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(*R*)-α-Lipoic Acid Reverses the Age-Associated Increase in Susceptibility of Hepatocytes to *tert*-Butylhydroperoxide both *In Vitro* and *In Vivo*

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ABSTRACT

Hepatocytes were isolated from young (3–5 months) and old (24–28 months) rats and incubated with various concentrations of *tert*-butylhydroperoxide (t-BuOOH). The t-BuOOH concentration that killed 50% of cells (LC_{50}) in 2 hr declined nearly two-fold from 721 ± 32 μM in cells from young rats to 391 ± 31 μM in cells from old rats. This increased sensitivity of hepatocytes from old rats may be due, in part, to changes in glutathione (GSH) levels, because total cellular and mitochondrial GSH were 37.7% and 58.3% lower, respectively, compared to cells from young rats. Cells from old animals were incubated with either (*R*)- or (*S*)-lipoic acid (100 μM) for 30 min prior to the addition of 300 μM t-BuOOH. The physiologically relevant (*R*)-form, a coenzyme in mitochondria, as opposed to the (*S*)-form significantly protected hepatocytes against t-BuOOH toxicity. Dietary supplementation of (*R*)-lipoic acid [0.5% (wt/wt)] for 2 weeks also completely reversed the age-related decline in hepatocellular GSH levels and the increased vulnerability to t-BuOOH as well. An identical supplemental diet fed to young rats did not enhance the resistance to t-BuOOH, indicating that antioxidant protection was already optimal in young rats. Thus, this study shows that cells from old animals are more susceptible to oxidant insult and (*R*)-lipoic acid, after reduction to an antioxidant in the mitochondria, effectively reverses this age-related increase in oxidant vulnerability. Antiox. Redox Signal. 2, 473–483.

INTRODUCTION

AGING APPEARS TO BE DUE, in good part, to the oxidants produced by mitochondria as by-products of normal metabolism (Shigenaga *et al.*, 1994; Hagen *et al.*, 1997, 1998, 1999; Beckman and Ames, 1998; Lykkesfeldt *et al.*, 1998). In old rats, compared to young rats, mitochondrial membrane potential, cardiolipin levels, respiratory control ratio and overall cellular O_2 consumption decline, and the level of oxidants (per unit O_2) increases (Hagen *et al.*, 1997, 1998). The level of mutagenic aldehydes from lipid peroxidation is also increased (Hagen *et al.*, 1999). Ambulatory activity of old rats is markedly lower than young rats (Hagen *et al.*, 1998). We have shown that (*R*)-α-lipoic acid, a coenzyme for pyruvate and α-ketoglutarate dehydrogenases in mitochondria, when fed to old rats for 2 weeks, significantly restored (compared to young rats) mitochondrial oxygen consumption and membrane potential, cellular ascorbate level, ascorbate recycling after *tert*-butylhyproperoxide (t-BuOOH) stress, and ambulatory activity (Hagen *et al.*, 1997, 1998, 1999; Lykkesfeldt *et al.*, 1998). Feeding (*R*)-α-lipoic acid also lowered the level of oxidants detected to that of a young rat.

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Mitochondrial decay with age may reduce the capacity of cells to respond to oxidative challenge due to lack of ATP to maintain defense systems or repair oxidative damage (Shigenaga *et al.*, 1994; Hagen *et al.*, 1997; Sastre *et al.*, 1996). Concentrations of cellular glutathione (Christen *et al.*, 1995; Loguercio *et al.*, 1996; Nakata *et al.*, 1996) and ascorbic acid decrease (Rikans and Moore, 1988; Yu *et al.*, 1989) in some tissues, which would lower overall antioxidant defenses and render cells more susceptible to oxidative injury. On the basis of these changes occurring at the basal metabolic level, it is plausible that the aging organism cannot adequately respond to external oxidative or toxicological insults.

Hepatocytes from old rats no longer adequately reduce dehydroascorbic acid when challenged with t-BuOOH (Lykkesfeldt *et al.*, 1998), and others have found a similarly diminished response to a variety of different oxidative stresses in tissues with age (Locke and Tanguay 1996; Ando *et al.*, 1997; Lawler *et al.*, 1997; Wachulec *et al.*, 1997). In contrast, one study demonstrates no age-related difference in the eye (Lou *et al.*, 1995) and another shows a better response to high oxygen stress than that seen in young rats (Choi *et al.*, 1995). Thus, the conclusion that there is a general decline in the ability of cells to respond to an added oxidant challenge with age is generally found, but is not necessarily true in all tissues.

This study seeks to determine whether hepatocytes from old rats are more susceptible to an oxidative stress than cells from young animals. For this analysis, we isolated rat liver parenchymal cells from young and old rats and subjected them to an oxidative challenge using t-BuOOH, a model alkyl peroxide that is known to cause lipid peroxidation, rapid loss of intracellular GSH, oxidation of pyridine nucleotides, and mitochondrial damage (Eklow *et al.*, 1984; Nieminen *et al.*, 1997; Morgan, 1995). The results presented in this paper show that hepatocytes from old rats are significantly more susceptible to t-BuOOH-induced toxicity with age. Moreover, this heightened sensitivity to toxic insult can be significantly reversed by dietary supplementation with (*R*)-lipoic acid, a disulfide compound that can be reduced by mitochondria into dihydrolipoic acid, a potent antioxidant. We also show that (*R*)-lipoic acid may exert its protective effect by increasing both cellular and mitochondrial GSH levels, thereby allowing detoxification reactions to continue to function.

MATERIALS AND METHODS

Materials

The following chemicals were used: trypan blue, digitonin, heparin (sodium salt), t-BuOOH (70% aqueous solution), iodoacetic acid, 2,4-dinitrofluorobenzene, glutathione, reduced form (GSH), glutathione, oxidized form (GSSG), Trizma Base (Tris [hydroxymethyl] aminomethane) (Sigma, St. Louis); 5-sulfosalicylic acid dihydrate (Aldrich, Milwaukee, WI); collagenase (type D) (Boehringer Mannheim, Indianapolis). (*R*)- and (*S*)-lipoic acid were a gift from Dr. H. Tritschler of Asta Medica (Frankfurt, Germany). All other chemicals were of reagent grade or better. Double-distilled/deionized water was used throughout.

Animals

Rats (Fisher 344, male; 3-5 mo) were obtained from Simonsen (Gilroy, CA). Old rats (Fisher 344, virgin male; 20–28 months) were from the National Institute on Aging animal colonies. All animals were acclimatized at the Northwest Animal Facilities on the Berkeley campus for at least 1 week prior to experimentation. Unsupplemented animals were fed Purina rodent chow and water *ad libitum*. (*R*)-lipoic acid [0.5% (wt/wt) in AIN-93M standard diet] chow was prepared by Dyets Inc. (Bethlehem, PA). The pellets were made into a mush and fed to some young and old rats for 2 weeks prior to cell isolation. Both young and old rats typically ate ~15 grams/rat per day (data not shown), which provides a daily (*R*)-lipoic acid dose of 43 grams/kg body weight for young rats and 33 grams/kg body weight for old rats.

Cell isolation and incubation

Rats were anesthetized using ethyl ether (Fisher Sci., Fair Lawn, NJ) and the liver was exposed by a midlateral incision in the abdomen. Rats were sacrificed by cutting through the diaphragm and severing the vena cava. The

animals were consistently sacrificed between 8:00 to 10:00 AM to minimize differences in food intake and diurnal variability. Liver tissue from young (3–5 months) and old (20–24 months) rats were dispersed into single cells by collagenase perfusion (Moldeus *et al.*, 1978). Cell number was assessed by using a hemocytometer, and viability was determined by trypan blue [0.2% (wt/vol) in phosphate-buffered saline (PBS)] exclusion. Viability was normally greater than 90% in both age groups.

To determine the effective LC_{50} concentration of t-BuOOH for hepatocytes from young and old-rats, cells (2.0×10^6/ml) were incubated at 37°C in the presence of 0, 300, 500, and 750 μM t-BuOOH and viability was assessed over a 2-hr time-course. Viability of cells was monitored by the ability of cells to exclude Trypan Blue. To ascertain further the accuracy of this assay, release of lactate dehydrogenase from hepatocytes were also monitored. The results from both means of measuring viability were essentially the same (data not shown). Because of the lack of differences, only the Trypan Blue exclusion data are reported due to larger N values.

(R)- and (S)-Lipoic acid

Hepatocytes from young and old unsupplemented rats were incubated with 100 μM (final conc.) (*R*)- or (*S*)-lipoic acid dissolved in dimethylformamide (DMF) for 30 min at 37°C with gentle shaking to maintain cells in suspension. For controls, an equal volume of DMF was added to cells, but otherwise treated similarly to those receiving lipoic acid. DMF treatment caused cell viability in all groups to decline initially by ~10%, but otherwise did not further affect cell morphology or viability (data not shown). The hepatocytes were pelleted at $100 \times g$ for 1 min and the supernatant aspirated. Cells were suspended with Krebs-Henseleit medium, pH 7.4, and pelleted again to remove extracellular lipoic acid. Cell pellets were resuspended in PBS (2.0×10^6 cells/ml, pH 7.4) and were immediately incubated with 300 μM t-BuOOH. Viability was determined (described above) over a 2-hr time course.

GSH analysis

GSH was measured by high-performance liquid chromatography (HPLC) as described by Reed *et al.* (1982). Briefly, cells were mixed with 5-sulfosalicylic acid (10% (wt/vol), final concentration) and the samples were spun for 1 min at 13,000 rpm in a microcentrifuge to remove denatured debris. *S*-Carboxymethylated, dinitrophenyl derivatives of the acid soluble thiols were prepared by addition of fresh aqueous iodoacetic acid (4 μmol) and 2,4-dinitrofluorobenzene (500 μl of a 1.5% [vol/vol] solution in absolute ethanol) to the acidified supernatant (final pH >10). Derivatives were separated by HPLC as described (Reed *et al.*, 1982) and oxidized and reduced GSH was quantified relative to standards.

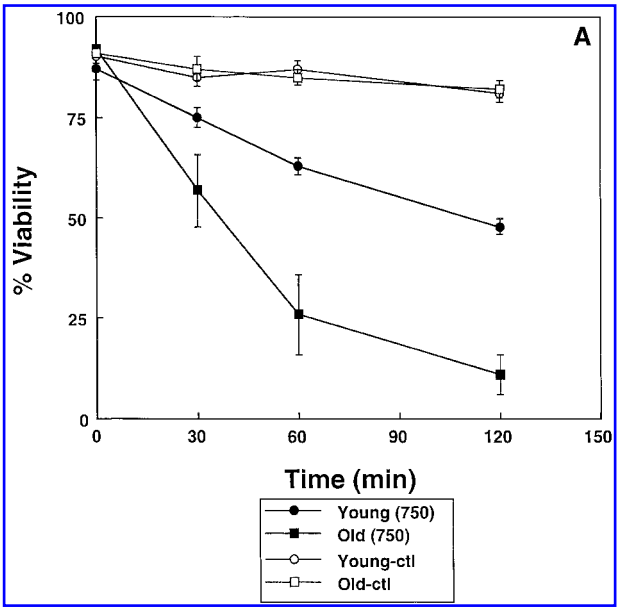
To measure mitochondrial GSH, hepatocytes were permeabilized with 1.2 and 0.9 mg/ml digitonin for cells from young and old rats, respectively, and immediately centrifuged for 30 sec at 13,000 rpm through a 25% (vol/vol) Percoll layer in Krebs-Henseleit medium, pH 7.4, using a microcentrifuge. The pellets were resuspended in 7.5% (wt/vol) 5-sulfosalicylic acid and analyzed for GSH content as described above.

Statistical analysis

Statistical significance was determined using the Students *t*-test or the alternate Welch test. Results are expressed as the mean ± SEM. A p value of less than 0.05 was considered significant.

RESULTS

To determine whether cells from old animals are more susceptible to oxidative injury, we incubated hepatocytes from young and old rats with t-BuOOH, an alkyl peroxide that causes lipid peroxidation, loss of mitochondrial GSH, and oxidation of pyridine nucleotides. As shown in Fig. 1A, incubation of cells from young rats with 750 μM t-BuOOH for 2 hr reduced viability by approximately 50%. This observed level of toxicity is consistent with other reports using t-BuOOH and hepatocytes (Jewell *et al.*, 1986; Fraga *et al.*, 1989; Bannach *et al.*, 1996). For cells from old rats, an equimolar concentration of t-BuOOH caused a more rapid ($p = 0.0005$) loss in cell viability with only 12% of cells remaining viable after 2 hr of incuba-



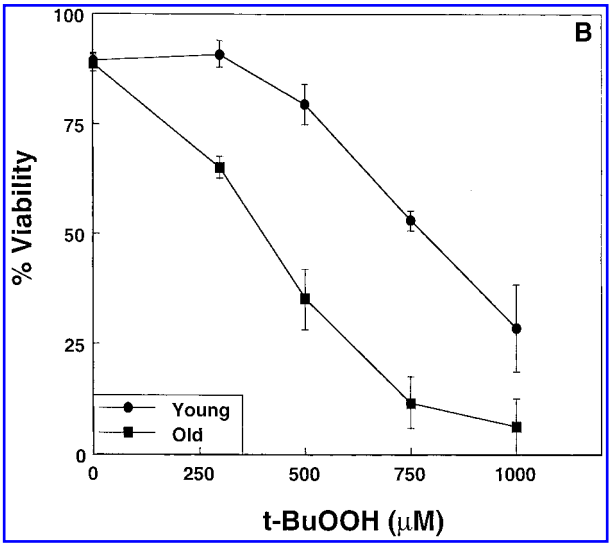


FIG. 1. Hepatocytes from old rats are markedly more susceptible to t-BuOOH than cells from young rats. (**A**) Isolated hepatocytes from young and old rats were incubated without (control) or with 750 μM t-BuOOH and viability was monitored over a 2-hr time course. Results show that hepatocytes from old rats are significantly more susceptible to t-BuOOH ($p < 0.01$) than cells from young rats. (**B**) Dose–response curve of t-BuOOH-mediated cytotoxicity to hepatocytes from young and old rats. Cells from young and old rats were incubated at various concentrations of t-BuOOH and viability determined after 2 hr. Cells from old rats are markedly more susceptible to t-BuOOH-mediated killing at all doses ($p < 0.002$).

tion. These results show that hepatocytes from old rats are markedly more susceptible to t-BuOOH-mediated toxicity than hepatocytes from young rats.

To characterize further the relative increase in susceptibility to toxic insult with age, a concentration dependence study with t-BuOOH was performed. Cells from old rats were markedly more susceptible to toxic insult by increasing concentrations of t-BuOOH than cells from young rats ($p < 0.002$; Fig. 1B). The concentration of t-BuOOH that killed 50% of the hepatocytes from young rats (LC_{50}) after 2 hr of incubation was 721.4 ± 31.6 μM ($n = 9$) versus 391.1 ± 30.8 μM for cells from old rats ($n = 10$). This indicates that hepatocytes from old rats are nearly twice as susceptible to t-BuOOH-induced toxicity than cells from young rats.

Because the extent of t-BuOOH toxicity is affected by cellular and mitochondrial GSH status, we determined whether the increased vulnerability to t-BuOOH was due to changes in GSH levels in old rats. As shown in Table 1, hepatocellular GSH concentrations were 37.7% lower in old versus young rats, a modest but significant decline ($p < 0.001$) with age. The GSH/GSSG ratio, an important indicator of oxidative stress, also declined significantly by almost 50% ($p = 0.034$) (Table 1). These results, along with our previous work showing an age-related loss in ascorbic acid levels (Hagen *et al.*, 1998; Lykkesfeldt *et al.*, 1998), reflect a significant perturbation in cellular redox status in rat hepatocytes with age.

Mitochondrial GSH concentrations, as measured in digitonin-permeabilized cells, also declined with age from 0.012 ± 0.002 to 0.005 ± 0.001 μmol/mg protein ($n = 3$). This reflects a significant ($p = 0.004$) loss of 58% and indicates that aging affects mitochondrial GSH pools even more severely than cytosolic GSH. Mitochondrial GSSG values were below the detection limits of the assay and, therefore, GSH/GSSG ratio could not be determined. The observed changes in both mitochondrial and total cellular GSH status indicate that the decline in this key antioxidant may be a significant factor in the increased susceptibility of hepatocytes to t-BuOOH.

Reversal of t-BuOOH susceptibility with R-*lipoic acid*

To investigate whether reversal of the decreased hepatocellular antioxidant capacity

TABLE 1. AGE-RELATED CHANGES TO HEPATOCELLULAR GSH STATUS

	GSH		*GSSG*		*GSH/GSSG*	
	Young	*Old*	*Young*	*Old*	*Young*	*Old*
Total	0.139 ± 0.008	0.087 ± 0.005[a]	0.005 ± 0.001	0.004 ± 0.012	31.6	18
MT[b]	0.012 ± 0.002	0.005 ± 0.001[a]	ND	ND	ND	ND

Values for GSH and GSSG are expressed as μmol/mg protein cells.
[a]$p < 0.05$.
[b]MT, Mitochondria.

could increase the resistance of hepatocytes from old rats to t-BuOOH, we incubated these cells with lipoic acid. Lipoic acid is a disulfide compound that is readily taken up into cells and is known to maintain or increase GSH levels (Han *et al.*, 1997). The (*R*)-enantiomer is the "physiologically relevant" isomer and is reduced in mitochondria to dihydrolipoic acid, a potent antioxidant (Suzuki *et al.*, 1991; Bustamante *et al.*, 1998). Dihydrolipoic acid may therefore augment mitochondrial antioxidant capacity as well as increase cellular antioxidant status following leakage or transport from the mitochondria. Cells from old rats were preincubated with 100 μM (*R*)-lipoic acid for 30 min prior to addition of 300 μM t-BuOOH. As shown in Fig. 2, (*R*)-lipoic acid completely protected the cells against the t-BuOOH-induced toxicity ($p = 0.024$ versus t-BuOOH-treated control cells, $n = 5$). Pretreatment with the same concentration (100 μM) of (*S*)-lipoic acid, however, did not protect the cells against t-BuOOH-mediated toxicity (Fig. 2). These results show that the physiologically relevant (*R*)-form of lipoic acid, when added to hepatocytes from old rats, reverses the age-associated increase in the susceptibility of hepatocytes to t-BuOOH.

Dietary interventions

Because addition of (*R*)-lipoic acid to cells from old rats prevented the increased t-BuOOH-mediated cytotoxicity *in vitro*, we sought to determine the effect of feeding old rats a diet supplemented with (*R*)-lipoic acid. Rats given [0.5% (wt/wt)] (*R*)-lipoic acid in the diet for 2 weeks had substantially higher cellular GSH levels compared to unsupplemented controls. Cellular GSH concentrations significantly increased ($p < 0.03$) by 47% and 54% over age-matched controls for both young and old rats, respectively (Fig. 3A). We also noted a significant ($p < 0.005$) increase in the hepatocellular GSH/GSSG ratio in both young and old rats treated with lipoic acid: 66.8 ± 7.5 ($n = 7$) and 69.9 ± 11.1 ($n = 4$), in cells from young and old rats supplemented with lipoic acid, respectively.

This feeding regimen also raised mitochondrial GSH levels in both young and old rats over their respective controls. The increase was more extensive than for cytosolic GSH, with mitochondrial concentrations over 2.8- and 35-fold higher in young and old cells, respectively (Fig. 3B). Overall, these results show that a diet

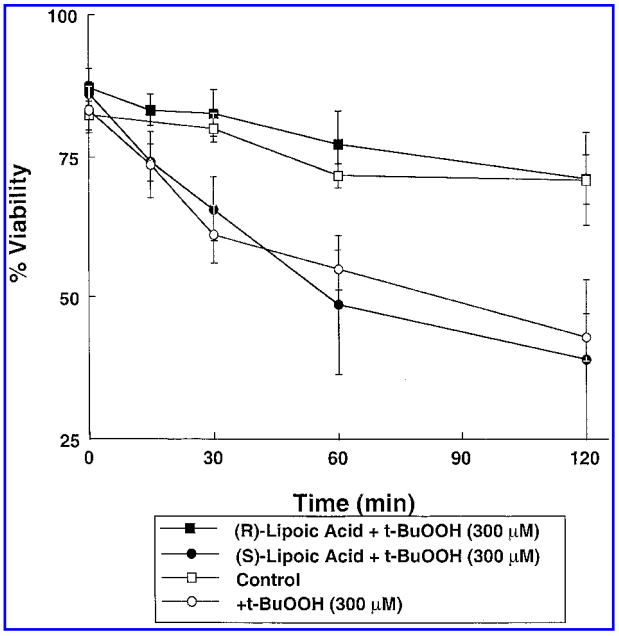


FIG. 2. (*R*)- but not (*S*)-lipoic acid protects against t-BuOOH toxicity in cells from old rats. Isolated hepatocytes from old rats were incubated with 100 μM (*R*)- or (*S*)-lipoic acid as described and then given a 300 μM dose of t-BuOOH. Results show that only the cells pretreated with (*R*)-lipoic acid were significantly protected against t-BuOOH-mediated cytotoxicity.

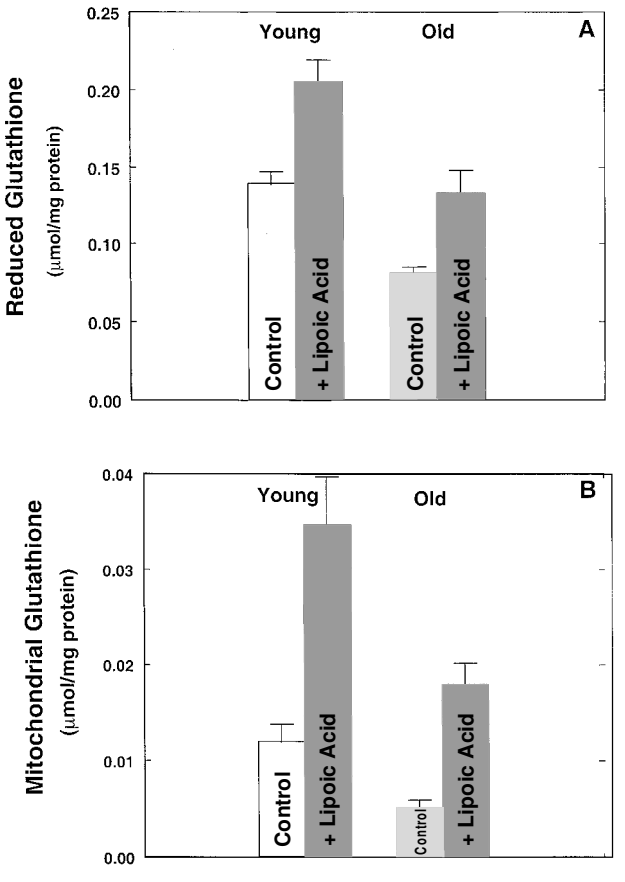


FIG. 3. Dietary supplementation with (*R*)-lipoic acid reverses the age-associated decline in hepatocellular GSH concentrations. (**A**) A 2-week feeding regimen of 0.5% (wt/wt) (*R*)-lipoic acid in the diet significantly increased total reduced GSH levels in hepatocytes of young and old rats by 47% and 54%, respectively, over age-matched unsupplemented controls. (**B**) Dietary supplementation with (*R*)-lipoic acid also significantly ($p < 0.01$) increased mitochondrial GSH over corresponding age-matched controls.

supplemented with (*R*)-lipoic acid substantially reverses the decline in hepatocellular glutathione status.

To determine the effect that this feeding regimen had on cellular susceptibility to t-BuOOH, we treated cells from rats given the (*R*)-lipoic acid supplemented diet with 750 μM t-BuOOH, the approximate LC_{50} for cells from young rats and a dose found to be lethal to hepatocytes from old rats (Fig. 1). As shown in Fig. 4, hepatocytes isolated from old rats on the (*R*)-lipoic acid diet was significantly protected against t-BuOOH mediated toxicity ($p < 0.03$) compared to unsupplemented old control rats and in fact identical to that of cells from unsupplemented young rats. In contrast, cells from young rats treated with (*R*)-lipoic acid did not show any increased resistance to t-BuOOH-mediated cytotoxicity (Fig. 5). A concentration dependence study confirmed that cells isolated from old rats on the supplemented diet were now equally resistant to t-BuOOH as those from unsupplemented young rats (Fig. 5). Thus, (*R*)-lipoic acid supplementation reverses the age-associated increase in the susceptibility to t-BuOOH-mediated toxicity.

DISCUSSION

Hepatocytes from old rats are significantly (about two-fold) more susceptible to oxidant insult from t-BuOOH than cells from young rats (Fig. 1A,B). We chose t-BuOOH for this study because this model alkyl hydroperoxide has been extensively used to examine the effects of oxidative stress in the liver (Eklow *et al.*, 1984; Jewell *et al.*, 1986; Fraga *et al.*, 1989; Morgan, 1995; Bannach *et al.*, 1996; Nieminen *et al.*, 1997; Lykkesfeldt *et al.*, 1998). t-BuOOH causes rapid loss of mitochondrial GSH (Shan *et al.*, 1993; Chernyak and Bernadi, 1996) and other low-molecular-weight antioxidants, oxidation of mitochondrial pyridine nucleotides (ATP and NADH), lipid peroxidation, and loss of calcium homeostasis (Eklow *et al.*, 1984; Morgan, 1995; Nieminen *et al.*, 1997). Thus, it would appear that aging compromises one or a number of antioxidant defense mechanisms of the cell.

At least part of the increased age-related susceptibility to t-BuOOH may be due to a decline in low-molecular-weight antioxidants in the aging rat liver, particularly GSH in the mitochondria. A significant decline in cellular GSH levels, including those in mitochondria (Table 1), indicates that aging results in severe loss of the low-molecular-weight antioxidants in the liver. The mitochondrial GSH pool showed a more pronounced age-related decline. This may be particularly important because the cytotoxic mechanism of t-BuOOH works through loss of mitochondrial GSH (Shan *et al.*, 1993; Chernyak and Bernardi, 1996). We found that mitochondrial GSH was over 43% lower in old compared to young digitonin-permeabilized

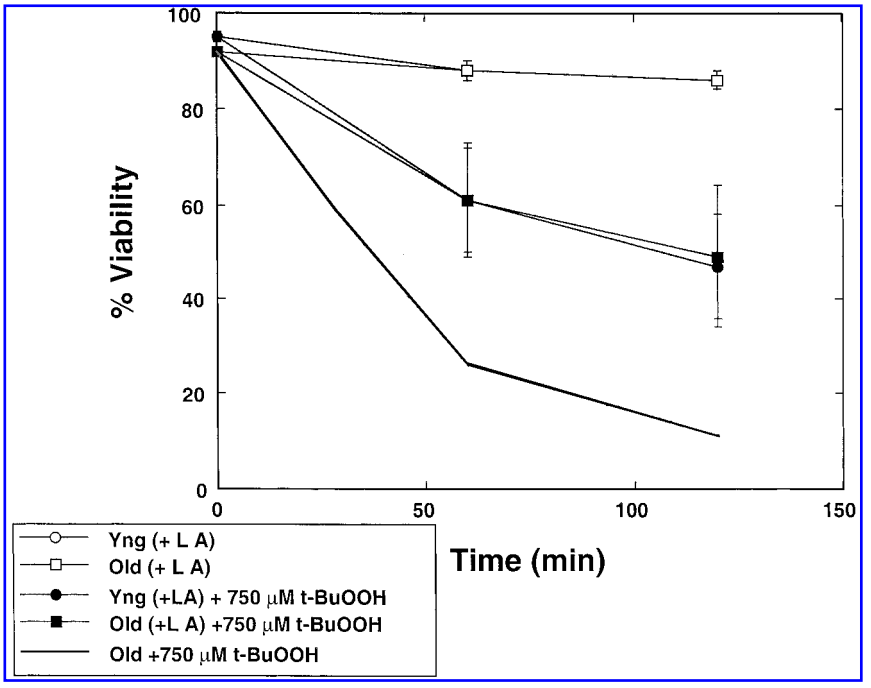


FIG. 4. Feeding (*R*)-lipoic acid to old rats reverses the age-related increase in susceptibility to t-BuOOH. Young and old rats were fed 0.5% (wt/wt) lipoic acid for 2 weeks prior to hepatocyte isolation. Cells were incubated with 750 μM t-BuOOH and viability assessed by trypan blue exclusion. Results show that cells from old rats were no longer more susceptible to t-BuOOH-induced cytotoxicity than cells from young rats. Lipoic acid treatment did not alter the extent of cytotoxicity seen in cells from young rats. The dashed line represents the level of viability for hepatocytes from old unsupplemented rats (see Fig. 1).

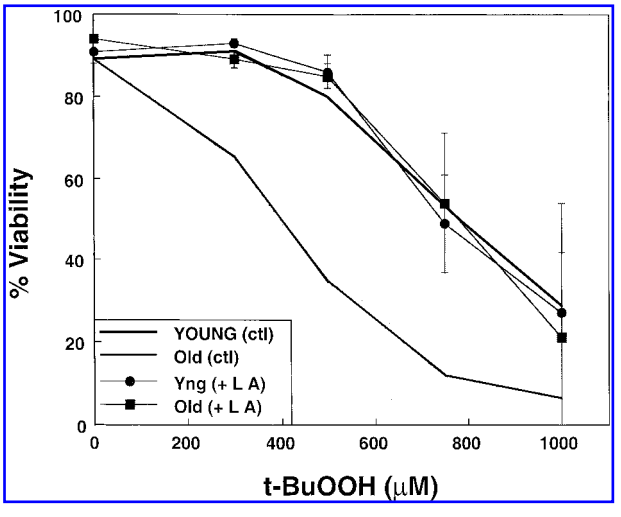


FIG. 5. Dose/response curve of t-BuOOH-induced cytotoxicity to hepatocytes from (*R*)-lipoic acid-treated rats. (*R*)-Lipoic acid supplementation to old rats reverses the age-associated increased susceptibility to t-BuOOH at all concentrations examined. The calculated LC_{50} concentrations were similar in cells from young and old supplemented rats to that found in cells from unsupplemented young rats.

cells. The underlying reasons for the extensive age-related decline in GSH status was not explored in the present study, but may reflect enhanced utilization of GSH because of increased oxidant production with age (Shigenaga *et al.*, 1994; Sohal and Weindruch, 1996; Hagen *et al.*, 1997, 1998) or may be due to a decline in GSH transport into the mitochondria (Chen and Lash, 1998) or both. Mitochondrial GSSG was below the detection limit and, therefore, we could not determine the GSH/GSSG ratio. However, Viña and colleagues found that mitochondrial GSH/GSSG declined significantly in kidney, liver, and brain of old rats and mice when compared to tissue from young animals (de la Asuncion *et al.*, 1996). These studies indicate that mitochondrial antioxidant capacity is significantly more attenuated than the cytosolic antioxidant defenses and may weaken the ability of cells to withstand insults from endogenous oxidants or toxins, such as t-BuOOH, that target mitochondria.

Decline in cellular enzymatic defense systems may also contribute to the increased vulnerability to t-BuOOH with age. GSH peroxi-

dase activity declines in a number of tissues, which could directly influence t-BuOOH cytotoxicity (Christen *et al.*, 1995; Nakata *et al.*, 1996). We and others have shown that hepatocellular ascorbate levels in old rats are significantly lower than those found in young animals (Rikans, *et al.*, 1996; Xu and Wells, 1996; Lykkesfeldt, *et al.*, 1998). Aging thus leaves cells vulnerable to a variety of oxidative or toxic insults (Lee and Werlin, 1995; Sotaniemi *et al.*, 1997; Rikans and Hornbrook, 1998).

The rate of hepatocellular GSH synthesis was not measured in this study and, therefore, it is not known whether the decline in GSH levels were due to lower synthetic capacity with age or from enhanced GSH utilization or both. We did find that preincubating cells from old rats with the amino acid precursors for GSH biosynthesis prior to t-BuOOH addition did not alter their increased susceptibility to t-BuOOH (data not shown). These results indicate that the capacity of cells from old rats to synthesize GSH was not sufficient to provide further protection against t-BuOOH-induced cytotoxicity.

Mitochondrial decay may influence the age-associated susceptibility of hepatocytes to t-BuOOH. Mitochondria are more prone to lipid peroxidation with age due to increased fatty acid unsaturation of inner membrane phospholipids (Shigenaga *et al.*, 1994), which could make them more susceptible to t-BuOOH-induced damage. Mitochondrial membrane potential also declines in the majority of rat hepatocytes with age (Hagen *et al.*, 1997), which may compromise ATP production, especially in times when more energy is required to allow detoxification reactions to continue. Decaying mitochondria may also be unable to supply important reducing equivalents (NADH, NADPH), which would limit recycling of GSH and further attenuate GSH-dependent detoxification reactions.

Some enzyme systems that may contribute to cellular antioxidant and xenobiotic defenses either do not change or actually increase with age. There is no age-related difference in GSSG reductase (Rikans and Moore, 1988), or in ascorbic acid recycling in rat liver (Xu and Wells, 1996; Lykkesfeldt *et al.*, 1998) and levels of Cu, Zn superoxide dismutase and catalase actually increase (Sanz *et al.*, 1997). However, measuring enzyme activities on a basal metabolic level may not accurately reflect how such detoxification systems function during oxidative stress. Like Xu and Wells (1996), we showed that the rate of ascorbate recycling was no different in rat hepatocytes with age on a basal metabolic level (Lykkesfeldt *et al.*, 1998). However, we showed that ascorbic acid recycling could not be maintained in hepatocytes from old animals treated with t-BuOOH (Lykkesfeldt *et al.*, 1998). This indicates that antioxidant enzyme defenses may be more compromised in aging during times of oxidative stress than measurement of their basal activities would indicate.

Dietary supplementation of (*R*)-lipoic acid to old rats reverses the decline in both cellular (Fig. 3A) and mitochondrial GSH (Fig. 3B), and ascorbic acid levels (Lykkesfeldt *et al.*, 1998), and also reverses the increased cytotoxicity to t-BuOOH (Figs. 4 and 5) seen in old rats. However, (*R*)-lipoic acid supplementation to young rats did not afford any greater protection against t-BuOOH (Figs. 4 and 5). This implies that (*R*)-lipoic acid does not act directly to protect hepatocytes against t-BuOOH, but indirectly influences its cytotoxicity through maintenance of other endogenous antioxidants, such as glutathione (Fig. 3A,B) and ascorbic acid (Lykkesfeldt *et al.*, 1998). However, hepatocellular levels of free lipoic acid or dihydrolipoic acid following dietary supplementation were not determined in the current study. Therefore, we do not know the extent that free lipoic acid may also contribute to reversing the age-related increase in hepatocellular susceptibility to t-BuOOH. Experiments are currently underway to determine the relative contribution of free lipoic acid/dihydrolipoic acid in reversing susceptibility to exogenous xenobiotic compounds. In either scenario, it would appear that dietary supplementation with antioxidants or agents that boost endogenous antioxidant levels is critical for lowering the age-related increase in vulnerability to oxidative damage.

R-lipoic acid, but not *S*-lipoic acid, significantly protected isolated hepatocytes from old rats against t-BuOOH-induced toxicity (Fig. 2). Addition of the same level of the racemic mix-

ture also significantly protected cells but to a lesser degree than for (*R*)-lipoic acid alone (data not shown). This difference in protection afforded by the (*R*)-enantiomer has been previously noted. Packer *et al.* showed that (*R*)-lipoic acid but not *S*-lipoic acid could protect rats against buthionine sulfoxide-induced cataract formation (Maitra *et al.*, 1996). The authors concluded that this was because (*R*)-lipoic acid substantially increased GSH levels in the lens while the (*S*)-form did not. Addition of (*R*)-lipoic acid also increases ATP synthesis and aortic blood flow in a working rat heart model during reoxygenation following hypoxia (Zimmer *et al.*, 1995). The (*S*-enantiomer had no effect on ATP synthesis and only improved blood flow at 10 times the effective dose of (*R*)-lipoic acid. In other studies, (*R*)-lipoic acid increased glucose uptake and the number of glucose transporters in muscle tissue much more effectively than (*S*)-lipoic acid (Estrada *et al.*, 1996) and, in addition, the (*R*)-enantiomer more effectively chelated copper and prevented copper-induced lipid peroxidation (Ou *et al.*, 1995). The (*R*)-form is specifically reduced via lipoate dehydrogenases in the mitochondria to dihydrolipoic acid, a potent antioxidant (Ou *et al.*, 1995). Dihydrolipoic acid can also diffuse out of the mitochondria and may thus act as a general cellular antioxidant. (*S*)-Lipoic acid is reduced more slowly and in a nonspecific manner by cytosolic GSSG reductase. Thus, (*R*)-lipoic acid may be more effective in preventing toxicity due to a more rapid conversion to dihydrolipoic acid. The (*R*)-enantiomer is the mitochondrial cofactor for pyruvate and α-ketoglutarate dehydrogenases, and may thus support greater mitochondrial ATP production. This, in turn, could maintain detoxification reactions that would otherwise be compromised in the aging rat liver.

The dose of (*R*)-lipoic acid given in this study was in the pharmacological range; however, it was well tolerated by the rats with no side effects noted. Extrapolating to humans would indicate that this dose is well above that expected from normal dietary sources. But supplemental use of α-lipoic acid (*R,S*) also seems to be well tolerated by people; daily doses of several hundred milligrams for 3 or 4 months have been given to diabetic patients in Germany with no signs of toxicity or side effects (Sachse and Willms, 1980; Zeigler *et al.*, 1995). High doses have also been administered to patients with diabetic polyneuropathies and have been equally well tolerated. We are currently determining whether supplementing rats with lower doses of lipoic acid are effective in reversing the increased susceptibility to t-BuOOH with age.

Lipoic acid supplementation to old rats partially reverses the general decline in basal metabolism and lowers increased oxidant production and oxidative damage seen in aging (Hagen *et al.*, 1999). Along with the present study, these results provide a rationale to examine whether dietary supplementation with (*R*)-lipoic acid (Hagen *et al.*, 1999) to elderly people would be a safe and effective means to reverse certain aspects of mitochondrial decay and metabolic decline as well as lower the vulnerability to oxidants and/or xenobiotics. Considering that the geriatric population is the fastest growing age group in this country, with disproportionately higher health-care costs, a diet enriched with lipoic acid may be a cost-effective means to maintain vigor as well as aid against increased cellular oxidative damage that occurs during aging.

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ABBREVIATIONS

DMF, dimethylformamide; GSH, glutathione; GSSG, oxidized GSH; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; t-BuOOH, *tert*-butyl hyproperoxide.

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