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 H₂O₂-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. H₂O₂-induced DCF fluorescence was inhibited by the monoclonal IgA-class anti-TfR antibody (42/6) that blocked TfR endocytosis and the iron uptake. H₂O₂-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. H₂O₂-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;92:56-63.)

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 H₂O₂ by numerous investigators.1–4 This assay was originally used to monitor intracellular oxidant produced during oxidative stress or apoptosis.5–7 The cell-permeable nonfluorescent probe DCFH-diacetate (DCFH-DA) is hydrolyzed by intracellular esterases to form the active probe DCFH.8,9 Previous studies implicated a role for redox-active iron in cellular oxidation of DCFH to DCF,5–7 although the origin of cellular iron was not known.5 In this study, we investigated whether H₂O₂-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.10,11 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.10–12 Reports also indicate that the cellular oxidative damage caused by reactive oxygen species and reactive nitrogen species is critically controlled by cellular iron homeostasis.12–15 Exposure of murine fibroblasts to H₂O₂ enhanced the expression of TfR mRNA12 and DCF fluorescence, implicating a potential link between oxidative stress and TfR-mediated iron uptake.

In this study, we tested the hypothesis that H₂O₂ 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 H₂O₂-induced intracellular DCFH oxidation. Treatment with anti-TfR antibody also inhibited H₂O₂ and lipid hydroperoxide–dependent apoptosis. Supplementation of cellular reduced glutathione (GSH) prevented H₂O₂-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 H₂O₂ and other hydroperoxides.

Materials and Methods

Materials

Glucose oxidase, glutathione monoethyl ester, hydrogen peroxide, and desferal (or desferrioxamine) were obtained from Sigma. 13-
Hydroperoxyoctadecadienoic 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) porphin (FeTBPAP) was synthesized according to published methods. Monoclonal antibody, 42/6, against human TIR (IgA class), was obtained from Dr Ian Trowbridge (Salk Institute, San Diego, Calif).

Endothelial Cell Culture
Bovine aortic endothelial cells (BAECs) were obtained from Clonetech. 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 mmol/L), or 13-HpODE (25 µmol/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-TIR antibody, iron chelator, and antioxidants were preincubated with cells for 1 hour before the addition of glucose oxidase. Cells pretreated with FeTBPAP 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 520 nm) equipped with a FITC filter. The intensity values were calibrated 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. On the day of the treatment, the medium was replaced with DMEM containing 2% FBS, and the cells were allowed to adjust to the medium conditions. ⁵⁵Fe (feric chloride; 0.1 µCi) was added to the medium for 0 to 6 hours, and its levels were measured as a function of time. Cells were washed twice with DPBS and lysed with PBS containing 0.1% Triton X-100, and the cell lysate was counted in a beta counter.

Electrophoretic Mobility Shift Assay
IRP binding to IRE was measured by electrophoretic mobility shift assay. ³²P-labeled IRE mRNA for the RNA band-shift assay was prepared using as a template a 1000-bp rat L ferritin pseudogene that contains the conserved IRE sequence. The plasmid (p66-L gene) containing this insert (which was generously provided by Dr Elizabeth Leibold, University of Utah, Salt Lake City, Utah) was linearized with SmaI (Life Technologies, Inc) and used for in vitro transcription of IRE mRNA. Transcription was carried out with Sp6 RNA polymerase using a Riboprobe transcription system from Promega.

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 nmol/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 nmol/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 Anti-TIR 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 peroxidases. Support for the intermediacy of iron in DCFH oxidation came from experiments using desferal, a well-established iron chelator. Pretreatment with desferal (10
H_2O_2) 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 O_2^- or H_2O_2 by FeTBAP. Although we observed a 30% reduction in the fluorescence intensity of DCF (10 \mu mol/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 H_2O_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 g/mL IgG-class immunoglobulin that does not bind to the extracellular domain of the TfR, DCFH oxidation was not inhibited in BAECs treated with H_2O_2. 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 fluorescence.
in TIR 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).²²,²³ 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 activity.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 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-mercaptoethenol (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-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 TIR, 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 TIR.²⁹ In the presence of 42/6, iron cannot enter the cell through TIR. Thus, a distinction between
TfR-independent and TfR-dependent $^{55}$Fe uptake was made. As shown in Figure 4F, $^{55}$Fe uptake was dramatically inhibited. H$_2$O$_2$-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.

Figure 5. H$_2$O$_2$-induced apoptosis. A, BAECs were treated with glucose/glucose oxidase (20 mU), and 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.

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.
induced caspase-3 activation in BAECs is mediated by the TIR-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_2O_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 activation 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 55Fe. Figure 6B shows the time course of 55Fe uptake in cells treated with 13-HpODE and 13-HODE. In 13-HpODE–treated cells, there was a 2-fold increase in 55Fe uptake between 2 and 6 hours. During the same period, caspase-3 activation was enhanced from 10 nmol pNA/mg protein to ~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_2O_2 or 13-HpODE to endothelial cells causes intracellular oxidation of the fluorescent probe DCFH to DCF that is regulated by anti-TIR–mediated uptake of transferrin-iron. Blockade of iron uptake by anti-TIR antibody abolishes H_2O_2 and 13-HpODE–induced DCF fluorescence and apoptosis. The present findings point to a critical role of TIR-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_2O_2–induced oxidative stress, iron signaling, DCF fluorescence, and apoptosis is shown in Figure 7. 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 TIR and ferritin mRNAs. The increased binding to TIR mRNA stabilizes the mRNA leading to enhanced mRNA translation and increased TIR 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. The proposed mechanism of activation of mitochondrial aconitase in cells involves either a direct interaction between the 4Fe–4S cluster and H_2O_2 (or derived oxidants) or an H_2O_2–dependent stress-response signaling pathway. The influx of iron and H_2O_2 causes oxidative damage to cellular lipid, protein, and DNA, ultimately resulting in apoptosis. 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 H_2O_2 or oxidative stress. However, H_2O_2 does not react with DCFH to DCF except in the presence of a catalyst (cytochromes, peroxidases, or redox-active metal ions). DCFH is oxidized to DCF by hydroxyl radical, nitrosonium cation (NO_2^+), thiyl radical, and bicarbonate radical anion. Superoxide anion does not appreciably react with DCFH to form DCF. In one of the pioneering studies in which DCFH was used to monitor intracellular oxidative stress during apoptosis, the investigators noted that H_2O_2–induced intracellular DCF fluorescence...
was dependent on GSH and inhibited by iron chelators.\textsuperscript{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.\textsuperscript{6,7} However, our interpretation is very different.

**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.\textsuperscript{38–42} Mitochondrial toxins that stimulate superoxide and H\textsubscript{2}O\textsubscript{2} formation cause excessive accumulation of cellular iron.\textsuperscript{43} For example, the redox-active menadione (2-methyl-1,4-napthoquinone or vitamin K\textsubscript{I}) induced iron signaling via activation of IRP-1 binding via increased generation of intracellular oxidants.\textsuperscript{13,14} Antioxidants inhibited quinone-induced IRr overexpression and the associated iron uptake, implicating a role for oxidant-induced iron signaling mechanism. Earlier reports showed that unsaturated fatty acid (e.g., docosahexaenoic acid; 22:6) supplementation significantly increased iron uptake and cell injury.\textsuperscript{44} Iron chelators markedly attenuated oxidized LDL-mediated apoptosis and toxicity in endothelial and epithelial cells.\textsuperscript{45,46} IRP-1 activation was demonstrated in rat lungs after lipopolysaccharide treatment.\textsuperscript{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.\textsuperscript{47} Recently, it was reported that H\textsubscript{2}O\textsubscript{2}-mediated endothelial cell toxicity is related to inhibition in mitochondrial respiration.\textsuperscript{48} The present results suggest that peroxide-induced TIR-mediated iron uptake is responsible for DCF fluorescence and apoptosis in endothelial cells. The present findings (i.e., peroxide-mediated iron signaling) may have broader implications in oxidant-mediated cell injury in cardiovascular diseases.

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**References**


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