10. P. F. Morgan and T. W. Stone, *IRCS Med. Sci.* **13**, 505 (1985).
11. P. F. Morgan, H. G. E. Lloyd and T. W. Stone, *Eur. J. Pharmac.* **87**, 121 (1983).
12. P. F. Morgan, H. G. E. Lloyd and T. W. Stone, *Neurosci. Lett.* **41**, 183 (1983).
13. W. Hunkeler, H. Mohler, L. Pieri, P. Polc, E. P. Bonetti, R. Cumin, R. Schaffner and W. Haefely, *Nature, Lond.* **290**, 514 (1981).
14. J. R. Hammond and A. S. Clanachan, *Br. J. Pharmac.* **76**, 301P (1982).
15. J. Patel, P. J. Marangos, P. Skolnick, S. M. Paul and A. M. Martino, *Neurosci. Lett.* **29**, 79 (1982).
16. A. S. Bender, P. H. Wu and J. W. Phillis, *J. Neurochem.* **35**, 629 (1980).
17. C. Barberis, A. Minn and J. Gayet, *J. Neurochem.* **36**, 347 (1981).
18. C. Braestrup, M. Nielsen and C. E. Olsen, *Proc. natn. Acad. Sci. U.S.A.* **77**, 2288 (1980).
19. H. Rommelspacher, C. Nanz, H. O. Borbe, K. J. Fehske, W. E. Muller and U. Wollert, *Archs Pharmac.* **314**, 97 (1980).
20. A. Concas, M. Salis, M. Serra, M. G. Corda and G. Biggio, *Eur. J. Pharmac.* **89**, 179 (1983).
21. L. P. de Carvalho, G. Greksch, G. Charpouthier and J. Rossier, *Nature, Lond.* **301**, 64 (1983).
22. M. Nielsen and C. Braestrup, *Nature, Lond.* **286**, 606 (1980).
23. D. J. Nutt, P. Cowen and H. J. Little, *Nature, Lond.* **295**, 436 (1982).
24. M. Schweri, M. Cain, J. Cook, S. Paul and P. Skolnick, *Pharmac. Biochem. Behav.* **17**, 457 (1982).
25. A. Valin, R. H. Dodd, D. R. Liston, P. Potier and J. Rossier, *Eur. J. Pharmac.* **85**, 93 (1982).

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Inhibition of phorbol ester-stimulated chemiluminescence and superoxide production in human neutrophils by fructose 1,6-diphosphate

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Fructose, 1,6-diphosphate (FDP) has been shown to influence cellular responses by interacting with several types of cell membranes and by regulating the glycolytic pathway of cells, with consequent ATP production, despite its inability to cross the cell membranes [1, 2]. Our previous experiments have demonstrated the effectiveness of FDP in preventing mast cell histamine release, induced by several agents [3, 4], and the ability of the drug to increase the ATP content, both in stimulated and unstimulated cells. The aim of the present experiment was to investigate the action of FDP on neutrophil oxidative metabolism in order to obtain further insight into the possible role of FDP in inflammatory processes, in the light of the stimulatory effects of the drug on the phagocytic activity of RES [5]. The generation of reactive oxygen radicals by human neutrophils plays an essential role in host defense, because together with their antimicrobial properties, they are capable of destroying a wide variety of biological targets [6]. These oxygen derivatives can also, in turn, modulate the inflammatory response, with host tissue destruction. The neutrophil oxidative metabolism can be detected through the chemiluminescent response (CL), arising from activated cells, which is an index of both the generation and the biochemical processes mediated by oxygen radicals [7]. Moreover in digitonin stimulated neutrophils activation is an energy-requiring process, which is linked to ongoing ATP synthesis [8]. On the other hand it has also been suggested that ATP might be a physiological regulator of the catalytic activity of the enzyme responsible for O_2^- production [9]. For this reason, investigations were also made into the effects of FDP, a glycolysis stimulator [1], on ATP intracellular variations, to test whether the drug could influence the neutrophil oxidative burst, by interfering with energy metabolism.

Materials and methods

Ferricytochrome C (type VI), bovine superoxide dismutase, phorbol myristate acetate (PMA), xanthine, xanthine-oxidase and luminol were obtained from Sigma (Saint Louis, MO). Kits for the bioluminescent assay for ATP were purchased from Packard (Milano, Italy). Fructose 1,6-diphosphate (Esafosfina) was kindly supplied by Biomedica Foscama. Eagle modified Dulbecco medium for chemiluminescence (MEM), phosphate-buffered saline (PBS) were from Boehringer Biochemia (Milano, Italy). Fycoll-Hypaque was obtained from Flow Lab. (Milano, Italy).

Cell isolation. Human neutrophils were isolated from heparinized peripheral blood of healthy adult donors and purified by one-step density gradient centrifugation on Ficoll-Hypaque following the method of Ferrante *et al.* [10].

Chemiluminescence. The luminol amplified chemiluminescent response induced by PMA stimulation of the cells was used to monitor neutrophil oxidative metabolism, after challenging the cell suspension (2×10^5 cells/ml) with 0.1 μg/ml of PMA in the presence or the absence of different concentrations of fructose 1,6-diphosphate (0.2 mM, 1 mM, 5 mM). A Packard Picolite Luminometer, thermostatted at 37°, operating in the dark was used to measure CL. Results are expressed as counts per minute. Statistical evaluation of the integrated area underlying the CL curves, was performed by Student's *t*-test for paired data.

O_2^- *assay.* Superoxide production was determined as SOD-inhibitable cytochrome *c* reduction at 37° in 1 ml cell suspension containing 10^6 neutrophils, 0.1 μg/ml PMA, FDP (0.2 mM, 1 mM, 5 mM), as described by Cohen and Chovaniec *et al.* [8]. The cell free superoxide generating system, xanthine–xanthine oxidase, was used to test whether FDP could act as O_2^- scavenger, following the method of Fridovich [11].

ATP determination. Samples for ATP determination were incubated for 5 min with FDP (0.2–5 mM) at 37°, before challenging the cells suspension with 0.1 μg/ml PMA. The cells were further incubated for 10 min and ATP intracellular levels were measured after extraction with Pico Ex S, using the bioluminescent luciferine–luciferase reaction.

Results and discussion

Incubation of neutrophils with FDP for 10 min before stimulation with PMA markedly affected the cell oxidative metabolism, as revealed by the degree of inhibition exerted on the luminol-amplified chemiluminescence. The inhibition was dose-dependent over the range 0.2 mM–5 mM, being maximal at the highest FDP concentration (80% inhibition) as shown in Fig. 1. The effect of FDP on the CL suggests that, possibly, the generation and efflux of a wide variety of reaction oxygen species is inhibited. Since

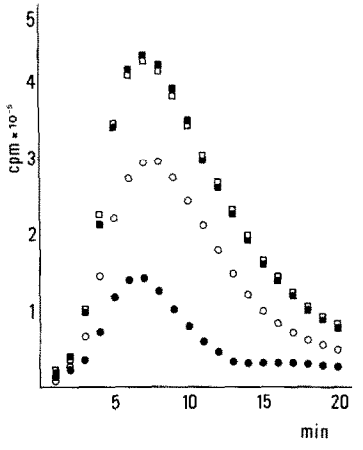

Fig. 1. The chemiluminescent response of PMA-stimulated human neutrophils in the absence (□) or the presence of increasing doses of fructose, 1,6-diphosphate: (■) 0.2 mM, (○) 1 mM, (●) 5 mM. The differences between FDP pretreated cells and PMA alone treated cells are significant ($P < 0.02$) at the 1 mM and 5 mM ($P < 0.01$) FDP concentrations.

luminol, which amplifies the photon emission during neutrophil stimulation is oxidized by several oxygen intermediates [12], practically any O_2 metabolite may be implicated in the production of CL. For this reason, little can be inferred about the free radical whose generation is antagonized by FDP. The cytochrome *c* reduction test revealed that FDP, in the dose range used in CL experiments, inhibited PMA-induced superoxide production more specifically (Fig. 2). Moreover, when the drug was exposed to the superoxide anions generated by the xanthine–xanthine oxidase enzymatic system, it was found to be devoid of any SOD-like activity. Consequently since FDP did not act as an O_2^- scavenger it should inhibit superoxide release by acting on the O_2^- generating system or by directly influencing PMA cell activation. Even if superoxides are not in themselves sufficient for light emission [12, 13], they appear to be involved in chemiluminescence production. As demonstrated by Kensler and Trush [14], it is possible to achieve an effective decrease of phorbol ester-induced CL by quenching superoxide. However, O_2^- can be converted to other highly reactive species such as $OH^{\cdot}$, $^1O_2$, H_2O_2 or OCl^-, which in turn may interact with excitable substrates amplifying the CL response. A membrane-mediated effect might be attributed to the action of FDP since the drug seems to be unable to enter the cells [15]. In several cell membranes, FDP binding is followed by FDP hydrolysis with P_i liberation, suggesting the involvement of a membrane-bound phosphatase [2]. In red blood cells, a Ca-ATPase has been suggested as the specific receptor of FDP [16], and a Ca^{2+}-ATPase also exists on the neutrophil plasma membrane [17]; however, it seems unlikely that the inhibitory effect of the drug could have resulted from a regulation of the calcium efflux because the cell response to PMA is not mediated by an increase in intracellular calcium. In fact, when PMA is used as a stimulator, neutrophil activation can be triggered even if $[Ca^{2+}]_i$ is lowered 10–20 times below the resting level [18]. Besides, as recently demonstrated, PMA itself induces calcium efflux, through the activation of the Ca^{2+}-ATPase [19] and as far as O_2^- is concerned, PMA-induced superoxide production is not inhibited but rather activated by the removal of extracellular calcium [20]. A primary effect of FDP on the enzyme controlling O_2^- generation seems to be improbable, even assuming an increase in the intracellular concentration of FDP as a consequence of drug binding to the cell surface, as suggested by the experiments of Lazzarino *et al.* [21]. As Badwey *et al.* have demonstrated, FDP is ineffective as an inhibitor, when tested *in vitro* on the solubilized oxidase [9]. Therefore a metabolic regulation of the cell oxidative metabolism carried out by FDP might take place (through ATP formation). In our experiments PMA addition to the cells caused a marked depletion of intracellular ATP (an 81% decrease, $P < 0.001$ compared with controls) which was counteracted by FDP pretreatment (Table 1). As Babior *et al.* have suggested [22], ATP might play a physiological role in regulating the catalytic activity of the NADPH-oxidase of human neutrophils. When added to the solubilized enzyme, ATP caused loss of activity within seconds. An ATP inhibition of the NADH-oxidase of guinea-pig neutrophils has also been reported [9] and free unchelated ATP has been proposed as the effector of the inhibitor exerted on the enzyme in resting cells; the cell stimulation relieves enzyme inhibition by diminishing the levels of free ATP. The authors suggest that the influx of calcium, when phagocytosable stimuli or ionophores are involved, could contribute to a decrease in free ATP, leading to oxidase activation. The trigger mechanism of PMA is independent from a rise in intracellular calcium, but our experiments have demonstrated that PMA treatment of the cells causes ATP to fall below the resting level, suggesting ATP depletion as part of the trigger mechanism. It should be observed that the maximally inhibitory dose of FDP (5 mM), both on O_2^- release and GL response (about 80% inhibition) is the one causing maximal stimulation of ATP synthesis. Even in unstimulated cells 5 mM FDP induced a highly significant variation in the ATP intracellular level ($P < 0.001$ compared with controls) (Table 1). The way the drug acts on

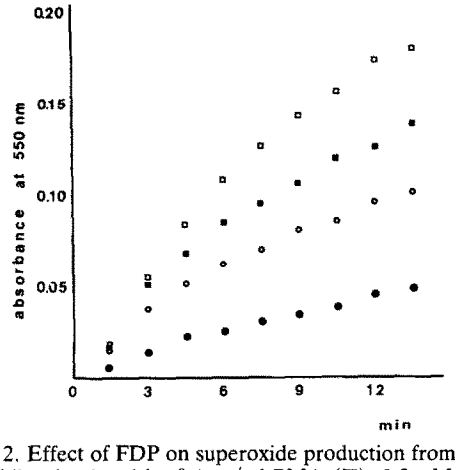

Fig. 2. Effect of FDP on superoxide production from neutrophils stimulated by 0.1 μg/ml PMA (□), 0.2 mM FDP (■), 1 mM FDP (○), 5 mM FDP (●).

Table 1. The dose related effect of FDP on ATP intracellular levels in resting and PMA stimulated cells

	PMA	
	0	0.1 μg/ml
	2.72 ± 0.19	0.574 ± 0.05
FDP 0.2 mM	2.132 ± 0.058	0.626 ± 0.040
FDP 1 mM	2.660 ± 0.072	1.036 ± 0.178*
FDP 5 mM	3.256 ± 0.109	1.859 ± 0.109*

ATP values are expressed as nmoles/10^6 cells as mean values (± S.E.M.) of 3 separate experiments, done in duplicate.

* $P < 0.001$ compared to PMA alone treated cells.

the whole oxidative metabolism might also suggest a direct interference with the signalling mechanism, either by directly interfering with the link of PMA with its receptor or by counteracting the depolarising effect of PMA on the plasma membrane [23]. In fact an FDP-induced membrane stabilizing effect could have resulted from the changes in ion permeability, with K^+ uptake, brought about by FDP binding to the cell surface [2].

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REFERENCES

1. M. E. Kirtley and M. McKay, *Mol. Cell Biochem.* **18**, 141 (1977).
2. M. P. Rigobello, M. Bianchi, R. Deana and L. Galzigna, *Aggressologie* **23**, 63 (1982).
3. M. L. Schinetti, R. Greco, G. Lazzarino and A. Bertelli, *Int. J. Tiss. Reac.* **V**, 55 (1983).
4. M. L. Schinetti, R. Greco, G. Lazzarino, G. Soldani and A. Bertelli, *Arzneim. Forsch./Drug Res.* **33**, 834 (1983).
5. R. Tarsi, L. Mulieri, F. Venturi and M. Simonetti, *J. Leukocyte Biol.* **37**, 72 (1985).
6. S. J. Weiss, in *Immunology of Inflammation*, (Ed. Peter Wards) p. 37, Elsevier, Amsterdam (1983).
7. M. A. Trush, M. E. Wilson and K. Van-Dyke, *Meth. Enzymol.* **LVII**, 462 (1978).
8. H. J. Cohen and M. E. Chovaniec, *J. clin. Invest.* **61**, 1088 (1978).
9. J. A. Badwey and M. L. Karnovsky, *J. biol. Chem.* **254**, 11530 (1979).
10. A. Ferrante and Y. N. Thong, *J. Immunol. Meth.* **24**, 389 (1978).
11. I. Fridovich, *J. biol. Chem.* **245**, 4053 (1970).
12. K. Cheung, A. C. Archibald and M. F. Robinson, *J. Immunol.* **130**, 2324 (1983).
13. L. C. McPhail, L. R. DeChatelet and R. B. Johnston, Jr., *J. clin. Invest.* **63**, 648 (1979).
14. T. W. Kensler and M. A. Trush, *Biochem. Pharmac.* **32**, 3485 (1983).
15. C. Clouverakis, *Metabolism* **17**, 703 (1968).
16. L. Galzigna, M. P. Rigobello, G. Scutari and L. Cattani, *Experientia*, in press.
17. H. Lagast, P. D. Lew and F. A. Waldvogel, *J. clin. Invest.* **73**, 107 (1983).
18. F. Di Virgilio, D. P. Lew and T. Pozzan, *Nature, Lond.* **310**, 691 (1984).
19. H. Lagast, T. Pozzan, F. A. Waldvogel and P. D. Lew, *J. clin. Invest.* **73**, 878 (1984).
20. N. Okamura, M. Uchida, T. Ohtsuka, M. Kawanishi and S. Ishibashi, *Biochem. biophys. Res. Comm.* **130**, 939 (1985).
21. G. Lazzarino, L. Cattani, R. Costrini, L. Mulieri, A. Candiani and L. Galzigna, *Clin. Biochem.* **17**, 42 (1984).
22. B. M. Babior and W. A. Peters, *J. biol. Chem.* **256**, 2321 (1981).
23. J. C. Whitin, C. E. Chapman, E. R. Simons, M. E. Chovaniec and H. J. Cohen, *J. biol. Chem.* **255**, 1874 (1980).

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The incorporation of palmitic acid into lipids in the rat after treatment with oleylanilide

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The potential toxicity of fatty acyl anilides in man was first raised in 1981 in connection with the Toxic Oil Syndrome [1]. Acyl anilides were found to be major contaminants of cooking oils, consumption of which was associated with mass poisoning in Spain. Suitable animal models for study of the syndrome have not, however, been generally established and there are conflicting reports on the toxicity of anilides [2]. Our studies in the rat have shown extensively hydrolysis of anilides after intragastric administration prior to or during absorption and we have been unable to detect anilides in body tissues [3]. Because of the toxicological importance of the general problem, we were therefore concerned by the detailed study of Casals *et al.* [4], showing significant disturbances in lipid metabolism in lung and adipose tissue after oral administration of oleylanilide to rats. We have repeated their dosing schedule and studied the incorporation of label into lipid fractions of lung, liver and epididymal fat following intravenous administration of labelled palmitic acid. This communication reports briefly on our failure to observe any significant disturbance in fatty acid incorporation into lipids following repeated oral dosing of oleylanilide.

Materials and methods

Chemicals. (1-14C) palmitic acid was obtained from the Radiochemical Centre (Amersham, U.K.). Oleic acid and aniline were obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.). Oleylanilide was prepared by heating oleic acid (1 g) and aniline (2 g) at 150° for 24 hr, followed by acid and bicarbonate washes and recrystallization from methanol. Purity (98%) was checked by capillary gas chromatography with a flame ionization detector and the product identified by mass spectrometry [3].

Rats and dosage. Male Porton-derived Wistar rats, weighing 149–175 g were used. Four rats received daily intragastric doses of 5 mg oleylanilide in olive oil (0.5 ml) for 13 days. Dosing was then stopped and the experiments carried out on day 18 [4]. Four control rats received olive oil alone. All rats had free access to food and water.

Experiment. Each rat received an injection via a tail vein of about 1.6 μCi (1-14C)-palmitic acid in rat serum (0.2 ml), prepared as described by Cunningham [5]. After 10 min the animals were decapitated and samples of lung, liver and of perirenal and epididymal fat were frozen in liquid nitrogen for subsequent lipid extraction.

Extraction of lipids. Samples of tissue were homogenized in chloroform/methanol (2:1 v:v) using an Ultra-Turrex homogenizer, filtered and the filtrate washed with chloroform/methanol. The total extract was washed by layering on an equal volume of water and left overnight to give final extraction volumes of about 15 ml/g lung and liver and 30 ml/g fat tissue. Samples (5–10 ml) of the