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APPARENT INVOLVEMENT OF PHOSPHOLIPASE A_2, BUT NOT PROTEIN KINASE C, IN THE PRO-OXIDATIVE INTERACTIONS OF CLOFAZIMINE WITH HUMAN PHAGOCYTES

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Abstract—The anti-leprosy agent, clofazimine, at concentrations of 0.1–5 μg/ml caused a dose-related, stimulus-non-specific (*N*-formyl-methionyl-leucyl-phenylalanine, calcium ionophore, opsonised zymosan, arachidonic acid and phorbol myristate acetate) potentiation of superoxide generation by human neutrohils *in vitro* without affecting basal oxidative responses. The pro-oxidative interactions of clofazimine with neutrophils were eliminated by the phospholipase A_2 inhibitor 4-*p*-bromophenacyl bromide but not by the protein kinase C (PKC) inhibitor H-7. In support of these observations clofazimine promoted the release of radiolabeled arachidonic acid from neutrophil membrane phospholipids but did not influence the activity of PKC in cytosolic extracts of neutrophils or of purified PKC from rat brain. Pro-oxidative interactions of clofazimine with human phagocytes may contribute to the intraphagocytic antimycobacterial activity of this agent.

Clofazimine, 3-(4-chloroanilino)-10-(4-chlorophenyl)-2,10-dihydro-2-phenazin-2-ylidene isopropylamine, is an anti-leprosy agent [1]. The biochemical mechanism of the antimicrobial activity of clofazimine has not been described. It has been proposed that clofazimine, which is strongly lipophilic and has a redox potential of −0.18 V, may penetrate the bacterial cell and initiate a redox cycle leading to generation of the antimicrobial oxidant H_2O_2 (1). Apart from its direct antimycobacterial properties clofazimine stimulates the phagocytic [2] and antimicrobial activities of human and murine neutrophils and macrophages by potentiating the activity of lysozomal enzymes [3] and by increasing the activity of the respiratory burst enzyme [4–7]. The stimulatory effects of clofazimine on the generation of antimicrobial reactive oxidants by human phagocytes during exposure to leucotactic [4] and phagocytic stimuli [5–7] may contribute to intracellular antimycobacterial activity. However, the molecular/pharmacological mechanism of the pro-oxidative interactions of clofazimine with human phagocytes has not been established.

The superoxide generating enzyme of phagocytes is a membrane-associated NADPH-oxidase which is activated by diverse stimuli such as the tumour promoter phorbol myristate acetate (PMA), diacylglycerols, lectins, *N*-formyl peptides, calcium ionophore, opsonised particles, anionic detergents and arachidonic acid [8–14]. These activators utilise different transductional mechanisms to activate NADPH-oxidase which involve activation of cytosolic protein kinase C (PKC) in the case of PMA and diacylglycerols [12, 15, 16]. In contrast stimuli such as *N*-formyl peptides and opsonised particles appear to bypass PKC and utilise a calcium and ATP-independent, magnesium requiring, arachidonate-dependent mechanism [17–19]. Clearly, however, the precise mechanisms of activation of NADPH-oxidase remain to be established. In this study we have investigated the stimulus specificity and biochemical mechanisms of the pro-oxidative interactions of clofazimine with human neutrophils.

MATERIALS AND METHODS

Clofazimine. This antimicrobial agent was kindly provided by Dr. J. F. O'Sullivan, Medical Research Council Laboratories, Department of Chemistry, Trinity College, University of Dublin, Republic of Ireland, and solubilised as previously described [20] to give a stock solution of 100 μg/ml in the appropriate buffer. Unless indicated all other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO).

Neutrophil preparation. Neutrophils were separated from mononuclear leucocytes by centrifugation of heparinised (5 units of preservative-free heparin per ml) venous blood from adult human volunteers for 15 min at 400 *g* on Ficoll Hypaque cushions (9% Ficoll–12% Hypaque; specific gravity, 1.072). The resultant pellet was resuspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatin for 30 min at 37° to remove most of the erythrocytes. The neutrophil 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 at 4° for 10 min. Neutrophils were centrifuged, washed once and resuspended to a concentration of 10^7/ml in

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indicator-free Hanks' balanced salt solution (HBSS, Grand Island Biological Co., Grand Island, NY) buffered with 4.2 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulphonic acid) to pH 7.4.

Measurement of superoxide generation by neutrophils. Neutrophils (1×10^6) were pre-incubated for 10 min at 37° in 5 ml polypropylene test tubes with 0.1 mM ferricytochrome C (cyt *c* type VI) with and without 200 units of superoxide dismutase (SOD) in HBSS. Clofazimine (0.1–10 μg/ml final concentration) or appropriate solvent controls were then added to each tube followed 5 min later by the addition of the following stimulants at the final concentrations shown in parenthesis: (i) *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP 0.1 and 1 μM); (ii) the calcium ionophore A23187 (1 μM); (iii) the tumour promoter phorbol 12-myristate 13 acetate (PMA 5 and 10 ng/ml); (iv) opsonised zymosan (1 mg/ml); and (v) arachidonic acid (50 and 200 μM). Appropriate unstimulated control systems (neutrophils only, neutrophils + clofazimine, neutrophils + solvent controls) were also included. The final volume in each tube was 3 ml and the tubes were placed in a water-bath maintained at 37° and incubated for the times shown in the results section. After incubation 0.5 ml aliquots were transferred immediately to test-tubes containing 2.5 ml ice-cold PBS, centrifuged in a refrigerated centrifuge to remove the neutrophils and the supernatants assayed spectrophotometrically at 550 nm for reduced cyt *c* [21]. The amount of reduced cyt *c* was calculated using an absorbance coefficient of 21.1 mM at 550 nm [22]. Superoxide-mediated reduction of cyt *c* is the difference between reaction mixtures with and without SOD and the results are expressed as nmoles reduced cyt $c/1 \times 10^6$ neutrophils. Neutrophil-free control systems were included to monitor possible interference of clofazimine with the assay system. Measurements of spontaneous and stimulus-activated superoxide production by clofazimine-treated neutrophils from three children with the autosomal recessive form of chronic granulomatous disease (CGD) were also performed.

To investigate the persistence of clofazimine effects on superoxide generation, neutrophils were treated with 1 μg/ml clofazimine for 5 min at 37°, washed twice, resuspended in HBSS and superoxide generation measured as above using FMLP (1 μM) as the stimulant. Time-matched, identically-processed, clofazimine-free neutrophils and time-matched, unprocessed neutrophils + clofazimine were included as control systems.

The effects on clofazimine-mediated enhancement of neutrophil membrane-associated oxidative metabolism of H-7 (1-(5-isoquinolinyl-sulphonyl-)-2-methylpiperazine) a potent inhibitor of C-kinase [23], and of BPB (4-p-bromophenacyl bromide) an inhibitor of phospholipase A_2 [24], were also investigated. H-7 was used at a fixed non-toxic, non-superoxide scavenging concentration of 100 μM [25], whilst BPB was used at concentrations of 0.5, 1, 5 and 10 μM [19].

To investigate the influence of clofazimine on a neutrophil free xanthine–xanthine oxidase superoxide generating system the antimicrobial agent was added to a reaction system containing 0.25 mM xanthine, 100 milliunits of xanthine oxidase, 0.1 mM cyt *c* in a final volume of 3 ml PBS with and without SOD and superoxide assayed as described above.

Protein kinase C (PKC) extraction from human neutrophils and from rat brain. A cytosolic extract of neutrophils was used as a source of PKC. Neutrophils, prepared as above, were exposed to 5 mM diisopropyl fluorophosphate (DFP) for 20 min on ice and then washed and resuspended in 20 mM Tris, 0.5 mM EGTA (ethyleneglycol-bis-(B-aminoethyl ether) *N,N'*-tetraaceticacid, 0.5 mM EDTA (ethylenediamine tetraacetic acid), 50 mM 2-mercaptoethanol in the presence of the protease inhibitors 1 mM phenylmethane sulphonyl fluoride, PMSF; 20.0 μg/ml soya bean trypsin inhibitor and 0.25 μg/ml leupeptin. The neutrophils were then disrupted by sonication and fractionated on gradients of Percoll (Pharmacia, Uppsala, Sweden) as previously described [26] and the cytosol fraction recovered, assayed for total protein and used (20 μl vol.) for measurements of PKC activity.

PKC was chromatographically purified from brain tissue by using DEAE ion exchange, phenyl sepharose and protamine agarose columns in sequence as previously described [27].

Assay for PKC. Activity of PKC was assayed by measuring the incorporation of ^{32}P-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, 20 mM $MgCl_2$ in the absence or presence of various amounts and combinations of phosphatidylserine (PS), Ca^{2+}, diolein and clofazimine as indicated. Reactions were initiated by the addition of 1 nmol ^{32}P-ATP (New England Nuclear, Boston, MA) containing $(0.5–1) \times 10^6$ cpm and allowed to incubate for 6 min at 30° before spotting onto Whatman 3 MM paper squares and washing in TCA. ^{32}P incorporation in the protein precipitates was estimated by Cerenkov counting and PKC activity was expressed as pmol ^{32}P transferred/min/mg enzyme protein by subtracting EGTA backgrounds from stimulated activity.

Measurement of the release of radiolabelled arachidonic acid by neutrophils. Neutrophils ($2–5 \times 10^7$/ml) were coincubated with 5 μCi/ml radiolabelled arachidonate [5, 6, 8, 9, 11, 12, 14, 15-^{3}H(N), 100 Ci/mmol; New England Nuclear Corp.] for 30 min at 37° then washed twice and resuspended in HBSS. The neutrophils ($2–5 \times 10^7$) were then incubated for 15 min at 37° prior to the addition of clofazimine (1, 5 and 10 μg/ml) or solvent controls and the tubes incubated for 1, 2 and 5 min at 37°. The final volume in each tube was 1 ml (HBSS). The reactions were terminated by the addition of 5 ml of *n*-hexane/isopropanolol/concentrated HCl (final concentration 0.1 M) 300:200:4 (v/v/v). Lipids were extracted as previously described [29]. The upper organic phase was separated, retained and dried under a stream of nitrogen. The lipids were dissolved in 100 μl of hexane/isopropanolol 3:2 (v/v). Aliquots of 10 μl were then spotted onto silica gel 60 F254 precoated thin layer chromatography (TLC) plates (Merck, Darmstadt, F.R.G.). Unlabelled arachidonate standard (2.7 μg) was added at the origin with the samples to facilitate the visual detection

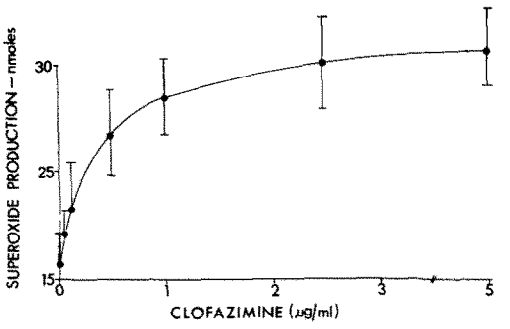

Fig. 1. Measurement of the effects of clofazimine (0.1–5 μg/ml) on superoxide generation by FMLP-activated neutrophils. Unstimulated background values have been subtracted and the results are expressed as nmoles reduced cyt c/1 × 10^6 neutrophils/5 min of incubation as the mean values ± SEM of 4 different experiments.

with iodine vapours. The plates were developed in chloroform/acetone 96:4 (v/v). After exposure to iodine vapours the arachidonate spots were localised and the silica removed and assayed for radioactivity. Control systems containing 0.2 μCi of ^{3}H-arachidonate and 2.7 μg of unlabelled arachidonate were included on each TLC plate. These results are expressed as fmoles ^{3}H-arachidonate/5 × 10^7 neutrophils.

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

RESULTS

Effects of clofazimine on spontaneous and FMLP-activated superoxide generation by neutrophils

The results of dose–response experiments with a fixed incubation time of 5 min are shown in Fig. 1 for clofazimine at concentrations of 0.1–5 μg/ml. At 10 μg/ml clofazimine interfered with the assay system. Clofazimine did not affect the spontaneous release of superoxide by neutrophils. However, the antimicrobial agent caused a dose-related potentiation of the FMLP-activated generation of superoxide by neutrophils. This effect achieved statistical significance with concentrations of 0.5 μg/ml clofazimine and greater. At concentrations of 0.1, 0.5, 1, 2.5 and 5 μg/ml clofazimine caused 33% ($P < 0.1$), 76% ($P < 0.025$), 99% ($P < 0.01$), 106% ($P < 0.005$) and 127% ($P < 0.005$) mean stimulation respectively of superoxide production by FMLP-activated neutrophils.

The effects of 1 μg/ml of clofazimine on maximal superoxide production by neutrophils activated with FMLP, calcium ionophore, opsonised zymosan, arachidonic acid and PMA are shown in Table 1. With the exception of the highest concentration of arachidonate (200 μM) clofazimine caused statistically significant ($P < 0.025$–$P < 0.005$) potentiation of superoxide production by neutrophils activated with the 5 different stimuli.

Pretreatment of neutrophils with 1 μg/ml clofazimine followed by washing (twice) and subsequent activation with 1 μM FMLP did not abrogate the priming effect of clofazimine on neutrophil superoxide generation. The stimulated values for unprocessed control and clofazimine-treated (5 min at 37°) neutrophils were 16.5 ± 1.3 and 28.7 ± 2 nmoles reduced cyt c respectively while the corresponding values for processed (washed) neutrophils were 16.7 ± 1.8 and 27.3 ± 1.2 nmoles respectively (mean value ± SEM of 3 experiments).

Clofazimine did not affect superoxide generation by a neutrophil-free xanthine/xanthine oxidase generating system. In the presence and absence of 1 μg/ml clofazimine the respective activities were 49.6 ± 1.4 and 47.6 ± 2.1 nmol reduced cyt c/min.

Inhibition studies

The effects of the PKC inhibitor H-7 (100 μM) on superoxide generation by neutrophils activated with FMLP (1 μM), calcium ionophore (1 μM), opsonised zymosan (1 mg/ml), arachidonate (50 μM) and PMA (5 ng/ml) are shown in Table 2. H-7 did not alter superoxide generation by neutrophils activated with FMLP, calcium ionophore, opsonised zymosan or arachidonate in the presence or absence of clofazimine (1 μg/ml). In the absence of clofazimine H-7 inhibited PMA-activated neutrophil superoxide generation by 60% (mean percentage inhibition; $P < 0.005$). The corresponding value in the presence of clofazimine was 26% ($P < 0.01$). The phospholipase A_2 inhibitor BPB caused a stimulus non-specific dose-related inhibition of superoxide generation by both control and clofazimine-treated neutrophils. The results obtained with 10 μM BPB are shown in Table 3.

Clofazimine interactions with PKC

Clofazimine at concentrations of up to 10 μg/ml did not affect the activity of purified PKC from rat brain or PKC activity in membrane-free cytosolic extracts of human neutrophils. PKC activities in the presence of calcium only or with optimal concentrations of all three physiological activators (100 μM Ca^{2+}, 5 μg/ml diolein and 25 μg/ml phosphatidylserine) were 7.2 ± 0.2 and 140.8 ± 4.6 nmol^{32}P/min/mg protein respectively. The corresponding values in the presence of 10 μg/ml clofazimine were 7.8 ± 0.7 and 147.3 ± 2.4 nmol^{32}P/min/mg protein. Varying the concentrations of the physiological activators was not associated with detectable effects of clofazimine on PKC activity.

Arachidonic acid release

The kinetics of release of ^{3}H-arachidonate by neutrophils co-incubated with 10 μg/ml of clofazimine are shown in Fig. 2. Relative to the corresponding drug-free control systems clofazimine-treatment increased the release of ^{3}H-arachidonate by neutrophils. These differences were statistically significant after 1 ($P < 0.025$), 2 ($P < 0.005$) and 5 min ($P < 0.005$) of incubation at 37°. In dose–response experiments using a fixed incubation period of 5 min at 37°, control, drug-free neutrophils and neutrophils treated with 1, 5 and 10 μg/ml clo-

Table 1. Measurement of the effects of clofazimine on superoxide generation by neutrophils activated with various stimuli

Stimulant	Superoxide generation as nmoles reduced cyt $c/1 \times 10^6$ neutrophils	
	Without clofazimine	With clofazimine (1 μg/ml)
0.1 μM FMLP	9.2 ± 1*	30.2 ± 2 (228)
1 μM FMLP	15.4 ± 2	37.6 ± 3 (144)
1 μM calcium ionophore	16.2 ± 3	29.0 ± 3 (79)
1 mg/ml opsonised zymosan	40.2 ± 2	58.8 ± 2 (46)
50 μM arachidonate	27.4 ± 3	48.8 ± 3 (78)
200 μM arachidonate	70.8 ± 6	79.6 ± 5 (12)
5 ng/ml PMA	178.0 ± 2	242.0 ± 3 (36)
10 ng/ml PMA	208.6 ± 5	228.6 ± 3 (10)

* Results are expressed as the mean values ± SEM in nmols reduced cyt *c* of three separate experiments. Background values for unstimulated neutrophils have been subtracted (3.5 ± 0.5 and 4.2 ± 0.5 nmoles for control and clofazimine-treated neutrophils respectively) and peak values (reached after 5 min for FMLP; 10 min for calcium ionophore, opsonised zymosan and arachidonate; 20 min for PMA) are shown.

Table 2. Measurement of the effects of H-7 on superoxide generation by neutrophils activated with various stimuli in the presence and absence of clofazimine

	Superoxide generation activated by the stimulants				
	FMLP	Calcium ionophore	Opsonised zymosan	Arachidonate	PMA
(a) Neutrophils only	18 ± 2*	12 ± 3	32 ± 2	28 ± 2	131 ± 8
(b) Neutrophils + clofazimine (1 μg/ml)	33 ± 3	24 ± 1	48 ± 3	59 ± 5	203 ± 5
(c) Neutrophils + H-7 (100 μM)	18 ± 2	9 ± 2	38 ± 6	24 ± 3	52 ± 11*
(d) Neutrophils + clofazimine + H-7	32 ± 4	21 ± 2	57 ± 5	53 ± 3	150 ± 7*

* Results are expressed as the mean values ± SEM in nmoles reduced cyt *c* of three different experiments. Peak values are shown for each stimulant following subtraction of background values (4.3 ± 0.6 and 5.7 ± 0.9 nmols for control and clofazimine-treated neutrophils respectively).

Table 3. Measurement of the effects of BPB on superoxide generation by neutrophils activated with various stimuli in the presence and absence of clofazimine

	Superoxide generation activated by the stimulants				
	FMLP	Calcium ionophore	Opsonised zymosan	Arachidonate	PMA
(a) Neutrophils only	24 ± 3*	20 ± 6	60 ± 2	50 ± 2	164 ± 4
(b) Neutrophils + clofazimine (1 μg/ml)	60 ± 2	41 ± 2	80 ± 3	80 ± 4	248 ± 5
(c) Neutrophils + 4 p-BPB (10 μM)	7 ± 1	2 ± 1	4 ± 2	17 ± 2	0
(d) Neutrophils + clofazimine + 4 p-BPB	14 ± 1	19 ± 1	9 ± 5	25 ± 3	0

* Results are expressed as the mean values ± SEM in nmoles reduced cyt *c* of 3 different experiments. Peak values are shown for each stimulant after subtraction of background values (2.6 ± 0.4 and 2.7 ± 0.3 nmols for control and clofazimine-treated neutrophils respectively).

fazimine released 94 ± 10, 117 ± 6 ($P < 0.05$), 131 ± 10 ($P < 0.025$) and 166 ± 1 ($P < 0.005$) fmoles of ^{3}H-arachidonate respectively (mean ± SEM of four determinations of a single representative experiment).

DISCUSSION

In the present study we have investigated the effects of clofazimine on the spontaneous and stimulus-activated generation of superoxide by human neutrophils. Five different stimulants of neutrophil

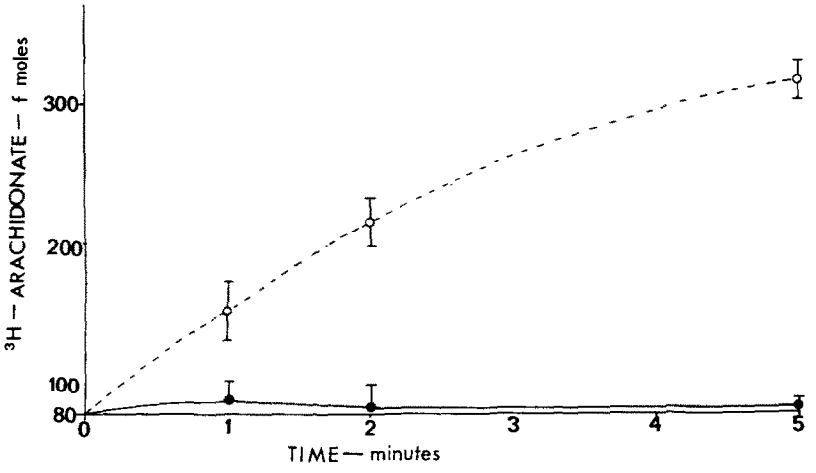

Fig. 2. Kinetics of release of ^{3}H-arachidonate by control neutrophils (●——●) and neutrophils incubated with 10 μg/ml of clofazimine (○- - -○). The results are expressed as the mean value ± SEM of quadruplicate determinations of a single representative experiment in fmoles ^{3}H-arachidonate/5 × 10^7 neutrophils.

membrane-associated oxidative metabolism with different potencies and transductional mechanisms were used, namely FMLP, calcium ionophore, opsonised zymosan, arachidonic acid and PMA. Spontaneous generation of superoxide, in the absence of stimulants, was not affected by clofazimine. However, the drug, at concentrations of 0.1–5 μg/ml, was found to cause a stimulus non-specific, dose-related enhancement of superoxide production by neutrophils. The magnitude of the clofazimine-mediated enhancement of superoxide generation by neutrophils was inversely related to the potency of the stimulus and was most effective with FMLP and calcium ionophore and least effective with PMA. Since PMA causes maximal activation of superoxide production by neutrophils the enhancing activity of clofazimine is understandably less with this stimulator. The sensitising effect of clofazimine on neutrophil membrane-associated oxidative metabolism was persistent and unaffected by washing of drug-treated neutrophils. In neutrophil-free control systems, or when normal neutrophils were substituted with those from individuals with CGD, the pro-oxidative properties of clofazimine were not observed. Likewise clofazimine did not influence SOD-inhibitable generation of superoxide by a neutrophil-free xanthine/xanthine oxidase system. These observations clearly show that the pro-oxidative interactions of clofazimine with human phagocytes are dependent on intact membrane-associated oxidative metabolism. Although clofazimine at the concentrations used here caused priming of stimulus-activated generation of superoxide by neutrophils it is probable that higher concentrations (>5 μg/ml), which could not be tested in our assay system, may directly activate membrane-associated oxidative metabolism. In support of this it has previously been reported that clofazimine at higher concentrations (30 μg/ml) causes spontaneous activation of luminol-enhanced chemiluminescence [7] as well as oxygen consumption [4] by neutrophils. However, since chemotherapy with clofazimine (200 mg daily) gives peak serum levels of 0.7–1 μg/ml [30], it is probable that the priming effect described here is pharmacologically relevant.

Some investigators have proposed a direct role for the cytosolic enzyme protein kinase C (PKC) in the activation of NADPH-oxidase [15, 16]. PKC is a calcium and phospholipid-dependent enzyme which is activated by diacylglycerol released from phosphoinositides by the action of phospholipase C [31]. Diacylglycerol is normally almost absent from cell membranes, but is transiently produced during membrane-receptor activation by extracellular signals [31]. Phorbol esters such as PMA function as agonists of diacylglycerol receptors for cytosolic PKC leading to direct activation of NADPH-oxidase [10, 15, 32]. PKC translocates from the cytosol to the cell membrane during neutrophil activation with PMA [15, 16] and mediates phosphorylation of an apparent oxidase component [33]. Although these observations imply a direct role for PKC in the activation of NADPH-oxidase other investigators using PKC and phospholipase A_2 inhibitors have clearly demonstrated the existence of an alternative PKC-independent, transductional mechanism which utilises arachidonic acid as a potential second messenger [17–19]. The selective PKC inhibitors H-7 and C-1 cause impressive inhibition of superoxide generation by neutrophils activated with PMA and diacylglycerol, but are largely ineffective in blocking the responses mediated by opsonised particles, FMLP and calcium ionophore [19, 33]. BPB on the other hand effectively blocks superoxide activation by various stimuli, including PMA [19]. Additionally arachidonic acid, a major phospholipid cleavage product of phospholipase A_2, activates superoxide generation in neutrophils and in neutrophil extracts [13, 17–19]. In membrane-containing extracts of unstimulated neutrophils arachidonate is a much more potent activator of NADPH-oxidase than PMA, is not inhibited by H-7, and requires the

participation of a non-kinase, magnesium-dependent cytosolic factor(s) of indeterminate molecular weight in the range 10–250 kD [17, 18]. These observations show that whilst PKC is involved in the activation of NADPH-oxidase by some stimuli of neutrophil membrane-associated oxidative metabolism such as PMA, it is not obligatory. It is possible that PKC-dependent activation of the oxidase is an indirect amplification mechanism involving phosphorylative inactivation of the membrane-located phospholipase A_2 inhibitor, lipomodulin, leading to release of arachidonate from membrane phospholipids and activation of NADPH-oxidase [19, 35, 36]. Nevertheless, the biochemical mechanism of arachidonic acid-mediated activation of NADPH-oxidase has not been established.

To establish the molecular/biochemical basis of the pro-oxidative interactions of clofazimine with neutrophils we identified PKC and phospholipase A_2 as possible targets. Clofazimine at concentrations of up to 10 μg/ml did not affect the activity of biochemically pure PKC from rat brain using optimal and suboptimal combinations of the physiological activators. Similar observations were made using unpurified PKC in membrane-free extracts of human neutrophils and platelets (data not shown). In support of these findings the selective PKC inhibitor H-7 (100 μM) did not eliminate clofazimine-mediated potentiation of superoxide generation by stimulus-activated neutrophils. On the other hand clofazimine caused a dose-related release of arachidonate from neutrophils, which is in agreement with our previous findings that this agent activates the spontaneous release of prostaglandin E_2 and leukotriene B_4 by neutrophils *in vitro* [20, 37]. The phospholipase A_2 inhibitor, BPB, was found to eliminate clofazimine-mediated enhancement of superoxide generation by activated neutrophils. These observations strongly suggest that the pro-oxidative interactions of clofazimine with neutrophils are due to drug-mediated activation of phospholipase A_2 leading to release of arachidonate at concentrations which prime neutrophil membrane-associated oxidative metabolism. Although arachidonate at high concentrations activates NADPH-oxidase in whole neutrophils [13, 14, 18, 19] it has been proposed [34] and recently demonstrated [38] that sub-optimal concentrations of arachidonate prime neutrophil membrane-associated oxidative metabolism to subsequent stimulation with FMLP and PMA. Nevertheless alternative mechanisms of clofazimine-mediated prooxidative activity may also exist as a consequence of the intracellular redox properties [1] or possible membrane-perturbating effects [13, 14] of this drug.

In conclusion we have found that clofazimine at therapeutic concentrations primes the membrane-associated oxidative metabolism of human neutrophils by apparent modulation of the activity of phospholipase A_2. This property may contribute to the intraphagocytic antimicrobial activity of clofazimine.

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