Effect of Ingested Pentoxifylline on Neutrophil Superoxide Anion Production

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Superoxide and other oxygen radicals produced by activated polymorphonuclear leukocytes (PMN) may be important causes of tissue damage in a number of inflammatory conditions. Therefore, a drug which suppresses PMN responses in vivo is potentially important. In vitro, pentoxifylline (PTOX) inhibits superoxide anion production when PMN are stimulated with an activated complement component (C5a Des Arg) or formyl peptides but only at concentrations not achieved in the circulation. The aim of this study was to determine whether PTOX has an effect on PMN responses in vivo. Superoxide anion production, monitored by lucigenin-enhanced chemiluminescence, was inhibited by 40.5% ± 8.0% ($n = 8$, $P < 0.009$) for C5a Des Arg and 47.7% ± 9.6% ($n = 8$, $P < 0.009$) for formyl-methionylleucylphenylalanine stimulation 1.5 h after ingestion of 400 mg of PTOX in a slow-release tablet, with some inhibitory effects persisting at 5 h. There was a strong correlation between reduced PMN response to activated complement and plasma concentrations of three PTOX metabolites ($P < 0.05$), but not with plasma concentrations of the parent drug. In vitro investigations with each of the four methylxanthines showed two of these metabolites to be most effective at reducing PMN respiratory burst activity, lactoferrin release, and the expression of CD11b and CD18 molecules. Furthermore, this in vitro inhibitory activity was achieved at concentrations of metabolites achievable in vivo. The results suggest that PTOX reduces oxygen radical production and protects against unwanted tissue damage in vivo by the action of its metabolites.

Polymorphonuclear leukocytes (PMN) have been implicated in tissue damage following myocardial infarction (19). These cells are also believed to cause tissue damage in a number of inflammatory conditions, one of the most extensively studied being adult respiratory distress syndrome (23). Consequently, a drug which reduces the response of PMN to physiological stimuli at sites of inflammation is potentially important. Among such stimuli are the complement component C5a Des Arg and formyl peptides secreted by bacteria (16). Among the possible toxins released by PMN are reactive oxygen radicals including superoxide anions, hydrogen peroxide, and hydroxyl radicals. Pentoxifylline (PTOX) is a methylxanthine usually prescribed for patients with peripheral or cerebral arterial insufficiency (24), but it is also effective in healing varicose ulcers, a condition in which PMN-mediated tissue damage may be important (4, 13). PTOX has been demonstrated in vitro to exert a number of effects on leukocyte function. These include increasing the filterability of whole blood through its effects on leukocytes and increasing the deformability of blood cells (14, 17). This effect is thought to be due to decreased actin polymerization in cells exposed to the drug. PTOX has been shown to reduce CR3 (CD11b), primary degranulation, and superoxide anion production (5) and inhibit C5a Des Arg-stimulated secondary degranulation (20). However, the concentrations of PTOX used in these investigations are not achieved in the plasma of patients taking normal therapeutic doses (1, 24). It is therefore important to establish whether normal doses of PTOX affect the function of circulating leukocytes and, if so, whether this is due to the parent drug or one or more of its metabolites.

MATERIALS AND METHODS

Subjects. Venous blood was obtained from normal healthy volunteers and placed into EDTA-dipotassium at a final concentration of 3 mM. Samples were taken just before ingestion of 400 mg of slow-release PTOX and then at 1.5, 5, 24, and 48 h. The blood was used immediately after venesection.

For in vitro investigations of PTOX and its metabolites, EDTA-anticoagulated blood was obtained from normal healthy volunteers, and the purified PMN were isolated and exposed to increasing concentrations of methylxanthines in vitro.

Preparation of PMN. The PMN were isolated by a rapid single-step technique. The blood was layered onto a Ficoll-Hypaque solution with a density of 1.114 g ml^{-1} (Mono-Poly Resolving Medium; Flow Laboratories). After centrifugation at 400 × g for 40 min at 22°C, the leukocytes resolved into two bands: mononuclear leukocytes formed a band at the interface, and PMN collected in a second band below the mononuclear cells. During centrifugation, the erythrocytes sedimented to the bottom of the tube. The PMN were collected from the second band and washed in sterile phosphate-buffered saline (PBS) (pH 7.2). PMN collected in this way were always of a purity greater than 97% with a viability greater than 99% as determined by trypan blue exclusion. Cells were suspended in sterile, low-endotoxin PBS at a concentration of 10^7 PMN ml^{-1} and were kept on ice until required.

Superoxide anion production. Superoxide anion production was determined by lucigenin-enhanced chemiluminescence with a Bio-Orbit 1251 luminometer. Lucigenin is reported as being a specific probe for the detection of superoxide anions involving a three-step reaction (9). Briefly, 350 μl of PBS (pH 7.2) containing 1 mM CaCl$_2$, 0.7 mM MgCl$_2$, and 0.1% bovine serum albumin (BSA) (Sigma Chemical Co.), 50 μl of 2.5 × 10^{-5} M lucigenin (Sigma

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Chemical Co.), and 50 μl of PMN suspension were added to luminometer cuvettes. The cuvettes were warmed in the luminometer to 37°C, and the PMN were stimulated with zymosan-activated serum (ZAS) at a final concentration of 10% (vol/vol) or by formyl-methionylleucylphenylalanine (FMLP) at a final concentration of 10^{-6} M. Chemiluminescence light units were recorded as millivolts, and readings were started immediately after the addition of the stimulus and were monitored continuously for up to 28 min. The integral chemiluminescence readings were taken as a measure of overall superoxide anions over this time. We chose 28 min because by this time peak superoxide anion production in response to both stimuli had been achieved. With chemotactic stimuli, the respiratory burst tends to be biphasic, consisting of a highly elevated first phase lasting only 1 to 3 min that is followed by a slower, less active phase which may last for more than 30 min (3). Therefore, taking the integral chemiluminescence after 28 min allowed for the measurement of both these phases.

To confirm that increased light output was associated with superoxide anion production, we did experiments with control cuvettes containing 50 μg of superoxide dismutase (Sigma Chemical Co.) ml^{-1}, which completely inhibited the chemiluminescence response.

Zymosan-activated serum. A standard supply of ZAS was produced by the method of Fernandez et al. (6). Briefly, blood was taken from 12 healthy volunteers, placed into serum tubes, and allowed to clot at room temperature. These samples were then centrifuged at 1,000 × g for 15 min, and the serum supernatants were harvested and then pooled together. The pooled serum was incubated with zymosan A (Sigma Chemical Co.) at a concentration of 1 mg ml^{-1} on a rotating wheel at 37°C for 60 min. The zymosan particles were removed by centrifugation at 1,000 × g for 15 min, and this was followed by filtration of the supernatant twice through 0.2-μm-pore-size, sterile, pyrogen-free filters (Sartorius). The filtration step was done twice to ensure removal of any residual opsonized zymosan particles. The ZAS was then aliquoted and stored at −70°C until required.

In vitro effects of methylxanthines. The effects of PTOX and its metabolites were determined after preincubation of PMN with each of the four methylxanthines tested diluted in PBS (pH 7.2) containing 0.1% (wt/vol) BSA at 37°C with constant agitation for 30 min. The lucigenin-enhanced chemiluminescent response to FMLP stimulation was determined as described above. The effect of these methylxanthines on lactoferrin (Lf) release was determined by using a sensitive enzyme-linked immunosorbent assay (ELISA) for determination of secreted Lf after preincubation with methylxanthines and then FMLP stimulation (cells were not treated with cytochalasin B).

Measurement of Lf. The ELISA method to measure Lf involved adding 200 μl of rabbit anti-human Lf polyclonal antibody (Dako) at a dilution of 1:1,000 in 50 mM sodium carbonate-bicarbonate coating buffer (pH 9.5) to each well of a 96-well microtiter plate (Dynatech). After incubation overnight at 4°C, the plates were washed with PBS (pH 7.4). Blocking buffer (2% BSA [wt/vol] in PBS [pH 7.4]) was added in a 200-μl volume to each well to block any unfilled binding sites; the plate was then incubated at room temperature for 30 min. After washing (four times for 4 min each) in PBS (pH 7.4) containing 0.01% Tween 20 (vol/vol; BDH), Lf standards were prepared from a stock solution of 10^{-6} M Lf (Calbiochem) to give a range of standards from 0.05 to 0.40 nmol. Standards were diluted in PBS (pH 7.4) containing 0.5% BSA (wt/vol) and 0.01% Tween 20 (vol/vol). Standards

and samples (diluted 1:5) (50 μl each) were added to each well and incubated on a continuous shaker for 2 h at room temperature. The plates were washed as previously described, and 50 μl of biotinylated anti-Lf at a dilution of 1:1,000 was added to each well. The plates were incubated for 1.5 h at room temperature with continuous shaking. After washing, 50 μl of a 1:1,000 dilution of avidin-peroxidase conjugate (ICN Biochemicals) in borate saline buffer (pH 8.6) was added to each well and incubated with continuous shaking for 1 h at room temperature. The plates were washed with borate saline (pH 8.6) containing 0.01% Tween 20 (four times for 4 min each time). 5-Aminosalicylic acid at 1 mg ml^{-1} in 0.02 M phosphate substrate buffer plus 0.01% (vol/vol) hydrogen peroxide in a 100-μl volume was added to each well and incubated with continuous shaking for 40 min at room temperature. The reaction was stopped by the addition of 50 μl of 3 M NaOH to each well. After vigorous mixing, the plate was read immediately at 455.5 nm against a 620-nm reference, using a Kontron SLT 210 plate reader.

Adhesion protein expression. CD11b and CD18 expression was determined by flow cytometry by an indirect immunofluorescence technique. PMN at 10^6 ml^{-1} in PBS-Ca^{2+}-Mg^{2+}-BSA were preincubated for 30 min at 37°C in the presence of methylxanthines at increasing concentrations. FMLP at a final concentration of 10^{-6} M was then added (or PBS [pH 7.2] was added as a control), and cells were incubated for a further 15 min. After this time, the PMN were centrifuged at 400 × g for 10 min and washed twice in cold sterile PBS (pH 7.2). Mouse monoclonal antibodies to human CD11b and CD18 (Dako) were added at a final dilution of 1:200 in PBS (pH 7.2) containing 0.1% BSA (wt/vol). The PMN were incubated at 4°C for 30 min. After centrifugation and washing in PBS (pH 7.2) (twice), the cells were then incubated with a rabbit anti-mouse fluorescein isothiocyanate-conjugated polyclonal immunoglobulin G_1 $F(ab')_2$ (Dako) at a final concentration of 1:30. The cells were centrifuged and washed twice before being analyzed with a Becton Dickinson FACScan. PMN were selectively gated by their light-scattering properties; a change in the expression of CD11b and CD18 was calculated as the percent shift in mean fluorescence over readings for unstimulated controls.

Measurement of plasma PTOX and metabolites. Plasma levels of the parent drug and its three metabolites were kindly measured by Hoechst UK Limited. Methylxanthines in the plasma were detected by automated capillary gas chromatography with nitrogen-selective detection by the method of Burrows (2). The metabolites measured were BL-194 (5'-hydroxyhexyl-3,7-dimethylxanthine), MET IV (4'-carboxybutyl-3,7-dimethylxanthine), and MET V (3'-carboxypropyl-3,7-dimethylxanthine).

Limulus **amoebocyte lysate assay.** All reagents were tested for the presence of endotoxin by the E-Toxate assay (Sigma Chemical Co.). The assay detected endotoxin down to 10 pg ml^{-1}. Reagents containing more than 20 pg of contaminating endotoxin ml^{-1} were not used in any of the experiments.

Statistics. Data are expressed as the means ± the standard errors of the mean. Data were compared by the Wilcoxon signed-rank nonparametric test; for determination of correlations between decreased chemiluminescence responses ex vivo after administration of the drug and levels of circulating methylxanthines, the Spearman rank correlation coefficient was used. Values of $P < 0.05$ for both tests were considered to be significant.
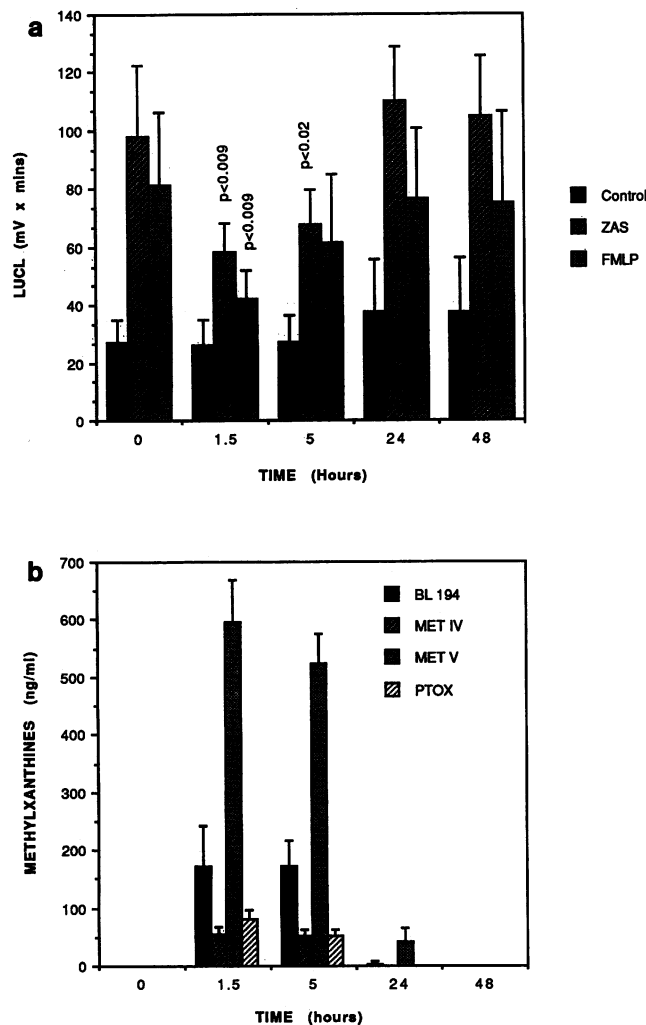
FIG. 1. (a) Effect of ingestion of 400 mg of slow-release PTOX on PMN lucigenin-enhanced chemiluminescence (LUCL) ex vivo. PMN once isolated were stimulated with either ZAS or FMLP or left unstimulated (control). (b) Concentrations of methylxanthines (PTOX and metabolites) in plasma. The results represent the means of observations from eight separate subjects ± SEM.

FIG. 2. In vitro effect of increasing concentrations of methylxanthines on lucigenin-enhanced chemiluminescence (LUCL) in response to 10^{-6} M FMLP stimulation. The PMN were incubated with methylxanthines for 30 min at 37°C before the addition of stimulus. The results represent the means of seven separate experiments ± SEM and were significant at $P < 0.05$ (*) and $P < 0.025$ (**).

RESULTS

Ex vivo superoxide anion production in response to ZAS and FMLP. Figure 1a shows that at 1.5 h after ingestion of 400 mg of PTOX, superoxide anion production was reduced in response to both ZAS and FMLP stimulation. This reduction in observed chemiluminescence was significant for both stimuli ($P < 0.009$) when compared with the results obtained at time zero. By 5 h, superoxide anion production in response to FMLP was no longer significantly different, but the response to ZAS remained significantly reduced ($P < 0.02$). At 24 h, there appeared to be a slight enhancement in superoxide anion production in response to both stimuli; however, this response was not significantly greater than that seen before ingestion of PTOX. By 48 h, the responses observed were the same as at time zero.

Plasma levels of PTOX and metabolites. As shown in Fig. 1b, the highest levels of the intact drug were detected 1.5 h postingestion. At 5 h, the circulating levels of all four methylxanthines were slightly reduced, and at 24 h, only
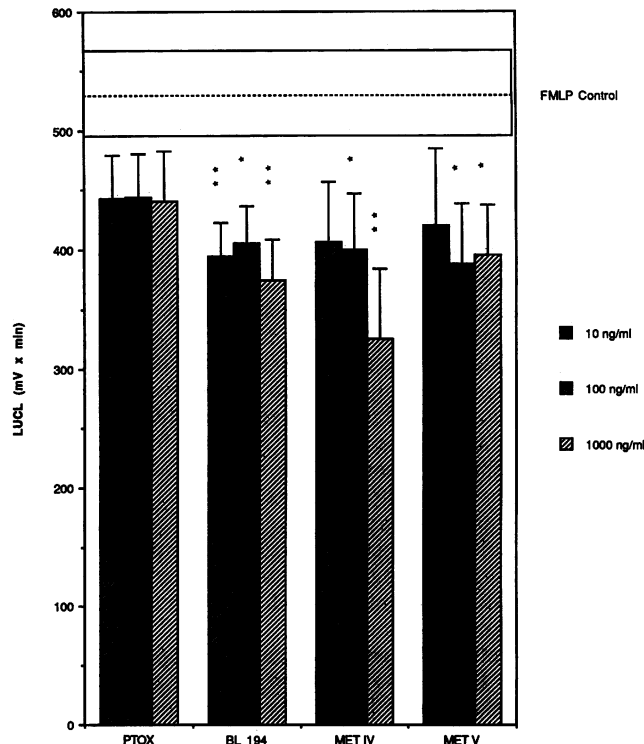
BL-194 and MET V could be detected in the plasma. By 48 h, there was no detectable drug or metabolites in the plasma. While decreased superoxide anion production was associated with peak levels of circulating methylxanthines, it was not possible to associate the effects with any one of the metabolites in particular. With all the metabolites, there was a correlation ($r_s = -0.298, P < 0.05$ for BL-194; $r_s = -0.312$, $P < 0.05$ for MET IV; and $r_s = -0.428, P < 0.04$ for MET V) between decreased chemiluminescence and the concentration of each metabolite detectable in the plasma; however, the correlation between superoxide anion production in response to ZAS and the levels of parent drug ($r_s = -0.208$) was not significant.

Effect of methylxanthines on PMN superoxide anion production in vitro. To determine whether one or all of the metabolites were more effective at inhibiting PMN function, we tested all four methylxanthines for their ability to affect respiratory burst activity (Fig. 2). When methylxanthines were added to luminometer cuvettes immediately before or after FMLP stimulation, no effect was observed (data not shown). However, when cells were preincubated with methylxanthines at 37°C for 30 min before stimulation with FMLP, all three metabolites tested induced a significant reduction in the amount of chemiluminescence observed at the physiologically relevant concentration of 100 ng ml^{-1}. None of the concentrations of PTOX used showed this inhibitory activity. These data therefore confirm the ex vivo results which indicated that it was the metabolites and not
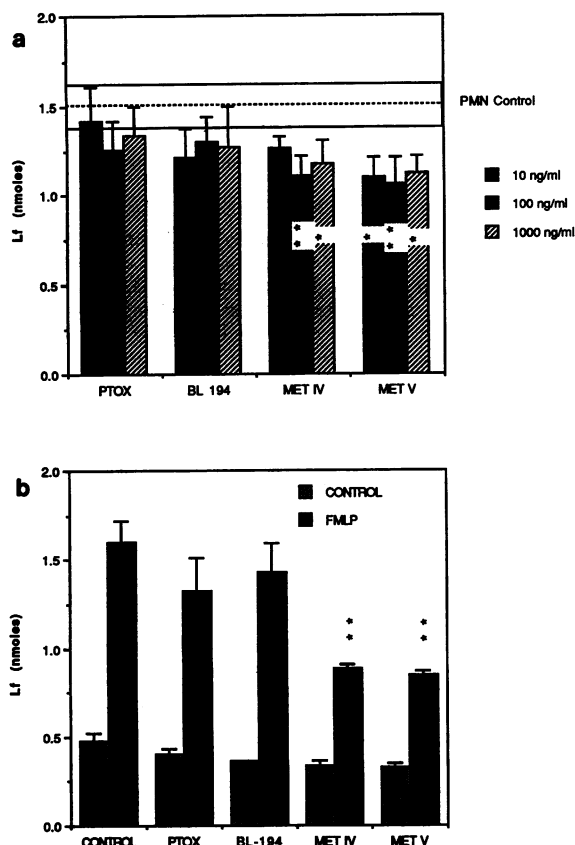
FIG. 3. (a) Effect of increasing concentrations of methylxan-thines on Lf released as a result of warming PMN from 4 to 37°C. (b) Effect of 100 ng of methylxanthines ml^{-1} with the addition of 10^{-6} M FMLP. The results represent the means of seven separate experiments ± SEM. Results were significant at $P < 0.04$ (*) and $p < 0.03$ (**).

FIG. 4. (a) Effect of increasing concentrations of methylxan-thines on CD11b and CD18 expression on PMN warmed from 4 to 37°C. (b) Effect of 100 ng of methylxanthines ml^{-1} on adhesion molecule expression in response to 10^{-6} M FMLP. The results represent the means of six separate experiments ± SEM and are calculated as the percent shift in mean fluorescence over controls.

the parent drug that were effective at reducing PMN respiratory burst activity.

Effect of methylxanthines on Lf release. Figure 3 shows that MET V was the most effective at reducing Lf secretion from cells stimulated by simply warming from 4 to 37°C, and with the additional stimulus of FMLP. Again, the parent drug had no effect on reducing Lf release at any of the concentrations used.

Effect of methylxanthines on CD11b and CD18 expression. As shown in Fig. 4, the most marked reduction in adhesion molecule expression was seen with MET V. PMN increase their expression of CD11b and CD18 after the cells are warmed (11), and MET V was effective at reducing this increased expression under these conditions. With the additional stimulus of FMLP, MET V again caused the most marked reduction in CD11b and CD18 expression. ·

DISCUSSION

Previous in vitro investigations have shown that PTOX is capable of inhibiting a number of aspects of neutrophil function including Lf release (20) and respiratory burst activity (5). PTOX has also been shown to inhibit tumor necrosis factor production from mononuclear cells stimulated with bacterial lipopolysaccharide (21). It would therefore appear that this particular methylxanthine is capable of
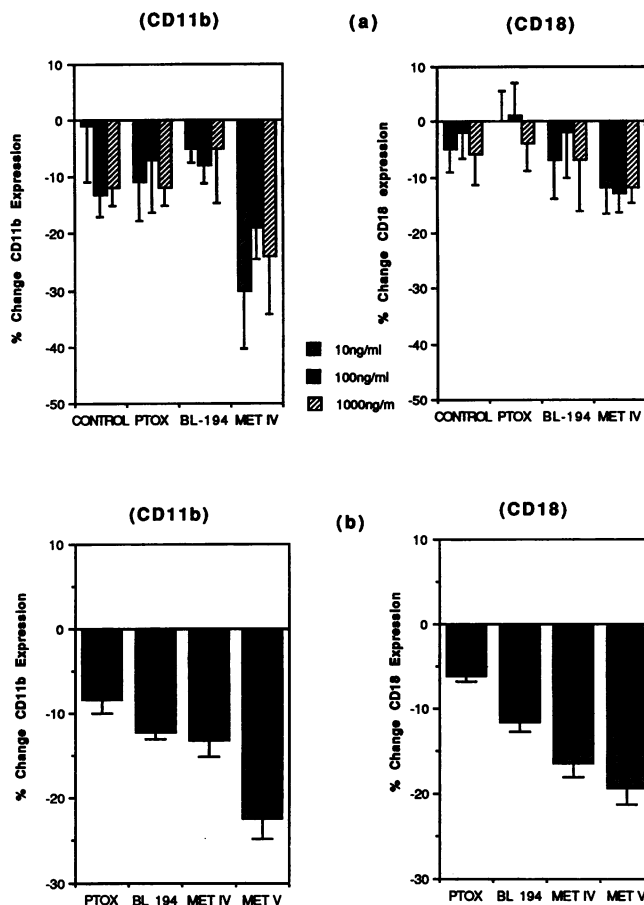
down-regulating a number of responses involved in promoting inflammation. However, these and a number of other studies (10, 15, 22) were done with concentrations of PTOX which are unachievable in vivo. In the present study, high concentrations of PTOX (10 μg to 1 mg ml^{-1}) could not be achieved in the circulation of subjects receiving one 400-mg slow-release tablet.

In the present study, plasma concentrations of about 100 ng of PTOX ml^{-1}, corresponding to approximately 3×10^{-7} M, were found together with significant depression of PMN response to both ZAS and FMLP stimulation. This conflicts with published in vitro data, which would predict either potentiation of responses or no effect at these concentrations (18). The slight enhancement seen at 24 h may be a result of the low levels of BL-194 and MET V detected in the plasma at this time point.

The implication of the ex vivo data presented here is that part of the effect of PTOX on circulating PMN is due to its metabolites. Of those measured, MET V reached the highest plasma concentrations at 600 ng ml^{-1}, approximately 2×10^{-6} M, but this is still much lower than the concentration of the parent drug required for the same effect in vitro. It would seem either that one or more of the metabolites are more active than the parent compound or that the drug and its

metabolites are more effective under in vivo conditions than in vitro conditions. One factor may be the time during which PMN are exposed to the drug, since in vitro, short incubation periods lasting only a few minutes are used (10, 22), while in vivo, the first observations were made after 1.5 h. To determine whether one or all of the metabolites were more effective at inhibiting PMN function, we tested all four methylxanthines in vitro for their ability to affect respiratory burst activity, Lf release, and adhesion molecule expression.

In vitro, all three metabolites induced a significant reduction of superoxide anion production at the physiologically relevant concentration of 100 ng ml^{-1}. None of the concentrations of PTOX used showed this inhibitory activity. These data therefore confirm the ex vivo results, which indicated that it is the metabolites and not the parent drug that are effective at reducing PMN respiratory burst activity.

PTOX has been used in the treatment of venous leg ulcers, in which it has been shown to improve ulcer healing when used in conjunction with compression bandaging (13). It is therefore possible that PTOX reduces tissue damage by reducing the production of toxic oxygen radicals. Methylxanthines may also reduce continued inflammation in leg ulcers by reducing the number of PMN in the marginated pool and those entering the tissues. Adhesion of PMN to endothelium is dependent on the expression of CD11b and CD18 molecules on the PMN surface (11, 25). Of the methylxanthine metabolites tested in vitro, MET V was the most effective at reducing both Lf release and adhesion molecule expression. This suggests that this particular metabolite is effective at reducing the adhesiveness of the PMN by reducing CD11b and CD18 expression and also by inhibiting Lf release. Since Lf is a strongly cationic protein, it may act to negate the electrostatic forces between the PMN and endothelium (8).

These investigations suggested that MET V is the most effective at reducing PMN activity both in vivo and in vitro because of its effectiveness in vitro and because it was found at the highest concentrations in the plasma.

The mechanism by which methylxanthines depress neutrophil responses is not known. It is usually assumed that they inhibit phosphodiesterase activity as determined by the measurement of increased cyclic AMP levels. However, this effect on cellular cyclic AMP only occurs at concentrations of methylxanthines far higher than those detected in the plasma. Methylxanthines can also act as antagonists of both A1 and A2 adenosine receptors (7), and it has been suggested that this is the mechanism by which they potentiate neutrophil responses at low concentrations (18). However, more recent evidence suggests that this is not the mechanism for inhibition of superoxide anion production from PMN when PTOX is used at higher concentrations (22). The most likely explanation remains that methylxanthines inhibit phosphodiesterase, as increased levels of cyclic AMP certainly reduce PMN responses (12).

The stimuli used in this study are physiologically relevant. ZAS contains C5a Des Arg, a potent stimulus to PMN via a specific receptor, and this complement component is generated during inflammation. FMLP is one of a family of formyl peptides shown to be secreted by certain bacteria (16) for which there are specific receptors on the surface of PMN. Consequently, it is probable that PTOX will depress PMN responses to inflammatory stimuli in vivo and reduce unwanted tissue damage. It is quite possible that this is the mechanism of action in potentiating the healing of varicose ulcers. There are a number of other agents such as the inflammatory mediators histamine and adrenalin and drugs

such as calcium channel antagonists which have effects similar to those of PTOX in vitro and whose in vivo influence on PMN should be explored.

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