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Mitochondrial Dysfunction in Lymphocytes from Old Mice: Enhanced Activation of the Permeability Transition

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Aging is associated with mitochondrial dysfunction in excitable tissues such as nerve and muscle. However, it is not known if immunosenescence is similarly associated with mitochondrial dysfunction in lymphocytes. We have found that spleen lymphocytes from old mice have lower respiration rates than lymphocytes from young mice. Cyclosporin, an inhibitor of the mitochondrial Permeability Transition, PT, restored normal respiration rates to lymphocytes from old mice, suggesting enhanced susceptibility to PT activation. Lymphocytes from old mice also had a lower mitochondrial membrane potential ($\Delta\psi_m$) than lymphocytes from young mice, which was also restored by cyclosporin. Oxidized FAD fluorescence was higher in lymphocytes from old mice suggesting a more oxidized state, which may be the cause of the enhanced activation of PT. Incubation of lymphocytes from old mice with the lipophilic cationic dye $DiOC_6(3)$, which inhibits electron transport, induced the appearence of apoptotic cells. These findings suggest that the mitochondrial PT is more susceptible to activation in lymphocytes from old mice. This activation may inhibit energy metabolism and enhance apoptosis, and may therefore contribute to immunosenescence.

Immunosenescence, the progressive dysfunction of the immune system in old age, is but one of several aging-related degenerative disorders. Studies of immunosenescence in humans and rodents have led to a detailed knowledge of immune system dysfunctions in the old (1). However, the underlying causes of aging-related immune dysfunction are not understood. Studies of aging-associated muscle degeneration and neurodegenerative diseases have suggested that mitochondrial dysfunction plays a major role in the aging-related degeneration of various tissues (2). However, to date there is no evidence for similar aging-associated mitochondrial dysfunctions in cells of the immune systems.

Recent studies on the mechanisms of cell death suggest that both necrosis (accidental cell death) and apoptosis (programmed cell death) can be mediated by mitochondrial events. In particular, the sustained activation of the mitochondrial permeability transition, PT, has been shown to be a controlling event in pathways leading to cell death (3–5).

In this study we examined the effect of aging on cell respiration, mitochondrial membrane potential, $\Delta\psi_m$, redox state, and the activation of PT in mouse lymphocytes. The results of our study suggest that lymphocytes from old mice are more succeptible to activation of the PT, most probably because of an increase in oxidative stress. PT activation could lead to inhibition of cell activation and prolferation, and may also induce apoptosis, and may therefore be an underlying cause of immunosenescence.

MATERIALS AND METHODS

Young (3 months) and old (24 months) male C57BL/6JNIA mice were obtained from Charles River Laboratories.

Isolation of spleen lymphocytes: The spleen was pressed through a nylon mesh in 5 ml HBSS medium (+0.3% BSA). The cell suspension was centrifuged at 800 g, for 6 min, at 4° C. The pellet was resuspended in 7 ml of the same medium, centrifuged again and washed once more the same way. The cells were resuspended in 5 ml of the same medium and layered on 5 ml Lympholyte M (Accurate), and centrifuged at 1500 g for 20 minutes, at 25° C. The lymphocytes were removed from the interphase, washed twice in HBSS and suspended in 2 ml of MEM medium (+1% FCS). The isolated lymphocytes were kept at room temperature for 1 to 3 h before the experiments. All experiments were preformed at 37° C in MEM (+1%FCS) medium(+ 1 mM Ca^{2+}, 5.5 mM glucose and 2.5 mM glutamine).

Cell respiration: respiration rates were measured by a polarographic oxygen electrode, usually with 1.5×10^7 cells in 1.5 ml, at 37° C.

Flow cytometry: Flow cytometry was carried out on a FACScan instrument (Becton Dickinson). For $\Delta\psi$m measurements, cells were incubated with $DiOC_6(3)$ (0.2 pmoles/10^6 cells, 37°C, 20 min). Then

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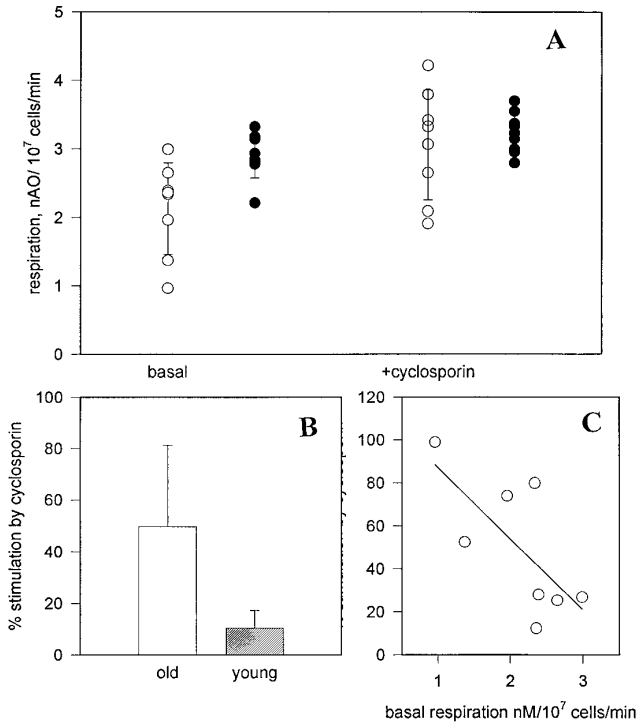

FIG. 1. The effect of aging and cyclosporin on lymphocytes' respiration. Assay conditions are described in "Material and Methods". Cyclosporin, when added, was 1 μM. (A) Rates of basal respiration and cyclosporin stimulated respiration in eight 24 month old mice (empty circles) and eight 3 month old mice (full circles). (B) Averaged (+ S.D.)% stimulation of respiration by cyclosporin in young and old mice (results are from Figure 1A). Figure 1C: Correlation between the % stimulation by cyclosporin and basal respiration in old mice (results from figure 1A).

divided into 3 fractions: controls (no further addition), cyclosporin treated (1 μM) and CCCP treated (40 μM). The cell suspensions were incubated for additional 20 min before the measurements (10^4 cells for each determination).

All the reagents were obtained from Sigma, except Lympholyte M that was was from Accurate, and DiOC6(3) that was from Molecular Probes. Cyclosporin was a gift from Sandoz.

RESULTS

Figure 1A shows the basal, steady-state, respiration rates of isolated spleen lymphocytes from 8 young (3 months) and 8 old (24 months) mice. The average respiration rate in lymphocytes from old mice was significantly lower than the average respiration rate in lymphocytes from young mice (2.13 nAO/10^7 cells/min $\pm$ 0.67(S.D.) vers 2.93 $\pm$ 0.36(S.D.), P=0.01). While the respiration rates of lymphocytes from young mice were very similar to each other (S.D. = $\pm$ 12%), the respiration rates of lymphocytes from old mice had a much larger variance (S.D. = $\pm$ 32%). Under conditions in which the mitochondrial respiration rate is limited by the concentrations of matrix substrates, triggering of the PT could inhibit respiration because respiratory substrates, which are accumulated in the matrix by $\Delta\Psi$ and/or ΔpH-driven transport systems, would be released from the matrix(6). Therefore, we tested the effect of cyclosporin, a potent inhibitor of the mitochondrial PT (6), on the rate of lymphocyte respiration. Cyclosporin stimulated respiration significantly in both old mice (P=0.0004) and young mice (P=.0002) (Figure 1A). This suggests that the PT is activated in a significant fraction of the mitochondria in both populations. However, the stimulation was much stronger in lymphocytes from old mice than in those from young mice (49.7% $\pm$ 31.4 stimulation in old vers. 10.4% $\pm$ 6.9 in young, P=0.001; figure 1B). After the addition of cyclosporin, there was no significant difference between the respiration rates of lymphocytes from old mice (3.06 $\pm$ 0.80) and young mice (3.22 $\pm$ 0.30). This suggests that the direct cause of the inhibition of respiration in lymphocytes from old mice is the enhanced activation of the PT. This conclusion can be verified by examining separately the effect of cyclosporin on lymphocyte respiration in each of the old mice (Figure 1C). It is seen that the magnitude of the effect of cyclosporin is inversely correlated with the rate of basal respiration (r=0.71). This is compatible with the conclusion that the low rate of respiration in lymphocytes from old mice is the result of PT activation. In contrast there was no correlation between the magnitude of the effect of cyclosporin and the basal respiration rates in lymphocytes from young mice (results not shown).

Since the collapse of $\Delta\Psi_m$ is a sensitive indicator of the activation of PT (6), we measured $\Delta\psi_m$ in lymphocytes from old and young mice with the fluorescence indicator $DiOC_6(3)$ using flow cytometry (7). Our studies of $\Delta\Psi_m$ in mouse lymphocytes (Wu and Rottenberg, in preparation), and in a malarial parasite (8), showed that this dye inhibits cell respiration at nM concentrations, and also that the response to $\Delta\psi_m$ is attenuated at nM concentrations. Therefore, we measured $\Delta\psi_m$ with the lowest possible dye concentration. The optimal concentration for $\Delta\Psi_m$ measurements in lymphocytes was determined to be 0.2 pmoles/10^6 cells) (Rottenberg and Wu, in preparation).

Figure 2A shows typical two dimensional histograms of $DIOC_6(3)$ fluorescence and forward light scattering (FSC) in lymphocytes from old (24 month) and young (3 month) mice with and without cyclosporin. The fluorescence intensity exhibits a much wider range of values in lymphocytes from old mice, with a much larger fraction of apparently apoptotic cells (i.e., having a low fluorescence and low FSC (5,7)). Addition of cyclosporin increased the fluorescence in lymphocytes from old mice much more than in young mice. In addition to reducing the size of the apoptotic fraction in lymphocytes from old mice, cyclosporin also eliminated the fraction of cells with low fluorescence but normal FSC. To estimate the

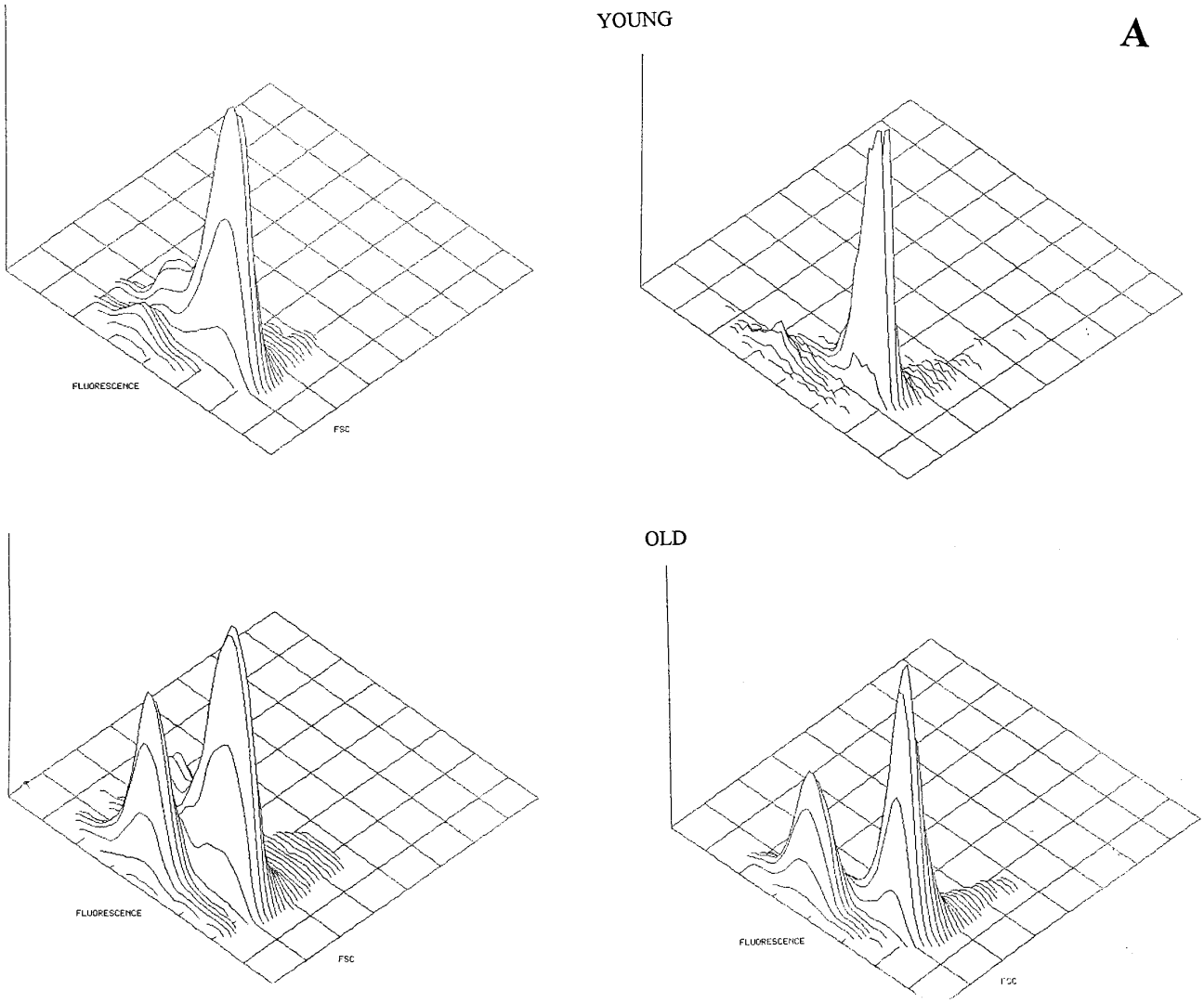

FIG. 2. The effect of aging on $\Delta\psi_m$ in spleen lymphocytes. (A) Two dimensional histograms, fluorescence vs forward light scattering (FSC), in young and old mice with and without cyclosporin. The protocol is described in "Material and Methods". $DioC_6(3)$ was 0.2 pM/10^6 cells. Cyclosporin was 1 μM. (B) The magnitude of membrane potential (i.e. F/Fcccp) in nine young mice(full symbols), and nine old mice(empty symbols), without cyclosporin (circles), and with cyclosporin (squares). Conditions are as in Figure 2A. CCCP when present was 40 μM.

magnitude of $\Delta\psi_m$, in each batch of cells, we compared the fluorescence of the cells to the fluorescence of the same batch of cells after treatment with the uncoupler CCCP, which colapses $\Delta\psi_m$. Figure 2B shows the average fluorescence ratio, F/Fcccp, which is proportional to the avearge $\Delta\psi_m$ (Rottenberg and Wu, in preperation), in lymphocytes from 8 young and 8 old mice, with and without cyclosporin. The ratio was significantly lower in lymphocytes from old mice (4.03 $\pm$ 0.53 in old mice vers. 4.98 $\pm$ 0.29 in young mice, p=0.0002). Cyclosporin increased the ratio(i.e. $\Delta\psi_m$) in lymphocytes from old mice more than in lymphocytes from young mice (57% $\pm$ 15.6 in old vers. 33.6% $\pm$ 10.1, P=0.0002), and the difference between old and young in the magnitude of the ratio F/Fcccp in the presence of cyclosporin (6.33 $\pm$ 1.09 in old mice vers. 6.66 $\pm$ 0.8 in young mice) was no longer significant. These findings are compatible with the interpetation of the results of the respiration experiments (Figure 1), which suggested increased activation of the PT in lymphocytes from old mice.

The large increase of the fraction with low FSC and low fluorescence (Figure 2A) suggests a large increase in the fraction of cells commited to apoptosis in lymphocytes from old mice (5,7). This increase was found to be largely due to the effect of $DiOC_6(3)$ on lymphocytes from old mice. Figure 3A shows typical histograms of forward light scattering in old and young mice. In the

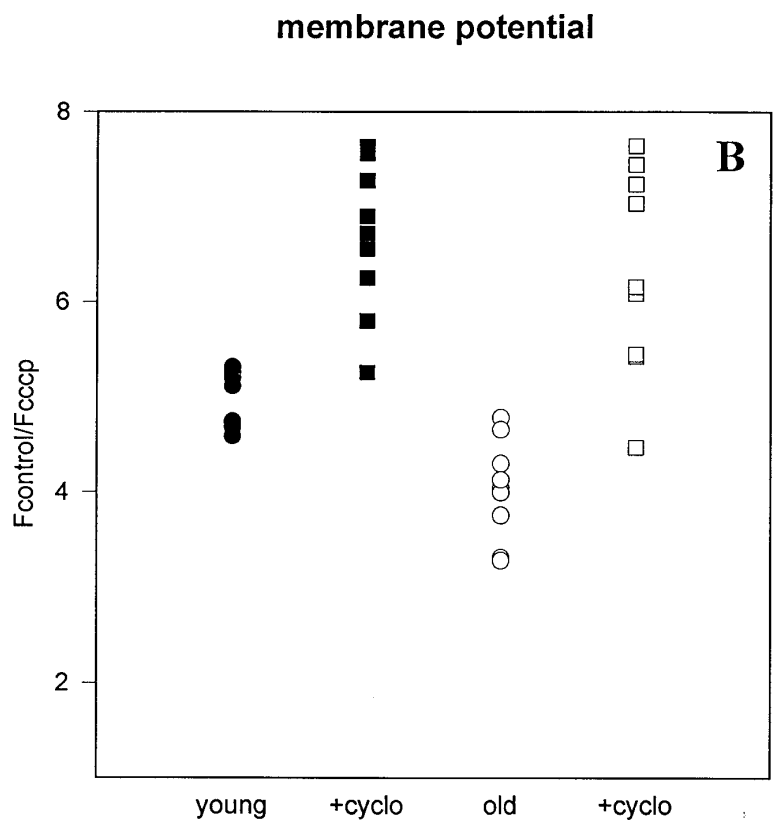

FIG. 2—*Continued*

absence of added dye there is a very small fraction of shrunken cells (low FSC) in lymphocytes from young mice. In old mice this fraction is larger. Moreover, in lymphocytes that were incubated for one hour with 0.2 nM $DIOC_6(3)$, there was a significant increase in the number of shrunken cells, and the $DIOC_6(3)$-induced increase is much larger in lymphocytes from old mice. Figure 3B shows the effect of $DIOC_6(3)$ on the % of cells with low FSC in lymphocytes from 6 old and 6 young mice. Without dye, only 3.7% ± 1.2 of lymphocytes from young mice were in the low FSC fraction compared with 8.0% ± 4.1 in old mice ($p=0.04$). Incubation for 1 hour with $DIOC_6(3)$ caused shrinkage in additional 4.8% of cells in young mice. In old mice an additional 17.4% of cell were shrunken. The difference between lymphocytes from young and old mice in their response to the effect of $DiOC_6(3)$ on forward light scattering was the largest difference we observed in this study and also the most significant ($P=3\times10^{-8}$). It has been reported that low concentrations of inhibitors of electron transport can induce apoptosis (9). Since $DIOC_6(3)$ is a potent inhibitor of electron transport (8,10, and Rottenberg and Wu, in preparation), it is likely that the increased number of apoptotic lymphocytes after 1 hour incubation with the dye indicates induction of apoptosis by $DIOC_6(3)$. It is therefore possible that lymphocytes from old mice are much more susceptible to induction of apoptosis by electron transport inhibitors, which could be directly related to their enhanced susceptibility to activation of PT.

In an effort to find out what is the basis of the enhanced susceptibility to activation of PT in lymphocytes from old mice, we compared the redox state of lymphocytes from young and old mice. It is well known that the mitochondrial redox potential is the most important parameter controling the activation of PT (11). A simple measure of the redox potential in mitochondria is the ratio FAD/FADH which is in equilibrium with the ratio NAD(P)/NAD(P)H in mitochondria(12). The latter ratio determines the ratio of oxidized/reduced glutathione in mitochondria through the action of glutathione reductase.

The intrinsic fluorescence of cells excited by argon laser (488 nm) is largly due to FAD fluorescence, of which the major fraction is contributed by the mitochondrial α-lipoamide dehydrogenase (13). Figure 4 summarizes the results of measurements of argon laser induced fluorescece in lymphocytes of 12 young and 12 old mice. The average intrinsic fluorescence of lymphocytes from old mice, 30.9 ± 5.8 is significantly higher than that of young mice, 25.5 ± 2.4 ($P=0.008$). This indicates that the mitochondrial FAD/FADH ratio is higher in lympho-

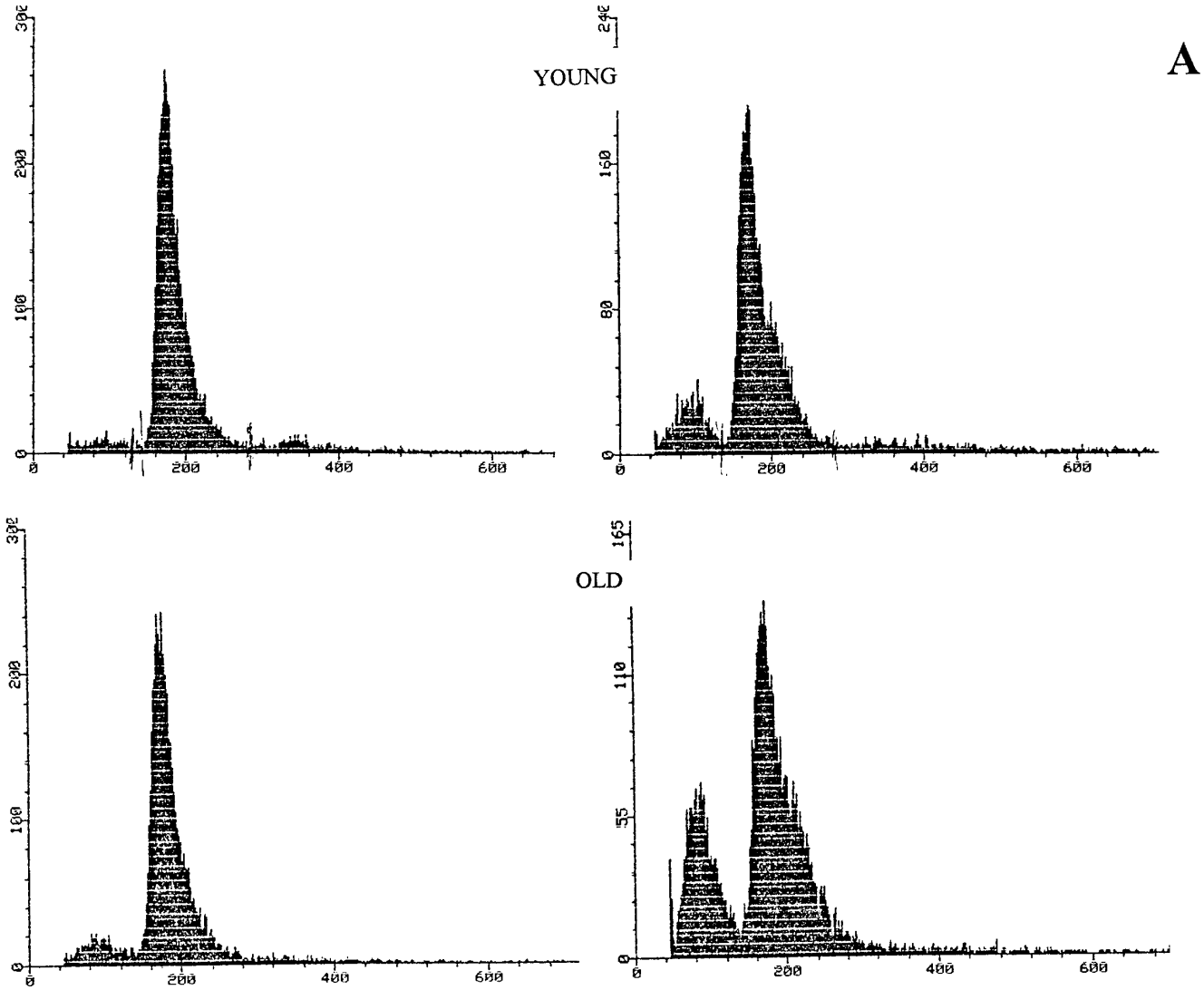

FIG. 3. Forward light scattering in lymphocytes from young and old mice. (A) Histograms of forward light scattering (FSC): lymphocytes from a young mouse incubated for 1 hour without dye (top left), or after 1 hour incubation with $DiOC_6(3)$ (top right); lymphocytes from old mouse incubated for 1 hour without dye (bottom left), and after 1 hour incubation with the dye (bottom right). Conditions are as in figure 2. (B) % cells with low FSC in six young mice (full circles), and six old mice (empty circles), without dye (background), and with dye (DIO). Conditions are as in Figure 3A.

cytes from old mice, suggesting a more oxidized state, which may be the cause of the increased susceptibility of lymphocytes from old mice to activation of PT.

DISCUSSION

This study is the first demonstration of aging-associated mitochondrial dysfunction in lymphocytes. The aging-associated mitochondrial dysfunction in muscle and nerve of rodents, primates, and humans was associated with attenuated activity of the electron transport complexes, and has been attributed to oxidative damage to mitochondrial proteins, lipids and DNA (2,14). Our findings that cyclosporin restores almost normal respiration rates and $\Delta\Psi_m$ to lymphocytes from old mice suggests that in this system, at least, the direct cause of inhibition of cell respiration and reduction of $\Delta\Psi_m$ is an activation of the mitochondrial PT.

It has been suggested previously that T cells from old mice exhibit lower $\Delta\psi_m$, on the basis of rhodamine 123 fluorescence (15). However, this response was shown later to result from increased activity of the P-glycoprotein multidrug transporter (16). We have used $DiOC_6(3)$ as a probe for $\Delta\psi_m$, and this dye is not as good a substrate for the P-glycoprotein transporter as rhodamine 123, because of its very high passive permeability (unpublished results, and cf. 17). Moreover, the difference in $\Delta\Psi$ between old and young in our study

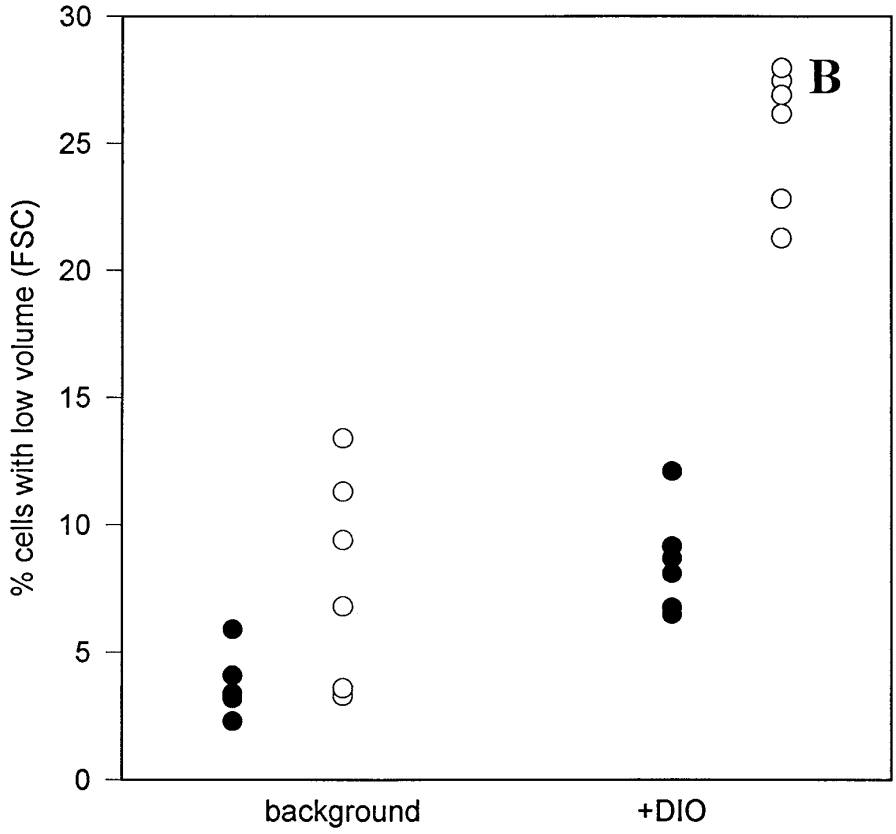

FIG. 3—*Continued*

was not affected by inhibition of the P-glycoprotein transporter by reserpine (results not shown).

It is likely that enhanced generation of Reactive Oxygen Species (ROS), perhaps as a result of damage to electron transport complexes (2,14), leads to increased oxidatative stress in lymphocytes from old mice, which renders their mitochondria more susceptible to PT activation (11). Our finding that lymphocytes from old mice exhibit higher FAD fluorescence, which suggests a more oxidized state of the mitochondrial redox couples(12), including glutathione, is compatible with this explanation.

It was reported recently that in liver cells from old rats $\Delta\psi_m$ declines and mitochondrial heterogeneity and oxidants increase (18), in complete agreement with our finding in mouse lymphocytes. Because PT activation has been shown to play a central role in apoptosis of lymphocytes (5,7), and most probably inhibits cell proliferation, it is possible that the susceptibility of lymphocyte mitochondria in old mice to the activation of PT is a major factor in immunosenescence. Increased susceptibility to apoptosis of naive T cells (19) may be the direct cause of the accumulation of memory T cells with age (1). It is also possible that similar activation of the mitochondrial PT occurs in the brain of the elderly and thus facilitates eventual neurodegeneration (3,20).

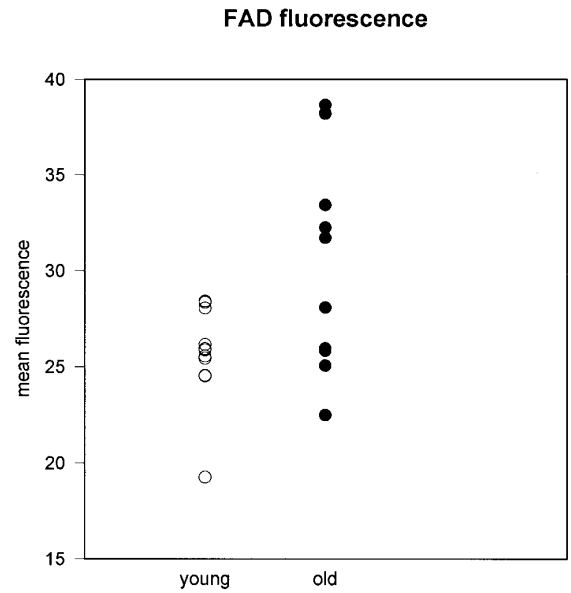

FIG. 4. FAD fluorescence in lymphocytes from Young and old mice. Intrinsic fluorescence, measured by flow cytometry (excitation = 488 nm, emission = 530 nm), from cells incubated for 40 min in MEM medium (+1% FCS).

In conclusion, our observation that a fraction of lymphocytes from old mice appear to be more susceptible to activation of the PT and induction of apoptosis by mitochondrial inhibitors is compatible with the suggestion that mitochondrial dysfunction contributes to immunosenescence.

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