Erythrocyte Vitamin E Is Oxidized at a Lower Peroxide Concentration in Neonates Than in Adults

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Erythrocytes of neonates and adults were incubated with increasing concentrations of \( H_2O_2 \) in the presence of a catalase inhibitor and in the absence of glucose; the pattern of oxidation of vitamin E was analyzed in relationship to that of glutathione, hemoglobin, and polyunsaturated fatty acids (PUFA), and in relationship to hemolysis. The changes of these various parameters were analyzed in function of \( H_2O_2 \) concentration and in relation to incubation time, and were compared in erythrocytes from neonates and adults. In the absence of \( H_2O_2 \), erythrocyte glutathione and tocopherol levels were similar in neonates and adults, despite fourfold lower serum vitamin E level in neonates: \( \alpha \)-tocopherolquinone, methemoglobin, and malondialdehyde (MDA) were not detectable. At 0.375 mmol/L of \( H_2O_2 \), glutathione was completely oxidized. Erythrocyte \( \alpha \)-tocopherol remained unchanged up to 0.75 mmol/L of \( H_2O_2 \), then decreased linearly, with increasing \( H_2O_2 \) concentrations to 10% of its initial value at 1.5 mmol/L of \( H_2O_2 \) in erythrocytes from neonates, whereas those from adults required 2.0 mmol/L of \( H_2O_2 \) (\( P < .05 \)) for the same level of oxidation. The formation of \( \alpha \)-tocopherolquinone appeared inversely related to the decrease of \( \alpha \)-tocopherol. The incubation time did not influence the level of vitamin E oxidation. MDA was generated autocatalytically and resulted in hemolysis at 1.5 mmol/L of \( H_2O_2 \) in erythrocytes from neonates and at 3.5 mmol/L of \( H_2O_2 \) in erythrocytes from adults (\( P < .001 \)). After four hours of incubation, MDA reached a plateau at a greater level (365 ± 46 nmol/L) in cells of neonates than in those of adults (206 ± 37 nmol/L/mL) (\( P < .001 \)). Hemoglobin was oxidized in the same pattern in erythrocytes of neonates and adults, and 90% of it was oxidized at 0.625 mmol/L of \( H_2O_2 \). In conclusion, in the experimental conditions used, oxidation of glutathione precedes that of vitamin E, and tocopherol is the last antioxidant to be consumed before the autocatalytic generation of MDA. Differences in the pattern of vitamin E oxidation, MDA generation, and hemolysis in erythrocytes from neonates and adults may be due to a lower erythrocyte vitamin E-PUFA ratio in neonates.
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30% (Merck Co, Darmstadt, West Germany) and dissolved in PBS to the required concentration. Both diluted RBCs and H$_2$O$_2$ at the desired concentration were preincubated separately for ten minutes at 37 °C, then rapidly mixed (vol/vol); the erythrocytes, at a final concentration of 5%, were incubated at 37 °C.

**MDA analysis.** MDA was determined by the thiorbarbituric method on a trichloroacetic acid extract of 4 mL of the incubation suspension, with absorbancy measurements at 532 and 600 nm.$^6$

**Tocopherols and α-tocopherolquinone analysis.** Tocopherols and α-tocopherolquinone were extracted in hexane without saponification, with pyrogallol as antioxidant (5:100/wt/vol) and $^{14}$C-tocopherol and γ-tocopherolquinone were extracted in hexane without saponification. Both diluted RBCs and H$_2$O$_2$ at the required concentration were preincubated separately for ten minutes at 37 °C, then rapidly mixed (vol/vol); the erythrocytes, at a final concentration of 5%, were incubated at 37 °C, then rapidly mixed (vol/vol); the erythrocytes, at a final concentration of 5%, were incubated at 37 °C.

**Retention time at 266 nm for a-tocopherolquinone.** Retention times of a-tocopherol and tocopherolquinone analysis were 9%. With the volume of sample used, the peak of γ-tocopherol was too small to permit precise quantification.

**Methemoglobin analysis.** The methemoglobin fraction of the reduced GSH content of erythrocytes was measured by the method of Beutler.$^17$

**Hemolysis estimation.** After exposure of the erythrocytes to H$_2$O$_2$ for the desired time, 100 μL of RBC were mixed with 200 μL PBS; a similar aliquot was mixed with 200 μL distilled water. Both aliquots were centrifuged, and the ratio of hemoglobin in the PBS supernatant to that in the distilled water supernatant was measured to determine the ratio of hemolysis.

**Reduced GSH analysis.** The reduced GSH content of erythrocytes was measured by the method of Lowry et al.$^14$

**Ghost cells.** Ghost cells were prepared from erythrocytes by hypotonic hemolysis$^{14}$; they were treated in the same manner as erythrocytes for the peroxide incubation; ghost protein content was measured by the method of Lowry et al.

**RESULTS**

Figure 1 shows a typical chromatogram of tocopherols and α-tocopherolquinone analysis in extracts of erythrocytes incubated in the absence and in the presence of H$_2$O$_2$. The retention times of α-tocopherol, α-tocopherolquinone, and γ-tocopherol were 4.85, 6.60, and 10.25 minutes, respectively. In the absence of H$_2$O$_2$, only the peaks of α-tocopherol and γ-tocopherol are identifiable. In the presence of H$_2$O$_2$, the peak of α-tocopherol has decreased to 10% of its initial value, whereas a peak of α-tocopherolquinone is identified. The pattern of oxidation of GSH in relation to that of vitamin E was analyzed in erythrocytes incubated with increasing concentrations of peroxide. Levels of significance of the difference between adults and neonates: glutathione: not significant at all H$_2$O$_2$ concentrations; α-tocopherol: $P < .05$ at 0.75 mmol/L; 0.875 mmol/L; 1.0 mmol/L H$_2$O$_2$ concentrations.
increasing concentrations of H$_2$O$_2$, erythrocyte α-tocopherol remained stable through a greater H$_2$O$_2$ concentration in adults (0.75 mmol/L) than in neonates (0.625 mmol/L); the erythrocyte α-tocopherol level was lower in neonates than in adults (0.625 mmol/L) than in neonates (0.75 mmol/L); the level of α-tocopherolquinone was higher in cells from neonates than from adults at 0.625 mmol/L and 1.0 mmol/L of H$_2$O$_2$. At higher H$_2$O$_2$ concentrations up to 5 mmol/L of H$_2$O$_2$ about 10% of the initial α-tocopherol remained unchanged.

No α-tocopherolquinone was detectable in erythrocytes of either group up to 0.375 mmol/L of H$_2$O$_2$ (Table 1); its level then increased in mirror image to that of α-tocopherol. The level of α-tocopherolquinone was higher in cells from neonates than from adults at 0.625 mmol/L (P < .05), 0.875 mmol/L (P < .05), and 1.0 mmol/L (P < .05) H$_2$O$_2$ concentrations. At higher H$_2$O$_2$ concentrations up to 5 mmol/L of H$_2$O$_2$ about 10% of the initial α-tocopherol remained unchanged.

The influence of incubation time on the erythrocyte vitamin E level was analyzed (Fig 3). At peroxide concentrations sufficient to initiate lipid peroxidation, the decrease of erythrocyte α-tocopherol was rapid: it took less time than that required for centrifugation of the cells before vitamin E extraction, corresponding to time zero. Subsequently, whatever the initial H$_2$O$_2$ concentration, erythrocyte α-tocopherol level remained stable for at least eight hours.

The pattern of oxidation of hemoglobin was compared in erythrocytes of neonates and adults (Table 1). Before addition of H$_2$O$_2$, methemoglobin was not detectable in erythrocytes of both groups; hemoglobin was oxidized at a similar rate in both groups, and was completely oxidized at 1.5 mmol/L of H$_2$O$_2$.

The pattern of lipid peroxidation was analyzed by measuring the formation of MDA in erythrocytes of neonates and adults (Fig 4; Table 1). MDA was absent in the erythrocytes in the absence of H$_2$O$_2$; a low but increasing amount of MDA was generated through 1.0 mmol/L and 1.5 mmol/L H$_2$O$_2$ concentrations in cells of neonates and adults, respectively. There was an abrupt increase in the formation of MDA at higher H$_2$O$_2$ concentrations, and the MDA level was higher in erythrocytes of neonates than adults at 1.5 mmol/L and higher H$_2$O$_2$ concentration (P < .001). The evolution with time of MDA formation is shown in the superimposed graph of Fig 4. At 5 mmol/L of H$_2$O$_2$, MDA increased more rapidly (P < .001 at 1, 2, 3, and 4 hours) and reached a plateau at a higher level in cells of neonates (365 ± 46 mmol/mL per milliliter) than of adults (208 ± 37 mmol/mL) (P < .001).

Hemolysis was absent at all H$_2$O$_2$ concentrations after 15 minutes of incubation. After eight hours of incubation with up to 1.0 mmol/L of H$_2$O$_2$ (Table 1), hemolysis was low (< 25%) and at a similar level in erythrocytes of both groups. At 1.5 mmol/L and 2.0 mmol/L of H$_2$O$_2$, after eight hours, hemolysis was complete in cord RBCs, but low in adult...
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Fig 4. Malondialdehyde level in erythrocytes incubated with increasing concentrations of H2O2: same populations, conditions, and symbols as in Fig 2. Level of significance of the difference between adults and neonates: P<.001 at 1.5 mmol/L and higher H2O2 concentrations. In superimposed graph: level of MDA in erythrocytes of both populations incubated for increasing time intervals at 5% hematocrit in phosphate-buffered saline with 5 mmol/L H2O2 concentration. Level of significance of the difference between adults and neonates: P<.001 at 1, 2, 3, and 4 hours.

erythrocytes (P<.001). At 5 mmol/L of H2O2, hemolysis was more rapid in neonates than in adults (P<.05 at 1 hour; P<.01 at 2, 3, and 4 hours) and complete after 8 hours in erythrocytes from adults and neonates (data not shown).

Ghost cells were incubated with H2O2 in the presence and absence of iron for eight hours (Table 2). In the absence of iron, after eight hours of incubation, H2O2 involved the oxidation of a small fraction of α-tocopherol and the formation of an amount of MDA slightly higher than in erythrocytes incubated with PBS. In the presence of iron, after eight hours of incubation with H2O2, the amount of oxidized α-tocopherol and of generated MDA was similar to that observed in erythrocytes.

**DISCUSSION**

*Relationship of vitamin E oxidation with GSH and lipid oxidation.* The mechanism of the hemolytic action of H2O2 has been extensively studied. H2O2 initiates the formation of free radicals in presence of iron by the Haber-Weiss reaction. These free radicals are highly unstable molecules that transfer their unpaired electron to PUFA in the cell membrane; with an unpaired electron, these PUFA become radicals themselves and, in the presence of oxygen, propagate a chain reaction of lipid peroxidation. Changes in membrane fatty acid composition contributes to alteration of membrane permeability by a direct toxic effect of lipid peroxidation on membrane integrity and by a decrease in membrane Na⁺-K⁺-ATPase activity, resulting in gradual colloidal hemolysis.

Vitamin E acts by interrupting the propagation phase of lipid peroxidation. When the peroxide hemolysis test was introduced in 1952 by Rose and Gyorgy, it was considered a specific index of cellular vitamin E content. Although there is no doubt that vitamin E deficiency is accompanied by increased sensitivity of the erythrocytes to H2O2, other antioxidants intervene in the cell before the utilization of vitamin E. Our results show that GSH is completely oxidized at H2O2 concentrations at which there is not yet significant vitamin E oxidation. Such a pattern is strongly suggestive of a redox system between vitamin E and GSH. Moreover, because there is a range of H2O2 concentrations at which all GSH has been oxidized and no vitamin E has undergone oxidation, other intermediate antioxidant(s) may intervene in a chain reaction between GSH and vitamin E. Such a role has been proposed recently by various authors for vitamin C in artificial systems. With PUFA in solution, the inhibition rate constant for the scavenging of lipid peroxide radicals of vitamin C is higher than that of vitamin E.

Our in vitro system demonstrates for the first time, to the best of our knowledge, vitamin E oxidation in a biological system. It was documented by the decrease of α-tocopherol and the increase of α-tocopherolquinone in erythrocytes incubated with increasing concentrations of H2O2. α-Tocopherolquinone accounted for only 40% of the initial value of α-tocopherol. Commercial unavailability of other oxidation products of vitamin E in pure form (tocopherol dimers and trimers) did not allow their proper identification on the chromatogram. The experiments with ghost cells demonstrate that α-tocopherol oxidation and MDA formation are mainly or exclusively located in the cell membrane; tocopherolquinone or MDA are not analytical artifacts of the interaction of H2O2 with components not located in the membrane; because iron was required to obtain the maximal oxidation, it is not H2O2 that initiates lipid peroxidation, but free radicals derived from H2O2 in the presence of trace of metals. Previously, other authors have attempted to demonstrate vitamin E oxidation in a biological system by incubating erythrocytes with fatty hydroperoxides and radiolabeled α-tocopherol; in these conditions, a fraction of radiolabeled α-tocopherolquinone was identified by thin-layer chromatography in extracts of erythrocytes after incubation; however, the incubation of radiolabeled α-tocopherol with lipid peroxides (in the absence of erythrocytes) also resulted in the formation of α-tocopherolquinone; there was thus no evi-

Table 2. Level of α-Tocopherol, α-Tocopherolquinone, and Malondialdehyde in Ghost Cells

<table>
<thead>
<tr>
<th></th>
<th>α-Tocopherol (µg/mL)</th>
<th>α-Tocopherolquinone (µg/mL)</th>
<th>Malondialdehyde (nmol/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before incubation</td>
<td>2.20</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>After 8-hour incubation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In PBS</td>
<td>2.13</td>
<td>0.06</td>
<td>8</td>
</tr>
<tr>
<td>In PBS-H2O2 (5 mmol/L)</td>
<td></td>
<td></td>
<td>34</td>
</tr>
<tr>
<td>In PBS-H2O2 + Fe²⁺ (100 mg/L)</td>
<td>0.15</td>
<td>1.02</td>
<td>350</td>
</tr>
</tbody>
</table>

Levels are expressed as µL/mL RBCs.
dence of oxidation of vitamin E by lipid peroxidation in the RBC membrane.25

In vivo, evidence of lipid peroxidation in normal or pathological states is still scarce and indirect. Our experimental data show that vitamin E oxidation precedes lipid peroxidation, and the detection of tocopherolquinone in cells could constitute a more sensitive method of detection of oxidant stress at the membrane level than do standard methods of measurement of lipid peroxidation.

Our in vitro system uses experimental conditions far from the physiologic ones: \( \text{H}_2\text{O}_2 \) concentrations were 100 times higher than those produced physiologically by stimulated phagocytes; regeneration of reduced GSH by the pentose phosphate pathway was impeded by the lack of glucose, and catalase was inhibited by azide. Nevertheless, in conditions close to physiologic conditions, when erythrocytes are exposed to stimulated phagocytes, it has been shown that cell lysis proceeds by lipid peroxidation.26 Our model of interaction between GSH and vitamin E could be reasonably extrapolated to these conditions.27

**Mechanism of the increased sensitivity of the cord erythrocyte to peroxide.** Increased peroxide hemolysis in cord RBCs has been attributed to their decreased GSH-Px activity;22 however, this relationship has been challenged by the fact that it is not the enzyme that is the limiting factor of the detoxification of \( \text{H}_2\text{O}_2 \) but the level of substrate.28 The level of GSH is similar in erythrocytes of adults and neonates, and it is oxidized in the same pattern in erythrocytes of both populations incubated with \( \text{H}_2\text{O}_2 \). Thus, the lower GSH-Px level in erythrocytes of neonates does not explain the increased sensitivity to \( \text{H}_2\text{O}_2 \). A potential lack of an intermediate antioxidant intervening between GSH and vitamin E in erythrocytes of neonates is highly improbable: in this case, hemoglobin would be oxidized at lower \( \text{H}_2\text{O}_2 \) concentrations in neonate erythrocytes than in those of adults.

Lower serum vitamin E in neonates has been considered a reflection of vitamin E deficiency at the cellular level.7 However, while serum \( \alpha \)-tocopherol is indeed four times lower in neonates than in adults of the present study, erythrocyte vitamin E is similar in both populations. This difference is due to the lower serum \( \beta \)-lipoprotein concentrations in neonates.11

Despite similar erythrocyte vitamin E level and despite similar patterns of oxidation of hemoglobin and GSH, \( \alpha \)-tocopherol was oxidized at lower \( \text{H}_2\text{O}_2 \) concentrations in neonate erythrocytes than in those of adults. This was associated with lipid peroxidation (MDA generation) at lower \( \text{H}_2\text{O}_2 \) concentrations in erythrocytes of neonates. The higher amount of MDA generated in newborn erythrocytes is a reflection of their higher PUFA content.29 Thus, the same amount of free radicals generated by the reaction of hemoglobin with \( \text{H}_2\text{O}_2 \) will result in the generation of a greater number of PUFA radicals; vitamin E then, is more rapidly oxidized once the intracellular antioxidant mechanisms have been depleted. The neonate represents another model of the increased requirement of vitamin E when the \( \text{PUFA} \) content at the cellular level is increased, as has been documented in other subjects.30 Another explanation for the different pattern of vitamin E oxidation would be that the protein moiety of fetal hemoglobin has greater free radical-scavenging properties than does adult hemoglobin.28

In conclusion, the peroxide hemolysis test should not be considered a specific index of vitamin E status. In neonates, there is no absolute vitamin E deficiency at the erythrocyte level; the requirement in vitamin E is nevertheless increased by the high \( \text{PUFA} \) content of the cord RBC and/or decrease in free radical-scavenging properties of fetal hemoglobin. The measurement of \( \alpha \)-tocopherol oxidation by determination of tocopherolquinone offers a new way to measure the oxidative stress at the membrane level. Such determinations should detect oxidative damage at a level at which there is no cytolyis and at which MDA generation is very low, at the limit of the sensitivity and reliability of the thiobarbituric analytical procedure.

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**REFERENCES**


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