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PROTECTIVE ROLE OF HEMOPEXIN ON HEME-DEPENDENT LUNG OXIDATIVE STRESS

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Tert-butylhydroperoxide(t-BOOH)-dependent chemiluminescence of perfused rat lungs increased by almost 500 % upon addition of heme (from 297 ± 28% to 1204 ± 125% of control, $p < 0.01$ *vs* no heme). Preincubation with hemopexin significantly decreased the heme-stimulated chemiluminescence to 779 ± 155% of control ($p < 0.01$) When hemopexin was replaced with rat albumin heme-stimulated chemiluminescence decreased to 1007 ± 104% (not significantly different from results with heme alone). *In vitro* studies showed that hemopexin did not decrease chemiluminescence from bovine serum albumin oxidation (493 ± 94% compared to 292 ± 81% of control in the absence of hemopexin) and that the heme-hemopexin mixture also emitted light when exposed to t-BOOH (264 ± 66% of control). These results suggest that hemopexin specifically inhibits some of the oxidative reactions catalyzed by free heme and may have a protect tissue oxidation in conditions in which heme release occurs (i.e., stroke).

Hemoglobin and other heme-containing proteins allow aerobic life to exist. Hemoglobin, myoglobin, and "free heme"[1] have also been shown to catalyze oxidative reactions in a number of models including "reperfusion injury" and stroke, (1-3) and to potentiate cellular damage and tissue injury (4-6).

This destructive potential of heme is mitigated by the ability of plasma proteins, including albumin, haptoglobin, and hemopexin, to bind heme. The plasma concentrations and binding affinities for heme (or heme-globin in the case of haptoglobin) of these proteins differ greatly. Albumin is present in the highest concentration (0.6 mM) and the dissociation constant for the heme-albumin complex is $K_d < 10^{-8}$, (7). The affinity of haptoglobin (8-24 μM) for heme-globin tetramers is even lower with a $K_d = 1.5 \times 10^{-6}$ (8). Hemopexin (6.7-25 μM) has the highest affinity for heme with a $K_d = 10^{-12}$ (7). The capacity of each of these proteins to prevent heme from participating as a catalyst in oxidative reactions varies. Heme-globin bound to haptoglobin (2) or heme bound to hemopexin (9) do not catalyze the formation of oxygen

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[1] The expression "free heme" is used throughout the manuscript representing all forms of heme, including hematin and hemin.

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radicals, whereas at least half of the catalytic ability of heme persists when bound to albumin (9), although there is species variability.

In the present study we examined the protective role of the heme-binding plasma protein hemopexin on heme-dependent chemiluminescence of isolated perfused rat lungs in the presence of tert-butyl hydroperoxide (t-BOOH).

METHODS

Sources of protein. Hemopexin was purified from rat plasma by heme agarose affinity chromatography (10). Only the first step of purification was performed as large quantities of protein were needed. Rat-hemopexin was also purified by R. Dee and D. Peyton (unpublished procedure), and the results obtained for the two proteins were comparable. Rat albumin was from Sigma Chemical Co. (St. Louis, MO).

Chemiluminescence measurement. Chemiluminescence was measured using a Thorn EMI Gencom (NJ) C-10 photon counter with an EMI 9658 RA photomultiplier as previously described (11).

Isolated perfused lung studies. Lungs were isolated from male Sprague-Dawley rats (300-500 g, Charles River) anesthetized with 65 mg/kg pentobarbital intraperitoneally. Lung were perfused at 37°C with 4% bovine serum albumin (BSA, Sigma Chemical Co., catalog # A7030; essentially fatty acid free) in Krebs-Henseleit buffer, pH 7.4 (117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.17 mM $MgSO_4$, 2.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11.1 mM glucose). Perfusion was carried out via the pulmonary artery and the left atrium, removed, covered with clear plastic, and positioned under the photomultiplier tube. A sheet of black plastic with a hole to expose the lungs' surface was used to minimize interference from non-lung light sources (i.e., lung support, tubing, etc.). Lungs were ventilated with room air (Harvard ventilator; 2.5 ml/breath, 60 breaths/min). After measuring baseline lung chemiluminescence, t-BOOH (1 mM) was added to the perfusate. Heme was added in the form of hemin (5 μM) and allowed to equilibrate 2-3 min before t-BOOH challenge. When either hemopexin (6.8-7.0 μM) or rat albumin (7.4 μM) were used, the proteins were pre-incubated with "free heme" for at least 30 min at 37°C before addition to the perfusate.

Data analysis. Responses were expressed as a percentage of control chemiluminescence to normalize the data both for exposed lung surface area as well as daily variability in the function of the measuring apparatus. Groups were compared employing analysis of variance with least significant differences used to compare means. The results were expressed as means ± standard errors.

RESULTS AND DISCUSSION

The destructive potential of heme released into the circulation is blunted by the plasma proteins which bind heme. The ability of heme to participate as a catalyst in oxidative reactions, however, varies greatly depending on the protein to which it is bound (9).

In the present study, we investigated the effect of hemopexin on t-BOOH dependent chemiluminescence in the presence of heme. Spontaneous chemilumines-cence was used as a direct measurement of the steady-state oxidative stress, as it reflects both lipid and protein oxidation (11-14).

Addition of 1 mM t-BOOH to isolated rat lungs perfused with 4% bovine serum albumin caused an increase in lung chemiluminescence, which occurred after a delay of 1-3 min (Figure 1, top tracing). This increased chemiluminescence reached a peak of 300% above control (Figure 2) within about five minutes, after which it returned to control levels. Addition of 5 μM "free heme" to the lung perfusate increased the intensity of the peak approximately five-fold (Figure 2); chemiluminescence still returned to control levels (Figure 1, middle tracing). Preincubation of heme with rat hemopexin attenuated the heme-enhanced intensity of the peak

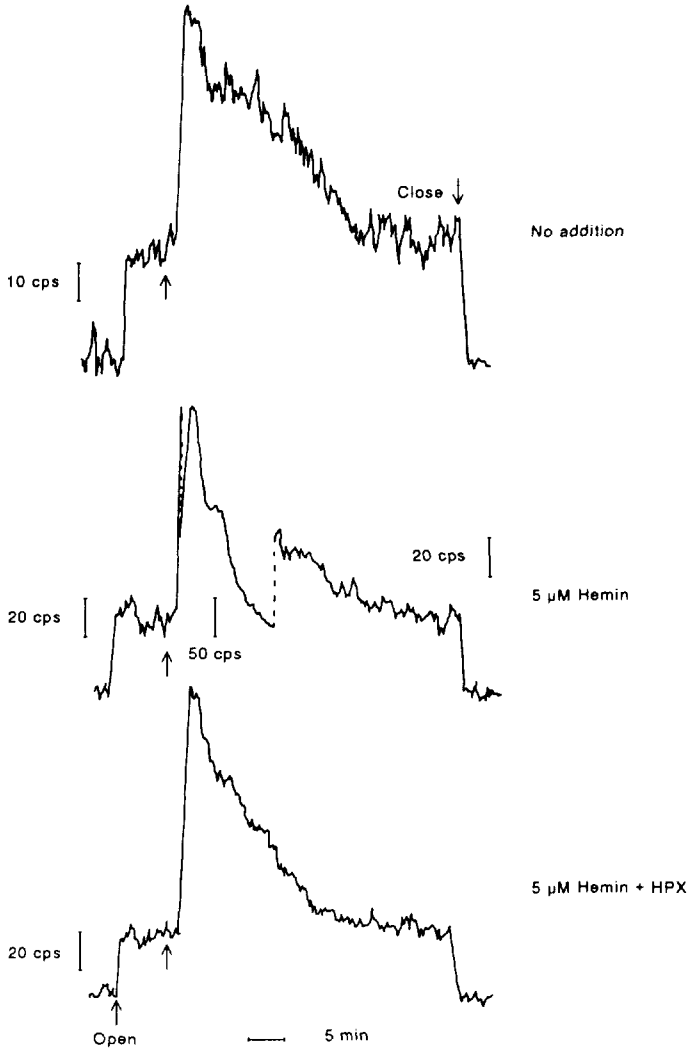

Figure 1. Shown are three representative tracings of lung chemiluminescence during oxidation with 1 mM t-BOOH. Arrows marked "Open" and "Close" represent the opening and closing of the shutter, respectively. Unmarked arrows on each trace represent the addition of 1 mM t-BOOH. Note that the scale in the first trace (No addition) is half that of the other two traces, and that the peak of the middle tracing (Heme) is on an increased scale. Dashed lines mark the change in scale.

chemiluminescence by almost 50% (Figure 1, bottom tracing, and Figure 2). Preincubation of heme with rat albumin, also resulted in a decreased peak intensity which was, however, not statistically significant (Figure 2). The chemiluminescence intensities of all samples was about the same after 30 minutes (not shown). These results support a previous report on the effects of these proteins on heme-dependent lipid peroxidation (9) and they parallel the protective effects of hemopexin in a model of heme-enhanced endothelial cytotoxicity (4).

The inhibition of chemiluminescence from isolated perfused lungs could be attributed to non-specific quenching by either rat albumin or hemopexin. Therefore, controls were

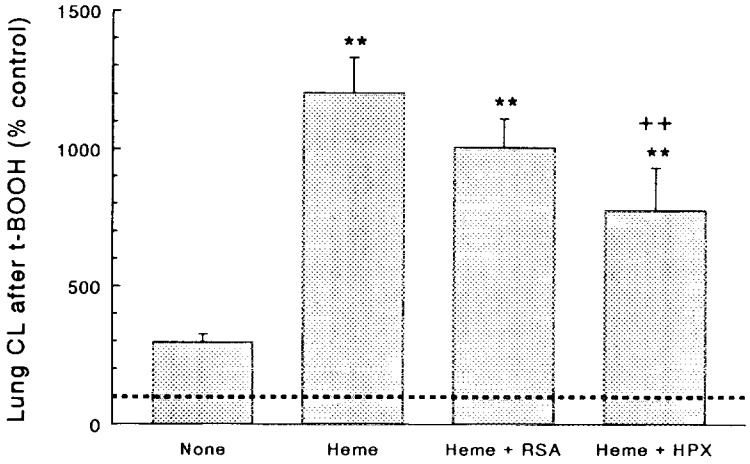

Figure 2. Lung chemiluminescence after addition of 1 mM t-BOOH to the perfusate. Lungs were perfused with 4% bovine serum albumin in Krebs-Henseleit bicarbonate buffer. Shown is the maximum change in chemiluminescence, expressed as a percentage of control to minimize daily variability. The horizontal line indicates 100% of control, which is equivalent to no change in chemiluminescence. None: No additions (other than the t-BOOH) to the lung perfusate. Heme: 5 μM heme added to perfusate before addition of t-BOOH. Heme + RSA: 0.148 μmol rat albumin was incubated with 0.1 μmol heme before addition to lung perfusate (final heme concentration 5 μM). Heme + HPX: Rat hemopexin (0.136-0.14 μmol) was incubated with 0.1 μmol heme before addition to lung perfusate (final heme concentration 5 μM). **: $p < 0.01$ *vs* None; ++: $p < 0.01$ vs Heme.

carried out *in vitro* to test the effect of these two proteins on light emission from the Krebs Henseleit buffer (with or without added 4% BSA) exposed to t-BOOH in the presence or absence of heme (Table 1). Addition of t-BOOH to heme in the absence of any protein did not cause significant decrease in chemiluminescence (85% of control, Table 1). The spontaneous chemiluminescence from either rat albumin or hemopexin incubated with t-BOOH was increased above control levels, although not significantly. In the presence of heme, however, chemiluminescence from both hemopexin and rat albumin were significantly increased 2.6- and 4.9-fold above control rates (Table 1). Finally, neither hemopexin nor rat-albumin were able to significantly inhibit chemiluminescence from BSA exposed to heme and t-BOOH (Table 1).

Both hemopexin and heme-hemopexin emit light when challenged with t-BOOH (Table 1), indicating that the protein is being oxidized. When exposed to t-BOOH, the heme-hemopexin complex emits more light than the sum of the intensities of heme or hemopexin alone. Since a previous report has indicated that heme, once bound to hemopexin, is unable to act as a catalyst (9), we should consider that heme is not responsible for this increase. One possibility is that a conformational change occurring after heme binding may favor the oxidation of some amino acids by t-BOOH. Although it was previously found that heme-hemopexin does not undergo protein oxidation (9), both the oxidant used and the measurement of protein oxidation (chemiluminescence *vs* tyrosine fluorescence) differed from the present study. Alternatively, hemopexin may be oxidized under these experimental conditions by contaminant metals or free iron through a process known as "metal catalyzed protein oxidation" which is enhanced in the presence of bicarbonate (present in the buffer) (12,15,16).

Table I. Increased chemiluminescence caused by t-BOOH *in vitro*. The ability of 1 mM t-BOOH to increase chemiluminescence of either Krebs-Henseleit buffer or 4% BSA in the same buffer was measured using 50 mm Pyrex petri dishes at a final volume of 2 ml. The results are expressed as % of the emission in the absence of t-BOOH for each experiment (n=4 for all groups). The values of chemiluminescence for these controls were between 13.5 to 25.9 counts per second. Chemiluminescence was measured as the light emission which remained constant for at least 5 minutes after the initial peak.

BSA in Krebs-Henseleit (g/100 ml)	Heme concentration (μM)	Heme-binding protein	t-BOOH-dependent Chemiluminescence (% of control)
0	0	none	85 ± 3
0	20	none	110 ± 12
0	0	hemopexin	137 ± 17
0	20	hemopexin	264 ± 66
0	0	rat albumin	135 ± 29
0	20	rat albumin	486 ± 111
4	0	none	442 ± 125
4	20	none	292 ± 81
4	20	hemopexin	493 ± 94
4	20	rat albumin	430 ± 129

These results indicate that the inhibition of chemiluminescence from isolated perfused rat lungs by either rat albumin or hemopexin was not caused by quenching, but by specifically preventing heme from leaving the capillary lumen and reaching other compartments (i.e., endothelial cell membranes) catalyzing t-BOOH-dependent oxidative reactions. These results may explain in part the incomplete abolition of chemiluminescence from perfused lungs in the presence of heme-hemopexin, since although hemopexin may protect the tissue from heme mediated oxidative injury, both albumin and hemopexin oxidation by t-BOOH may contribute to the overall emission of light (12).

In summary, the ability of hemopexin to bind heme with high affinity reduces the catalytic activity of heme in stimulating oxidation of lung tissue. Therefore, this plasma protein may play a protective role in preventing heme released into the circulation from promoting tissue oxidation.

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