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Age-related Changes in the Mitochondrial Depolarization Induced by Oxidative Injury in Human Peripheral Blood Leukocytes

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Aging is associated with impaired immunity and reduced host defenses. Mitochondrial bioenergetic dysfunctions and reduced antioxidative ability of immunocompetent cells may contribute to this phenomenon. In this study, 60 healthy volunteers of different age groups donated their blood after overnight fasting. Leukocytes were subjected to oxidative injuries by exposure to *t*-butylhydroperoxide, and were labeled with fluorochromes for measuring mitochondria transmembrane potential ($\Delta\psi_m$), membrane peroxidation and mitochondrial oxidant formation. $\Delta\psi_m$ declined after *t*-butylhydroperoxide exposure, and the change was more prominent in leukocytes from older individuals. Cyclosporin A partly restored $\Delta\psi_m$, implying the contributing role of mitochondrial permeability transition pores. The mitochondrial depolarization was accompanied by increased oxidant formation and oxidation of pyridine nucleotides, which were more prominent in older subjects. The results support the view that the bioenergetic functions of mitochondria are more susceptible to oxidative injury in aged individuals. The decreased ability of leukocytes to resist oxidative stress may contribute to immunosenescence in humans.

Keywords: Aging, leukocytes, mitochondria, oxidants, cyclosporin, immunity

Abbreviations: $\Delta\psi_m$, mitochondria transmembrane potential; PT, permeability transition; ROS, reactive oxygen species; DCFH-DA, 2′-7′-dichlorofluorescein diacetate; NAO, 10-n-nonyl acridine orange; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide; Fluor-DHPE, fluoresceinated phosphoethanolamine; PI, propidium iodide; *t*-BuOOH, *tert*-butylhydroperoxide; CSA, cyclosporin A

INTRODUCTION

Progressive dysfunction of the immune system may contribute to the higher rate of morbidity and mortality from infectious disorders in elderly individuals. Impaired leukocyte phagocytic functions and superoxide productions, reduced

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neutrophils chemotaxis, and defects in leukocyte signal transduction have been associated with immunosenescence.[1,2] However, the nature of the mechanisms causing these age-related alterations is not well understood and remains under intense investigation.

Tissues of older organisms are more vulnerable to free radical-induced damage, which may be attributed to progressive accumulation of oxidative damage[3] or a decline in the competence of antioxidants. One of the potential targets of oxidant-related injury is the energy factory of cells, mitochondria. Several lines of evidences indicate that the bioenergetic functions of mitochondria deteriorate during normal aging.[4] The mitochondria dysfunctions during aging are multifarious, including increased frequency of mitochondrial DNA mutagenesis,[5] decline in electron transport activity,[6,7] and decreased mitochondrial transmembrane potential ($\Delta\psi_m$). [7–9] $\Delta\psi_m$ is the driving force of cellular ATP formation, and the reduction in $\Delta\psi_m$ can lead to ATP depletion and cell deenergization. Recent evidence showed that oxidants may induce $\Delta\psi_m$ reduction and mitochondrial depolarization by promoting mitochondrial permeability transition (PT) due to oxidation of mitochondrial pyridine nucleotides and glutathione.[10,11]

Because immune cells especially phagocytes generate large amounts of reactive oxygen species (ROS) as part of their normal functions, they are particularly sensitive to changes in the antioxidant status. The favorable effects of various antioxidants on the natural killer activity in mononuclear cells from aging subjects may render support to this notion.[12] In view of the fact that oxidative damage accumulates during aging process,[13] the ability of immune cells to resist against the age-associated oxidative injury is potentially important to the maintenance of immune functions.

Lymphocytes from old mice were shown to have enhanced activation of mitochondrial PT and impairments in energy metabolism.[7] It is tempting to speculate that the age-related changes in leukocyte mitochondria functions may be related to their different vulnerabilities to oxidative injury. In the current study, we show that the peripheral blood leukocytes display age-related variability in their responses to oxidative injuries with respect to $\Delta\psi_m$, mitochondrial redox status and oxidants production. This knowledge may be important to clarify the nature of immunosenescence and may suggest potential pathways for its modulation.

METHODS

Subjects

Sixty healthy subjects from three age groups (group 1: 20–30 years; group 2: 45–65 years; group 3: beyond 75 years) were recruited into the study, where each donated 10 ml of blood samples after overnight fasting (Table I). All subjects were free of cardiopulmonary, infectious, malignancy, immune or inflammatory disease with potential for disturbed immune status or oxidative stress. None of the recruited subjects had taken anti-inflammatory drugs, corticosteroids or antioxidants for one month before the study entry. Written informed consent was obtained from all study participants, and the performance of this study was approved by the research ethics committee at the Taipei Veterans General Hospital, Taiwan.

Reagents

2′,7′-dichlorofluorescein diacetate (DCFH-DA), 10-n-nonyl acridine orange (NAO), 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazol carbocyanine

TABLE I Patient characteristics

Group	1	2	3
Number, *n*	20	20	20
Age, years	23.5 ± 0.65	54.64 ± 1.2	79.7 ± 0.67
Gender (M/F), *n*[a]	13/7	12/8	10/10

[a]$p < .05$, compared among groups.

iodide (JC-1), fluoresceinated phosphoethanolamine (fluor-DHPE) and propidium iodide (PI) were obtained from Molecular Probes (Eugene, OR). *Tert*-butylhydroperoxide (*t*-BuOOH) was purchased from Sigma (St. Louis, MO). Cyclosporin A (CSA) was obtained from CalBiochem (San Diego, CA).

Cell Isolation and Treatment

Approximately 10 ml heparinzed whole blood was treated with 90 ml of 1:10 erythrocyte lysing buffer (PharMingen, San Diego, CA) for 10 min. The buffer did not contain a fixative agent, so leukocytes remained viable after red blood cell lysis.[14] The supernatant was discarded and the cells were washed once with PBS at 200 g for 5 min. The cell pellet was resuspended with Hank's balanced salt solution (Gibco BRL, Paisley, Scotland, UK) to approximately 10^5 cells/ml and separated into two aliquots for subsequent cell labeling. The viability of leukocytes was confirmed to be more than 95% as assessed by trypan blue exclusion test.

One aliquot of leukocyte suspension was incubated with 2 mM *t*-BuOOH for 15 min at 37 °C. The other aliquot of leukocyte was incubated under the same circumstances without adding *t*-BuOOH. After incubation, the cells were washed twice with PBS at 200 g for 5 min. The pellet was resuspended with Hank's balanced salt solution to approximately 10^5 cells/ml and separated into several 200 ml aliquots for subsequent staining.

Cell Labeling

All aliquots of leukocytes, with or without *t*-BuOOH exposure, were labeled separately with the following fluorescent probes.

Because the mitochondria mass of different populations of leukocytes may vary greatly at different points, a more reliable method to specify $\Delta\psi_m$, using the fluorochrome JC-1, was employed in the present study.[15] The leukocyte suspension was incubated with 5 μM JC-1 for 20 min at 37 °C. After staining, JC-1 incorporates into mitochondria, where it either forms monomers (green fluorescence, 527 nm) or, at high transmembrane potential, aggregates (red fluorescence, 590 nm). The ratio between fluorescence intensity of JC-1 aggregates and monomers can reliably reflect $\Delta\psi_m$, independent of changes in the number and mass of mitochondria. In separate experiments, another aliquot of leukocyte suspension was pre-incubated with 5 μM CSA for 15 min at 37 °C[16] and was stained later with JC-1 using the same procedure.

In addition, we also measured leukocyte mitochondrial mass by staining the cells with 1 μg/ml NAO for 15 min at 37 °C. NAO interacts with mitochondria cardiolipin, which is not influenced by the mitochondria energy state.[17]

For measuring intracellular oxidants, cells are incubated for 20 min at 37 °C with 20 μM DCFH-DA. Upon reaction with intracellular oxidants, DCFH-DA is converted to fluorescent 2′,7′-dichlorofluorescein (DCF).[18] To exclude the fluorescence of dead cells, the samples stained with DCFH-DA are also counterstained with 5 μl of 3 mM PI for 3 min before the measurement by flow cytometry.

The extent of membrane peroxidation was evaluated by incubating leukocyte suspensions with 1 μM fluor-DHPE for 1 h at 37 °C. Fluor-DHPE decreases its fluorescence upon exposure to membrane peroxides,[19] and is an ideal probe for measuring single cell lipid peroxidation as it is not exchangeable among cells.

Mitochondria Redox Status

The intrinsic fluorescence of leukocytes excited by a 325-nm UV laser was used as a reflection of mitochondrial reduced pyridine nucleotides (NADH and NADPH), as previously described.[20] Oxidized pyridine nucleotides are nonfluorescent and the change in the intrinsic fluorescence accounts for the redox state of pyridine nucleotides.

Flow-cytometric Evaluation

Fluorescence was analyzed by cytometry using a FACScan (Becton Dickinson, San Jose, CA) fitted with an air-cooled argon laser emitting at 488 nm, and an air-cooled helium cadmium laser emitting at 325 nm (for the measurement of autofluorescence of leukocytes). All data were analyzed using CellQuest flow cytometric analysis software. During analysis a gate was set on the dot plot of forward and side scatter to include polymorphonuclear (PMN) leukocytes, monocytes and lymphocytes, and exclude red blood cells and debris. The identity of cell populations in each analysis was verified by comparison with FSC/SSC profiles obtained from respective cell populations separated from the whole blood by density gradient centrifugation and confirmed by counterstaining with CD45 and CD14 antibody reagents.[21] The total number of events from each sample was made such that at least 5,000 events were collected for the three leukocyte subpopulations. To ensure consistency of data among different measurements, an appropriate voltage setting of the photomultiplier for each type of fluorochrome, based on the data from a control subject, was employed throughout all the experiments. The mean fluorescence of each cell was determined to permit comparisons among data. For the cells labeled with JC-1, the ratio of mean red fluorescence intensity (FL2: 590 nm) over green fluorescence intensity (FL1: 527 nm) was calculated and quoted as an index of $\Delta\psi_m$. The fluorescence from cells labeled with NAO, DCFH-DA or fluor-DHPE was collected through the FL1 channel. All the procedures were completed within 3 hours after blood sampling.

Statistics

All continuous data were expressed as mean ± SEM. Group comparison was judged by χ^2-test, Mann-Whitney U-test or ANOVA where appropriate. A $p < .05$ was considered statistically significant.

RESULTS

Comparison of Untreated Leukocytes among Different Age Groups

Figure 1 shows $\Delta\psi_m$, mitochondria mass, intracellular oxidants and reduced pyridine nucleotides of untreated leukocytes in the three age groups. $\Delta\psi_m$, as reflected by the ratio of JC-1 red and green fluorescence intensity, did not differ significantly among different age groups ($p > .05$ by ANOVA) (Figure 1A). Likewise, mitochondrial mass remained unchanged among different age groups ($p > .05$ by ANOVA) (Figure 1B). PMN had relatively lower $\Delta\psi_m$ value than that of monocytes or lymphocytes. In comparison, lymphocytes had lowest mitochondria mass, as indicated by lowest NAO fluorescence intensity under the same assay circumstances.

Intracellular oxidants, as indicated by DCF fluorescence, increased in elder subjects (Figure 1C). DCF fluorescence altered less prominently in lymphocytes, while the value of age group 3 (> 75 years) was still higher than that of age group 20–30. In contrast, intrinsic fluorescence of leukocytes declined significantly in the elder groups (Figure 1D). Similarly, lymphocytes showed fewer changes among three age groups.

Leukocyte $\Delta\psi_m$ Changes after Exposure to *t*-BuOOH

Exposure of leukocytes to *t*-BuOOH resulted in a different degree of $\Delta\psi_m$ reduction in the three leukocyte subpopulations (Figure 2, black columns). An age-dependent disruption in $\Delta\psi_m$ was noted in monocytes and lymphocytes, with the elder subjects exhibiting more pronounced $\Delta\psi_m$ reduction (Figure 2B and 2C). In contrast, the *t*-BuOOH-induced $\Delta\psi_m$ reduction in PMN was less prominent, with the difference among three age groups being statistically indiscriminate ($p > .05$ by ANOVA).

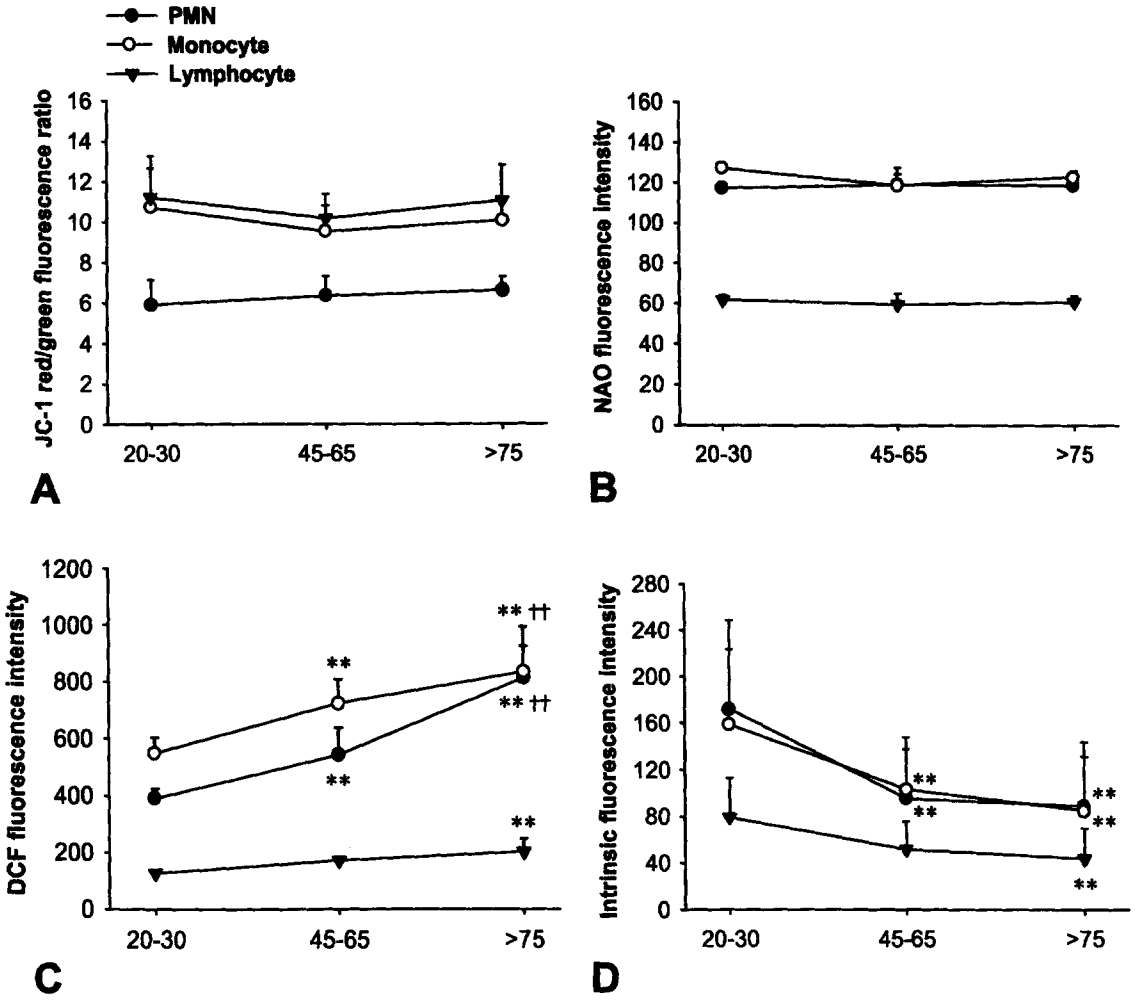

FIGURE 1 Distribution of $\Delta\psi_m$ (A), mitochondrial mass (B), mitochondrial oxidants (C) and redox status (D) with age in peripheral blood leukocytes. Value is expressed as mean ± SEM. $^{**}p < .01$ compared with age group 20–30; $^{\dagger\dagger}p < .01$ compared with age group 45–65.

Reversibility of $\Delta\psi_m$ by Cyclosporin A

When *t*-BuOOH-treated leukocytes were additionally incubated with CSA, $\Delta\psi_m$ was recovered to variable extents (Figure 2, white columns). CSA completely restored $\Delta\psi_m$ in PMN (Figure 2A). The effect of CSA on $\Delta\psi_m$ was less striking in monocytes and lymphocytes, with the elder subjects showing poorer responses (Figure 2B and 2C). Nevertheless, the increase in $\Delta\psi_m$ was statistically significant ($p < .01$).

Intracellular Oxidants and Membrane Peroxidation

As shown in Figure 3A, *t*-BuOOH caused an increase in the DCF fluorescence in the three subsets of leukocytes, indicating increased intracellular oxidant production. The increase was more prominent in PMN than in monocytes or lymphocytes. Also apparent is a significant trend toward higher DCF fluorescence in the elder subjects ($p < .01$ by ANOVA). As shown

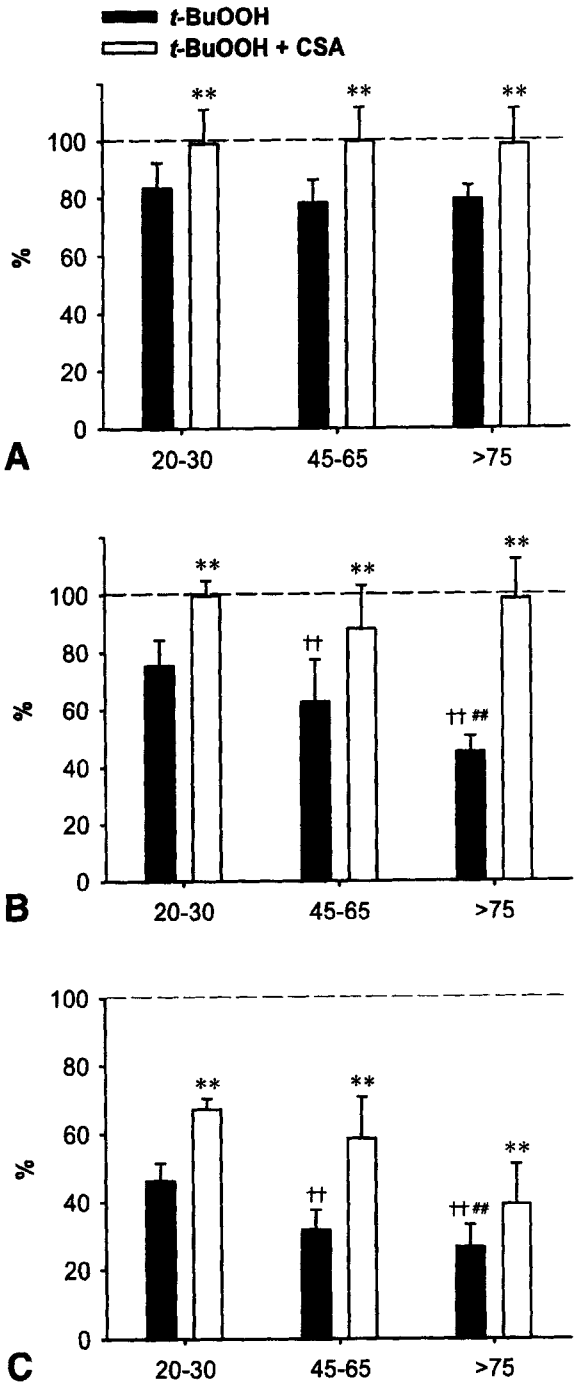

FIGURE 2 Percentage change of $\Delta\psi_m$ after *t*-BuOOH exposure with (white columns) or without (black columns) CSA in PMN (A), monocytes (B) and lymphocytes (C). The data are expressed as the percentage of $\Delta\psi_m$ value without *t*-BuOOH exposure. $^{**}p < .01$ compared with $\Delta\psi_m$ value obtained without CSA exposure; $^{\dagger\dagger}p < .01$ compared with age group 20–30; $^{\#\#}p < .01$ compared with age group 45–65.

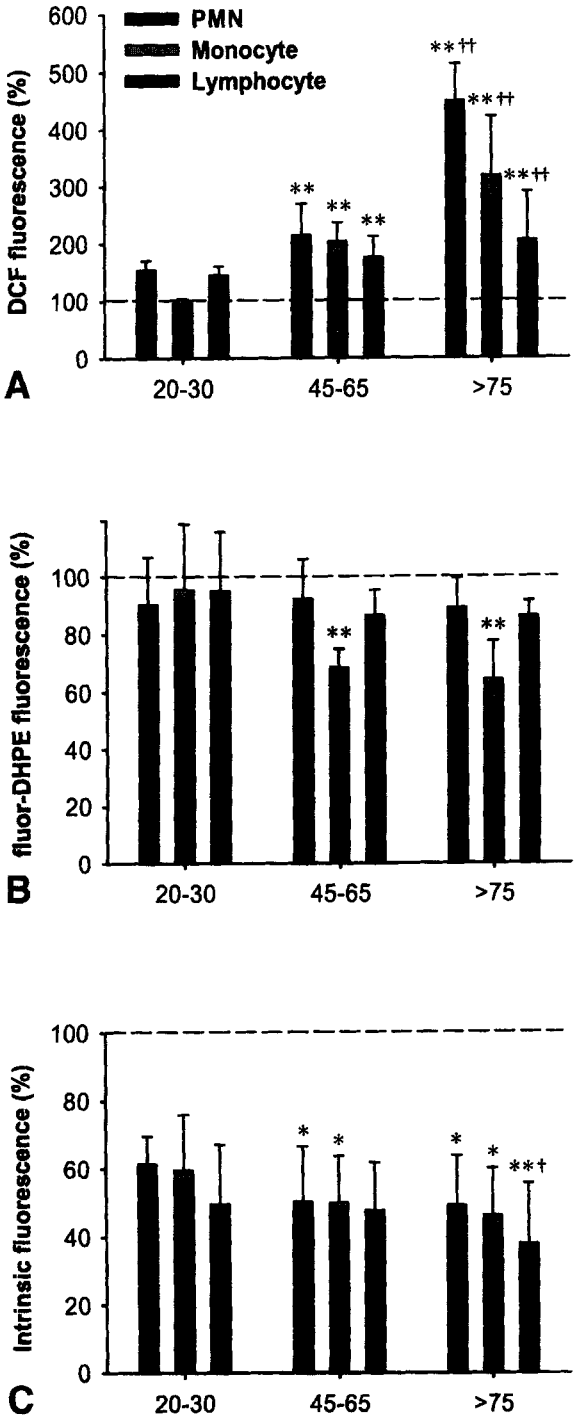

FIGURE 3 Comparisons of changes of intracellular oxidants (A), membrane peroxidation (B) and reduced pyridine nucleotides (C) after *t*-BuOOH exposure among three age groups. $^{*}p < .05$, $^{**}p < .01$ compared with age group 20–30; $^{\dagger}p < .05$, $^{\dagger\dagger}p < .01$ compared with age group 45–65.

in Figure 3B, *t*-BuOOH also caused reductions in the fluor-DHPE fluorescence, indicating increased peroxidation of cell and organelle membranes. However, only monocytes showed significant age-dependent changes.

Reduced Pyridine Nucleotide and Mitochondrial Redox Status

t-BuOOH caused a decline of leukocyte intrinsic fluorescence excited by 325-nm UV light (Figure 3C). Though less prominent, the decline still showed significant age-dependent alterations ($p < .05$ by ANOVA in the three leukocyte subpopulations, respectively). Leukocytes from group 3 subjects had the lowest intrinsic fluorescence after *t*-BuOOH treatment.

DISCUSSION

The accumulation of oxidative damage to proteins or DNA is hypothesized to play a key role in the aging process. A heightened rate of ROS generation and an increase in the susceptibility of tissues or cells to oxidative stress may account for this phenomenon.[13] Age-related changes in plasma antioxidant molecules and the effects of antioxidants on the functions of immune cells[12,22,23] have been evaluated previously, but few studies investigated the age-related alterations in the oxidant-resisting ability of immune cells. The present study is the first demonstration of the effect of age on the oxidant-induced mitochondrial dysfunctions in peripheral blood leukocytes in humans. The results may shed a new light on the pathogenesis of immunosenescence of elderly individuals.

Contrary to *in vitro* cell line studies, caution must be taken in interpreting the alterations of leukocyte function in the present clinical investigation. It is not due to the changes of the same cell population, but due to leukocyte subpopulational changes. This is especially relevant in the measurement of $\Delta\psi_m$, as the variation in mitochondria mass may alter the fluorescent intensity of other fluorochromes such as rhodamine 123 or $DiOC_6$ and, thus, interfere with the interpretation of $\Delta\psi_m$. The use of the ratio between red and green fluorescence of JC-1 obviously obviated the above problem and proved to be a better estimation of $\Delta\psi_m$ due to its excellent correlation with that which is measured by using electrochemical method.[15]

Age-related mitochondrial dysfunction has been demonstrated in muscle cells, nerve, and hepatocytes.[3,4] A decline in $\Delta\psi_m$ has been reported in hepatocytes,[8] fibroblasts,[24] and lymphocytes,[16] but not in epithelial cells.[25] In the present study, we did not find an age-dependent decline in $\Delta\psi_m$ in the three subsets of peripheral blood leukocytes. There is no clear explanation for this discrepancy, but it is possible that cell types with rapid turnover such as epithelial cells or blood cells may be able to remove damaged mitochondria before phenotypic changes occur. Probably for the same reason, the mitochondria mass, as measured by cell cardiolipin content, did not differ among different age groups.

t-BuOOH is an analogue of short-chain lipid hydroperoxides formed during oxidative stress.[26] The mechanisms by which *t*-BuOOH induce mitochondrial PT have previously been investigated in hepatocytes.[27,28] Upon exposure to *t*-BuOOH, membrane lipids and mitochondria pyridine nucleotides (NADH and NADPH) become rapidly peroxidized, as confirmed by the decline in the fluor-DHPE and intrinsic fluorescence of leukocytes (Figure 3). The susceptibility of leukocytes to oxidation increased with age as the changes of both markers became more prominent in older groups. The oxidation of mitochondrial pyridine nucleotides may in turn trigger the opening of the redox-sensitive PT pores by oxidizing dithiols in its structure.[10,28] PT pores opening will cause mitochondria depolarization and impairment of ATP formation, which eventually lead to cell deenergization and dysfunction.

Several previous investigations proposed that the opening of mitochondria PT pores was selectively blocked by CSA, by inhibiting the enzymic activity of cyclophilin-D, a constitutive component of the PT pore.[29] In the present study, CSA completely restored $\Delta\psi_m$ in PMN in all age groups, corroborating the paradigm that mitochondrial PT contributes to the mitochondrial depolarization induced by oxidative injury. In contrast, CSA only partially restored $\Delta\psi_m$ in monocytes and lymphocytes. It is possible that alternative mechanisms, such as the oxidation of carrier molecules in the respiratory chain that may hamper respiratory coupling, may also contribute to the oxidant-related mitochondrial depolarization. Another finding worth mentioning is that the recovery effect of CSA on $\Delta\psi_m$ became attenuated in older subjects (Figure 2B and 2C). Another plausible explanation would be the age-related attenuation in the functional activity of adenine nucleotide translocase (ANT).[30] ANT is one of the major constituents of mitochondrial PT pore complex.[10] The decrease in ANT activity in older subjects may aggravate oxidant-induced mitochondrial depolarization either by promoting PT pore opening or by giving rise to decreased availability of ADP in mitochondria, leading to a depression of state 3 respiration and ATP synthesis.

As consistent with previous reports,[8] mitochondria oxidant production was higher in leukocytes from older subjects (Figure 1C), which might be a reflection of an impaired coupling of mitochondrial electron transport to ATP production. A plausible explanation is that the age-related mitochondrial DNA mutagenesis may lead to inhibition of the electron transport chain, which in turn leads to increased superoxide generation by reaction of ubisemiquinone radical with molecular oxygen.[4] The age-dependent decrease in the mitochondrial ANT activity may also promote mitochondrial ROS generation due to enhanced autoxidizability of electron transport molecules.[31] Furthermore, older individuals showed more increase in leukocyte DCF fluorescence after exposure to *t*-BuOOH, which was shown to be solely due to ROS generation in mitochondria.[28] A greater magnitude of mitochondrial pyridine nucleotide loss and increased probability of PT pore opening, which in itself promotes ROS formation,[32] in the leukocytes from older subjects may contribute to this phenomenon.

In conclusion, our results support the view that the bioenergetic functions of leukocyte mitochondria are more susceptible to oxidative injury in aged individuals. More inducible mitochondrial PT and increased mitochondrial oxidant formation may have causal relationships with this phenomenon. The decreased ability of leukocytes to resist oxidative stress may be responsible for immunosenescence in humans.

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