Oxidative Stress–Induced Iron Signaling Is Responsible for Peroxide-Dependent Oxidation of Dichlorodihydrofluorescein in Endothelial Cells
Role of Transferrin Receptor–Dependent Iron Uptake in Apoptosis

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Abstract—Dichlorodihydrofluorescein (DCFH) is one of the most frequently used probes for detecting intracellular oxidative stress. In this study, we report that H\textsubscript{2}O\textsubscript{2}-dependent intracellular oxidation of DCFH to a green fluorescent product, 2',7'-dichlorofluorescein (DCF), required the uptake of extracellular iron transported through a transferrin receptor (TfR) in endothelial cells. H\textsubscript{2}O\textsubscript{2}-induced DCF fluorescence was inhibited by the monoclonal IgA–class anti-TfR antibody (42/6) that blocked TfR endocytosis and the iron uptake. H\textsubscript{2}O\textsubscript{2}-mediated inactivation of cytosolic aconitase was responsible for activation of iron regulatory protein-1 and increased expression of TfR, resulting in an increased iron uptake into endothelial cells. H\textsubscript{2}O\textsubscript{2}-mediated caspase-3 proteolytic activation was inhibited by anti-TfR antibody. Similar results were obtained in the presence of a lipid hydroperoxide. We conclude that hydroperoxide-induced DCFH oxidation and endothelial cell apoptosis required the uptake of extracellular iron by the TfR-dependent iron transport mechanism and that the peroxide-induced iron signaling, in general, has broader implications in oxidative vascular biology. (Circ Res. 2003;92:1111–1119.)

Key Words: transferrin receptor • dichlorodihydrofluorescein • caspase activation • apoptosis • oxidative stress

The oxidation of 2',7'-dichlorodihydrofluorescein (DCFH), a nonfluorescent probe, to a green fluorescent product, 2',7'-dichlorofluorescein (DCF), has been used to measure intracellular H\textsubscript{2}O\textsubscript{2} by numerous investigators. This assay was originally used to monitor intracellular oxidant produced during oxidative stress or apoptosis. The cell-permeable nonfluorescent probe DCFH-diacetate (DCFH-DA) is hydrolyzed by intracellular esterases to form the active probe DCFH. Previous studies implicated a role for redox-active iron in cellular oxidation of DCFH to DCF, although the origin of cellular iron was not known. In this study, we investigated whether H\textsubscript{2}O\textsubscript{2}-induced iron signaling mechanism is responsible for DCFH oxidation to DCF.

Published reports indicate that the amount of iron in cells is controlled by the cell surface transferrin receptor (TfR)–mediated uptake of iron as transferrin iron. The TfR synthesis is regulated by interaction of the iron regulatory protein (IRP) with the iron-responsive element (IRE) present on the 3'-untranslated region of TfR mRNA. IRPs serve as sensors of cellular iron. Reports also indicate that the cellular oxidative damage caused by reactive oxygen species and reactive nitrogen species is critically controlled by cellular iron homeostasis. Exposure of murine fibroblasts to H\textsubscript{2}O\textsubscript{2} enhanced the expression of TfR mRNA\textsuperscript{12} and DCF fluorescence, implicating a potential link between oxidative stress and TfR-mediated iron uptake.

In this study, we tested the hypothesis that H\textsubscript{2}O\textsubscript{2} and lipid hydroperoxide–induced intracellular DCFH oxidation to DCF is mediated by TfR-dependent uptake of iron. Results show that DCFH oxidation in endothelial cells was inhibited by an anti-TfR antibody, 42/6, that blocked iron uptake, hence suggesting that the iron transported into endothelial cells via TfR initiates H\textsubscript{2}O\textsubscript{2}-induced intracellular DCFH oxidation. Treatment with TfR antibody also inhibited H\textsubscript{2}O\textsubscript{2} and lipid hydroperoxide–dependent apoptosis. Supplementation of cellular reduced glutathione (GSH) prevented H\textsubscript{2}O\textsubscript{2}-induced DCFH oxidation and apoptosis. We conclude that intracellular oxidative stress, accompanied by GSH depletion and aconitase inactivation (as shown in Figure 7), is responsible for iron signaling and DCFH oxidation in endothelial cells exposed to H\textsubscript{2}O\textsubscript{2} and other hydroperoxides.

Materials and Methods

Materials
Glucose oxidase, glutathione monoethyl ester, hydrogen peroxide, and desferal (or desferrioxamine) were obtained from Sigma.

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Hydroperoxyctadecadienoic acid (13-HpODE) and 13-hydroxyoctadecadienoic acid (13-HODE) were from Cayman Chemical Company. DCFH-DA was purchased from Molecular Probes Inc. Fe(III) tetrakis (4-benzoic acid) porphyrin (FeTBAP) was synthesized according to published methods. Monoclonal antibody, 42/6, against human TfR (IgA class), was obtained from Dr Ian Trowbridge (Salk Institute, San Diego, Calif).

Endothelial Cell Culture

Bovine aortic endothelial cells (BAECs) were obtained from Clonetics. Cells were obtained at the third passage; transferred to 75-cm² filter vent flasks (Costar) and grown to confluence (5.2 × 10⁶ cells/75 cm²) in DMEM containing 10% FBS, L-glutamine (4 mmol/L), penicillin (100 U/mL), and streptomycin (100 µg/mL); and incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were passaged as described by Balla et al. and used between passages 4 and 14. On the day of the treatment, the medium was replaced with DMEM containing 2% FBS, which contains ~25 to 30 µg transferrin/mL.

Treatment of Endothelial Cells With Peroxides

H₂O₂ (reagent, 1 mmol/L), glucose oxidase (5 to 50 mU/mL), or 13-HpODE (25 mmol/L) was added to cells in the medium (which contained 25 mmol/L glucose as substrate for glucose oxidase). The level of H₂O₂ generated from glucose/glucose oxidase was measured using a YSI model 25 oxidase meter fitted with a YSI 2510 oxidase probe (Yellow Springs Instrument Co). Anti-TfR antibody, iron chelator, and antioxidants were preincubated with cells for 1 hour before the addition of glucose oxidase. Cells pretreated with FeT-BAP were washed twice with Dulbecco’s PBS (DPBS), and glucose oxidase was subsequently added to cells.

Measurement of Oxidative Stress

After treatment of BAECs with H₂O₂ and glucose/glucose oxidase, the medium was aspirated and cells were washed with DPBS and incubated in 2 mL of fresh culture medium without FBS. DCFH-DA was added at a final concentration of 10 µmol/L and incubated for 20 minutes. The cells were then washed twice with DPBS and maintained in 1 mL culture medium. Fluorescence was monitored using a Nikon fluorescence microscope (excitation 488 nm, emission 510 nm) equipped with a FITC filter. The intensity values were calculated using the Metamorph software.

Measurement of Glutathione

The level of GSH was measured by HPLC as the o-phthalaldehyde adduct at pH 8.0.

Measurement of ⁵⁵Fe Uptake in Endothelial Cells

BAECs were grown in DMEM containing 10% FBS until confluence (5.2 × 10⁶ cells/75 cm²) in DMEM containing 10% FBS, L-glutamine (4 mmol/L), and streptomycin (100 µg/mL); and incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were passaged as described by Balla et al. and used between passages 4 and 14. On the day of the treatment, the medium was replaced with DMEM containing 2% FBS, which contains ~25 to 30 µg transferrin/mL.

Measurement of Caspase-3 Activity

After treatment with glucose/glucose oxidase and antioxidants, cells were washed twice with DPBS and lysed with cell lysis buffer (caspase-3 assay kit, Clontech). The caspase-3 activity in the 12 000g supernatant was measured in a spectrophotometer using DEVD-pNA (acetyl Asp-Glu-Val-Asp p-nitroanilide) as a substrate according to the manufacturer’s instructions provided with the assay kit.

Mitochondrial Cytochrome c Release

To investigate the role of mitochondria in glucose/glucose oxidase–induced apoptosis, the release of mitochondrial cytochrome c into the cytosol was measured as described previously by Western analysis.

Results

H₂O₂-Induced Intracellular Oxidation of DCFH: Effect of GSH

BAECs were treated with H₂O₂ (1 mmol/L) for different time periods, and DCFH-DA, a nonfluorescent probe, was added at the end of the treatment as described in Materials and Methods. DCFH-DA is hydrolyzed intracellularly to form the active probe, DCFH. The oxidation of DCFH to DCF, a green fluorescent oxidation product, was measured as a function of time. As shown in Figure 1A, the DCF fluorescence intensity was 1000-fold greater, as compared with control, in BAECs exposed to H₂O₂ for 4 hours. Similar results were obtained when BAECs were treated with H₂O₂ (1 µmol/L per minute) generated from glucose/glucose oxidase (Figure 1B). Figure 1C shows a dose-dependent increase in DCF green fluorescence. As with the bolus addition of H₂O₂, there was a lag time in the appearance of DCF green fluorescence.

To fully understand the mechanism of lag time, we measured intracellular GSH, a major H₂O₂ detoxifying antioxidant. Exposure of BAECs to glucose/glucose oxidase (generating 1 µmol/L per minute H₂O₂) caused a decrease in GSH levels from 4.8 nmol/mg protein to <1.9 mol/mg protein in 4 hours. Only after nearly 60% of intracellular GSH had been depleted did DCF fluorescence begin to accumulate (Figures 2A and 2B). The lag time correlated well with the rate of depletion of GSH. When cells were treated with GSH ester, intracellular GSH levels increased to 8.3 nmol/mg protein as a result of intracellular esterase–mediated hydrolysis of GSH ester to GSH (Figure 2C). In the presence of GSH ester, even after 6 hours of exposure to glucose/glucose oxidase, intracellular GSH remained at 5.5 mol/mg protein. At this GSH concentration, DCFH fluorescence was not detected directly oxidize DCFH and that H₂O₂-dependent oxidation of DCFH caused a decrease in GSH levels from 4.8 nmol/mg protein to <1.9 mol/mg protein in 4 hours. Only after nearly 60% of intracellular GSH had been depleted did DCF fluorescence begin to accumulate (Figures 2A and 2B). The lag time correlated well with the rate of depletion of GSH. When cells were treated with GSH ester, intracellular GSH levels increased to 8.3 nmol/mg protein as a result of intracellular esterase–mediated hydrolysis of GSH ester to GSH (Figure 2C). In the presence of GSH ester, even after 6 hours of exposure to glucose/glucose oxidase, intracellular GSH remained at 5.5 mol/mg protein. At this GSH concentration, DCFH oxidation was not detected in BAECs treated with H₂O₂. With the bolus addition of H₂O₂ (1 mmol/L), a similar trend (cf Figure 2A) was observed. Again, only after intracellular GSH levels were decreased by 70% after a 4-hour treatment with H₂O₂ did DCF fluorescence start to increase (Figure 2C, bottom).

Effect of Iron Chelator, Metalloporphyrin, and TfR Antibody on DCFH Oxidation

Published results show that neither H₂O₂ nor O₂⁻ could directly oxidize DCFH and that H₂O₂-dependent oxidation of DCFH required the presence of redox metals or peroxides. Support for the intermediacy of iron in DCFH oxidation came from experiments using desferal, a well-established iron chelator. Pretreatment with desferal (10...
mMol/L) for 1 hour greatly inhibited DCFH oxidation induced by glucose/glucose oxidase (Figure 3A). FeTBAP, a cell-permeable metalloporphyrin antioxidant, inhibited DCFH oxidation (Figure 3A). This could be due to a nonspecific quenching of DCF fluorescence by FeTBAP that is paramagnetic or to scavenging of $\text{O}_2^-$ or $\text{H}_2\text{O}_2$ by FeTBAP. Although we observed a 30% reduction in the fluorescence intensity of DCF (10 mMol/L) in the presence of FeTBAP under in vitro conditions (not shown), it is highly unlikely that FeTBAP inhibits intracellular DCF fluorescence via a nonspecific physical quenching mechanism. Pretreatment with FeTBAP prevented the depletion of GSH levels in BAECs treated with glucose/glucose oxidase (Figure 3C). Thus, we attribute the inhibitory effect of FeTBAP to intracellular scavenging of reactive oxygen species. Treatment of cells with the anti-TfR antibody (42/6) totally inhibited DCF fluorescence (Figure 3), suggesting that $\text{H}_2\text{O}_2$-dependent oxidation of DCFH to DCF was mediated by TfR-dependent iron uptake. In control experiments in which BAECs were incubated with 12 $\mu\text{g/mL}$ IgG-class immunoglobulin that

**Figure 1.** $\text{H}_2\text{O}_2$-induced oxidation of DCFH in endothelial cells. A, BAECs were treated with a bolus of $\text{H}_2\text{O}_2$ (1 mMol/L) for various time periods, and cells were washed with DPBS and incubated with DCFH-DA (10 mMol/L) for 20 minutes. Cells were then washed twice with DPBS and kept in culture medium. Green fluorescence due to DCF, the oxidation product of DCFH, was monitored with time (left). B, Same as panel A, except that BAECs were treated continuously with $\text{H}_2\text{O}_2$ (1 mMol/L per minute) generated from glucose/glucose oxidase (20 mU) (left). C, Same as panel B, except that cells were treated with increasing concentrations of glucose oxidase for 4 hours. A through C, Graphs (right) show average fluorescence intensity as calculated using Metamorph software. Data shown represent 3 separate experiments.
does not bind to the extracellular domain of the TfR, DCFH oxidation was not inhibited in BAECs treated with H₂O₂. To investigate the effect of protein expression on DCFH oxidation, BAECs were treated with cycloheximide for 20 hours before being treated with glucose/glucose oxidase. As shown in Figure 3A (g and h), cycloheximide inhibited DCF fluorescence, suggesting that increased protein expression is associated with TfR-mediated DCFH oxidation. As shown in Figure 3B, cycloheximide treatment counteracted the increase in TfR protein expression in BAECs exposed to glucose/glucose oxidase. To further corroborate the role of intracellular oxidative stress in DCFH oxidation, catalase (1000 U/mL) was added to cells that had been treated with glucose/glucose oxidase (20 mU) for 4 hours and washed with DPBS. Under these conditions, intracellular oxidation of DCFH to DCF was unaffected (Figure 3A, i).

**H₂O₂-Induced Changes in Aconitase and IRP-1 Activities: Increased Cellular Iron Uptake and Caspase-3 Proteolytic Activation**

Exposure of BAECs to glucose/glucose oxidase caused a steady decrease in aconitase activity (Figure 4A). Within
6 hours, the aconitase activity was decreased by >80%. Inactivation of aconitase was prevented (Figure 4B) in the presence of GSH ester (5 mmol/L). GSH ester supplementation also inhibited DCFH oxidation in H2O2-treated BAECs.

Previous investigators have shown that oxidant-induced inactivation of aconitase is accompanied by an increase in IRP-1 activation.14–17 Oxidatively activated IRP-1 has been shown to bind to mRNA IRE. Treatment of cells with glucose/glucose oxidase for 4 hours caused a dose-dependent increase in IRP-1 activity (Figures 4C and 4D). At least a 10-fold increase in IRP-1 activation was observed in BAECs treated with glucose/glucose oxidase. To determine whether the increase in IRP-1 activity in response to H2O2 treatment was due to an increase in total IRP-1 activity and not simply to increased protein synthesis, lysates were treated with 1% 2-mercaptoethanol (2-ME), which activates IRP-1 to the high-affinity RNA binding form.28 In the presence of 2-ME, IRP binding to IRE was the same in control and in H2O2-

Figure 3. Inhibition of H2O2-induced DCFH oxidation and GSH depletion in endothelial cells. BAECs were treated with glucose/glucose oxidase (20 mU) and intracellular oxidation of DCFH and depletion of GSH measured after 4 hours, as described in Figures 1 and 2. A, BAECs were pretreated with desferal (10 μmol/L) (c), FeTBAP (25 μmol/L) (d), anti-TfR antibody, IgA class (12 μg/mL) (e), and anti-TfR antibody, IgG class (12 μg/mL) (f) for 1 hour, before addition of glucose/glucose oxidase (Glu/GO, 20 mU) (b); green fluorescence images due to DCF are shown. After treatment with FeTBAP, cells were washed with DPBS before addition of glucose/glucose oxidase. BAECs were treated with cycloheximide (10 μg/mL) for 20 hours before treating with glucose/glucose oxidase (20 mU) before and after washing with DPBS (g and h). Catalase (1000 U/mL) was added to cells that had been treated with glucose/glucose oxidase for 4 hours and washed (i). B, Effect of glucose/glucose oxidase on TfR levels in BAECs and in BAECs pretreated with cycloheximide (10 μg/mL) for 20 hours. C, Effect of pretreatment of FeTBAP (●) and desferal (C) on depletion of intracellular GSH in glucose/glucose oxidase-treated BAECs. Data are mean ± SD of 3 experiments.
Figure 4. H$_2$O$_2$-induced inactivation of aconitase and activation of IRP-1, and $^{55}$Fe uptake in endothelial cells: effect of antioxidants. A, BAECs were treated with glucose/glucose oxidase (20 mU), and the total aconitase activity was measured in cell lysates at different time points, as described in Materials and Methods. B, Cells were preincubated for 1 hour with FeTBAP (25 μmol/L) or GSH ester (5 mmol/L) and washed free of antioxidants, and glucose/glucose oxidase (20 mU) was added and incubated for an additional 4 hours. Aconitase activity was subsequently measured in cell lysates in control BAECs and antioxidant-treated BAECs. C and D, BAECs were treated with glucose/glucose oxidase for 4 hours as described in panel B, and cytoplasmic extracts were analyzed by gel-shift assay with and without 2-ME. Conditions were as follows: control (a), glucose/5 mU glucose oxidase (b), glucose/20 mU glucose oxidase (c), glucose/100 mU glucose oxidase (d), and glucose/20 mU glucose oxidase + 5 mmol/L GSH ester (e). E, H$_2$O$_2$-induced $^{55}$Fe uptake was measured in BAECs treated with glucose/glucose oxidase (50 mU) as a function of time. F, Effect of GSH ester and anti-TfR antibody on $^{55}$Fe uptake in cells treated with glucose/glucose oxidase (50 mU) for 4 hours. Note that TfR antibody treatment drastically lowered H$_2$O$_2$-induced $^{55}$Fe uptake. Data are mean±SD of 3 independent experiments.
treated cells (Figure 4C). Thus, experiments performed with and without 2-ME indicate that the IRP-1 activity was stimulated by H₂O₂. Incubation of BAECs with glucose/glucose oxidase caused an increase in the cellular uptake of ⁵⁵Fe. Figure 4E shows a time-dependent increase in ⁵⁵Fe uptake by cells. To examine the involvement of TfR, we used the monoclonal (IgA) anti-TfR antibody (42/6), which specifically binds to the extracellular domain of the TfR and blocks receptor endocytosis.²⁹ This antibody recognizes both human and bovine TfR.²⁹ In the presence of 42/6, iron cannot enter the cell through TfR. Thus, a distinction between TfR-independent and TfR-dependent ⁵⁵Fe uptake was made. As shown in Figure 4F, ⁵⁵Fe uptake was dramatically inhibited. H₂O₂-induced iron uptake was inhibited in BAECs pretreated with GSH ester (Figure 4F). This finding suggests that intracellular oxidative stress or GSH depletion is pivotal to stimulating the iron signaling mechanism.

Increase in caspase-3 activity occurs through a protease cascade during the early stages of apoptosis.²¹,²² As shown in Figure 5A, when BAECs were incubated with glucose/glucose oxidase, caspase-3 proteolytic activity increased by 4-fold after 8 hours. In the presence of TfR antibody, H₂O₂-induced caspase-3 activation was significantly inhibited (Figure 5B). Pretreatment of BAECs with FeTBAP dimin-

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**Figure 5.** H₂O₂-induced apoptosis. A, BAECs were treated with glucose/glucose oxidase (20 mU), and caspase-3 activity was measured as a function of time. Caspase-3 activity was measured by monitoring the release of p-nitroanilide as described in Materials and Methods. B, Caspase-3 activity was measured after 8 hours in BAECs treated with glucose/glucose oxidase (GO) (20 mU) in the presence and absence of anti-TfR antibody (IgA, 12 µg/mL), FeTBAP (25 µmol/L), and GSH ester (5 mmol/L) (with pretreatment for 1 hour). Values are mean±SD of 3 separate experiments. C, BAECs were treated for 8 hours with glucose/glucose oxidase (GO) (b) and other agents as indicated (c and d), stained for TUNEL–positive cells, and examined by fluorescence microscopy (original magnification, ×100). Photographs are overlaid images of propidium iodide– and FITC-stained cells (TUNEL–positive cells). Yellow and red denote apoptotic and nonapoptotic cells, respectively. D, BAECs were treated as in panel C, and the cytosolic fraction (10 µg of protein) was subjected to 14% SDS-polyacrylamide gel electrophoresis followed by Western analysis using anti–cytochrome c antibody.
ished caspase-3 activation by 170% (Figure 5B). Iron porphyrins (ie, FeTBAP) were reported to rapidly react with H$_2$O$_2$. Taking these results together, these results indicate that H$_2$O$_2$-induced caspase-3 activation in BAECs is mediated by the TfR-dependent uptake of iron.

The actual extent of apoptosis in glucose/glucose oxidase–treated cells in the presence and absence of antioxidants was quantified using the terminal deoxynucleotidyltransferase-mediated nick-end labeling (TUNEL) technique. As shown in Figure 5C, exposure of BAECs to glucose/glucose oxidase increased the fraction of TUNEL-positive BAECs from 2% to 65%. Preincubation with FeTBAP and GSH ester substantially decreased the fraction of TUNEL-positive BAECs. To confirm mitochondrial damage in response to H$_2$O$_2$ treatment, we monitored the efflux of cytochrome c from the intermembrane space of mitochondria into the cytosolic compartment. As shown in Figure 5D, glucose/glucose oxidase treatment of BAECs resulted in the release of cytochrome c from mitochondria, which was inhibited by GSH ester and FeTBAP pretreatment.

**Lipid Hydroperoxide–Induced Iron Uptake and Caspase-3 Activation**

Endothelial cells were treated with 13-HpODE (lipoxygenase-catalyzed oxidative metabolite of linoleic acid) and 13-HODE (2-electron reduction product of 13-HpODE) as a function of time, and caspase-3 activity was measured. Figure 6A shows the time course of caspase-3 activation induced by 13-HpODE in BAECs. As shown in Figure 6A, 13-HODE did not enhance caspase-3 activity, whereas 13-HpODE stimulated caspase-3 proteolytic activity 5-fold. In the presence of anti-TfR antibody, 13-HpODE–induced caspase-3 activation was significantly inhibited (Figure 6C). Next, we investigated whether 13-HpODE treatment caused an increase in the cellular iron uptake of $^{55}$Fe. Figure 6B shows the time course of $^{55}$Fe uptake in cells treated with 13-HpODE and 13-HODE. In 13-HpODE–treated cells, there was a 2-fold increase in $^{55}$Fe uptake between 2 and 6 hours. During the same period, caspase-3 activation was enhanced from 10 nmol pNA/mg protein to $\approx$40 nmol pNA/mg protein. In the presence of DEVD-CHO, a specific inhibitor of caspase-3, 13-HpODE–induced caspase-3 proteolytic activity was totally inhibited (Figure 6D).

**Discussion**

Our findings demonstrate that the exogenous addition of bolus or continuously generated H$_2$O$_2$ or 13-HpODE to endothelial cells causes intracellular oxidation of the fluorescent probe DCFH to DCF that is regulated by TfR-mediated uptake of transferrin-iron. Blockade of iron uptake by TfR antibody abolishes H$_2$O$_2$ and 13-HpODE–induced DCF fluorescence and apoptosis. The present findings point to a critical role of TfR-dependent iron uptake in peroxide-mediated DCF fluorescence and apoptosis.

**Proposed Pathway for Peroxide-Induced DCFH Oxidation and Apoptosis**

The proposed sequence of events linking H$_2$O$_2$-induced oxidative stress, iron signaling, DCF fluorescence, and apoptosis is shown in Figure 1. The cellular iron–sensing mechanism is triggered by intracellular iron deprivation or when the 4Fe-4S cluster in aconitase is disassembled; inactivation of aconitase and subsequent activation of IRPs act as sensors of cellular iron status. The IRPs bind with a high affinity to IRE present...
on TfR and ferritin mRNAs. The increased binding to TfR mRNA stabilizes the mRNA leading to enhanced mRNA translation and increased TfR synthesis. A major portion of cellular iron is utilized for the assembly of iron-sulfur clusters and heme biosynthesis in mitochondria. The oxidant-induced inactivation of mitochondrial iron-sulfur proteins (ie, aconitase) is, thus, sufficient to stimulate cellular iron signaling.31 The proposed mechanism of inactivation of mitochondrial aconitase in cells involves either a direct interaction between the 4Fe-4S cluster and H2O2 (or derived oxidants)32 or an H2O2-dependent stress-response signaling pathway.13 The influx of iron and H2O2 causes oxidative damage to cellular lipid, protein, and DNA, ultimately resulting in apoptosis.33 Iron chelators (eg, desferal) that are endocytosed into cells, or diffused into cells, inhibited peroxide-induced apoptosis.

**Intracellular Oxidation of DCFH Probe**

The assay based on DCFH oxidation to DCF has often been used to measure intracellular H2O2 or oxidative stress.1-7 However, H2O2 does not react with DCFH to DCF except in the presence of a catalyst (cytochromes, peroxidases, or redox-active metal ions).24-27 DCFH is oxidized to DCF by hydroxyl radical, nitrogen dioxide radical (NO2·), thyl radical, and bicarbonate radical anion.34 Superoxide anion does not appreciably react with DCFH to form DCF.26 In one of the pioneering studies in which DCFH was used to monitor intracellular oxidative stress during apoptosis, the investigators noted that H2O2-induced intracellular DCF fluorescence was dependent on GSH and inhibited by iron chelators.6,7 It was concluded that intracellular hydroxyl radical formed via the Fenton reaction was responsible for DCFH oxidation to DCF. The present findings are similar to those reported previously.6,7 However, our interpretation is very different. Results obtained with anti-TfR antibody (Figure 3A) strongly suggest that iron transported into cells via TfR is responsible for H2O2-induced DCFH oxidation, and that intracellular oxidative stress, caused by GSH depletion, triggers iron signaling and apoptosis in this model (Figure 7). However, reports also indicate that stimulation of vascular smooth muscle cells with mitogenic growth factors induces a large increase in DCF fluorescence minutes after ligand stimulation.35,36 In these studies, DCFH oxidation is unlikely to be mediated by oxidant-induced cellular iron signaling. Recent data suggest that DCFH oxidation in cells may be caused by oxidants formed from the interaction between heme proteins (ie, myoglobin and cytochrome c) and H2O2.57 Additional studies are clearly warranted to fully elucidate mechanisms of growth factor-induced oxidant formation.

**Oxidant-Induced Iron Signaling: A More Prevalent Mechanism in Vascular Oxidative Pathologies?**

The role of oxidant-induced cellular iron signaling and the subsequent occurrence of free radical–mediated oxidative damage are becoming increasingly relevant in cardiovascular and neurodegenerative mechanisms.38-42 Mitochondrial toxins that stimulate superoxide and H2O2 formation cause excessive accumulation of cellular iron.43 For example, the redox-active menadione (2-methyl-1,4-naphthoquinone or vitamin K1) induced iron signaling via activation of IRP-1 binding via increased generation of intracellular oxidants.13,14 Antioxidants inhibited quinone-induced TfR overexpression and the associated iron uptake, implicating a role for oxidant-induced iron signaling mechanism. Earlier reports showed that unsaturated fatty acid (eg, docosahexaenoic acid; 22:6) supplementation significantly increased iron uptake and cell injury.44 Iron chelators markedly attenuated oxidized LDL-mediated apoptosis and toxicity in endothelial and epithelial cells.35,46 IRP-1 activation was demonstrated in rat lungs after lipopolysaccharide treatment.42 Thus, oxidant-induced iron signaling is more common than previously recognized in vascular oxidative biology. A recent report revealed that the 75-kDa Fe-S subunit of mitochondrial complex I is regulated by a novel IRE-IRP system.47 Recently, it was reported that H2O2-mediated endothelial cell toxicity is related to inhibition in mitochondrial respiration.48 The present results suggest that peroxide-induced TfR-mediated iron uptake is responsible for DCF fluorescence and apoptosis in endothelial cells. The present findings (ie, peroxide-mediated iron signaling) may have broader implications in oxidant-mediated cell injury in cardiovascular diseases.

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