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CO-OXIDATION OF LUMINOL BY HYPOCHLORITE AND HYDROGEN PEROXIDE IMPLICATIONS FOR NEUTROPHIL CHEMILUMINESCENCE

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Stimulated neutrophils produce several potent oxidants including H_2O_2, O_2^- and HOCl. Previous studies have revealed all of these compounds to be capable of oxidizing luminol, a reagent often used to indicate, by its chemiluminescence, the oxidative burst of neutrophils. Data presented in this paper indicate that H_2O_2 and HOCl spontaneously react at physiologic pH to produce luminol-dependent chemiluminescence 100 times the sum of the chemiluminescence of either reagent alone. This enhancement is due to a cooxidation by HOCl and H_2O_2, or to a novel oxidant generated by the interaction of HOCl and H_2O_2. The HOCl scavenger, taurine, inhibits the chemiluminescence. Evidence is presented against the participation of hydroxyl radical, O_2^- or singlet oxygen in the oxidation of luminol by HOCl and H_2O_2. These findings have implications for potential anti-inflammatory compounds.

Stimulated polymorphonuclear leukocytes (PMNs) generate hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-), and hypochlorous acid (HOCl) (1,2,3,4). Simultaneous with this oxidative burst luminol-enhanced chemiluminescence (CL) is observed. The marked reduction of luminol-enhanced CL in stimulated PMNs from patients with myeloperoxidase (MPO) deficiency indicates the importance of a product of MPO as the luminol oxidant (5). Harrison and Schultz have shown that HOCl is a product of the MPO-catalyzed reaction of chloride and H_2O_2 (6). The importance of the MPO-H_2O_2-chloride system has been demonstrated in its cytotoxicity for prokaryotic and eukaryotic cells (7,8). Thus, scavengers of HOCl might have antiinflammatory potential. Recent investigators have proposed that HOCl is the major luminol oxidant produced by stimulated PMNs (9). However, HOCl reacts rapidly with H_2O_2 to produce O_2 + Cl^- + H_2O. Some of the oxygen is believed to exist transiently in the singlet ($^1\Delta_g$) state, and is derived by a transfer of two electrons from H_2O_2 to HOCl

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(10). This study was undertaken to quantitate the relative ability of H_2O_2, HOCl or a product of their interaction to oxidize luminol and produce CL.

MATERIALS AND METHODS

NaOCl (5% solution) and 30% H_2O_2 were purchased from J.T. Baker Chemical Company, Phillipsburg, NJ. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), horse heart ferricytochrome c (type IV), xanthine, xanthine oxidase (from buttermilk), superoxide dismutase (SOD), deuterium oxide, and taurine were purchased from Sigma Chemical Company, St. Louis, MO. D-mannitol and formic acid (91%) were purchased from Fisher Scientific Company, Fair Lawn, N.J.

The concentration of NaOCl was determined spectrophotometrically at pH 12 with $E_{290} = 350\ M^{-1}$ (11). The concentration of H_2O_2 was also determined spectrophotometrically with $E_{230} = 80\ M^{-1}$ (12). Each reagent was prepared at 4×10^{-3} M in 0.01 M phosphate buffer, pH 7.2. Luminol was prepared at 10^{-7} M in the buffer.

SOD was assayed by its ability to inhibit the reduction of ferricytochrome c by xanthine-xanthine oxidase (13). One milligram of SOD had 2000 U of activity. SOD heated at 100°C for twenty minutes lost ninety percent of its activity.

CL was measured in a Turner Designs Photometer (Model 20), Mountain View, CA. One set of reagents was premixed in a 8x50 mm polyethylene tube and placed in the photometer's counting chamber. A pipet injector assembly was placed over the tube and the injection of the initiating reactant simultaneously triggered a shutter exposing the photomultiplier. This assembly enabled reproducible measurements of rapidly progressive reactions. The photometer's sensitivity may be adjusted over a one thousand-fold range.

The importance of the sequence of reagent addition was determined first. In one set of experiments H_2O_2 and luminol were premixed for five and sixty seconds prior to the addition of the NaOCl. The addition of the NaOCl triggered the operation of the photometer. In another set of experiments NaOCl and luminol were premixed for five and fifteen seconds prior to the addition of H_2O_2.

The effect of each reagent (NaOCl and H_2O_2) and their combination on luminol CL was tested. Either 0.1 ml H_2O_2 or NaOCl was added to a mixture of 0.1 ml of the appropriate buffer plus 0.1 ml luminol. The photometer was activated when the H_2O_2 or NaOCl was injected into the luminol-buffer mixture. Results were tabulated as integrated counts over a 15 second period and simultaneous tracings were made with a Gilford, model 6040A, pen recorder. The effect of combined H_2O_2 and NaOCl was determined by premixing 0.1 ml each of H_2O_2 and luminol. The photometer was activated by injecting 0.1 ml NaOCl.

In one set of experiments luminol was replaced with phosphate buffer to determine the amount of spontaneous CL from a mixture of H_2O_2 and NaOCl.

Compounds were tested for their ability to inhibit luminol-dependent CL generated by H_2O_2 plus NaOCl. Equal volumes (0.1 ml) of 4×10^{-3} M H_2O_2, 10^{-7} M luminol and inhibitor or buffer were premixed and placed in the photometer's counting chamber. The photometer was activated by the injection of 4×10^{-3} M NaOCl. All reagents were in 0.01 M phosphate buffer, pH 7.2. One set of experiments was run with reagents in 90% deuterium oxide (D_2O) to test if singlet oxygen participates in the CL.

RESULTS

When NaOCl was added to H_2O_2 and luminol premixed for five and sixty seconds, the same degree of CL was obtained. Thus, H_2O_2 did not oxidize the luminol during preincubations of a minute or less. However, the addition H_2O_2 to NaOCl and luminol premixed for five and fifteen seconds gave no detectable light. This indicated NaOCl oxidized all of the luminol in less than five seconds. Thus, when testing the oxidant activity of NaOCl plus H_2O_2, NaOCl was always added to premixed solutions of H_2O_2 and luminol.

Table 1 demonstrates the relative CL (thus, luminol oxidant activity) of H_2O_2, NaOCl and their combination. It is clear that NaOCl plus H_2O_2 is a far more potent CL system than either reagent alone. The kinetics of this CL response is shown in Figure 1. It should be noted that the light curve for NaOCl alone is at a sensitivity setting 100 times greater than that for the combination of the reagents. H_2O_2 and NaOCl produced no detectable amount of CL without luminol present. The pH of all final reaction mixtures was 7.2, ruling out an effect of pH change on the luminol-dependent CL.

Table 2 demonstrates the inhibitory action of SOD to be nonspecific, as the inactivated enzyme (10% activity of fresh) was more inhibitory than the fresh. The inhibitory effect of human albumin suggests the property is due to a scavenging of the oxidant by the protein. This may be due to free amino groups scavenging the hypochlorite, as taurine was a highly effective inhibitor. Mannitol, a scavenger of hydroxyl radical ($^{\cdot}OH$), was a poor inhibitor, and formate, which helps convert $^{\cdot}OH$ to $HCOO^{\cdot}$ (14), also did not inhibit the CL. These data suggest that the oxidant of luminol in this system

Table 1. Luminol-dependent CL of H_2O_2, NaOCl and H_2O_2 plus NaOCl

Condition	Relative CL
H_2O_2 + NaOCl	0.000
H_2O_2 + luminol	0.000
NaOCl + luminol	0.214
H_2O_2 + luminol + NaOCl	27.2

Results are expressed as the mean values of triplicate experiments integrated over ten seconds.

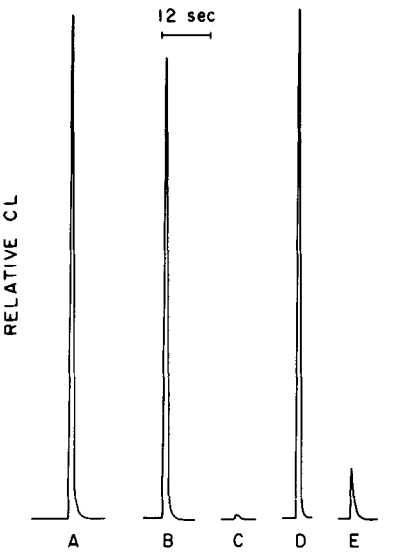


Figure 1. Kinetics of CL response of luminol oxidation. A, H_2O_2 + NaOCl; B, NaOCl; C, H_2O_2; D, H_2O_2 + NaOCl + buffer; E, H_2O_2 + NaOCl + taurine. Luminol was present in all cases. See Materials and Methods section for reagent concentrations and volumes. Curves were taken simultaneously with representative experiments of Tables 1 and 2. The sensitivity setting for curves B and C was 100 times greater than that for curves A, D and E.

(H_2O_2 + NaOCl) is not O_2^- or $^{\cdot}OH$. The luminol-dependent CL of H_2O_2 + NaOCl in D_2O was 72 percent of the CL in H_2O, suggesting singlet oxygen did not participate in the oxidation of luminol.

Table 2. Effect of antioxidant compounds on luminol-dependent CL of H_2O_2 plus NaOCl

Inhibitor	Percent Control
SOD (80 μg/ml):	
Fresh	75
Boiled	39
Formate (10 mM)	103
Mannitol (25 mM)	82
Taurine:	
2.5×10^{-3}M	0.1
2.5×10^{-4}M	0.8
2.5×10^{-5}M	17
2.5×10^{-6}M	89
Albumin (25 μg/ml)	0.5
Buffer	100

DISCUSSION

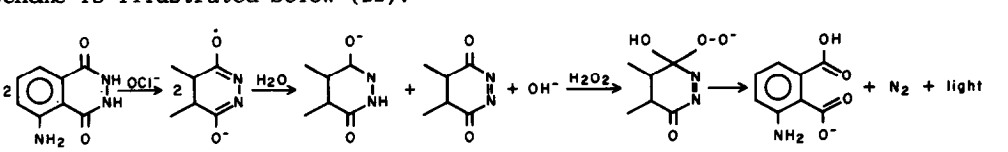
The reduction of dioxygen by PMNs results in the production of H_2O_2, itself a potent oxidant. However, in the presence of chloride and MPO, HOCl is rapidly formed (6). Its availability as an oxidant of biomolecules, such as taurine and o-tolidine, has been demonstrated (4,6). It has been proposed that HOCl is the major oxidant of luminol and responsible for the CL of stimulated PMNs (9).

However, in this study it is demonstrated that NaOCl plus H_2O_2 is a more potent CL-generating system than either reagent alone. One would expect such a condition to be generated by intact PMNs which produce H_2O_2 and HOCl simultaneously.

Kasha and Khan have proposed singlet oxygen to be a product of the reaction between H_2O_2 and OCl^- (15). Others have confirmed this and found it to be a thermodynamically favorable mechanism (10). However, other studies have demonstrated singlet oxygen generated in the presence of luminol gives little CL (16). The failure of D_2O to enhance CL in this study is further evidence against the participation of singlet oxygen.

The poor inhibition of the CL by mannitol and formate suggest the participation of $^{\cdot}OH$ is unlikely. Additionally, Kozlov, et.al., have produced evidence against $^{\cdot}OH$ formation from $H_2O_2 + OCl^-$, and find $^{\cdot}OH$ production to be thermodynamically unlikely (10).

This is not the first study to demonstrate that H_2O_2 and OCl^- react to form an oxidant of luminol more potent than either alone (17,18,19). However, in previous studies reactions were carried out under alkaline conditions. It has been proposed that the oxidation of luminol by OCl^- and H_2O_2 is sequential (20,21). That is, OCl^- oxidizes luminol to a compound which subsequently reacts with H_2O_2 resulting in the chemiluminescent reaction. A possible scheme is illustrated below (22):



Hodgson and Fridovich have found CL induced by OCl^- and persulfate to be inhibitable by superoxide dismutase (23). The findings in this study that SOD was without specific inhibitory effect (Table 2) very likely rules out the participation of O_2^-, since 10^{-5}M H_2O_2 did not significantly reduce the ability of SOD to inhibit ferricytochrome c reduction by xanthine-xanthine oxidase (data not shown). Others have also shown that H_2O_2 at this concentration does not inhibit SOD (24).

The results of this study, together with data from the literature, suggest that in systems where OCl^- and H_2O_2 coexist, luminol-dependent CL is a cooxidative process. The possibility that another species generated by the reaction of H_2O_2 with OCl^- (such as $COCl^-$) is the oxidant has not been ruled out. However, it appears that scavengers of OCl^- can be detected as inhibitors of CL. Therefore, luminol-dependent CL by PMNs or MPO-H_2O_2-chloride may be used to screen for hypochlorite scavengers with anti-inflammatory potential. One might expect compounds that possess free amine groups (to form chloramines from OCl^-), such as taurine, to be capable of protecting tissues from oxidant-induced injury from the MPO-H_2O_2-chloride system (25). Another property of taurine that may enhance its efficiency as an OCl^- scavenger in the MPO-H_2O_2-chloride system is its sulfonate group. This anionic charge may serve to concentrate taurine in the cationic region of the enzyme's heme where HOCl is produced. Such a mechanism has been proposed for the inhibition of CL in PMNs by heparin, a polyanionic (sulfated) polysaccharide (26). Thus, one way to select for compounds with anti-inflammatory potential is to predict their reactivity with OCl^-. From the data presented here it can be expected that inhibitors of luminol-dependent CL of the MPO-H_2O_2-chloride system act by scavenging OCl^-. It is unlikely that the oxidants $^{\cdot}OH$, O_2^- and singlet oxygen are involved in producing CL.

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