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Original Article

Luminol-amplified chemiluminescence detects mainly superoxide anion produced by human neutrophils

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Abstract: Reactive oxygen species (ROS) are produced by numerous biological systems and by several phagocytes such as neutrophils and macrophages. ROS include mostly superoxide anion, hydrogen peroxide, singlet oxygen and hydroxyl radical, which are involved in a variety of biological processes such as immunity, inflammation, apoptosis and cell signaling. Thus, there is a need for a sensitive and reliable method to measure ROS. The luminol-amplified chemiluminescence technique is widely used to measure ROS production by neutrophils; however, it is unclear which ROS species are detected by this technique. In this study, we show that Xanthine/Xanthine oxidase (XXO), a known superoxide-producing system, stimulated a luminol-amplified chemiluminescence in the absence of horseradish peroxidase (HRPO), while the presence of HRPO enhanced the response. Both reactions were inhibited by superoxide dismutase (SOD), but not by catalase, confirming that superoxide anion, and not hydrogen peroxide, is the species oxidizing luminol to produce chemiluminescence. Glucose/Glucose oxidase (GGO), a known hydrogen peroxide-producing system, did not induce luminol-amplified chemiluminescence in the absence of HRPO; however, addition of HRPO resulted in a chemiluminescence response, which was inhibited by catalase, but not by SOD. Myeloperoxidase (MPO), isolated from human neutrophils, was also able to enhance the superoxide- and hydrogen peroxide-dependent luminol-amplified chemiluminescence. The production of ROS by stimulated human neutrophils was detected by luminol-amplified chemiluminescence, which was only partially inhibited by SOD and catalase. Interestingly, adding HRPO to stimulated neutrophils increased the luminol-amplified chemiluminescence, which was strongly inhibited by SOD, but not by catalase. These results show that (a) luminol-amplified chemiluminescence is able to detect superoxide anion in the absence of peroxidases, but not hydrogen peroxide; (b) in the presence of peroxidases, luminol-amplified chemiluminescence is able to detect both superoxide anion and hydrogen peroxide; and (c) luminol-amplified chemiluminescence detects mainly superoxide anion produced by neutrophils, especially in the presence of HRPO.

Keywords: ROS, superoxide anion, hydrogen peroxide, luminol-amplified chemiluminescence, neutrophils

Introduction

Production of reactive oxygen species (ROS) by phagocytes such as neutrophils, monocytes and macrophages is essential for host defense against pathogens [1-3]. This is demonstrated by the genetic disease, chronic granulomatous disease (CGD) where patients suffering from this disease have recurrent infections because their phagocytes are unable to produce ROS, and thus cannot kill and eliminate pathogens [4, 5].

The phagocyte NADPH oxidase (NOX2) is responsible for the production of superoxide anion ($O_2^{\bullet -}$) [2, 6], which generates hydrogen peroxide (H_2O_2) in the presence of protons. H_2O_2 reacts with $O_2^{\bullet -}$ to form hydroxyl radical ($OH^{\bullet}$) [2, 6, 7], and is used by myeloperoxidase to produce hypochlorous acid (HOCl) [1, 7], a powerful killing agent. NOX2 is composed of two membrane proteins (gp91phox, p22phox) and four cytosolic proteins (p47phox, p67phox, p40phox and the small GTPase Rac1/2) that assemble with activation [6, 8]. Although the

physiological ROS production is important for innate immunity, excessive phagocyte activation releasing excessive ROS can induce oxidative stress and damage membrane lipids, DNA and proteins, leading to cell death and tissue injury [9-12]. ROS are known to be involved in numerous pathologies, notably inflammatory diseases such as rheumatoid arthritis, inflammatory bowel diseases (IBD), atherosclerosis, diabetes, neurodegenerative diseases, cancer, and during the aging process [13-15].

A large number of methods have been developed for measure ROS production, which are based on detection by colorimetry, fluorescence or chemiluminescence, depending on the probe used [16-20]. The ideal technique should be highly sensitive, specific for one ROS species, and should not interfere with cellular functions. Among these techniques, luminol-amplified chemiluminescence has been largely used to detect ROS production [16-19]. Luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione) is a redox-sensitive compound that emits blue luminescence when oxidized. This technique has been reported to be peroxidase-dependent and to detect all ROS species. It has the advantages to be very sensitive and to detect both intra- and extra-cellular ROS as it can diffuse into cells.

To better understand the ROS species that are detected by luminol-amplified chemiluminescence, we first used *in vitro* known systems that can specifically generate superoxide anion or hydrogen peroxide, and compared the chemiluminescence response in the presence of different enzymes known to interact with ROS. We then used the same approach with neutrophils stimulated to produce ROS. We report here that luminol is mainly oxidized by superoxide anion.

Methods

Reagents

Luminol (5-amine-2,3-dihydro-1,4-phtalazinedione), xanthine (X), xanthine oxidase (XO), SOD, human leukocyte MPO (EC 1.7.1.11), HRPO, diisopropyl fluoro phosphate (DFP), Ficoll, Dextran, cytochrome c, trypan blue, Dulbecco's Phosphate-buffered saline (PBS), Hanks' balanced salt solution (HBSS), 4-phorbol-12-myristate-13-acetate (PMA) were all purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany). Solutions were prepared by dilution in phosphate-buffered saline (PBS) immediately before use.

Isolation of human neutrophils

Neutrophils were isolated from heparinized venous fresh blood from healthy volunteers using Dextran (T500) to remove red blood cells, followed by centrifugation over Ficoll-Paque to remove mononuclear cells, and hypotonic lysis to remove any remaining contaminating red blood cells [21, 22]. The neutrophils were washed in PBS by centrifugation and suspended in the same buffer.

Purification of MPO-rich azurophilic granules

Neutrophils were treated with DFP, a protease inhibitor, and were lysed by nitrogen cavitation. Granules were isolated by Percoll gradient using standard techniques [23]. Azurophilic granules were lysed in and the resulting solubilized content used in the chemiluminescence assay [24].

Cytochrome c reduction assay

Xanthine-xanthine oxidase (XXO)-derived superoxide production was determined in 1 mL PBS pH 7.4 containing xanthine (100 μM) and xanthine oxidase (10 mU) in the presence of cytochrome c (1 mg/mL) and with or without SOD or catalase. Glucose (5 mM) and Glucose oxidase (2.5 mU) (GGO) were used to produce hydrogen peroxide and assayed similarly. Superoxide anion production was determined by measuring ferricytochrome c reduction with a UVIKON 860 spectrophotometer at 550 nm over 10 min. For the neutrophils, cells (1×10^6) were pre-incubated 10 min at 37°C with cytochrome c (1 mg/mL) in PBS, and were then stimulated with PMA (100 ng/ml) at 37°C, with or without SOD or catalase. Superoxide anion production was determined as above [25].

Luminol-amplified chemiluminescence assay

Superoxide anion and hydrogen peroxide were produced by the XXO and GGO systems, respectively, as described above. Chemiluminescence was evaluated with a luminometer (Auto Lumat LB953 model, EG & G Berthold), where light emission was recorded in c.p.m (counted pho-

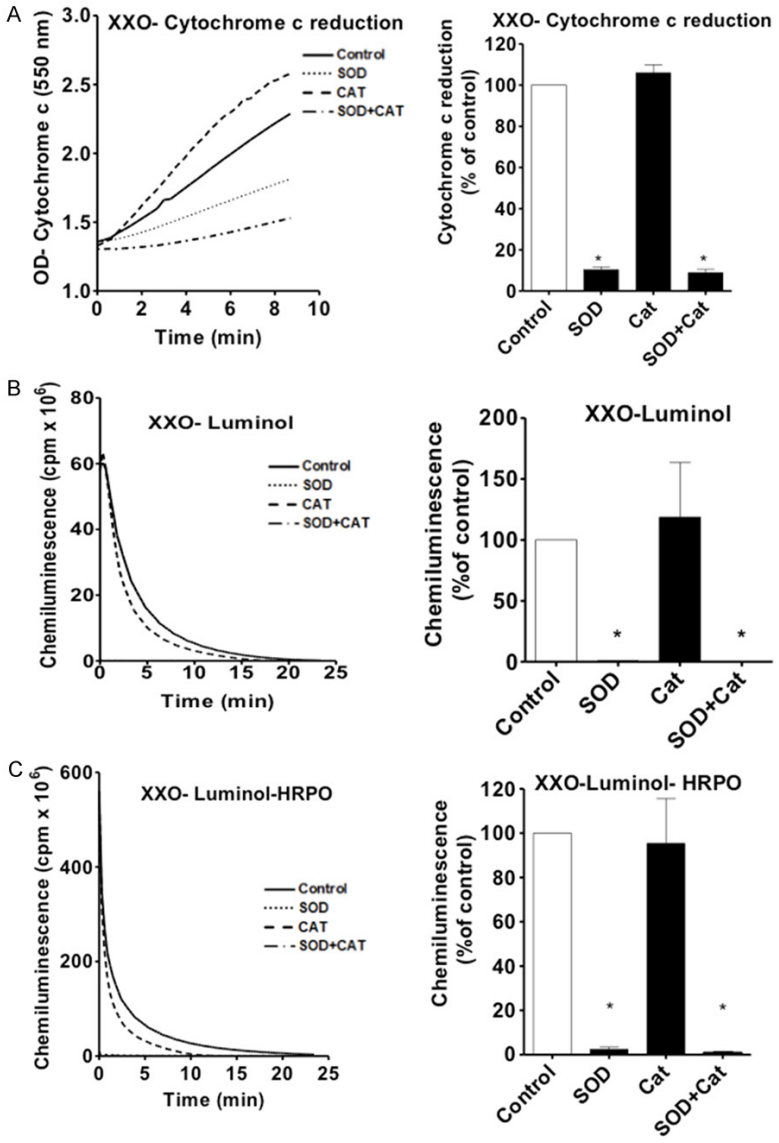

Figure 1. Production of superoxide anion by the xanthine-xanthine oxidase (XXO) system and detection by different methods. XXO-derived superoxide production was determined in 1 mL PBS containing xanthine (100 μM) and xanthine oxidase (10 mU) in the presence of cytochrome c (A), luminol (10 μM) alone (B) or luminol and HRPO (5 mU) (C). Absorbance of reduced cytochrome c and luminol-amplified chemiluminescence were measured over 15 min. When indicated SOD (30 mU) or catalase (186 mU) or both were added before starting the reaction. Left panels show one representative experiment and right panels show a quantification analysis of 5 experiments. Results are expressed as means ± SEM; n=5, *P<0.01.

tons per minute) during 30 min at 37°C [25, 26]. Neutrophils (5 × 10^5/0.5 ml) were suspended in HBSS in the presence of luminol (10 μM) for 10 min at 37°C. Cells were then stimulated with PMA (100 ng/ml), with or without HRPO (5 mU), SOD (30 mU) or catalase (186 mU). Chemiluminescence was measured as above.

Statistical analyses

Statistical analyses between were performed using one-way ANOVA test with Tukey's Multiple Comparison post-test. *P<0.05, **P<0.01, and ***P<0.001 values were considered as significant.

Results

Superoxide anion is detected by luminol-amplified chemiluminescence in the absence or presence of HRPO

We first used Xanthine-xanthine oxidase (XXO) to produce superoxide anion and different assays to detect its production. As expected, XXO was able to induce the reduction of cytochrome c at 550 nm, and SOD inhibited this reaction and not catalase (**Figure 1A**). XXO was also able to induce a luminol-amplified chemiluminescence response in the absence of HRPO (**Figure 1B**). Surprisingly, the presence of HRPO enhanced the chemiluminescence response by approx. 10 fold (Total ROS production: 1.83±0.22 × 10^8 cpm with luminol alone compared to 17.99±0.55 × 10^8 cpm with luminol + HRPO, n=5, P<0.01) (**Figure 1C**). Interestingly, luminol-amplified chemiluminescence in the absence or presence of HRPO was inhibited by SOD, but not by catalase, confirming that superoxide anion, and not hydrogen peroxide, is responsible for luminol oxidation in these reactions. These results show that superoxide anion alone is able to induce a luminol-dependent chemiluminescence response,

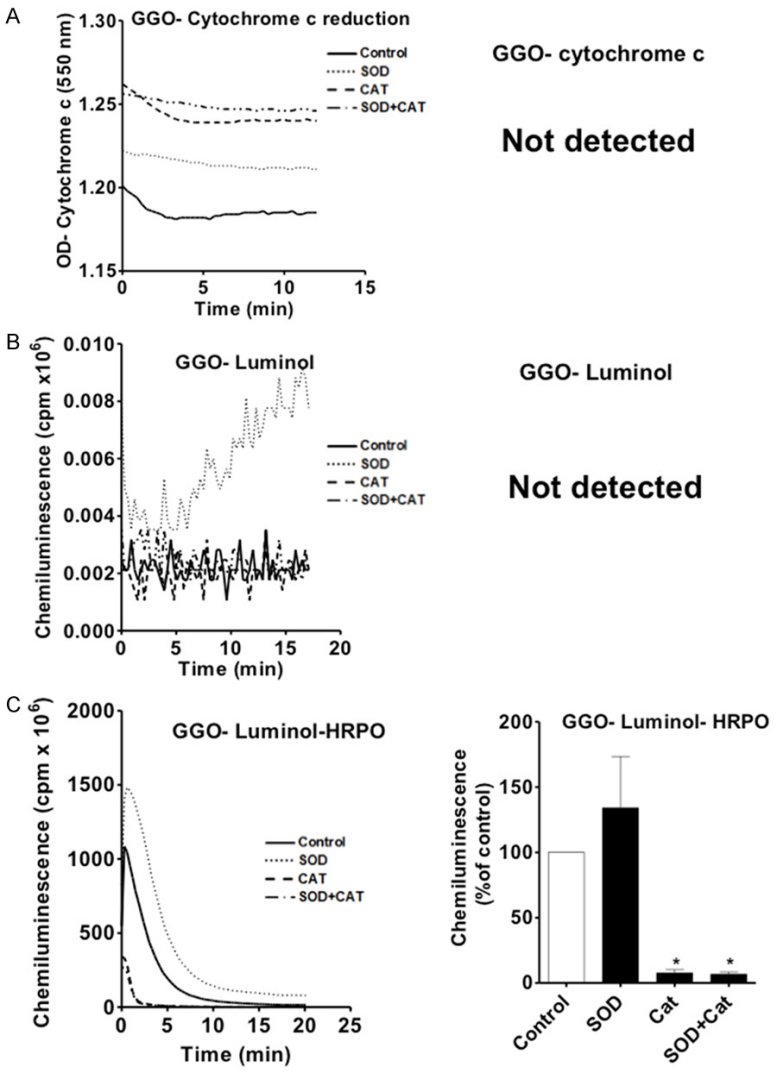

Figure 2. Production of hydrogen peroxide by the glucose-glucose Oxidase (GGO) and detection by different methods. GGO-derived hydrogen peroxide production was determined in 1 mL PBS containing glucose (5 mM) and GGO (2.5 mU) in the presence of cytochrome c (A) luminol (10 μM) alone (B), or luminol and HRPO (5 mU) (C). Absorbance of reduced cytochrome c and luminol-amplified chemiluminescence were measured over 15 min. When indicated SOD (30 U) or catalase (186 U) or both were added before starting the reaction. Left panels show one representative experiment and right panels show a quantification analysis of 5 experiments. Results are expressed as means ± SEM; n=5, *P<0.01.

and that HRPO enhances this reaction independently of H_2O_2.

Hydrogen peroxide is detected by luminol-amplified chemiluminescence only in presence of HRPO

We next used Glucose-Glucose oxidase (GGO) to produce hydrogen peroxide, which did not reduce cytochrome c, as expected (**Figure 2A**). In the absence of HRPO, GGO did not oxidize luminol (**Figure 2B**), but was able to induce a chemiluminescence response when HRPO was added to the reaction (**Figure 2C**). This reaction was inhibited by catalase, but not by SOD, confirming that hydrogen peroxide, and not superoxide anion, reacts with HRPO to oxidize the luminol. The same results were obtained using commercial hydrogen peroxide (data not shown). Thus, hydrogen peroxide alone cannot induce a luminol-amplified chemiluminescence signal, contrary to superoxide anion, and HRPO is required for hydrogen peroxide-induced chemiluminescence.

The neutrophil MPO is able to use superoxide anion as well as hydrogen peroxide to generate luminol-dependent chemiluminescence

As HRPO induced the amplification of superoxide and hydrogen peroxide chemiluminescence signals, we wanted to check if another peroxidase, i.e., the neutrophil MPO, had the same effect. Results show that MPO present in azurophilic granules enhanced the superoxide-dependent chemiluminescence response generated by XXO (Total ROS production: $1.77\pm0.42 \times 10^8$ cpm with luminol alone as compared to $14.59\pm0.32 \times 10^8$ cpm with luminol + MPO, n=5, P<0.01). The luminol and MPO-amplified chemiluminescence was inhibited by SOD, but not catalase, confirming the role of superoxide anion (**Figures 1B** and **3A**). The GGO system generated a chemiluminescence response only in the presence of MPO, and this reaction was inhibited by catalase, but not by SOD, confirming the role of hydrogen peroxide (**Figures 2B** and **3B**). These results show that MPO is able

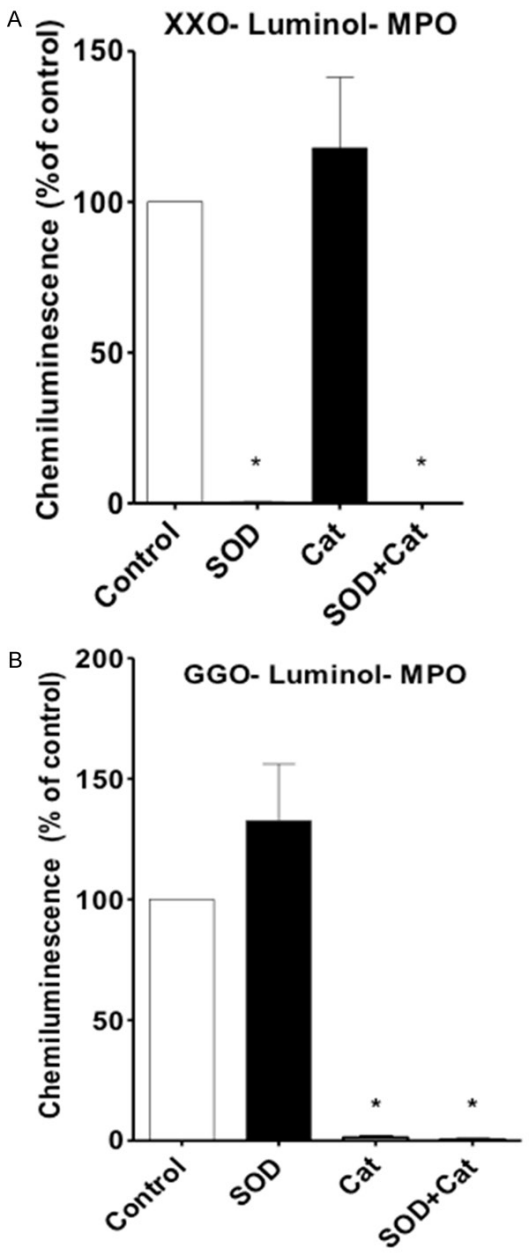

Figure 3. Human MPO is able to enhance superoxide anion-dependent chemiluminescence. XXO-derived superoxide production was determined in 1 mL PBS containing xanthine (100 μM) and xanthine oxidase (10 mU) in the presence of luminol (10 μM) alone or luminol and MPO-containing granules, and chemiluminescence was measured during 15 min, total chemiluminescence of each condition was quantified and compared to control (A). GGO-derived hydrogen peroxide production was determined in 1 mL PBS containing glucose (5 mM) and GGO (2.5 mU) in the presence of luminol (10 μM) alone or luminol and MPO-containing granules, and chemiluminescence was measured during 15 min, total chemiluminescence of each condition was quantified and compared to control (B). When indicated SOD (30 mU) or catalase (186 mU) or both were added before starting the reaction. Results are expressed as means ± SEM; n=5, *P<0.01.

to use superoxide anion or hydrogen peroxide to generate a luminol-amplified chemiluminescence response.

Detection of superoxide anion and hydrogen peroxide produced by neutrophils

We then used the same techniques to assess neutrophil ROS production. As expected, PMA-activated neutrophils induced the reduction of cytochrome c, which was inhibited by SOD, but not by catalase (**Figure 4A**). On the other hand, both SOD and catalase inhibited the chemiluminescence generated by PMA-activated neutrophils (**Figure 4B**). The SOD inhibition was greater than the catalase inhibition, suggesting that mainly superoxide anion produced by neutrophils oxidized the luminol to generate a chemiluminescence response. Interestingly, HRPO enhanced the chemiluminescence response, which was only inhibited by SOD, but not by catalase (**Figure 4C**). Thus, in the presence of HRPO, the luminol-amplified chemiluminescence assay mainly detects superoxide anion when produced by neutrophils.

Discussion

Luminol-amplified chemiluminescence has been widely used for the detection of ROS produced by a variety of cells and enzyme systems. However, the exact nature of the ROS species detected by this technique is not entirely clear. In this study, we used different approaches to answer this question, such as enzymes known to produce a given type of ROS species (XXO for superoxide anion and GGO for hydrogen peroxide), and enzymes known to specifically catabolize them (SOD for superoxide anion and catalase for hydrogen peroxide) in order to compare the signals generated with the luminol-amplified chemiluminescence. The results presented here revealed that luminol-amplified chemiluminescence is able to detect superoxide anion in the absence of peroxidases, while in the presence of peroxidases, both superoxide anion and hydrogen peroxide were detected by the assay. Notably, both HRPO and MPO peroxidases enhanced the superoxide-dependent luminol-amplified chemiluminescence.

It was believed for a long time that chemiluminescence with luminol is mainly due to the reaction of hydrogen peroxide with the MPO present in phagocytes [27, 28]. Because of the complexity of the ROS reactions, we first used cell-free enzyme systems to produce a given

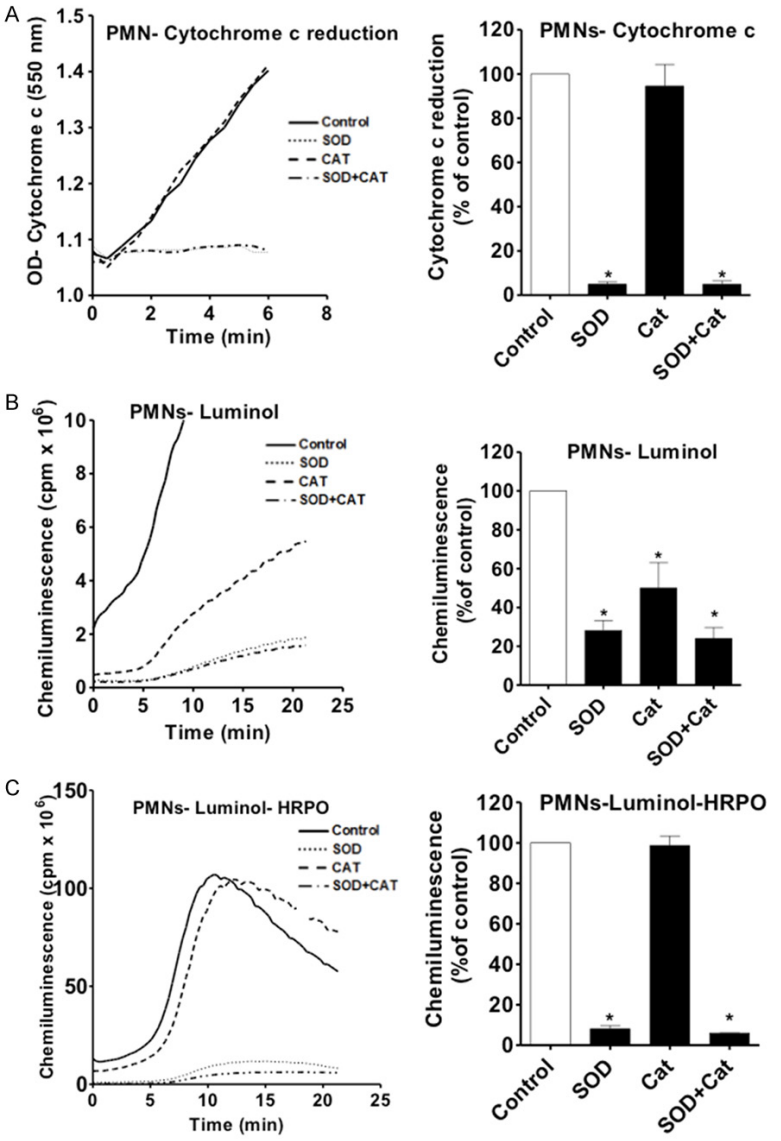

Figure 4. Superoxide and hydrogen peroxide production by neutrophils measured by different approaches. Neutrophils (10^6 cells in 1 ml) were pre-incubated for 10 min at 37°C with cytochrome c (1 mg/ml) in HBSS, and stimulated with PMA (100 ng/ml) with or without SOD (30 U) or/and catalase (186 U). Reduction of cytochrome c was determined by measuring the OD at 550 nm using a UVIKON 860 spectrophotometer at 550 nm (A). Neutrophils (10^6 cells) were re-suspended in 0.5 ml HBSS in the presence of luminol (10 μM) for 10 min at 37°C. Cells were stimulated with PMA (100 ng/ml), without (B) or with HRPO (5 mU) (C), and chemiluminescence was measured. Where indicated SOD (30 mU) or/and catalase (186 mU) were added before starting the reaction. Left panels show one representative experiment and right panels show a quantification analysis of 5 experiments. Results are expressed as means ± SEM; n=5, *P<0.01.

type of ROS species. The XXO system, known to produce superoxide anion, was able to generate a chemiluminescence signal in the absence of peroxidases; however, HRPO and MPO did enhance this signal. This chemiluminescence was not due to hydrogen peroxide, which is generated via superoxide anion dismutation, as SOD, but not catalase, inhibited this signal. In addition, hydrogen peroxide alone, produced by GGO, could not generate a chemiluminescence signal, indicating that the presence of peroxidases is absolutely required for hydrogen peroxide to oxidize luminol and generate chemiluminescence. These results also suggest that HRPO and MPO can use both superoxide anion or hydrogen peroxide as substrate.

Interestingly, when using PMA-stimulated human neutrophils, luminol-amplified chemiluminescence was inhibited by SOD and to a lesser extent by catalase. As superoxide anion does not diffuse from the site of production, and added SOD is extracellular, the results obtained in the presence of SOD suggest that the superoxide anion produced by stimulated neutrophils was mainly released extracellularly. The ROS portion that was SOD-insensitive may be due to the superoxide anion produced intracellularly as luminol can cross the plasma and granules membranes and detect intracellular ROS production, or to the extracellular MPO/H_2O_2 reaction as intracellular hydrogen peroxide can diffuse extracellularly. Interestingly, the addition of extracellular HRPO enhanced the luminol-amplified chemiluminescence response and SOD almost completely inhibited this reaction without any effect of catalase. This observation was also reported by Lundqvist and Dahlgren using isoluminol, which only detects extracellular ROS [29].

The SOD-inhibitable cytochrome c reduction technique has been the standard assay to measure superoxide production, especially by stimulated neutrophils, and it has the advantages to be specific for extracellular superoxide anion and quantitative [20]. However, this assay is not very sensitive, and thus cannot be used to measure superoxide anion in systems producing low levels of ROS, such as with the other members of the NOX family, unless using cumulative end point measurements. In contrast, the SOD-inhibitable luminol-amplified chemiluminescence (with or without HRPO) is very sensitive and can be used as a NOX assay. Another advantage of the luminol is that it is not toxic and can be used *in vivo* to measure superoxide anion and hydrogen peroxide in different pathological models [30-32]. Contrary to the cytochrome c-reduction assay, luminol-amplified chemiluminescence does not reflect the exact amount of superoxide anion produced, as luminol by itself can generate this molecule [33].

In summary, the luminol-amplified chemiluminescence assay is very easy to use and is a high sensitivity method. Luminol is also cost effective compared to cytochrome c. Thus, luminol-amplified chemiluminescence can be used to specifically detect superoxide anion in the absence of peroxidases such as HRPO and MPO. However, in the presence of peroxidases, luminol-amplified chemiluminescence is even more sensitive, but SOD and catalase must be used as controls to discriminate between superoxide anion and hydrogen peroxide as the luminol oxidizing agent.

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Disclosure of conflict of interest

None.

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