Impaired Glutathione Peroxidase Activity Accounts for the Age-Related Accumulation of Hydrogen Peroxide in Activated Human Neutrophils

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Background. As assessed by flow cytometry, the increase in hydrogen peroxide in individual neutrophils from old volunteers was significantly greater than in neutrophils from young volunteers. To explain the discrepancy in previous reports that showed reduced superoxide generation with age and our finding, we measured the kinetics of antioxidative enzymes.

Methods. Neutrophils were obtained from young (ages 21–34) and old (ages over 65) volunteers. The increase in hydrogen peroxide following stimulation with formyl peptide in individual neutrophils was assessed by flow cytometry by using dihydrorhodamine 123. The enzyme kinetics was determined from the best fit curve using Michaelis-Menten equations.

Results. Aging was associated with a significant reduction in the Vm» for glutathione peroxidase. The decreased activity was not due to selenium deficiency as the serum and neutrophil concentrations were identical with age. Following activation, a significant increase in the Km was noted in neutrophils from young but not from old volunteers.

Conclusions. These results account for the increased intracellular accumulation of hydrogen peroxide as a function of age in stimulated neutrophils. These results provide evidence in humans of an age-related impairment in antioxidative defense mechanisms that support the free radical theory of aging.

An accumulation of free radicals is thought to contribute to age-related declines in cellular function (1). In phagocytic cells, however, the generation of large concentrations of oxygen radicals plays a central role in host defense against bacteria.

Earlier studies by us have suggested an age-related decline in the ability of neutrophils from older human volunteers to generate superoxide following activation by the chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (FMLP) (2,3). The decline in the ability of neutrophils to generate superoxide is accompanied by a significant reduction in agonist-induced generation of the second messengers inositol triphosphate and diacylglycerol (3). Mobilization of intracellular calcium stores and influx from the extracellular medium following activation are also decreased in neutrophils obtained from old volunteers (2).

The initial purpose of this study was to define whether the age-dependent decline in neutrophil function reflected changes in the neutrophil cell population as a whole or changes in a subpopulation of cells that had a reduced ability to respond to stimulation. Heterogeneity, accounting for declines in function with age, has been reported in other cellular systems (4). We therefore measured intracellular hydrogen peroxide (H2O2) production in individual cells by flow cytometry. Paradoxically, we demonstrated an increase in H2O2 accumulation with age and no evidence of heterogeneity in responsiveness. To explain the discrepancy between the previously reported age-related decline in superoxide generation and increased accumulation of H2O2 in FMLP-stimulated neutrophils, we examined the activity and kinetic characteristics of key enzymes involved in the generation and neutralization of H2O2 in neutrophils. In this study, we found a significant impairment with age in the activity of the enzyme selenium-dependent glutathione peroxidase, which accounts for the age-related increase in free radical accumulation in neutrophils.

METHODS

Blood
Venous blood from healthy male and female volunteers was drawn in acid-citrate-dextrose solution. All young (ages...
21–34 years) and old (ages over 65 years) volunteers were screened to exclude the presence of disease or medications.

**Neutrophil Intracellular H$_2$O$_2$ Accumulation**

The preparation of neutrophils and the measurement of intracellular H$_2$O$_2$ accumulation were carried out using dihydrodorhodamine 123 (Molecular Probes Inc., Eugene, OR) as previously described (5). Neutrophils were preincubated for 10 minutes at 37°C and then treated with 10$^{-5}$–10$^{-5}$ M FMLP. The emitted fluorescence was monitored using a fluorescence activated cell sorting (FACS) scan (Becton Dickinson, San Jose, CA). The H$_2$O$_2$ accumulation was quantified as a ratio of fluorescence intensity to control as described previously (6).

**Preparation of Samples for Enzyme Assays**

Neutrophils were isolated and purified as described previously (7). Neutrophils were resuspended in Krebs Ringer phosphate (KRP) containing 5.5 mM glucose at 4 × 10$^5$ cells/ml. After incubation at 37°C for 10 min, neutrophils were stimulated by 10$^{-4}$ M FMLP for 5 or 20 min. Neutrophils were sonicated and centrifuged as previously described (8). Superoxide dismutase activity was measured in the pellet. Supernatant samples were used for the other enzymes. To exclude the effects of sonication we conducted the following experiment. One unit of glutathione peroxidase (GPX) was added to cell suspensions of neutrophils obtained from young and old volunteers. Enzyme recovery was identical in both, averaging .95 ± .06 U in the young and .97 ± .05 U in the old.

**Enzyme Assays**

Total superoxide dismutase activity was measured by a Gilford Response II spectrophotometer (Ciba Corning Diagnostic Corp., Oberlin, OH) using kit SOD-525 purchased from Bioxytech (Cedex, France) (9).

Glutathione peroxidase activity was measured as described previously (10,11), except that the final reaction mixture contained 63.5 mM potassium phosphate buffer (pH 7.0), 1.19 mM reduced glutathione (GSH), 1.58 U/ml glutathione reductase, .178 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) (previously diluted in 0.1% NaHCO$_3$), and 0.635 mM ethylenediaminetetraacetic acid (EDTA). The reaction was started by adding various concentrations of H$_2$O$_2$, and the absorbance in the pellet. Supernatant samples were used for the other enzymes. To exclude the effects of sonication we conducted the following experiment. One unit of glutathione peroxidase (GPX) was added to cell suspensions of neutrophils obtained from young and old volunteers. Enzyme recovery was identical in both, averaging .95 ± .06 U in the young and .97 ± .05 U in the old.

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**Calculation of Enzyme Kinetics**

Enzyme activity was expressed as units (IU). Maximum velocity (V$_{max}$) and Michaelis constant (K$_m$) obtained from the activity of each individual were calculated from the best fitting curves of Michaelis-Menten equations by using a nonlinear curve fitting program in Sigma Plot 5.0 (Jandel Scientific, Corte Madera, CA).

**Selenium Level in Neutrophils and Serum**

Neutrophils were digested with nitric acid as described previously (15). Selenium content was measured as described previously (16).

**Immunoblotting of Glutathione Peroxidase**

Neutrophils were lysed as described previously (17). The lysates were equalized for protein and subjected to 12% SDS-polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose membranes. The membrane was immunoblotted as described previously (18) using monoclonal antibody to glutathione peroxidase (GPX347, gift from Dr. T. Moriuchi, Tokai University, Isehara, Japan), and developed using enhanced chemiluminescence (ECL) reagents (Amer sham, Buckinghamshire, U.K.). Immunoblots were scanned and analyzed by a computing densitometer model 300A using the program ImageQuant (Molecular Dynamics, Sunnyvale, CA). The amount of glutathione peroxidase in the individual samples was calculated based on the density of a blot containing 125 ng glutathione peroxidase standard. Values were expressed as ng glutathione peroxidase/mg total neutrophil protein.

**Statistical Analysis**

Data are expressed as means ± SEM. The Student’s independent or paired t-test, or the Mann-Whitney U-test were used; a p-value of .05 or less was considered significant. The power of the test to detect effects of age for V$_{max}$, K$_m$, and V$_{max}$/K$_m$ was increased by combining the effects across stimulation groups. That is the consistency of the effects for age across stimulation groups contributed to the statistical significance.

**RESULTS**

**Neutrophil Intracellular H$_2$O$_2$ Accumulation by Dihydrodorhodamine 123**

Intracellular H$_2$O$_2$ accumulation (expressed as the ratio of median fluorescence intensity) in individual neutrophils from old volunteers was significantly higher than in the young at all concentrations of FMLP tested (Figure 1B). For example, 5 min after stimulation with 10$^{-4}$ M FMLP, H$_2$O$_2$ accumulation averaged 14.67 ± 3.21 in neutrophils from the old and 6.27 ± 1.10 in the young volunteers (p < .05, Figure 1A). No obvious subpopulations of cells with
Figure 1. FMLP-induced intracellular H$_2$O$_2$ accumulation in individual neutrophils measured by flow cytometry using dihydrorhodamine 123. H$_2$O$_2$ accumulation was expressed as the ratio of median fluorescence intensity of individual neutrophils as indicated in Materials and Methods. (A) Results at various time points following exposure of neutrophils to 10$^{-7}$ M FMLP. (B) Results obtained after exposure to increasing concentrations of FMLP for 5 min. The results represent the mean (±SEM) measured in each of 10 young (circles) and 10 old volunteers (triangles). At all time points studied, measurements obtained in neutrophils from old volunteers were significantly greater than values obtained in the young volunteers (p < .05).

either increased or decreased responsiveness were noted in cells from either young or old volunteers. We eliminated the possibility that the increase in fluorescence noted in neutrophils from old volunteers was due to an increased accumulation of nitric oxide by showing that it was not detected by the fluorescent dye we employed in this study (data not shown).

**Enzyme Assays in Neutrophils**

We measured the activity of superoxide dismutase in neutrophil pellets prior to and following stimulation by FMLP. There was no significant difference between measurements, either basally or following stimulation, in neutrophils from young or old volunteers (Figure 2).

The activity versus substrate plot of glutathione peroxidase was significantly different with age following activation of neutrophils by FMLP (Figure 3). Five minutes after stimulation with FMLP, a $V_{\text{max}}$ for glutathione peroxidase of 16.47 ± 5.36 IU/g protein in the old volunteers was significantly lower than a value of 36.03 ± 10.41 IU/g protein in the young volunteers (p < .05, Table 1). Basal $K_m$ for glutathione peroxidase was identical in the young and in the old volunteers. In the young volunteers, $K_m$ increased from a value of 1.79 ± .76 μM in the basal state to 3.03 ± .95 μM 20 min after stimulation with FMLP. In neutrophils obtained from old volunteers, $K_m$ remained constant averaging 1.80 ± .44 and 1.67 ± .28 μM prior to and 20 min after stimulation with FMLP.
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NADP (pM)
220
H2O2 (mM)

Figure 3. The activity versus substrate plots of glutathione peroxidase (A, n = 9), glutathione reductase (B, n = 7), G-6PD (C, n = 10), and catalase (D, n = 9) were calculated 5 min after exposure of neutrophils to FMLP. Neutrophils were obtained from young (circles) and old (triangles) volunteers. *The activity of glutathione peroxidase in the old volunteers is significantly lower than the young volunteers (p < .05).

stimulation with FMLP. In the basal state the $V_{\text{max}}/K_m$ ratio averaged $11.02 \pm 2.63$ L/min/g protein in neutrophils from old volunteers, which was significantly lower than a value of $22.53 \pm 3.49$ L/min/g protein in the young (Table 1). Following activation the ratio decreased significantly in neutrophils from young volunteers to a value of $14.09 \pm 2.09$ L/min/g protein. In neutrophils from old volunteers, stimulation did not result in a significant change in the $V_{\text{max}}/K_m$ ratio. Similar age-related reductions in glutathione peroxidase activity were noted when we used t-butyl hydroperoxide as the substrate to measure enzyme activity (data not shown).

We then studied the activity versus substrate plot of glutathione reductase (Figure 3). We found no significant differences in the $V_{\text{max}}$, $K_m$, or $V_{\text{max}}/K_m$ between the young and the old volunteers, either prior to or serially after stimulation with FMLP (Table 1).

Employing different concentrations of NADP as a substrate, the kinetics of G-6PD was not compromised with age. Although the $V_{\text{max}}$ at each time point prior to and following stimulation with FMLP was not significantly different, the $V_{\text{max}}$ for G-6PD was significantly increased with age ($p < .05$) when all the values were analyzed together (Table 1).

Finally we examined the enzyme catalase. We did not find any significant differences in the $V_{\text{max}}$ or $K_m$ with age in either nonstimulated or FMLP-stimulated cells (Table 2).

Selenium Concentrations in Neutrophils and Serum

Glutathione peroxidase is a selenium-dependent enzyme. To determine if the age-related reduction in enzyme activity was due to selenium deficiency, we measured selenium concentrations in serum and neutrophils from 10 young and 10 old volunteers. Neutrophil selenium levels averaged $118.8 \pm 10.8$ in the young and $117.2 \pm 10.1$ pg/10^6 cells in the old volunteers. Serum levels averaged $79.1 \pm 3.6$ and $81.2 \pm 2.4$ ng/ml in the young and old volunteers, respectively.

Immunoblotting of Glutathione Peroxidase

There was no significant age-related difference in the concentration of glutathione peroxidase in neutrophils from nine young and nine old volunteers, either prior to or following stimulation with FMLP (Figure 4). Relative density units for individual samples were obtained following development and normalized to control glutathione peroxidase. Values expressed as ng glutathione peroxidase/mg protein averaged $241.9 \pm 65.2$ in the young and $314.9 \pm 135.4$ in
Table 1. The Kinetic Parameters of the Glutathione Redox Enzymes in Neutrophils from Young and Old Volunteers

<table>
<thead>
<tr>
<th>Time After Stimulation (min)</th>
<th>Glutathione Peroxidase</th>
<th>Glutathione Reductase</th>
<th>G-6PD</th>
</tr>
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<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>29.71 ± 10.64</td>
<td>15.55 ± 3.21</td>
<td>22.53 ± 3.49</td>
</tr>
<tr>
<td>5</td>
<td>36.03 ± 10.41</td>
<td>16.47 ± 5.36*</td>
<td>20.63 ± 3.75</td>
</tr>
<tr>
<td>20</td>
<td>35.22 ± 11.44</td>
<td>19.76 ± 4.63**</td>
<td>14.09 ± 2.09**</td>
</tr>
</tbody>
</table>

**Notes:** Cells were stimulated with 10^{-7} M FMLP. \( V_m \) was expressed as IU/g protein. \( K_m \) was expressed as \( \mu \)M. \( V_m/K_m \) ratio was expressed as L/min/g protein. * = 9 (glutathione peroxidase), 7 (glutathione reductase), or 10 (G-6PD), respectively.

• Indicates that the old volunteers are significantly different from the young volunteers (p < .05).

****When measurements at all time points are analyzed together, the values for the old volunteers are significantly different from the young volunteers (p < .05).

••• Indicates that the value with stimulation is significantly different from the value of control (p < .05).

Table 2. The Kinetic Parameters of Catalase in Neutrophils Obtained from Young and Old Volunteers

<table>
<thead>
<tr>
<th>Time After Stimulation (min)</th>
<th>Catalase</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>907.0 ± 73.3</td>
</tr>
<tr>
<td>5</td>
<td>925.3 ± 72.0</td>
</tr>
<tr>
<td>20</td>
<td>925.3 ± 72.0</td>
</tr>
</tbody>
</table>

**Notes:** Cells were stimulated with 10^{-7} M FMLP. \( V_m \) was expressed as IU/g protein. \( K_m \) was expressed as mM. Neutrophils were obtained from nine young and nine old volunteers.

Figure 4. Four cases of immunoblots for glutathione peroxidase were given. The 0 indicates that cells before stimulation with FMLP. The numbers 5 or 20 indicate minutes after stimulation.
measured by the fluorescent dye we employed. In this study, we did not demonstrate any age-related alteration in the activity of superoxide dismutase in the neutrophil. This indicates no impairment in the ability to produce H$_2$O$_2$ from superoxide. An alternative explanation for the persistently high level of H$_2$O$_2$ could be related to an impaired ability to neutralize H$_2$O$_2$.

In this report we found a highly significant decrease with age in the $V_{\text{max}}$ for glutathione peroxidase. However, the protein level of this enzyme was not affected with age, suggesting that decreased activity may be due to modification of protein structure or inability to produce an activated form of the enzyme (19). A similar explanation could apply to the changes in $V_{\text{max}}$ and $K_n$ with FMLP stimulation as no changes in protein level occurred following activation of the oxidative burst.

Prior to stimulation with FMLP, neutrophil glutathione peroxidase $V_{\text{max}}/K_n$ ratio was significantly higher in neutrophils from young volunteers as compared to the old. This first order rate constant approximates the fraction of H$_2$O$_2$ that is converted to H$_2$O and O$_2$ per unit time. A higher $V_{\text{max}}/K_n$ ratio suggests a distinct advantage for neutrophils from young volunteers as compared to the old. The significant decrease over time in the $V_{\text{max}}/K_n$ ratio probably indicates that reactive oxygen species are efficiently removed in a short time frame in neutrophils from the young volunteers. Further, the lack of change following stimulation with FMLP in the old volunteers might indicate a lack of response to increased free radical exposure and resultant persistence of these reactive species.

An examination of reaction mechanisms using Michaelis-Menten kinetic values provided additional evidence of impaired glutathione peroxidase activity with age. The reaction mechanism for glutathione peroxidase is "ping pong," where a product (H$_2$O) is released between the addition of two substrates, H$_2$O$_2$ and GSH (20). The ratio of the concentrations of two substrates has been shown to affect the enzyme $K_n$ and $V_{\text{max}}/K_n$ but not to affect the $V_{\text{max}}$ (21). In neutrophils from young volunteers, activation with FMLP resulted in a significant decrease of the $V_{\text{max}}/K_n$, a significant increase of the $K_n$ for H$_2$O$_2$, and no change of the $V_{\text{max}}$. The increase in $K_n$ for H$_2$O$_2$ and the decrease of the $V_{\text{max}}/K_n$ could be the result of increasing H$_2$O$_2$ concentrations and decreasing GSH concentrations yielding a decrease in the GSH/[H$_2$O$_2$] ratio as indicated for the ping-pong mechanism (21). The excess H$_2$O$_2$ would be removed rapidly by the addition of glutathione peroxidase as indicated by the high $V_{\text{max}}$ in neutrophils from young volunteers. In neutrophils from old volunteers, activation with FMLP was not associated with any significant decrease in the $V_{\text{max}}/K_n$ ratio or increase in the $K_n$. This suggests minimal change of the GSH/[H$_2$O$_2$] due to the lack of GSH oxidation and an increase in H$_2$O$_2$ production during the oxidative burst. However, H$_2$O$_2$ is ineflectively removed due to the low activity of glutathione peroxidase in neutrophils from old volunteers as indicated by the significantly lower $V_{\text{max}}$. The lower activity of glutathione peroxidase also reduced the rate of GSH oxidation. This likelihood is supported by a previous report which showed no change following stimulation in GSH or GSSG in neutrophils from old volunteers (22). By contrast, in neutrophils from young subjects stimulation was associated with significant reductions in GSH and increases in GSSG (22). These conclusions apply to kinetic changes noted in an in vitro assay in which substrate concentrations are controlled. A similar circumstance may not occur in vivo.

The kinetic parameters of neutrophil glutathione reductase following activation by FMLP were similar in the young and the old volunteers. However, in the presence of lower concentrations of GSSG as a result of the reduced $V_{\text{max}}$ for glutathione peroxidase in neutrophils from old volunteers, substrate would be reduced to levels significantly below the $K_n$ for glutathione reductase sooner, because the $V_{\text{max}}$ or the $K_n$ for glutathione reductase in neutrophils are unaffected by donor age. Thus, resupply of GSH to significant levels is more easily achieved in neutrophils from young volunteers.

The GSSG reduction by glutathione reductase requires NADPH that is generated by G-6PD. Although the differences for the $V_{\text{max}}$ and $K_n$ were not significant at individual time points prior to and following stimulation with FMLP, it is of interest to note that they were higher in neutrophils obtained from old as compared to young volunteers. This applied particularly to time points after stimulation with FMLP when reducing potential should be maximized. In addition, when the data were summed, the $V_{\text{max}}$ was significantly higher in the old volunteers. These findings suggest that aging is associated with an increase in the concentration of the enzyme, and the trend toward age-related increase in the $K_n$ suggests that the enzyme is less efficient. This would necessitate a compensatory increase in its concentration. Interestingly, these observations are consistent with those found in aging rodents suggesting similar mechanisms (23).

It is unlikely that normal catalase activity, which also neutralizes H$_2$O$_2$, can compensate for the age-related impairment in glutathione peroxidase. The $K_n$ for catalase is significantly larger than the $K_n$ for glutathione peroxidase (Tables 1 and 2), even though deviation from normal Michaelis-Menten kinetics makes absolute definition of the catalase $K_n$ difficult. This indicates that catalase has a much lower affinity for H$_2$O$_2$ than does glutathione peroxidase. High levels of H$_2$O$_2$ stimulate the oxidation of catalase to the inactive compound II (24). Thus, the range of substrate concentration at which catalase is active is limited.

Our findings demonstrate a highly specific age-related reduction in the activity of a selenium-dependent enzyme. Selenium deficiency is known to result in impairments of glutathione peroxidase activity (25). We found no age-related differences in selenium concentrations, making it extremely unlikely that deficiency of this trace element accounts for the age-related impairment in glutathione peroxidase activity in neutrophils. In this study we clearly demonstrate increased accumulation of H$_2$O$_2$ in neutrophils from old volunteers. Recently much attention has focused on the role of free radical accumulation as a contributing mechanism to many age-dependent diseases. It has also been widely proposed that free radical-induced damage might explain many of the physiological and cellular declines that accompany the
aging process (1). An age-related impairment in neutralizing capability of H$_2$O$_2$ by inflammatory cells could contribute significantly to age-related increases in cellular damage. Most studies have thus far been primarily derived from animal studies or in vitro cell culture models. This report provides compelling evidence in humans for an age-related compromise in the defense mechanisms against oxidant injury, and that these defects exist in vivo. Monitoring neutrophil function may prove to be useful in assessing the efficacy of interventions aimed at minimizing age-related declines in free radical accumulation. It may also be a valuable biomarker of physiological aging.

Acknowledgments

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References


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