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A real-time-based *in vitro* assessment of the oxidative antimicrobial mechanisms of the myeloperoxidase-hydrogen peroxide-halide system

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ABSTRACT

Mammals have evolved a special cellular mechanism for killing invading microbes, which is called the phagocytosis. Neutrophils are the first phagocytosing cells that migrate into the site of infection. In these cells, hypochlorite (HOCl) and other hypohalites, generated in the myeloperoxidase (MPO)-hydrogen peroxide (H_2O_2)-halide system is primarily responsible for oxidative killing. Here, we present a method for assessing these oxidative mechanisms in an *in vitro* cell-free system in a kinetical real-time-based manner by utilizing a bioluminescent bacterial probe called *Escherichia coli*-lux. The *E. coli*-lux method provides a practical tool for assessing the effects of various elementary factors in the MPO-H_2O_2-halide system. Due to the reported versatile intracellular pH and halide concentration during the formation of the phagolysosome and respiratory burst, the antimicrobial activity of the MPO-H_2O_2-halide system undergoes extensive alterations. Here, we show that at a physiological pH or lower, the antimicrobial activity of MPO is high, and the system effectively enhances the H_2O_2-dependent oxidative killing of *E. coli* by chlorination. The HOCl formed in this reaction is a prominent microbe killer. During the respiratory burst, there is a shift to a more alkaline environment. At pH 7.8, the chlorinating activity of MPO was shown to be absent, and the activity of the HOCl decreased. At this higher pH, the activity of H_2O_2 is enhanced and high enough to kill *E. coli* without the participation of MPO, and the lowered chloride concentration seemed still to enhance the H_2O_2-dependent killing capacity.

1. Introduction

During phagocytosis in neutrophils, microbicidal reactive oxygen species (ROS) are generated in a process called the respiratory burst due to their highly increasing oxygen consumption and uptake (Babior, 1984). Microbes are first internalized by an active, receptor-mediated process by creating a membrane-bound vacuole, called a phagosome. Shortly after this, the phagolysosome is formed by the fusion of lysosomes to the newly formed phagosome. The hexose monophosphate shunt is then activated leading to the formation of nicotinamide adenine dinucleotide phosphate (NADPH) acting as a substrate for the NADPH-oxidase. Oxidase accepts electrons from NADPH and reduces molecular oxygen, O_2, forming superoxide anion radical O_2^-, which is then dismutated into H_2O_2. The myeloperoxidase (MPO) enzyme, which can utilize H_2O_2 to generate hypohalites, enters into the phagolysosome by the fusion of the azurophilic granules. MPO converts H_2O_2 and chloride and other halides in a two-electron oxidation step of Cl^- and other halides to hypochlorous acid (HOCl) and other hypohalous acids (Hurst, 2012). Numerous reports demonstrate the involvement of the MPO-H_2O_2-halide system in the antimicrobial activities in the neutrophil phagolysome, and it has been shown that hypohalides are a much more potent killer than H_2O_2 alone, and therefore, the MPO-H_2O_2-halide system has generally been considered essential in killing (Babior, 1984; Hurst, 2012; Roos and Winterbourn, 2002; Thrasher and Segal, 2011; Klebanoff et al., 2013; Nauseef, 2014). Chloride has been reported to be the main physiological halide for MPO (Klebanoff et al., 2013). Alongside with MPO, fusing granules contain an abundant cocktail of other antimicrobials like lysozyme, defensins, and proteases.

Conventional microbe killing experiments have been made using relatively large amounts of microbes (10^6-10^9 cells), and their viability has been estimated using plate counting (Klebanoff et al., 2013; Reeves et al., 2002; Segal, 2005; Decleva et al., 2006). The method is, however, time consuming and moreover, it is laborious to reveal the detailed

Abbreviations: BL, bioluminescence; CFU, colony forming unit; FMN/$FMNH_2$, flavin mononucleotide; HOCl, hypochlorous acid, hypochlorite; H_2O_2, hydrogen peroxide; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxidant species

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kinetics of killing. We have transformed a wild type *Escherichia coli* K-12 with a plasmid including a modified bacterial luciferase gene *lux ABCDE* originating from the chromosomal *luxCDABE* gene of the gram-negative soil bacterium *Photorhabdus luminescens*. Expression of the whole operon, under the control of Lac promoter, produces the luciferase holoenzyme complex resulting in bacterial cells called *E. coli*-lux that constitutively emit bioluminescence (BL) without the addition of any substrate, which provides a convenient indicator of metabolic activity and viability of the cell (Atosuo et al., 2013). The oxidation of a long fatty acid aldehyde and reduced flavin mononucleotide (FMN/$FMNH_2$) is catalyzed by the bacterial luciferase enzyme complex. This system simultaneously emits a BL signal with an emission maximum at 490 nm (Atosuo et al., 2013). The dynamic measurement range of BL appears to be strictly comparable with the plate counting of 10^1–10^7 colony-forming units (CFU) (Atosuo et al., 2013). Because the decrease in the BL signal correlates with the decrease in CFU, *i.e.*, with the number of bacterial cells killed, it proves to be very suitable for assessing antibacterial effects. We have previously studied the antimicrobial activity of intact neutrophils during phagocytosis *in vivo*, the hydrogen peroxide and lactoperoxidase system (Atosuo and Lilius, 2011; Schlorke et al., 2016), the serum complement system, the effects of antimicrobial agents like various chemicals and toxins (Atosuo et al., 2013; Lilley et al., 2013), and the antimicrobial activity of the insect hemolymph utilizing *E. coli*-lux as a probe cell (Vojtek et al., 2014).

In this study, we present a functional and a real-time-based tool for analysing the effects of altering pH, changing chloride concentration, and various bacterial numbers on the activity of the MPO–H_2O_2 antimicrobial system *in vitro*.

2. Materials and methods

2.1. E. coli-lux

Antimicrobial assays were performed using *E. coli*-lux, an *E. coli* K12 strain transformed with a plasmid including the modified bacterial luciferase gene, called *luxABCDE*, originating from the chromosomal *luxABCDE* gene of *Photorhabdus luminescens* (Atosuo et al., 2013). The expression of the whole operon produces the luciferase holoenzyme complex resulting in bacterial cells emitting BL without the addition of substrate providing a convenient indicator of the metabolic activity in the cell.

A freezer stock preparation of the bacteria was made by cultivating the *E. coli*-lux in Luria Bertani Broth (LB_{amp}) [10 *g* tryptone (Neogen, Lansing, MI, USA), 5 *g* of yeast extract (Neogen, Lansing, Michigan, USA), 5 *g* NaCl (Sigma, St. Louis, Missouri, USA), and 100 µg/ml ampicillin (Sigma, St. Louis, Missouri, USA), pH 7.4 and by incubating in a shaker (250 rpm) at 37 °C, until the bacteria was in their stationary phase defined by the turbidity (optical density, OD) measurement at 620 nm (UV-1601 Shimadzu Spectrophotometer, Shimadzu Corp., Tokyo, Japan). Cells were washed and harvested by centrifugation (3000 rpm, 10 min), resuspended in LB_{amp} containing 25% glycerol, and placed in deep freeze (− 80 °C) in appropriate containers or directly in the microtiter wells. All *E. coli*-lux cultivation media contained ampicillin (Sigma, St. Louis, Missouri, USA) (100 µg/ml) to maintain the selection pressure.

2.2. MPO-H_2O_2-HOCl reactions and viability assessment

The measurements of killing activities of MPO (Planta, Wien, Austria), H_2O_2 (Merck KGaA, Darmstadt, Germany), and HOCl (NaOCl) (Sigma, St. Louis, Missouri, USA) at various pH values were performed in a phosphate buffer (67 mM) containing various concentrations of Cl^- (NaCl) (Sigma, St. Louis, Missouri, USA) by adding 25 µl *E. coli*-lux bacterial suspension diluted (phosphate buffer at 67 mM) from a freezer stock, and 25 µl of MPO diluted in a phosphate buffer (67 mM) to the desired concentration of Cl^- (NaCl) at the desired pH into the wells of a transparent bottom 96-well microtiter plate (96-well plate, Greiner One, *Düsseldorf* Germany). The reaction was started by adding 25 µl of H_2O_2 diluted at a desired concentration in a phosphate buffer (67 mM). HOCl killing was assessed by adding 50 µl of a NaOCl dilution (phosphate buffer at 67 mM) to the wells containing 50 µl of *E. coli*-lux dilution (10^5 cells). The final reaction volume was 100 µl. The desired bacterial concentration was pipetted into a well, and BL was assessed by incubating in the plate reader luminometer (Hidex Sense Plate Reader, Hidex, Turku, Finland) at 37 °C and by measuring the BL signal at 2 min intervals during the 30 min period. Results are shown as the average CPS/well (counts per second/well) of three parallel plates counted.

In all experiments, the viability of *E. coli*-lux was measured by utilizing two applications. The first application was by assessing the BL signal and then the next application was by measuring simultaneously from the results of the CFU samples from the parallel wells at the incubation time points 0, 15, and 30 min.

For the colony-forming unit (CFU) measurement, parallel microtiter plates were incubated in the 37 °C incubator, and 50 µl samples were taken at designated time points from the reaction wells. Six times 1/10 dilutions were made in the LB medium containing 100 µg/ml ampicillin, and 100 µl and 20 µl of each dilutions, sampled from the microtiter wells, were spread into the Petri dishes containing LB agar with 100 µg/ml ampicillin. Colonies were counted after an overnight incubation at 37 °C. Results are shown as the average CFU/well (colony-forming unit/well) of three parallel plates counted.

In experiments presented in Figs. 1A, , 100% killing was defined (after 30 min of incubation) as the bioluminescence level descending to the background level of 110 CPS. The signal from the intact bacteria wells with no added killing agents was defined as 0% of killing.

2.3. Data handling and statistical analysis

Raw data was analyzed in Excel, version 2013 (Microsoft, Redmond, Washington, USA), Q-Q-plotting with SPSS, version 25 (IBM, New York, USA) and all graphs were prepared with Origin, version 2015 (Microcal, OriginLab, Northampton, Massachusetts, USA).

3. Results

The effects of the H_2O_2, the MPO-H_2O_2 system, and hypochloride acid on the viability of *E. coli*-lux (10^5 cells) were assessed by measuring the BL signal and by simultaneously collecting samples from the parallel wells for the overnight CFU analysis (Fig. 1A). The BL signal of the viable *E. coli*-lux correlated with the CFU plate counts, and the diminishment of the BL signal correlated with the non-viable bacterial cells. The zero-time point (0 min) signified the onset of the reaction by the addition of the H_2O_2 or NaOCl into the microtiter wells and initiating the plate reader. The toxic effect of these oxidizing agents was very rapid, and the diminishment of the BL signal by these burst-phase kinetics was already visible at the first measurement cycle (Fig. 1A). The comparable loss of viability was observed in overnight CFU data. At 0 min of incubation, the presence of 250 µM H_2O_2 caused the killing of 45% of bacterial cells measured by the CFU plate counting and 52% killing when assessed with BL (Fig. 1A). After 15 min of incubation, these figures were 80% (CFU) and 72% (CPS) and after 30 min, 90% (CFU) and 80% (CPS). The MPO-derived reaction (33 µM H_2O_2 + 2.1 nM MPO) diminished CFU values 93% and CPS 87% at 0 min and 99% (CFU) and 96% (CPS) at 15 min (Fig. 1A). The entire BL signal decreased to baseline (*i.e.*, background signal was 110 CPS) after 25 min of incubation, and no colonies were observed on the plates from the time point of 30 min. No BL signal was observed after 6 min of incubation, when HOCl (NaOCl at 4 µM) was added, and only the 0-minute plates had colonies (Fig. 1A). HOCl decreased the CFU signal 99% and CPS signal 97% already at the onset of the measurement.

The viability of *E. coli*-lux was measured in the presence of different H_2O_2 concentrations with and without MPO (0.7 nM MPO) presented in

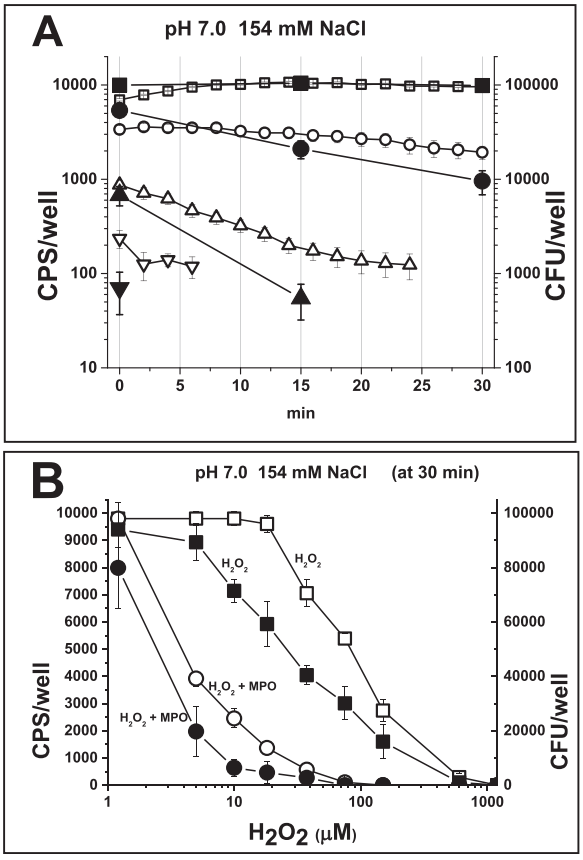

Fig. 1. A: Viability assessment of *E. coli*-lux cells (10^5 cells/well) (square) with the addition of 250 μM H_2O_2 (circle), 33 μM H_2O_2 + 2.1 nM MPO (upwards triangle) and 4 μM NaOCl (downwards triangle) incubated (60 min) in phosphate buffer, pH 7.0, containing 154 mM NaCl at 37 °C. Viability is presented as bioluminescence, counts per second (CPS/well; open symbols) or in colony forming units (CFU; closed symbols). **B:** Viability of *E. coli*-lux (10^5 cells/well) presented as a function of various H_2O_2 concentrations added in the absence (square) and in the presence of 0.7 nM MPO (circle). Killing was assessed after 30 min of incubation in phosphate buffer, pH 7.0, containing 154 mM NaCl at 37 °C. Viability is presented as bioluminescence, counts per second (CPS/well; open symbols) or in colony forming units (CFU; closed symbols). Values are shown as a mean ± SD from three parallel measurements.

Fig. 1B as BL and CFU after 30 min of incubation. It was observed that 100% killing (*e.g.*, no colonies on agar plates and BL 110 CPS, which is the noise of the background signal of the instrument) was achieved with the same H_2O_2 concentrations. However, somewhat more efficient killing was manifested at suboptimal H_2O_2 concentrations in plate counting compared with BL (Atosuo et al., 2013; Atosuo and Lilius, 2011).

Thus, unlike with plate counting, the kinetics of killing can be monitored in a real-time basis using the BL measurements of *E. coli*-lux. Fig. 2 shows the kinetics of the killing of 10^5 *E. coli*-lux cells by H_2O_2, NaOCl, and MPO-H_2O_2-Cl^- at pH 7.0. The higher the H_2O_2 or NaOCl concentration, the faster was the killing. More than 1000 μM of H_2O_2 (Fig. 2A) or 3.5 μM of HOCl (Fig. 2B) was needed for killing all bacterial cells in 30 min and doubling the concentration of the antimicrobial agent, halved the time needed. H_2O_2 at a concentration of 2 μM did not support a substantial killing even in the presence of MPO (Fig. 2C). On the other hand, when the concentration was 33 μM, the addition of 2.1 nM MPO killed all bacterial cells in less than 30 min (Fig. 2D). Again, doubling the amount of MPO, doubled the velocity of killing.

The difference between H_2O_2, in the absence and presence of 0.7 and 7.0 nM MPO, and NaOCl concentrations in the ability to kill *E. coli*-lux within 30 min is shown in Fig. 3. During phagocytosis, the phagolysosomal chloride concentration is reported to be 70–80 mM (Painter and Wang, 2006), and we compared the killing of *E. coli*-lux at two different chloride concentrations, 154 mM (*i.e.*, being the physiological concentration; Fig. 3A and C) and 77 mM (*i.e.*, being the phagolysosomal concentration; Fig. 3B and D). Neither the concentration of Cl^- nor the used pH alone had an effect to the BL signal of the probe bacteria (this data is not shown).

As Fig. 3A and B show, there were differences in killing rates between these two chloride concentrations at pH 7.8. At 77 mM chloride, the killing by H_2O_2 became more efficient and HOCl killing slightly less efficient. At pH 7.8 and a 77 mM chloride concentration (Fig. 3B), the killing of *E. coli*-lux was solely dependent on H_2O_2.

When in turn, the pH was reduced to 7.0, dramatic changes in the killing occurred (Fig. 3C and D). About a 100-fold higher concentration of H_2O_2 compared with that of NaOCl was needed to kill the same number of bacteria at pH 7.0 (Fig. 3C and D) at both chloride concentrations. The most impressive shift was, however, the activation of MPO (both 0.7 nM and 7 nM) independent of chloride concentration.

Subsequently, in a reaction mixture containing 10^5 *E. coli*-lux in the presence of 77 mM NaCl and 2 μM NaOCl or 50 μM H_2O_2 in the absence or presence of 0.7 nM MPO, the killing experiments were performed at different pH values varying from pH 6.0 to pH 7.8 (Fig. 4). The results show that the killing activity of a MPO-H_2O_2-Cl^- system followed the proportion of HOCl killing until pH 7.0(Dychdala, 2001). At pH 7.8, 50% of the killing activity of HOCl was lost compared to that of a pH 7.0. Similarly, at pH values higher than 6.8, the MPO-H_2O_2-Cl^- system started to lose its activity. On the other hand, the killing activity of H_2O_2 alone started to increase at pH values above 6.5. Finally, at pH 7.8, the killing by H_2O_2 alone was the same as by MPO-H_2O_2-Cl^- indicating that the chlorination activity of MPO at this pH was absent. Also, the killing kinetics of H_2O_2 alone and of MPO-H_2O_2-Cl^- were identical at this pH (data not shown).

Finally, we determined that the killing rates with *E. coli*-lux cell numbers varied between 10^3 and 10^7. About 100-fold less H_2O_2 was needed to kill 10^3 cells compared with 10^7 cells (Fig. 5A). The linear graph was generated by plotting the number of killed bacteria *versus* H_2O_2 or HOCl concentrations. By extrapolating the graph to one-killed bacterial cell, we found that at pH 7.0 and in the presence of 154 mM chloride, the needed H_2O_2 concentration for killing one cell was approximately 10 μM, while that of HOCl (or H_2O_2 + 7 nM MPO) was only 2 nM (Fig. 5B). On the other hand, when the pH was raised to 7.8 and the chloride level reduced to 77 mM, the needed H_2O_2 concentration decreased to 200 nM. MPO at these conditions was inactive (Fig. 5C).

4. Discussion

The *E. coli*-lux method provides a tool for assessing the effects of different factors influencing the *in vitro* MPO-H_2O_2-system. Earlier, we have utilized this system testing the antimicrobial activity of the serum complement system (Atosuo et al., 2013), lactoperoxidase activity, and antimicrobial activity of the intact neutrophils (*in vivo*) (Atosuo and Lilius, 2011; Schlorke et al., 2016). It is a convenient method for testing elementary factors such as pH and halide concentrations and to assess the influence of pharmaceuticals such as paracetamol and taurine on the MPO-H_2O_2-halide system (data not yet published). However, caution must be exercised when extrapolating isolated *in vitro* systems to very complicated phagolysosome conditions, because (Koelsch et al., 2010; Peskin and Winterbourn, 2006) results may vary depending on the altering factors as presented by Winterbourn et al. (Winterbourn et al., 2006).

CFU counting, a golden standard in microbiology, was used as a reference tool for the BL data and, although the results were in good correlation, the CFU counts manifested more efficient killing at lower H_2O_2 concentrations (Fig. 1B). Previously, when we compared CFU and BL in the assessment of serum complement activity, we noticed the

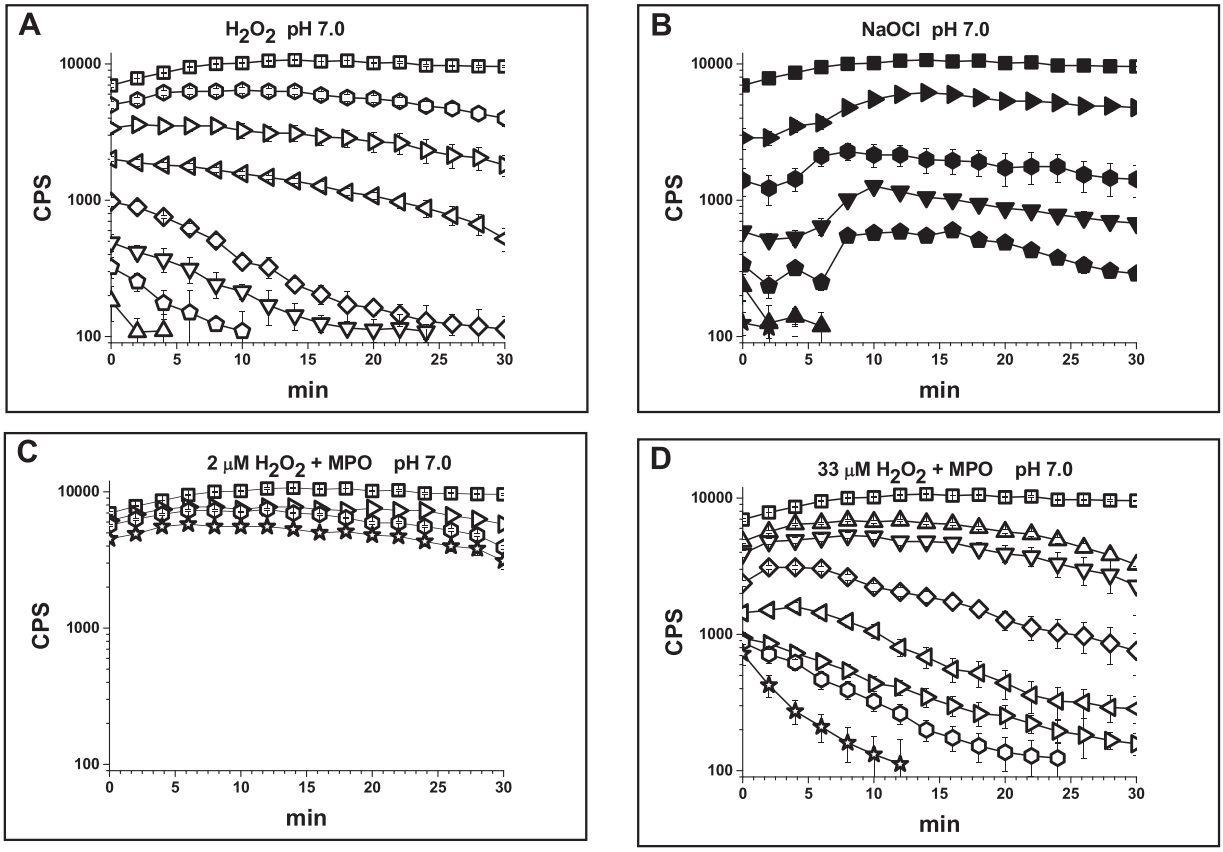

Fig. 2. Kinetics of the bioluminescence emission (CPS) of 10^5 *E. coli*-lux cells at 37 °C. The reaction mixture contained in a total volume of 100 µl of phosphate buffer, pH 7.0, 154 mM NaCl and **A**: (△) 8000, (○) 4000, (▽) 2000, (◇) 1000, (◁) 500, (▷) 250, (○) 125, and (fx8) 0 µM H_2O_2; **B**: (★) 5, (▲) 4, (fx) 3.5, (▼) 3, (fx) 2, (►) 1, and (■) 0 µM NaOCl; **C**: 2 µM H_2O_2; **D**: 33 µM H_2O_2. **C** and **D** in the presence of (●) 4.3, (○) 2.1, (▷) 1.1, (◁) 0.7, (◇) 0.5, (▽) 0.3, (△) 0.1 nM MPO, and (fx6) bacterial cells only. Values are shown as the mean ± SD from three parallel measurements.

same phenomena. Despite the high dilution of the tested antimicrobial agents before plating, the reaction continued in samples transferred to agar plates for overnight incubations (Atosuo et al., 2013). We believe that the same may apply to the oxidative killing. The effect caused by these agents continue during the incubation, because the agents themselves are not present in active concentrations after the dilutions. It has been reported that plate counting demonstrated more efficient killing than another indicator of metabolic activity, adenylate energy charge, when *Streptococcus lactis* and *Pseudomonas aeruginosa* were exposed to HOCl (Barrette et al., 1989).

Our results support the theory that the MPO-system is an essential antimicrobial factor during the respiratory burst by producing highly microbicidal hypohalides such as HOCl. At pH 7.0, the MPO-H_2O_2 system possessed an active antimicrobial reaction apparently by efficiently oxidizing Cl- to hypochlorous acid (Figs. 1–5). In an alkaline environment (pH 7.8), this peroxidase activity is, however, inhibited (Figs. 3) (Kettle and Winterbourn, 1989; Spalteholz et al., 2006). It is interesting that at a phagolysosomal chloride concentration of pH 7.8, H_2O_2 killing capacity seems to increase (Fig. 3B). The basis of the chloride dependence of H_2O_2 killing is potentially interesting but strictly a phenomenon with no known mechanism. Determining the concentration dependence, and whether this dependence is specific for chloride is needed, before validity can be given to the phenomenon. The highest H_2O_2 concentration used was 1000 µM, which is much higher than the reported physiological concentration. It was first used in initial testing and we decided to keep using it still in the actual test protocols in order to show the whole range of the H_2O_2 activity.

The killing kinetics seem to be active in two phases (Figs. 1A and 2), because already at 0 min revealed a substantial loss of viability in both measurements (BL and CFU, Fig. 1A). It has been reported that chlorination of monochlorodimedon catalyzed by MPO follows burst-phase kinetics interpreted as a rate-limiting turnover of an accumulating enzyme intermediate (Kettle and Winterbourn, 1988). The killing of *E. coli*-lux by H_2O_2 or NaOCl, even in the absence of MPO, turned out to follow similar burst-phase kinetics (Figs. 1A, 2 A, and B) suggesting that this type of kinetics may generally apply to oxidative reactions. Winterbourn et al. have modeled the oxidative reactions in the neutrophil phagosome (Winterbourn et al., 2006). Their simulations reveal that under standard conditions, the H_2O_2 concentration is only 2 µM, whereas in the absence of MPO, it is 33 µM (Fig. 2C and D). During the 30-minute incubation, all of the 33 µM H_2O_2 bolus present in the reaction mixture was converted to HOCl by MPO concentrations higher than 1 nM (data not shown). Thus, the higher MPO concentrations did not increase the killing capacity, but the concentrations up to 100 nM increased the velocity of killing (data not shown). It should be noted that when testing the reactions, the MPO is progressively inactivated, when there is a large excess of H_2O_2 present contributing to the results. In future studies, instead of adding H_2O_2 bolus, the enzymatic production of the H_2O_2 *via* glucose-glucose oxidase system will bring the test system closer to the *in vivo* activity. This enzyme system mimics the phagolysosomal production of H_2O_2 trough the NADPH superoxide anion radical production (Weibel and Bright, 1971, Wong, Wong et al., 2008).

Chloride is the main physiological halide for the MPO (Klebanoff et al., 2013), and we demonstrated the role for HOCl as a tenacious microbe killer (Figs. 1–5). The activity optimal of MPO is reported to be at pH 6 (Foote et al., 2017). After the onset of the phagocytosis, during the first three minutes, the pH tentatively elevates close to 7.8, but during the next 10 min, falls to near 7.0 (Winterbourn et al., 1985; Cech and Lehrer, 1984). The pH elevation in the neutrophil phagolysosome is

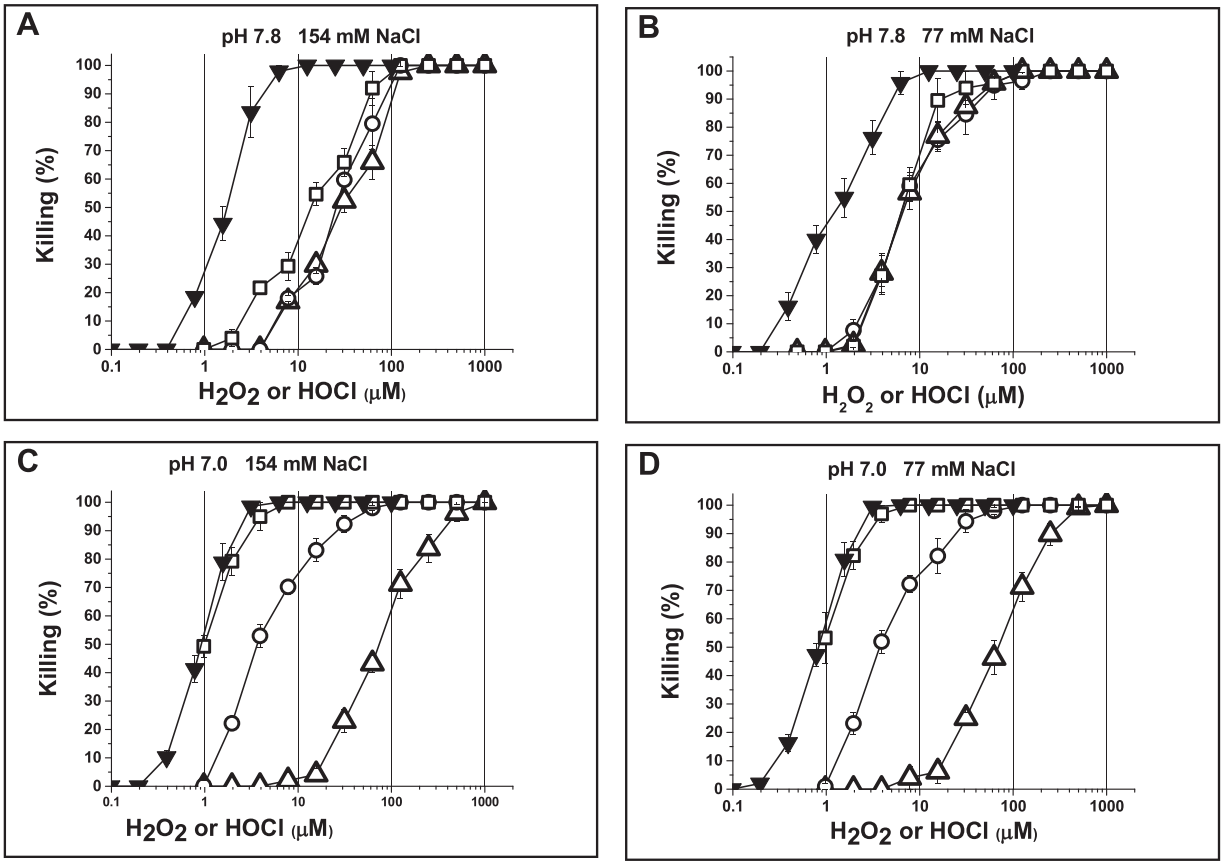

Fig. 3. *E. coli*-lux (10^5 cells/well) killing by various H_2O_2 or HOCl concentrations in the absence and in the presence of 0.7 nM MPO. Killing was assessed after 30 min of incubation at 37 °C in 100 µl of phosphate buffer. **A**: pH 7.8, 154 mM NaCl; **B**: pH 7.8, 77 mM NaCl; **C**: pH 7.0, 154 mM NaCl; **D**: pH 7.0, 77 mM NaCl. (△) H_2O_2, (●) H_2O_2 + 0.7 nM MPO, (□) H_2O_2 + 7 nM MPO, and (▼) NaOCl. 100% killing was defined as the bioluminescence level reaching the background signal of 110 CPS. Values are shown as the mean ± SD from three parallel measurements.

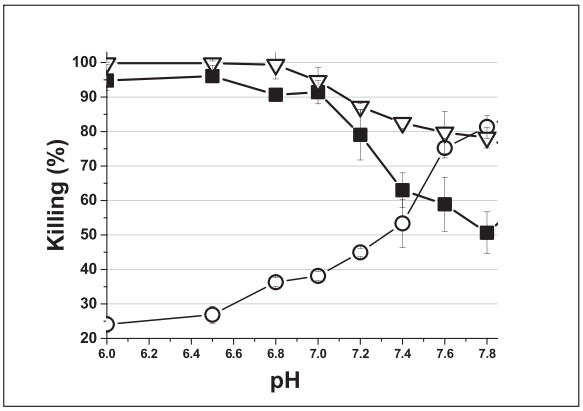

Fig. 4. *E. coli*-lux (10^5 cells/well) killing by H_2O_2 (50 µM) (●); H_2O_2 (50 µM) + MPO (0.7 nM) (▽); NaOCl (2 µM) (■) at various pH values. Killing was assessed after 30 min of incubation at 37 °C in 100 µl of phosphate buffer (77 mM NaCl). 100% killing was defined as the bioluminescence level reaching the background signal of 110 CPS. Values are shown as the mean ± SD from three parallel measurements. Solid line follows the percentual proportion of HOCl killing.

caused by the production of superoxide anion radical by the respiratory-burst activity into the phagosome, which by dismutation to hydrogen peroxide, consumes H^+ ions and rises the phagolysosomal pH transiently to 7.8–8.0 or even higher to pH 9 (Segal, 2005; Cech and Lehrer, 1984; Geisow et al., 1981; Segal et al., 1981; Jiang and Griffin, 1997; Dri et al., 2002; Reeves et al., 2003; Levine et al., 2015). It is noteworthy that also HOCl partially lost activity in an alkaline environment at pH 7.8 (*i.e.*, about a 30% to 50% decrease from that at pH 7.0, Figs. 3–5). These are potentially significant observations that require validation, such as by also carrying out plate assay experiments.

The number of bacteria in the reaction mixture has a large impact on the concentrations of agents needed for the killing. The concentration of oxidants required to kill 100% of *E. coli*-lux cells was found to be dependent on the bacterial cell numbers. At pH 7.0, the approximated H_2O_2 concentration varied from 100 µM to 4 mM, when the cell numbers increased from 10^3 to 10^7 in the reaction mixture, while the concentration of HOCl varied from 0.3 µM to 300 µM. When pH was raised to 7.8, the needed H_2O_2 concentration decreased varying from 10 µM to 3 mM, while the HOCl concentration required stayed roughly the same.

About 20 particles are assumed to be ingested by one neutrophil, and about 20 phagolysosomes are formed (Winterbourn et al., 2006), suggesting that one phagolysosome contains one ingested bacterial cell. Because the H_2O_2 concentration in a phagolysosome is, according to simulations, 2 µM (Winterbourn et al., 2006) or in the absence of MPO even higher (33 µM), the phagolysosomal concentration is, according to our rough estimations (Fig. 5), at least 10-fold higher than the observed concentration needed for *E. coli* killing. H_2O_2 is known to be a competent microbe killer and an efficient antimicrobial activity still occurs in the absence of MPO. It has been reported that the generation of ROS is higher in the MPO-deficient mouse neutrophils augmenting the role of H_2O_2 (Doi et al., 2007; Aratani et al., 2012). Gained data supports the claim that H_2O_2 at reported phagolysosomal concentrations during the respiratory burst would be high enough to support the killing (Fig. 5C).

Previous studies with *E. coli, Pseudomonas aeruginosa, Streptococcus lactis, S. aureus, Burkholderia cepacia, K. pneumoniae*, and *Saccharomyces cerevisiae* showed that 100% killing of 5×10^8 microbial cells/ml was

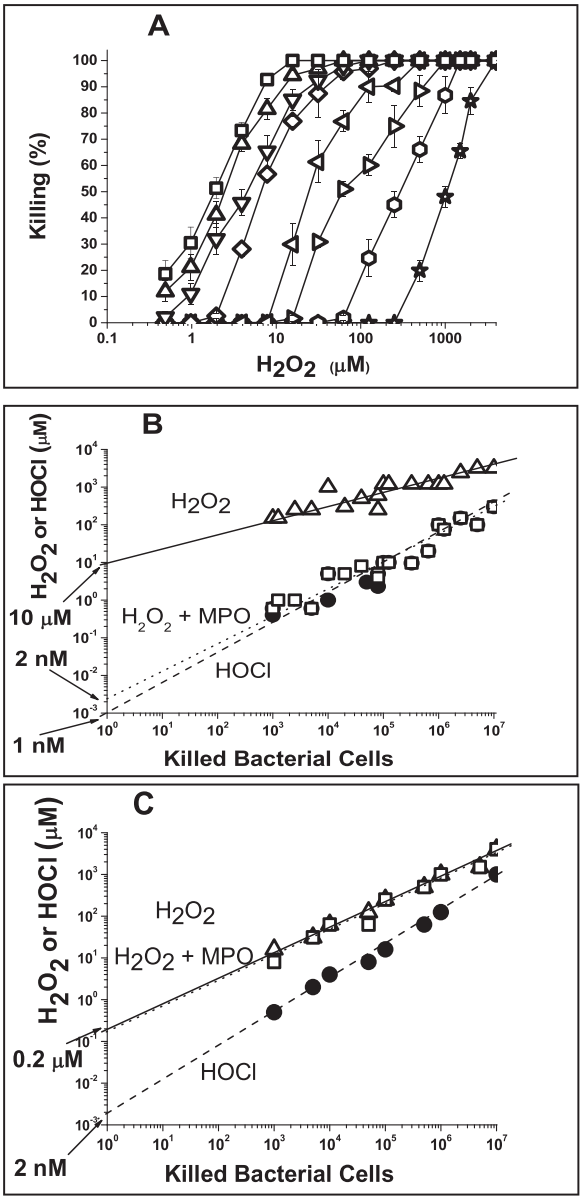

Fig. 5. Dependence of the killing of various numbers of *E. coli*-lux cells after 30 min of incubation at 37 °C in 100 μl of phosphate buffer upon H_2O_2 or HOCl concentration; **A**: pH 7.8; 77 mM NaCl; initial number of the bacterial cells in the reaction: (□) 10^3, (△) 10^4, (▽) 5×10^4, (◇) 10^5, (◁) 5×10^5, (▷) 10^6, (○) 5×10^6, and (⬟) 10^7. **B**: pH 7.0, 154 mM NaCl. **C**: pH 7.8; 77 mM NaCl; H_2O_2 (△); H_2O_2 and 7 nM MPO (□); HOCl (●). Number of killed bacteria is 100% killing of the initial number in the reaction. Values are shown as the mean ± SD from three parallel measurement. The approximations (lines) were gained by using the SPSS Q-Q-.

achieved with 100–1000 μM HOCl (Klebanoff et al., 2013; Barrette et al., 1989; King et al., 2004; Barrette et al., 1987; Green et al., 2017). By extrapolating the cell density, our results show the concentration of HOCl needed to be about 700 μM. The respective H_2O_2 concentration for killing 2×10^7 cells is reported to be 0.5–5 mM, while our results show 2 mM. Earlier studies also showed that the oxidant levels required for equivalent killing increased about 20-fold with increasing the cell densities of the microbial suspensions (*i.e.*, *E. coli, P. aeruginosa, S. lactis, S. cerevisiae*) over the range investigated (2×10^7-2×10^9 cfu/ml). The toxic effect of oxidizing agents seems to be somewhat universal among the microbes.

Our test system utilizing the real-time-based BL method seems to agree with earlier studies. Results demonstrate that CFU values overestimate the killing rates, while the method relying on the metabolic activities of the bacterial cells express more realistic rates (Atosuo et al., 2013).

In this context, we must bear in mind that the phagolysosome environment is a microbicidal cocktail, a combination of various antimicrobial systems including the phagolysosolmal oxygen independent agents like cationic proteins, lysozyme, an active complement system, cathepsin G, and elastase participating in the microbe killing. Currently, our group is testing these antimicrobial agents utilizing the *E. coli*-lux system. Cathepsin G and elastase have a higher optimal pH (pH 7–9 and pH 8–10, respectively) (Foote et al., 2017) than MPO (pH 6), and these compounds play a vital role in bacterial killing. Bactericidal/Permeability Increasing Protein (BIP) is known to negatively affect *E. coli* (Chockalingam et al., 2007b, a). Chronic Granulomatous Disease (CGD) patients with impaired NADPH-oxidase functions are reported to have no more common *E. coli* infections than healthy individuals (Rosen and Michel, 1997; Marciano et al., 2015). However, CGD patients suffer frequently from the gram-positive *Staphylococcus aureus* infections, because their neutrophils are not able to destroy these pathogens (Falcone and Holland, 2013), and the oxidative arm including MPO is required.

Various studies (Thrasher and Segal, 2011; Klebanoff et al., 2013; Homme et al., 2013) describe the significant and vital involvement of MPO in the host defense against invading pathogens like *S. aureus*, *Candida albicans*, and *Klebsiella pneumoniae* by human neutrophils. In the case of *E. coli,* this is not the case, and an MPO-dependent oxidative host defense system seems to have variations in efficiency depending on the pathogen in question. According to our unpublished data, paracetamol, a known MPO inhibitor, and taurine, a HOCl antagonist, did not have any influence on the *in vivo* neutrophil killing of the *E. coli* cells. MPO may thus play a less significant role in killing gram-negative bacteria like *E. coli* by neutrophils (Segal, 2005; Levine et al., 2015; Segal, 2006), at least this is a case in various *in vivo* and *in vitro* studies (Klebanoff et al., 2013; Decleva et al., 2006; Rosen and Michel, 1997; Brovkovych et al., 2008). Consequently, *E. coli*-lux is not the perfect probe microbe for assessing the complete physiological role of the MPO-H_2O_2-halide system in intracellular killing and in the future, alongside with the *E. coli*-lux, a gram-positive reference biosensor like *S. aureus* with similar BL properties would be feasible (Francis et al., 2000). Nevertheless, *E. coli*-lux is still a practical tool for testing the basic activities of the neutrophil antimicrobial system. It is a feasible and easy-to-use *in vitro* model for assessing the effects of various agents and circumstances in intracellular killing and also practical in *iv vivo* assessment utilizing intact neutrophils (Atosuo and Lilius, 2011).

Statement of ethics

The authors have no ethical conflicts to disclose.

Disclosure statement

The authors have no conflicts of interest to declare.

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Author contributions

Janne Atosuo is the main author of the manuscript and he has conducted the main laboratory work and data analysis. Eetu Suominen has contributed to the laboratory work and data analysis.

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