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The Mitochondrial Network of Human Neutrophils: Role in Chemotaxis, Phagocytosis, Respiratory Burst Activation, and Commitment to Apoptosis¹

Gianluca Fossati, Dale A. Moulding, David G. Spiller, Robert J. Moots, Michael R. H. White, and Steven W. Edwards²

It is commonly assumed that human neutrophils possess few, if any, functional mitochondria and that they do not depend on these organelles for cell function. We have used the fluorescent mitochondrial indicators, JC-1, MitoTracker Red, and dihydrorhodamine 123 to show that live neutrophils possess a complex mitochondrial network that extends through the cytoplasm. The membrane potential of these mitochondria was rapidly (within 2 min) disrupted by the addition of FCCP ($IC_{50} = 20$ nM), but not by the Fo-ATPase inhibitor, oligomycin (at up to 7 μ g/ml). However, inhibition of mitochondrial function with both agents resulted in cell shape changes. Neither activation of the respiratory burst nor phagocytosis of either latex particles or serum-opsonized *Staphylococcus aureus* was affected by the addition of FCCP or oligomycin. However, FCCP inhibited chemotaxis at concentrations that paralleled disruption of mitochondrial membrane potential. Furthermore, prolonged (2-h) incubation with oligomycin resulted in an impaired ability to activate a respiratory burst and also inhibited chemotaxis. These observations indicate that intact mitochondrial function is required to sustain some neutrophil functions, but not for the rapid initiation of the respiratory burst or phagocytosis. Loss of mitochondrial membrane potential was a very early marker for commitment of neutrophils into apoptosis and preceded the appearance of phosphatidylserine on the cell surface. However, inhibition of mitochondrial function did not accelerate the rate of neutrophil apoptosis. These data shed important insights into the hitherto unrecognized importance of mitochondria in the function of neutrophils during infection and inflammation. *The Journal of Immunology*, 2003, 170: 1964–1972.

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 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.

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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 5×10^6 /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 μ g/ml, FCCP³ (Sigma-Aldrich) added at up to 5 μ M. PMA (final concentration, 0.1 μ g/ml) and fMet-Leu-Phe (final concentration, 1 μ M; 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 $\leq 0.2\%$ (v/v), which was without effect on any of the parameters measured.

Morphological estimation of apoptosis

Following culture, a 20- μ l aliquot of suspension was made up to 200 μ l with RPMI 1640, and cells were cytocentrifuged using a Shandon Cytospin3 (Runcorn, Cheshire, U.K.). May-Grünwald-Giemsa staining of cytopins 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 1×10^6 neutrophils were removed from culture, and MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) or JC-1 (Molecular Probes) were added to final concentrations of 25 nM and 7.7 μ M, 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 μ M and then incubated for 20 min at 37°C before pelleting at $400 \times g$ for 3 min and resuspension in 50 μ l of PBS (pH 7.4) before analysis. As indicated in the figure legends, neutrophils were either stained while in suspension or allowed to adhere to glass slides for 10 min before addition of mitochondrial dyes.

Annexin V-FITC staining

Neutrophils (10^6) 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 $400 \times g$ and resuspended in HBSS without phenol red before analysis by flow cytometry using a Cytoson 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 μ g/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 μ l 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 $\times 63$ 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 505- to 550-nm band-pass filter (green fluorescence) and through a 545-nm dichroic mirror and a 560-nm long-pass filter (j-aggregate red fluorescence). DHR-123 fluorescence was collected through a 505- to 550-nm band-pass filter. Alternatively, MitoTracker Red fluorescence was also analyzed on a Zeiss Axiovert S100 TV fluorescence microscope through a $\times 40$ 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 ($1 \times 10^6/200 \mu$ l) and incubated with JC-1 (7.7 μ M) or DHR-123 (2 μ M) 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 μ M 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 (5×10^8 /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 whirlmixing 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 microchamber using a modified Boyden chamber (18). *Escherichia coli* endotoxin-treated human serum and fMLP (10^{-8} M), were used as positive chemotactic stimuli. Neutrophils (5×10^6 /ml) were incubated with different concentrations of inhibitor (as described in the figure legends) before measurement of chemotaxis. Cells (1×10^6 /well) were incubated at 37°C for 60 min before fixation with ethanol and staining with hematoxylin. The distance traveled into 3- μ m 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 $\times 400$ magnification for each filter.

Reactive oxygen metabolite production

Chemiluminescence was measured at 37°C in neutrophil suspensions (1×10^6 /ml) in HBSS medium that was supplemented with 10 μ M luminol using an LKB 1251 luminometer (Gaithersburg, MD) (15, 19). Cells were stimulated by the addition of PMA (0.1 μ g/ml) or unopsonized latex particles (1- μ m diameter).

Statistics

Statistical analysis was performed on datasets using ANOVA. Significant differences between datasets were defined as $p \leq 0.05$ (\dagger) and $p \leq 0.01$ (*). All data are presented as the mean \pm 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

³ Abbreviations used in this paper: FCCP, ; DHR-123, dihydrorhodamine 123; DPI, diphenylene iodonium.

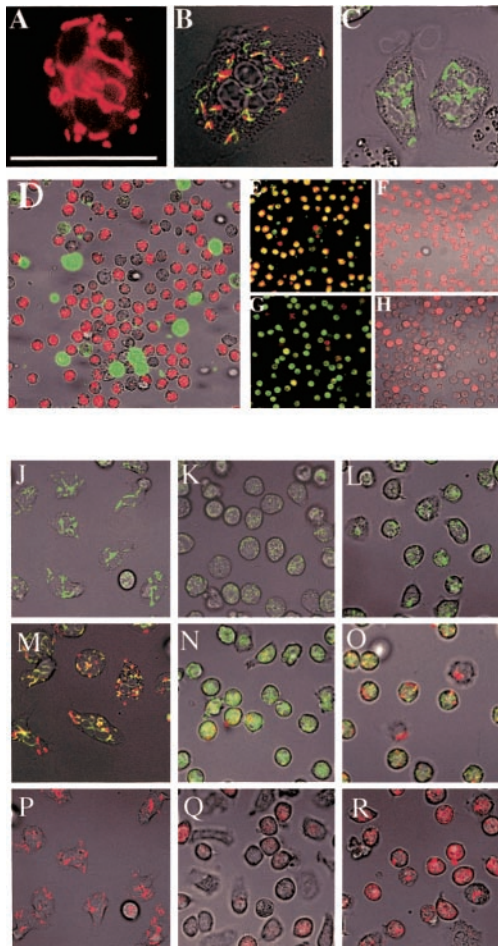


FIGURE 1. Neutrophils possess a complex mitochondrial network whose membrane potential is disrupted by FCCP, but not oligomycin. *A, F, H, and P–R*, Neutrophils were stained by the addition of MitoTracker Red (25 nM); *B, E, G, and M–O*, neutrophils were stained by JC-1 (7.7 μ M); *C and J–L* neutrophils were stained with DHR-123 (2 μ M). *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 μ g/ml oligomycin. All images were obtained by confocal microscopy. *A–C and J–R*, Neutrophils were allowed to adhere to glass slides for 10 min before addition of fluorescent dyes. *D–H*, Neutrophils were stained while in suspension, before analysis by confocal microscopy. The bar marker in *A* represent 10 μ m.

and red fluorescence (Fig. 1*B*). 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 Ca^{2+} inside mitochondria (21). DHR-123 is colorless, but becomes fluorescent upon oxidation, for example in the presence of H_2O_2 . 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. 1*C*). The development of this latter fluorescence was seen after an incubation of \sim 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. 1*E*) and MitoTracker Red fluorescence (Fig. 1*F*). 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. 1*H*), and the JC-1 fluorescence was green in most cells (Fig. 1*G*), 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. 1*D* 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.

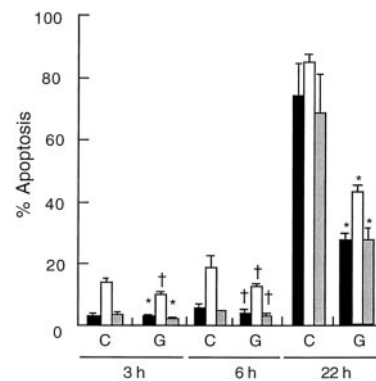


FIGURE 2. Decreases in mitochondrial membrane potential precede changes in morphology and phosphatidylserine exposure during neutrophil apoptosis. Neutrophils were incubated in the absence (C) or the presence (G) of 50 ng/ml GM-CSF. At 3, 6, and 22 h samples were removed for assessment of apoptosis by 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 (▒). *, $p \leq 0.05$; †, $p \leq 0.01$. Values shown are the means (\pm 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

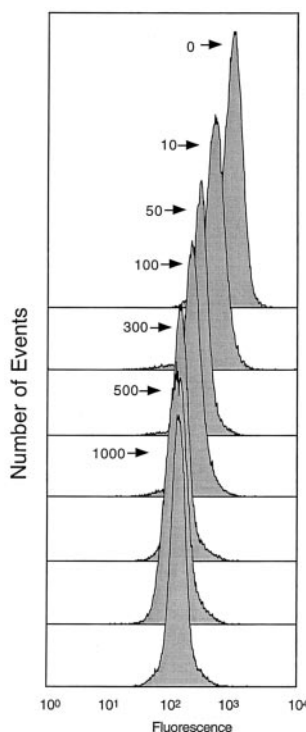


FIGURE 3. FCCP decreases rhodamine 123 fluorescence. Neutrophils were incubated for 20 min with DHR-123 (2 μ M) and then incubated in the absence (0) and the presence of FCCP at the indicated concentrations (nanomolar). After 15-min incubation, rhodamine 123 fluorescence was determined by flow cytometry.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

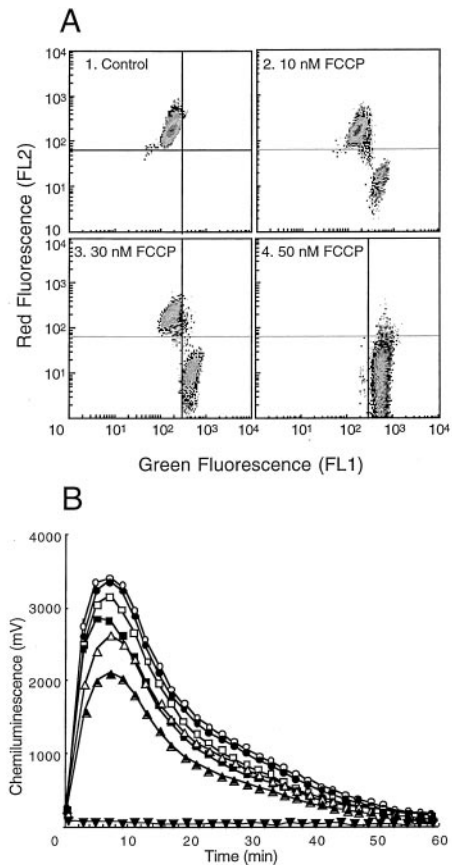


FIGURE 4. FCCP decreases mitochondrial membrane potential measured by JC-1 fluorescence. *A*, Neutrophils were loaded with JC-1 (7.7 μ M) and then incubated in the absence (control) and the presence of FCCP at the indicated concentration. After 15-min incubation, red (high mitochondrial membrane potential) 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 (\circ ; control) 10 nM (\bullet), 100 nM (\square), 300 nM (\blacksquare), 400 nM (\triangle), or 500 nM (\blacktriangle), or with 10 μ M DPI (\blacktriangledown). They were then stimulated by the addition of 0.1 μ g/ml PMA, and luminol chemiluminescence was measured. A typical result of six separate experiments is shown.

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 μ M (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 μ M) 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 μ M 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 μ M (data not shown).

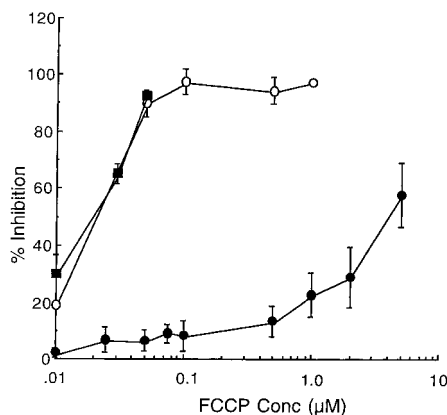


FIGURE 5. FCCP inhibits chemotaxis and decreases the mitochondrial membrane potential at similar concentrations, but only inhibits the respiratory burst at much higher concentrations. FCCP was added to neutrophil suspensions at the indicated concentrations before the measurement of chemotaxis (■), mitochondrial membrane potential (○; as a red-green shift in JC-1 fluorescence as in Fig. 4), and PMA-stimulated respiratory burst activity (●). Values presented are the means (\pm SD; $n = 3-6$).

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 mitochondr-

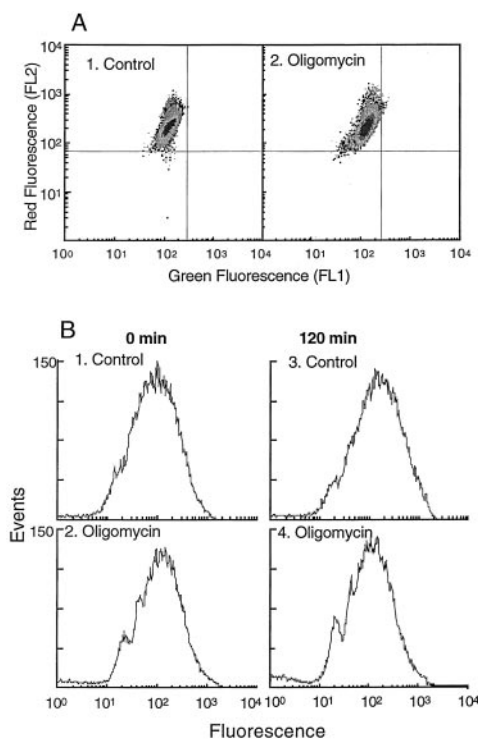


FIGURE 6. Oligomycin does not affect the mitochondrial membrane of neutrophils, measured by JC-1 fluorescence. *A*, Neutrophils were incubated as described in Fig. 4, except that they were incubated in the absence (*panel 1*) or the presence (*panel 2*) of oligomycin ($2 \mu\text{g/ml}$) before analysis of JC-1 fluorescence. A typical result of four separate experiments is shown. *B*, Neutrophils were incubated for 0 min (*panels 1* and *2*) or 120 min (*panels 3* and *4*) in the absence (*panels 1* and *3*) or the presence (*panels 2* and *4*) of oligomycin. After this, they were incubated with serum-opsinized, propidium iodide-stained *S. aureus* (prepared as described in *Materials and Methods*) for 30 min, and then bacterial uptake was determined by flow cytometry. A typical result of four separate experiments is shown.

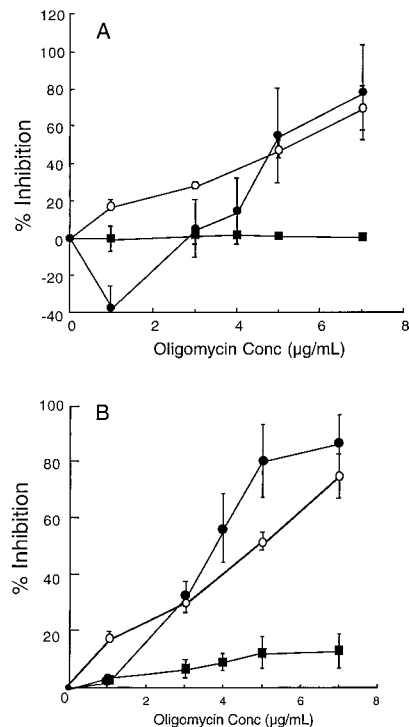


FIGURE 7. Long term, but not short term, incubation of neutrophils with oligomycin decreases their ability to generate a respiratory burst in response to PMA or latex particles. Neutrophils were incubated with and without oligomycin at the indicated concentrations for either 5 min (■) or 120 min (●) and then stimulated by the addition of either PMA at $0.1 \mu\text{g/ml}$ (*A*) or latex particles (*B*). Inhibition was determined as the decrease in peak chemiluminescence responses of control cells obtained after stimulation. Values presented are the mean \pm SD ($n = 5$). Also shown is inhibition of chemotaxis by oligomycin measured in response to fMLP or LPS-activated serum (○), shown as the mean \pm SD ($n = 6$).

drial 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. 6*A*). 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. 6*B*) or to activate a respiratory burst in response to PMA (Fig. 7*A*) or latex particles (Fig. 7*B*). 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. 7*A*) and latex particles (Fig. 7*B*). Half-maximal inhibition of these responses occurred at $\sim 4 \mu\text{g/ml}$ oligomycin. Curiously, after incubation of neutrophils with low concentrations of oligomycin ($1 \mu\text{g/ml}$), an enhancement of the respiratory burst by $28 \pm 14\%$ ($n = 5$; $p < 0.01$) was observed (Fig. 7*A*). 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 $\mu\text{g/ml}$) 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.

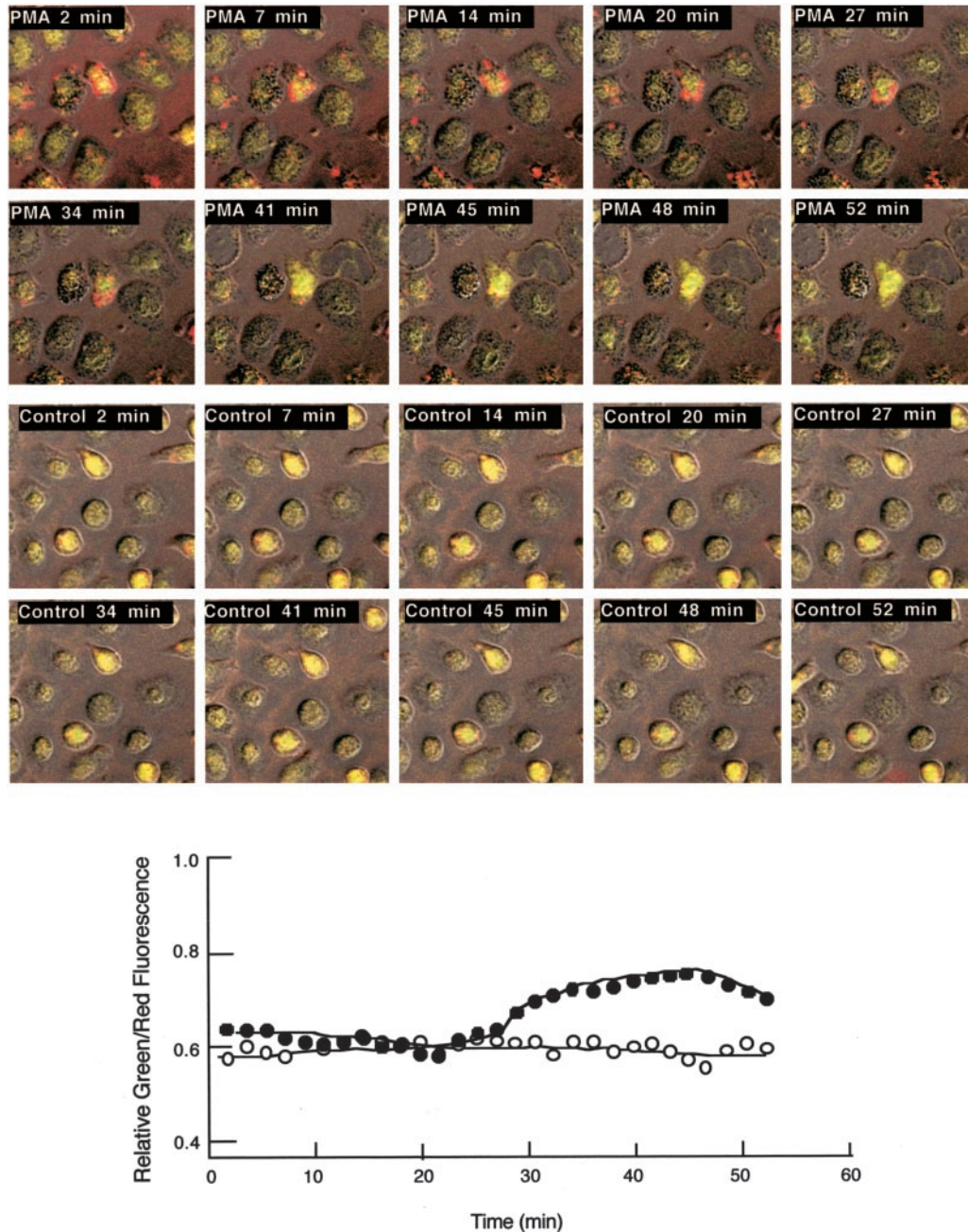


FIGURE 8. PMA treatment results in loss of mitochondrial function. Freshly isolated neutrophils were allowed to adhere to glass slides for 10 min and were stained with JC-1 for an additional 10 min. They were then incubated over a 60-min period on a temperature- and gas-controlled microscope slide, and confocal images were obtained every 2 min. Half the cell suspension was treated with PMA (0.1 $\mu\text{g/ml}$), 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.

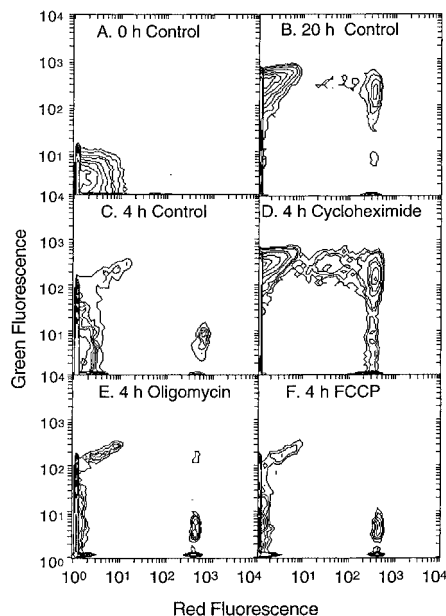


FIGURE 9. Oligomycin and FCCP do not accelerate the rate at which neutrophils progress into apoptosis. Neutrophils were stained with annexin V-FITC and propidium iodide (as described in *Materials and Methods*) after incubation as follows: A, 0-h incubation (freshly isolated cells); B, 20-h incubation, no additions; C, 4-h incubation, no additions; D, 4-h incubation with 10 $\mu\text{g/ml}$ cycloheximide; E, 4-h incubation with 2 $\mu\text{g/ml}$ 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.

No such changes in mitochondrial function or cell shape were seen in control suspensions or fMLP-treated cells (data not shown).

Effects of oligomycin and FCCP on neutrophil apoptosis

We then determined whether disruption of mitochondrial function with either FCCP or oligomycin accelerated the rate at which neutrophils progress into apoptosis. Freshly isolated neutrophils showed very low binding to annexin V (indicating only very low levels of phosphatidylserine exposed on their cell surface) and low staining with propidium iodide (indicating an intact plasma membrane), as shown in Fig. 9A. When cells aged during culture in vitro for 20 h, apoptosis was detected as cells with high annexin V-FITC binding and high staining with propidium iodide (Fig. 9B). After 4-h incubation in culture in HBSS, $\sim 15\%$ of the neutrophils were apoptotic (Fig. 9C), and this number increased to $\sim 50\%$ when cells were incubated with the protein synthesis inhibitor, cycloheximide (Fig. 9D), in agreement with previous reports (15). However, 4-h incubation with either oligomycin (at 2 $\mu\text{g/ml}$) or FCCP (at 50 nM) did not accelerate apoptosis compared with levels seen in control suspensions (Fig. 9, E and F). Note that in these experiments neutrophils were incubated in HBSS in the absence of serum to accelerate the rate at which they undergo apoptosis, enabling the early events controlling this process to be studied.

Discussion

In this report we show, for the first time that human neutrophils possess an intricate mitochondrial network that is required for key cellular processes such as chemotaxis and maintenance of cell shape. We used three separate fluorescent indicators of mitochondrial function, each with different chemical properties, to stain mitochondrial-like structures that extend throughout the neutrophil

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 mitochondrion-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 ~ 20 nM, in agreement with the known effects of this compound on uncoupling of mitochondrial respiration (35). At higher concentrations of FCCP ($>1 \mu\text{M}$), 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 $\sim 4 \mu\text{g/ml}$, 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

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 $\mu\text{g}/\text{ml}$ 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 H_2O_2 , 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 oxidase activity. This oxidase 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 oxidant 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 oxidant 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 oxidant 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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