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Binding of Iron and Inhibition of Iron-Dependent Oxidative Cell Injury by the "Calcium Chelator" 1,2-Bis(2-Aminophenoxy)Ethane *N*,*N*,*N′*,*N′*-tetraacetic Acid (BAPTA)

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Abstract. A role for increases in intracellular calcium (Ca^{2+}) has been suggested in the pathophysiology of various forms of oxidant-mediated cell injury. In recent studies, we found that iron bound to the *Pseudomonas aeruginosa* siderophore, pyochelin, augments oxidant-mediated endothelial cell injury by catalyzing the formation of hydroxyl radical ($HO^{\bullet}$). To investigate the role of Ca^{2+} in this process, the effects of two Ca^{2+} chelating agents, Fura-2 and 1,2-bis(2-aminophenoxy)ethane *N*,*N*,*N′*,*N′*-tetraacetic acid (BAPTA), were assessed. BAPTA, but not Fura-2, was protective against H_2O_2/ferripyochelin-mediated injury. Subsequent data suggested that chelation of iron rather than Ca^{2+} by BAPTA was most likely responsible. Spectrophotometry demonstrated that both ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron formed a complex with BAPTA. The affinity of BAPTA for the metals was $Fe^{3+} > Ca^{2+} > Fe^{2+}$. BAPTA was found to decrease markedly iron-catalyzed production of $HO^{\bullet}$ and/or ferryl species when analyzed by spin trapping. Although our results do not definitively prove that BAPTA protects endothelial cells from ferripyochelin-associated damage by chelating iron, these data indicate that caution must be exercised in utilizing protective effects of intracellular "Ca^{2+} chelating agents" as evidence for a role of alterations in cellular Ca^{2+} levels in experimental conditions in which iron-mediated oxidant production is also occurring. BIOCHEM PHARMACOL **55**;3:287–295, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. BAPTA; calcium; iron; hydroxyl radical; ferryl species; endothelial cell; spin trapping; pyochelin

Oxidant-mediated cell injury has been implicated in a variety of pathologic processes [1, 2]. Sources for these oxidants can be exogenous (e.g. reactive oxygen intermediates released by phagocytic cells) or endogenous (e.g. oxidant products generated by intracellular xanthine oxidase or release from mitochondria) [1, 2]. Among the reactive oxygen species felt to play a key role in cell injury is $HO^{\bullet}$‖ [1, 2]. Most studies [1, 2] implicate the Fenton reaction, in which ferrous iron (Fe^{2+}) reduces H_2O_2 to $HO^{\bullet}$, as the primary mechanism for $HO^{\bullet}$ formation under such circumstances:

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + Fe^{3+} + OH^-$$

For sustained $HO^{\bullet}$ production to occur, an additional source of reducing equivalents must be present to convert the ferric iron (Fe^{3+}) back to its reactive ferrous form. In many biologic systems, $O_2^{\bullet -}$, which also can serve as the source of H_2O_2 via a dismutation reaction, serves this function. The resulting reaction scheme, referred to as the superoxide-driven Fenton reaction or the Haber–Weiss reaction, is as follows:

$$\begin{aligned} O_2^{\bullet -} + Fe^{3+} &\rightarrow O_2 + Fe^{2+} \\ H_2O_2 + Fe^{2+} &\rightarrow HO^{\bullet} + Fe^{3+} + OH^- \\ \hline O_2^{\bullet -} + H_2O_2 &\rightarrow HO^{\bullet} + O_2 + OH^- \end{aligned}$$

Under some circumstances, other oxidizing species of iron (ferryl species) have been shown to be produced via this reaction [3–7]. The relative proportion of hydroxyl radical and ferryl species resulting from the interaction of H_2O_2 and Fe^{2+} appears to be related, in part, to the agent to which the iron is chelated, as well as the ratio of H_2O_2 and Fe^{2+} present [3, 4]. In biologic systems, cells are protected, in part, from the deleterious consequences of untoward production of $HO^{\bullet}$and ferryl species by the fact that iron is usually maintained both intra- and extracellularly com-

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‖ *Abbreviations:* BAPTA, 1,2-bis(2-aminophenoxy)ethane *N*,*N*,*N′*,*N′*-tetraacetic acid; DMPO, 5,5 dimethylpyrroline-*N*-oxide; DTPA, diethylenetriaminepentaacetic acid; $HO^{\bullet}$, hydroxyl radical; $O_2^{\bullet -}$, superoxide; PBN, α-phenyl-*N*-*t*-butyl-nitrone; 4-POBN, α-(4-pyridyl-1-oxide)-*N*-*t*-butyl-nitrone; PPAEC, porcine pulmonary artery endothelial cells.

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plexed to other molecules in a form that is not redox active [2, 8, 9].

Vascular endothelial cells are among the cell types that are particularly susceptible to oxidant-mediated injury [10–16]. Such endothelial cell damage has been linked to the pathophysiology of various types of lung injury [13–16]. Over the last several years, our laboratory has obtained *in vitro* evidence that a novel iron chelate present at sites of pulmonary infection with *Pseudomonas aeruginosa*, iron bound to the *P. aeruginosa*-derived siderophore pyochelin, augments oxidant-mediated damage to pulmonary artery endothelial cells via its ability to act as a catalyst of the Haber–Weiss reaction [17, 18].

The site and/or mechanism whereby ferripyochelin initiates endothelial cell injury is not known. Other laboratories have suggested that an oxidant-induced rise in intracellular Ca^{2+} levels may play a key role in cellular damage resulting from exposure to various oxidant species [19–25]. These conclusions are derived, in part, from the ability of various chelators of intracellular Ca^{2+} (e.g. BAPTA, Fura-2, Quin-2) to inhibit cell injury [26–29].

Based on the above, we initiated studies to investigate the role of changes in intracellular Ca^{2+} in the process of ferripyochelin-dependent endothelial cell injury. Although loading cells with BAPTA markedly protected them from such injury, implying a role of Ca^{2+} modulation, subsequent work reported herein suggests that the protective effect of BAPTA results, instead, from its ability to bind iron in a non-redox active form. This work, along with recent studies with other Ca^{2+} chelators [30–32], indicates the need for caution when attributing protective effects of intracellular Ca^{2+} chelating agents as experimental evidence for a role for Ca^{2+} fluxes in cellular processes in which the Fenton reaction may also be involved.

MATERIALS AND METHODS

Cell Culture

PPAEC were maintained in monolayer culture as previously described [17, 18]. Briefly, PPAEC were seeded (5×10^4 cells/well) in 24-well tissue culture plates containing 0.5 mL medium 199 (University of Iowa Cancer Center) plus 10% fetal bovine serum, 2× basal medium amino acids, basal minimal essential vitamins, 2 mM L-glutamine, and 10 U/mL penicillin/streptomycin (GIBCO). The cells were incubated at 37°, 5% CO_2 until 2–3 days post confluence. With each experiment, control cells of the same passage were studied in parallel to avoid any contribution of a cell line or passage number to the results.

Cell Injury Assay

The specific release of ^{51}Cr from previously loaded endothelial cell monolayers was utilized as the indicator of cellular injury. The *P. aeruginosa* siderophore pyochelin was purified to uniformity from broth cultures of *P. aeruginosa* strain PA01, as described previously [33]. PPAEC, whose intracellular content had been labeled with ^{51}Cr as previously detailed [17, 18], were incubated (37°) for 30 min in the presence of iron-loaded pyochelin (ferripyochelin, 2.5 μM) or the same concentration of its ethanol vehicle. Cells were then exposed to the desired concentration of H_2O_2 (50 μM) for 90 min. At the end of the incubation, supernatant was removed, and its content of ^{51}Cr was determined by a gamma counter. Specific release of ^{51}Cr resulting from oxidant exposure was calculated as follows:

$$\frac{\text{Test well } ^{51}\text{Cr cpm} - \text{spontaneous release } ^{51}\text{Cr cpm}}{\text{Maximum release } ^{51}\text{Cr cpm} - \text{spontaneous release } ^{51}\text{Cr cpm}} \times 100\%$$

Spontaneous release was defined as ^{51}Cr release with cells suspended in buffer only. Maximum release was determined by lysing the cells with 10% Triton X-100. To assess the effect of intracellular BAPTA or Fura-2, the cell monolayer was first incubated in the presence of a 50 μM concentration of the methyl esters of each agent (BAPTA-AM or Fura-2-AM; Sigma Chemical Co.) for 30 min at 37° following which the monolayer was washed three times.

Formation of Iron-BAPTA Complex with Divalent and Trivalent Cations

The binding of Ca^{2+} to BAPTA results in a shift absorbance spectrum of the compounds [34]. Evidence for chelation of Fe^{2+} or Fe^{3+} was gathered by obtaining the absorbance spectrum of BAPTA in the presence or absence of Fe^{2+} or Fe^{3+}. BAPTA (50 μM) was suspended in DMSO to which was added either buffer (0.15 M NaCl) or buffer containing either: 10 μM–10 mM $CaCl_2$, 1 μM–10 mM $FeSO_4$ or 5 μM–10 mM $FeCl_3$. The resulting solution was placed in a cuvette, and absorbance was determined (220–350 nm) by spectrophotometer. The ranges of calcium and iron were employed in the experiments designed to estimate K_d of BAPTA for these metals. Calculations of K_d for BAPTA and Fe^{2+} or Fe^{3+} were based on the relative ability of these cations to inhibit formation of the Ca^{2+}–BAPTA complex using a K_d for that complex of 1.1×10^{-7} M [34, 35]. K_d was estimated using the concentration of BAPTA needed to decrease the formation of the Fura-2–Ca^{2+} complex by 50% using the formula:

$$\frac{[\text{BAPTA}]}{[\text{Fura-2}]} = \frac{1.1 \times 10^{-7}\ \text{M}}{\text{BAPTA } K_d}$$

Spin Trapping

Formation of free radicals was quantitated using previously described techniques of spin trapping in conjunction with EPR spectrometry [36]. Desired reactions were allowed to take place in the presence of: (1) 100 mM DMPO or (2) 10 mM 4-POBN (both compounds were obtained from the Oklahoma Medical Research Foundation). Reaction mix-

tures also contained 1–4% DMSO (Fischer Scientific). The solutions were placed in a quartz EPR flat cell that was, in turn, placed in the cavity of a Brüker ESP300 EPR spectrometer (Brüker Instruments). Then EPR spectra were recorded at 25°. Unless otherwise stated, instrument settings were: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 0.892 G; sweep time, 0.238 G/min; and response time, 0.655 sec. In some experiments, signal averaging was performed to enhance the signal/noise ratio.

Statistics

Experimental data were analyzed by ANOVA analysis using Epistat software with statistical significance defined as a P value ≤ 0.05.

RESULTS

Role of Calcium in Ferripyochelin-Mediated Endothelial Cell Injury

Consistent with our earlier work [17, 18], ferripyochelin (2.5 μM) significantly enhanced injury to PPAEC monolayers resulting from their exposure to H_2O_2 as assessed by the specific release of ^{51}Cr (Fig. 1). Injury was not augmented if pyochelin that did not contain iron (apopyochelin) was substituted for ferripyochelin ($P > 0.05$), indicating that iron was required for the ferripyochelin-mediated enhancement of injury and that the ethanol vehicle itself did not contribute to the effect. Given some evidence in other cell systems that elevations in intracellular Ca^{2+} are involved in the pathogenesis of oxidant-induced cell injury [22, 23, 37, 38], we sought to explore the potential link between alterations in intracellular Ca^{2+} and ferripyochelin-dependent PPAEC injury.

One potential mechanism whereby intracellular Ca^{2+} can increase following oxidant exposure is via influx from the extracellular environment [22, 23, 37, 38]. However, we found no difference in the magnitude of ^{51}Cr release resulting from exposure to H_2O_2 and ferripyochelin regardless of whether or not Ca^{2+} was present in or absent from the extracellular buffer (Fig. 1). In addition, inclusion of the extracellular Ca^{2+} chelator EGTA (10 mM) or $LaCl_3$, (10 μM), an agent that interferes with Ca^{2+} influx through channels [39], also failed to provide protection (Fig. 1).

An alternative source of Ca^{2+} that could increase intracellular levels of this cation following oxidant exposure is release from intracellular stores [22, 23, 37, 38]. One means of assessing this possibility is to load cells with a high affinity Ca^{2+} chelator that can buffer potential elevations of intracellular Ca^{2+} [26–28, 32]. Accordingly, PPAEC were incubated with methyl ester derivatives of BAPTA or Fura-2 (i.e. BAPTA-AM and Fura-2 AM), which are membrane permeable and partition into the intracellular space. Once inside the cells, the ester linkage is cleaved by intracellular enzymes releasing free BAPTA or Fura-2 that is trapped in the intracellular space and capable of rapidly binding free Ca^{2+} [34, 35, 40].

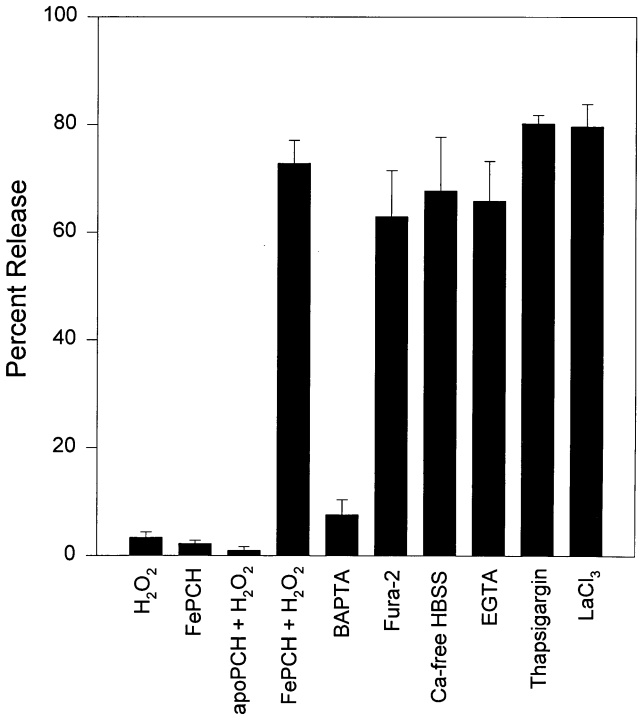

FIG. 1. Effect of BAPTA on H_2O_2/ferripyochelin injury of PPAEC. Shown is specific ^{51}Cr release from PPAEC monolayers resulting from exposure of control PPAEC to H_2O_2 (50 μM) or H_2O_2 following a 30-min preincubation with 2.5 μM ferripyochelin (Fe-PCH + H_2O_2) or apo-pyochelin (apo-PCH + H_2O_2). Also shown are results obtained when PPAEC were preloaded with Fura-2 (50 μM) or BAPTA (50 μM) or the experiments were performed in the presence of: EGTA (10 mM); $LaCl_3$ (10 μM); in Ca^{2+}-free HBSS; or after pretreatment of the cells with thapsigargin (2 μM). The difference in ^{51}Cr release between H_2O_2-treated cells and those treated with H_2O_2 and Fe-PCH was significant at $P < 0.00001$. BAPTA produced a statistically significant decrease in ^{51}Cr release from cells exposed to H_2O_2 and ferripyochelin with $P < 0.001$. Values of P with Fura 2, EGTA, $LaCl_3$, thapsigargin, and Ca^{2+}-free HBSS were all > 0.05. Results are means ± SD (N = 3 or 4).

When the ability of exposure to H_2O_2 and ferripyochelin to damage Fura-loaded or BAPTA-loaded PPAEC was compared with control cells, there was a significant decrease in the magnitude of ^{51}Cr release observed with BAPTA, but not Fura-2 loaded cells (Fig. 1). The intracellular location of BAPTA appeared to be critical, as we found no difference in cellular protection if the cells were washed free of extracellular BAPTA or if it was allowed to remain during cellular exposure to H_2O_2 and ferripyochelin (Fig. 2). As assessed spectrophotometrically, there was no evidence that ^{51}Cr bound to BAPTA (data not shown) and, hence, the result was not related to interference with the ^{51}Cr release assay. The ability of BAPTA to protect the PPAEC from H_2O_2/ferripyochelin-mediated injury was consistent with a role for alterations in intracellular Ca^{2+} in this process. However, pretreatment of the cells with

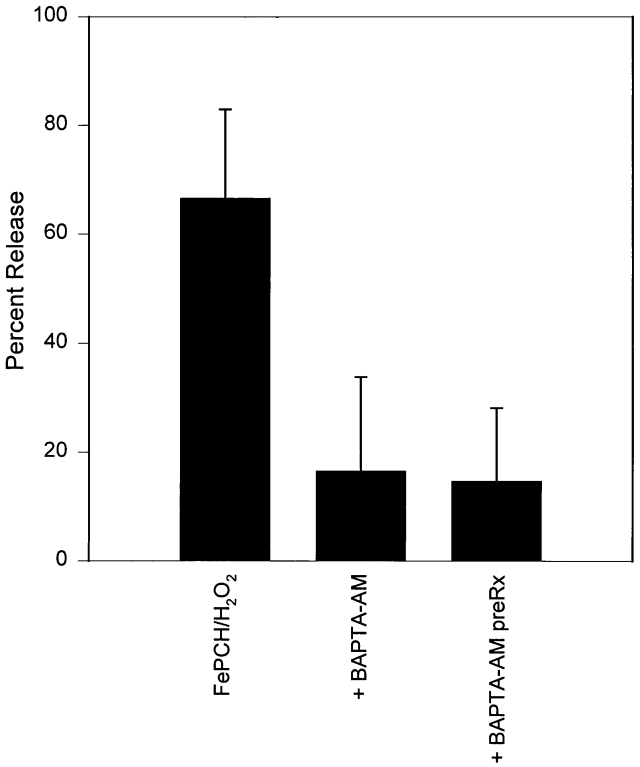

FIG. 2. Lack of requirement of extracellular BAPTA for PPAEC protection. Shown is specific ^{51}Cr release resulting from exposure of PPAEC to 50 μM H_2O_2 and 2.5 μM ferripyochelin alone (H_2O_2 + Fe-PCH), or in the presence of 50 μM BAPTA-AM (+ BAPTA-AM), or following preincubation with 50 μM BAPTA-AM after which the cells were washed free of extracellular BAPTA-AM (BAPTA-AM pre-Rx). There was no statistically significant difference ($P > 0.05$) between results obtained when BAPTA-AM was present during H_2O_2/ferripyochelin exposure compared to when it had been removed prior to such exposure. Results are means ± SD (N = 3).

thapsigargin (2 μM), an agent that depletes intracellular Ca^{2+} stores [41], had no effect on H_2O_2/ferripyochelin-mediated injury (Fig. 1). Based on these data and the fact that the other Ca^{2+} chelating agent, Fura-2, had no effect, we sought other possible explanations for the protection afforded by BAPTA.

Chelation of Iron by BAPTA

Given the role of catalytic iron in ferripyochelin-dependent injury, we examined the possibility that BAPTA was functioning as an iron rather than a Ca^{2+} chelator. As shown in Fig. 3, addition of Fe^{2+} or Fe^{3+} to a solution of BAPTA resulted in new absorbance spectra that were consistent with the formation of iron–BAPTA complexes. Iron by itself did not absorb at these wavelengths (data not shown). The absorbance spectrum was distinct from that of the BAPTA–Ca^{2+} complex (Fig. 3). Given the differences in the character of those various spectra, we were able to estimate the relative affinity of Fe^{2+} and Fe^{3+} for BAPTA by assessing its ability to compete with Ca^{2+} whose K_d is

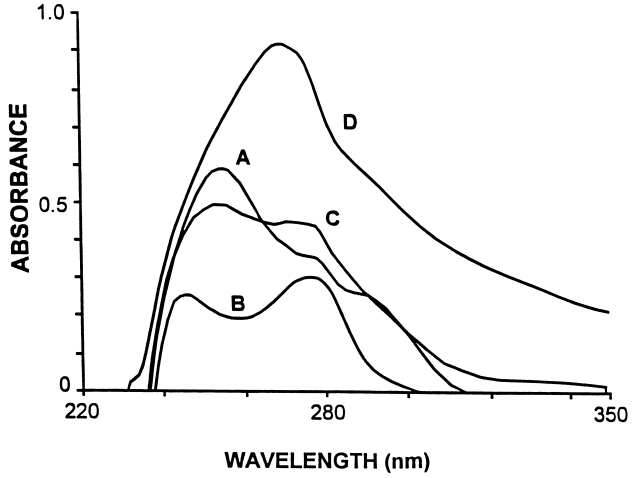

FIG. 3. Iron binding by BAPTA. Shown are UV spectra of 50 μM BAPTA alone (A) or following the addition of 100 μM $CaCl_2$ (B), 100 μM ferrous sulfate (Fe_2SO_4) (C), or 100 μM ferric chloride ($FeCl_3$) (D). At the concentration employed (100 μM), neither $CaCl_2$, Fe_2SO_4, nor $FeCl_3$ alone yielded a detectable spectrum. Results are representative of three separate experiments.

known [34, 35]. Based on these data, we estimate a K_d of BAPTA for Fe^{2+} and Fe^{3+} of 1×10^{-6} and 5×10^{-9} M, respectively. These compare with a K_d of 1.1×10^{-7} M for Ca^{2+}.

BAPTA and Iron-Dependent Hydroxyl Radical Formation

The above data are consistent with a possible role for iron chelation in the ability of BAPTA to decrease H_2O_2/ferripyochelin-mediated PPAEC injury. However, in order for this to be a viable hypothesis, BAPTA would not only have to chelate iron, but do so in such a way that the iron was less capable of acting as a catalyst for $HO^{\bullet}$ production or reacting to form ferryl species. Therefore, using spin-trapping techniques as a means of quantitating oxidant formation, we assessed the ability of BAPTA to decrease iron-mediated production of $HO^{\bullet}$ or ferryl species. As shown in Fig. 4, the presence of BAPTA decreased in a concentration-dependent manner DMPO/$^{\bullet}$OH detection resulting from the reaction of H_2O_2 and Fe^{2+} in the presence of DMP0 and DMSO. This was also observed with a slightly higher concentration of Fura-2 (Fig. 4). Even in the presence of a high concentration of DMSO, little DMPO/$^{\bullet}CH_3$ was detectable (Fig. 4). These results are consistent with the work of Yamazaki and Piette [3, 4] and suggest that under these experimental conditions, ferryl species, rather than $HO^{\bullet}$ contributed to the majority of DMPO/$^{\bullet}$OH produced.

Consistent with the earlier data suggesting a greater affinity of BAPTA for Fe^{3+} relative to Fe^{2+}, an even greater level of inhibition was observed in the amount of $HO^{\bullet}$ formed by the reaction of xanthine oxidase to xanthine in the presence of Fe^{3+}, DMSO, and 4-POBN

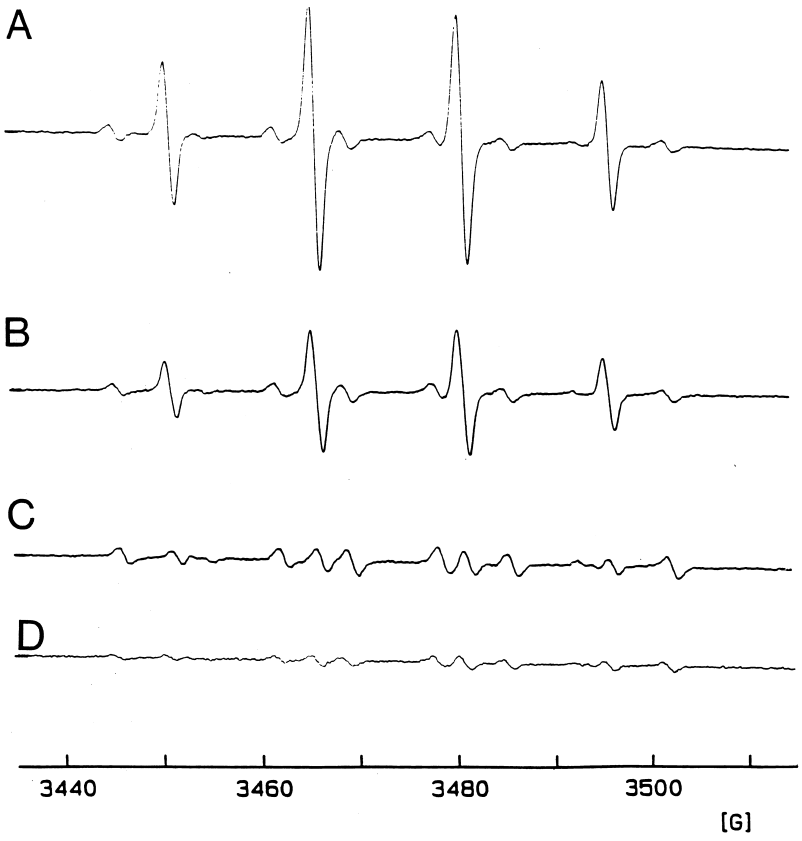

FIG. 4. Effect of BAPTA on iron-catalyzed oxidant production. Shown are EPR spectra from experiments in which H_2O_2 (100 μM) was added to Fe^{2+} (10 μM) in the presence of 100 mM DMPO and 4% DMSO (A) and when the same reaction was allowed to occur in the presence of 100 μM BAPTA (B), 200 μM BAPTA (C), or 400 μM Fura-2 (D). Each spectrum was obtained immediately after the addition of H_2O_2. The spectra reveal the presence of two species: DMPO/$^{\bullet}$OH ($A_N = A_H = 14.9$ G), the largest peaks, and DMPO/$^{\bullet}CH_3$ ($A_N = 16.1$ G and $A_H = 23.8$ G), the smallest. Results are representative of three separate experiments.

(Fig. 5). Once again, Fura-2 also inhibited spin adduct formation to a similar extent (Fig. 5). 4-POBN was substituted for DMPO as the spin trap because, in contrast to DMPO, O_2^- does not react with 4-POBN to form long-lived spin adducts. Thus, with the 4-POBN system, the confounding effect of superoxide-derived spin adducts is eliminated. Since ferryl species react less well with DMSO than does HO$^{\bullet}$ [3, 4], this spin-trapping system would be expected to primarily detect HO$^{\bullet}$ production. The EPR spectra obtained was comprised of two spin adducts (Fig. 5). The splitting constants of the least prevalent species (A_N = 15.8 G, A_H = 2.8 G) are consistent with previous reports of 4-POBN/$^{\bullet}CH_3$ [42]. The splitting constants of the more prominent species (A_N = 14.7 G, A_H = 2.4 G) suggest an oxygen-centered radical, but a 4-POBN spin adduct with such splitting constants has not been described to our knowledge. As expected for a product of the Haber–Weiss reaction, catalase and SOD almost totally inhibited formation of both species (Fig. 5). Based on previous experience with a PBN- and DMSO-based spin-trapping system, in which the predominant species generated was PBN/$^{\bullet}OCH_3$ [43, 44], we believe this spin adduct to be the 4-POBN/$^{\bullet}OCH_3$ spin adduct. Confirming this assignment, when hydroxyl radical formation occurred under conditions of limited O_2 availability (N_2 bubbling), the spectrum of 4-POBN/$^{\bullet}CH_3$ predominated whereas the second spectrum was diminished markedly (Fig. 6).

The effect of BAPTA was not due to decreasing spin adduct stability as addition of BAPTA immediately after reaction of H_2O_2 and Fe^{2+} in the presence of the spin traps had no effect (data not shown). In addition, BAPTA previously incubated with equimolar iron lost its inhibitory effect, arguing against direct scavenging of free radicals by BAPTA (data not shown). These data are consistent with BAPTA inhibition of iron-dependent oxidant formation via its ability to chelate ferrous and ferric iron.

Protective Effect of BAPTA Relative to Other Iron-Chelating Agents

Finally, we sought to compare the ability of BAPTA-AM to prevent injury in relationship to other chelators (deferoxamine and DTPA) that bind iron in a form that results in a poor catalyst for HO$^{\bullet}$ production. At the concentration employed (100 μM), DTPA showed no protective effect: 68 ± 13% (control) vs 77 + 18% (DTPA-treated) ^{51}Cr release ($P > 0.05$, N = 3). BAPTA was similar to 100 μM deferoxamine: 16 ± 15% (N = 5) for BAPTA vs 21 ± 7% (N = 3) for deferoxamine ($P < 0.05$).

DISCUSSION

An increase in intracellular Ca^{2+} may contribute to various forms of oxidant-mediated cell injury [19–25]. Consistent with these earlier data, we found that loading PPAEC with the high affinity Ca^{2+} chelating agent BAPTA markedly decreased PPAEC injury resulting from exposure to a combination of H_2O_2 and iron chelated to the *P. aerugi-*

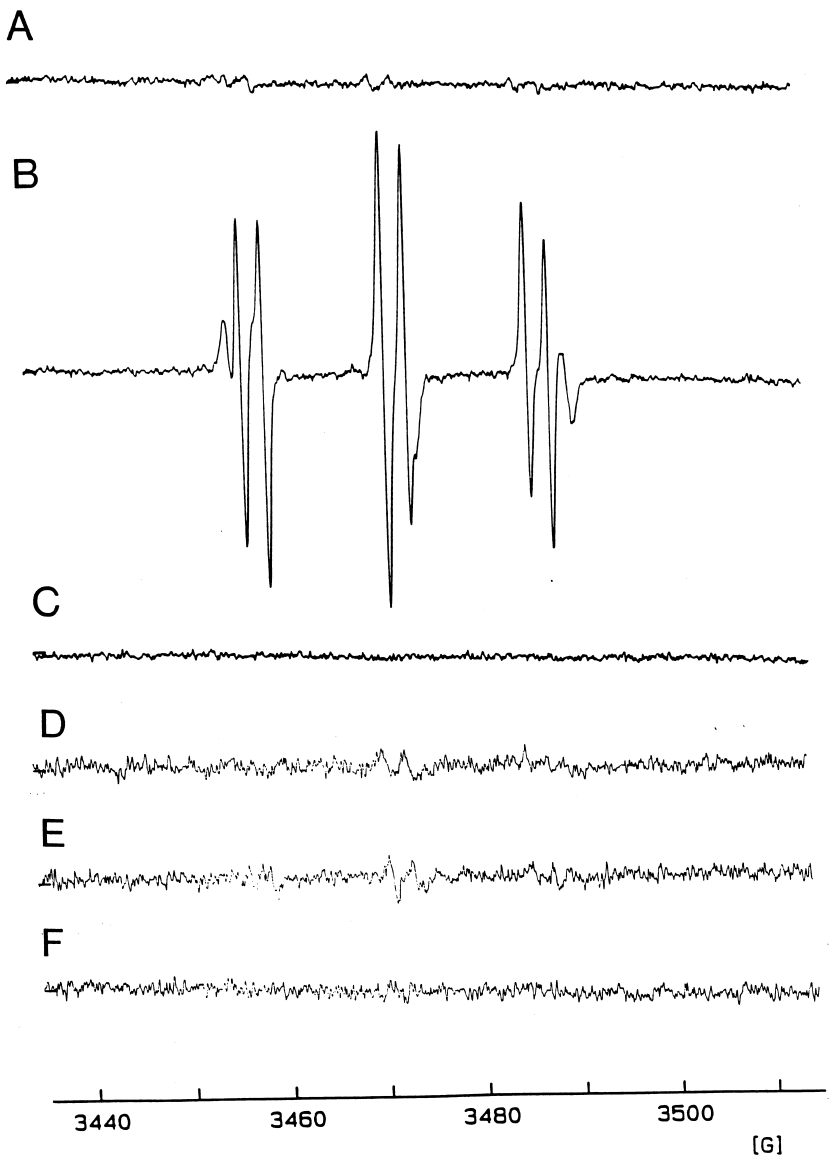

FIG. 5. Effect of BAPTA on the Haber–Weiss reaction. In the top EPR tracing, xanthine oxidase (2.5 mU/mL) was added to a solution of xanthine (0.1 mM), DPTA (0.1 mM), 4-POBN (10 mM), and DMSO (140 mM) (A). Tracing B was obtained under the same conditions as the top except that 10 μM $FeCl_3$ was present as well. The predominant species detected had splitting constants of A_N = 14.7 G, A_H = 2.4 G, which we believe to represent 4-POBN/$^{\bullet}OCH_3$ (see text). The second species of lesser magnitude that was observed (A_N = 15.8 G, A_H = 2.8 G) was consistent with previous reports of 4-POBN/$^{\bullet}CH_3$ (see text). Tracings C and D were obtained under the same conditions as Tracing B except that 0.1 mM BAPTA or 0.1 mM Fura-2 was also included, respectively. Tracings E and F were also obtained under the same conditions as Tracing B except that SOD (30 U/mL) and catalase (500 U/mL) were present, respectively. In each case, spectra were obtained immediately after the addition of xanthine oxidase to the reaction mixture. Results are representative of three separate experiments.

nosa-derived siderophore pyochelin. We have shown previously that this injury is linked to the iron-catalyzed oxidant production of $HO^{\bullet}$ [17, 18].

Although these results suggested that Ca^{2+} could be involved in the process of H_2O_2/ferripyochelin-induced injury, subsequent data provided a more likely explanation. In contrast to the BAPTA results, a different Ca^{2+} chelator, Fura-2, whose affinity for Ca^{2+} is similar to BAPTA [34, 35], failed to exhibit any protective effect. Other experimental manipulations that removed intracellular and extracellular Ca^{2+} also failed to provide protection. This suggested that BAPTA could be acting through an ability to chelate another molecule known to be involved in H_2O_2/ferripyochelin-mediated cell injury, iron.

Consistent with the above, BAPTA was shown to bind iron, both Fe^{2+} and Fe^{3+}. Our data indicate that the relative affinities for BAPTA are $Fe^{3+} > Ca^{2+} > Fe^{2+}$. BAPTA has been studied previously for its iron-binding capabilities. For example, Smith and colleagues [45], using NMR, provided evidence for the ability of BAPTA to bind Fe^{2+}. Under their conditions, BAPTA binding of Fe^{2+} was somewhat greater than Ca^{2+}, which differs from our data.

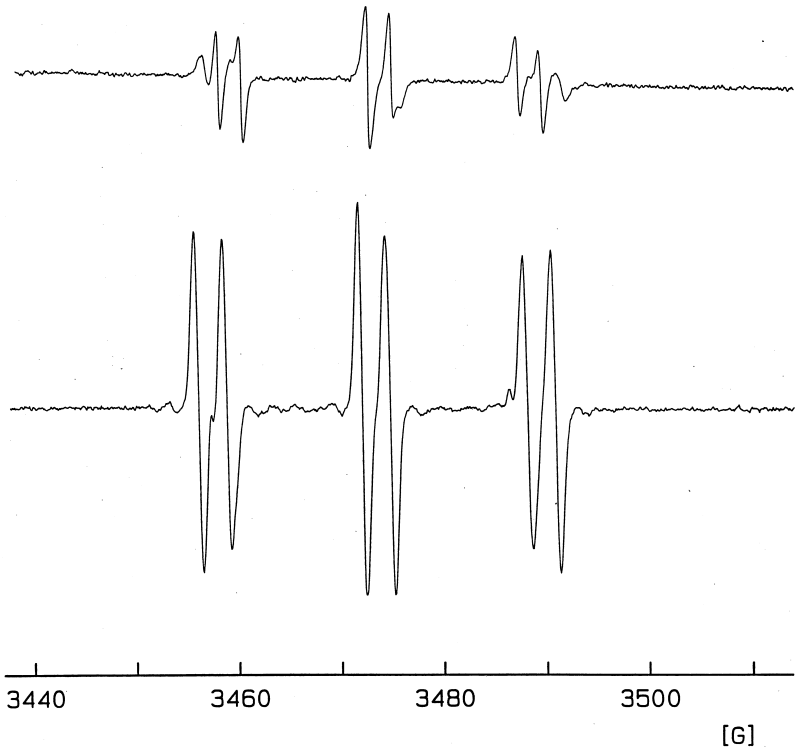

FIG. 6. Characterization of the novel 4-POBN spin adduct. Shown in the top tracing is an EPR spectrum in which H_2O_2 (100 μM) was added to Fe^{2+} (10 μM) in the presence of 4-POBN (10 mM) and DMSO (140 mM). The resulting EPR spectrum was composed of two separate species whose splitting constants were identical to those seen in Fig. 5 resulting from the reaction of xanthine, xanthine oxidase, and Fe^{3+}. The dominant species exhibited splitting constants of A_N = 14.7 G, A_H = 2.4 G. The bottom tracing was performed under the same conditions as the top except that the solution was bubbled with N_2 for 15 min before and after the addition of H_2O_2. This resulted in an increase in 4-POBN/$^{\bullet}CH_3$ (A_N = 15.8 G, A_H = 2.8 G) with a marked diminution of the other species. Results are representative of three separate experiments.

Golconda *et al.* [46] reported that BAPTA does not bind iron. However, the assay they employed for the detection of iron chelation (bleomycin assay) is dependent on the complex resulting in the presence of redox active iron. Since our spin-trapping results indicate that the BAPTA–Fe complex is not redox active, the bleomycin assay would miss the formation of a BAPTA–Fe complex. Work by others has shown that Quin-2 and Fura-2, other agents used to measure intracellular Ca^{2+} via their ability to form complexes with Ca^{2+} with fluorescent properties, also can chelate both ferric and ferrous iron [30, 31, 35, 47, 48]. Other chelating agents (e.g. EDTA) also bind both calcium and iron.

Consistent with our findings with BAPTA and those of others with Quin-2 [30, 31, 47], Fura-2 also appears to be capable of inhibiting iron-catalyzed oxidant formation. It is not clear why, if both BAPTA and Fura-2 share this property, only BAPTA protected endothelial cells from ferripyochelin-mediated injury. Interestingly, Schnellmann [32] also found that BAPTA was more effective than Fura-2 in protecting renal tubular cells from oxidative injury mediated by ferric nitrilotriacetate. Why such a difference should exist is unexplained at present. The K_d of Fura-2 for iron appears similar to that of BAPTA [35]. It seems possible that the intracellular concentrations achieved by BAPTA and Fura-2 may be different. In addition, there may be unrecognized differences in the way in which BAPTA and Fura-2 partition into the, as yet undefined, subcellular compartments at which ferripyochelin mediates its cytotoxicity. Further work is needed in this regard.

In addition to its ability to bind iron, in order for a compound to limit $HO^{\bullet}$-mediated cell injury the resulting iron complex must exhibit decreased ability to participate in Fenton and/or Haber–Weiss chemistry. Addition of BAPTA to a mixture of Fe^{2+} and H_2O_2 or Fe^{3+} and xanthine/xanthine oxidase decreased the magnitude of $HO^{\bullet}$ and/or ferryl species that were detected by ESR. This was not explainable on the basis of "scavenging" or a non-specific effect of BAPTA on the spin-trapping system used to assess free radical formation.

Although we cannot exclude a role for the Ca^{2+}-chelating properties of BAPTA in its ability to protect PPAEC from H_2O_2/ferripyochelin-mediated injury, the data appear to be more consistent with a role for iron chelation in these events. Relative to other iron chelators used experimentally for their antioxidant properties (deferoxamine and DTPA), BAPTA proved to have greater (DTPA) or similar (deferoxamine) protective efficacy in spite of the fact that extracellular BAPTA was not present. This may reflect, in part, the relative achievable intracellular concentration of these various agents.

In summary, our data provide the most extensive assessment to date of the iron-chelating properties of BAPTA and their effect on biologic systems. Although our data do not definitively prove that BAPTA protects endothelial cells from ferripyochelin-associated injury via chelating iron, they indicate that it is essential that investigators consider iron chelation when interpreting experimental data resulting from loading cells with "calcium chelating" agents such as BAPTA. Although earlier works with other "calcium chelators" [30–32, 47] have raised similar concerns, this potential has not been investigated extensively with BAPTA, and it has not been routinely considered by investigators in interpreting the results of studies using

these agents. The greater or equal potency of BAPTA-AM relative to other iron-chelating agents that we observed also suggests that BAPTA should undergo further investigation as a possible agent to protect cells from injury resulting from the intracellular generation of oxidants produced via the Fenton/Haber–Weiss reaction.

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