Age-associated decline in ascorbic acid concentration, recycling, and biosynthesis in rat hepatocytes—reversal with (R)-α-lipoic acid supplementation

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ABSTRACT Ascorbic acid recycling from dehydroascorbic acid and biosynthesis from gulono-1,4-lactone were used as measures of cellular response capacity to increased oxidative stress induced by tert-butylylhydroperoxide. The hepatic ascorbic acid concentration was 54% lower in cells from old rats when compared to cells isolated from young rats (P < 0.0005). Freshly isolated hepatocytes from old rats exhibited a significantly decreased ascorbic acid recycling capacity in response to oxidative stress (P < 0.005) compared to cells from young rats. Ascorbic acid synthesis in these cells from old animals was unaffected by various concentrations of tert-butylylhydroperoxide, but amounted to only approximately half of the biosynthetic rate when compared to cells from young animals (P < 0.001). Cells from young animals were not significantly affected by the tert-butylylhydroperoxide treatments. The results demonstrate a declining ability with age to respond to increased oxidative stress. (R)-α-Lipoic acid, a mitochondrial coenzyme, is a powerful antioxidant. A two-week dietary supplementation of old animals with 0.5% (R)-α-lipoic acid prior to cell isolation almost completely reversed the age-associated effects on ascorbic acid concentration (P < 0.0001), recycling (P < 0.05) and biosynthesis after oxidative stress. These results provide further evidence for the potential of α-lipoic acid in treatment of diseases related to oxidative stress. Furthermore, the study extends the value of ascorbic acid as a biomarker of oxidative stress. Lykkefeldt, J., Hagen, T. M., Vinarsky, V., Ames, B. N. Age-associated decline in ascorbic acid concentration, recycling, and biosynthesis in rat hepatocytes—reversal with (R)-α-lipoic acid supplementation. FASEB J. 12, 1183–1189 (1998)

Key Words: biomarker · oxidative stress · aging

DURING AGING, MITOCHONDRIA DECAY (1), rates of oxidant production increase (2, 3), and oxidative damage to important biomolecules increase (4–6) and may in part be responsible for aging as well as age-associated degenerative diseases such as cancer and atherosclerosis (7–9). In this light, it is important to understand whether the cellular distribution and bioavailability of key antioxidants have become altered with age.

One of the most important cellular antioxidants is ascorbic acid (AA) (10), a valuable biomarker of oxidative stress (11–13). No clear relationship between AA and aging has been established (14). Some studies (15–17), but not others (18), found an overall decline in hepatic AA level of male rats with age. In female rats, one study found increased hepatic AA concentrations with age (19) while no change was observed in another (17).

In contrast to measuring tissue AA concentrations, the age-associated changes in AA recycling capacity, i.e. the intracellular reduction of dehydroascorbic acid (DHA) to AA by enzymatic or chemical means (Fig. 1A), has not been studied extensively. In a recent study of the NADPH-dehydroascorbic acid reductase activity, no effect of aging was observed in livers from male or female rats (17). This study examined only the recycling capacity at the basal metabolic level. To date, little is known about AA recycling and synthesis in mammalian cells subjected to environmental stresses that would place an added burden on the antioxidant pools.

In the present study, we report an age-associated decline in ascorbic acid metabolism as measured by the ability of freshly isolated rat hepatocytes to respond to elevated oxidative stress. Various levels of oxidative stress were induced by different concentrations of tert-butylylhydroperoxide (t-BuOOH), a model alkyl peroxide that preferentially acts in mitochondria and causes a lipid peroxidation cascade and loss

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4 Abbreviations: AA, ascorbic acid; DHA, dehydroascorbic acid; t-BuOOH, tert-butylylhydroperoxide; MPA, meta-phosphoric acid; GL, L-gluono-1,4-lactone; PBS, phosphate-buffered saline.
of antioxidant capacity (20). As parameters of response, hepatic recycling and biosynthetic capacity of AA were measured after addition of DHA and L-gulono-1,4-lactone (GL), respectively, to \( \text{tBuOOH} \) treated cells. Basal hepatic AA concentrations were also measured. Our results demonstrate that cells isolated from old rats are less able to respond to an elevated oxidant load compared to hepatocytes isolated from young rats. Furthermore, we show that the observed age-associated decline in ascorbic acid concentration and metabolism can be reversed by supplementation with \((R)\)-lipoic acid (Fig. 1B), a powerful antioxidant that has been shown to maintain levels of other endogenous antioxidants even in times of oxidative insult (21–27).

**MATERIALS AND METHODS**

L-Ascorbic acid (AA), \( \alpha \)-dehydroascorbic acid (dimer) (DHA), L-gulono-1,4-lactone (GL) and meta-phosphoric acid (MPA) were from Fluka. \((R)\)-\( \alpha \)-Lipoic acid was a gift from Dr. H. Tritschler of Asta Medica (Frankfurt, Germany). \( \text{tBuOOH} \) has been shown not to change the AA recovery significantly (11).

**Hepatocyte isolation**

Liver parenchymal cells were isolated from young (3–5 months, Simonsen, Gilroy, Calif.) and old (28 months, National Institute of Aging animal colonies) male rats (Fischer 344, outbred albino) by collagenase perfusion as described (3, 28). This isolation method typically results in a cell suspension that maintains the proper representation of the cell types found in the intact organ. Three consecutive washes with 150 mL Krebs-Henseleit buffer, pH 7.4, were used followed by aspiration to remove nonparenchymal cells, extracellular ascorbic acid, and excessive volume. The cells were kept in suspension in a pear-shaped flask rotating at 50 rpm at room temperature until used for the experiments described below. Cell number was assessed by using a hemocytometer, and viability was determined by the ability of cells to exclude Trypan Blue (0.2% in phosphate buffered saline (PBS)). In general, about \( 6 \times 10^6 \) parenchymal cells were obtained from the isolation procedure. Viability was usually greater than 90% in both age groups.

**Diet and supplementation**

All rats were kept in the Berkeley animal facilities for at least 1 month before the experiments on an amino acid-defined diet (AIN-93M) and water *ad libitum*. In addition, for animals supplemented with \((R)\)-lipoic acid, 0.5% (w/w) was added to the food for 2 wk before cell isolation (Dyets Inc., Bethlehem, Pa.). As measured by food consumption, this supplementation corresponds to about 100 mg of \((R)\)-lipoic acid per day. No overall differences in food consumption were noted between animals fed the supplemented or control diets.

**Ascorbic acid recycling**

Hepatocytes isolated from young or old rats were diluted with PBS, as cultured for 1 h at 37°C in the presence 0, 300, and 500 \( \mu \text{M} \) \( \text{tBuOOH} \). To 975 \( \mu \text{L} \) of the cell suspensions (2 \( \times \) 10^6 cells/mL) was added 25 \( \mu \text{L} \) of DHA (2 mg/mL \( \sim \) 280 \( \mu \text{M} \) final concentration) and immediately after gentle mixing, a 200 \( \mu \text{L} \) aliquot of sample was added to an equal volume of freshly prepared 10% (w/v) MPA, vortex-mixed, centrifuged for 1 min at high speed, and frozen to \(-20^\circ\text{C}\) until analysis. The DHA solution was prepared immediately before use in 5 mM sodium acetate buffer pH 4.0 and kept on ice. Meanwhile, the remainder of the sample was incubated for 5 min in an incubator with gentle rocking set at 37°C, after which a second 200 \( \mu \text{L} \) aliquot was quenched with MPA and treated as above. Experiments showed (unpublished observations) that the cells become permeable to ascorbic acid upon treatment with MPA and that additional measures to disrupt the cells, e.g., by homogenization or lysing, only decrease the recovery of this labile compound. Furthermore, since we consistently did not find AA in the extracellular fluid (unpublished observations), a step involving centrifugation and resuspension before acidification was omitted from the assay prior to the work presented here. The samples were typically stored less than 1 wk before analysis, but storage up to at least 6 months at \(-20^\circ\text{C}\) has been shown not to change the AA recovery significantly (11).

**Ascorbic acid synthesis**

The biosynthesis of AA was measured as described above for AA recycling except for the addition of GL. (25 \( \mu \text{L} \), 2 mg/ml \( \sim \) 280 \( \mu \text{M} \) final conc.) instead of DHA and the use of a 10 min incubation period. Similar techniques have been used by others (29, 30).
pressed as nmoles per minute per million cells. Total AA analysis was performed after reduction with 3.3 mM DTT for 5 min as previously described (11).

**Statistics**

Statistical analysis of variance (ANOVA) and probability were done with Statistica 5.1 (StatSoft, Tulsa, Okla.). After ANOVA homogeneity by Levene’s test, differences were tested between groups by using two-tailed t-test. Linear regression was done by the method of least squares. A P-value of less than 0.05 was considered statistically significant. Values are given as means ± SD.

**RESULTS**

Total AA concentrations (AA + DHA) in hepatocytes isolated from young and old rats are displayed in Table 1. DHA concentrations, assessed as the difference between AA and total AA measurements, were negligible in both supplemented and control rats (data not shown). However, a significant 54% decrease in total AA with age was observed (P<0.0005). A 2 wk supplementation regimen with (R)-lipoic acid (0.5% (w/w) in the diet) resulted in a highly significant 127% increase in hepatic AA concentration in cells from old rats (P<0.0001), completely restoring the levels to those found in cells from untreated young rats. In cells from young animals, a significant 44% increase in AA concentration was also observed with (R)-lipoic acid supplementation (P<0.05).

AA recycling and synthesis as measured by reduction of DHA and biosynthesis from GL were used as parameters of response to an oxidative insult imposed by t-BuOOH. Hepatocytes isolated from young and old rats were incubated with 0, 300, or 500 μM t-BuOOH for 1 h at 37°C before the experiments.

The results of the AA recycling experiments are summarized in Fig. 2. For cells from young rats, there was no significant difference in recycling capacity between cells preincubated with 300 or 500 μM t-BuOOH compared to controls, indicating that cells from young animals can adequately respond to the t-BuOOH-induced insult at these concentrations. In contrast, cells isolated from old rats lacked the ability to efficiently reduce DHA to AA with increasing concentrations of t-BuOOH (Fig. 2). In agreement with the individual comparisons, linear regression of AA recycling on t-BuOOH concentration showed no effect for cells from young rats (intercept=2.55; slope=-0.0002; r=0.07; P=0.80) but a significant inverse correlation with t-BuOOH concentration for cells from old rats (intercept=2.51; slope=-0.002; r=0.82, P<0.005). No ascorbic acid production was observed without cells, without DHA addition or with dead cells (following 150 s of boiling), and all measurements were consequently corrected for changes in viability. The viability decreased with age and with increasing levels of oxidative stress and was lowest among cells from untreated old rats incubated with the highest concentration of t-BuOOH where it averaged 52 ± 32% (n=5). Therefore, if anything, this correction for viability would underestimate the age-associated effect.

A 2 wk feeding regimen of 0.5% (R)-lipoic acid in the diet caused a significant increase in recycling of AA in cells isolated from old animals in all cases when compared to the equivalent unsupplemented group (P<0.05 in all individual comparisons, data not shown). No significant effects were observed for cells from young animals. Again, linear regression analysis showed no significant effect of increasing t-BuOOH concentration for cells from young animals (intercept=3.03; slope=-0.0017; r=0.35; P=0.20) while a significant inverse correlation with t-BuOOH concentration persisted for cells from old animals (intercept=3.34; slope=-0.0015; r=0.56; P<0.05). How-

![Figure 2. Recycling capacity for cells isolated from old animals and young control.](Image 305x599 to 544x767)

TABLE 1. Total ascorbic acid concentration in hepatocytes isolated from young and old rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Supplementation</th>
<th>n</th>
<th>Total ascorbic acid* (nmoles/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>None</td>
<td>15</td>
<td>7.29 ± 2.97</td>
</tr>
<tr>
<td></td>
<td>(R)-Lipoic acid</td>
<td>6</td>
<td>10.5 ± 2.38**</td>
</tr>
<tr>
<td>Old</td>
<td>None</td>
<td>11</td>
<td>3.38 ± 0.67**</td>
</tr>
<tr>
<td></td>
<td>(R)-Lipoic acid</td>
<td>6</td>
<td>7.68 ± 1.09***</td>
</tr>
</tbody>
</table>

*Mean ± sd; significance of difference compared to young control: * P < 0.05, ** P < 0.0005, *** P < 0.0001 compared to old control, n.s. compared to young control.
however, the reduction capacity in supplemented cells from old animals was consistently higher than the equivalent unsupplemented young group (Fig. 2).

The results of the AA biosynthesis experiments are summarized in Fig. 3. AA biosynthesis declined by 37% in cells from old animals compared to young ($P<0.001$). Neither cells from young (intercept $= 0.60$; slope $= -0.0002$; $r=0.46$; $P=0.22$) nor old (intercept $= 0.39$; slope $= -0.0002$; $r=0.44$; $P=0.24$) untreated animals showed a significant effect of increasing oxidative stress on the biosynthesis of AA although both had decreasing trends. No biosynthesis of ascorbic acid was observed during the incubation period when no cells were added but the ability to form AA was not directly related to the viability. Interpreting the results is complicated because the hepatocytes continued to produce AA from GL although they were considered dead according to the Trypan Blue exclusion method. After a 150 s boiling treatment, no AA was produced. We have therefore found it more appropriate not to correct the biosynthetic data for changes in viability since it would introduce artificially high values for the samples with lowest viability as measured by Trypan Blue exclusion.

In rats supplemented with (R)-lipoic acid, linear regression analysis showed a significant positive correlation with $t$-BuOOH concentration for cells from young animals (intercept $=0.36$; slope $=0.0004$; $r=0.72$; $P<0.05$) while the now-positive trend for cells from old animals was not statistically significant (intercept $=0.37$; slope $=0.0001$; $r=0.34$; $P=0.37$). In the case of (R)-lipoic acid-supplemented animals, there was no difference between the levels of biosynthesis. However, this was primarily due to a nonsignificant drop among the lipoic acid-supplemented young rats compared to untreated young rats.

**DISCUSSION**

In the present study, we found that ascorbic acid concentration as well as the ability to recycle DHA to AA in response to increased oxidative stress declines with age in rat hepatocytes and that this effect can be reversed by supplementation with (R)-lipoic acid.

During aging, mitochondria decay (1), oxidants increase (2, 3) and oxidative damage to important biomolecules increases (4–6). We therefore questioned the methodology of previous reports that only examined AA metabolism in metabolically quiescent cells. In the present system, experiments were carried out under different levels of oxidative stress, and this design enabled us to assess the flexibility and responsiveness of the antioxidant defense in cells isolated from young and old animals. The similar levels of recycling capacity among unstressed cells from young and old animals as well as the difference in actual hepatic AA concentration found in the present study agree with earlier findings (17). However, we believe our results with cells under stressed conditions emphasize the importance of extending the investigations to include a stressed environment. $t$-BuOOH is a commonly used model alkyl peroxide, which causes extensive lipid peroxidation as well as loss of low molecular weight antioxidants in hepatocytes (20). The choice of $t$-BuOOH as stressing agent was based on the literature that has characterized its toxicity as being due to membrane damage that results in perturbation of cellular calcium homeostasis (31, 32) and oxidation of cellular and mitochondrial pyridine nucleotides (33). Cells from old rats had a significantly lower ascorbic acid concentration compared to cells from young animals ($P<0.0005$, Table 1). The recycling capacity of AA in hepatocytes from young rats was unaffected by the preincubation with up to 500 $\mu$M $t$-BuOOH for 1 h. In contrast, hepatocytes from old rats showed a significantly decreasing ability to reduce DHA with increasing concentrations of $t$-BuOOH ($P<0.005$, Fig. 2). There was no difference between young and old controls. Biosynthesis of AA was not induced or otherwise affected by increasing oxidative stress. However, the biosynthetic rate of AA was 37% lower in cells from old animals compared to young ($P<0.001$).

These results demonstrate that cells isolated from old rat livers generally have a lower antioxidant capacity compared to young as measured by AA concentration, biosynthesis, and recycling. Consequently, they are more susceptible to increased oxidative stress and lack the ability to make the necessary...
adjustments to or sufficiently mobilize their defense systems. This is in agreement with the higher level of oxidative damage that has been observed with age in several models (1, 9). AA and GSH represent the lowest level of the antioxidant hierarchy and recycling cascade. Presumably, therefore, oxidative stress both in membrane and cytosolic environments is similarly reflected in the oxidation level of both of these antioxidants, which explains their value as biomarkers in this type of study. Indeed, preliminary data suggest that GSH levels match those of AA in our model (data not shown). A comprehensive study of this is under way.

α-Lipoic acid is a powerful antioxidant and possesses numerous important cellular functions as well as beneficial effects in situations with elevated oxidative stress (21–27). This compound has previously been shown to facilitate recycling of AA from DHA and other antioxidants in vitro (34–37). Xu and Wells found a fourfold increase in AA regeneration from DHA when they incubated rat liver mitochondria in the presence of (R,S)-lipoic acid. They attributed the positive effect of lipoic acid to its ability to mediate the reduction of DHA (35). Another possibility is that dihydrolipoic acid, a strong reductant (Eº′ = −320 mV) (38), spares AA through its separate but overlapping radical-scavenging effect (25).

In the present study we used dietary supplementation of lipoic acid and examined the effect in vivo on our biomarkers of oxidative stress: AA concentration, recycling, and biosynthesis. Of the two biologically active stereoisomers, (R)-lipoic acid was chosen as a supplement because it is the natural stereoisomer. (R)-Lipoic acid is preferentially reduced to its active dihydro form within the mitochondria by the NADH-dependent dihydrolipoamide dehydrogenase (39).

(R)-Lipoic acid supplementation of old rats for 2 wk caused a complete reversal of the AA depletion observed in cells from old compared to young rats—a highly significant increase of 127% compared to unsupplemented animals. In cells from young rats, a smaller though significant increase in hepatic AA concentration was also observed.

For AA recycling, (R)-lipoic acid supplementation resulted in a significant positive effect in cells from old rats where the increases ranged from 36 to 84% compared to the equivalent unsupplemented group. Although the slope of the linear regression analysis of AA recycling on t-BuOOH was significantly negative both with and without lipoic acid supplementation, the recovery is significant. With (R)-lipoic acid supplementation, the recycling capacity of cells from old animals was restored to a level that was above that of cells from unsupplemented young animals, regardless of t-BuOOH concentration. The more efficient recycling of AA can explain the large increase in AA concentration observed in cells from old rats. AA metabolism goes via the oxidized form DHA (40), and by keeping the AA pool more efficiently reduced, the half-life of AA, which is measured in hours (41), can be extended. In cells isolated from young rat livers, there was no significant effect on the recycling capacity, indicating that AA metabolism was adequate to meet the enhanced oxidative insult that t-BuOOH provided.

The results from the AA synthesis experiments are more complex (Fig. 3). AA synthesis was generally positively affected by (R)-lipoic acid supplementation. In hepatocytes from young rats, linear regression analysis revealed a significantly positive correlation between AA synthesis and t-BuOOH concentration. In cells from old animals, AA synthesis increased with increasing t-BuOOH concentration, but the correlation did not reach significance. Although the significant difference between young and old in the biosynthetic rate no longer persisted, this apparently was due mainly to a decrease among the cells from young animals rather than an increase among the old. The drop in biosynthesis among cells from (R)-lipoic acid supplemented old rats compared to untreated can be explained by the generally better antioxidant status in this group. Thus, in the case of (R)-lipoic acid supplementation, there is less need for de novo synthesis of AA with the higher tissue concentration of AA (Table 1). This is consistent with Tsao and Young, who found decreasing biosynthesis in mouse liver homogenates with increasing concentrations of AA obtained from dietary AA supplementation (42). The equivalent drop among old animals is not observed because of the already significantly lowered synthesis compared to young.

Presumably, the cells would benefit from being able to induce AA biosynthesis with increasing oxidative stress. That negative trends for the untreated animals turn to positive trends among the (R)-lipoic acid supplemented rats suggests the importance of sulfhydryl groups for the proper activity of L-gulono-1,4-lactone oxidase. This agrees with Sato et al., who found that dithiothreitol and 2-mercaptoethanol activated this microsomal enzyme (43). Nishikimi concluded that the enzyme has a sulfhydryl group, which is essential for activity (44). Lipoic acid supplementation does result in a marked increase in, for example, cellular GSH (45–47). However, further investigations are needed to clarify the influence of (R)-lipoic acid on AA biosynthesis.

In the present paper, we have investigated the relationship between aging and ascorbic acid metabolism. By using recycling and biosynthesis as biomarkers of responsiveness to different levels of oxidative stress, we demonstrate a declining capacity for hepatocytes to respond to oxidative stress with age. Dietary supplementation of old rats with (R)-lipoic acid reverses the overall age-associated decline in ascorbic acid concentration, recycling, and biosynthesis in iso-
lated hepatic parenchymal cells. These results provide further evidence for the potential of $\alpha$-lipoic acid in treatment of diseases related to oxidative stress. We have shown that AA recycling can provide more information about the cellular capacity to respond to oxidative stress. Thus, this study extends the value of AA as a biomarker of oxidative stress and the possible importance of AA recycling as a biomarker in humans is under investigation.

Declining mitochondrial function is associated with aging in general (2, 8, 48). Although a causal relationship remains to be established, we have hypothesized that improving mitochondrial function would improve cellular function in general (1, 3). The effect of ($R$)-lipoic acid demonstrated here could be due to specific mitochondrial improvements. We are working on further characterizing the cellular mechanisms that cause this effect as well as the role of mitochondria in aging.

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REFERENCES

33. Bellomo, G., Thor, H., and Orrenius, S. (1984) Increase in cystolic $Ca^{2+}$ concentration during $\beta$-butyl hydroperoxide metabolism by isolated hepatocytes involves NADPH oxidasi-
tion and mobilization of intracellular Ca^{2+} stores. *FEBS Lett.* **168**, 38–42

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