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Effects of non-steroidal anti-inflammatory drugs on the luminol and lucigenin amplified chemiluminescence of human neutrophils

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Abstract

A panel of non-steroidal anti-inflammatory drugs commonly used for therapeutic purposes was assessed for their effects on the respiratory burst of isolated human polymorphonuclear neutrophils. Cells were stimulated with opsonised yeast and the production of reactive oxygen species was measured by amplified chemiluminescence with luminol and lucigenin which are two luminogenic agents measuring different cellular events. A special attention was devoted to the establishment of dose–effect curves and calculation of ED_{50}. Some of the drugs tested (acemetacine, diclofenac, flufenamic acid and niflumic acid) were able to decrease both luminol and lucigenin chemiluminescence in a dose-dependent manner reflecting an inhibitory effect on the respiratory burst. The most potent derivative was flufenamic acid (ED_{50} 8 and 78 μM, respectively, with luminol and lucigenin), followed by diclofenac (21 and 98 μM), niflumic acid (97 and 227 μM) and acemetacine (585 and 427 μM). In contrast, several other drugs (flurbiprofen, ibuprofen, ketoprofen, piroxicam) stimulated both luminol and lucigenin chemiluminescence, suggesting a pro-oxidant activity. Acetylsalicylic acid (up to 1250 μM) was a modest inhibitor (maximum 25% inhibition) showing no dose-dependant effect and tolmetin (up to 125 μM) had no significant effect in both systems. The results were in agreement using both luminogenic agents, except for indomethacin, naproxen and tenoxicam which showed different kinds of effects. The unspecific and complex nature of the measurement systems used did not allow to give a complete mechanistic interpretation of the results, but the comparison with literature data gave some pertinent explanations for both anti- and pro-oxidant effects.

Keywords: Free radical; NSAID (non-steroidal anti-inflammatory drug; Chemiluminescence; Luminol; Lucigenin; Opsonised yeast; Polymorphonuclear neutrophil

1. Introduction

Reactive oxygen species are generated by polymorphonuclear neutrophils in physiological conditions during the respiratory burst of phagocytosis. The cells can be activated by a variety of biological or synthetic molecules such as fMLP (formylmethionyl-leucyl-phenylalanine), the complement fragment C3a and PMA (phorbol- myristate-acetate) or by opsonised particles. Independent of their nature, the stimuli activate protein kinase C which phosphorylates NADPH oxidase leading to the production of the superoxide anion radical ($O_2^{-\cdot}$). This species further dismutates to hydrogen peroxide (H_2O_2), either spontaneously or enzymatically by the superoxide dismutase, and both $O_2^{-\cdot}$ and H_2O_2 then leads to the formation of the highly damaging hydroxyl radicals ($OH^{\cdot}$) via a Fenton reaction (Haliwell et al., 1985). In addition, the myeloperoxidase enzyme contained in some phagocytic cells catalyses the formation of hypochlorous acid (HClO) which is a potent bactericidal agent. Besides this O_2-dependent system, phagocytic cells also use an O_2-independent pathway constituted by proteolytic enzymes contained in secondary granules, such as elastase or collagenase. Both systems actually are indispensable to the bactericidal activity and act synergistically in the destruction of phagocytosed particles (Weiss, 1989). If reactive oxygen-derived species may be considered as beneficial intermediates in this respect, it is also, nowadays, largely admitted that they can become destructive for the host tissues in certain conditions. These species together with granule enzymes indeed contribute to the pathogeny of various immune and non-immune chronic inflammatory diseases, including some rheumatic disorders. They cause damages to physiologically important

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molecules, cells and tissues, therefore, aggravating the inflammatory response (Weiss and Lobuglio, 1984; Black, 1989).

In these conditions, it is of interest in the pharmacologi-cal treatment of chronic inflammatory disorders to use non-steroidal anti-inflammatory drugs (NSAIDs) that are able to react directly with these species (scavenging effects) or to inhibit their generation in the respiratory burst by specific mechanisms, in addition to their classical inhibition of cyclooxygenase (Black, 1989). As polymor-

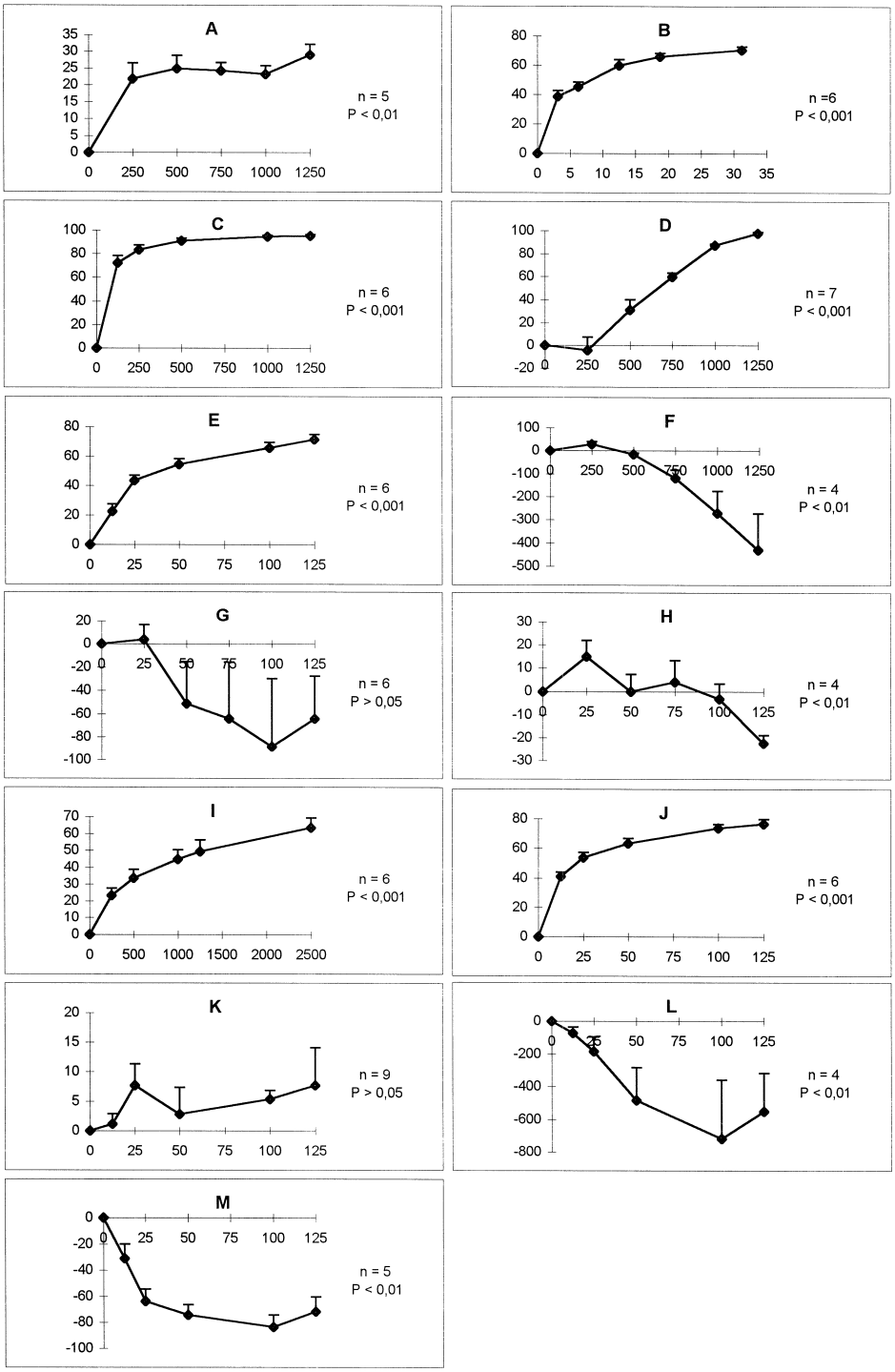

Fig. 1. Effect of NSAIDs on luminol chemiluminescence. *X*-axis represents the drug concentration (μM) in the reaction mixture and *Y*-axis represents the percentage of inhibition calculated as described in Section 2. The drugs corresponding to the graphs are: (A) acetylsalicylic acid; (B) flufenamic acid; (C) niflumic acid; (D) acemetacin; (E) indomethacin; (F) ibuprofen; (G) flurbiprofen; (H) ketoprofen; (I) naproxen; (J) diclofenac; (K) tolmetin; (L) piroxicam; (M) tenoxicam. Experimental points are mean + S.E.M.; *n* is the number of experiments and *P* is the significance of the global effect calculated by the Friedman's test.

phonuclear neutrophils are known to be the major infiltrating phagocytes at the inflammatory site, e.g., in the synovia of inflamed joints in rheumatic disorders (Brown et al., 1988), it seems important to study the ability of NSAIDs to inhibit the oxidative metabolism of these cells. In continuation to our previous investigations on the antioxidant effects of NSAIDs (Parij et al., 1995; Parij and Nève, 1996), we describe in the present paper the use of suspensions of purified human polymorphonuclear neutrophils and an amplified chemiluminescence test to compare the effects of NSAIDs on the respiratory burst activated by opsonised yeast. This event is indeed accompanied by the emission of a natural luminescence. The latter could directly be measured but we preferred to use chemical light amplificators such as luminol and lucigenin that offer a better sensitivity which allows the use of a simpler equipment and a reduced number of cells to be examined. Even if inhibitory effects have already been reported for some NSAIDs using different activators, the originality of our study relies on (i) the comparison of a large panel of drugs in an optimised system using two different light amplificators and (ii) the establishment of complete dose–effect curves, with determination of inhibitory median doses (ED_{50}).

2. Materials and methods

2.1. Chemicals and apparatus

The following reagents were used: calparine (Ca heparinate 5000 IU/ml) from Sanofi-Labaz (Brussels, Belgium), Ficoll–Hypaque gradient of density 1.07 (Pharmacia, Uppsala, Sweden), Hank's balanced salt solution (HBSS) without phenol red with or without Ca^{2+} and Mg^{2+} from Life Technologies (Merelbeke, Belgium), phosphate buffer solution (PBS) 150 mM pH 7.2 from Bio Mérieux (Marcy-l'Etoile, France). The lysing solution at pH 7.4 was composed of a mixture of NH_4Cl 150 mM, $KHCO_3$ 33 mM and EDTA 2.4 mM (all from Merck, Overijse, Belgium). Stock solutions of luminol 10^{-2} M and lucigenin $5 \cdot 10^{-3}$ M (Sigma, Bornem, Belgium) were aliquoted, frozen and freshly diluted in HBSS with Ca^{2+} and Mg^{2+}. Opsonised yeast was prepared with *Saccharomyces cerevisae* (baker's yeast) suspensions and serum from healthy volunteers (see Section 2.3). The drugs were kindly provided by their respective manufacturers: flurbiprofen and ibuprofen (Boots Pharmaceuticals, Nottingham, UK), ketoprofen and acemetacin (Rhône-Poulenc Rorer, Paris, France), naproxen (Sarva Syntex, Brussels, Belgium), diclofenac Na (Ciba Geigy, Groot-Bijgaarden, Belgium), flufenamic acid (Trenker, Brussels, Belgium), indomethacin (Merck, Sharp & Dohme, Rahway, NJ, USA), niflumic acid (Upsa Medica, Brussels, Belgium), tolmetin Na (Cilag, Schaffhausen, Switzerland), piroxicam (Pfizer, Brussels, Belgium), tenoxicam (Roche, Basle, Switzerland), and acetylsalicylic acid (Bayer, Leverkussen, FRG). The poorly water soluble drugs were dissolved by addition to the aqueous suspension of the minimum volume of a sodium carbonate solution (0.4 M) and the pH was, thereafter, rapidly adjusted to 7.4. The solutions were made daily in deoxygenated water. Chemiluminescence measurements were performed on a LKB 1251 BioOrbit luminometer (Turku, Finland).

2.2. Preparation of cell suspensions

Venous heparinised blood was collected daily from healthy human volunteers. Purified preparations of polymorphonuclear neutrophils were obtained by the following procedure. Blood was centrifuged once at $170 \times g$ to

Table 1
Effects of NSAIDs on the amplified chemiluminescence test

Drug	Luminol-CL		Lucigenin-CL	
	Effect	$ED_{50} \pm$ S.E.M. (μM)	Effect	$ED_{50} \pm$ S.E.M. (μM)
Acetylsalicylic acid	↓	25% of inhibition at 1250 μM without dose effect	↓	22% of inhibition at 1250 μM with dose effect
Acemetacine	↓	585 ± 38	↓	427 ± 54
Diclofenac	↓	21 ± 4	↓	98 ± 21
Flufenamic acid	↓	8 ± 2	↓	78 ± 11
Flurbiprofen	↑	(−)	↑	(−)
Ibuprofen	↑	(−)	↑	(−)
Indomethacin	↓	45 ± 9	↑	(−)
Ketoprofen	↑	(−)	↑	(−)
Naproxen	↓	1490 ± 352	NS	(−)
Niflumic acid	↓	93 ± 13	↓	227 ± 35
Piroxicam	↑	(−)	↑	(−)
Tenoxicam	↑	(−)	NS	(−)
Tolmetin	NS	(−)	NS	(−)

Effect: (↑) significant increase and (↓) significant decrease of chemiluminescence as compared to control. NS—no significant effect observed.
ED_{50}: (−) means that ED_{50} could not be calculated.

eliminate the platelet rich plasma and then at $1000 \times g$ to eliminate platelet poor plasma. The buffy coat containing white cells was pipetted off and diluted in PBS pH 7.2. The polymorphonuclear neutrophils and the remaining red cells were isolated from monocytes and lymphocytes using a Ficoll–Hypaque gradient (Boyum, 1968), followed by dextran sedimentation. The hypotonic lysis of the remaining erythrocytes was subsequently performed and a pellet of neutrophils was obtained after centrifugation. Polymorphonuclear neutrophils were washed twice with HBSS without Ca^{2+} and Mg^{2+} and resuspended in HBSS with Ca^{2+} and Mg^{2+}. Cell suspensions were counted with a Technicon H2 counter and diluted with HBSS with Ca^{2+} and Mg^{2+} to obtain a final cell suspension of about 5000 cells/μl. The cell suspensions obtained by this way contained 97.9% of polymorphonuclear neutrophils ($n = 30$)

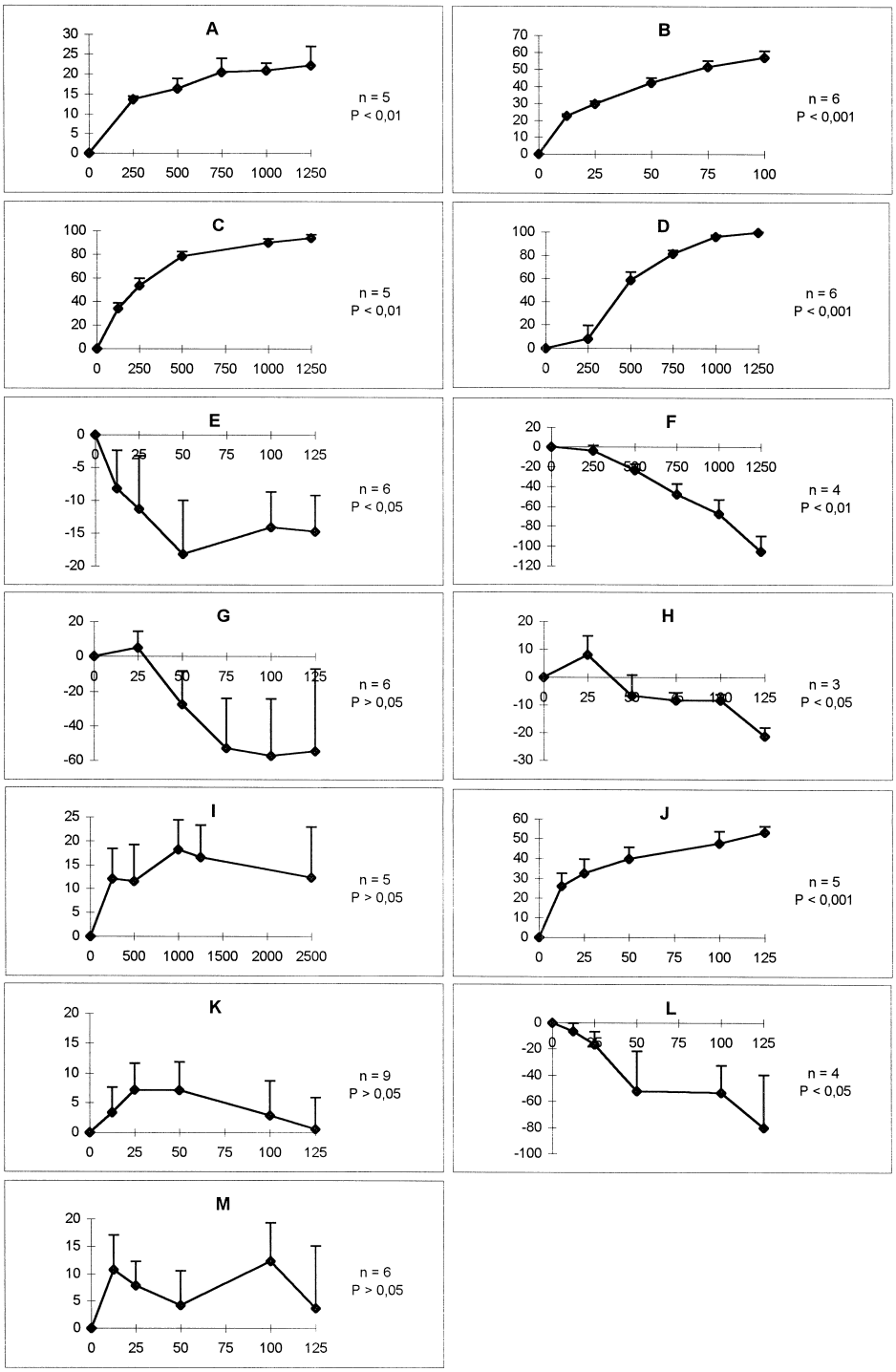

Fig. 2. Effect of NSAIDs on lucigenin chemiluminescence. For explanations, see the legend to Fig. 1.

and the control of their viability showed that 98.9% of the cells excluded Trypan blue.

2.3. Preparation of opsonised yeast

A total of 2 g of yeast were incubated with 10% of fresh human serum at 37°C for 30 min with stirring in order to cover particles with antibodies and complement. After opsonisation, the suspension was heated at 100°C for 30 min to kill possible bacteria. The suspension was then centrifuged and the supernatant discarded. The pellet was washed twice with HBSS, resuspended in HBSS, counted with a Technicon H2 counter, diluted in HBSS to obtain a final concentration of 25 000 cells/μl and finally stored frozen in aliquots.

2.4. Amplification and measurement of chemiluminescence

An incubation of the cells with the amplifying agent was found necessary as both light amplificators caused a transitory excitation of the cells (increase in chemiluminescence) that returned to their basal state in less than 1 h. Therefore, 250 μl of the polymorphonuclear neutrophils suspension (5000 cells/μl) were incubated at 37°C with 250 μl of a luminol 10^{-6} M or a lucigenin 10^{-5} M solution for a period of 1 h during which light emission was continuously measured. A total of 500 μl of a solution containing the drug at a fixed concentration or 500 μl of HBSS (for the blank, see later section) were added in the reaction mixture. The cells were then immediately stimulated with 500 μl of opsonised yeast (25 000 cells/μl) and chemiluminescence further recorded for 30 min. The intensity of chemiluminescence was determined by integrating the area under the chemiluminescence curve after 20 min. These values were compared to those of the control curve (containing no drug, i.e., cells/luminol or lucigenin/no drug/opsonised yeast) which was taken as 100% chemiluminescence. Percentage of chemiluminescence response at each drug concentration was expressed as the percent of the control value and the percent inhibition was then calculated by subtracting this value from 100. A value was also determined for the blank and subtracted from all chemiluminescence values before any calculation. The blank (i.e., cells/luminol or lucigenin/no drug/no opsonised yeast) accounts for the basal chemiluminescence obtained with the reaction mixture without any stimulation.

2.5. Calculation of ED_{50} and statistical analysis

The significance of the mean dose–effect curve was established by a Friedman non-parametric two-way analysis. Results were considered statistically significant at $P < 0.05$. Median inhibitory doses (ED_{50}), i.e., dose of NSAID producing 50% inhibition of control chemiluminescence, were calculated using a log–plot transformation of the data.

3. Results

3.1. Luminol-amplified chemiluminescence

A significant dose-dependent inhibition of the intensity of luminol-enhanced chemiluminescence was observed with half of investigated drugs: acemetacin, diclofenac, flufenamic acid, indomethacin, naproxen and niflumic acid. Fig. 1 illustrates the curves obtained for each derivative while Table 1 reports ED_{50} values. Acetylsalicylic acid (250 to 1250 μM) produced about 25% of inhibition without a dose–response effect and tolmetin (12.5 to 125 μM) had no significant effect. In contrast, flurbiprofen, ibuprofen, ketoprofen, piroxicam and tenoxicam increased the intensity of emitted chemiluminescence measured in comparison to a control without drug.

3.2. Lucigenin-amplified chemiluminescence

Only four of investigated drugs (acemetacine, diclofenac, flufenamic acid and niflumic acid) were able to inhibit in a significant and dose-dependent manner the intensity of lucigenin-enhanced chemiluminescence in this system. Fig. 2 illustrates the curves obtained for each derivative while Table 1 reports ED_{50} values. Acetylsalicylic acid (250 to 1250 μM) produced only 22% inhibition at 1250 μM, but with a moderate dose–response effect. Naproxen (250 to 2500 μM), tenoxicam (12.5 to 125 μM) and tolmetin (12.5 to 125 μM) had no significant effect. Some drugs like flurbiprofen, ibuprofen, indomethacin, ketoprofen and piroxicam increased the intensity of emitted chemiluminescence.

4. Discussion

It is generally admitted that luminol chemiluminescence largely depends on the reactions of the myeloperoxidase–H_2O_2–Cl^- system (Dahlgren and Stendhal, 1983; Edwards, 1987). However, the role of $O_2^{-\cdot}$ seems also essential (Hodgson and Fridovich, 1973; Misra and Squatrito, 1982; Muller-Peddinghaus, 1984; Falck, 1986). Luminol is a relatively small molecule which is able to enter the cell (Bender and Van Epps, 1983; Dahlgren et al., 1985) and, therefore, reflects both extra- and intracellular events. Apart from measuring intra and extracellular production of $O_2^{-\cdot}$, H_2O_2 and HClO produced by the myeloperoxidase–H_2O_2 system, the role of luminol in relation to other reactive oxygen species such as hydroxyl radical and singlet oxygen remains unclear (Muller-Peddinghaus, 1984). On the other hand, the lucigenin chemiluminescence is generally

attributed to extracellular $O_2^{-\cdot}$ (Gyllenhammar, 1987). Being a larger molecule unable to enter the cell, lucigenin only reflects extracellular events (Dahlgren et al., 1985).

For the present study, only opsonised yeast was selected among the panel of the existing activators because our priority was to determine the active doses of NSAIDs with good precision in one-well standardised system instead of investigating their precise mode of action. Indeed, the majority of previous studies only reported partial results (e.g., the inhibition at one concentration) that could not be used for the determination of important pharmacological parameters such as ED_{50}. Our study was successful to this purpose as several NSAIDs were able to modulate the respiratory burst of phagocytes in a dose- dependent manner allowing the calculation of quantitative data. However, some of them either disclosed no significant effect in the concentration range examined or even increased the intensity of chemiluminescence, suggesting a pro-oxidant effect rather than an anti-oxidant one. Concerning the magnitude and the type of effect (anti- or pro-oxidant), luminol and lucigenin chemiluminescence delivered quite comparable results for the majority of examined drugs. There were, however, some discrepancies between the results of both systems concerning indomethacin, naproxen and tenoxicam.

Concerning the drugs that decrease chemiluminescence in both systems, comparison of the present data with those of literature is not easy, mainly because of the small number of publications dealing with similar conditions. Our results for acetylsalicylic acid are in good agreement with those of Mazzulo et al. (1985) who used both luminogenic agents. However, the same authors reported an ED_{50} of 0.5 μM for diclofenac in a luminol chemiluminescence system, which is much less than the 21-μM we obtained.

The observation that indomethacin inhibits the luminol chemiluminescence while increasing the lucigenin one confirms previous results (Horan et al., 1983; Mazzulo et al., 1985). It was suggested that indo-methacin exerts a part of its effect by inhibiting the reactions of the myeloperoxidase–H_2O_2–Cl^- system (Pekoe et al., 1981). The increase in the lucigenin chemiluminescence should, therefore, be an indirect effect of the locking of the myeloperoxidase–H_2O_2–Cl^- system and is thought to reflect an increase in the superoxide anion concentration (Gay et al., 1984, 1985). Naproxen similarly inhibits the luminol chemiluminescence but to a lesser extent than indomethacin, and it has no effect on the lucigenin chemiluminescence. This is consistent with the demonstration that naproxen is able to inhibit the reactions of the myeloperoxidase–H_2O_2–Cl^- system but with a potency 100 times lower than that of indomethacin (Pekoe et al., 1982).

Several NSAIDs quite systematically show pro-oxidant effects in both systems. This increase in the luminol and lucigenin chemiluminescence is probably due to a stimulating effect on some reactive oxygen generating systems like the NADPH oxidase complex and/or the myeloperoxidase–H_2O_2–Cl^- system. The scavenging effects documented in some studies (Vapaatalo, 1986; Wasil et al., 1987; Aruoma and Haliwell, 1988; Parij et al., 1995) should, therefore, have a minimal impact on the global effect such as attested by the huge increase in chemiluminescence, sometimes as much as 6 times more elevated than the control. The pro-oxidant effects of piroxicam and tenoxicam in our luminol system is in total contradiction with the results reported by Mazzulo et al. (1985). Interestingly, we recently documented similar pro-oxidant effects for the two oxicams in the deoxyribose assay for hydroxyl radical scavenging (Parij et al., 1995) and in a horseradish peroxidase assay for reaction with H_2O_2 (Parij and Nève, 1996). The fact that chemiluminescence curves show a significant rise at 125 μM could indicate an opposite effect on reactive oxygen species production at higher concentrations. Concerning lucigenin chemiluminescence, our results are in some way in contradiction with those of Colli et al. (1991) who used another system to measure O_2^- production, but the discrepancy between the two studies could probably be related to the difference in concentrations tested.

The present study supports the idea that the relative ability of the drugs to inhibit the luminol chemiluminescence of polymorphonuclear neutrophils can be put in parallel with their ability to inhibit the luminol chemiluminescence produced by a myeloperoxidase–H_2O_2–Cl^- system (Pekoe et al., 1982). Concerning the lucigenin chemiluminescence on the other hand, we do not have arguments that it reflects the extracellular production of O_2^- due to the lack of results from literature showing an inhibition by NSAIDs on $O_2^{-\cdot}$ production of polymorphonuclear neutrophils stimulated by opsonised yeast.

In conclusion, some of the drugs examined are good inhibitors of the respiratory burst of polymorphonuclear neutrophils induced in vitro by opsonised yeast. However, it is clear that NSAIDs present a large diversity of effects that are not easy to explain. A comprehensive mechanistic interpretation of the results is not possible because of the unspecific and complex nature of the measurement systems used. Only global effects of drugs are indeed measured in such systems: they represent the result of complex interactions such as the inhibition of cyclooxygenase and prostaglandin synthesis, the scavenging of reactive oxygen species produced during the respiratory burst of cells, the inhibition of mechanisms leading to the activation of the respiratory burst (depending themselves on the nature and the concentration of the stimulus used) and probably the ability of the drug to enter the cell and act on intracellular events in addition to the activity on surface receptors. We can meanwhile suggest that NSAIDs act rather by inhibiting at different levels the mechanisms of reactive oxygen species production than by scavenging free radical species. Although the effects here described occur at relatively high concentrations as compared to those reported in plasma

and synovial fluid of healthy humans receiving the drugs in pre-clinical tests (Netter et al., 1989; Mustofa et al., 1991; Urquhart, 1991), it is difficult to extrapolate the present data to the situation in inflamed tissues where some NSAIDs were reported to accumulate, leading for example to concentrations in the synovial fluid about 3 to 8 times higher than in control joints (Graf et al., 1975).

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