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Inhibition of Neutrophil Respiratory Burst by Nimesulide Independence from Cytoplasmic pH-Regulating Mechanisms

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Summary

Activation of NADPH oxidase and superoxide anion release in human neutrophils has been shown to be dependent on Na^+/H^+ exchange, and inhibition of intracellular alkalinisation has been proposed as a mechanism of action for some anti-inflammatory drugs. The effect of nimesulide, a novel nonsteroidal anti-inflammatory drug, on intracellular pH (pH_i) regulation by the Na^+/H^+ antiport in human neutrophils was examined using the pH-sensitive fluorescent probe 2,7-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF). When stimulated by formylmethionyl-leucyl-phenylalanine (fMLP) or phorbol-12-myristate-13-acetate (PMA), neutrophils displayed changes in pH_i consisting of an initial acidification followed by a sustained alkalinising phase indicative of antiport activation. Nimesulide did not change the pH_i of resting neutrophils and did not affect the pH_i response to stimulation. It is concluded that the anti-inflammatory effect of nimesulide is not directly linked to interactions with the Na^+/H^+ antiport or other pathways affecting the regulation of pH_i .

The stimulation of human neutrophils by various substances induces the so-called respiratory burst, a series of metabolic events leading to the release of highly reactive oxygen derivatives (i.e. the oxygen singlet, the superoxide anion and hydrogen peroxide) via a membrane-bound NADPH oxidase (Babior 1984). This process, the energy for which is derived mainly from the hexose-monophosphate shunt (Borreagaard et al. 1984), produces hydrogen ions (protons) that accumulate within the cytoplasm, thereby lowering the intracellular pH (pH_i). The principal mechanism for the removal of excess protons from the neutrophil is the amiloride-sensitive Na^+/H^+ exchange effected by a transmembrane glycoprotein (Seifter & Aronson 1986). The inability of the neutrophil to prevent its pH_i from shifting towards acidic values has several det-

rimental effects on cell metabolism. Respiratory burst activity seems to be particularly sensitive to cytoplasmic acidification: inactivation of the Na^+/H^+ antiport by amiloride or its derivatives strongly inhibits superoxide anion generation (Simchowitz 1985; Wright et al. 1986).

Nimesulide is a novel anti-inflammatory drug, the mechanisms of action of which are not completely understood. We have previously reported that nimesulide has a strong inhibitory effect on the superoxide anion release induced by phorbol 12-myristate-13-acetate (PMA) and formylmethionyl-leucyl-phenylalanine (fMLP) *in vitro* in human neutrophils (Vago et al. 1990). The aim of the present study was to investigate the possible effect of nimesulide on the Na^+/H^+ antiport, the predominant system of pH_i regulation in neutrophils.

1. Methods

Neutrophils were separated from freshly drawn human blood using a modification of established methods (Boyum 1968) involving dextran sedimentation, density gradient centrifugation, and hypotonic lysis of contaminating red cells. Once isolated, neutrophils were resuspended in Hank's balanced salt solution (HBSS) at a concentration of 1 to 5×10^7 cells/ml.

Measurement of pH_i was achieved by loading the cells with fluorescent pH-sensitive dye 2,7-bis-carboxyethyl-5(6)-carboxyfluorescein (BCECF), according to established methods (Grinstein & Furuya 1986). In brief, cells in HBSS were incubated in the dark for 30 minutes at 37°C with the membrane-permeant acetoxymethyl ester of BCECF ($5 \mu\text{mol/L}$ in 0.1% dimethyl sulfoxide), and then washed to remove the unincorporated dye. In order to minimise leakage of the dye, the BCECF-loaded cells were maintained at 4°C until the subsequent procedures, which occurred within 4 hours of loading. Cells were next transferred in disposable fluorimeter cuvettes and diluted to yield a final concentration of 2.5×10^6 cells/ml.

The neutrophil respiratory burst was stimulated by adding fMLP 100 nmol/L or PMA 10 nmol/L to cells pre-warmed at 37°C . The following drugs were added 5 minutes before respiratory burst stimulation: nimesulide 0.1 mmol/L , 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid (DIDS) 0.1 mmol/L or dimethylamiloride (DMA) 0.1 mmol/L . Dimethyl sulfoxide, the solvent for each drug used, was present at a maximum concentration of 0.2% and did not interfere with the detection system.

Fluorescence changes were recorded using a spectrofluorometer equipped with a stirred and water bath-thermostated cuvette holder. The method of fluorescence ratio measurement was used (Wright et al. 1988), with dual excitation wavelengths of 506 and 455 nm and an emission wavelength of 530 nm ; the ratio was automatically calculated by the instrument. The pH_i measurement system was calibrated for each cell batch by the addition of nigericin $1 \mu\text{mol/L}$ to BCECF-loaded neutrophils resuspended in medium at pH values

ranging from 6.5 to 8.0 , in either the absence or presence of drug, in order to correct values for any fluorescent activity of the drugs.

2. Results

Upon resuspension in HBSS, the pH_i of resting neutrophils in the preparations was in the range 7.1 to 7.3 . Addition of fMLP to resting neutrophils induced a slight reduction in cytoplasmic pH within a few seconds, followed by a sustained increase in alkalinity (fig. 1). Similar pH_i changes were observed after stimulation by PMA (fig. 2), except that the variations were less prominent. The described stimulus-induced shifts of pH_i were not affected by the simultaneous presence of DIDS, a blocker of $\text{Cl}^-/\text{HCO}_3^-$ cotransport (data not shown). By contrast, DMA produced a marked acidification and no subsequent alkalisation (data not shown). This confirms existing data indicating that the regulation of pH_i in neutrophils is largely dependent on the Na^+/H^+ antiport (Wright et al. 1986), whereas $\text{Cl}^-/\text{HCO}_3^-$ cotransport, an important contributor to pH regulation in other cells, plays a minor role, if any, in the neutrophil.

The addition of nimesulide to neutrophil suspensions did not induce changes in pH_i by itself. Furthermore, the changes in the pH_i of neutrophils induced by fMLP and PMA were not affected by the presence of nimesulide (figs 1 and 2).

3. Discussion

The activity of NADPH oxidase is inhibited by cellular acidification, which leads to a decrease in induced superoxide anion release (Simchowicz 1985). This may represent a cellular autoregulatory mechanism guarding against a proton overload, and it may also explain in part the anti-inflammatory effect of drugs such as piroxicam that inhibit the alkalisation of cultured renal tubular cells (Tønnessen et al. 1989).

In the present work, we examined whether or not the inhibitory effect of nimesulide on the neutrophil respiratory burst could result from an effect on pH_i regulation via the Na^+/H^+ antiport. The

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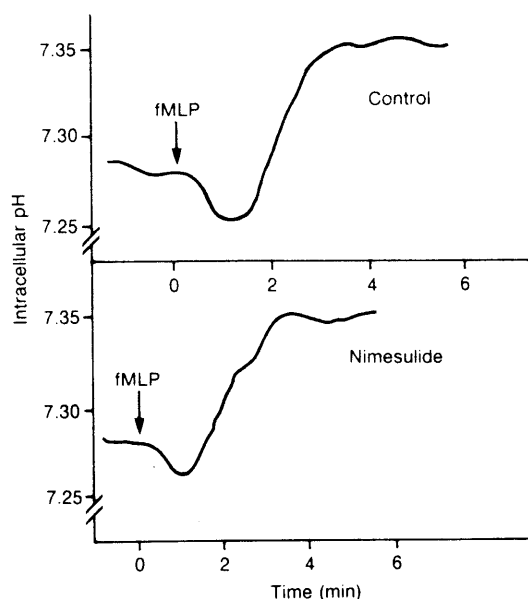


Fig. 1. Intracellular pH changes in human neutrophils (2.5×10^6 /ml) after stimulation with formylmethionyl-leucyl-phenylalanine (fMLP) 100 nmol/L (arrow). Upper panel: control cells. Lower panel: cells treated with nimesulide 0.1 mmol/L.

results do not support the hypothesis that nimesulide inhibits superoxide anion release by directly inhibiting neutrophil alkalisation. The drug does not seem to interfere with the interaction between fMLP and the Na^+/H^+ antiport, and the resultant shift of the 'set point' of the latter that generates the pH_i overshoot normally seen in this setting (Grinstein et al. 1985), nor does it seem to affect the course and extent of pH_i changes in PMA-treated cells. Therefore, other possible mechanisms for this effect of nimesulide should be evaluated.

The action of nimesulide on neutrophil metabolism is currently being investigated. The drug was shown to act as a scavenger of hypochlorous acid generated outside the cells (Dallegrì et al. 1989, 1991), but not of superoxide anions. The reduction in superoxide anion release produced by nimesulide is an intracellular action that might be expected to involve protein kinase C (PKC), in view of the central role played by this enzyme in the events leading to respiratory burst activation. The

current belief that PKC is implicated in the activation of the Na^+/H^+ antiport is supported by the finding that PKC inhibitors such as H-7 inhibit late alkalisation in neutrophils stimulated with fMLP or influenza A virus (Hartshorn et al. 1990). Results from studies by our group (Bevilacqua et al. 1991) give no convincing evidence that PKC activity is directly altered by nimesulide. The results of this study support this conclusion in that the cytoplasmic alkalisation of neutrophils, which can be considered a PKC-dependent phenomenon, is unaffected by nimesulide treatment.

The lack of effect of nimesulide on the neutrophil Na^+/H^+ antiport has clinical implications. The inhibition of cellular mechanisms that protect against acid overload by several nonsteroidal anti-inflammatory drugs contributes to their production of adverse effects such as gastric mucosal damage (peptic ulcer) and hyperstimulation of ventilatory centres (Tønnessen 1991; Tønnessen et al. 1989). Since it does not affect pH_i regulatory

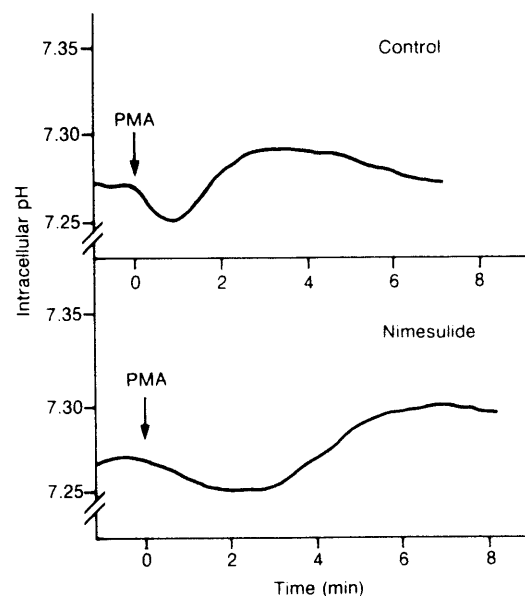


Fig. 2. Intracellular pH changes observed in human neutrophils (2.5×10^6 /ml) after stimulation with phorbol-12-myristate-13-acetate (PMA) 10 nmol/L (arrow). Upper panel: control cells. Lower panel: cells treated with nimesulide 0.1 mmol/L.

mechanisms, nimesulide would be expected to have a relatively low propensity to produce such acid-related effects. Clinical data support this assertion.

In conclusion, the *in vitro* inhibition of neutrophil oxidant production by nimesulide is unique and does not involve an interaction with mechanisms of pH_i regulation in these cells. Thus, protection from the proton overload that accompanies the respiratory burst is maintained in the presence of the drug.

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