-1 Activity—Inactivation of aconitase has been used as a physiologically relevant indicator of the intracellular oxidation (43–45). As shown in Fig. 6A, DOX (0.5 μM) treatment inhibited aconitase activity (40%) within 2–3 h. Pretreatment with antioxidants (FeTBAP, PBN, and ebselen) almost fully restored aconitase activity (Fig. 6B). Fig. 6C shows that anti-TfR antibody and pretreatment with cell-permeable iron chelators restored the activity of aconitase, indicating that TfR-mediated cellular iron transport exacerbates DOX-induced aconitase inactivation. Earlier studies (24, 25) have shown that IRP-1, a central cytoplasmic regulator of cellular iron metabolism, is oxidatively activated to bind to mRNA IRE. Treatment of cells with 0.5 μM DOX for 0–8 h caused a dose-dependent increase in IRP-1 activity. This activity was significantly increased within 2 h of DOX treatment (Fig. 6D, right panel) with respect to total IRP-1 (active and inactive). To determine whether the increase in IRP-1 activity with DOX treatment was due to an increase in total IRP-1, lysates were treated with 1% 2-mercaptoethanol, which activates IRP-1 to the high affinity RNA-binding form (46). Under these conditions, major differences in IRP binding to IRE were not observed between control and DOX-treated cells (Fig. 6D, left panel). This suggests that DOX treatment clearly induces activation of IRP-1. The present findings are in agreement with a recent study reporting that DOX at low concentrations (∼1 μM) activates IRP-1 in cardiomyocytes (47). However, at higher concentrations (>5 μM), DOX irreversibly inactivates IRP-1 (47).

Next, we determined the effect of anti-TfR antibody on DOX-induced ethidium staining (Fig. 7B), which reportedly detects intracellular superoxide generation (38, 52). As shown in Fig.
there was a dose-dependent increase in the intensity of red fluorescence with DOX, indicative of enhanced superoxide generation. However, anti-TfR antibody did not have any effect on DOX-induced ethidium fluorescence (Fig. 7B). This suggests that DOX-induced superoxide generation in BAEC is not affected by $^{55}$Fe transport. This result also reveals that anti-TfR antibody does not interfere with the cellular uptake and reductive activation of DOX (Fig. 7B). In addition, iron chelators had no effect on the intensity of red fluorescence, indicating that superoxide production remained unchanged (Fig. 7B).

DISCUSSION

Our results demonstrate that DOX-mediated apoptosis in endothelial cells is accompanied by a significant increase in TfR-mediated uptake of transferrin iron and that blockade of iron uptake by an anti-transferrin receptor antibody abolishes DOX-induced apoptosis. In addition, TfR-mediated iron uptake and apoptotic signaling are mitigated by antioxidants that inhibit DOX-dependent intracellular oxidant generation. These findings place the TfR as a "gatekeeper" for iron uptake by BAEC and act as an effective modulator of apoptotic signaling initiated by DOX.

Role of Iron in DOX-induced Toxicity and Apoptosis: Old and New Perspectives—The possible involvement of iron in DOX-induced cardiotoxicity was demonstrated in the early 1970s when a series of nonpolar derivatives of EDTA, including a bis-ketopiperazine derivative (ICRF-187 or dexrazoxane), was reported to prevent DOX-mediated cardiac damage and myocardial dysfunction in an isolated perfused heart model and in whole animals. More recently, it was reported that the secondary alcohol metabolite of DOX could cause the release of iron from cytosolic fractions of myocardial tissues. DOX itself can form iron complexes under acidic conditions, and this preformed complex has been shown to oxidize lipids, protein, and DNA. The current thinking is that DOX modifies the cellular iron-induced redox signaling through oxidative modification of aconitase. Published reports on redox signaling of iron support this view. Exposure of hydroperoxides to murine B6 fibroblasts increased the expression of the TfR mRNA, because of the induction of a 98-kDa, cytosolic iron regulatory protein (IRP-1). This process also leads to the reduced synthesis of ferritin, an intracellular iron storage protein, which triggers a signal for increased iron uptake. Quinone-induced oxidative stress has also been reported to induce iron signaling via IRP-1. Treatment of murine B6 fibroblasts with a redox-active quinone such as menadione activated IRP-1 binding to IREs through increased generation of intracellular oxidants. However, the IRP-1 response in cells treated with extracellular and intracellular H$_2$O$_2$ has been reported to be different.

In the present study we have shown that DOX causes an induction in TfR expression and increases iron influx into cells through TfR. Antioxidants inhibit DOX-induced TfR overexpression and the associated iron uptake, implicating a role for oxidant-induced iron signaling mechanism in DOX toxicity.

Effect of DOX on the Molecular Regulation of Transferrin Receptor Expression by IRPs—The proposed model linking iron and DOX-induced changes in aconitase and IRP-1 activities. A, BAEC were treated with 0.5 $\mu$M DOX for different time periods, and the total aconitase activity was measured as described under "Experimental Procedures." B, cells were preincubated for 2 h with 20 $\mu$M FeTBAP, 100 $\mu$M PBN, or 50 $\mu$M ebselen, and 0.5 $\mu$M DOX was then added to cells and incubated further for an additional 6 h. C, BAEC were treated with 10 $\mu$M ICRF-187 or anti-TfR antibody (12 $\mu$g/ml) and 0.5 $\mu$M DOX for 6 h prior to measuring total aconitase activity in cell lysates. D, BAEC were treated with 0.5 $\mu$M DOX for different time intervals, and cytoplasmic extracts were analyzed by gel shift assay with and without 1% 2-mercaptoethanol. The gels are representative of three independent experiments, and the values are the means ± S.D. of three separate experiments.
DOX-induced oxidative stress and iron signaling is shown in Scheme 1. As shown in Scheme 1, the TfR plays a key role in regulating the entry of iron into cells. The cellular iron-sensing mechanism enables synchronized regulation of TfR and ferritin levels in cells. TfR and ferritin syntheses are regulated by iron at the mRNA translation level by the interaction of cytoplasmic regulatory proteins (IRPs) with their respective mRNAs (57–59). The IRPs (IRP-1 and-2) function as sensors of cellular iron status. Under conditions of iron deprivation or when the [4Fe-4S] cluster in aconitase is disassembled, the IRPs bind with high affinity to IRE present on TfR and ferritin mRNAs. The increased binding to TfR mRNA stabilizes the mRNA, resulting in increased mRNA translation and increased receptor synthesis (Scheme 1). On the other hand, when cellular iron is in excess, IRP-1/IRE binding is decreased, leading to rapid degradation of TfR mRNA and to efficient translation of ferritin mRNA. IRP-1 senses iron levels by switching between cytoplasmic aconitase and IRP-1, an IRE-binding protein (Scheme 1). Because a large portion of the iron needs of the cell is for the assembly of iron sulfur clusters and heme biosynthesis in the mitochondria, the partial inactivation of the mitochondrial iron sulfur protein (e.g., aconitase) is presumably sufficient to stimulate cellular iron signaling (60). Although the exact mechanism of oxidant-induced activation of IRP-1 remains unknown, it has been proposed that either a direct interaction between the 4Fe-4S cluster of IRP-1 and H2O2 (or derived oxidant) or an H2O2-dependent stress-response signaling pathway is operative (23). Scheme 1 shows the relationship between DOX-

**Fig. 7.** The effect of anti-transferrin receptor antibody and iron chelators on DOX-induced oxidative stress, as measured by DCF and HE staining. BAEC were treated with 0.5 μM DOX alone or in the presence of anti-TfR antibody (12 μg/ml) or iron chelators as indicated for 4 h. The medium was then aspirated, and the cells were washed twice with DPBS and subsequently incubated with 10 μM DCF-DA (A) or 5 μM dihydroethidium (B) for 20 min. The cells were then washed with DPBS and maintained in 1 ml of the culture medium. The green fluorescence characteristic of DCF and red fluorescence caused by ethidium binding to DNA were measured using fluorescein isothiocyanate and rhodamine filters, respectively. The data shown are representative of three separate experiments.
Oxidant-induced Iron Signaling

H2O2 production in nonphagocytic cells (67). Recently, molecular oxygen generates O2–/H2O2, which dismutates to form H2O2 and peroxide. The enzyme involved in the activation of DOX in mitochondria appears to be the NADH dehydrogenase (10). The one-electron reduction of DOX to its semiquinone radical by NADH dehydrogenase, followed by its redox cycling in the presence of molecular oxygen generates O2–, which dismutates to form H2O2 in the mitochondria (10). Recently, we reported that DOX-generated H2O2 enhances endothelial nitric-oxide synthase expression in endothelial cells and that the reductase domain of endothelial nitric-oxide synthase amplifies O2– and H2O2 generation from DOX (62). It is possible that an increase in ROS levels may diminish the putative iron pool (i.e. complexes of Fe with low molecular weight ligands in the cell) leading to a decrease in the cytosolic aconitase activity and enhanced TIR-mediated iron signaling (Scheme 1).

Role of Cell-permeable Antioxidants and Iron Chelators in DOX-induced Apoptosis—We previously reported that BAEC incubated with ICRF-187, a clinically well established iron chelator, did not inhibit DOX-induced apoptosis (20). These results differed from those previously reported for myocytes, where ICRF-187 significantly inhibited DOX-induced apoptosis (19). In the present study, we show that preincubation of BAEC with three different iron chelators (desferral (or deferoxamine), HBED, and ICRF-187) is absolutely essential for inhibiting DOX-induced apoptosis (Fig. 5B). The three iron chelators are presumably transported into cells by different mechanisms. Deferoxamine is hydrophilic and easily endocytosed into cells, whereas the lipophilic HBED readily diffuses into cells and chelates intracellular iron (63). ICRF-187 is metabolized intracellularly to generate an in situ iron chelator (64). Because treatment with ebosen completely inhibits DOX-induced apoptosis in endothelial cells and myocytes, it appears that both H2O2 and iron are responsible for DOX apoptosis (19, 20).

Oxidative Stress, Iron Signaling, and Endothelial Apoptosis—The role of oxidant-induced iron signaling may have broader applications in oxidative vascular biology. Endothelial cell injury is presumed to be an early oxidative insult in the development of atherosclerosis (65). It was proposed that H2O2 generated in leukocytes and macrophages caused endothelial dysfunction (66). H2O2 and other peroxides including a lipid hydroperoxide were shown to induce NAD(P)H oxidase-dependent O2•− production in nonphagocytic cells (67). Recently, it was reported that hyperglycemia could promote transition metal-catalyzed hydroxyl radical reactions in the microenvironment of the diabetic artery wall (68, 69). Another clinical study found that redox-active iron might contribute to endothelial dysfunction in atherosclerotic patients and demonstrated the beneficial effects of iron chelation with defersulf (70). From these reports, it is evident that the role of cellular iron signaling and iron-mediated oxidative damage is relevant in cardiovascular and lung diseases (68, 71, 72) that should perhaps be more fully explored from a new perspective (i.e. oxidant-induced iron signaling mechanism).

REFERENCES
21. Klauser, R. D., Ashwell, G., Van Renswoude, J., Harford, J. B., and Bridges,
transferrin receptor-dependent iron uptake is responsible for doxorubicin-mediated apoptosis in endothelial cells: role of oxidant-induced iron signaling in apoptosis

Srigiridhar Kotamraju, Christopher R. Chitambar, Shasi V. Kalivendi, Joy Joseph and B. Kalyanaraman

doi: 10.1074/jbc.M111604200 originally published online February 20, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M111604200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 71 references, 35 of which can be accessed free at http://www.jbc.org/content/277/19/17179.full.html#ref-list-1