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HEMOGLOBIN POTENTIATES THE PRODUCTION OF REACTIVE OXYGEN SPECIES BY ALVEOLAR MACROPHAGES

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☐ *The objectives of this investigation were (1) to determine the effects of hemoglobin on the production of reactive oxygen species by activated rat alveolar macrophages, (2) to determine a possible mechanism for these effects, and (3) to determine which part of the hemoglobin molecule is responsible for these effects. Production of reactive oxygen species by phorbol myristate acetate (PMA)-stimulated cells was assessed by measuring luminol-enhanced chemiluminescence (CL). Hemoglobin enhances PMA-stimulated CL in a dose-dependent manner. The effect is maximal at 0.5–1.0 μM hemoglobin where PMA-induced CL is increased by approximately 20-fold. Superoxide anion release from PMA-stimulated cells is not affected by hemoglobin. However, the hemoglobin-induced enhancement of PMA-stimulated CL is inhibited by superoxide dismutase, catalase, dimethylthiourea, or deferoxamine. These results suggest that hydroxyl radical may be formed from hydrogen peroxide which is derived from superoxide anion. Measurements of electron spin resonance spectra following spin trapping of radicals verify that hydroxyl radicals are produced by the cells in the presence of PMA and hemoglobin. The hemoglobin effects appear to require iron in a protoporphyrin complex, because hemin stimulates PMA-induced CL, whereas neither ferrous nor ferric iron has any effect. These findings taken together suggest that hemoglobin can act as a biological Fenton reagent to enhance the production of reactive oxygen species from alveolar macrophages and potentially contribute to lung damage during leakage of blood into the alveolar spaces.*

Keywords *chemiluminescence, electron spin resonance, Fenton reagent*

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Alveolar macrophages are phagocytic cells located on the alveolar surface and in the lower airways of the lungs. A primary function of these cells is to provide a first line of defense against the inhalation of bacteria and foreign particles. When alveolar macrophages come into contact with foreign debris, the cells attempt to kill and ingest the particles. In this regard, a primary response of these cells is the release of reactive oxygen species, such as superoxide anion and hydrogen peroxide. These reactive products have microbicidal activity and can participate in inflammatory processes [1–3]. In general, these activities of alveolar macrophages are beneficial in that they help to maintain the sterility of the alveolar region. However, continuous release of reactive oxygen species or enhancement of their activity, as may occur during chronic inflammatory states, can be harmful; i.e., reactive oxygen species can damage lung tissue.

There are some lung injuries which can result in destruction of the alveolar-capillary barrier and, consequently, leakage of blood into the alveolar spaces. For example, this may occur as a result of pulmonary oxidant injury caused by exposures to high concentrations of either ozone or nitrogen dioxide [4, 5] or by inhalation of particles, such as silica [6]. Also, there is damage to the air-blood barrier in patients with acute respiratory distress syndrome [7]. Once the alveolar-capillary barrier is damaged, red blood cells may then leak into the alveolar spaces. Lysis of these cells may also occur, leading to the presence of free hemoglobin in the alveolar region. There is some evidence to indicate that there are enhanced levels of toxicity following oxidant-induced lung injuries in which hemoglobin is present in the alveolar spaces. For example, the oxidant injury produced by *tert*-butyl hydroperoxide in isolated rat lungs is potentiated in the presence of hemoglobin [8]. In addition, there is some evidence to indicate that hemoglobin is involved in promoting the oxidative stress produced during air blast–induced lung injury in rats [9]. These results suggest that hemoglobin potentiates oxidant-induced lung damage.

One way by which hemoglobin may potentiate oxidant injury is via interactions with alveolar macrophages. For example, in conditions where lung injury leads to increased levels of hemoglobin in the alveolar spaces, alveolar macrophages may be exposed to this substance. Because alveolar macrophages release reactive oxygen species and because hemoglobin has been shown to potentiate oxidant injury, it is possible that exposure of alveolar macrophages to hemoglobin may lead to an enhanced production of reactive oxygen species which, in turn, may result in increased lung injury. Therefore, the objectives of this investigation were (1) to determine the effects of hemoglobin on the production of reactive oxygen species by alveolar macrophages, (2) to determine a possible mechanism for these effects, and (3) to determine which part(s) of the hemoglobin molecule is (are) responsible for the effects.

MATERIALS AND METHODS

Isolation of Alveolar Macrophages

Specific pathogen–free male Sprague-Dawley rats (250–350 g; Hilltop Laboratories; Scottdale, PA) were used in all experiments. The animals were treated with pentobarbital sodium (150 mg/kg body weight; intraperitoneally [IP]) and exsanguinated by cutting the abdominal aorta. The trachea, heart, and lungs were then removed from the animals intact. Alveolar macrophages were obtained via bronchoalveolar lavage according to the method of Myrvik and colleagues [10]. The lungs from each animal were lavaged 8 times with 5 mL of phosphate-buffered medium (145 mM NaCl, 5 mM KCl, 9.4 mM Na_2HPO_4, and 1.9 mM NaH_2PO_4; pH 7.4). The cells were separated from the lavage fluid by centrifugation at 300 *g* for 5 minutes, washed 3 times by alternate centrifugation and resuspension in the lavage medium. After the washing procedure, the cells were resuspended in HEPES-buffered medium (145 mM NaCl, 5 mM KCl, 10 mM *N*-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid [HEPES], 1.0 mM $CaCl_2$, and 5 mM glucose; pH 7.4) for use in all experiments. The number of alveolar macrophages in the suspensions was determined by using an electronic cell counter (Coulter Model ZBI; Coulter Electronics, Hialeah, FL). The cells used in these experiments were 98 (± 1)% alveolar macrophages, with leukocytes as the contaminating cells. Viability of the alveolar macrophages, as assessed by measuring oxygen consumption and trypan blue exclusion, did not change during the incubation periods used in these experiments. The cell numbers reported in the results refer to viable cells.

Measurement of Chemiluminescence

In order to estimate the release of reactive oxygen species from alveolar macrophages, the measurement of chemiluminescence was employed. Chemiluminescence is the emission of light from chemical reactions. It has been shown that alveolar macrophages emit light when exposed to zymosan, an extract from the cell walls of *Saccharomyces cerevisiae* yeast [11]. In that system, the chemiluminescence was due to the extracellular reaction between zymosan and various reactive forms of oxygen. The data from that study [11] suggest that at least superoxide anion, hydrogen peroxide, and hydroxyl radical are involved in the chemiluminescence response. One problem with that system is that the level of light produced is relatively low. Therefore, it is useful to use luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) in order to amplify the production of light [12]. Luminol is a cyclic hydrazide which produces chemiluminescence when oxidized by a variety of agents. In this reaction, luminol is oxidized to an electronically excited ion, aminophthalate, which relaxes to the ground state by photon emission [13]. The chemiluminescence produced is in the visible range with a peak at 425 nm. This reaction has been utilized by other investigators to study release of oxygen radicals from phagocytic cells [12] and to study enzymes and subcellular fractions involved in generating oxidizing species [14, 15]. In our experiments, luminol was added to the incubation medium containing alveolar macrophage preparations. The luminol-enhanced chemiluminescence produced was due to the oxidation of luminol by reactive oxygen species released by the cells.

The effects of hemoglobin on luminol-enhanced chemiluminescence from alveolar macrophages were determined in the absence or presence of a soluble stimulus, phorbol myristate acetate (PMA). PMA interacts with cell membranes to stimulate respiratory burst activity in alveolar macrophages. The cells (5×10^5) were preincubated at 37°C for 10 minutes in HEPES-buffered medium. After this preincubation period, luminol (final concentration = 80 ng/mL; Sigma Chemical, St Louis, MO) was added to all incubation mixtures. In addition, various concentrations of rat hemoglobin (Sigma) or PMA (final concentration = 3 μM; Sigma) were added to some incubation mixtures. This concentration of PMA was used because it produces a near-maximal response. Both luminol and PMA were dissolved in small amounts of DMSO for delivery to the cells. DMSO had no adverse effects on the cells. Following the addition of these substances, the incubation mixtures were placed in a luminometer (Berthold Model LB953; Wallac, Gaithersburg, MD), and chemiluminescence was measured for 30 minutes at wavelengths between 390 and 620 nm. The total amount of chemiluminescence produced during the 30-minute incubation period was measured as the integral of chemiluminescence versus time. The results were expressed as counts per minute (cpm) per 5×10^5 cells.

In other experiments, the effects of some other substances on PMA-stimulated chemiluminescence from alveolar macrophages were studied. Experiments were performed in order to study which reactive oxygen species may be involved in the production of chemiluminescence. For these experiments, cells were preincubated for 10 minutes in HEPES-buffered medium. Then luminol, PMA, hemoglobin, and either superoxide dismutase (SOD; 1000 U/mL; Sigma), catalase (2000 U/mL; Sigma), dimethylthiourea (DMTU; 10 mM; Aldrich, Milwaukee, WI), or deferoxamine (100 μM; Sigma) were added to some incubation mixtures and chemiluminescence was measured for 30 minutes. In an attempt to determine which component(s) of the hemoglobin molecule is (are) responsible for its effects, hemin (4 μM; Porphyrin Products, Salt Lake City, UT), zinc protoporphyrin (4 μM; Porphyrin Products), $FeCl_3$ (4 μM), or $FeSO_4$ (4 μM) were used. All of these substances are soluble in the HEPES-buffered medium with the exception of hemin. Hemin was first dissolved in dilute NaOH and the pH was adjusted to 7.4 with HCl for delivery to the cells. Alveolar macrophages were preincubated in HEPES-buffered medium for 10 minutes. After the preincubation period, luminol, PMA and/or one of the substances listed above were added to some incubation mixtures and chemiluminescence was measured for an additional 30-minute period. None of the substances used in these experiments affected cell viability as assessed by measuring oxygen consumption and lactate dehydrogenase (LDH) release.

Measurement of Superoxide Anion Release

The release of superoxide anions from alveolar macrophages was measured in order to determine if exposure to hemoglobin led to an increase in superoxide anion release from unstimulated or from PMA-stimulated cells. The method used was a modification of the one described by Johnston [16]. Alveolar macrophages (5×10^5 cells) were preincubated for 10 minutes at 37°C in HEPES-buffered medium containing 0.12 mM cytochrome *c* (Type VI; Sigma). Then either hemoglobin (1 μM) alone, PMA (3 μM) alone, or hemoglobin and PMA were added to the appropriate incubation mixtures. One-milliliter samples were taken immediately (zero time) and after an additional 30-minute incubation period. The samples were centrifuged at 6000 *g* for 30 seconds at 4°C and the optical densities of the supernatants measured at 550 nm with a spectrophotometer (Model DU 640; Beckman Instruments, Fullerton, CA). SOD (1000 U/mL) was added to some samples. The difference in the amount of cytochrome *c* reduced in the absence and presence of SOD was used to estimate superoxide anion release. The results were expressed as nmoles superoxide anion released per 5×10^5 cells per 30 minutes by using an extinction coefficient of 21 $\text{mM}^{-1}\ \text{cm}^{-1}$ [17].

Electron Spin Resonance Measurements

Experiments were performed in an attempt to identify the reactive oxygen species released by PMA-stimulated alveolar macrophages in the presence of hemoglobin. For these experiments, the radicals were trapped with a spin trapping agent, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO; Sigma) and then identified by using electron spin resonance (ESR). The DMPO was purified by charcoal decolorization and vacuum distillation so that it did not contain any ESR-detectable impurities. All incubation mixtures (final volume = 1 mL) contained alveolar macrophages (1×10^6 cells) in HEPES-buffered medium with PMA (3 μM), hemoglobin (1 μM), and DMPO (100 mM). Under our experimental conditions, the DMPO had no effect on cell viability as measured by release of LDH. The mixtures were incubated at 37°C for 10 minutes and then transferred to the flat cells used for ESR measurements. ESR spectra were recorded immediately following the 10-minute incubation period. All ESR measurements were made at room temperature with a Varian E9 ESR spectrometer (Varian Associates, Palo

Alto, CA). Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate and 1,1-diphenyl-2-picrylhydrazyl as reference standards. The spectrometer settings were: receiver gain = 2.5×10^4, time constant = 0.5 seconds, modulation amplitude = 1.0 G, and magnetic field = 3360 ± 50 G. The radicals produced in our incubations were identified by comparing the ESR spectra with spectra obtained in the presence of known radicals. These comparisons were made with the computer by using a software package, the Electron Paramagnetic Resonance Data Analysis Program (U.S. EPR, Clarksville, MD).

Statistics

Analysis of variance procedures were used to determine differences among groups (SAS, Cary, NC). The level of significance was set at $P \leq .05$.

RESULTS

Effects of Hemoglobin on the Production of Reactive Oxygen Species by Alveolar Macrophages

The effects of hemoglobin on the production of reactive oxygen species as measured by chemiluminescence (CL) were determined in unstimulated cells and in alveolar macrophages stimulated with phorbol myristate acetate (PMA). The results are shown in Table 1. Unstimulated alveolar macrophages produce a small amount of CL, i.e., $\sim 3 \times 10^5$ cpm/5×10^5 cells/30 min. When these unstimulated cells are exposed to 1 μM hemoglobin, a concentration which produces a maximal effect, there is a 2- to 3-fold increase in

TABLE 1 Effects of Hemoglobin (Hb), PMA, or PMA and Hemoglobin on Chemiluminescence (CL) from Alveolar Macrophages

Treatment (concentration)	CL (cpm $\times 10^{-5}/5 \times 10^5$ cells)
Control	3.1 (±0.3)
Hb (1 μM)	8.6 (±1.2)[a]
PMA (3 μM)	20 (±3)[a]
PMA + Hb	428 (±50)[a,b]

Values are means ± *SE* for 6 experiments. Alveolar macrophages (5×10^5 cells) were preincubated (37°C) for 10 minutes in HEPES-buffered medium. After this preincubation period, luminol (80 ng/mL) was added to all samples, and Hb, PMA, or PMA + Hb was added to the appropriate sample. The control sample did not contain either Hb or PMA. Incubation mixtures were placed in a luminometer (37°C), and chemiluminescence was measured for 30 minutes as described in Materials and Methods. Results are expressed as cpm $\times 10^{-5}$ per 5×10^5 cells.

[a] Values are significantly different from control ($P < .05$).

[b] Value is significantly different from Hb or PMA alone ($P < .05$).

CL. One way to stimulate alveolar macrophages to release reactive oxygen species is to expose them to PMA, a cell surface membrane activator. In the presence of PMA alone, CL is increased by almost 7-fold over that in unstimulated cells. However, when the cells are exposed to PMA in the presence of hemoglobin, there is a marked increase in CL; i.e., the response is approximately 20-fold greater than in the presence of PMA alone. This effect of hemoglobin on PMA-stimulated CL is dose-dependent and is maximal at a hemoglobin concentration of 0.5 to 1.0 μM (Figure 1). In other experiments (data not presented), we show that alveolar macrophages are required for these responses. If alveolar macrophages are not present, or if the cells are heated to 100°C for 30 minutes prior to the experiment, the combination of hemoglobin and PMA is not associated with increased CL. These results show that hemoglobin enhances the formation of reactive oxygen species from PMA-stimulated cells. The experiments described in the remainder of this paper were designed to study either the mechanism by which hemoglobin enhances PMA-stimulated CL or the component(s) of hemoglobin responsible for these effects. For these experiments, the concentration of hemoglobin used was 1 μM.

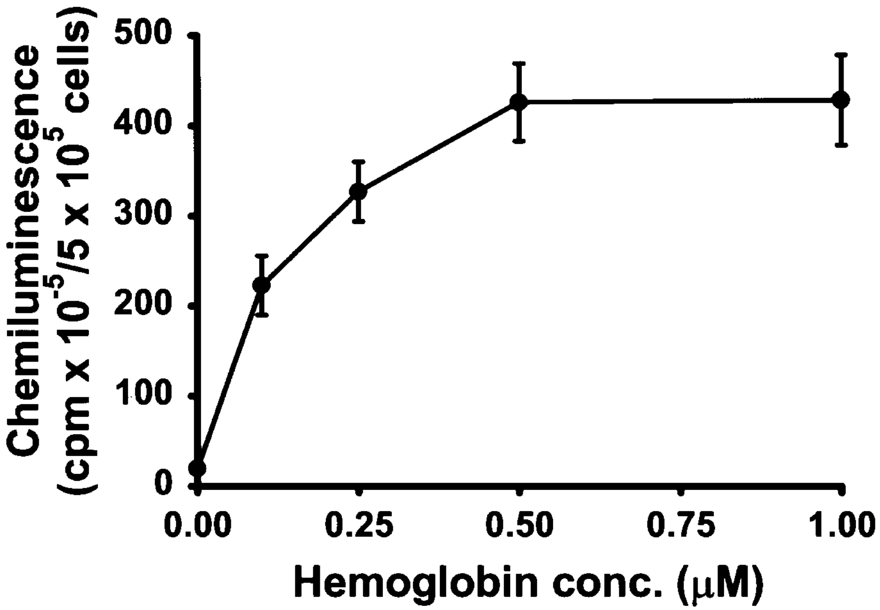

FIGURE 1 Effects of hemoglobin on PMA-stimulated chemiluminescence from alveolar macrophages. Cells (5×10^5) were preincubated (37°C) for 10 minutes in HEPES-buffered medium. After this preincubation period, luminol (80 ng/mL) and various concentrations of hemoglobin were added to the incubation mixtures. Then PMA (3 μM) was added to all samples. Incubation mixtures were placed in a luminometer (37°C) and chemiluminescence was measured for 30 minutes as described in Materials and Methods. The results are expressed as cpm $\times$ $10^{-5}/5 \times 10^5$ cells. Points are mean values for 6 experiments and bars represent *SE*.

Effects of Hemoglobin on the Release of Superoxide Anions from Alveolar Macrophages

It has been known for some time that superoxide anions are released from alveolar macrophages following stimulation of the cells. Then at least 2 other reactive oxygen species, hydrogen peroxide and hydroxyl radical, may be formed from superoxide by various reactions. Therefore, we performed experiments to determine if hemoglobin affects superoxide release from unstimulated or PMA-stimulated cells. The results are shown in Table 2. There is a some superoxide release from unstimulated cells. When the cells are exposed to hemoglobin alone, there is no effect on the release of superoxide anions. Stimulation with PMA leads to a greater than 3-fold increase in release of superoxide relative to that in unstimulated cells. Exposure of the cells to PMA in the presence of hemoglobin results in no change in superoxide release relative to that caused by PMA alone. These results suggest that the hemoglobin-induced enhancement of PMA-stimulated CL is not primarily due to an increase in the release of superoxide anions from alveolar macrophages.

Effects of Radical Scavengers or Inhibitors of Reactive Oxygen Species

The results presented above suggest that the activity of reactive oxygen species produced by PMA-stimulated alveolar macrophages is somehow

TABLE 2 Effects of Hemoglobin (Hb), PMA, or PMA and Hemoglobin on Superoxide Anion Release from Alveolar Macrophages

Treatment (concentration)	Superoxide anion release (nmoles/5×10^5 cells/30 minutes)
Control	8.9 (± 1.0)
Hb (1 μM)	8.4 (± 1.2)
PMA (3 μM)	29.0 (± 4.5)*
PMA + Hb	24.5 (± 4.2)*

Values are means± *SE* for 6 experiments. Alveolar macrophages (5×10^5 cells) were preincubated in HEPES-buffered medium containing 0.12 mM cytochrome *c*. After this preincubation period, either hemoglobin alone, PMA alone, or PMA and hemoglobin were added to the appropriate incubation mixtures. One-milliliter samples were taken immediately (zero time) and after an additional 30-minute incubation period. Superoxide anion release was then measured by using the supernatants as described in Materials and Methods. Results are expressed as nmoles superoxide anion released per 5×10^5 cells during the 30-minute incubation. * Values are significantly different from control ($P < .05$).

enhanced when hemoglobin is present and that this effect is probably not due to increased release of superoxide anions. It is known that alveolar macrophages release superoxide anions in response to stimulation by PMA and that the superoxide can then dismutate to hydrogen peroxide. If iron is present, it can then act as a Fenton reagent and react with hydrogen peroxide to form hydroxyl radical. In an attempt to determine if any of these reactive oxygen species and/or iron are involved in the responses, we evaluated the effects of SOD, catalase, deferoxamine, or DMTU on PMA-stimulated CL in the presence of hemoglobin. The results are shown in Table 3. Inclusion of SOD, a substance which removes superoxide anions, in the medium leads to a > 98% reduction in CL. Catalase, an enzyme which breaks down hydrogen peroxide, also causes a > 98% reduction in CL. Both deferoxamine, a chelator of iron, and DMTU, a hydroxyl radical scavenger, lead to > 98% and 86% reductions in CL, respectively. These results suggest that the removal of superoxide anion, hydrogen peroxide, or hydroxyl radical inhibits the CL produced by alveolar macrophages in the presence of PMA and hemoglobin. Furthermore, these data suggest that iron is an important factor in this response. These results taken together suggest that the iron-containing hemoglobin molecule may act as a biological catalyst to promote the formation of hydroxyl radicals from hydrogen peroxide which is derived from superoxide anions. It should be noted that superoxide-producing enzymes, e.g., NADPH-dependent oxidoreductases, contain functional iron sites and the activity of such enzymes may be decreased in the presence of deferoxamine. Therefore, the possibility that deferoxamine inhibits CL, in part, by this mechanism cannot be excluded.

TABLE 3 Effects of Superoxide Dismutase (SOD), Catalase, Deferoxamine, and Dimethyl Thiourea (DMTU) on Chemiluminescence (CL) from Alveolar Macrophages in the Presence of PMA and Hemoglobin (Hb)

Treatment (concentration)	CL (cpm × 10^{-5}/5 × 10^5 cells)
PMA (3 μM) + Hb (1 μM)	428 (± 50)
PMA + Hb + SOD (1000 U/mL)	1.2 (± 0.2)*
PMA + Hb + catalase (2000 U/mL)	1.9 (± 0.2)*
PMA + Hb + deferoxamine (100 μM)	1.2 (± 0.2)*
PMA + Hb + DMTU (10 mM)	14 (± 2)*

Values are means ± *SE* for 6 experiments. Alveolar macrophages (5 × 10^5 cells) were preincubated (37°C) for 10 minutes in HEPES-buffered medium. After this preincubation period, luminol (80 ng/mL) was added to all samples, and SOD, catalase, deferoxamine, or DMTU was added to the appropriate sample. Then hemoglobin and PMA were added to all samples, the samples were placed in the luminometer (37°C), and chemiluminescence was measured for 30 minutes as described in Materials and Methods. Results are expressed as cpm × 10^{-5} per 5 × 10^5 cells. * Values are significantly different from PMA + Hb ($P < .05$).

Effects of Hemin, Zinc Protoporphyrin, Ferrous Iron, or Ferric Iron on the Production of Reactive Oxygen Species by Alveolar Macrophages

The data presented above suggest that iron may somehow be involved in the enhancement of PMA-induced CL by hemoglobin. Iron is a component of the hemoglobin molecule. Therefore, experiments were performed in an attempt to identify the component(s) of the hemoglobin molecule which is (are) responsible for its effects. One important part of the molecule is heme, an iron-containing protoporphyrin compound. Therefore, we compared the effects of two different protoporphyrin complexes. One is hemin, an iron-based complex, and the other is zinc-based protoporphyrin. The effects of hemoglobin were also determined in these experiments, and the results are shown in Table 4. The concentrations of the porphyrin compounds used (4 μM) were 4 times the concentration of hemoglobin (1 μM) because there are 4 heme groups per hemoglobin molecule. Hemin is as effective as hemoglobin in increasing CL from PMA-stimulated alveolar macrophages, on the other hand, zinc protoporphyrin actually inhibits PMA-stimulated CL by approximately 80%. Because these results show that hemin can mimic the effects of hemoglobin and because hemin contains iron, we also studied the effects of iron alone. These results are also presented in Table 4. Again, we compared the effects of hemoglobin (1 μM) to 4-fold more iron (4 μM), either in a ferric or ferrous form. These results show that neither form of iron alone can mimic the effects of hemoglobin. All of these results taken together indicate that iron

TABLE 4 Effects of Hemoglobin (Hb), Hemin, Zinc Protoporphyrin (ZnPP), Ferrous Iron, or Ferric Iron on PMA-stimulated Chemiluminescence (CL) from Alveolar Macrophages

Treatment (concentration)	CL (cpm $\times 10^{-5}/5 \times 10^5$ cells)
PMA (3 μM)	20 (± 3)[b]
PMA + Hb (1 μM)	471 (± 42)[a]
PMA + hemin (4 μM)	451 (± 55)[a]
PMA + ZnPP (4 μM)	3 (± 1)[a,b]
PMA + ferrous sulfate (4 μM)	16 (± 3)[b]
PMA + ferric chloride (4μM)	25 (± 4)[b]

Values are means ± *SE* for 6 experiments. Alveolar macrophages (5×10^5 cells) were preincubated (37°C) for 10 minutes in HEPES-buffered medium. After this preincubation period, luminol (80 ng/mL) was added to all samples, and Hb, hemin, ZnPP, ferrous sulfate, or ferric chloride was added to the appropriate sample. Then PMA was added to all samples, the samples were placed in the luminometer (37°C), and chemiluminescence was measured for 30 minutes as described in Materials and Methods. Results are expressed as cpm $\times 10^{-5}$ per 5×10^5 cells. [a] Values are significantly different from PMA alone ($P < .05$). [b] Values are significantly different from PMA + Hb ($P < .05$).

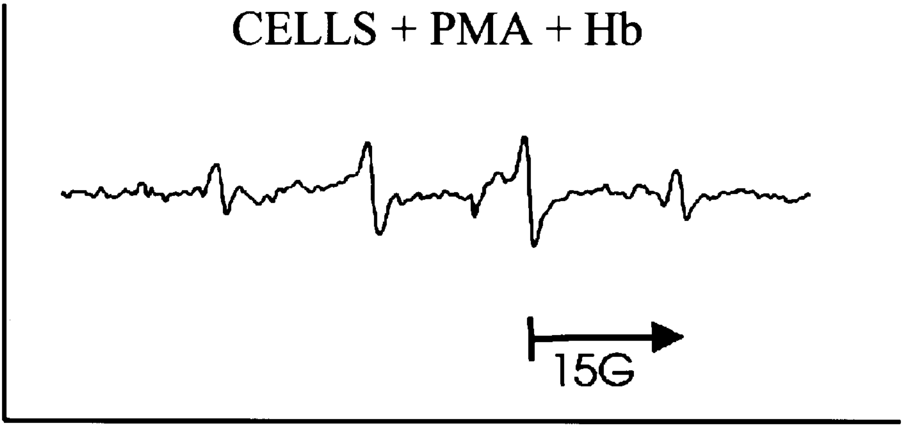

FIGURE 2 Spectrum obtained from electron spin resonance (ESR) measurements following incubation of alveolar macrophages with PMA and hemoglobin. The cells (1×10^6) were incubated for 10 minutes at 37°C in HEPES-buffered medium containing PMA (3 μM), hemoglobin (1 μM), and the spin trapping agent, DMPO (100 mM). Following the 10-minute incubation period, the mixture was transferred to a flat cell used for ESR measurements, and the spectrum was recorded immediately at room temperature. Spectrometer settings were: receiver gain = 2.5×10^4, time constant = 0.5 seconds, modulation amplitude = 1.0 G, and magnetic field = 3,360 ± 50 G. This spectrum is representative of those obtained from 6 different experiments.

in a protoporphyrin complex is necessary for the enhancement of reactive oxygen species production by alveolar macrophages and support the notion that hemoglobin may act as a biological Fenton reagent.

Evaluation of Reactive Oxygen Species Production by ESR

In an attempt to identify the reactive oxygen species produced by alveolar macrophages in the presence of PMA and hemoglobin, we used electron spin resonance (ESR) measurements in combination with spin trapping. For these experiments, an ESR spectrum was recorded following 10-minute incubations (37°C) of alveolar macrophages in the presence of PMA, hemoglobin, and the spin trapping agent, DMPO. A typical ESR spectrum obtained under these conditions is shown in Figure 2. Shown in this spectrum is a 1 : 2 : 2 : 1 quartet with hyperfine splitting constants of $a_H = a_N = 14.9$ G. Based on these splitting constants, this 1 : 2 : 2 : 1 quartet was assigned to a DMPO/hydroxyl radical adduct [18]. Thus, the ESR spectrum in Figure 2 is evidence for the generation of hydroxyl radicals when alveolar macrophages are incubated with PMA in the presence of hemoglobin.

DISCUSSION

The results of our experiments demonstrate that the PMA-induced production of reactive oxygen species by alveolar macrophages is dramatically increased in the presence of hemoglobin. This effect of hemoglobin does not appear to be due to an increase in the release of superoxide anions from the cells, but rather to an increase in the formation of other reactive oxygen species produced from superoxide, i.e., hydrogen peroxide and hydroxyl radicals. The data suggest that hemoglobin acts as a Fenton reagent, like iron in nonbiological systems, to produce hydroxyl radicals from its reaction with hydrogen peroxide. Furthermore, the presence of iron in a protoporphyrin complex, i.e., heme, appears to be the component of hemoglobin which is responsible for these effects.

There is evidence from our work for the involvement of hydroxyl radicals ($\cdot OH$) in the enhancement of PMA-stimulated CL by hemoglobin. It is well known that alveolar macrophages release superoxide anions (O_2^-) in response to stimulation. However, our data show that PMA-induced O_2^- release is not affected in the presence of hemoglobin (Table 2). It is also well known that other reactive oxygen species are produced from O_2^- as shown schematically below:

$$2O_2^- + O_2 + 2H^+ \rightarrow H_2O_2 + 2O_2$$

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

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

We have shown that interfering with this chain of events at any point inhibits PMA-stimulated CL in the presence of hemoglobin, i.e., CL in the presence of hemoglobin is inhibited when superoxide dismutase removes O_2^-, catalase destroys hydrogen peroxide (H_2O_2), deferoxamine chelates iron, or DMTU scavenges $\cdot OH$ (Table 3). The observation that superoxide dismutase inhibits PMA-stimulated CL in the presence of hemoglobin suggests that superoxide anion, in addition to serving as a substrate for the formation of hydrogen peroxide, plays a role in modulating the valence state of iron in our system. In addition, there is direct evidence from ESR studies indicating that $\cdot OH$ is involved in the response (Figure 2). All of these data taken together suggest that hemoglobin functions as a biological Fenton reagent, like iron in the scheme shown above, to increase the production of hydroxyl radicals. In this regard, it has been shown that hemoglobin can function as a Fenton reagent in nonbiological, chemically defined systems. In these reactions, hydroxyl radicals are formed in the presence of hydrogen peroxide and hemoglobin [19, 20].

The component of the hemoglobin molecule responsible for enhancement of PMA-induced CL appears to be the iron porphyrin compound, heme. Our data show that the effects of hemin on PMA-induced CL are the same as the effects of hemoglobin. These effects of hemin and hemoglobin are not duplicated by ferrous or ferric iron, i.e., iron salts have no effect on PMA-stimulated CL (Table 4). Therefore, it would seem that iron in a complex, such as heme or hemoglobin, provides a favorable structural environment for the catalytic reactions of iron. In this regard, Graf and colleagues [21] have reported that the production of hydroxyl radicals from the reaction of iron with hydrogen peroxide is greatly facilitated when iron is complexed such that there is a vacant site for hydrogen peroxide coordination.

Although only data in which PMA, a soluble stimulus, was used to stimulate alveolar macrophages is shown in this paper, we also used 3 particulate stimuli, i.e., zymosan (an extract from the cell walls of *S. cerivisiae* yeast), asbestos, and silica. Exposure of the cells to either zymosan, asbestos, or silica alone increases CL. Inclusion of hemoglobin in the incubation medium is associated with enhanced zymosan-, asbestos-, and silica-induced CL (data not presented). Thus, hemoglobin potentiates the production of reactive oxygen species from alveolar macrophages in the presence of both soluble and particulate stimuli.

There is evidence from other studies to indicate that the lungs are susceptible to increased damage when hemoglobin is present. For instance, hemoglobin potentiates oxidant-induced lung injury in an isolated rat lung model as evidenced by increases in the permeability of pulmonary capillaries [8]. Hemoglobin has also been implicated in contributing to the pulmonary oxidative stress which follows air blast–induced lung injury [9]. However, these studies did not specifically address the role that alveolar macrophages might play in the accentuation of lung injury when hemoglobin is present. The results from our study suggest that alveolar macrophages may be a cell type which could significantly contribute to the potentiation of lung damage when exposed to hemoglobin. The presence of hemoglobin is also associated with increased damage in other tissues. For instance, the incubation of brain homogenates with hemoglobin is associated with increased lipid peroxidation [22]. At the kidney, hemoglobinuria may be associated with nephrotoxic effects and the development of acute renal failure [23, 24]. It may be that resident macrophages or macrophage-like cells which are present in these tissues contribute to the observed toxicity of hemoglobin.

The finding that hemoglobin enhances the production of reactive oxygen species from alveolar macrophages may have important implications for lung injury and disease states. Hemoglobin is normally compartmentalized and sequestered in red blood cells and separated from contact with alveolar macrophages. However, there are some lung injuries which can cause leakage of blood into the alveolar spaces. For instance, damage to the barrier may occur in patients with acute respiratory distress syndrome, as a result of pulmonary injury following oxidant gas exposure, or following the inhalation of particles such as silica [4–7]. Alveolar macrophages could then be exposed to hemoglobin and such exposure could be associated with a potentiation of the production of reactive oxygen species by these cells. This source of increased oxygen-based free radical production could contribute to increased tissue damage, because it is well known that reactive oxygen species play a significant role in the pathogenesis of lung injury and disease.

In summary, the results obtained from the present study support the following conclusions: (1) hemoglobin potentiates the generation of reactive oxygen species by stimulated alveolar macrophages; (2) the reactive oxygen species produced appears to be hydroxyl radical; (3) the formation of hydroxyl radicals appears to be due to hemoglobin acting as a Fenton reagent; (4) the heme portion of the hemoglobin molecule is responsible for these effects; and (5) alveolar macrophages may be a cell type which contributes to the potentiation of lung damage when hemoglobin is present.

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