Extracellular Iron(II) Can Protect Cells from Hydrogen Peroxide

Stephen L. Hempel, Garry R. Buettner, Duane A. Wessels, George M. Galvan, and Yunxia Q. O'Malley
Department of Veterans Affairs Medical Center, Department of Medicine, and ESR Facility, University of Iowa, Iowa City, Iowa 52242

Received January 8, 1996, and in revised form March 27, 1996

We hypothesized that exposure of cells to \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \) 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 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) followed immediately by addition of 0–200 \( \mu \text{M} \) \( \text{Fe}^{2+} \). After oxidant exposure, cells were stimulated with 20 \( \mu \text{M} \) arachidonic acid to induce prostaglandin I\(_2\) (PGI\(_2\)) synthesis. Adding 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) prior to arachidonic acid decreased PGI\(_2\) synthesis by 80%. However, in our surprise, the addition of \( \text{Fe}^{2+} \) protected PGI\(_2\) synthesis against the harmful effects of \( \text{H}_2\text{O}_2 \). A ratio of one part \( \text{H}_2\text{O}_2 \) to two parts \( \text{Fe}^{2+} \) offered almost complete protection, whereas \( \text{Fe}^{2+} \) did not protect PGI\(_2\) synthesis from \( \text{H}_2\text{O}_2 \). We found that 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) was not cytolytic; however, 250 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) was cytolytic; \( \text{Fe}^{2+} \) protected against this cytotoxicity. In addition, extracellular \( \text{Fe}^{2+} \) prevented the rise in intracellular calcium caused by \( \text{H}_2\text{O}_2 \) and extracellular \( \text{Fe}^{2+} \) preserved intracellular glutathione in \( \text{H}_2\text{O}_2 \)-exposed cells. Electron paramagnetic resonance spin trapping demonstrated that extracellular \( \text{Fe}^{2+} \) generated the hydroxyl free radical, HO•, outside the cell. We speculate that extracellular \( \text{Fe}^{2+} \) protects the intracellular space from \( \text{H}_2\text{O}_2 \) by initiating the Fenton reaction outside the cell. This reductive cleavage of \( \text{H}_2\text{O}_2 \) generates HO• in the extracellular space, where much of the HO• 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.

It is well known that \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \) will generate oxidants that initiate free radical reactions and that these radicals may result in cell injury (1, 2). The primary oxidant generated by \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \) is the hydroxyl radical (HO•) (3).

\( \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{HO}^• \) (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•, the reaction of \( \text{H}_2\text{O}_2 \) with \( \text{Fe}^{2+} \) will generate \( \text{Fe}^{2+}/\text{Fe}^{3+} \) ratios that may increase lipid oxidation (7, 8). A 1:1 ratio of \( \text{Fe}^{2+}/\text{Fe}^{3+} \) has been reported as ideal for maximizing lipid peroxidation (7–9). If a reaction mixture contains two parts \( \text{Fe}^{2+} \) to one part \( \text{H}_2\text{O}_2 \), the products would be HO•, plus a 1:1 ratio of \( \text{Fe}^{2+}/\text{Fe}^{3+} \). The resulting combination of HO• plus a 1:1 ratio of \( \text{Fe}^{2+}/\text{Fe}^{3+} \) might generate more lipid peroxides than HO• alone. In addition, the \( \text{Fe}^{2+}/\text{Fe}^{3+} \) ratios may generate the peroxy radical, \( \text{Fe}^{2+}\text{OO}^- = \text{Fe}^{3+}\text{O}_2^- \), which may generate additional lipid peroxides (2, 10, 11). Therefore, it seems likely that a ratio of two parts \( \text{Fe}^{2+} \) to one part \( \text{H}_2\text{O}_2 \) 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; PGI2, prostaglandin I2; 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 tri-oxalato chromate; POBN, a-(4-pyridyl-1-oxide)-N-tert-butyl nitrite.
is highly sensitive to lipid peroxides and H$_2$O$_2$ (12-14). Injury of PGHS by peroxides results in decreased synthesis of prostaglandin I$_2$ (PGI$_2$, prostacyclin) (12-14).

We hypothesized that H$_2$O$_2$ plus Fe$^{2+}$ would generate HO', 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$_2$O$_2$. Though it has been previously speculated (16) and reported that extracellular Fe$^{2+}$ may protect cells from H$_2$O$_2$ (17, 18), this possibility is not widely understood. Rather, the predominant view is that 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 apologize for the errors in our previous work. The correct interpretation is that extracellular Fe$^{2+}$ protected endothelial PGHS activity against H$_2$O$_2$. Though it has been previously speculated (16) and reported that extracellular Fe$^{2+}$ may protect cells from H$_2$O$_2$ (17, 18), this possibility is not widely understood. Rather, the predominant view is that 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.

**MATERIALS AND METHODS**

Reagents

Powdered medium 199 (with Earle's salts and L-glutamine but without NaHCO$_3$), Hanks' balanced salt solution (HBSS)(10x), Eagle's basal medium (EBM) amino acid solution (100x), and EBM vitamin solution (100x) were purchased from Grand Island Biological Co. (Grand Island, NY). Heps buffer, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), and (4-pyridyl-1-oxide)-tert-butyl nitronate (POBN), reduced glutathione, glutathione reductase (#G4751), nicotinamide adenine dinucleotide phosphate-reduced (NADPH, #NI530), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and (4-pyridyl-1-oxide)-tert-butyl nitronate (POBN) 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$_2$O$_2$ solution, Fisher Scientific (Fair Lawn, NJ); Na$_2$CrO$_4$ (Cr), and [$^3$H]6-keto-PGF$_{1{\alpha}}$, New England Nuclear (Boston, MA); and human fibroblast from Collaborative Research (Bedford, MA). All other chemicals were reagent grade.

Solutions

Medium 199 (M199) 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 g/ml streptomycin, and 300 g/ml L-glutamine, pH 7.4. HBSS contained 2.2 m NaHCO$_3$ plus 15 m Heps, pH 7.4. HBSS without calcium and magnesium was used for cell washes. Radioimmunoassay (RIA) buffer contained per liter 1.56 g K$_2$HPO$_4$. 0.33 g KH$_2$PO$_4$, 9 g NaCl, 3.33 g bovine gamma globulin, 1 g Na$_3$Na$_2$, and 10 g bovine serum albumin (BSA), pH 7.4.

Fe$^{2+}$ solutions. To create iron stock solutions, FeSO$_4$ (4 m final concentration) was added to 18 M H$_2$O 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$_2$O was added to cells to serve as a control.

H$_2$O$_2$. H$_2$O$_2$ 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$_2$O$_2$ solution, so the concentration was adjusted by absorption spectroscopy according to the formula $a_{320} = 88$ L mol$^{-1}$ cm$^{-1}$. Arachidonic acid in ethanol was diluted in HBSS from a 20% stock solution. This was prepared fresh for each day's experiments. DTNB buffer contained per liter 7.81 g Na$_2$HPO$_4$. 2.76 g NaH$_2$PO$_4$. 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$_2$HPO$_4$. 2.76 g NaH$_2$PO$_4$. 0.372 g EDTA, 3.4 g imidazole, 1.2 ml HCl (15 m), 200 mg BSA, 500 mg NADPH, and 1200 U glutathione reductase to one liter final H$_2$O$_2$. 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 [6$^3$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% PGI$_2$; 2% PGI$_{2\alpha}$; 1.6% PGE$_2$; > 1% PGE$_1$, PGD$_1$, PGD$_2$: 0.2% PGI$_2$ (25, 26).

**51Cr Release**

Endothelial monolayers were labeled with $7 \mu$Ci Na$_2$CrO$_4$ for 1 h at 37°C in M199, then washed and resuspended in HBSS. Cells were then exposed to control buffer, H$_2$O$_2$, Fe$^{2+}$, or H$_2$O$_2$ 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$_2$CO$_3$:0.1 n NaOH for 2 h at 37°C, followed by a Na$_2$CO$_3$ wash, designated Count C. Radioactivity was measured with a gamma counter. Percentage release was determined by the formula

\[ \text{Percentage release} = \frac{\text{Count A} - \text{Count B}}{\text{Count A}} \times 100\% \]

U937

U937 cells were cultured in RPMI with 10% bovine serum, 100,000 U/liter penicillin G, 100 mg/ml streptomycin, and 300 mg/ml L-glutamine, pH 7.4. Prior to electron paramagnetic resonance (EPR) studies, cells were centrifuged at 50g, 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\(_2\)O\(_4\))\(_3\)]·3H\(_2\)O), used in some of the EPR studies, was synthesized in our lab using the method of Bailor and J ones (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, 28, 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\(_2\)O\(_2\) plus Fe\(^{2+}\) and Prostaglandin Synthesis

PGHS is an oxidant-sensitive intracellular enzyme that loses activity when exposed to extracellular H\(_2\)O\(_2\) (12, 14). To determine if H\(_2\)O\(_2\) plus Fe\(^{2+}\) alters the activity of PGHS, H\(_2\)O\(_2\) was added to confluent human endothelial cell cultures. Then, Fe\(^{2+}\), at 0, 0.5, 1, and 2 times the concentration of H\(_2\)O\(_2\), 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\(_2\)O\(_2\), 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\(_2\)O\(_2\). At an Fe\(^{2+}\) to H\(_2\)O\(_2\) ratio of 2:1 there is near-complete protection from the effects of H\(_2\)O\(_2\). 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\(_2\)O\(_2\) (data not shown). These results demonstrate that extracellular Fe\(^{2+}\) protects PGI\(_2\) synthesis from the effects of extracellular H\(_2\)O\(_2\).

Other Intracellular Effects of H\(_2\)O\(_2\) 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\(_2\)O\(_2\), or H\(_2\)O\(_2\) 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\(_2\)O\(_2\) decreased mitochondrial reduction of MTT, but Fe\(^{2+}\) protected from the effects of H\(_2\)O\(_2). These findings indicate that extracellular Fe\(^{2+}\) protects mitochondrial succinate dehydrogenase, in addition to PGH synthase, from extracellular H\(_2\)O\(_2\).

Intracellular calcium is reported to be released by H\(_2\)O\(_2\) (31). To test if extracellular Fe\(^{2+}\) would prevent H\(_2\)O\(_2\) from releasing intracellular calcium, endothelial
generates a DMPO signal that is consistent with HO·. This HO· signal occurs with or without cells and broadens with 25 mM CrOx, leaving no evidence of the previous HO· 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·, detected by DMPO, and the cell-dependent carbon-centered radical, detected by POBN, are generated outside the cell.

Since the reactants H2O2 plus Fe2+/Fe2+ 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 51Cr loaded and then ex-

FIG. 2. Fe2+ protects mitochondria from H2O2. Cells were incubated 1 h at 37°C with HBSS control, H2O2, or H2O2 plus Fe2+ 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 H2O2 or H2O2 plus Fe2+. Results, shown in Fig. 3, demonstrate that 250 μM H2O2 causes a progressive increase in intracellular calcium. However, Fe2+ prevents this increase. H2O2 concentrations below 250 μM did not cause calcium release (data not shown). The protective effect of Fe2+ was demonstrated at H2O2 concentrations up to 5 mM. This result is further evidence that extracellular Fe2+ protects the cell interior from H2O2.

Extracellular Radicals

To explain these results, we hypothesized that the extracellular Fe2+ caused a Fenton reaction outside the cell, thus protecting oxidant-sensitive processes in the cell interior. To confirm that extracellular Fe2+ reacted with H2O2 to generate HO· in the extracellular buffer, EPR was utilized. Potassium trioxalato chromiate (K3[Cr(C2O4)3]·3H2O) (CrOx) is an EPR line-broadening agent that effectively broadens extracellular nitroxide EPR signals (27, 32, 33). The EPR spin traps DMPO and a-(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· and O2·− (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 H2O2 plus Fe2+ (data not shown). Therefore, the monocyte cell line U937, which grows in suspension, was used. Figure 4 shows that H2O2 plus Fe2+ generates a DMPO signal that is consistent with HO·. This HO· signal occurs with or without cells and broadens with 25 mM CrOx, leaving no evidence of the previous HO· 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·, detected by DMPO, and the cell-dependent carbon-centered radical, detected by POBN, are generated outside the cell.

Since the reactants H2O2 plus Fe2+/Fe2+ 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 51Cr loaded and then ex-

FIG. 3. Fe2+ protects from H2O2-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 H2O2 or Fe2+/Fe2+ plus H2O2. 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 H2O2. (B) 250 μM H2O2 plus 500 μM Fe2+. 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 Fe2+ (data not shown).
FIG. 4. Spin-trapping radicals in cells exposed to \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \). U937 cells were incubated with the spin traps for 5 min at 20°C. 100 \( \mu\text{M} \) \( \text{H}_2\text{O}_2 \) (final), then 200 \( \mu\text{M} \) \( \text{Fe}^{2+} \) (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 \( \mu\text{M} \) DMPO, \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \). Splitting constants are \( a_h = 14.87 \text{ gauss} \) and \( a_v = 14.98 \text{ gauss} \), consistent with hydroxyl radical. (B) \( 8 \times 10^5 \) cells, 25 \( \mu\text{M} \) DMPO, \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \). (C) \( 8 \times 10^5 \) cells, 25 \( \mu\text{M} \) DMPO, \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \), 25 \( \mu\text{M} \) CrOx. (D) \( 2 \times 10^5 \) cells, 12 \( \mu\text{M} \) POBN, \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \). Splitting constants are \( a_h = 15.61 \text{ gauss} \) and \( a_v = 2.79 \text{ gauss} \), consistent with a carbon-centered lipid-derived radical. (E) No cells, 12 \( \mu\text{M} \) POBN, \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \). (F) \( 2 \times 10^5 \) cells, 12 \( \mu\text{M} \) POBN, \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \), 25 \( \mu\text{M} \) CrOx. There was no detectable DMPO signal from cells plus HBSS alone, \( \text{H}_2\text{O}_2 \) alone, or \( \text{Fe}^{2+} \) alone with the instrument conditions used (data not shown).

![FIG. 4](image)

FIG. 5. \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \) does not enhance endothelial cytology. \( ^{51}\text{Cr} \) leak was measured at 1, 3, and 6 h. There was no increase in \( ^{51}\text{Cr} \) leak up to 6 h in cells exposed to either buffer, \( \text{Fe}^{2+} \), \( \text{H}_2\text{O}_2 \), or \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \). The 6-h data are shown in Fig. 5. These findings demonstrate that \( \text{Fe}^{2+} \) plus \( \text{H}_2\text{O}_2 \) does not enhance cytology. The results also confirm previous observations that show 100 \( \mu\text{M} \) \( \text{H}_2\text{O}_2 \) alone does not cause lysis of endothelial cells (12, 14). Additional experiments at concentrations of 250 \( \mu\text{M} \) \( \text{H}_2\text{O}_2 \) plus 500 \( \mu\text{M} \) \( \text{Fe}^{2+} \) showed cytology from \( \text{H}_2\text{O}_2 \) alone, but no cytolysis from \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \) (data not shown), consistent with previous reports (17, 18).

![FIG. 5](image)

**Diffusion of \( \text{H}_2\text{O}_2 \) into the Cell**

We wished to confirm that extracellular \( \text{Fe}^{2+} \) prevents \( \text{H}_2\text{O}_2 \) from entering the cell. However, directly measuring intracellular \( \text{H}_2\text{O}_2 \), or other oxygen-centered species, proved to be technically difficult. As an alternate approach, our previous work had demonstrated that small concentrations of \( \text{H}_2\text{O}_2 \) would cause measurable decreases in intracellular glutathione in endothelium (12). Therefore, glutathione was measured to indirectly assess intracellular \( \text{H}_2\text{O}_2 \). Results in Table I show that 100 \( \mu\text{M} \) \( \text{H}_2\text{O}_2 \) decreased intracellular \( \text{glutathione} \) 27%. \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \) caused no decrease in intracellular glutathione. These observations strengthen our hypothesis that extracellular \( \text{Fe}^{2+} \) protects the cell from \( \text{H}_2\text{O}_2 \) by reacting with \( \text{H}_2\text{O}_2 \) outside the cell, thereby preventing the diffusion of \( \text{H}_2\text{O}_2 \) into the cell interior.

**Limited Cell Injury by \( \text{Fe}^{2+} \) plus \( \text{H}_2\text{O}_2 \)**

The above studies demonstrate that the intracellular sites are protected from \( \text{H}_2\text{O}_2 \) by \( \text{Fe}^{2+} \). 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 \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \) are generated. Therefore, the thrombin receptor, or components related to the thrombin receptor, might be altered by \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \). To explore this, cells were stimulated

### Table I

<table>
<thead>
<tr>
<th>Reagents added</th>
<th>Glutathione (pmol/( \mu\text{g \text{protein}} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>99 ± 4.7</td>
</tr>
<tr>
<td>( \text{Fe}^{2+} )</td>
<td>105 ± 13</td>
</tr>
<tr>
<td>( \text{H}_2\text{O}_2 )</td>
<td>72 ± 5.1</td>
</tr>
<tr>
<td>( \text{H}_2\text{O}_2 ) + ( \text{Fe}^{2+} )</td>
<td>100 ± 11</td>
</tr>
</tbody>
</table>

Note. HUVEC were washed with HBSS then incubated at 37°C with HBSS, 200 \( \mu\text{M} \) \( \text{Fe}^{2+} \), 100 \( \mu\text{M} \) \( \text{H}_2\text{O}_2 \), or 100 \( \mu\text{M} \) \( \text{H}_2\text{O}_2 \) plus 200 \( \mu\text{M} \) \( \text{Fe}^{2+} \). 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.
FIG. 6. \( \text{Fe}^{2+} \) protects the thrombin pathway from \( \text{H}_2\text{O}_2 \). Cells were incubated 15 min with HBSS (control) or 100 \( \mu\text{M} \) \( \text{H}_2\text{O}_2 \) plus 200 \( \mu\text{M} \) \( \text{Fe}^{2+} \) as in Fig. 1. The 0 h cells were then washed with HBSS and stimulated with 1 \( \mu\text{M} \) 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 \( \pm \), 127 \( \pm \), 25, and 160 \( \pm \) pmol/ml for the 0, 1.5, 6, and 24-h recovery cells, respectively. \( n = 3 \), \( \pm \text{SD} \).

with 1 \( \mu\text{U} / \text{ml} \) thrombin following exposure to \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \). \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \) 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 \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \) and then stimulated with arachidonic acid was not decreased, consistent with results in Fig. 1 (data not shown). In addition, \( \text{H}_2\text{O}_2 \) 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 \( \text{Fe}^{2+} \) affords protection of the thrombin signal pathway from \( \text{H}_2\text{O}_2 \), 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 \( \text{H}_2\text{O}_2 \) is decreased by extracellular \( \text{Fe}^{2+} \). 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 \( \text{Fe}^{2+} \) reacts with \( \text{H}_2\text{O}_2 \). In addition, \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \) does not cause delayed cytolysis at concentrations up to 250 \( \mu\text{M} \) \( \text{H}_2\text{O}_2 \) and 500 \( \mu\text{M} \) \( \text{Fe}^{2+} \).

However, \( \text{Fe}^{2+} \) does not completely protect all cell functions from \( \text{H}_2\text{O}_2 \). The thrombin signal pathway loses 22% of its activity, but recovers in 24 h. Further, the observation in Fig. 4 that \( \text{Fe}^{2+} \) plus \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) are decreased by extracellular \( \text{Fe}^{2+} \) and the absence of cytolysis supports the view that extracellular \( \text{Fe}^{2+} \) protects the cell from \( \text{H}_2\text{O}_2 \).

Our interpretation that the EPR experiments in Figs. 4D–4F demonstrate that \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \) generates lipid radicals (L·) 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·, since the POBN/L· 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· generated in the cell interior. The authors believe that the absence of intracellular injury from \( \text{H}_2\text{O}_2 \) plus \( \text{Fe}^{2+} \) favors the interpretation that most, or all, of the POBN/L· signal is generated at the outer cell membrane.

One question raised by our observations is why excess \( \text{Fe}^{2+} \) is required for complete protection from \( \text{H}_2\text{O}_2 \), since the stoichiometry of the Fenton reaction is one part \( \text{Fe}^{2+} \) to one part \( \text{H}_2\text{O}_2 \) (3). Two potential reasons are (i) \( \text{Fe}^{2+} \) rapidly oxidizes to \( \text{Fe}^{3+} \) in physiologic buffers, with a \( \text{l}_{1/2} \) of \( \approx 20 \text{ s} \) (42); and (ii) the reaction \( \text{HO}^\cdot + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{H}_2\text{O} \) will consume some of the \( \text{Fe}^{2+} \). Thus, a requirement for excess \( \text{Fe}^{2+} \) is expected.

The rate of \( \text{HO}^\cdot \) reaction with other molecules, such as cell constituents, is nearly diffusion limited. This makes direct cellular defense against \( \text{HO}^\cdot \) difficult. Therefore, numerous strategies are used by the cell to prevent \( \text{HO}^\cdot \) 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 \( \text{HO}^\cdot \) 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· (50).

The experimental findings we obtained were in specific cell culture systems in which the addition of H2O2 and Fe2+ 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 Fe2+ and peroxide do react, our findings indicate that the resultant HO· 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·, including buffer salts, proteins, amino acids, and specific antioxidants.

In summary, the findings in this report demonstrate that extracellular Fe2+ can protect the cell from extracellular H2O2. The resultant HO·, formed by the reductive cleavage of H2O2, is generated outside the cell where there are numerous noncellular targets. This provides protection of vital intracellular functions.

ACKNOWLEDGMENTS

This work was performed during tenure of an American Heart Association—Genentech Clinician-Scientist Award, by an American Heart Association Grant-in-Aid Award, by a grant from the American Lung Association of Iowa, and by funds from a Department of Veterans Affairs Merit Review Award to Dr. Hempel. Dr. Buettner was supported in part by NIH HL-49264. Funds for The University of Iowa EPR Center are provided in part by the College of Medicine, The University of Iowa. Funds for the Calcium Core Facility are provided in part by the Department of Veterans Affairs Medical Center (Iowa City, IA). Gerene Denning, Ph.D., Director, Calcium Core Facility, provided assistance with the calcium measurements.

REFERENCES