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Extracellular Iron(II) Can Protect Cells from Hydrogen Peroxide

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We hypothesized that exposure of cells to H_2O_2 plus Fe^{2+} would increase formation of cell-derived lipid peroxides that would inactivate prostaglandin H synthase, resulting in decreased prostaglandin synthesis. Therefore, we treated human endothelial cells with 0–100 μM H_2O_2 followed immediately by addition of 0–200 μM Fe^{2+}. After oxidant exposure, cells were stimulated with 20 μM arachidonic acid to induce prostaglandin I_2 (PGI_2) synthesis. Adding 100 μM H_2O_2 prior to arachidonic acid decreased PGI_2 synthesis more than 80%. However, to our surprise, the addition of Fe^{2+}, in increasing amounts, progressively protected PGI_2 synthesis against the harmful effects of H_2O_2. A ratio of one part H_2O_2 to two parts Fe^{2+} offered almost complete protection, whereas Fe^{3+} did not protect PGI_2 synthesis from H_2O_2. We found that 100 μM H_2O_2 was not cytolytic; however, 250 μM H_2O_2 was cytolytic; Fe^{2+} protected against this cytotoxicity. In addition, extracellular Fe^{2+} prevented the rise in intracellular calcium caused by H_2O_2 and extracellular Fe^{2+} preserved intracellular glutathione in H_2O_2-exposed cells. Electron paramagnetic resonance spin trapping demonstrated that extracellular Fe^{2+} generated the hydroxyl free radical, $HO^{\bullet}$, outside the cell. We speculate that extracellular Fe^{2+} protects the intracellular space from H_2O_2 by initiating the Fenton reaction outside the cell. This reductive cleavage of H_2O_2 generates $HO^{\bullet}$ in the extracellular space, where much of the $HO^{\bullet}$ will react with noncellular components, thereby protecting the cell interior.

Key Words: prostaglandin endoperoxide synthase; prostaglandins; oxidant stress; free radicals; reactive oxygen species; hydroxyl radical; electron paramagnetic resonance.

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It is well known that H_2O_2 plus Fe^{2+} will generate oxidants that initiate free radical reactions and that these radicals may result in cell injury (1, 2). The primary oxidant generated by H_2O_2 plus Fe^{2+} is the hydroxyl radical ($HO^{\bullet}$) (3).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet} \text{ (Fenton reaction)}$$

The hydroxyl radical is highly reactive and is believed to cause cytolysis by initiating peroxidation of cell lipids (4–6).

In addition to $HO^{\bullet}$, the reaction of H_2O_2 with Fe^{2+} will generate Fe^{2+}/Fe^{3+} ratios that may increase lipid oxidation (7, 8). A 1:1 ratio of Fe^{2+} to Fe^{3+} has been reported as ideal for maximizing lipid peroxidation (7–9). If a reaction mixture contains two parts Fe^{2+} to one part H_2O_2, the products would be $HO^{\bullet}$, plus a 1:1 ratio of Fe^{2+}:Fe^{3+}. The resulting combination of $HO^{\bullet}$ plus a 1:1 ratio of Fe^{2+}:Fe^{3+} might generate more lipid peroxides than $HO^{\bullet}$ alone. In addition, the Fe^{2+}:Fe^{3+} ratios may generate the perferryl ion, $Fe^{2+}OO^{\bullet} \rightleftarrows Fe^{3+}O_2^{\cdot -}$, which may generate additional lipid peroxides (2, 10, 11). Therefore, it seems likely that a ratio of two parts Fe^{2+} to one part H_2O_2 would increase cytolysis over either agent alone.

Lipid peroxidation products may also injure specific cell functions without causing cytolysis. For example, prostaglandin H synthase (PGHS)[2] is an enzyme that

[2] Abbreviations used: PGHS, prostaglandin H synthase; PGI_2, prostaglandin I_2; HBSS, Hanks' balanced salt solution; EBM, Eagle's basal medium; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; FBS, fetal bovine serum; PGF; prostaglandin F; M199, medium 199; RIA, radioimmunoassay; BSA, bovine serum albumin; EPR, electron paramagnetic resonance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; CrOx, potassium trioxalato chromiate; POBN, a-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone.

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is highly sensitive to lipid peroxides and H_2O_2 (12–14). Injury of PGHS by peroxides results in decreased synthesis of prostaglandin I_2 (PGI_2, prostacyclin) (12–14).

We hypothesized that H_2O_2 plus Fe^{2+} would generate $HO^{\bullet}$, Fe^{2+}/Fe^{3+} ratios capable of oxidizing lipids, and perferryl ions, all contributing to increased formation of lipid peroxides (2, 4–6, 7–11). These peroxides would then injure PGHS and, perhaps, prostacyclin synthase (15), resulting in decreased PGI_2 synthesis.

We were surprised to discover that extracellular Fe^{2+} protected endothelial PGHS activity against H_2O_2. Though it has been previously speculated (16) and reported that extracellular Fe^{2+} may protect cells from H_2O_2 (17, 18), this possibility is not widely understood. Rather, the predominant view is that Fe^{2+} plus H_2O_2 is cytotoxic (19–23). In this report we demonstrate that extracellular Fe^{2+} "detoxifies" H_2O_2, protecting intracellular molecules from oxidant injury (17, 18).

MATERIALS AND METHODS

Reagents

Powdered medium 199 (with Earle's salts and L-glutamine but without $NaHCO_3$), Hanks' balanced salt solution (HBSS)(10×), Eagle's basal medium (EBM) amino acid solution (100×), and EBM vitamin solution (100×) were purchased from Grand Island Biological Co. (Grand Island, NY). Hepes buffer, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and (4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN), reduced glutathione, glutathione reductase (#G4751), nicotinamide adenine dinucleotide phosphate-reduced (NADPH, #Nl530), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and *tert*-butyl hydroperoxide (*t*-BuOOH, #2633) were purchased from Sigma Chemical Co. (St. Louis, MO); Fura-2 was purchased from Molecular Probes (Junction City, OR); thrombin (bovine topical) from Armour (Kankakee, IL). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). Additional materials were obtained from the following suppliers: 6-keto-$PGF_{1\alpha}$, Upjohn Co. (Kalamazoo, MI); arachidonic acid, Nu Check Prep (Elysian, MN); 30% H_2O_2 solution, Fisher Scientific (Fair Lawn, NJ); $Na_2{}^{51}CrO_4$ (^{51}Cr) and [^{3}H]-6-keto-$PGF_{1\alpha}$, New England Nuclear (Boston, MA); and human fibronectin from Collaborative Research (Bedford, MA). All other chemicals were reagent grade.

Solutions

Medium 199 (Ml99) with 20% FBS was eight parts of 9.87 g/liter medium 199 powder plus 2.2 g/liter $NaHCO_3$, 10 ml each EBM vitamins and amino acids, with two parts FBS, then 100,000 U/liter penicillin G, 100 mg/liter streptomycin, and 300 mg/liter L-glutamine, pH 7.4. HBSS contained 2.2 mM $NaHCO_3$ plus 15 mM Hepes, pH 7.4. HBSS without calcium and magnesium was used for cell washes. Radioimmunoassay (RIA) buffer contained per liter 1.56 g K_2HPO_4, 0.33 g KH_2PO_4, 9 g NaCl, 3.33 g bovine gammaglobulin, 1 g NaN_3, and 10 g bovine serum albumin (BSA), pH 7.4.

Fe^{2+} solutions. To create iron stock solutions, $FeSO_4$ (4 mM final concentration) was added to 18 MΩ H_2O that had been purged with argon for 15 min. This solution was stored sealed under argon until use. Fresh Fe^{2+} solution was prepared for each day's experiments. In experiments that had no added Fe^{2+}, an equal volume of argon-purged H_2O was added to cells to serve as a control.

H_2O_2. H_2O_2 was mixed with HBSS prior to each experiment. In spite of cold storage in the dark, there was some decay of the stock 30% H_2O_2 solution, so the concentration was adjusted by absorption spectroscopy according to the formula $\epsilon_{230} = 88\ L\ mol^{-1}\ cm^{-1}$. Arachidonic acid in ethanol was diluted in HBSS from a 20 mM stock solution. This was prepared fresh for each day's experiments. DTNB buffer contained per liter 7.81 g Na_2HPO_4, 2.76 g NaH_2PO_4, 2.79 g EDTA, 0.04% BSA, and 120 mg DTNB, pH 7.2. Glutathione reductase buffer was prepared by adding 7.81 g Na_2HPO_4, 2.76 g NaH_2PO_2, 0.372 g EDTA, 3.4 g imidazole, 1.2 ml HCl (15 mM), 200 mg BSA, 500 mg NADPH, and 1200 U glutathione reductase to one liter final H_2O, pH 7.2.

Endothelial Cultures

Human umbilical vein endothelial cells were isolated from fresh umbilical cords and cultured in M199 with 20% FBS (24, 25). Cells were seeded on human fibronectin-coated 12-well plates at a density of 450,000 cells per well. The medium was changed after 2–4 h to remove red blood cells and nonadherent tissue cells. Previous studies have shown that this technique yields cultures of high purity with minimal variability in cell number or total protein from well to well (12, 25). All experiments were performed with primary cultures 96 h after initial seeding. Cultures were examined by phase-contrast microscopy prior to use to verify confluence and culture purity.

PGI_2 Determination

The buffer is assayed for the stable product of PGI_2, 6-keto-$PGF_{1\alpha}$, by RIA, as previously described (25, 26) with the following modifications: 100-μl samples of media from the cell cultures were incubated for 16 h at 25°C with 100 μl of [^{3}H]6-keto-$PGF_{1\alpha}$:anti-6-keto-$PGF_{1\alpha}$ antibody mixture in RIA buffer. Two hundred and fifty microliters of saturated ammonium sulfate was added, and the mixture was vortexed and after 15 min centrifuged at 4000*g* for 10 min. Four hundred microliters of the supernatant solution was then added to 5 ml scintillation cocktail, and the radioactivity was detected with a Packard Tri-Carb 4530 scintillation counter. Concentrations were determined from a standard curve. Samples from each well were measured in duplicate. Cross-reactivity of the antibody has been determined as 4% $PGF_{2\alpha}$; 2% $PGF_{1\alpha}$; 1.6% PGE_2; > 1% PGE_1, PGD_1, PGD_2; 0.2% PGH_2 (25, 26).

^{51}Cr Release

Endothelial monolayers were labeled with 7 μCi $Na_2{}^{51}CrO_4$ for 1 h at 37°C in M199, then washed and resuspended in HBSS. Cells were then exposed to control buffer, H_2O_2, Fe^{2+}, or H_2O_2 plus Fe^{2+} as described in the text. The ^{51}Cr released into the buffer during this exposure is designated Count A. The cells were then incubated for 1 to 6 h in M199 plus 20% FBS. The ^{51}Cr released into the medium during this period is designated Count B. The cells were then lysed with 2% Na_2CO_3:0.1 N NaOH for 2 h at 37°C, followed by a Na_2CO_3 wash, designated Count C. Radioactivity was measured with a gamma counter. Percentage release was determined by the formula 100% × (Count A + Count B)/(Count A + Count B + Count C) (12, 25).

U937

U937 cells were cultured in RPMI with 10% bovine serum, 100,000 U/liter penicillin G, 100 mg/liter streptomycin, and 300 mg/liter L-glutamine, pH 7.4. Prior to electron paramagnetic resonance (EPR), cells were centrifuged at 50*g*, then resuspended in HBSS at the cell concentrations depicted in the text.

EPR

The EPR studies utilized a Bruker ESP-300 EPR spectrometer equipped with a TM cavity and an aqueous flat cell. The instrument conditions were 9.79 GHz with a modulation frequency of 100 kHz; modulation amplitude 1 Gauss; sweep rate 60 Gauss/167 s; time constant 327 ms; and nominal microwave power 40 mW. Potassium trioxalato chromiate ($K_3[Cr(C_2O_4)_3] \cdot 3H_2O$), used in some of the EPR studies, was synthesized in our lab using the method of Bailor and Jones (27).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT) Assay

Cells were incubated for 2 h at 37°C in culture medium containing 0.5 mg/ml MTT. The medium was then removed and the intracellular diformazan precipitate dissolved by 2 ml of acid–isopropanol (0.04 N HCl in 100% isopropanol). The absorption was measured at 540 nm against an acid–isopropanol blank (28, 29).

Glutathione Assay

Total glutathione was extracted by adding 500 μl of ice-cold 0.01 N HCl to cells on 12-well plates, followed by freeze-thawing, scraping with a rubber policeman, and diluting 1:10 (dilution varied as needed) with 0.01 N HCl. Four hundred microliters of the diluted cell material was mixed with 400 μl of DTNB buffer in a 1-ml cuvette at 37°C. Four hundred microliters of glutathione reductase buffer at 37°C was then added with mixing. One hundred microliters of the resulting solution was added to a 96-well ELISA plate. The absorbance was followed at 405 nm with an ELISA plate reader (12, 30). Absorbance was compared to a standard curve prepared from stock GSH. Samples from each well were measured in triplicate. This assay measures both glutathione and glutathione disulfide (GSH plus GSSG). Previous studies show that 99% of the glutathione is in the reduced form (12).

RESULTS

H_2O_2 plus Fe^{2+} and Prostaglandin Synthesis

PGHS is an oxidant-sensitive intracellular enzyme that loses activity when exposed to extracellular H_2O_2 (12, 14). To determine if H_2O_2 plus Fe^{2+} alters the activity of PGHS, H_2O_2 was added to confluent human endothelial cell cultures. Then, Fe^{2+}, at 0, 0.5, 1, and 2 times the concentration of H_2O_2, was immediately added while swirling the culture dish. Cells were incubated for 15 min at 37°C. After washing, 20 μM arachidonic acid was added and the buffer assayed for PGI_2. The assay results demonstrate that PGI_2 synthesis is decreased by exposure to H_2O_2, shown by the black bars in Fig. 1A, consistent with previous observations (12, 14). However, the addition of Fe^{2+} protected PGI_2 synthesis from the effects of H_2O_2. At an Fe^{2+} to H_2O_2 ratio of 2:1 there is near-complete protection from the effects of H_2O_2. Fe^{2+}, by itself, has no effect on PGI_2 synthesis (Fig. 1B). To confirm that the protective effects were specific to Fe^{2+}, these studies were repeated with Fe^{3+} [as $Fe_2(SO_4)_3$]. Fe^{3+} had no effect on PGI_2 synthesis in the presence (or absence) of H_2O_2 (data not shown). These results demonstrate that extracellular Fe^{2+} protects PGI_2 synthesis from the effects of extracellular H_2O_2.

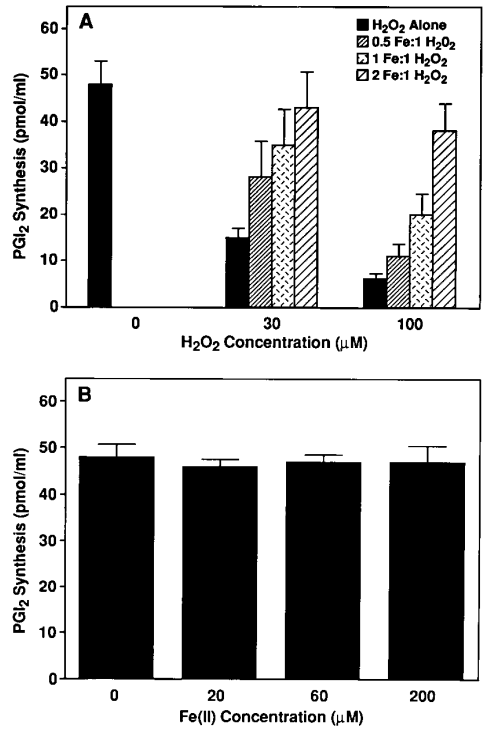

FIG. 1. Fe^{2+} protects endothelial cell prostaglandin synthesis from H_2O_2. (A) Confluent endothelium were washed with HBSS. H_2O_2 in HBSS was added while swirling the culture dish. Fe^{2+}, in pure water, was quickly added with continued swirling. Cells were incubated 15 min at 37°C, washed with HBSS, then stimulated with 20 μM arachidonic acid for 15 min. PGI_2 was measured by RIA for 6-keto-$PGF_{1\alpha}$. (B) Endothelium were treated as in A, but without H_2O_2. $n = 3$ in A and B. Error bars are ±SD. The 0 μM H_2O_2 results in A are used as the 0 μM Fe^{2+} in B. The same cell preparation was used in both A and B.

Other Intracellular Effects of H_2O_2 plus Fe^{2+}

To extend these findings, succinate dehydrogenase, an oxidant sensitive mitochondrial electron transport enzyme, was studied. Endothelial cells were exposed to control buffer, Fe^{2+}, H_2O_2, or H_2O_2 plus Fe^{2+}. Cells were then loaded with MTT. This compound is reduced to a blue water-insoluble diformazan by mitochondrial succinate dehydrogenase (28, 29). As shown in Fig. 2, H_2O_2 decreased mitochondrial reduction of MTT, but Fe^{2+} protected from the effects of H_2O_2. These findings indicate that extracellular Fe^{2+} protects mitochondrial succinate dehydrogenase, in addition to PGH synthase, from extracellular H_2O_2.

Intracellular calcium is reported to be released by H_2O_2 (31). To test if extracellular Fe^{2+} would prevent H_2O_2 from releasing intracellular calcium, endothelial

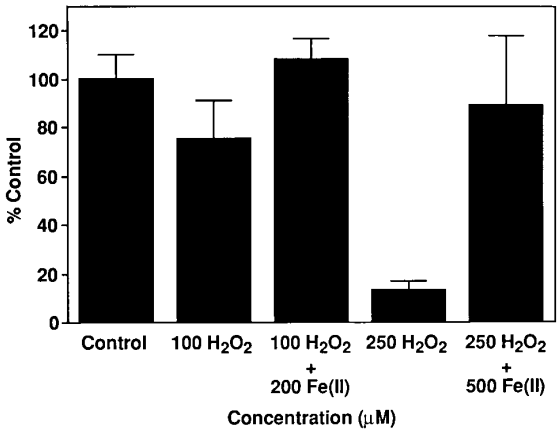

FIG. 2. Fe^{2+} protects mitochondria from H_2O_2. Cells were incubated 1 h at 37°C with HBSS control, H_2O_2, or H_2O_2 plus Fe^{2+} at the concentrations (μM) shown on the *x*-axis. After 1 h, cells were washed and new medium containing MTT was added for 2 h. After lysis in HCl–isopropanol, the reduced diformazan was measured at 540 nm. The *y*-axis is percentage of the untreated control cells. $n = 6$, ±SD.

cells grown on glass coverslips were loaded with Fura-2 and then exposed to H_2O_2 or H_2O_2 plus Fe^{2+}. Results, shown in Fig. 3, demonstrate that 250 μM H_2O_2 causes a progressive increase in intracellular calcium. However, Fe^{2+} prevents this increase. H_2O_2 concentrations below 250 μM did not cause calcium release (data not shown). The protective effect of Fe^{2+} was demonstrated at H_2O_2 concentrations up to 5 mM. This result is further evidence that extracellular Fe^{2+} protects the cell interior from H_2O_2.

Extracellular Radicals

To explain these results, we hypothesized that the extracellular Fe^{2+} caused a Fenton reaction outside the cell, thus protecting oxidant-sensitive processes in the cell interior. To confirm that extracellular Fe^{2+} reacted with H_2O_2 to generate $HO^{\bullet}$ in the extracellular buffer, EPR was utilized. Potassium trioxalato chromiate ($K_3[Cr(C_2O_4)_3]\cdot 3H_2O$) (CrOx) is an EPR line-broadening agent that effectively broadens extracellular nitroxide EPR signals (27, 32, 33). The EPR spin traps DMPO and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) were used for signal detection. Both of these agents diffuse into the cell (33–35). DMPO reacts with oxygen species such as $HO^{\bullet}$ and $O_2^{\cdot -}$ (33–35). POBN is more specific for carbon-centered radicals (36–38). Initial EPR studies with endothelium, which were grown on starch beads, were unsuccessful because the starch generated a carbon-centered radical following exposure to H_2O_2 plus Fe^{2+} (data not shown). Therefore, the monocyte cell line U937, which grows in suspension, was used. Figure 4 shows that H_2O_2 plus Fe^{2+} generates a DMPO signal that is consistent with $HO^{\bullet}$. This $HO^{\bullet}$ signal occurs with or without cells and broadens with 25 mM CrOx, leaving no evidence of the previous $HO^{\bullet}$ signal. POBN also generates a signal which disappears with CrOx. However, the POBN signal is not present in the absence of cells. These results imply that the $HO^{\bullet}$, detected by DMPO, and the cell-dependent carbon-centered radical, detected by POBN, are generated outside the cell.

Since the reactants H_2O_2 plus Fe^{2+} do not generate a carbon-centered radical in the absence of cells, this means the carbon-centered radical detected by POBN is cell-derived (37, 39). The most likely source is the cell outer membrane. This raised the question of whether there might be protection of the cell interior, but cytolysis due to membrane injury later (41). To investigate this, cells were ^{51}Cr loaded and then ex-

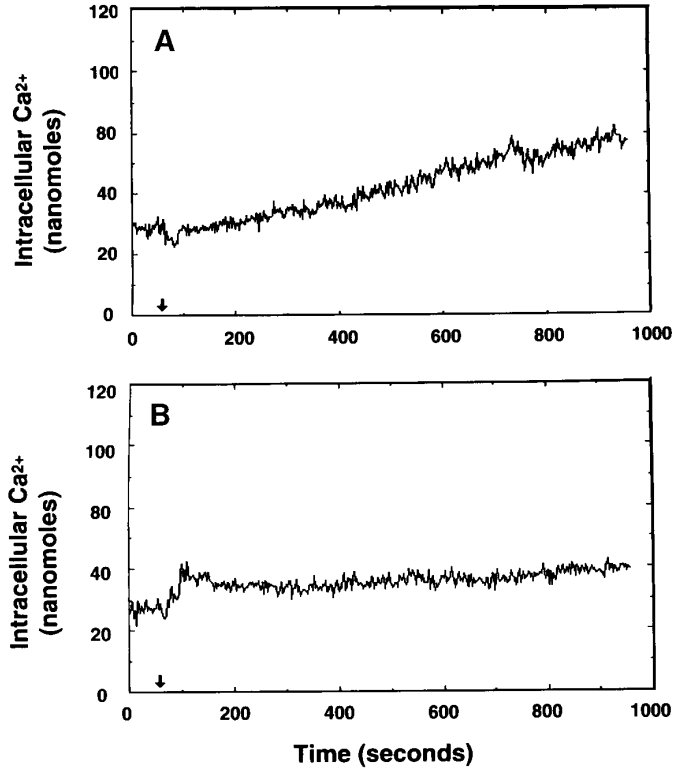

FIG. 3. Fe^{2+} protects from H_2O_2-induced increases in intracellular calcium. Endothelial cells were loaded with Fura-2 in HBSS for 30 min at 37°C. Cells were gently washed twice, then treated with H_2O_2 or Fe^{2+} plus H_2O_2. Calcium was determined on a fluoroscope at 25°C. Excitation frequency was 340 nM for calcium-bound Fura-2 and 380 nM for unbound Fura-2. Emission was measured at 510 nM. Intracellular calcium was measured by the ratio of emission as the excitation frequency was switched from 340 to 380 nM. The arrow in each figure represents the time of reagent addition. (A) 250 μM H_2O_2. (B) 250 μM H_2O_2 plus 500 μM Fe^{2+}. The small rise in calcium after reagent addition in B was not present in all tracings. There was no rise in calcium with buffer alone or with Fe^{2+} (data not shown).

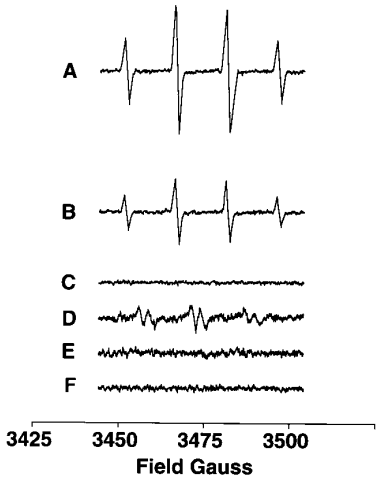

FIG. 4. Spin-trapping radicals in cells exposed to H_2O_2 plus Fe^{2+}. U937 cells were incubated with the spin traps for 5 min at 20°C. 100 μM H_2O_2 (final), then 200 μM Fe^{2+} (final), was added to the cells. 0.5 ml of the cells was then added to an EPR cuvette and spectra were collected as described under Materials and Methods. (A) No cells, 25 mM DMPO, H_2O_2 plus Fe^{2+}. Splitting constants are $a^N = 14.87$ gauss and $a^H = 14.98$ gauss, consistent with hydroxyl radical. (B) 8×10^5 cells, 25 mM DMPO, H_2O_2 plus Fe^{2+}. (C) 8×10^5 cells, 25 mM DMPO, H_2O_2 plus Fe^{2+}, 25 mM CrOx. (D) 2×10^6 cells, 12 mM POBN, H_2O_2 plus Fe^{2+}. Splitting constants are $a^N = 15.61$ and $a^H = 2.79$, consistent with a carbon-centered lipid-derived radical. (E) No cells, 12 mM POBN, H_2O_2 plus Fe^{2+}. (F) 2×10^6 cells, 12 mM POBN, H_2O_2 plus Fe^{2+}, 25 mM CrOx. There was no detectable DMPO signal from cells plus HBSS alone, H_2O_2 alone, or Fe^{2+} alone with the instrument conditions used (data not shown).

posed to H_2O_2 and Fe^{2+} for 1 h at 37°C. ^{51}Cr leak was measured at 1, 3, and 6 h. There was no increase in ^{51}Cr leak up to 6 h in cells exposed to either buffer, Fe^{2+}, H_2O_2, or H_2O_2 plus Fe^{2+}. The 6-h data are shown in Fig. 5. These findings demonstrate that Fe^{2+} plus H_2O_2 does not enhance cytolysis. The results also con-

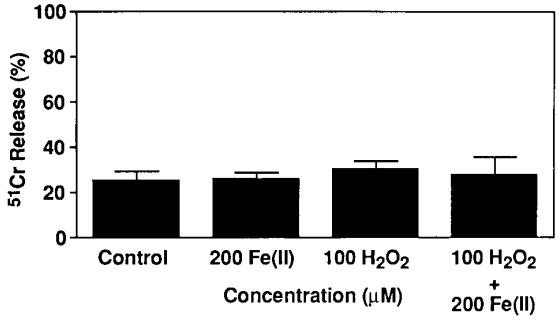

FIG. 5. H_2O_2 plus Fe^{2+} does not enhance endothelial cytolysis. ^{51}Cr-loaded cells were incubated at 37°C with HBSS, 200 μM Fe^{2+}, 100 μM H_2O_2, or 100 μM H_2O_2 plus 200 μM Fe^{2+}. After 1 h cells were washed and new medium was added. Cells were cultured in the new medium an additional 6 h, then assessed for ^{51}Cr release. $n = 6$, error bars are ±SD.

TABLE I

Fe^{2+} Protects Intracellular Glutathione from H_2O_2

Reagents added	Glutathione (pmol/μg protein)
Buffer	99 ± 4.7
Fe^{2+}	105 ± 13
H_2O_2	72 ± 5.1
H_2O_2 + Fe^{2+}	100 ± 11

Note. HUVEC were washed with HBSS then incubated at 37°C with HBSS, 200 μM Fe^{2+}, 100 μM H_2O_2, or 100 μM H_2O_2 plus 200 μM Fe^{2+}. After 30 min cells were washed once with HBSS and then fixed with ice-cold 0.01 N HCl. Glutathione was measured by the recycling method of Tietze (12, 30) as described under Materials and Methods. Protein was measured by the Bradford method (51); $n = 9$, ±SD.

firm previous observations that show 100 μM H_2O_2 alone does not cause lysis of endothelial cells (12, 14). Additional experiments at concentrations of 250 μM H_2O_2 plus 500 μM Fe^{2+} showed cytolysis from H_2O_2 alone, but no cytolysis from H_2O_2 plus Fe^{2+} (data not shown), consistent with previous reports (17, 18).

Diffusion of H_2O_2 into the Cell

We wished to confirm that extracellular Fe^{2+} prevented H_2O_2 from entering the cell. However, directly measuring intracellular H_2O_2, or other oxygen-centered species, proved to be technically difficult. As an alternate approach, our previous work had demonstrated that small concentrations of H_2O_2 would cause measurable decreases in intracellular glutathione in endothelium (12). Therefore, glutathione was measured to indirectly assess intracellular H_2O_2. Results in Table I show that 100 μM H_2O_2 decreased intracellular glutathione 27%. H_2O_2 plus Fe^{2+} caused no decrease in intracellular glutathione. These observations strengthen our hypothesis that extracellular Fe^{2+} protects the cell from H_2O_2 by reacting with H_2O_2 outside the cell, thereby preventing the diffusion of H_2O_2 into the cell interior.

Limited Cell Injury by Fe^{2+} plus H_2O_2

The above studies demonstrate that the intracellular sites are protected from H_2O_2 by Fe^{2+}. However, the cell-derived carbon-centered radical detected by the spin trap POBN (Fig. 4) suggested that noncytolytic injury to the extracellular membrane leaflet was occurring. This implied that injury to membrane-associated extracellular proteins might be detectable. The thrombin receptor interfaces with the aqueous phase where the products of H_2O_2 plus Fe^{2+} are generated. Therefore, the thrombin receptor, or components related to the thrombin receptor, might be altered by H_2O_2 plus Fe^{2+}. To explore this, cells were stimulated

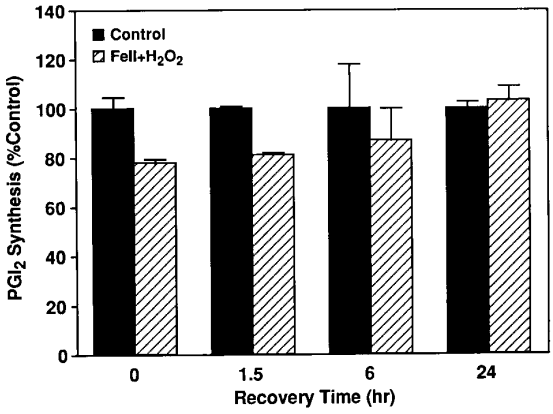

FIG. 6. Fe^{2+} protects the thrombin pathway from H_2O_2. Cells were incubated 15 min with HBSS (control) or 100 μM H_2O_2 plus 200 μM Fe^{2+} as in Fig. 1. The 0-h cells were then washed with HBSS and stimulated with 1 U/ml thrombin. The remaining cells had new M199 added and were incubated for 1.5, 6, or 24 h, then washed and stimulated with thrombin. Results are expressed as percentage control. Measured PGI_2 syntheses in the controls were 88 ± 4, 127 ± 1.2, 141 ± 25, and 160 ± 4 pmol/ml for the 0−, 1.5−, 6−, and 24-h recovery cells, respectively. n = 3, ±SD.

with 1 U/ml thrombin following exposure to H_2O_2 plus Fe^{2+}. H_2O_2 plus Fe^{2+} caused a 22% decrease in PGI_2 synthesis as shown in Fig. 6. PGI_2 synthesis by endothelial cells from this same preparation exposed to H_2O_2 plus Fe^{2+} and then stimulated with arachidonic acid was not decreased, consistent with results in Fig. 1 (data not shown). In addition, H_2O_2 alone decreased thrombin-stimulated PGI_2 synthesis 76% (data not shown); these results are also consistent with the findings in Fig. 1. Recovery of the thrombin pathway occurred in 24 h (Fig. 6). These findings demonstrate that Fe^{2+} affords protection of the thrombin signal pathway from H_2O_2, that this protection is not complete, that the alteration of the thrombin pathway is prior to prostaglandin H synthase, and that recovery of the thrombin pathway occurs in 24 h.

DISCUSSION

The findings in this study are important because they indicate that intracellular injury from extracellular H_2O_2 is decreased by extracellular Fe^{2+}. As discussed in the introduction, this finding is not intuitively obvious and is not widely understood. PGH synthase activity, mitochondrial electron transport, cell calcium, and intracellular glutathione are preserved when extracellular Fe^{2+} reacts with H_2O_2. In addition, H_2O_2 plus Fe^{2+} does not cause delayed cytolysis at concentrations up to 250 μM H_2O_2 and 500 μM Fe^{2+}.

However, Fe^{2+} does not completely protect all cell functions from H_2O_2. The thrombin signal pathway loses 22% of its activity, but recovers in 24 h. Further, the observation in Fig. 4 that Fe^{2+} plus H_2O_2 generates a POBN EPR signal only in the presence of cells indicates that cellular constituents are reacting with components in the extracellular buffer. The fact that this signal disappears with CrOx implies that the signal is generated outside the cell. The most likely site is the outer cell membrane. However, our demonstration that the intracellular effects of H_2O_2 are decreased by extracellular Fe^{2+} and the absence of cytolysis supports the view that extracellular Fe^{2+} protects the cell from H_2O_2.

Our interpretation that the EPR experiments in Figs. 4D–4F demonstrate that H_2O_2 plus Fe^{2+} generates lipid radicals ($L^{\bullet}$) in the outer cell membrane rests on the idea that CrOx does not diffuse into cells. This fact has been adequately demonstrated by Samuni *et al.* (33). However, the absence of a detectable signal by POBN in the CrOx-exposed cells does not indicate absence of intracellular $L^{\bullet}$, since the POBN/$L^{\bullet}$ spin adduct may diffuse out of the cell. In addition, the magnitude of the intracellular signal may be below the detection limit of the instrument. Therefore, while our findings indicate that the large extracellular signal is broadened by CrOx, they do not prove that there is no $L^{\bullet}$ generated in the cell interior. The authors believe that the absence of intracellular injury from H_2O_2 plus Fe^{2+} favors the interpretation that most, or all, of the POBN/$L^{\bullet}$ signal is generated at the outer cell membrane.

One question raised by our observations is why excess Fe^{2+} is required for complete protection from H_2O_2, since the stoichiometry of the Fenton reaction is one part Fe^{2+} to one part H_2O_2 (3). Two potential reasons are (i) Fe^{2+} rapidly oxidizes to Fe^{3+} in physiologic buffers, with a $t^{1/2}$ of ≈20 s (42); and (ii) the reaction $HO^{\bullet} + Fe^{2+} \rightarrow Fe^{3+} + H_2O$ will consume some of the Fe^{2+}. Thus, a requirement for excess Fe^{2+} is expected.

The rate of $HO^{\bullet}$ reaction with other molecules, such as cell constituents, is nearly diffusion limited. This makes direct cellular defense against $HO^{\bullet}$ difficult. Therefore, numerous strategies are used by the cell to prevent $HO^{\bullet}$ formation. The strategies include chelation of iron to prevent the Fenton reaction (6, 43) and removal of peroxides by cytoplasmic and mitochondrial glutathione peroxidase and peroxisomal catalase (44, 45). In addition, the cell can increase levels of ferritin and antioxidant enzymes following exposure to sublethal oxidant stress (42, 46). All of these methods involve removing intracellular peroxides or iron, thereby removing the substrates needed for $HO^{\bullet}$ and alkoxyl radical production. In addition to these intracellular defenses, the blood and extracellular fluid defend cells against extracellular oxidants by a combination of ascorbate, reduced sulfhydryl compounds, red cell catalase and glutathione peroxidase, and iron chelation by

transferrin (40, 47–50). When these antioxidants fail to control extracellular oxidant production, there is ample *in vitro* evidence of cellular injury, including injury from $HO^{\bullet}$ (50).

The experimental findings we obtained were in specific cell culture systems in which the addition of H_2O_2 and Fe^{2+} was carefully controlled. *In vivo,* where iron and peroxide are tightly regulated, this scenario is but one of many possible complex interactions between the cell and iron or peroxide. Nevertheless, in situations where extracellular Fe^{2+} and peroxide do react, our findings indicate that the resultant $HO^{\bullet}$ may be less toxic for the cell than the peroxide alone (19–21, 52, 53). Inside the cell, peroxide has only cell constituents to react with, while outside there are numerous targets for the highly reactive $HO^{\bullet}$, including buffer salts, proteins, amino acids, and specific antioxidants.

In summary, the findings in this report demonstrate that extracellular Fe^{2+} can protect the cell from extracellular H_2O_2. The resultant $HO^{\bullet}$, formed by the reductive cleavage of H_2O_2, is generated outside the cell where there are numerous noncellular targets. This provides protection of vital intracellular functions.

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