Mechanisms of Inhibition of Chemiluminescence in the Oxidation of Luminol by Sodium Hypochlorite

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Two different mechanisms of inhibition of chemiluminescence in the oxidation of luminol by sodium hypochlorite were found. Most substances investigated in these experiments acted by scavenging NaOCl. This mechanism was independent of the concentration of hydrogen peroxide and the incubation time between luminol and inhibitors. The most potent inhibitors were substances containing SH groups. Compounds with amino groups as a target for $HOCl/OCl^-$ to yield chloramines were much less effective inhibitors. Another mechanism of inhibition was found for catalase. It depended on the presence of hydrogen peroxide in the incubation medium and the incubation time between luminol and catalase. The enzyme inhibited the luminescence by removing H_2O_2 at molar concentrations much smaller than those found for all other inhibitors. Our results confirm the present models of the mechanism of generation of luminescence in luminol oxidation.

Keywords: Luminol; sodium hypochlorite; hydrogen peroxide; inhibition of chemiluminescence

INTRODUCTION

Luminol-amplified chemiluminescence (LCL) is often used to examine the process of stimulation in polymorphonuclear leukocytes (PMN). The light emission is due to the formation of reactive oxygen species in stimulated cells (1–3). There have been many attempts to use LCL of cells for clinical investigation of a number of diseases (4). As well as pathological states with deficiencies in PMN enzymes, the role of neutrophils in such common diseases as myocardial injury and rheumatoid arthritis has been studied. However, the quantification of the cellular chemiluminescence in PMN is complicated by the complex nature of the luminol luminescence, and the presence of various intercellular and intracellular inhibitors.

Although the exact mechanism of luminol che-

miluminescence during the oxygen burst in PMN remains unknown, it is assumed that the light emission is mainly due to the oxidation of luminol by hypochlorous acid formed in a myeloperoxidase-catalysed reaction between hydrogen peroxide and chloride anions (5–7). Other candidates for luminol chemiluminescence in cells are reactive oxygen species derived from reactions catalysed by cyclooxygenase (8), and lipoxygenase (9), and peroxynitrite (10, 11).

Regardless of the existence of other pathways for chemiluminescence generation, we focused on the luminol–hypochlorous acid system in this paper. The mechanism of light generation in this reaction is complex. First luminol is oxidized by $HOCl/OCl^-$ to a diazaquinone which is unstable in aqueous solutions. This intermediate is further converted in the presence of hydrogen peroxide anion to an excited aminophthalate (12, 13). Consequently, luminescence is drastically amplified by H_2O_2 in the system luminol–NaOCl (14). A linear

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relationship between light intensity and concentration of hydrogen peroxide was recently observed by our group (15). Therefore, luminescence intensity in the oxidation of luminol depends on both concentrations of hypochlorous acid and hydrogen peroxide.

Hypochlorous acid $HOCl/OCl^-$ is a very reactive compound. NaOCl as a source for $HOCl/OCl^-$ easily oxidizes sulphydryl and thioether groups in proteins and smaller molecules as shown by inhibition experiments of chlorination of monodichlordimedon, whereas under these conditions the reaction of $HOCl/OCl^-$ with amino groups to yield chloramines occurs with a much more lower rate (16). In a number of systems (HSA, LDL, human plasma) we have found that first $HOCl/OCl^-$ oxidizes all SH groups even under conditions where other potential targets have higher concentrations (17–19). The reactivity of luminol with $HOCl/OCl^-$ is similar to those of amino groups (17).

In stimulated cells the light generation occurs in the presence of cellular components, proteins and other compounds. Therefore, many other targets for hypochlorous acid and hydrogen peroxide exist in cells that can influence the yield of luminol chemiluminescence. The aim of this study was to compare inhibitory effects of proteins and smaller molecules on chemiluminescence obtained in the oxidation of luminol by sodium hypochlorite and its dependence on the concentration of hydrogen peroxide.

MATERIALS AND METHODS

Solutions

Luminol was purchased from Boehringer (Mannheim, Germany). Catalase, superoxide dismutase (SOD), oxidized glutathione, and taurine were from Serva (Heidelberg, Germany). Desferal was obtained from Ciba-Geigy (Basle, Switzerland). Mannitol, dimethylurea, and acetylcysteine were purchased from Sigma (Deisenhofen, Germany). All other reagents were from Laborchemie Apolda (Germany). All solutions were prepared using tridistilled water.

Stock solutions of luminol and inhibitor substances were made in 0.14 mol/L NaCl, 10 mmol/L phosphate buffer and adjusted to pH 7.40. Stock solutions of NaOCl and H_2O_2 were prepared in water. Their concentrations were determined spec-

trophotometrically immediately prior to use ($\epsilon_{290} = 350\,M^{-1}\,cm^{-1}$ at pH 12 (20) and $\epsilon_{230} = 74\,M^{-1}\,cm^{-1}$ (21) for NaOCl and H_2O_2, respectively). Solutions of NaOCl and H_2O_2 were freshly prepared daily.

Chemiluminescence measurements

Luminescence measurements were performed using a computer-driven luminometer, Autolumat LB 953 (Fa. Berthold, Wildbad, Germany). Luminol, inhibitors, and isotonic phosphate buffer were preincubated in the presence or absence of hydrogen peroxide in a final volume of 950 μL and then 50 μL of NaOCl injected. The final concentrations of luminol and NaOCl in the vial were 10^{-5} mol/L and 2.5×10^{-6} mol/L, respectively. Control measurements revealed that the pH value of 7.40 was unchanged by addition of NaOCl under these conditions.

All measurements were repeated four times and performed at 37°C. In all measurements the number of photons was counted during ten seconds after injection of NaOCl.

RESULTS

Within one second, the injection of NaOCl into luminol solution results in a luminescence burst declining to a zero level during the next few seconds.

First, inhibitory effects on light generation during the oxidation of luminol by sodium hypochlorite were investigated without addition of hydrogen peroxide. The dependence of luminescence counts on molar concentrations of selected proteins and smaller molecules is given in Fig. 1. The majority of substances tested showed similar curve profiles. At low inhibitor concentrations the number of counts remained almost unchanged compared to the pure luminol–NaOCl system. Then they declined in a certain range of concentration to zero, except for catalase. In all cases, a similar slope for the decline of luminescence with increasing inhibitor concentration was found.

In order to compare different inhibitors the concentrations of 50% inhibition ($c_{In,50}$) were determined. These values are given in Table 1. The lowest $c_{In,50}$ value was found for catalase with 3.2×10^{-11} mol/L using an incubation time of 1 min. That is more than four orders of magnitude
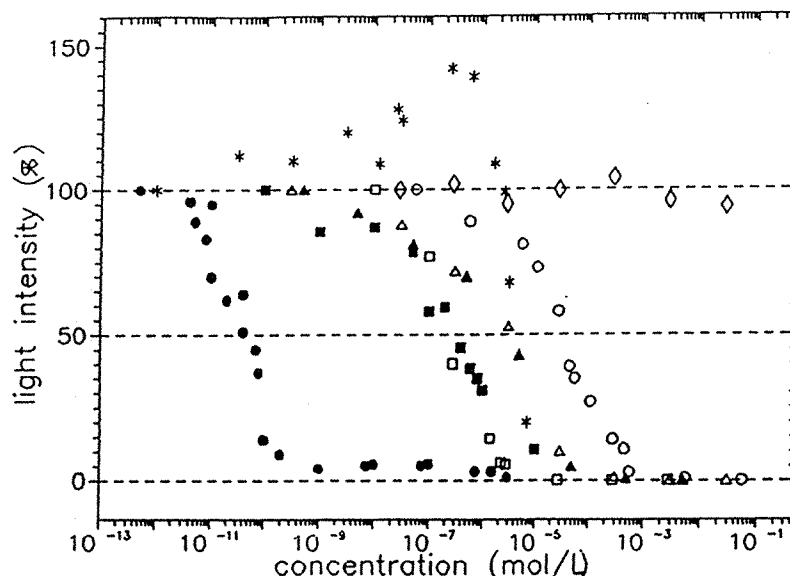
Figure 1. Inhibition profiles of luminescence in luminol oxidation by NaOCl. Luminol (10^{-5} mol/L) and inhibitor substances in 0.14 mol/L NaCl, 10 mmol/L phosphate buffer, pH 7.40 were preincubated 1 to 5 min (in the case of catalase and SOD 3 min) and then NaOCl (2.5×10^{-6} mol/L) was injected. Results are given for catalase (●), cysteine (□), human serum albumin (■), superoxide dismutase (∗), lysine (△), valine (▲), alanine (○), and glucose (◇). The luminescence generated in the absence of inhibitors was set 100%. (All values are the means (SD < 8%) of four experiments)

lower than the final concentration of NaOCl used in these experiments. All other compounds showed an inhibition of chemiluminescence at concentrations higher than 5×10^{-8} mol/L.

Substances containing one free SH group had $c_{In,50}$ values between 1.2×10^{-7} mol/L and 8.5×10^{-7} mol/L. Chemiluminescence was completely inhibited by these substances at 2.5×10^{-6} mol/L, the final concentration of hypochlorous acid in these experiments. Methionine and ascorbic acid had $c_{In,50}$ values of 1.1×10^{-6} mol/L and 1.8×10^{-6} mol/L, respectively. Various amino acids with one or two amino groups, oxidized glutathione, and desferal were effective between 2.5×10^{-6} mol/L and 3.2×10^{-5} mol/L. The lowest value was found for lysine (2.5×10^{-6} mol/L) that contains two amino groups. Finally, glucose, mannitol, and dimethylurea did not show any inhibition of luminescence up to 10^{-2} mol/L.

Deviations from the inhibition profiles given above are found for catalase and SOD. Although catalase strongly inhibited chemiluminescence at low concentrations, a residual luminescence of nearly 5% of the original value was found up to 10^{-7} mol/L. This residual light emission disappeared at about 10^{-6} mol/L catalase. Small

Table 1. Values for 50% inhibition of luminescence in luminol oxidation by NaOCl

| Inhibitor	c_{In50} (mol/L)
catalase	3.2×10^{-11}
SOD	5.0×10^{-6}
thiourea	1.2×10^{-7}
cysteine	2.5×10^{-7}
human serum albumin	2.9×10^{-7}
glutathione reduced	5.4×10^{-7}
N-acetyl-L-cysteine	8.5×10^{-7}
methionine	1.1×10^{-6}
ascorbic acid	1.8×10^{-6}
taurine	3.6×10^{-6}
desferal	5.3×10^{-6}
lysine	2.5×10^{-6}
valine	4.5×10^{-6}
glutathione oxidized	8.3×10^{-6}
tryptophan	1.5×10^{-5}
alanine	3.2×10^{-5}
glucose mannitol dimethylurea }	no inhibition until 10^{-2} mol/L
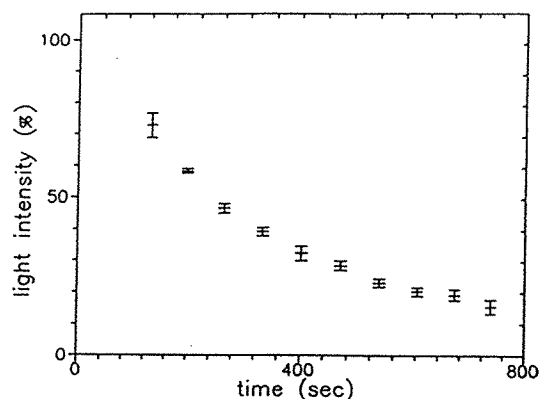
Figure 2. Chemiluminescence intensities in luminol oxidation by NaOCl as a function of incubation time between luminol and catalase (3×10^{-10} mol/L). Other experimental conditions given in Fig. 1. The light intensity obtained without catalase was set 100%. (The values with error bars are the result of at least four experiments)

Figure 3. Values for 50% inhibition ($c_{In,50}$) of luminescence in luminol oxidation by NaOCl for cysteine ($\square$), lysine ($\triangle$), alanine ($\bigcirc$), and catalase ($\bullet$) as a function of concentration of hydrogen peroxide. Inhibition profiles to determine $c_{In,50}$ were obtained as given in Fig. 1

amounts of SOD caused an increase of the original luminescence and only at higher concentrations was an inhibition found ($c_{In,50}$ value of 5.0×10^{-6} mol/L).

A dependence of light intensities on the time of preincubation between luminol and catalase was observed (Fig. 2), whereas all other inhibitor substances showed no dependence on preincubation time. The longer catalase was preincubated with luminol the lower was the luminescence burst.

The inhibition of luminescence in the system luminol–NaOCl was further investigated in the presence of hydrogen peroxide. Luminol, hydrogen peroxide, and inhibitor substances were preincubated and then sodium hypochlorite was added. In all cases the luminescence values increased at low inhibitor concentrations with increasing concentration of hydrogen peroxide (data not shown). This result agrees with the previously reported amplification of luminescence by hydrogen peroxide in the system luminol–NaOCl (4, 15).

Higher concentrations of inhibitors also caused a decrease of light production in the presence of hydrogen peroxide similar to the results shown in Fig. 1. The majority of inhibitor compounds had the same $C_{In,50}$ value as determined using different concentrations of H_2O_2. Examples are given for cysteine, lysine, and alanine in Fig. 3. For catalase there was a displacement of the inhibition curve at higher concentrations of hydrogen peroxide (Fig. 3): The concentration of 50% inhibition
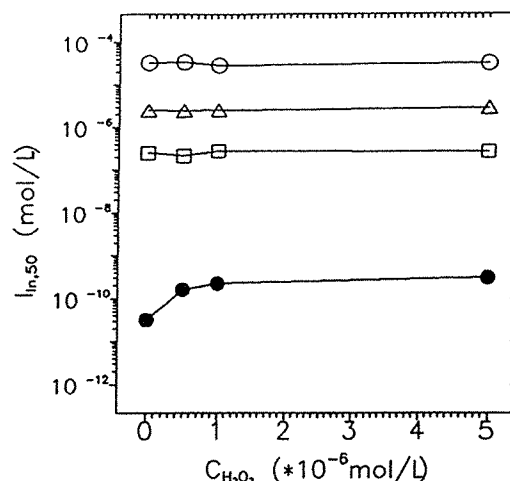
increased as the amount of hydrogen peroxide increased.

DISCUSSION

Our data reveal two different mechanisms for the inhibition of luminescence in the oxidation of luminol by sodium hypochlorite. One mechanism found for the majority of substances was independent of the concentration of hydrogen peroxide and the time of preincubation between luminol and inhibitors, whereas the action of catalase, and to some extent SOD, depended on the concentration of H_2O_2 and incubation conditions.

The first mechanism of inhibition seems to be related to a competition between inhibitors and luminol for NaOCl. This agrees with the sequence of reactions in luminol oxidation. The reaction of HOCl/OCl$^-$ with luminol is the first step in this cascade. Hydrogen peroxide, however, reacts with oxidized luminol, the diazaquinone intermediate, at a later stage in the reaction (12, 13). Therefore, any removal of HOCl/OCl$^-$ by inhibitors should decrease luminescence independent of the concentration of hydrogen peroxide. Furthermore, the order of concentrations for 50% inhibition ($c_{In,50}$) correlates well with their known reactivities against HOCl/OCl (16). With the exception of catalase, the lowest values were observed for mole-

cules containing SH groups. This corresponds to observations of other authors that HOCl/OCl⁻ very easily oxidizes SH groups (16, 22, 23). Moreover, reactions of HOCl/OCl⁻ with other targets starts only after the complete oxidation of sulphydryl groups in protein samples (17–19). Our data reveal a total inhibition of light generation by HSA, cysteine, and other substances of this group at inhibitor concentrations equal or higher than 2.5×10^{-6} mol/L (the final concentration of NaOCl in our experiments).

All other substances have lower reactivities with HOCl/OCl⁻ according to previous findings (16). The next group of substances consists of methionine and ascorbic acid followed by a group of molecules containing amino groups as the main target for HOCl/OCl⁻. Relatively low reactivities compared to SH groups were found between HOCl/OCl⁻ and amino groups to yield chloramines. However, the formation of chloramines should be considered in competition with the oxidation of luminol because some compounds of this group, such as lysine or taurine, inhibit the luminescence at a concentration of 10^{-5} mol/L (the final concentration of luminol in these experiments) by more than 70%. Taurine is present in high amounts in PMN and known to yield the long-lived oxidant taurinechloramine (24).

The absence of any inhibition of light generation by pure hydroxyl radical scavengers (mannitol, glucose, dimethylurea) reveals that hydroxyl radicals do not play any role in the oxidation of luminol by hypochlorous acid. This is in agreement with published data (14, 25).

The present data agree with findings of others (14) that the formation of singlet oxygen by a direct reaction between HOCl/OCl⁻ and H_2O_2 can be neglected under these experimental conditions. The reactivity between NaOCl and H_2O_2 is known to be low (26). An excess of luminol over NaOCl was always used. Finally, substances competing with luminol for HOCl/OCl⁻ have identical inhibition profiles at different concentrations of hydrogen peroxide. The formation of singlet oxygen in substantial amounts would change the inhibition profile with increasing H_2O_2 concentration.

Catalase shows a second mechanism of luminescence inhibition. This enzyme influences the luminescence by removing hydrogen peroxide. According to the scheme of luminol oxidation hydrogen peroxide is a necessary component for light generation (12, 13). We showed previously

that even in the absence of hydrogen peroxide, luminescence is produced in the luminol–NaOCl system, because the solutions are contaminated by traces of hydrogen peroxide or small amounts of H_2O_2 are formed in a side reaction during the oxidation of luminol (15). Indeed, incubation experiments with catalase demonstrate that small amounts of hydrogen peroxide are present in the incubation mixture. They can be scavenged by an incubation with catalase. However, the residual luminescence of nearly 5% after incubation with catalase in the concentration range 10^{-9} to 10^{-7} mol/L seems to be caused by traces of H_2O_2 formed in a side reaction during the oxidation of luminol by NaOCl. This residual chemiluminescence disappears at concentrations of catalase where a direct scavenging of HOCl/OCl⁻ by catalase is possible.

Results obtained with SOD also support the view that hydrogen peroxide is necessary for the development of chemiluminescence in the system luminol–NaOCl. The enhancement at low SOD concentrations (Fig. 2) is explained by H_2O_2 formation in trace amounts due to SOD-catalysed superoxide dismutation.

LCL is developed inside and outside the neutrophils because luminol is known to permeate easily through the plasma membrane and parts of azurophilic granula containing myeloperoxidase are released during PMN stimulation. Recently a total inhibition of extracellular chemiluminescence in fMLP-stimulated PMN by addition of plasma has been demonstrated (17). Plasma pretreated with NaOCl to block reactive groups caused a lower inhibition of luminescence.

According to the present results the luminol-amplified chemiluminescence in PMN should be mainly dependent on the presence of such potent inhibitors as catalase and substances containing SH groups. Because a distinct amount of hydrogen peroxide is always generated during the first few minutes after PMN stimulation and the formation of H_2O_2 exceeds its removal in this period (27) substances containing SH groups should strongly influence the inhibition pattern of LCL.

SH groups occur mainly protein-bound or in glutathione. The concentration of free SH groups in biological systems depends on the oxidative state. It is diminished under stress situations. Probably, a relationship exists between the oxidative state and the enhanced values in LCL of PMN isolated from synovial fluid of patients with rheuma-

toid arthritis (28, 29), or of PMN for peripheral blood of patients with ischaemic heart disease or acute myocardial infarction (30).

Acknowledgements

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