Promotion of DNA Strand Breaks in Cocultured Mononuclear Leukocytes by Protein Kinase C-dependent Prooxidative Interactions of Benoxaprofen, Human Polymorphonuclear Leukocytes, and Ultraviolet Radiation

Gwen Schwalb, Albertus D. Beyers, Ronald Anderson, and Andre E. Nel

Division of Immunology, Department of Medical Microbiology, Institute of Pathology, University of Pretoria [G. S., R. A.]; and the Departments of Medical Physiology (A. D. B.) and Internal Medicine (A. E. N.), Faculty of Medicine, University of Stellenbosch, Tygerberg; Republic of South Africa

ABSTRACT

At concentrations of 5 µg/ml and greater the nonsteroidal antinflammatory drug benoxaprofen caused dose-related activation of lucigenin-enhanced chemiluminescence in human polymorphonuclear leukocytes (PMNL). Benoxaprofen-mediated activation of lucigenin-enhanced chemiluminescence by PMNL was increased by UV radiation and was particularly sensitive to inhibition by the selective protein kinase C inhibitor H-7. To identify the molecular mechanism of the prooxidative activity of benoxaprofen, the effects of the nonsteroidal antinflammatory drug on the activity of purified protein kinase C in a cell-free system were investigated. Benoxaprofen caused a dose-related activation of protein kinase C by interaction with the binding site for the physiological activator phosphatidylserine, but could not replace diacylglycerol. When autologous mononuclear leukocytes (MNL) were cocultured with PMNL and benoxaprofen in combination, but not individually, the frequency of DNA strand breaks in MNL was markedly increased. UV radiation significantly potentiated damage to DNA mediated by benoxaprofen and PMNL. Inclusion of superoxide dismutase, H-7, and, to a much lesser extent, catalase during exposure of MNL to benoxaprofen-activated PMNL prevented oxidant damage to DNA. These results clearly demonstrate that potentially carcinogenic prooxidative interactions, which are unlikely to be detected by conventional assays of mutagenicity, may occur between phagocytes, UV radiation, and certain pharmacological agents.

INTRODUCTION

Highly reactive forms of molecular oxygen may play an important role in tumorogenesis, apparently by acting at the level of tumor promotion (1). These oxygen species, such as superoxide, hydroperoxy radical, singlet oxygen, hydroxyl radical and hydrogen peroxide are potent inducers of DNA strand breaks and chromosomal aberrations (2–7). For this reason activated phagocytes are potential carcinogens since they generate unstable and stable reactive oxidants (8) which are mutagenic (9) and promote chromosomal abnormalities and malignant transformation in cocultured eukaryotic cells (10, 11). The superoxide-generating enzyme of phagocytes is a membrane-associated NADPH-oxidase which is activated by diverse stimuli such as the tumor promoter phorbol myristate acetate, lectins, calcium ionophore, opsonized particles, and complement components (12). These oxidases utilize different transductional mechanisms to activate the NADPH oxidase such as PKC (8) in the case of phorbol myristate acetate (8, 12–14).

Theoretically pharmacological agents which directly activate membrane-associated oxidative responses in human phagocytes present the potential hazard of oxygen toxicity to host tissues. We have previously reported that the NSAID benoxaprofen activates superoxide generation by human PMNL in the absence of physiological stimuli and that the prooxidative interactions of this NSAID are potentiated by UV radiation (15–18). Clinical experience with benoxaprofen was relatively brief since the drug was withdrawn from the international market in 1982 due to drug-related deaths and a particularly high incidence of cutaneous side effects (19) including eruptive skin tumors on sun-exposed skin (20). Nevertheless we believe that continued laboratory research on benoxaprofen is warranted and may contribute to the documentation of novel mechanisms of drug-mediated toxicity and carcinogenesis. In this study we have investigated the effects of benoxaprofen and human PMNL individually and in combination on the frequency of DNA strand breaks in cocultured autologous lymphocytes as well as the molecular mechanism of the prooxidative activity of this NSAID.

MATERIALS AND METHODS

Chemicals and Reagents. Unless indicated chemicals and reagents were purchased from Sigma Chemical Co., St. Louis, Mo. Benoxaprofen, 2,4-(chlorophenyl)-α-methyl-benzoxazoleacetate, was obtained from the Lilly Research Centre Ltd., Earl Wood Manor, Windlesham, Surrey, England, dissolved at a concentration of 6 mg/ml in 0.05 N NaOH then diluted to a stock solution of 600 µg/ml with restoration to pH 7.4. The concentration range tested was 0.1–60 µg/ml which is well within the range of serum concentrations which were achieved during chemotherapy with this NSAID (21, 22). [32P]ATP was synthesized by interacting [32P]orthophosphate (Amersham, England) with Gamma-prep (Promega Biotec.).

Preparation of PMNL and MNL. PMNL and MNL were prepared from venous blood taken from healthy adult human volunteers and treated with preservative-free heparin (5 units/ml). PMNL and MNL were separated by centrifugation, at 400 × g for 15 min, of heparinized blood on cushions of Ficoll (Pharmacia, Uppsala, Sweden) metrizoate. Residual erythrocytes in the PMNL preparations were removed by sequential sedimentation with 3% gelatin and selective lysis with 0.85% ammonium chloride. After washing, the PMNL and MNL were resuspended to 2 × 107/ml in 4.2 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered, indicator-free HBSS, pH 7.4. Measurement of Oxidant Generation by PMNL. This was measured using LECL (23). PMNL (107) were preincubated for 15 min at 37°C with 0.2 mM lucigenin in 900 µl HBSS. LECL was then measured in an LKB Wallace (Turku, Finland) luminometer (Model 1251) after addition of benoxaprofen (0.1–60 µg/ml final concentrations). LECL readings were integrated for 5-s intervals and plotted as mV·s−1. Appropriate benoxaprofen-free and PMNL-free control systems were included. The effects of UV radiation (as described below) and H-7, a potent inhibitor of protein kinase C (24) on benoxaprofen-induced oxidant generation by PMNL were investigated. PMNL were preincu-
bated for 30 min at 37°C with 12.5–100 µM of H-7, a concentration range which proved to be nontoxic to PMNL and free of scavenging effects (25). Benoxaprofen (30 µg/ml) was then added and LECL recorded as described above.

Measurement of DNA Single-Strand Breaks. The formation of single-strand breaks in DNA was measured by alkaline unwinding and determination of ethidium bromide fluorescence using a Hitachi (Tokyo, Japan) fluorescence spectrophotometer (Model 650-10) with excitation at 520 nm and emission at 590 nm according to the method of Birnbom and Jevcak (26). Under the conditions employed ethidium bromide binds preferentially to double-stranded DNA. PMNL (4 × 10⁷) were preincubated at 37°C for 15 min in a volume of 3.4 ml HBSS in 9-mm plastic Petri dishes, followed by addition of benoxaprofen and MNL (2 × 10⁶) in a final volume of 6 ml HBSS. PMNL-free and benoxaprofen-free control systems were included. The dishes were incubated for 15 min at 37°C after which the nonadherent MNL were separated from adherent PMNL, enumerated, and resuspended to 10⁷/ml in 250 mM mesosinol, 10 mM sodium phosphate, 1 mM MgCl₂ (pH 7.2). 200 µl of MNL suspension were lysed in alkaline medium containing 7 nM urea, 10 mM NaOH, 2.5 mM cyclohexanediaminetetraacetic acid, and 0.1% sodium dodecyl sulfate. Ethidium bromide fluorescence was determined after a 60-min incubation period at 15°C. The results were calculated according to the formula, D (percentage of double-stranded DNA) = (F - Fmin)/(Fmax - Fmin) × 100, where F is the fluorescence of the sample, Fmax the background fluorescence determined in samples that were sonicated at the beginning of the unwinding period in order to induce maximal unwinding, and Fmin is the fluorescence of samples kept at pH 11.0, which is below the pH level needed to induce unwinding of double-stranded DNA. This system was also used for testing the effect of H-7 (50 and 100 µM), catalase (200 units/ml), and SOD (100 units/ml) on the frequency of DNA single-strand breaks in MNL exposed to benoxaprofen (30 µg/ml) and PMNL individually and in combination, with and without exposure to UV radiation.

UV Irradiation of PMNL and MNL. The UV source was a Philips MLV irradiation lamp, 300 W (Philips Electronics Ltd, Johannesburg, South Africa), emitting at 50 cm from the lamp UVA 1.69 mW/cm², UVB 0.88 mW/cm², and UVC 0.01 mW/cm². On the basis of preliminary experiments a 1-min exposure time was chosen which corresponded to 0.13 J/cm² UVA. Briefly, the Petri dishes containing the PMNL were irradiated for 1 min immediately after the addition of MNL and benoxaprofen. Thereafter subsequent incubation and assays for DNA strand breaks in cocultured MNL were performed as described above. The following control systems were included: (a) irradiated MNL only, (b) irradiated PMNL and MNL, and (c) irradiated MNL and benoxaprofen. For these investigations benoxaprofen was used at concentrations of 10 and 30 µg/ml.

Protein Kinase C Purification and Assay. PKC was chromatographically purified from brain tissue by using DEAE ion-exchange-, phenyl sepharose-, and protamine agarose columns in sequence as previously described (27).

The activity of PKC was assayed by measuring the incorporation of [³²P]ATP into lysine-rich histone (type 111S) as previously described with minor modifications (28). Briefly, the 100-µl reaction mixture contained 20 mM Tris (pH 7.5), 20 µg histone, 2.5 mM EGTA, 10 mM MgCl₂ in the absence or presence of various amounts and combinations of PS, Ca²⁺, dienol, and benoxaprofen as indicated. Reactions were initiated by the addition of 1 nmol [³²P]ATP containing (0.5-1) × 10⁶ cpm and allowed to incubate for 6 min at 30°C before spotting onto Whatman 3MM paper squares and washing in trichloroacetic acid. [³²P] Incorporation in the protein precipitates were estimated by Cerenkov counting and PKC activity was expressed as nmol [³²P] transferred/min/mg enzyme protein by subtracting EGTA backgrounds from stimulated activity.

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

RESULTS

Chemiluminescence Responses of PMNL. The kinetics of benoxaprofen induced activation of LECL as compared with control are shown in Fig. 1. The response was linear for 2–3 min, peaked at 6 min, and thereafter subsided. A dose-response effect was also obtained by recording the LECL peak at 6 min after the addition of varying concentrations of benoxaprofen (Fig. 2). This activation was statistically significant for benoxaprofen concentrations >7.5 µg/ml and yielded stimulation indices of 2.4, 3.7, 6.5, and 12 for benoxaprofen concentrations of 7.5, 15, 30, and 60 µg/ml, respectively. Benoxaprofen-mediated stimulation of LECL was completely eliminated by the inclusion of 100 units/ml of SOD.

Effects of H-7 on Benoxaprofen-activation of LECL. H-7 caused dose-related inhibition of the LECL responses of PMNL activated with 30 µg/ml benoxaprofen (Fig. 3). The inhibition obtained with 12.5, 25, 50, and 100 µM H-7 was 47, 63, 79, and 95% of the maximal response, respectively. This translates
into an IC₅₀ value of 13 µM as indicated and compares well with the IC₅₀ value of 7 µM for PKC inhibition in vitro (29).

Effects of Benoxaprofen and PMNL on DNA Strand Breaks in MNL. Exposure of MNL to autologous PMNL or varying concentrations of benoxaprofen individually did not affect the frequency of DNA strand breaks in MNL. Percentages of intact dsDNA for MNL only, MNL + PMNL, and MNL + 60 µg/ml benoxaprofen were 91 ± 4, 89 ± 3, and 88 ± 4%, respectively (mean value ± SEM of three separate experiments). In contrast, coinubation with PMNL and benoxaprofen markedly increased the frequency of mononuclear DNA strand breaks (Fig. 4). Moreover, this effect was dependent on the concentration of benoxaprofen, yielding 91 ± 4, 76 ± 4, 64 ± 4, and 29 ± 5% intact dsDNA in control systems and systems containing benoxaprofen concentrations of 5, 10, 15, 30, and 60 µg/ml, respectively.

Effects of UV Radiation on Benoxaprofen-activated PMNL. These results are shown in Tables 1 and 2. Exposure of benoxaprofen-activated PMNL to UV radiation significantly potentiated the frequency of DNA single strand breaks in cocultured MNL. In PMNL-free control systems containing MNL only or MNL and benoxaprofen no effects of UV radiation were observed. However in the benoxaprofen-free control system containing PMNL and MNL a slight, but statistically significant (p < 0.01) increase in the frequency of DNA strand breaks was observed. UV radiation exposure also potentiated the LECL responses of benoxaprofen-activated PMNL (Table 2) and this effect was eliminated by H-7 (IC₅₀ = 11 µM) and SOD.

Effects of Catalase, Superoxide Dismutase, and H-7 on the Frequency of DNA Strand Breaks in MNL Exposed to PMNL and Benoxaprofen. These results are shown in Table 3. SOD caused statistically significant protection of DNA from damage mediated by PMNL and benoxaprofen (30 µg/ml), while catalase had a modest protective effect. The combination of catalase and SOD was not significantly better than SOD alone. H-7 at concentrations tested (50 and 100 µM) protected MNL from DNA single strand breaks inflicted by PMNL and benoxaprofen. Corresponding results with UV radiation-exposed, benoxaprofen-treated PMNL are shown in Table 4. SOD and H-7, but not catalase, protected cocultured MNL from oxidant damage to DNA mediated UV radiation-exposed, benoxaprofen-activated PMNL.

Interactions of Benoxaprofen with PKC. Benoxaprofen (30 µg/ml) failed to stimulate PKC activity by itself in the absence of benoxaprofen concentrations of 5, 10, 15, 30, and 60 µg/ml (all p < 0.005), respectively. However in the benoxaprofen-free control system containing PMNL and MNL a slight, but statistically significant (p < 0.01) increase in the frequency of DNA strand breaks was observed. UV radiation exposure also potentiated the LECL responses of benoxaprofen-activated PMNL (Table 2) and this effect was eliminated by H-7 (IC₅₀ = 11 µM) and SOD.

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of Ca²⁺ and diolein (not shown). When these two allosteric effectors were present alone or in combination, however, activity was consistently (n = 3 experiments) enhanced in the presence of benoxaprofen (Table 5, a, c, and e). This apparent effect was negated in the presence of saturating amounts of PS (Table 5, d and f), but could again be seen if nonsaturating amounts of this phospholipid were present (Fig. 5A). These results would argue that benoxaprofen operates via the PS binding site and of H-7 provided statistically significant protection (p < 0.005).

**Table 4 Measurement of the protective effects of SOD, catalase, and H-7 on the structural integrity of ds DNA in MNL exposed to irradiated, benoxaprofen-activated (30 µg/ml) PMNL.**

Results of triplicate determinations of three different experiments are expressed as the mean percentage (±SEM) of ds DNA. SOD and both concentrations of H-7 provided statistically significant protection (p < 0.005).

<table>
<thead>
<tr>
<th>Test system</th>
<th>Percentage of ds DNA remaining in MNL</th>
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<tbody>
<tr>
<td>a. MNL + PMNL</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>b. MNL + PMNL + benoxaprofen</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>c. MNL + PMNL + benoxaprofen + UV radiation</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>d. MNL + PMNL + benoxaprofen + UV radiation + catalase</td>
<td>11 ± 5</td>
</tr>
<tr>
<td>e. MNL + PMNL + benoxaprofen + UV radiation + SOD</td>
<td>76 ± 6</td>
</tr>
<tr>
<td>f. MNL + PMNL + benoxaprofen + UV radiation + catalase + SOD</td>
<td>82 ± 2</td>
</tr>
<tr>
<td>g. MNL + PMNL + benoxaprofen + UV radiation + 50 µM H-7</td>
<td>51 ± 2</td>
</tr>
<tr>
<td>h. MNL + PMNL + benoxaprofen + UV radiation + 100 µM H-7</td>
<td>59 ± 2</td>
</tr>
</tbody>
</table>

**Table 5 Measurement of PKC activity in the presence of various combinations of allosteric effectors with or without 30 µg/ml benoxaprofen.**

Results are expressed as the mean values ± SEM of triplicate assays. Percentage of change is calculated as increase in PKC activity with benoxaprofen above activity with allosteric effectors alone.

<table>
<thead>
<tr>
<th>Allosteric effectors in assay system</th>
<th>PKC activity (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>benoxaprofen</td>
</tr>
<tr>
<td>a. Ca²⁺, 100 µM</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>b. PS, 25 µg/ml</td>
<td>4.4 ± 1.4</td>
</tr>
<tr>
<td>c. Diolein, 5 µg/ml</td>
<td>12.2 ± 0.7</td>
</tr>
<tr>
<td>d. Ca²⁺ + PS (same amounts)</td>
<td>79.9 ± 3.4</td>
</tr>
<tr>
<td>e. Ca²⁺ + diolein (same amounts)</td>
<td>30.5 ± 0.3</td>
</tr>
<tr>
<td>f. Ca²⁺ + PS + diolein (same amounts)</td>
<td>83.9 ± 1</td>
</tr>
</tbody>
</table>

**Fig. 5. A, determination of PKC activity in the presence of various amounts of PS without (C) or with (B) benoxaprofen. Free Ca²⁺ was 100 µM. Data, mean values ± SEM of three triplicate measurements at every point. Background activity of control tubes incubated in the absence of Ca²⁺ and PS has been subtracted. B, PKC activity in the presence of various amounts of benoxaprofen. The reaction vessels contained 100 µM Ca²⁺ 10 µg/ml diolein, but PS was absent. Data, means ± SEM of triplicate measurements.**

**DISCUSSION**

The mutagenic and carcinogenic potential of phagocyte-derived reactive oxidants (9–11) and the association of chronic inflammation and cancer (30–33) have been documented. PMNL may also contribute to drug toxicity and carcinogenesis by prooxidative mechanisms. Interactions of the phagocyte-derived oxidants hypochlorous acid, sodium hypochlorite, and taurine chloramine with chlorpromazine, aminopyrine, and phenylhydrazine lead to the generation of potentially toxic free radical forms of these agents (34). In the present study we have described an alternative mechanism of drug-mediated carcinogenesis due to prooxidative interactions of benoxaprofen, phagocytes, and UV radiation.

Prior to its withdrawal from the international market in 1982 clinical experience with benoxaprofen had been brief, but eventful. The drug was reported to be efficacious in the treatment of rheumatoid arthritis (22) and various chronic inflammatory skin disorders (19). However, reports of drug-related fatal hepatorenal toxicity and a particularly high incidence of cutaneous side effects, especially phototoxicity, led to its withdrawal (19). It could be argued that further research on benoxaprofen would generate little interest. However it is noteworthy that conventional assays of drug toxicity failed to detect the unusually high incidence of cutaneous side effects (phototoxicity, onycholyisis, and eruptive skin tumors on sun-exposed areas) which were associated with benoxaprofen chemotherapy (19, 20). We believe that important insight into novel mechanisms of drug-mediated toxicity and carcinogenesis can be learned from the benoxaprofen experience.

The results of this investigation clearly demonstrate the potential of benoxaprofen to stimulate membrane-associated oxidative metabolism in human phagocytes leading to the release of reactive oxidants which damage DNA in bystander cells. We have previously described the activation of superoxide generation by benoxaprofen in PMNL using a ferricytochrome c reduction assay (17). In the present investigation we have used a lucigenin-enhanced chemiluminescence method (23) which...
has been reported to detect superoxide and to be approximately 10-fold more sensitive than the cytochrome c method (35). In additional control experiments (data not shown) we were unable to demonstrate stimulatory effects on the LECL responses of PMNL of supernatants from MNL which had been pulsed with benoxaprofen (60 µg/ml). This clearly demonstrates that the observed effects on DNA strand breaks are due to PMNL/ benoxaprofen interactions and not to stimulatory factors released by drug-treated MNL. The primary mediator of DNA strand breaks in MNL cocultured with benoxaprofen-activated PMNL appears to be superoxide since inclusion of SOD and H-7, an inhibitor of PKC (24) and superoxide generation (25), protected the MNL. Catalase, on the other hand, was largely ineffective. PMNL and benoxaprofen individually did not alter the structure of DNA in cocultured MNL. The prooxidative interactions of benoxaprofen and human PMNL were significantly potentiated by UV radiation. We have previously reported that this is due to the UVA component of UV radiation and is due to a sensitizing effect on PMNL and apparently not to photostabilization of the benoxaprofen molecule (17). In support of this others have reported that UV radiation exposure primes the membrane-associated oxidative and degranulation responses of PMNL to hyperreact to receptor-mediated activation with the synthetic N-formylated chemotactic tripeptide N-formyl-methionyl-leucyl-phenylalanine (36). UV radiation exposure of benoxaprofen-activated PMNL was associated with a significantly greater frequency of DNA strand breaks in cocultured MNL relative to the corresponding nonirradiated systems. A small, but statistically significant increase in DNA strand breaks was also observed in benoxaprofen-free control systems containing PMNL and MNL and is probably attributable to the slight increase in oxidant generation observed in PMNL irradiated in the absence of benoxaprofen. The association of benoxaprofen chemotherapy with the appearance of eruptive skin tumors on sun-exposed areas could have been a direct consequence of prooxidative interactions of skin phagocytes with the NSAID and UV radiation leading to the generation of tumor-promoting reactive oxidants. Importantly the concentrations used in the present investigation did not exceed 60 µg/ml. During chemotherapy with benoxaprofen (600 mg daily) the mean serum concentrations recorded in 10 patients with rheumatoid arthritis after 3 and 6 months of treatment were 66 ± 16 and 81 ± 18 µg/ml, respectively (22).

Benoxaprofen-mediated activation of oxidant generation in PMNL was extremely sensitive to inhibition by H-7 with an IC_{50} value of 13 µM. We have previously reported that benoxaprofen activates PKC activity in crude extracts of human platelets (18), Although we proposed that benoxaprofen may act at the phosphatidylycerine binding site, our data were inconclusive (18). In the present study, we have extended our previous investigations by measuring the effects of benoxaprofen on the activity of highly purified PKC in the presence of varying amounts of allosteric effectors.

Benoxaprofen stimulated PKC activity in a dose-dependent manner in the presence of diolen and nonsaturating amounts of PS. No further stimulation was observed if the PS concentration exceeded 25 µg/ml, which suggests that the drug may enhance a PS effect at its allosteric site. Whether this is due to competitive binding or to some other more complex stoichiometric interaction between enzyme-lipid vesicles, substrate and allosteric effectors is unknown at this stage. It has recently been suggested that with histone, substrate aggregates may form with lipid vesicles in such a way that access of calcium-dependent, membrane-bound enzyme to its substrate is facilitated (37). Conceivably, benoxaprofen may exert more than one effect in this complex stoichiometrical environment; this may depend on whether the drug is primarily intercalated in the vesicle membrane or not. This is clearly important in the intact cell level where benoxaprofen is able to stimulate pro-oxidative activity despite the presence of saturating amounts of PS. The mechanism of priming of PMNL membrane-associated oxidative metabolism by UV radiation remains to be established.

In conclusion we have shown that UV-potentiated prooxidative interactions which occur between human phagocytes and benoxaprofen lead to the generation of tumor-promoting reactive oxidants. Although benoxaprofen is no longer available these observations are relevant to the early detection of the toxic and carcinogenic potential of new pharmacological agents.

REFERENCES

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