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The Mitochondrial Network of Human Neutrophils: Role in Chemotaxis, Phagocytosis, Respiratory Burst Activation, and Commitment to Apoptosis

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It is commonly assumed that mature human neutrophils have few, if any, functional mitochondria. This assumption is based on the fact that electron microscopy usually fails to identify intact mitochondria, and any that are seen are small with poorly defined cristae and inner mitochondrial membrane definition (1–7). Furthermore, it is established that neutrophils rely heavily on glycolysis for their energy production (8), and rates of mitochondrial respiration are very low. This O$_2$-independent mechanism for energy generation is beneficial in that it allows for neutrophils to function at inflamed or infected sites where local O$_2$ tensions may be very low (9, 10). During phagocytosis neutrophils use large quantities of molecular O$_2$ not for mitochondrial respiration, but, rather, to generate O$_2^-$ and other oxidants via a respiratory burst catalyzed by NADPH oxidase (11, 12). This ability of neutrophils to phagocytose efficiently under anaerobic conditions contrasts with that of macrophages, in which phagocytosis is inhibited under anaerobic conditions (8). From this morphological and biochemical evidence it would appear that neutrophils have neither the capacity nor the requirement for active mitochondrial functions.

However, evidence is now emerging that mitochondrial function may play a role in the control of neutrophil apoptosis. For example, cytochrome c was shown to move from a membrane-bound fraction to the cytosol during apoptosis, and this molecule colocalized with 70-kDa heat shock protein in fixed (nonapoptotic) neutrophils (13). More recently, it was shown that during spontaneous neutrophil apoptosis, Bax translocated from the cytosol to the mitochondrial structures before caspase 3 activation (14). The purpose of this work, therefore, was to reinvestigate the nature of the mitochondrial network in neutrophils and to define its function in cell physiology. We show, using fluorescent indicators of mitochondrial function in live cells, that neutrophils possess a highly developed mitochondrial network. The membrane potential of these mitochondria can be disrupted by chemical uncouplers of electron transport, and in parallel with this disruption in membrane potential, neutrophil chemotaxis and cell shape are grossly perturbed. Mitochondria are not involved in the rapid initiation of the respiratory burst or phagocytosis, but respiratory burst activity was severely diminished in neutrophils pretreated with mitochondrial inhibitors for 2 h. These novel data point to previously unidentified roles for active mitochondria in neutrophils, which have wide implications for our understanding of the physiological and pathological functions of these cells in infection and inflammation.

Materials and Methods

Neutrophil isolation and culture

Neutrophils were isolated from heparinized venous blood from healthy volunteers by one-step centrifugation through NIM (Cardinal Associates, Santa Fe, NM) as described in the manufacturer’s instructions (15). After hypotonic lysis to remove contaminating erythrocytes, cells were resuspended in RPMI 1640 medium supplemented with 5% pooled human male
AB serum (Sigma-Aldrich, Poole, U.K.) at 5 × 10⁶/ml. Culture was performed at 37°C with gentle agitation. Purity and viability were routinely >95%, assessed by May-Grünwald-Giemsa staining and trypan blue exclusion, respectively. GM-CSF (Roche, East Sussex, U.K.) was added at 50 ng/ml. Oligomycin (Sigma-Aldrich) was added up to 7 μg/ml. FCCP3 (Sigma-Aldrich) added at up to 5 μM. PMA (final concentration, 0.1 μg/ml) and fMet-Leu-Phe (final concentration, 1 μM; both from Sigma-Aldrich) were used as cell stimulants. These solutions were made as concentrated stocks in DMSO, such that the final amount of DMSO used was ≤0.2% (v/v), which was without effect on any of the parameters measured.

**Morphological estimation of apoptosis**

Following culture, a 20-μl aliquot of suspension was made up to 200 μl with RPMI 1640, and cells were cytocentrifuged using a Shandon CytoSpin 3 (Runcorn, Cheshire, U.K.). May-Grünwald-Giemsa staining of cytoplasmic terminals allowed apoptosis to be scored by morphology as previously described (16). This method correlates well with other markers of apoptosis (17).

**Mitochondrial staining**

For mitochondrial staining 1 × 10⁶ neutrophils were removed from culture, and MitoTracker Red CMXRsos (Molecular Probes, Eugene, OR) or MitoTracker Green Fluo (Molecular Probes) were added to final concentrations of 25 nM and 7.7 μM, respectively. Cells were incubated at 37°C for 10 min before analysis. Dihydrorhodamine 123 (DHR-123; Molecular Probes) was added to cells at a concentration of 2 μM and then incubated for 20 min at 37°C before pelleting at 400 × g for 3 min and resuspension in 50 μl of PBS (pH 7.4) before analysis. As indicated in the figure legends, neutrophils were either stained before addition of annexin V-FITC or allowed to adhere to glass slides for 10 min before addition of mitochondrial dyes.

**Annexin V-FITC staining**

Neutrophils (10⁶) were removed from culture and resuspended in HBSS without phenol red (Life Technologies, Gaithersburg, MD) before the addition of annexin V-FITC (Sigma-Aldrich) at a 1:100 dilution. After 10 min on ice, cells were pelleted at 400 × g and resuspended in HBSS without phenol red before analysis by flow cytometry using a Cytoron Absolute bench top flow cytometer system (Ortho Diagnostics, Raritan, NJ) using a protocol that samples a precisely known volume. Neutrophils were also dual-stained with annexin V-FITC and propidium iodide (after incubation with 10 μg/ml for 5 min on ice). Red and green fluorescence was measured using an EPICS ALTRA (Beckman-Coulter, Palo Alto, CA) flow cytometer (see below). For dual MitoTracker Red/annexin V-FITC staining, neutrophils were stained first with MitoTracker Red, then resuspended in HBSS and stained with annexin V-FITC for 10 min at room temperature. Cells were then resuspended in 50 μl of HBSS and examined by confocal microscopy (see below) after allowing them to settle onto glass slides.

**Confocal microscopy**

Cells were visualized using a LSM510 confocal microscope (Carl Zeiss, New York, NY) at 1024 × 1024 pixel resolution through a ×63 Plan Apochromat (na 1.4) objective with 2 times averaging. Tracking mode was used to eliminate spillover between fluorescence channels. Excitation was at 543 nm for MitoTracker Red and at 488 nm for JC-1, DHR-123, and annexin V-FITC. Fluorescein fluorescence was collected through a 505- to 550-nm band-pass filter. MitoTracker Red fluorescence was collected through a 585-nm long-pass filter. JC-1 fluorescence was captured through a ×40 objective and captured by a Hamamatsu XC-77CE CCD camera using filter set 00 (excitation through a 530- to 585-nm band-pass filter, emission through a 615-nm long-pass filter). At least three fields were captured for each treatment.

**Flow cytometry**

Neutrophils were resuspended in HBSS (1 × 10⁶/200 μl) and incubated with JC-1 (7.7 μM) or DHR-123 (2 μM) at 37°C for 20 min, followed by incubation with FCCP (for 15 min 37°C) or oligomycin (for 15 min 37°C) at the indicated concentrations. Cells were then washed and resuspended in 1 ml of HBSS and analyzed immediately. An EPICS ALTRA (Beckman-Coulter) flow cytometer equipped with a 488-nm argon laser was used to detect both JC-1 and DHR-123 fluorescence. A total of 50,000 neutrophils were analyzed. The green fluorescence from DHR-123 and JC-1 was collected through a 505- to 525-nm band-pass filter. The red fluorescence from JC-1 was collected through a 610-nm band-pass filter. The red and green fluorescence distributions from JC-1 were displayed by two-color contour plot analysis. The data are expressed as the mean fluorescence intensity.

**Phagocytosis of heat-killed bacteria**

*Staphylococcus aureus* were heat-killed by incubation at 60°C for 30 min, washed twice, and then resuspended in PBS containing 30 μM propidium iodide. The suspension was incubated in the dark at 4°C for 2 h. The fluorescent bacteria were washed three times in HBSS containing 0.1% gelatin and opsonized. Opsonization, using pooled human serum from healthy donors (stored in aliquots at −20°C), was achieved by incubating bacteria (5 × 10⁶/ml) with 10% heat-inactivated human serum (v/v; final concentration) for 30 min at 37°C before the addition of 3 vol of cold sterile PBS and while mixing for 30 s. They were then incubated with neutrophils at a ratio of 10 bacteria/1 neutrophil in the dark for 30 min at 37°C with gentle agitation. After incubation the neutrophils were pelleted by centrifugation, washed twice, and then suspended in PBS containing 5 mM EDTA, 3 mM sodium azide, and 1% paraformaldehyde. Cells were analyzed immediately by flow cytometry, and red fluorescence was collected through a 620-nm long-pass filter. Ten thousand gated events were collected in the neutrophil gate. Phagocytosis of propidium iodide-stained bacteria was verified by confocal microscopy.

**Chemotaxis**

Chemotaxis and random migration were evaluated with a 10-well micro-well chamber using a modified Boyden chamber (18). *Escherichia coli* endotoxin-treated human serum and FMLP (10⁻⁹ M), were used as positive chemotactic stimuli. Neutrophils (5 × 10⁶/ml) were incubated with different concentrations of inhibitor (as described in the figure legends) before measurement of chemotaxis. Cells (1 × 10⁶/well) were incubated at 37°C for 60 min before fixation with ethanol and staining with hematoxylin. The distance traveled into 3-μm pore size nitrocellulose filters (Millipore, Bedford, MA) by cells (in micrometers) was evaluated by the leading front method as determined from 10 randomly chosen fields at ×400 magnification for each filter.

**Reactive oxygen metabolism production**

Chemiluminescence was measured at 37°C in neutrophil suspensions (1 × 10⁶/ml) in HBSS medium that was supplemented with 10 μM luminol using an LKB 1251 luminometer (Gaithersburg, MD) (15, 19). Cells were stimulated by the addition of PMA (0.1 μg/ml) or unopsonized latex particles (1-μm diameter).

**Statistics**

Statistical analysis was performed on datasets using ANOVA. Significant differences between datasets were defined as p ≤ 0.05 (*) and p ≤ 0.01 (**). All data are presented as the mean ± SD, where n is the number of experiments.

**Results**

**Neutrophils possess a complex mitochondrial network**

We used three different fluorescent dyes that can stain active mitochondria in live, unfixed cells. MitoTracker Red (20, 21) fluorescence is indicative of mitochondria with active membrane potential, and its fluorescence is diminished in cells that have lost their membrane potential. MitoTracker Red staining revealed an extensive mitochondrial network throughout the cytosol, surrounding the nuclear lobes that are seen as unstained regions in unfixed neutrophils (Fig. 1A). JC-1 fluorescence has two emission peaks (21, 22), with red fluorescence of j-aggregates indicating hyperpolarized mitochondria (high membrane potential) and green fluorescence (JC-1 monomers) due to low mitochondrial membrane potential. The ratio of red to green fluorescence is thus a measure of the mitochondrial membrane potential. Freshly isolated neutrophils showed complex staining with JC-1, again indicating an intricate network of mitochondrial structures exhibiting both green

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3 Abbreviations used in this paper: FCCP, DHR-123, dihydorhodamine 123, DPI, diphenylene iodonium.
FIGURE 1. Neutrophils possess a complex mitochondrial network whose membrane potential is disrupted by FCCP, but not oligomycin. A, F, H, and P–R. Neutrophils were stained by the addition of MitoTracker Red (25 nM); B, E, G, and M–O, neutrophils were stained by JC-1 (7.7 μM); C and J–L, neutrophils were stained with DHR-123 (2 μM). D, Neutrophils were costained with MitoTracker Red and FITC-annexin V. A–C, E, G, J, M, and P, Freshly isolated neutrophils; D, F, and H, neutrophils cultured for 18 h at 37°C before staining; K, N, and Q, neutrophils incubated for 1 min with 50 nM FCCP; L, O, and R, neutrophils treated for 15 min with 2 μg/ml oligomycin. All images were obtained by confocal microscopy. The bar marker in A represents 10 μm.

and red fluorescence (Fig. 1B). This pattern of regions of hyperpolarized and depolarized mitochondria has been reported in other cell types and is considered to reflect uneven distribution of proton circuits, respiration, ATP synthesis, and localized Ca2+ inside mitochondria (21). DHR-123 is colorless, but becomes fluorescent upon oxidation, for example in the presence of H2O2. It can stain mitochondria, but has also been used to measure reactive oxygen metabolite production by neutrophils (23–26). Rhodamine 123 fluorescence (due to intracellular oxidation of the colorless DHR-123) was clearly seen in mitochondrial-like structures within the neutrophil cytoplasm (Fig. 1C). The development of this latter fluorescence was seen after an incubation of ~15 min.

Changes in mitochondrial function during apoptosis

When neutrophils are cultured in vitro, they rapidly undergo spontaneous apoptosis (27, 28). In freshly isolated neutrophils >99% of the cells exhibited high mitochondrial membrane potential, as indicated by JC-1 staining revealing J-aggregates (red) and JC-1 monomers (green; Fig. 1E) and MitoTracker Red fluorescence (Fig. 1F). This would indicate that immediately after isolation virtually all neutrophils in the population have active mitochondrial function. However, after 22 h in culture, >80% of the cells had lost their MitoTracker Red staining (Fig. 1H), and the JC-1 fluorescence was green in most cells (Fig. 1G), indicating loss of mitochondrial membrane potential. In other experiments, dual MitoTracker Red staining and FITC-annexin V binding were measured during time-course experiments in which neutrophils were incubated in the presence or the absence of GM-CSF, a cytokine known to delay the progression of neutrophils into apoptosis (16, 29–31). Fig. 1D shows that the loss of mitochondrial membrane potential (as indicated by loss of MitoTracker Red staining) precedes the appearance of annexin V binding on the cell surface due to phosphatidylserine exposure (32). In this image there are clearly many cells that have lost their mitochondrial membrane potential before phosphatidylserine becomes exposed on the cell surface.

That loss of mitochondrial membrane potential during apoptosis precedes phosphatidylserine exposure on the cell surface is also apparent from the fact that in time-course experiments (Fig. 2), there were always a greater number of cells that were MitoTracker Red negative than that were annexin V positive. Loss of mitochondrial membrane potential also preceded the appearance of morphological indicators of neutrophil apoptosis (Fig. 2), as at all time points there were always more cells that were MitoTracker Red negative than that showed apoptotic morphology. All cells that were apoptotic as determined by annexin V binding were always MitoTracker Red negative. However, cells that were MitoTracker Red negative were not necessarily annexin V positive. GM-CSF delayed neutrophil apoptosis and preserved mitochondrial membrane potential. It is noteworthy that at early time points, significant changes in neutrophil apoptosis can be seen when measuring mitochondrial membrane potential, but these are not apparent when measuring morphology or annexin V binding. Thus, measurement of mitochondrial membrane potential is a very early indicator of commitment of neutrophils to apoptosis.

FIGURE 2. Decreases in mitochondrial membrane potential precede changes in morphology and phosphatidylserine exposure during neutrophil apoptosis. Neutrophils were incubated in the absence (C) or the presence (G) of 50 ng/ml GM-CSF. At 3, 6, and 22 h samples were removed for assessment of apoptosis by morphology (■), decreased mitochondrial membrane potential, as measured by loss of MitoTracker Red fluorescence and fluorescence microscopy (□), and phosphatidylserine exposure of the cell surface, as measured by FITC-annexin V binding and flow cytometry (■). *, p ≤ 0.05; †, p ≤ 0.01. Values shown are the means (±SD) of six separate experiments.
Disruption of mitochondrial function by FCCP and effects on cell function

Having shown that neutrophils possess an intricate mitochondrial network, it was then necessary to determine whether their mitochondrial function could be disrupted pharmacologically. Neutrophils were loaded with DHR-123, and then rhodamine 123 fluorescence was allowed to develop during a 15-min incubation at 37°C due to intracellular reactive oxidant production. Cells were then incubated with varying concentrations of the uncoupler, FCCP, and its effects on rhodamine 123 fluorescence were determined. The data shown in Fig. 3 indicate that the uncoupler resulted in a dose-dependent decrease in mitochondrial fluorescence, as detected by flow cytometry (Fig. 3). The concentration of FCCP that resulted in a 50% decrease in this fluorescence was ~20 nM. When these effects of FCCP were viewed by confocal microscopy (Fig. 1, J and K), again a marked loss of rhodamine 123 staining of mitochondria was seen. After uncoupler treatment the green fluorescence was fainter and more diffuse throughout the cell. Interestingly, cell morphology was greatly affected by uncoupler treatment, and the adherent cells became more rounded.

Similarly, in JC-1-loaded neutrophils, FCCP treatment resulted in the loss of red fluorescence (due to high mitochondrial membrane potential) with the corresponding increase in green fluorescence indicating the loss of membrane potential (Fig. 4A). The concentration of FCCP resulting in a 50% decrease in red fluorescence was 20 nM (Fig. 5). This was also observed by microscopic examination of JC-1-stained cells. After uncoupler treatment, mostly green JC-1 staining was observed, which was more diffuse throughout the cell (Fig. 1, M and N). Again, the change in neutrophil morphology was apparent after FCCP treatment, with the cells adopting a rounded morphology. Similarly, MitoTracker Red fluorescence was rapidly lost in neutrophils treated with uncoupler, indicating loss of mitochondrial membrane potential (Fig. 1, P and Q).

FCCP also resulted in dose-dependent decrease in respiratory burst activation in response to PMA (Fig. 4B). However, this decrease in respiratory burst activity was only detected at concentrations of FCCP >100 nM. Indeed, the concentration of FCCP required for half-maximal inhibition of the respiratory burst was ~4 μM (Fig. 5), which was 200-fold higher than that required to disrupt mitochondrial membrane potential. Thus, we conclude that inhibition of the respiratory burst at high (>1 μM) concentrations of FCCP is due to nonspecific effects of this agent, perhaps by disturbing plasma membrane function. It is also apparent that mitochondrial function is not required for this rapid activation of the NADPH oxidase, because concentrations of FCCP that completely disrupt mitochondrial membrane potential had no effect on respiratory burst activation. Diphenylene iodonium (DPI) used at 10 μM completely inhibited the respiratory burst activated by PMA (Fig. 4B). No effect of FCCP on phagocytosis of serum-opsonized or IgG opsonized S. aureus was detected at concentrations up to 5 μM (data not shown).

**FIGURE 3.** FCCP decreases rhodamine 123 fluorescence. Neutrophils were incubated for 20 min with DHR-123 (2 μM) and then incubated in the absence (0) and the presence of FCCP at the indicated concentrations (nanomolar). After 15-min incubation, rhodamine 123 fluorescence was determined by flow cytometry. A typical result of three separate experiments is shown.

**FIGURE 4.** FCCP decreases mitochondrial membrane potential measured by JC-1 fluorescence. A, Neutrophils were loaded with JC-1 (7.7 μM) and then incubated in the absence (control) and the presence of FCCP at the indicated concentration. After 15-min incubation, red (high mitochondrial membrane potential) and green (low mitochondrial membrane potential) fluorescence were measured by flow cytometry. A typical result of four separate experiments is shown. B, Neutrophils were incubated in the absence and the presence of FCCP as follows: 0 nM (○; control) 10 nM (●), 100 nM (□), 300 nM (■), 400 nM (△), or 500 nM (▲), or with 10 μM DPI (▼). They were then stimulated by the addition of 0.1 μg/ml PMA, and luminol chemiluminescence was measured. A typical result of six separate experiments is shown.
In contrast to the effects of FCCP on phagocytosis and respiratory burst activation, chemotaxis in response to either fMLP or activated serum was inhibited by FCCP (Fig. 5). The concentration dependencies of inhibition of chemotaxis and loss of mitochondrial membrane potential overlapped (Fig. 5) and were both maximal at 50 nM. Thus, we show a remarkable correlation between loss of mitochondrial membrane potential and inhibition of chemotaxis. We conclude, therefore, that neutrophil chemotaxis requires active mitochondrial function.

Disruption of mitochondrial function by oligomycin and effects on cell function

Unlike the uncoupler FCCP, the mitochondrial ATPase inhibitor oligomycin had no effect on JC-1 fluorescence from neutrophil mitochondria, as determined by flow cytometry (Fig. 6A). Likewise it had no effect on the fluorescence of rhodamine 123, JC-1, or MitoTracker Red as determined by confocal microscopy (Fig. 1, L, O, and R). However, oligomycin treatment resulted in a change in cell shape, with the cells becoming more rounded. Short term (5-min) treatment with oligomycin also did not affect the ability of neutrophils to phagocytose bacteria (Fig. 6B) or to activate a respiratory burst in response to PMA (Fig. 7A) or latex particles (Fig. 7B). However, when neutrophils were preincubated for 2 h with oligomycin, this mitochondrial ATPase inhibitor resulted in a dose-dependent inhibition of the respiratory burst in response to both PMA (Fig. 7A) and latex particles (Fig. 7B). Half-maximal inhibition of these responses occurred at ~4 μg/ml oligomycin. Curiously, after incubation of neutrophils with low concentrations of oligomycin (1 μg/ml), an enhancement of the respiratory burst by 28 ± 14% (n = 5; p < 0.01) was observed (Fig. 7A). While the respiratory burst was inhibited by long term (2-h) treatment of...
neutrophils, phagocytosis was unaffected by oligomycin treatment (Fig. 6B). Thus, we conclude that long term (2-h) treatment of neutrophils with oligomycin results in an inability to generate a respiratory burst, but does not affect phagocytosis. We found that oligomycin treatment resulted in dose-dependent inhibition of chemotaxis, and the dose dependency of this inhibition closely matched the concentration dependence of inhibition of respiratory burst activity seen after 2-h treatment with the inhibitor (Fig. 7).

The effects of PMA, an activator of the respiratory burst, on mitochondrial function were then determined. Freshly isolated neutrophils were allowed to adhere to glass slides and then were stained with JC-1 on a temperature- and CO₂-controlled microscope chamber, divided into two sections such that cells in each section could receive different treatments. To one section of the chamber PMA (0.1 μg/ml) was added, whereas the other section received no treatment and served as a control. Images taken over a 60-min period revealed that PMA has little effect on mitochondrial function up to ~30 min after addition (Fig. 8). Thereafter, the cells began to lose their red fluorescence and increase their green fluorescence, indicative of loss of mitochondrial function. They then began to dramatically change shape, becoming very flattened and enlarged, and gross changes in nuclear structure were evident.

**FIGURE 8.** PMA treatment results in loss of mitochondrial function. Freshly isolated neutrophils were allowed to adhere to glass slides for 10 min and were stained with JC-1 for an additional 10 min. They were then incubated over a 60-min period on a temperature- and gas-controlled microscope slide, and confocal images were obtained every 2 min. Half the cell suspension was treated with PMA (0.1 μg/ml), while the other half (control) received no further addition. The upper panels show representative images obtained during incubation over this 60-min period. A typical result of six separate experiments is shown. The lower panel shows quantitative data obtained showing ratio of green to red fluorescence of control cells (○) and PMA-treated cells (●) of entire microscopic fields. A typical result of six separate experiments is shown.
cytoplasm. The distinct staining patterns associated with each of these fluorescent indicators was rapidly and extensively disrupted by a well-characterized uncoupler of mitochondrial function, FCCP, which dissipates the electron transport-derived proton gradient (33). Dissipation of this proton gradient of the inner mitochondrial membrane by FCCP allows respiration to proceed, but prevents ATP synthesis that is normally driven by this proton gradient. This uncoupling will disrupt the membrane potential of the inner mitochondrial membrane. Oligomycin, on the other hand, inhibits proton pumping through Fo-ATPase on the inner mitochondrial membrane (33, 34), thereby preventing ATP synthesis. This inhibitor would not perturb the potential of the inner mitochondrial membrane. Our experiments show that FCCP, but not oligomycin, rapidly disrupts the mitochondrial membrane potential and hence the intracellular staining patterns of the three indicator dyes used in this study (Fig. 1). Thus, the fluorescent structures that we see by confocal microscopy are genuine mitochondria, based on their staining properties and susceptibility to established pharmacological inhibitors.

Disruption of mitochondrial function by either FCCP or oligomycin had no effect on phagocytosis of serum-opsonized bacteria or latex particles or on the rapid initiation of the respiratory burst that results in the generation of reactive oxygen metabolites. Both of these are ATP-requiring processes, the latter using NADPH that is generated via increased activity of the hexose monophosphate shunt (11, 12). It is generally assumed (35) that increased rates of glycolysis, an O$_2$-independent and mitochondrial-independent process, can supply the ATP required for these processes. Our data do not argue against this idea, because short term incubation of neutrophils with FCCP or oligomycin did not affect phagocytosis or respiratory burst activity. However, these pharmacological inhibitors of mitochondrial function exerted three completely unexpected effects on neutrophil function, indicating an underlying, fundamental requirement for mitochondrial activity previously unrecognized in these cells.

First, both FCCP and oligomycin induced rapid changes in cell shape of adherent neutrophils. These shape changes were seen by confocal microscopy when previously adhered, flattened cells rounded up (but did not detach) within 1 and 15 min of addition of FCCP or oligomycin, respectively. The mechanisms responsible for these changes in cell shape are not known, but are likely to reflect changes in cytoskeletal arrangement and function as a consequence of impaired mitochondrial function. Second, both FCCP and oligomycin inhibited chemotaxis of neutrophils in response to either fMLP- or LPS-activated serum. For FCCP, there was a remarkable correlation between the concentration required to disrupt the mitochondrial membrane potential and the inhibition of chemotaxis. The IC$_{50}$ for inhibition of both these functions was $\sim$20 nM, in agreement with the known effects of this compound on uncoupling of mitochondrial respiration (35). At higher concentrations of FCCP ($\geq$1 $\mu$M), the respiratory burst was inhibited, but we believe that this inhibition was due to effects unrelated to uncoupling of mitochondrial function. The NADPH oxidase is associated with a proton pump (36, 37), and these high concentrations of FCCP may be affecting the activity of this pump on the plasma membrane. Oligomycin similarly inhibited neutrophil chemotaxis with an IC$_{50}$ of $\sim$4 $\mu$g/ml, again in broad agreement with the established effects of this compound on inhibition of Fo-ATPase (34).

The third unexpected result of these studies was that while oligomycin did not affect initiation of the respiratory burst when added a few minutes before cell stimulation, significant inhibition was seen following a 120-min preincubation with this inhibitor. After this period of incubation with oligomycin, respiratory burst

![FIGURE 9. Oligomycin and FCCP do not accelerate the rate at which neutrophils progress into apoptosis. Neutrophils were stained with annexin V-FITC and propidium iodide (as described in Materials and Methods) after incubation as follows: A, 0-h incubation (freshly isolated cells); B, 20-h incubation, no additions; C, 4-h incubation, no additions; D, 4-h incubation with 10 $\mu$g/ml cycloheximide; E, 4-h incubation with 2 $\mu$g/ml oligomycin; and F, 4-h incubation with 100 nM FCCP. After incubation and staining, cells were analyzed by flow cytometry. A typical result of five separate experiments is shown.](http://www.jimmunol.org/)
activation in response to either latex particles (a phagocytic stimulus) or PMA (which directly activates protein kinase C) was decreased in a dose-dependent manner. The concentration dependence of this inhibition closely matched that of inhibition of chemotaxis. The chemotaxis assay requires incubation with inhibitors or stimuli for ~60 min before migration through filters can be measured. This perturbation of neutrophil function by long term incubation with oligomycin may be due to a slow depletion of cellular ATP levels via oxidative phosphorylation. Functions such as chemotaxis and respiratory burst activation may then not be efficiently activated under these circumstances. It is also possible that the effects of FCCP and oligomycin are not directly related to impaired ATP generation, but instead result from perturbation of other mitochondrial functions such as impaired ion channel activity. This possibility clearly requires further investigation. An unexplained finding was the consistent enhancement of respiratory burst activity after 120-min incubation with 1 µg/ml oligomycin. This was only observed when cells were stimulated with PMA and not with latex particles, suggesting modification of a signaling pathway rather than a fundamental alteration in the activation process of the oxidase.

The finding that incubation of neutrophils with DHR-123 revealed mitochondrial structures in neutrophils is intriguing for several reasons. First, it indicates that neutrophil mitochondria are generating significant levels of H₂O₂, as this molecule is required to convert the nonfluorescent molecule into the fluorescent rhodamine 123 derivative (26, 38). Second, this dye is commonly used to measure respiratory burst activation in neutrophils (23–25), by its very nature measuring intracellular oxidative activity. This oxidative activity has been assumed to be that of the NADPH oxidase, but mitochondrial function may now contribute to intracellular neutrophil oxidant production. Indeed, the most commonly used NADPH oxidase inhibitor is DPI (39), but this is also a known inhibitor of mitochondria (40). Third, it is known that neutrophils can generate reactive oxygen species intracellularly, which has again been assumed to be via internal NADPH oxidase activity (41–44). A mitochondrial contribution to this activity cannot be ruled out. Finally, it is established that neutrophil-derived reactive oxygen species can regulate the progression of these cells into apoptosis (45–47). Oxidants derived from either NADPH oxidase or mitochondria may be involved in this process. It will be important to distinguish the importance of these two routes of oxidative production for the design of new ways to manipulate neutrophil apoptosis for therapeutic benefit. Mitochondria have recently been identified in human eosinophils (48).

In summary, the data presented in this report describe novel and extremely important insights into our understanding of the control of neutrophil function in infection and inflammation. Neutrophils that infiltrate inflamed sites can function for much longer periods of time than neutrophils in the bloodstream, because inflammatory neutrophils have delayed apoptosis. Our experiments show that this extended neutrophil activity also requires intact mitochondrial function and that perturbation of this function would greatly decrease the ability of inflammatory neutrophils to mount an effective antimicrobial challenge.

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