Br. J. Pharmacol. (1988), **93**, 289–294

Benoxaprofen activates membrane-associated oxidative metabolism in human polymorphonuclear leucocytes by apparent modulation of protein kinase C

Pauline T. Lukey, [1]Ronald Anderson & Ursula H. Dippenaar

Division of Immunology, Department of Medical Microbiology, Institute for Pathology, University of Pretoria, Republic of South Africa

1 The non-steroidal anti-inflammatory drug (NSAID) benoxaprofen at concentrations of 15, 30 and 60 $\mu g\ ml^{-1}$ caused a dose-related activation of superoxide generation by human polymorphonuclear leucocytes (PMNL) *in vitro*.

2 The protein kinase C (PKC) inhibitor H-7 prevented benoxaprofen-mediated activation of superoxide generation by PMNL.

3 Benoxaprofen, by apparent substitution for phosphatidylserine, caused a dose-related activation of purified PKC from rat brain and in cytosolic extracts from human platelets.

4 Benoxaprofen-mediated stimulation of PMNL membrane-associated oxidative metabolism is due to apparent activation of PKC by this NSAID. These findings establish the molecular basis of the pro-oxidative properties of benoxaprofen.

Introduction

Prior to the withdrawal of benoxaprofen from the international market, a number of reports appeared which described the beneficial, therapeutic effects of the drug in rheumatoid arthritis and inflammatory diseases of the skin (reviewed by Allen, 1983). Novel mechanisms of anti-inflammatory activity such as preferential inhibition of 5-lipoxygenase and selective inhibition of the migration of mononuclear leucocytes (MNL) were attributed to benoxaprofen (Walker & Dawson, 1979; Meacock & Kitchen, 1979). The clinical significance of the inhibitory effects of the drug on leukotriene production was questioned by some (Salmon *et al.*, 1984) whilst others reported that the migratory responses of both human polymorphonuclear leucocytes (PMNL) and MNL were equally sensitive to the inhibitory effects of benoxaprofen (Naude *et al.*, 1983; Anderson *et al.*, 1984). We reported that benoxaprofen, unlike other commonly-used non-steroidal anti-inflammatory drugs (NSAIDs), possessed pro-oxidative properties by activating the membrane-associated oxidative metabolism of both PMNL and MNL (Anderson *et al.*, 1984; Eftychis & Anderson 1984). The drug promoted the auto-oxidative inhibition of PMNL and MNL migration and induced non-specific suppressor activity in MNL (Anderson *et al.*, 1984; Eftychis & Anderson, 1984). We proposed a direct relationship between the pro-oxidative properties of benoxaprofen and drug-mediated anti-inflammatory, immunosuppressive mechanisms. However, the molecular basis of the activation of membrane-associated oxidative metabolism of human phagocytes by benoxaprofen has not been described.

Recent studies have demonstrated the involvement of protein kinase C (PKC) in the activation of membrane-associated oxidative metabolism of human PMNL (Cox *et al.*, 1985). In the present study we have investigated interactions which occur between benoxaprofen and PKC in the activation of PMNL membrane-associated oxidative metabolism in human PMNL *in vitro*.

[1]Author for correspondence.

Methods

Benoxaprofen

Benoxaprofen (2,4 (chlorophenyl)-α-methyl-5-benzoxazoleacetic acid) was supplied by the Lilly Research Centre Ltd, Erl Wood Manor, Windlesham, Surrey and dissolved in 0.05 N NaOH at a stock solution of 3 $mg\ ml^{-1}$. Subsequent dilutions were made in Hanks'

balanced salt solution (HBSS, Grand Island Biological Co., Paisley, Scotland) with restoration of the pH to 7.4. Benoxaprofen was tested at concentrations of 0.1–60 $\mu g\,ml^{-1}$ which are well within the therapeutic range. During benoxaprofen chemotherapy with a single oral daily dose of 600 mg of the NSAID, peak serum values ranged from 50–300 $\mu g\,ml^{-1}$ (Kamal & Koch, 1982; Naude & Anderson, 1982). Appropriate solvent controls were included for each concentration of benoxaprofen.

Preparation of polymorphonuclear leucocytes

PMNL were prepared from heparinised venous blood (5 units of preservative-free heparin ml^{-1} blood) taken from normal adult human volunteers. PMNL were separated by centrifugation of blood at 400 *g* for 15 min on cushions of Ficoll (Pharmacia, Uppsala, Sweden) metrizoate. The resultant erythrocyte/PMNL fraction was sedimented with 3% gelatin for 30 min at 37°C to remove most of the erythrocytes. The PMNL-rich supernatant was centrifuged at 250 *g* for 10 min and the residual erythrocytes in the cell pellet were lysed by exposure to 0.85% ammonium chloride. PMNL were centrifuged, washed once, and resuspended to a concentration of $10^7\,ml^{-1}$ in HBSS.

Measurement of superoxide generation by PMNL

This was measured using lucigenin (*bis*-N-methylacridinium nitrate, Sigma Chemical Co., St Louis, Mo, U.S.A.)-enhanced chemiluminescence (LECL) as previously described (Dahlgren *et al.*, 1985). PMNL (10^6) were pre-incubated for 15 min at 37°C with 200 μM lucigenin in 0.9 ml of HBSS. LECL was then measured in an LKB Wallac 1251 luminometer (Turku, Finland) after addition of 0.1 ml of benoxaprofen (15, 30 and 60 $\mu g\,ml^{-1}$ final concentrations). LECL readings were integrated for 5 s intervals and plotted as millivolts (mV) $s.^{-1}$ Superoxide-mediated oxidation of lucigenin is expressed as the mean values of 4 separate experiments with standard errors (s.e.mean) shown for peak readings. Benoxaprofen-free control systems were included.

Investigations with H-7

The effects of H-7 (1-(5-isoquinoline-sulphonyl)-2-methylpiperazine, Sigma Chemical Co.), a selective inhibitor of protein kinase C (Hidaka *et al.*, 1984), on benoxaprofen-mediated activation of superoxide generation by PMNL were also investigated. PMNL were pre-incubated at 37°C for 30 min with various concentrations of H-7 (6.25, 12.5, 25, 50 and 100 μM). Benoxaprofen (30 $\mu g\,ml^{-1}$) was then added and LECL responses of PMNL measured as above. H-7 was omitted from control systems. At the concentrations used here (6.25–100 μM) H-7 is non-toxic to PMNL and does not scavenge superoxide (Fujita *et al.*, 1986).

Protein kinase C (PKC) extraction

(i) A crude extract from platelets isolated from heparinised fresh blood was used as a source of PKC. Briefly, platelet-rich plasma was removed from whole blood and diluted 1:2 in 0.15 M phosphate buffered saline/50 mM EGTA (ethyleneglycol-*bis*-(β-aminoethyl ether) N,N'-tetraacetic acid, pH 7.4) and centrifuged at 5,000 *g* for 25 min at 4°C. Platelets were then resuspended in 20 mM Tris, 0.5 mM EGTA, 0.5 mM EDTA (ethylenediamine tetraacetic acid) 50 mM 2-mercaptoethanol, 250 mM sucrose in the presence of protease inhibitors (1 mM phenylmethanesulphonyl-fluoride, PMSF; 200 $\mu g\,ml^{-1}$ soya bean trypsin inhibitor and 0.25 $\mu g\,ml^{-1}$ leupeptin). Platelets were disrupted by three 10 s bursts of sonication at an amplitude of 15 microns peak to peak. Debris was pelletted at 120,000 *g* for 45 min at 4°C. Supernates were assayed for protein by use of a Biorad (R) protein assay, held in 20% glycerol and stored at 4°C (for a maximum of 3 days) for use in PKC assays. (ii) Supplementary experiments using purified PKC were performed on a rat brain preparation purified by the method of Wooten *et al.* (1987). Briefly, 25 g of Sprague Dawley rat brains were homogenized in 8 vol of 20 mM Tris, 10 mM EGTA, 2 mM EDTA, 250 mM sucrose (pH 7.4) and protease inhibitors (as above). Debris was pelletted at 100,000 *g* for 30 min at 4°C. Supernatant was applied to a DE52 column equilibrated in buffer as above without sucrose but containing 50 mM 2-mercaptoethanol. PKC was eluted with a 0–0.3 M NaCl linear gradient. Fractions were assayed for PKC and enzyme-containing fractions pooled, conductivity adjusted and applied to a phenylsepharose column equilibrated in buffer as above containing 1.5 M NaCl. PKC was eluted in a linear NaCl gradient (0.6 M–0.0M). Fractions were collected, assayed, pooled and applied to a protamine agarose column (Sigma Chemical Co.) equilibrated with 20 mM Tris, 0.5 mM EDTA, 0.5 mM EGTA, 50 mM 2-mercaptoethanol. Fraction collection started on loading. PKC was concentrated by ultrafiltration and stored in 30% glycerol at −70°C. Purity was assessed by SDS-PAGE and Biorad protein assay.

Protein kinase C assay

Enzyme assays were carried out in 0.1 ml volumes containing 10 mM Pipes (pH 6.4, piperazine-N-N'-*bis*(2-ethane sulphonic acid), Sigma Chemical Co.) 10 mM $MgCl_2$, 200 $\mu g\,ml^{-1}$ histone (histone H1, type III-S, Sigma Chemical Co.), 0.25 mM EGTA (pH 7.4) and where indicated 0.5 mM $CaCl_2$, 50 $\mu g\,ml^{-1}$ phosphatidylserine (PS, Sigma Chemical Co.) and

25 μg ml^{-1} 1,2-diolein (Dio; Sigma Chemical Co.). PS and Dio were dissolved at 2 mg ml^{-1} in chloroform dispensed, dried under nitrogen and stored at −20°C. Prior to use, 15 mM Tris (pH 7.4) was added and PS and Dio resuspended by sonication. Benoxaprofen was added immediately before assay to the concentrations indicated in results. Crude PKC preparations were added at 30 μg protein per assay and purified PKC at 2 μg protein per assay. The reaction was started by the addition of 5 nmol ATP (containing 5×10^5 c.p.m, gamma ^{32}P-ATP, New England Nuclear Corp., Boston, Mass., USA). Incubation was at 30°C for 5 min. The reaction was stopped by spotting 0.1 ml on to filter paper and precipitating in 25% trichloroacetic acid (TCA). The filter paper was dried and Cherenkov counts used in calculation of PKC specific activity. In all cases protein phosphorylation occurring in the absence of Ca^{2+}, PS and Dio has been subtracted from results and therefore only phosphorylation attributable to PKC is presented. The results are expressed as pmol ^{32}P min^{-1}mg^{-1} protein.

Expression and statistical analyses of results

The results are expressed as the mean values ± s.e.mean for each series of experiments. The numbers of experiments are indicated in the table and Figures. Statistical analyses were performed by the Student's *t* test (paired *t* statistic) by comparison of systems containing benoxaprofen with the corresponding matched benoxaprofen-free control system.

Results

Effects of benoxaprofen on PMNL lucigen-enhanced luminescence

These results are shown in Figure 1. At the 3 concentrations tested (15, 30 and 60 μg ml^{-1}) benoxaprofen caused a dose-related activation of superoxide generation by PMNL which was linear to 3 min, peaked at 6 min and subsided thereafter. The peak responses (after 6 min) for PMNL co-incubated with 15, 30 and 60 μg ml^{-1} of benoxaprofen were 5.4 ($P < 0.025$) 9.5 ($P < 0.025$) and 16 fold ($P < 0.005$) greater than the corresponding values for the benoxaprofen-free control systems. Benoxaprofen-mediated stimulation of LECL was completely eliminated by the inclusion of 200 units of superoxide dismutase ml^{-1} (Sigma Chemical Co.).

Effects of H-7 on benoxaprofen-activation of lucigen-enhanced luminescence

These results are shown in Figure 2. H-7 caused a dose-related inhibition of the LECL responses of PMNL

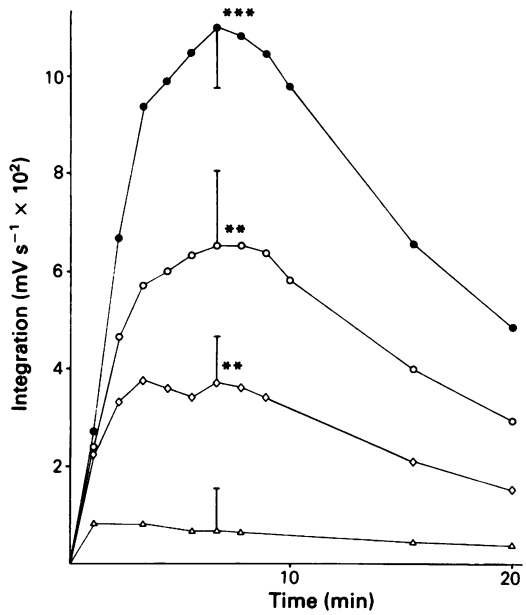

Figure 1 Kinetics of superoxide generation by PMNL co-incubated in the absence (△) and in the presence of benoxaprofen 15 μg ml^{-1} (◇), 30 μg ml^{-1} (○) and 60 μg ml^{-1} (●). Superoxide was assayed by lucigenin-enhanced chemiluminescence and the results are expressed as the mean value in mV s^{-1} of 4 separate experiments; vertical lines show s.e.mean. ***$P < 0.005$; **$P < 0.025$.

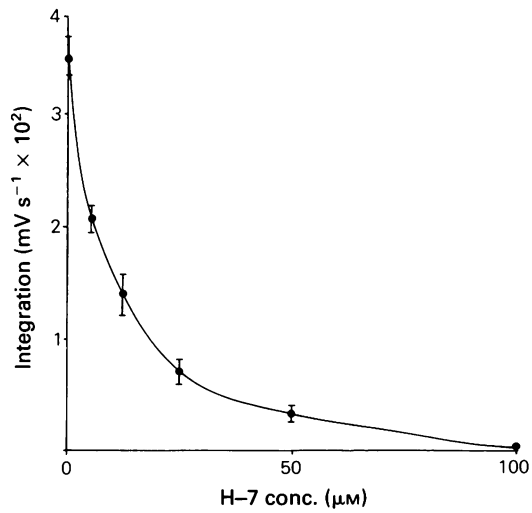

Figure 2 Effects of various concentrations (6.25–100 μM) of the protein kinase C inhibitor H-7 on the lucigenin-enhanced chemiluminescence responses of PMNL activated with 30 μg ml^{-1} benoxaprofen. Background values in the absence of benoxaprofen have been subtracted from the results which are expressed as the mean value in mV s^{-1} of 4 experiments; s.e.mean shown by vertical lines.

activated with 30 μg ml^{-1} benoxaprofen. Relative to H-7-free control systems the mean percentages inhibition observed with H-7 at concentrations of 6.25, 12.5, 25, 50 and 100 μM were 39.5 ± 4.1% ($P < 0.005$), 59.8 ± 3.4% ($P < 0.005$) 80.8 ± 1.9% ($P < 0.005$) 90.3 ± 1.2% ($P < 0.005$) and 100% ($P < 0.005$). The concentration of H-7 which caused 50% inhibition (IC_{50}) of benoxaprofen-mediated activation of LECL in PMNL was 10 μM.

Effects of benoxaprofen on protein kinase C activity

With the crude platelet-derived PKC preparation, benoxaprofen at concentrations of 0.1 μg ml^{-1} ($P < 0.025$), 1 μg ml^{-1} ($P < 0.005$) and 10 μg ml^{-1} ($P < 0.005$) caused a dose-related stimulation of PKC activity in the presence of Ca^{2+}, PS and Dio (Figure 3). To determine the site on the PKC molecule at which benoxaprofen was active, enzyme assays were performed in the presence of 10 μg ml^{-1} benoxaprofen and various combinations of the physiological activators (Table 1). The combination of Ca^{2+} and Dio caused 202.52 ± 0.56 pmol ^{32}P precipitated min^{-1} mg^{-1} protein whilst inclusion of benoxaprofen increased the level of phosphorylation to 458.48 ± 39.40 pmol ^{32}P min^{-1} mg^{-1} protein ($P < 0.05$). Benoxaprofen had no significant effect on any other combination of physiological activators (Table 1). When partially purified PKC was used, confirmation of these results was obtained for 10 μg ml^{-1} benoxaprofen in the presence of all three physiological activators. Control systems containing the Ca^{2+}, PS and Dio caused TCA precipitation of 53.81 ± 6.72 nmol ^{32}P min^{-1} mg^{-1} protein. The inclusion of benoxaprofen increased protein phosphorylaton to 72.32 ± 2.6 nmol ^{32}P min^{-1} mg^{-1} protein ($P < 0.025$).

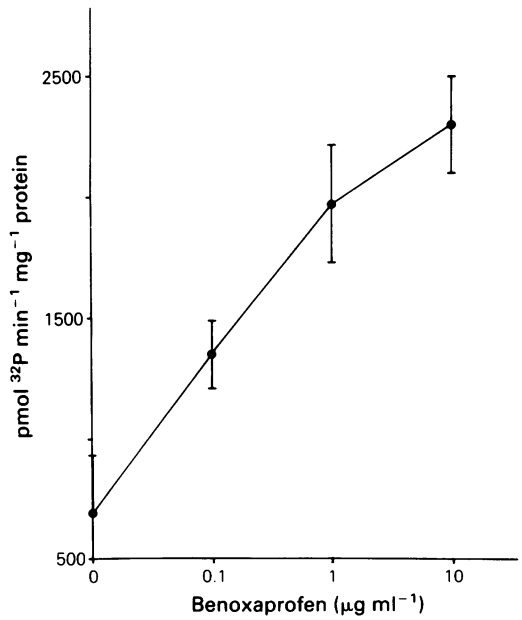

Figure 3 Dose-response of benoxaprofen-mediated stimulation of protein kinase C (PKC) in the presence of Ca^{2+}, phosphatidylserine (PS) and 1,2-diolein (Dio). PKC was assayed as explained in the text in the presence of Ca^{2+}, PS and Dio and various concentrations of benoxaprofen (0.1–10 μg ml^{-1}). Background values in the absence of Ca^{2+}, PS and Dio have been subtracted. Results are expressed as the mean value of 3 experiments; s.e.mean shown by vertical lines.

Discussion

Activation of phagocytes by soluble and particulate stimuli of membrane-associated oxidative metabolism is accompanied by a metabolic burst and increased oxygen consumption which lead to the formation of a series of reactive oxidants derived from superoxide (reviewed by Babior, 1984). Although primarily antimicrobial, phagocyte-derived reactive toxic oxidants are indiscriminate and when released extracellularly are potentially carcinogenic (Weitzman *et al.*, 1985) and can damage bystander host cells and tissues (Fantone & Ward, 1982; Ward *et al.*, 1983; Fox, 1984; Rehan *et al.*, 1984; Shandall *et al.*, 1986). The superoxide-generating enzyme is a membrane-bound NADPH-oxidase (Babior, 1984) which is activated by PKC translocated from the cytoplasm to the PMNL membrane (Cox *et al.*, 1985; Gennaro *et al.*, 1986). PKC is a calcium- and phospholipid-dependent enzyme that is activated by diacylglycerol released from phosphoinositides by the action of phospholipase C (Nishizuka, 1984). Diacylglycerol is normally almost absent from cell membranes but is transiently produced during membrane receptor activation by extracellular signals (Nishizuka, 1984). Some agents bypass the requirement for membrane-receptor-mediated activation of PKC and NADPH oxidase in PMNL. Phorbol esters such as phorbol myristate acetate (PMA) function as agonists of diacylglycerol receptors for cytosolic PKC and directly activate NADPH oxidase in PMNL (Tauber *et al.*, 1982; Leach *et al.*, 1983; Cox *et al.*, 1985). Unsaturated fatty acids, especially arachidonate, activate PKC and NADPH oxidase by a different mechanism which requires the participation of 1,2-diolein (synthetic diacylglycerol) and calcium, but not phosphatidylserine, for full expression (Bromberg & Pick, 1984; McPhail *et al.*, 1984).

In the present study we have shown that benoxaprofen, at therapeutic concentrations (Kamal & Koch, 1982; Naude & Anderson, 1982), causes dose-related activation of superoxide generation in human

Table 1 Measurement of protein kinase C (PKC) stimulation by benoxaprofen in the presence of combinations of the physiological stimulators Ca^{2+}, phosphatidylserine (PS) and 1, 2-diolein (Dio)

Combination of activators	*PKC activity*	
	without benoxaprofen	*with benoxaprofen (10 μg ml⁻¹)*
Ca^{2+} only	289.49 ± 8.05	311.88 ± 14.62
PS only	221.31 ± 38.19	230.10 ± 41.74
Dio only	201.30 ± 42.4	203.11 ± 51.47
Ca^{2+}, PS	247.64 ± 98.77	207.39 ± 76.97
Ca^{2+}, Dio	202.52 ± 70.56	458.48 ± 39.40*
PS, Dio	340.26 ± 37.60	318.56 ± 11.20
Ca^{2+}, PS, Dio	630.40 ± 109.29	1730.39 ± 168.62**

Results are expressed as the mean value ± s.e.mean as pmol ^{32}P precipitated $min^{-1} mg^{-1}$ protein of 3 experiments. *$P < 0.05$; **$P < 0.005$.

PMNL *in vitro*. Superoxide is the precursor of various reactive oxidants, which, if released extracellularly, possess wide-ranging immunosuppressive activities. Phagocyte-derived reactive oxidants inhibit lymphocyte proliferation (Zoschke & Messner, 1984) and leucocyte migration (Baehner *et al.*, 1977) and promote the oxidative inactivation of both leukotrienes (Henderson *et al.*, 1982) and leucoattractants (Clark, 1982). We have previously proposed that reactive oxidants released by benoxaprofen-activated phagocytes are the probable mediators of the immunosuppressive properties of this NSAID (Anderson *et al.*, 1984; Eftychis & Anderson, 1984).

The benoxaprofen-mediated activation of superoxide generation in PMNL was inhibited by the selective PKC inhibitor H-7 (Hidaka *et al.*, 1984) with an IC_{50} of 10 μM. Interestingly, the reported K_i value of H-7 for pure PKC is about 6 μM (Hidaka *et al.*, 1984). The reported IC_{50} concentrations of H-7 for PMA, FMLP and calcium ionophore-activation of superoxide generation by PMNL are 68, 120 and 110 μM respectively (Fujita *et al.*, 1986). Relative to the other activators the benoxaprofen-mediated stimulation of superoxide generation is extremely sensitive to the inhibitory effects of H-7 which indicates possible involvement of the NSAID in activation of PKC.

In an attempt to identify the molecular/biochemical mechanism of benoxaprofen-mediated activation of PMNL membrane-associated oxidative metabolism, we investigated the effects of this agent on PKC activity in a cytosolic extract of human platelets as well as a partially purified rat brain extract. Both of these enzyme sources contain considerably higher PKC activity and less protease activity than PMNL (Kikkawa *et al.*, 1983). Benoxaprofen at concentrations of 0.1 μg–10 μg ml^{-1} caused a dose-related stimulation of PKC which was detectable in the presence of all three physiological activators Ca^{2+}, PS and Dio. Furthermore, benoxaprofen stimulated PKC in the absence of PS and apparently substitutes for this physiological activator. However, other mechanisms can not be excluded since benoxaprofen and phosphatidylserine in the presence of diolein and Ca^{2+} caused apparent synergistic activation of PKC. These observations suggest that the direct stimulation by benoxaprofen of membrane-associated oxidative metabolism in human phagocytes occurs as a consequence of apparent activation of PKC by this NSAID. Molecular structure/function studies with analogues of benoxaprofen will be required to identify the physico-chemical properties of the molecule which are linked to stimulation of PKC and pro-oxidative activity.

Chemotherapy with benoxaprofen was associated with an unusually high incidence of cutaneous side-effects including phototoxic reactions, onycholysis and eruptive skin tumours on sun-exposed areas (reviewed by Allen, 1983). Reactive oxidants released extracellularly by benoxaprofen-activated phagocytes in the skin are potential mediators of these side-effects of the NSAID. In support of this we have recently observed striking synergism between benoxaprofen and ultraviolet radiation on the release of reactive oxidants by human phagocytes *in vitro* (Anderson & Eftychis, 1986). It is probable that the therapeutic, immunosuppressive mechanisms as well as the cutaneous side-effects of benoxaprofen were related to pro-oxidative interactions of the NSAID with human phagocytes.

In conclusion, the results presented in this study demonstrate that benoxaprofen apparently activates cytosolic PKC in human PMNL and establish the biochemical mechanism of the pro-oxidative activity of this agent.

This investigation was supported by a research grant from The South African Arthritis Foundation.

References

ALLEN, B.R. (1983). Benoxaprofen and the skin. *Br. J. Dermatol.*, **109**, 361–364.

ANDERSON, R. & EFTYCHIS, H.A. (1986). Potentiation of the generation of reactive oxidants by human phagocytes during exposure to benoxaprofen and ultraviolet radiation *in vitro*. *Br. J. Dermatol.*, **115**, 285–295.

ANDERSON, R., LUKEY, P.T., NAUDE, S.P.E. & JOONE, G. (1984). Benoxaprofen: a pro-oxidant anti-inflammatory drug? *Agents & Actions*, **14**, 238–246.

BABIOR, B.M. (1984). Oxidants from phagocytes: agents of defence and destruction. *Blood*, **64**, 959–966.

BAEHNER, R.L., BOXER, L.A., ALLEN, J.M. & DAVIS, J. (1977). Auto-oxidation as a basis for altered function by polymorphonuclear leucocytes. *Blood*, **50**, 327–335.

BROMBERG, Y. & PICK, E. (1984). Unsaturated fatty acids stimulate NADPH-dependent superoxide production by cell-free systems derived from macrophages. *Cell. Immunol.*, **88**, 213–221.

CLARK, R.A. (1982). Chemotactic factors trigger their own oxidative inactivation by neutrophils. *J. Immunol.*, **129**, 2725–2728.

COX, J.A., JENG, A.Y., SHARKEY, N.A., BLUMBERG, P.M. & TAUBER, A.I. (1985). Activation of human neutrophil nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase by protein kinase C. *J. Clin. Invest.*, **76**, 1932–1938.

DAHLGREN, C., ANIANSSON, H. & MAGNUSSON, K. (1985). Pattern of formyl-methionyl-leucyl-phenylalanine-induced luminol and lucigenin dependent chemiluminescence in human neutrophils. *Infect. Immun.*, **45**, 1–5.

EFTYCHIS, H. & ANDERSON, R. (1984). Benoxaprofen activation of suppressor activity in mononuclear leucocytes by a pro-oxidative mechanism *in vitro*. *Agents and Actions*, **14**, 649–653.

FANTONE, J.C. & WARD, P.A. (1982). Role of oxygen-derived free radicals and metabolites in leucocyte-dependent inflammatory reactions. *Am. J. Pathol.*, **107**, 397–418.

FOX, R.B. (1984). Prevention of granulocyte-mediated oxidant lung injury in rats by a hydroxyl radical scavenger, dimethylurea. *J. Clin. Invest.*, **74**, 1456–1464.

FUJITA, I., TAKESHIGE, K. & MINAKAMI, S. (1986). Inhibition of neutrophil superoxide formation by 1-(5-isoquinoline-sulphonyl)-2-methylpiperazine (H-7), an inhibitor of protein kinase C. *Biochem. Pharmacol.*, **35**, 4555–4562.

GENNARO, R., FLORIO, C. & ROMEO, D. (1986). Co-activation of protein kinase C and NADPH oxidase in the plasma membrane of neutrophil cytoplasts. *Biochem. Biophys. Res. Commun.*, **134**, 305–312.

HENDERSON, W.R., JORG, A. & KLEBANOFF, S.J. (1982). Eosinophil peroxidase mediated inactivation of leukotrienes B4, C4 and D4. *J. Immunol.*, **128**, 2609–2613.

HIDAKA, H., INAGAKI, M., KAWAMOTO, S. & SASAKI, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide-dependent protein kinase and protein kinase C. *Biochemistry*, **23**, 5036–5041.

KAMAL, A. & KOCH, I.M. (1982). Pharmacokinetic studies of benoxaprofen in geriatric patients. *Eur. J. Rheumatol. Inflamm.*, **5**, 76–81.

KIKKAWA, U., MINAKUCHI, R., TAKAI, Y. & NISHIZUKA, Y. (1983). Calcium-activated, phospholipid-dependent protein kinase (protein kinase C) from rat brain. *Methods Enzymol*, **99**, 288–298.

LEACH, K.L., JAMES, M.L. & BLUMBERG, P.M. (1983). Characterisation of a specific phorbol ester aporeceptor in mouse brain cytosol. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 4208–4212.

McPHAIL, L.C., CLAYTON, C.L. & SNYDERMAN, R. (1984). A potential second messenger role for unsaturated fatty acids: activation of Ca^{2+}-dependent protein kinase. *Science*, **224**, 622–625.

MEACOCK, S.C.R. & KITCHEN, E.A. (1979). Effects of the non-steroidal anti-inflammatory drug benoxaprofen on leucocyte migration. *J. Pharm. Pharmacol.*, **31**, 366–370.

NAUDE, S.P.E. & ANDERSON, R. (1982). Benoxaprofen: could the side effects have been prevented? *S. Afr. Med. J.*, **62**, 427.

NAUDE, S.P.E., ANDERSON, R., EFTYCHIS, H., THERON, A., VAN WYK, H. & JOONE, G. (1983). The effects of benoxaprofen on mononuclear and polymorphonuclear leucocyte motility. An *in vitro* and *in vivo* study. *S. Afr. Med. J.*, **63**, 926–930.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature*, **308**, 693–698.

REHAN, A., JOHNSON, K.J., WIGGINS, R.C., KUNKEL, R.G. & WARD, P.A. (1984). Evidence for the role of oxygen radicals in acute nephrotoxic nephritis. *Lab. Invest.*, **51**, 396–415.

SALMON, J.A., HIGGS, G.S., TILLING, L., MONCADA, S. & VANE, J.R. (1984). Mode of action of benoxaprofen. *Lancet*, **i**, 848.

SHANDALL, A.A., WILLIAMS, G.T., HALLETT, M.B. & YOUNG, H.L. (1986). Colonic healing: a role for polymorphonuclear leucocytes and oxygen radical production. *Br. J. Surg.*, **73**, 225–228.

TAUBER, A.I., BRETTLER, D.B., KENNINGTON, E.A. & BLUMBERG, P.M. (1982). Relation of human neutrophil phorbol ester occupancy and NADPH-oxidase activity. *Blood*, **60**, 333–339.

WALKER, J.R. & DAWSON, W. (1979). Inhibition of rabbit PMN lipoxygenase activity by benoxaprofen. *J. Pharm. Pharmacol.*, **31**, 778–780.

WARD, P.A., TILL, G.O., KUNKEL, R. & BEAUCHAMP, C. (1983). Evidence for the role of hydroxyl radical in complement and neutrophil-dependent tissue injury. *J. Clin. Invest.*, **72**, 789–801.

WEITZMAN, S.A., WEITBERG, A.B., CLARK, E.P. & STOSSEL, T.P. (1985). Phagocytes as carcinogens: malignant transformation produced by human neutrophils. *Science*, **227**, 1231–1233.

WOOTEN, M.W., VAN DEN PLAAS, M. & NEL, A.E. (1987). Rapid purification of protein kinase C from rat brain; a novel method employing protamine agarose affinity column chromatography. *Eur. J. Biochem*, **164**, 461–467.

ZOSCHKE, D.C. & MESSNER, R.P. (1984). Suppression of human lymphocyte mitogenesis mediated by phagocyte-released reactive oxygen species: comparative activities in normals and in chronic granulomatous disease. *Clin. Immunol. Immunopathol.*, **32**, 29–40.

(Received May 18, 1987.
Revised September 4, 1987.
Accepted October 13, 1987.)