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Auranofin Inactivates Phosphofructokinase in Human Neutrophils, Leading to Depletion of Intracellular ATP and Inhibition of Superoxide Generation and Locomotion

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SUMMARY

The effects of the oral gold compound auranofin (AF), at concentrations well within the therapeutic range (0.04–1.5 μM), on human neutrophil functions and energy metabolism were investigated *in vitro*. At the concentrations tested, this agent had minimal effects on neutrophil degranulation and phagocytosis. However, AF caused dose-related inhibition of neutrophil chemotaxis and stimulus-activated generation of superoxide, which was evident at concentrations as low as 0.04 μM. Inhibition of superoxide generation by activated neutrophils increased with the time of preincubation of the cells with AF at 37°. At low concentrations of AF (<0.75 μM), early events (within 5 min) involved in the transduction, assembly, and activity of the neutrophil superoxide-generating enzyme NADPH oxidase appeared to be normal, but the cells were unable to sustain the level of oxygen consumption, superoxide production, and NADPH oxidase activity of the corresponding drug-free control cells. On a mechanistic level, coincubation of neutrophils with AF was associated with decreased glycolytic activity and depletion of intracellular ATP, apparently due to drug-mediated, dose-related inactivation of the glycolytic enzyme phosphofructokinase (PFK). Using purified PFK, the triethylphosphine gold (TEPG) moiety of AF, but not AF *per se*, caused dose-related inactivation of enzyme activity. These data indicate that the potent inhibition of neutrophil migration and reactive oxidant generation observed during treatment of neutrophils with low, therapeutically attainable concentrations of AF is related to TEPG-mediated inactivation of PFK and consequent interference with cellular energy metabolism and functions.

AF is an orally administered chrysotherapeutic agent with immunosuppressive and anti-inflammatory properties (1). This agent is useful in the treatment of rheumatoid arthritis (2) and possibly severe bronchial asthma (3). AF is extremely lipophilic and is promptly (within 10 min) and avidly concentrated by eukaryotic cells (4). Cell-associated drug is located in the nuclear, cytosolic, and membrane fractions (4). The association of AF with cells involves the displacement of the TATG component by membrane-localized thiol groups, and the net intracellular accumulation of the drug results from the sequential shuttling of the TEPG or gold moieties between cellular sulfhydryl groups (4–6).

AF is a potent inhibitor of phagocyte functions *in vitro*, and it has been proposed that macrophages and neutrophils are the primary targets of this agent (6). At therapeutic or suprather-apeutic concentrations, AF *in vitro* has been reported to suppress neutrophil phagocytosis, chemotaxis, superoxide generation, and degranulation (7–16). AF also inhibits the corresponding functions of macrophages (6), in addition to suppression of antigen-presenting functions (17), production of interleukin-1 (18), and generation of leukotriene B_4 (19). The precise biochemical mechanisms of these multiple inhibitory effects of AF on phagocyte functions have not been established. However, it has recently been reported that AF inhibits the activity of cytosolic PKC in neutrophils (16, 20) and depletes ATP in macrophages, leading to secondary inhibition of the ATP-dependent enzyme 5-lipoxygenase (19). These properties of the drug may be related to chemotherapeutic activity, because PKC appears to be involved in the activation of some neutrophil functions by certain stimuli (21), whereas sustained generation of ATP is essential for cellular activation and function (22, 23).

In the present study, the interactions of AF with human neutrophils *in vitro* have been investigated, and data are presented that clearly identify ATP depletion, due to drug-me-

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ABBREVIATIONS: AF, auranofin (2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato-*S*)(triethylphosphine)gold(I); TATG, tetraacetyl thioglucose; TEPG, triethylphosphine gold; TEP, triethylphosphine; PKC, protein kinase C; DMSO, dimethyl sulfoxide; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; EAS, endotoxin-activated serum; PMA, phorbol 12-myristate 13-acetate; OZ, opsonized zymosan; DTT, dithiothreitol; LECL, lucigenin-enhanced chemiluminescence; lucigenin, bis-*N*-methylacridinium nitrate; CB, cytochalasin B; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBSS, Hanks' balanced salt solution; PFK, phosphofructokinase; BSA, bovine serum albumin.

diated inactivation of the glycolytic enzyme PFK, as a primary mechanism of inhibition of neutrophil function.

Experimental Procedures

Materials. AF was kindly provided by Smith, Kline & French Laboratories (Philadelphia, PA), whereas TEPG and TEP were purchased from the Aldrich Chemical Co. (Milwaukee, WI) and TATG from the Sigma Chemical Co. (St. Louis, MO). These agents were dissolved in DMSO at stock concentrations of 5 mg/ml and investigated for effects on neutrophil functions at a final concentration range of 0.04–1.5 μM (corresponding to approximately 25–1000 ng/ml). In preliminary experiments, it was observed that dilution of AF and TEPG in aqueous media (relative to dilution in DMSO) was associated with some loss of activity. For this reason, all dilutions were made in DMSO, and 10 μl of DMSO with or without test agents were added directly to 1×10^6 neutrophils/ml of cell-suspending medium. DMSO, at the final concentration of 1% used throughout, did not adversely influence neutrophil functions. Other chemicals and reagents were purchased from Sigma, with the exception of reagents used for assays of neutrophil glycolytic enzymes and intracellular lactate, which were obtained from Boehringer Mannheim (Germany). Indicator-free HBSS buffered with 4.2 mM HEPES, pH 7.4, was the cell-suspending medium.

Neutrophils. Venous blood from adult human volunteers was treated with preservative-free heparin, and the neutrophils were separated and suspended to 1×10^7/ml of HBSS, as previously described (24). For all the assays described below, the neutrophils were prewarmed at 37° for 15 min, followed by addition of the test compounds and an additional 30-min period of preincubation at 37° before activation of the cells. The final number of neutrophils (10^6/ml) relative to the final drug concentration (0.04–1.5 μM) was constant throughout. For assays in which higher neutrophil concentrations (>10^6/ml) were required, the neutrophils were concentrated by centrifugation after preincubation with the test agents.

Measurement of superoxide generation by neutrophils. This was measured using LECL, as previously described (25). Neutrophils (10^6) were preincubated, as described above, in 900 μl of HBSS containing 0.2 mM lucigenin and AF (0.04–1.5 μM) or DMSO only (1%). Spontaneous and stimulus-activated LECL was then recorded in an LKB Wallac 1251 luminometer (Turku, Finland), after the addition of 100 μl of the stimulus of neutrophil membrane-associated oxidative metabolism. Four different stimuli were used at predetermined optimal concentrations, the synthetic chemotactic tripeptide FMLP (1 μM) potentiated by CB (1 μg/ml), the calcium ionophore A23187 (1 μM), PMA (10 ng/ml), and OZ (1 mg/ml). LECL readings were integrated for 5-sec intervals and recorded as mV/sec. Additional experiments were performed to investigate the effects of the following on AF-mediated inhibition of the LECL responses of OZ-activated neutrophils: (a) various times of preincubation at 37° (30, 60, and 90 min) of cells with 0.075, 0.15, and 0.375 μM AF, (b) preincubation of cells and AF (1.5 μM) at 37° for 30 min followed by washing (twice) of the neutrophils, and (c) addition of AF (1.5 μM) to neutrophils 5 min after activation with OZ.

To control for possible superoxide-scavenging activity, the effects of AF (0.75 and 1.5 μM) on superoxide generation (LECL) by a cell-free hypoxanthine (0.1 mM)/xanthine oxidase (100 milliunits) system were investigated.

Oxygen consumption by activated neutrophils. This was measured using a three-channel, Clark-type oxygen electrode (model DW1; Hansatech Ltd, King's Lynn, Norfolk, UK). Neutrophils (10^6) were preincubated with AF (0.15 μM), followed by addition of OZ. The reduction in pO_2 was monitored for an additional 20 min after addition of the stimulus.

Measurement of NADPH oxidase activity in neutrophil membrane fractions. In these experiments, neutrophils (5×10^7 in 50 ml of HBSS) were preincubated with 0.375 or 1.5 μM AF or DMSO solvent, followed by activation with PMA (10 ng/ml). After 5 and 15 min of incubation at 37°, the tubes were placed on ice to terminate activation, and the neutrophils were pelleted by centrifugation. Neutrophil membranes were then prepared as previously described (26). Briefly, the neutrophils were resuspended in 3 ml of sucrose (0.34 M), supplemented with 0.5 mM phenylmethylsulfonyl fluoride, and were disrupted by sonication. Cellular debris was removed by centrifugation at 1400 rpm for 10 min at 4°, and the membrane fractions in the supernatants were harvested by centrifugation at 25,000 rpm for 30 min. The membrane pellets were resuspended and dispersed in 2 ml of sucrose and assayed for NADPH oxidase activity using LECL. To 700 μl of HBSS were added lucigenin and 100 μl of membrane fractions. LECL was then recorded, as described above, after the addition of 100 μl of NADPH (2 mM) to initiate superoxide generation. In an extension of these experiments, AF (1.5 μM) was added to membrane fractions from PMA-activated control neutrophils, incubated for 30 min at 37°, and assayed for NADPH oxidase activity.

Neutrophil migration. This was measured using modified Boyden chambers with 5-μm pore size nitrocellulose filters (Sartorius Membranfilter, Germany). After preincubation with AF, neutrophils were concentrated to 5×10^6/ml and the HBSS was supplemented with 0.1% BSA. Neutrophils (200 μl) were then introduced into the upper compartment of the Boyden chambers, and 1 ml of leukoattractant in BSA-supplemented HBSS was placed in the lower compartment. Two leukoattractants were used, (a) FMLP at a final concentration of 20 nM and (b) EAS generated by incubation of fresh autologous serum with 500 μg/ml bacterial endotoxin (*Escherichia coli* 0127:B8; Difco Laboratories, Detroit, MI) for 30 min at 37° and used at a final concentration of 10%. In random migration systems, 1 ml of leukoattractant-free, BSA-supplemented HBSS was placed in the lower chambers. The chambers were incubated for 40–60 min at 37°, after which the filters were removed, fixed, stained, and processed. The number of neutrophils that had completely traversed the filter was counted microscopically, and the results are expressed as the number of neutrophils/microscope high-powered field.

Degranulation and phagocytosis. Neutrophils were coincubated with AF or DMSO, concentrated to 2×10^6/ml, and then activated with FMLP/CB. After incubation for 15 min at 37°, the neutrophils were removed by centrifugation and the supernatants were assayed for myeloperoxidase (primary granules), lysozyme (primary and secondary granules), and vitamin B_{12}-binding protein (secondary granules), using previously described colorimetric (27), turbidometric (28), and radiometric (29) procedures, respectively. The results are expressed as the percentage of total cellular enzymes.

The effects of AF on the phagocytic activity of neutrophils were measured by a previously described method based on the uptake of opsonized *Candida albicans* (30).

Assay of uptake of radiolabeled glucose by neutrophils. Neutrophils were preincubated with AF and concentrated to 2×10^7/ml, and 2×10^6 cells were coincubated for 30 min at 37° with 1 μCi of D-[3-^{3}H]glucose (15.5 Ci/mmol; Du Pont NEN Research Products, Boston, MA), in a final volume of 100 μl of HBSS containing 10 μM carrier glucose, on 150 μl of silicone oil in microcentrifuge tubes. Cell-associated and free [^{3}H]glucose were separated by centrifuging the neutrophils through the oil at 12,000 × *g* for 3 min. After freezing at −70°, the neutrophil pellets were sliced from the bottom of each tube and solubilized in Protosol (Du Pont NEN). Solubilized pellets and unbound glucose were quantitated by liquid scintillation counting, and results are expressed as nmol of [^{3}H]glucose/2×10^6 neutrophils.

Measurements of neutrophil glycolysis, ATP, and activity of glycolytic enzymes. Neutrophils were exposed to AF or DMSO and then concentrated to 1×10^7/ml. Intracellular lactate concentration was used as an index of glycolytic activity and assayed as previously described (31), whereas intracellular ATP was measured by a luciferin/luciferase chemiluminescence method in cell lysates (32). The activities of the glycolytic enzymes hexokinase, glucose phosphate isomerase, PFK, aldolase, triose phosphate isomerase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, monophosphoglyceromutase, enolase, pyruvate kinase, and lactate dehydrogenase were assayed in

the cytoplasmic extracts of control and AF-treated neutrophils, as previously described (31). The effects of AF and TEPG on the activity of purified PFK from rabbit muscle (400 milliunits/assay, which approximated the total PFK activity in 10^7 neutrophils) were also investigated. For assays of the activity of purified PFK, the enzyme was preincubated with AF or TEPG for 5 min, in 100 μl of HBSS containing 50% DMSO. This concentration of DMSO was necessary to optimize the activity of TEPG and did not affect PFK activity. Thereafter, the various components of the PFK assay system were added in a final volume of 3 ml (31).

Reversibility of AF-mediated neutrophil dysfunction. To assess the reversibility of AF-mediated inhibition of neutrophil functions, OZ-stimulated LECL responses and activity of cellular PFK were measured in cells that had been treated with AF (1.5 μM) followed by exposure to 20 or 40 mM DTT for 15 min, with subsequent washing (two times). The responses of these cells were compared with those of identically processed neutrophils that had not been exposed to DTT. Similar experiments were performed with purified PFK.

Investigation of the cytotoxic potential of AF. This was measured according to the release of lactate dehydrogenase by drug-treated cells. Neutrophils (1×10^6/ml) were coincubated with 1% DMSO only (control) or 1.5 μM AF for 60 min at 37°. After incubation, the cells were removed by centrifugation, and the supernatant was assayed spectrophotometrically for lactate dehydrogenase (31).

Expression and statistical analysis of results. The results are expressed as the mean value ± the standard error for each series of experiments. Levels of statistical significance were calculated using Student's *t* test (paired *t* statistic).

Results

Effects of AF on superoxide generation by activated neutrophils. These results are shown in Fig. 1. AF at concentrations of 0.075 μM and upwards inhibited the LECL responses of neutrophils activated by all four stimuli (FMLP, calcium ionophore, PMA, and OZ). Statistically significant inhibition ($p < 0.05$–0.005) was observed with 0.15 μM AF (21, 36, 25, and 18% mean inhibition for FMLP, calcium ionophore, PMA, and OZ, respectively). The concentrations of AF that caused 50% inhibition of LECL were approximately 0.375 μM with calcium ionophore and 0.75 μM with FMLP/CB, PMA, or OZ. AF also caused dose-related inhibition of the spontaneous LECL responses of unstimulated neutrophils. The spontaneous LECL responses of control and AF (0.375 μM)-treated neutrophils were 46 ± 10 and 28 ± 7 mV/sec, respectively (mean ± standard error of six experiments; mean inhibition, 39%; $p < 0.01$). The kinetics of AF (0.15 μM)-mediated inhibition of OZ-activated LECL are shown in Fig. 2. Interestingly, the initial responses (up to 5 min after the addition of the stimulus) of the AF-treated neutrophils at least equalled those of the untreated control cells. However, the LECL responses of the AF-treated cells peaked prematurely, were not sustained, and subsided rapidly (Fig. 2). This phenomenon was also observed with PMA (data not shown). However, AF at concentrations of 0.75 μM and greater caused immediate inhibition of OZ-activated LECL responses of activated neutrophils (Fig. 2).

The extent of inhibition of LECL increased with the duration of preincubation of neutrophils with AF at 37°. The mean percentages of inhibition of the LECL responses of OZ-activated cells preincubated with 0.15 μM AF for 30, 60, and 90 min at 37° were 15.8, 23.4, and 30%, respectively. Washing of neutrophils after coincubation with AF did not lessen the degree of inhibition of OZ-activated LECL. The mean percentages of inhibition of unwashed and washed neutrophils preincubated with 1.5 μM AF for 30 min at 37° were 73 and 81%, respectively. Addition of AF to neutrophils (1.5 μM) 5 min after addition of OZ was accompanied by inhibition of LECL (30%), which was somewhat less than the corresponding inhibition (57%) observed with cells preincubated with AF for 30 min at 37°.

AF, at concentrations of 0.75 and 1.5 μM, did not affect superoxide generation by a cell-free hypoxanthine/xanthine oxidase system. The peak LECL responses of drug-free and AF-containing systems were 295 ± 26 and 317 ± 21 mV/sec, respectively (background value without substrate, 12 ± 1 mV/sec).

Oxygen consumption. The kinetics of O_2 consumption by OZ-activated neutrophils in the presence and absence of 0.15 μM AF are shown in Fig. 3. As with superoxide generation, AF, at this concentration, did not interfere with initial consumption of O_2 by activated neutrophils but, rather, inhibited the ability of the cells to sustain a respiratory burst. The mean percentage of inhibition of O_2 consumption of AF (0.15 μM)-treated, OZ-activated neutrophils was 24 ± 4% (data from six different experiments; $p < 0.005$).

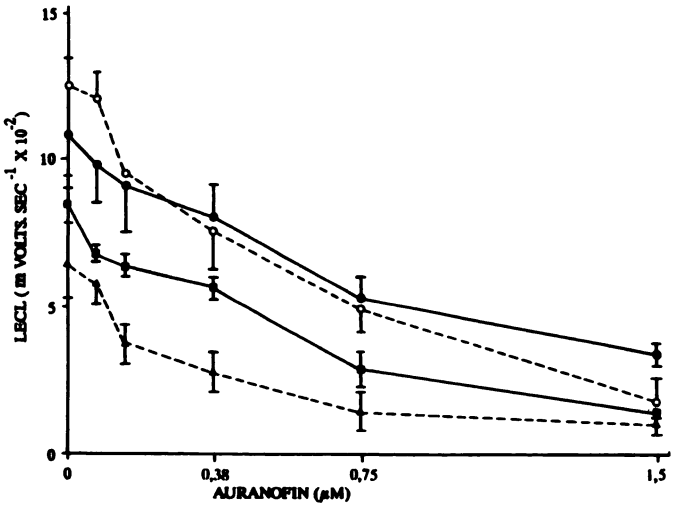

Fig. 1. Effects of AF (0.075–1.5 μM) on the peak LECL responses of neutrophils activated with OZ (●), PMA (○), FMLP/CB (■), and calcium ionophore (▲). The average times at which the peak values were reached were 12, 20, 2, and 17 min, respectively. The results of six experiments are expressed as the mean ± standard error, in mV/sec.

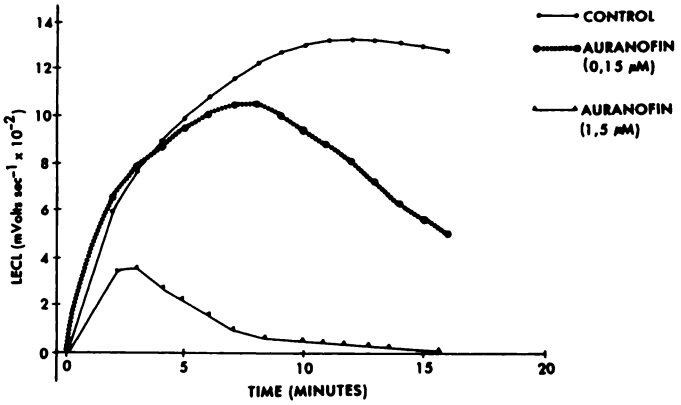

Fig. 2. Kinetics of LECL by OZ-activated control neutrophils (●) and cells treated with 0.15 μM (○) or 1.5 μM AF (▲). Data for each time point are presented as the mean values of triplicate determinations from a single representative experiment.

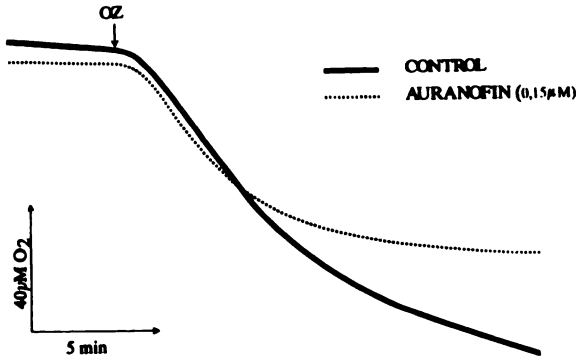

Fig. 3. Data from a single representative experiment showing the kinetics of O_2 consumption by OZ-activated control neutrophils (——) and cells treated with 0.15 μM AF (· · · · · ·).

TABLE 1

Measurement of NADPH oxidase activity in membrane fractions isolated from control and AF-treated neutrophils at varying times after activation of the cells with PMA

Data from three experiments (each performed in triplicate) are expressed as the mean peak LECL values ± standard errors. The peak LECL responses occurred 2–4 min after the addition of NADPH. The peak LECL responses of membranes prepared from control unstimulated neutrophils after 5- and 15-min incubations of the cells at 37° in the absence of PMA were 27 ± 1 and 30 ± 1 mV/sec, respectively.

Membranes prepared from	Peak LECL responses of membranes prepared	
	5 min after addition of PMA	15 min after addition of PMA
	mV/sec	
Control, PMA-activated neutrophils	284 ± 79	675 ± 89
AF (0.375 μM)-treated, PMA-activated neutrophils	363 ± 129	162 ± 85[a]
AF (1.5 μM)-treated, PMA-activated neutrophils	61 ± 12[a]	35 ± 10[a]

[a] $p < 0.005$.

NADPH oxidase activity in membranes from control and AF-treated, PMA-activated neutrophils. These data are shown in Table 1. AF (0.375 μM) treatment of neutrophils did not appear to inhibit either the early transduction or activity (5 min after addition of PMA) of NADPH oxidase in neutrophil membranes. However, the level of oxidase activity in membranes isolated from AF (0.375 μM)-treated neutrophils 15 min after the addition of PMA was, on average, 75% less ($p < 0.005$) than that observed with membranes prepared from the corresponding drug-free, PMA-activated control cells. Coincubation of neutrophils with 1.5 μM AF caused striking inhibition of NADPH oxidase activity in membranes prepared from neutrophils 5 and 15 min after treatment with PMA. Addition of AF (1.5 μM) to membranes prepared from PMA-activated control neutrophils (15 min after addition of PMA) did not significantly alter LECL responses (data not shown), indicating that this agent interferes minimally with the activity of fully assembled NADPH oxidase.

Neutrophil migration. These results are shown in Fig. 4. AF caused dose-related inhibition of neutrophil migration activated by both leukoattractants, with statistical significance achieved at a threshold concentration of 0.15 μM drug ($p < 0.005$ for both FMLP and EAS). Inhibition of random migration was observed with neutrophils treated with 0.375 μM AF

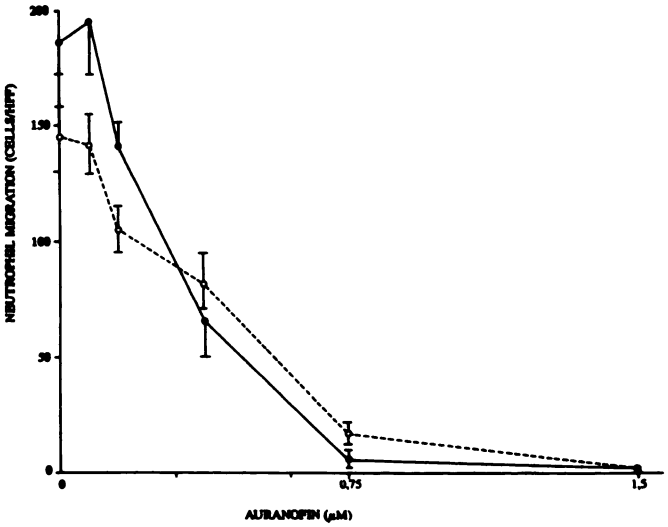

Fig. 4. Effects of AF (0.075–1.5 μM) on neutrophil migration activated by FMLP (●) and EAS (○). The results of three experiments are expressed as the mean number of neutrophils/microscope high-powered field ± standard error.

(8.2 ± 1 versus 4.2 ± 1 neutrophils/microscope high-powered field for control and AF-treated cells respectively; $p < 0.01$).

Degranulation and phagocytosis. Control, drug-free neutrophils activated with FMLP/CB released, on average, 19, 30, and 35% of total lysozyme, vitamin B_{12}-binding protein, and myeloperoxidase, respectively. The corresponding respective values for neutrophils coincubated with 1.5 μM AF were 20, 29, and 36%. Treatment of neutrophils with 1.5 μM AF, but not lower concentrations, caused slight, but statistically significant, inhibition of ingestion of opsonized *C. albicans* (92 ± 1% versus 84 ± 2% uptake of opsonized microorganisms for control and AF-treated neutrophils, respectively; $p < 0.01$).

Uptake of radiolabeled glucose. AF (1.5 μM) did not affect the uptake of glucose by neutrophils. The uptake by control and AF-treated cells was 0.32 ± 0.4 and 0.32 ± 0.6 nmol of glucose/2 × 10^6 neutrophils/30 min, respectively (three experiments).

Intracellular levels of ATP and lactate and activities of the glycolytic enzymes. The effects of AF on intracellular ATP levels are shown in Fig. 5. This agent caused a dose-related inhibition of neutrophil ATP levels that was statistically significant at concentrations of 0.15 μM ($p < 0.01$) and upwards. Neutrophil glycolytic activity, measured according to intracellular lactate concentration, was also decreased during treatment of the cells with AF. The mean intracellular lactate concentrations of control and AF (0.75 μM)-treated neutrophils were 48 ± 12 and 32 ± 6 μg of lactate/10^7 neutrophils, respectively (data from three separate experiments; mean inhibitory value, 33%; $p < 0.01$).

AF caused dose-related inhibition of neutrophil PFK, and these data are shown in Fig. 6. However, at the concentrations tested, the drug did not influence the activities of the other glycolytic enzymes (data not shown). Surprisingly, AF did not inhibit the activity of purified PFK from rabbit muscle. However, TEPG was a potent inhibitor of purified PFK, and these data are also shown in Fig. 6.

Comparison of the inhibitory effects of AF and TEPG on neutrophil LECL, ATP generation, lactate production, and PFK activity. These data for a fixed concentration

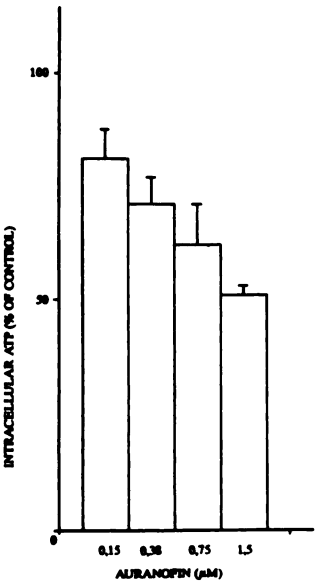

Fig. 5. Effects of AF (0.15–1.5 μM) on intracellular ATP concentrations. The results of six experiments are expressed as the mean percentages ± standard errors of the corresponding drug-free control systems. The absolute value for control neutrophils was 2.4 ± 0.2 nmol of ATP/10^7 cells.

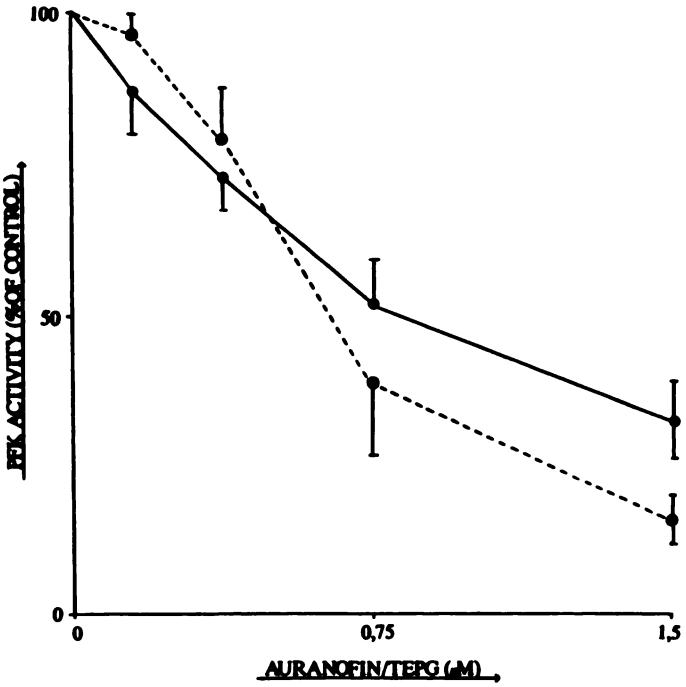

Fig. 6. Effects of treatment of neutrophils with AF (0.04–1.5 μM) on the activity of cytosolic PFK (●) and of exposure to TEPG (0.04–1.5 μM) on the activity of purified PFK (○). Data from six (AF) or three (TEPG) experiments are expressed as the mean percentages ± standard errors of the corresponding drug-free control systems.

of 0.75 μM AF and 1.5 μM TEPG are shown in Table 2. TEPG-mediated inhibition of these parameters was approximately double that observed with AF, which can be accounted for on the basis of simple stoichiometry, because at equal weights the gold content of TEPG is approximately double that of AF (5). Of the other agents tested, neither TATG nor TEP, at concentrations of up to 1.5 μM, inhibited any of the neutrophil functions tested (data not shown).

TABLE 2

Comparison of the effects of 0.75 μM AF and 1.5 μM TEPG on neutrophil OZ-activated LECL responses, intracellular levels of lactate and ATP, and activity of PFK

The results of three experiments are expressed as the mean percentages of inhibition ± standard errors for each parameter, tested relative to the corresponding drug-free control systems, for which the absolute values for LECL, intracellular lactate, ATP, and activity of PFK were 1404 ± 283 mV/sec, 53 ± 9 μg of lactate/10^7 cells, 3.2 ± 0.8 nmol of ATP/10^7 cells, and 6.4 ± 1.6 μM NAD/min, respectively.

	Inhibition observed with	
	AF	TEPG
	%	
LECL	45 ± 8	76 ± 3
Lactate	33 ± 6	66 ± 6
ATP	39 ± 4	79 ± 2
PFK	40 ± 8	72 ± 4

Reversal of AF-mediated inhibition of OZ-activated neutrophil LECL and PFK activity by DTT. Coincubation of neutrophils with 1.5 μM AF caused 63 ± 3% and 48 ± 3% inhibition of OZ-activated LECL responses and PFK activity, respectively. The corresponding values for AF-treated cells exposed to 20 mM DTT for 15 min were 23 ± 8% and 14 ± 2%, respectively. With 40 mM DTT, PFK activity was almost completely restored, and inhibition of peak OZ-activated LECL responses was negligible (9%). Exposure of purified PFK (400 milliunits) to 1.5 μM TEPG was associated with 97% loss of enzyme activity, which was completely restored after an exposure (5 min) to 20 mM DTT.

Lactate dehydrogenase release. Control neutrophils and cells treated with 1.5 μM AF released 4.7 ± 1.1% and 3.3 ± 1% of total lactate dehydrogenase, respectively. These data demonstrate that AF is not cytotoxic for neutrophils at the concentrations used in these short term assay procedures.

Discussion

The data presented in this report confirm the well established inhibitory effects of AF on neutrophil functions *in vitro* (7–15). However, a considerable amount of new data are presented that provide insight into the biochemical mechanisms and probable molecular target of AF-mediated suppression of neutrophil function. In many respects, the effects of the drug on neutrophil membrane-associated oxidative metabolism described here resemble those previously reported by Hafström *et al.* (11). For example, these authors also observed the following: (a) dose-related inhibition of stimulus-mediated superoxide generation by AF in the range of 0.15–1.5 μM, (b) the persistence of these inhibitory effects after washing of drug-treated cells, or when AF was added after the stimulus of membrane-associated oxidative metabolism, (c) the premature termination of superoxide generation by drug-treated cells, preceded (within the first 5 min of stimulus addition) by essentially normal responses, and (d) the absence of superoxide scavenging by concentrations of AF up to 1.5 μM. However, Hafström *et al.* (11) did not detect inhibitory effects of AF on superoxide generation in neutrophils activated with calcium ionophore. This discrepancy is probably related to differences in the duration of preincubation at 37° of neutrophils with AF, methods of solubilization of the drug, and/or the high sensitivity of the LECL method used here (25), relative to the ferricytochrome *c* reduction technique used by Hafström and colleagues (11). In the present study, it was also observed that AF (0.15 μM) decreased O_2 consumption by

activated neutrophils with kinetics that are almost identical to those of AF-mediated inhibition of superoxide generation i.e., normal initial responses followed by a premature subnormal peak and rapid decline thereafter. However, at concentrations of 0.75 μM and greater, AF-mediated inhibition of the LECL responses of activated neutrophils was evident throughout the entire time course of the assay.

These concentration-related differences in the inhibitory effects of AF on the kinetics of superoxide generation by activated neutrophils appeared to be related to the level of transduction of NADPH oxidase. At low concentrations of AF (<0.75 μM), the early kinetics of assembly (within 5 min) and activity of NADPH oxidase in the membranes of PMA-activated neutrophils were normal. However, the level of NADPH oxidase activity in cells treated with 0.375 μM AF was significantly decreased 15 min after activation with PMA. Clearly, at these concentrations (<0.75 μM), AF does not inhibit the early transduction and activity of NADPH oxidase but affects metabolic events necessary for sustained transduction and/or activity of the respiratory burst enzyme. At higher concentrations (>0.375 μM), the drug also inhibited the early transduction of NADPH oxidase, as well as processes necessary for sustained activity. These effects may be explained on the basis of differential concentration-related effects of AF on the generation of ATP by neutrophils. At low concentrations of the drug, cellular ATP reserves, although depleted, are adequate for early transductional requirements and cellular functional responses. However, the combination of increased consumption and compromised generation of ATP lead to premature exhaustion of neutrophil functions. Continuous generation of ATP is a requirement for both transduction and sustained activity of NADPH oxidase (33). At higher concentrations, AF causes severe depletion of intracellular ATP pools, leading to failure of transductional events as an additional inhibitory mechanism. Importantly, AF (1.5 μM), when added retrospectively to isolated membrane fractions from PMA-activated neutrophils, did not affect the activity of NADPH oxidase, confirming that activity of the oxidase in neutrophils treated with high dose AF (>0.375 μM) is due to a decrease in the amount of enzyme transduced and not to altered enzyme reaction kinetics.

The extent of inhibition of superoxide generation by OZ-activated neutrophils coincubated with a fixed concentration of AF (0.15 μM) for 30–90 min was found to increase according to the duration of preincubation at 37°. Because cellular uptake of AF is essentially complete within 10 min of incubation at 37° (4), the observed time-related progressive inhibition of superoxide generation is probably not attributable to increased cellular accumulation of the drug. Ongoing time- and temperature-related depletion of cellular ATP reserves is a more likely explanation for the increased level of neutrophil dysfunction during prolonged exposure to AF.

Of the other neutrophil functions, leukoattractant-activated migration was comparable to membrane-associated oxidative metabolism, in terms of sensitivity to AF-mediated inhibition. On the other hand, phagocytic activity was less sensitive, whereas no effects on degranulation were observed. However, progressive dose-dependent suppression of phagocytosis and degranulation were observed with higher drug concentrations (1.5–15 μM) (data not shown). The apparent selective sensitivity of neutrophil chemotaxis and superoxide generation to the inhibitory effects of AF has also been described for other sulfhydryl-reactive agents that deplete intracellular ATP (23).

Those concentrations of AF that inhibited neutrophil migration and superoxide production also depressed intracellular levels of lactate and ATP, indicating that glycolysis is a probable target of the drug. Because uptake of radiolabeled glucose was unaffected during exposure of neutrophils to AF, a primary effect of the drug on the activity of a glycolytic enzyme(s) was suspected. At the concentrations tested, a selective decrease in the activity of PFK was observed in AF-treated neutrophils. AF-mediated inactivation of PFK was dose related and correlated well with inhibition of glycolysis, ATP generation, and neutrophil chemotaxis and superoxide generation. At higher concentrations of AF (7.5–15 μM), inhibition of hexokinase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and pyruvate kinase was also observed in drug-treated cells (data not shown). However, these concentrations of AF are supratherapeutic, and the observed inhibitory effects of the drug on these enzymes (hexokinase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and pyruvate kinase) in intact neutrophils are of doubtful therapeutic relevance. Surprisingly, biochemically purified PFK was not inhibited by AF, although striking, dose-dependent inhibition of the enzyme was observed when AF was substituted with TEPG. These data demonstrate that AF-mediated inactivation of PFK in cells is dependent on the release of the TEPG moiety of the drug. This is in agreement with previously published reports that TEPG is the bioactive component of AF (5, 6). This agent (TEPG) was also found to be a potent inhibitor of neutrophil functions and energy metabolism.

AF-mediated inhibition of neutrophil superoxide generation and PFK activity, as well as TEPG-mediated inactivation of purified PFK, were reversed by exposure of the cells or enzyme to DTT. These observations implicate a critical sulfhydryl(s) on PFK as the molecular target of AF/TEPG. Only 3 of the 16 sulfhydryl groups of PFK react with the strong thiol oxidant 5,5-dithiobis(2-nitrobenzoate) under nondenaturing conditions at physiological pH (34). Two of these appear to be located at, or near, the active site, and oxidation, leading to the formation of intramolecular disulfides or interaction with sulfhydryl-reactive agents, results in the reversible loss of >90% of enzyme activity (35, 36). PFK, however, is not the only sulfhydryl-dependent glycolytic enzyme. Glyceraldehyde-3-phosphate dehydrogenase has a critical active-site sulfhydryl group that is vulnerable to inactivation by sulfhydryl-reactive agents (37). However, PFK in neutrophils, and probably other cell types, appears to be particularly susceptible to AF/TEPG-mediated inactivation. Inhibition of superoxide generation by activated macrophages pretreated with AF or related compounds, and reversibility by DTT, have been described previously (38, 39).

On the basis of these data, it is possible to devise a model of AF-mediated inhibition of neutrophil functions. During exposure of these cells to AF, TATG is displaced by interaction of TEPG with membrane-localized thiol groups (6). Due to sequential ligand-exchange reactions, TEPG reaches the cytosol (4), where it reacts preferentially with PFK and promotes dose-dependent inactivation of this glycolytic enzyme. The consequence is interference with cellular energy metabolism, depletion of ATP, and multifunctional inhibition, manifested as premature exhaustion, because the neutrophil relies exclusively on anaerobic glycolysis for ATP (22). Although inhibitory

effects on PKC have been described, it seems unlikely, at least with lower concentrations of AF, that direct interference with transductional mechanisms involved in stimulus-mediated neutrophil activation is a primary mechanism of action of the drug. Inhibition of transductional events is probably a secondary consequence of AF-mediated depletion of ATP. Interference with cellular energy metabolism *in vivo* should lead to decreased accumulation of phagocytes and generation of toxic reactive oxidants by these cells (40) at sites of inflammation, and this represents a probable mechanism of pharmacotherapeutic activity.

It seems unlikely that these effects are unique to neutrophils, and AF-mediated depletion of ATP by an undefined mechanism has, indeed, been described in macrophages (19), whereas TEPG complexes deplete ATP in cultured hepatocytes *in vitro* (41–43). These inhibitory effects on the energy metabolism of hepatic cells were observed with 25–50 μM concentrations of the test TEPG complexes and were due to interference with mitochondrial function and oxidative phosphorylation, leading to cell death within 60–180 min (41, 42). Clearly, the mechanisms of AF-induced cell dysfunction are different in various cell types, with those that utilize anaerobic glycolysis as a primary energy source being particularly susceptible to the inhibitory effects of low therapeutic doses (<1.5 μM) of the drug. Furthermore, AF-induced disturbances of energy metabolism in enterocytes may explain the well documented gastrointestinal side effects of this agent (44). Although the transient nature of these side effects could be ascribed to the increased synthesis of protective metallothioneins by enterocytes (6, 45), compensatory production of PFK during administration of AF is a possible alternative cellular adaptive mechanism. Preexisting PFK levels in phagocytes or adaptive increases in the production of this enzyme by neutrophil/monocyte precursors or mature macrophages may be a determinant of the therapeutic efficacy of AF.

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