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HYDROGEN PEROXIDE MODULATION OF THE RESPIRATORY BURST OF HUMAN NEUTROPHILS

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Abstract—Addition of micromolar concentrations of hydrogen peroxide (H_2O_2) to human neutrophils resulted in a dose-dependent luminol-enhanced chemiluminescent response. Pretreatment of neutrophils with micromolar concentrations of H_2O_2 altered their response to the surface acting stimulants serum-treated zymosan (STZ) and formyl-methionyl-leucyl-phenylalanine (fMLP), but not to the intracellular stimulant phorbol myristate acetate (PMA). The alterations were partially reversible by catalase, but exacerbated by superoxide dismutase. These results suggest a modulatory role for H_2O_2 in the respiratory burst of neutrophils.

Hydrogen peroxide (H_2O_2) is a major product of the respiratory burst of neutrophils [1, 2]. Neutrophil phagocytosis [3] activates the respiratory burst enzyme complex, NADPH–oxidase, which generates large quantities of superoxide free radicals and thereby initiates the production of an array of reactive oxygen intermediates [2, 4]. Superoxide dismutates spontaneously or enzymatically to H_2O_2, which can act directly as a cytotoxic agent [5–8]. H_2O_2 is also a substrate of the granule component myeloperoxidase (MPO) [1, 9], which is released into phagosomes and the extracellular environment during degranulation. When supplied with H_2O_2 and chloride ions, MPO produces hypochlorous acid (HOCl) [10, 11].

Neutrophils possess cytosolic enzymes for the metabolism of H_2O_2 [6], which suggests that this potential oxidant is likely to be shortlived within the cell [12]. Enzymes for the removal of H_2O_2 may be present outside the cell, but their concentrations and activities are too low to remove extracellular H_2O_2 efficiently [13, 14]. Although the extracellular concentration of H_2O_2 is limited by its passive equilibrium across the cell membrane [12], it has been reported to accumulate to a concentration of 10 μM per million stimulated neutrophils [15]. However, where neutrophils adhere to cellular substratum, enclosed regions allow the extracellular concentration of H_2O_2 to rise even higher [6, 16].

Several functions of neutrophils are affected by millimolar concentrations of H_2O_2. Rajkovic and Williams [6] found that phagocytosis, bacterial killing and degranulation were reduced by H_2O_2. Hexose monophosphate shunt activity was enhanced and glutathione (GSH) stores depleted by H_2O_2 treatment. During active phagocytosis, neutrophils are constantly exposed to micromolar rather than millimolar concentrations of self-generated H_2O_2, but there has been no attempt to investigate thoroughly the consequences of such physiological concentrations of H_2O_2 on their function. In particular, little information is available on the effects of micromolar concentrations of H_2O_2 on the respiratory burst of the neutrophil.

The neutrophil respiratory burst is accompanied by weak chemiluminescence (CL), which can be amplified for experimental measurement by luminol or lucigenin [17]. Luminol-enhanced CL is an indicator of the intracellular and extracellular activity of the MPO system of the neutrophil [18, 19], whereas lucigenin-enhanced CL detects extracellular production of superoxide by the NADPH–oxidase complex [20].

We investigated the effects of physiologically plausible micromolar concentrations of H_2O_2 on some neutrophil functions and found that it induced a luminol-enhanced CL response, not previously reported. Furthermore, H_2O_2 altered the response of neutrophils to two stimulants of the respiratory burst, which act via surface receptors, serum-treated zymosan (STZ) and formyl-methionyl-leucyl-phenylalanine (fMLP).

MATERIALS AND METHODS

Reagents. Heparin was obtained from Fisons Pty. Ltd (Sydney, Australia). Zymosan A, formyl-methionyl-leucyl-phenylalanine (fMLP) phorbol myristate acetate (PMA), superoxide dismutase (bovine blood) (SOD) and *N*-ethylmaleimide (NEM) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Dextran ($M_r > 200{,}000$) was obtained from BDH Chemicals Ltd (Poole, U.K.). Lucigenin, luminol and catalase (bovine liver) were supplied by Boehringer Mannheim GmbH (Mannheim, F.R.G.). Dulbecco's phosphate-buffered saline (PBS), with and without calcium (1.79 mM) and magnesium (0.95 mM), was obtained from the Commonwealth Serum Laboratories (Melbourne, Australia). Ficoll-Hypaque (Monopoly Resolving Medium, Density 1-114) was obtained from Flow Laboratories (Sydney, Australia).

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Dimethylsulfoxide (DMSO) was obtained from Fluka AG (Buchs, F.R.G.).

Zymosan was opsonized with normal human serum, and suspended in PBS with magnesium and calcium. PMA was dissolved in DMSO. All other reagent solutions were made up in PBS with calcium and magnesium.

Isolation of neutrophils. Neutrophils were isolated from fresh venous blood of healthy volunteers. Heparinized blood was incubated for 45 min with 6% Dextran, to sediment erythrocytes. The leukocyte rich plasma was decanted onto Ficoll-Hypaque and centrifuged at 500 *g* for 25 min. The neutrophil fraction was washed twice in Dulbecco's PBS without calcium or magnesium.

Neutrophils were counted using a standard hemocytometer, after staining with Gentian Violet or Trypan Blue. Cell viability was estimated by Trypan Blue exclusion and always found to be greater than 95%.

Neutrophils were suspended to a stock concentration of 5.0×10^6 cells/mL in Dulbecco's PBS with calcium and magnesium.

Chemiluminescence assay. The respiratory burst events of the neutrophils were measured by chemiluminescence (CL), in a Packard Pikolite 400 luminometer, with temperature control at 37°. All reactions were carried out in acid washed vials, with a final volume of 1 mL. Final cell suspension contained 0.5×10^6 cells/mL. Both luminol (final concentrations 2 μg/mL) and lucigenin (final concentration 1 mg/mL) were used to amplify neutrophil CL.

Neutrophils were allowed to equilibrate in the luminometer for 15 min prior to treatments and/or addition of H_2O_2. Chemiluminescent counts were taken over 5 sec at 1 or 2 min intervals for each sample. The reading taken 10 sec after H_2O_2 addition was recorded as zero time. Counting was discontinued when the counts approached the prestimulant resting baseline.

Chemiluminescent measurement of the respiratory burst of neutrophils is a bioassay that is quantitatively variable between neutrophil samples from different individuals and also between samples obtained from the same individual on different days. Consequently, each experiment was carried out four to ten times, and the results presented are averages of triplicates from a single representative experiment.

Stimulation of neutrophils. H_2O_2 (final concentration 6–126 μM) was added to the equilibrated neutrophil suspensions in the luminometer. CL counts were recorded every 1 or 2 min, over a period of up to 10 min or until the cells returned to the basal state.

Within 10 min of the addition of H_2O_2, neutrophils were stimulated with STZ (final concentration 1 μg/mL), fMLP (final concentration 0.1 μM) or PMA (final concentration 0.1 μg/mL). CL counts were recorded until the cells returned close to the resting state.

In some experiments, catalase (final concentration 2600 Units/mL) was added after the initial H_2O_2-induced CL response had returned to baseline. After a further 10 min at baseline, neutrophils were challenged with STZ. In further experiments, superoxide dismutase (SOD) (final concentration 150 Units/mL) was added to the neutrophils immediately prior to STZ stimulation.

RESULTS

Exposure of neutrophils to 6–126 μM H_2O_2 resulted in a dose-dependent luminol-enhanced CL response (Fig. 1a). CL was not observed in the absence of neutrophils, indicating that the CL was not due to direct interaction between H_2O_2 and luminol.

To determine whether the observed luminol-enhanced CL response involved generation of superoxide by NADPH–oxidase, H_2O_2 was added to neutrophils in the presence of lucigenin. In contrast to the luminol results, no CL was observed.

We then investigated whether luminol-enhanced CL was the result of H_2O_2 acting simply as a substrate for neutrophil MPO. In the presence of luminol, repeated exposure of the neutrophils to 6.3 μM H_2O_2 induced CL responses of increasing peak heights (Fig. 2). This suggested a priming effect of H_2O_2, rather than simple substrate utilization by MPO.

To determine whether thiol groups, in particular GSH, were necessary for the H_2O_2-induced CL response, neutrophils were preincubated with 50 μM *N*-ethylmaleimide (NEM) for 30 min at room temperature, immediately prior to H_2O_2 addition and subsequent STZ stimulation. No inhibition of the H_2O_2-induced CL response was observed. However, the CL response to STZ was absolutely dependent on thiol groups, as this was abolished by NEM preincubation.

When neutrophils were preatreated with micromolar concentrations of H_2O_2 (Fig. 1a), and then challenged with STZ, it was noted that the following CL response was suppressed in proportion to the concentration of H_2O_2 present (Fig. 1b). Suppression of the CL response of neutrophils to STZ was observed in the presence of both luminol and lucigenin (Fig. 3a and b).

In order to investigate whether the suppression was a surface receptor effect of H_2O_2 or an indication of damage to later events of the respiratory burst, H_2O_2 addition to neutrophils was followed by PMA stimulation. PMA bypasses membrane events and acts as a diacylglycerol mimic to activate NADPH–oxidase via intracellular receptors [21]. Preincubation of neutrophils with 31 μM H_2O_2 had no effect on the magnitude of their subsequent CL response to PMA.

The absence of any effect of H_2O_2 on the response of neutrophils to PMA led us to conclude that H_2O_2 suppressed the response to STZ by acting at the level of surface receptors. We therefore investigated the effect of H_2O_2 on the response of neutrophils to fMLP, another surface-acting stimulant of the respiratory burst. When neutrophils were preincubated with 6–126 μM H_2O_2 and then challenged with fMLP, the subsequent CL response was enhanced significantly (Fig. 4). Luminol-enhanced CL showed a marked increase that was dependent on H_2O_2 concentration. The fMLP-induced lucigenin-enhanced CL was also increased, but to a smaller degree, after exposure to H_2O_2.

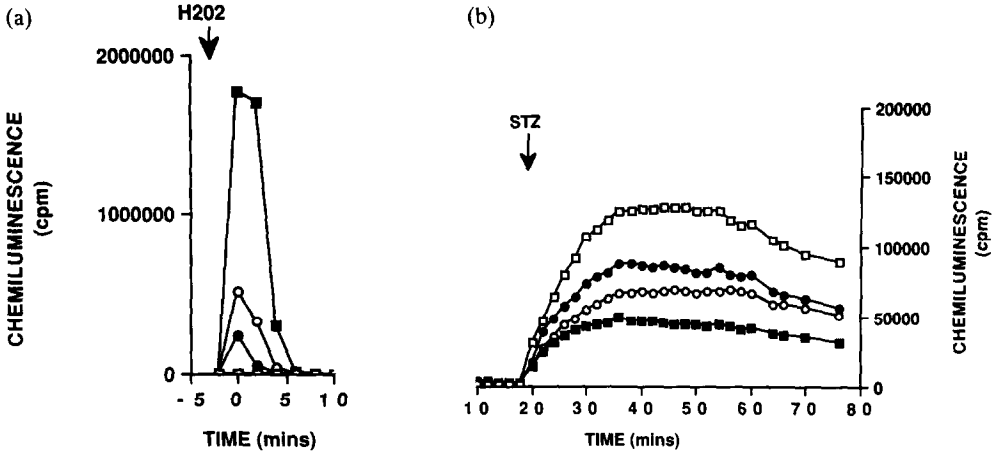

Fig. 1. Luminol-enhanced CL response of neutrophils to H_2O_2. (a) CL response to 6 (●), 31 (○) and 126 μM (■) H_2O_2. Control cells (□) received no H_2O_2. (b) CL response of the same neutrophils to subsequent STZ challenge, showing suppression of response to STZ dependent on H_2O_2 concentration.

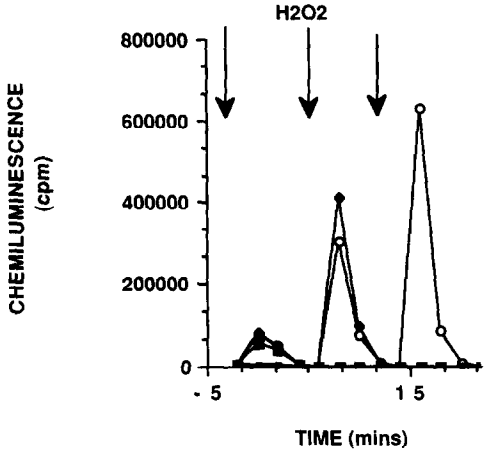

Fig. 2. Luminol-enhanced CL response of neutrophils to repeated additions of 6 μM H_2O_2. Control cells (□) received no H_2O_2. One group of neutrophils received one addition of 6 μM H_2O_2 (■); a second group received two additions (◆); a third group received three additions (○). Each successive addition of H_2O_2 produced an increased CL response.

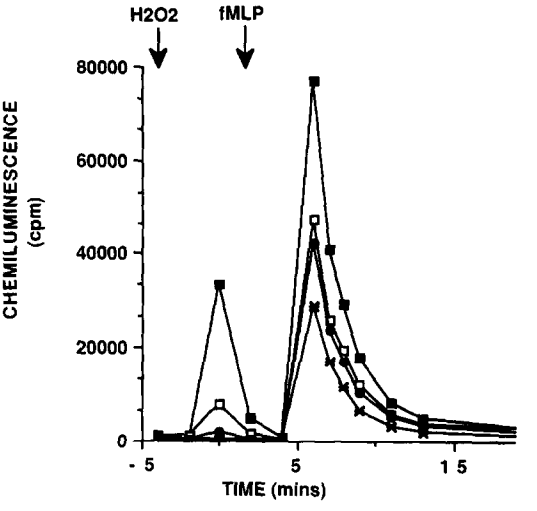

Fig. 4. Luminol-enhanced CL response of neutrophils to 6 (●), 31 (□) and 126 μM (■) H_2O_2 followed by fMLP challenge. The neutrophil response to fMLP increased in a manner dependent on the concentration of H_2O_2. Control cells (*) received no H_2O_2 pretreatment.

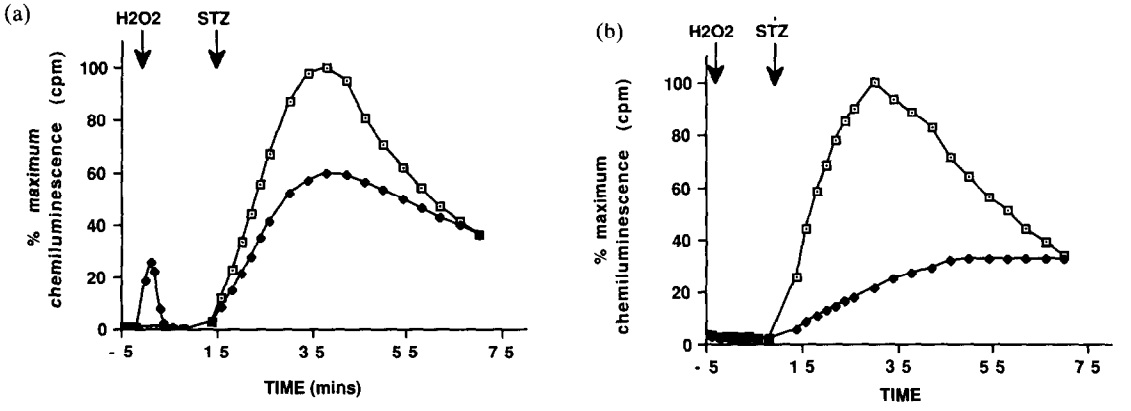

Fig. 3. (a) Luminol-enhanced CL and (b) Lucigenin-enhanced CL responses of neutrophils to STZ, after exposure to 12 μM H_2O_2 (◆). Control cells (⊡) received no H_2O_2. Due to the different sensitivities of luminol and lucigenin amplification of neutrophil CL, these results are expressed as a percentage of the maximum response.

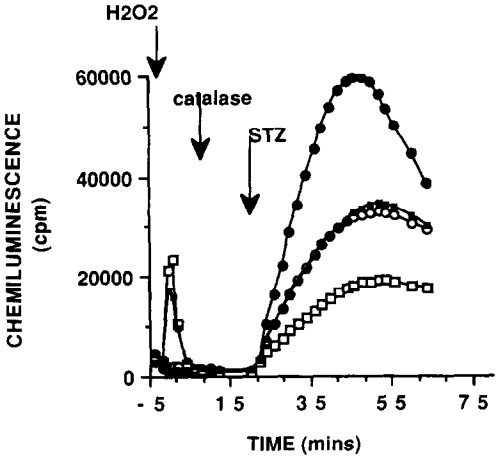

Fig. 5. Effect of H_2O_2 and catalase on the luminol-enhanced CL response of neutrophils to STZ. H_2O_2 was added to neutrophils, followed by catalase incubation and then challenged with STZ. Control cells (○) received no H_2O_2 or catalase. Neutrophils treated only with H_2O_2 (□) exhibited a suppressed response to STZ. Neutrophils treated with H_2O_2 and then with catalase (■) showed a restored response to STZ. Neutrophils incubated with catalase alone (●) underwent a response to STZ that was enhanced above that of control cells.

The nature of the H_2O_2-induced suppression of neutrophil CL response to STZ was examined by incubating the neutrophils with catalase, after exposure to H_2O_2. In neutrophils pre-exposed to 31 μM H_2O_2, catalase treatment restored the STZ-induced CL to that of control cells that had not been pretreated with H_2O_2 (Fig. 5). When catalase was added to neutrophils not previously exposed to H_2O_2, their CL response to STZ was also enhanced, well above the level of control cells (Fig. 5).

Pretreatment of neutrophils with SOD also caused suppression of the CL response to STZ, not only in H_2O_2 pretreated cells but also in control cells.

DISCUSSION

Since the addition of micromolar concentrations of H_2O_2 to neutrophils resulted in a dose-dependent CL response in the presence of luminol, but not in the presence of lucigenin, H_2O_2 alone was not able to induce activation of the NADPH–oxidase in human neutrophils, but may have acted directly on their MPO system. Luminol-enhanced CL of neutrophils reflects the activity of the granule enzyme MPO [18, 19], while the lucigenin-enhanced CL detects production of superoxide by the NADPH–oxidase of the neutrophil [20].

The luminol-enhanced CL response may have been due to H_2O_2 penetration of the intact neutrophil azurophil granules and reaction with MPO within the granules. Alternatively, H_2O_2 may have promoted neutrophil degranulation and release of MPO into the extracellular medium, where the H_2O_2 was utilized by the MPO to produce a luminol-enhanced CL. H_2O_2 can diffuse freely across membranes [12], but in order to react directly with MPO within the granules of the neutrophil, exogenous H_2O_2 would need to survive the intracellular gauntlet of catalase and glutathione peroxidase [15]. Thus, it is more likely that the CL produced in response to H_2O_2 requires MPO to be released into the extracellular medium. Degranulation due to micromolar concentrations of H_2O_2 has been observed in eosinophils [22], and mast cells have also been shown to release histamine on exposure to H_2O_2 [23]. Degranulation and extracellular release of MPO by the neutrophils may have occurred as a result of H_2O_2-induced membrane perturbations and may involve calcium mobilization from plasma membrane-bound stores.

Cytosolic glutathione peroxidase was not involved in the luminol-enhanced CL, since treatment of the neutrophils with NEM, to remove the co-substrate of glutathione peroxidase, GSH [15], did not diminish the neutrophil CL response to H_2O_2.

We are currently investigating the possibility that the addition of micromolar H_2O_2 leads to degranulation and release of MPO from human neutrophils.

Exposure to 6 μM H_2O_2 sensitized the neutrophils to subsequent additions of H_2O_2, as indicated by increasing peaks of luminol-enhanced CL observed with each successive addition. The effect was suggestive of "priming". The first addition of H_2O_2 may have initiated a small amount of degranulation, releasing MPO into the extracellular medium, where it reacted with the H_2O_2 to produce luminol-enhanced CL. Further additions of H_2O_2 may have acted synergistically with the already available extracellular MPO, or its products, to induce further degranulation and MPO release, thereby resulting in an increased CL response. In effect, each addition of H_2O_2 may have "primed" the MPO activity of the neutrophils by accelerating degranulation. A similar mechanism has been found in eosinophils, in which H_2O_2 induces degranulation, and this is further enhanced by the presence of extracellular eosinophil peroxidase [22].

Exposure to 6–126 μM H_2O_2 suppressed the subsequent luminol—and lucigenin—enhanced CL response of neutrophils to STZ. Binding of STZ to the $C3_b$ and F_c receptors on the surface of the neutrophil induces phagocytosis, the production of superoxide by NADPH–oxidase and degranulation, releasing MPO and proteolytic enzymes into phagosomes and into the extracellular medium [9]. In contrast to the STZ response, the PMA-stimulated CL response of neutrophils was unimpaired by preincubation with micromolar H_2O_2. PMA diffuses across the neutrophil plasma membrane and binds to intracellular receptors to activate NADPH–oxidase and degranulation [24]. Therefore, the unimpaired response of neutrophils to PMA and their suppressed response to STZ suggest that micromolar H_2O_2 affects membrane receptor events necessary for activation of the respiratory burst by STZ, rather than a direct effect on NADPH–oxidase generation of superoxide or MPO release. H_2O_2 may interfere with the binding of STZ to either the

$C3_b$ or F_c receptors or prevent ligand–receptor signal transduction into the cell by oxidation of chemical groups essential for receptor activity. Alternatively, H_2O_2 may perturb the cell membrane, thereby altering the conformation of the receptor and masking its binding site or preventing its coupling with second messenger components within the membrane.

Exogenous catalase increased the CL response of neutrophils to STZ. In neutrophils pretreated with H_2O_2, catalase removed H_2O_2 inhibition of the STZ response, indicating that this inhibition requires the continued presence of H_2O_2. In neutrophils with no prior H_2O_2 exposure, catalase also enhanced the response to STZ. This result suggests that H_2O_2 produced extracellularly during the respiratory burst would normally exert a negative feedback, reducing the magnitude of the neutrophil response to STZ. The restored response to STZ in the neutrophils pretreated with H_2O_2, and then incubated with catalase, was less than the response to STZ of the control cells treated with catalase, however. This may indicate that some permanent damage was incurred by the STZ-activated signalling system of the neutrophils when exposed to micromolar H_2O_2. Very low concentrations of H_2O_2 are not scavenged efficiently by catalase [12], so that the incomplete restoration by catalase of the STZ response in the H_2O_2 treated cells may also be due to the presence of a small amount of H_2O_2 remaining after catalase incubation. The STZ-induced luminol-enhanced CL of neutrophils comprises intracellularly-generated CL, which will remain unaffected by extracellular catalase, and a smaller proportion of extracellularly-generated CL, which is likely to be inhibited by catalase removal of H_2O_2. Consequently, the observed enhancement of CL in the presence of catalase may be an underestimation.

Unlike the STZ response, the fMLP-induced CL response was enhanced by pretreatment of the neutrophils with micromolar H_2O_2. fMLP stimulates the respiratory burst of neutrophils by binding to a specific membrane-bound fMLP receptor [25]. It induces a response distinctively different from that produced by PMA or STZ stimulation, in that it is shorter-lived and releases less superoxide [25]. The enhancement of the response of neutrophils to fMLP by H_2O_2 provided further evidence that the superoxide generating capacity and degranulation of the neutrophils were not directly damaged by micromolar H_2O_2. Rather, modulation of the neutrophil response to STZ and fMLP by H_2O_2 occurred at the level of their specific membrane receptors. Our results suggested that the fMLP receptor may be primed by the presence of micromolar amounts of H_2O_2. Such activation was shown for insulin receptor [26]. H_2O_2 may induce release of calcium bound to the inner surface of the plasma membrane and thereby cause intracellular stores of fMLP receptors to be brought to the surface of the neutrophil, ready for an increased response to fMLP.

This work has provided evidence that physiological concentrations of H_2O_2, commonly produced by activated neutrophils, are able to elicit a luminol-enhanced CL response from neutrophils, most likely via the induction of degranulation. Micromolar H_2O_2 also modulates neutrophil responses to two surface-acting stimulants of the respiratory burst, STZ and fMLP. Both exogenously added H_2O_2 and H_2O_2 generated by the activated neutrophil itself were able to produce altered responses to STZ. At sites of infection or inflammatory disease, micromolar concentrations of H_2O_2 produced by activated neutrophils may thus be an important modulator of their own respiratory burst activities.

Inflammatory disease states are characterized by large influxes of neutrophils into the affected area [3]. By comparison with normal neutrophils, neutrophils from patients with inflammatory conditions such as rheumatoid arthritis and osteoarthritis or patients with hypertension, myeloperoxidase deficiency or acatalesemia generate more superoxide [27–30]. Consequently, higher concentrations of H_2O_2 are able to accumulate in the extracellular medium and the modulatory effect of this oxidant may become important to the course of the disease. When neutrophils are recruited to a site of inflammation, early arrivals may have already undergone activation of the respiratory burst, producing micromolar concentrations of H_2O_2. The H_2O_2 may induce degranulation of new neutrophils arriving at the site, releasing MPO into the environment, initiating production of cytotoxic hypochlorous acid and priming further degranulation. fMLP receptors may also be primed by the presence of the H_2O_2, but $C3_b$ or F_c receptors may be damaged. Depending on the balance of stimuli available at the inflammation site, neutrophil responses to surface stimulants may be altered. Micromolar concentrations of H_2O_2 may thus be an important autoregulator of the neutrophil respiratory burst.

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