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Original Contribution

EFFECTS OF AGING ON THE SUSCEPTIBILITY TO THE TOXIC EFFECTS OF CYCLOSPORIN A IN RATS. CHANGES IN LIVER GLUTATHIONE AND ANTIOXIDANT ENZYMES

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Abstract—Free radicals are involved in aging and cyclosporin A-induced toxicity. The age-related changes in the liver oxidative status of glutathione, lipid peroxidation, and the activity of the enzymatic antioxidant defense system, as well as the influence of aging on the susceptibility to the hepatotoxic effects of cyclosporin (CyA) were investigated in rats of different ages (1, 2, 4, and 24 months). The hepatic content of reduced glutathione (GSH) increased with aging, peaked at 4 months, and decreased in senescent rats. By contrast, glutathione disulfide (GSSG) and thiobarbituric acid-reactive substances (TBARS) concentrations and superoxide dismutase, catalase, and glutathione peroxidase activities were higher in the oldest than in the youngest rats. CyA treatment, besides inducing the well-known cholestatic syndrome, increased liver GSSG and TBARS contents and the GSSG/GSH molar ratio, and altered the nonenzymatic and enzymatic antioxidant defense systems. The CyA-induced cholestasis and hepatic depletion of GSH, and the increases in the GSSG/GSH ratio, and in GSSG and TBARS concentrations were higher in the older than the mature rats. Moreover, superoxide dismutase and catalase activities were found to be significantly decreased only in treated senescent rats. The higher CyA-induced oxidative stress, lipoperoxidation, and decreases in the antioxidant defense systems in the aged animals render them more susceptible to the hepatotoxic effects of cyclosporin. © 2001 Elsevier Science Inc.

Keywords—Aging, Antioxidant enzymes, Cyclosporin A, Free radicals, Glutathione, Hepatotoxicity, Lipoperoxidation, Liver, Oxidative stress

INTRODUCTION

An imbalance between the formation and removal of reactive oxygen species (ROS) and the development of oxidative stress has been widely purported to play a important role in drug toxicity, ischemic damage, neoplastic transformation and metastasis, and cardiovascular, neurodegenerative, and age-associated diseases [1], as well as in differentiation, development, and aging [1–3].

CyA is a key immunosuppressant drug used in medical transplantation, but its use is often limited by the induction of nephrotoxicity, neurotoxicity, and hepatotoxicity [4–11]. Regarding hepatotoxicity, it has been reported that CyA interferes with different important processes and functions in the liver, mainly the vectorial transport of biliary components from blood to bile, thus causing their accumulation in blood, the inhibition of bile formation and, hence, the development of a cholestatic syndrome in rat [7–9,12, 13] and man [4,13–17]. Over the last several years there has been accumulating evidence for the role of oxidative stress, free radicals, and lipid peroxidation in the pathogenesis of adverse CyA-induced side effects. It has been reported that CyA increases superoxide anion and/or H_2O_2 formation in cultured rat hepatocytes [18] and different kidney cells [19], and that it induces oxidative stress and lipoperoxidation in microsomal preparations from humans [10], rats [20], and rabbits [21], as well as in in vitro assays using

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different organs from the rat, such as the liver [5] or kidney [22]. In vivo, CyA treatment increases lipoperoxidation in the rat kidney [23,24] and liver [24] and in transplanted patients [11], depletes the hepatic pool of glutathione and reduces its biliary secretion in rats [5,6,9], and significantly reduces the glutathione contents in the kidney [6], while glutathione depletion increases the susceptibility to drug-induced toxicity [3,25].

Aging is usually associated with increasing levels of oxidation and it has been suggested that senescence may result from the accumulation of unrepaired structural damage to cells, which disrupts the cellular functions when the organism enters into contact with different endogenous and exogenous agents, ROS being the most important members of these toxins [1,26]. ROS alter proteins, carbohydrates, and lipids, and inactivate enzymes and transporters; they damage DNA and the transcriptional machinery [27,28], and initiate the chain reactions that peroxidize polyunsaturated fatty acids in membrane phospholipids [3,29].

It is well known that the vulnerability of organs to the toxic effects of xenobiotics and the incidence and severity of drug-induced toxicity in humans and other species vary with age [2,3,25], and knowledge of the factors that contribute to age-associated differences in sensitivity to toxic agents is important for predicting the effects of xenobiotics compounds on aged populations. Although age-related variations in the antioxidant defenses of the organism have been indicated to be the cause of increased susceptibility to drugs and diseases in advanced age [3,25,26,30], contradictory results regarding the age-related behavior of the antioxidant capacity and oxidative status of the rat liver have been reported [3,29,31–33]. By contrast, although oxidative stress seems to play an important role in CyA-induced liver injury, to date no information exists about the age-dependent variations that occur in the hepatic antioxidant defense systems of organisms subjected to CyA therapy, nor about the influence of age on the susceptibility and vulnerability of the liver to the toxic effect of the drug.

With this background, the present study was undertaken to investigate the influence of aging on the susceptibility to the toxic effects of CyA and the age-related changes in the oxidative status and antioxidant defense systems in rats after a short-term CyA treatment. Young, young-adult, mature, and senescent male Wistar rats were treated for 1 week with CyA or the CyA-vehicle, and the oxidative status in the liver (GSSG/GSH molar ratio and levels of TBARS) and antioxidant-related parameters (GSH and GSSG content, and antioxidant enzyme activities) were evaluated.

MATERIALS AND METHODS

Animals and treatment

Male Wistar rats were obtained from Charles River, Barcelona, Spain. Ages at the beginning of treatment with CyA or its vehicle were 1 month (young), 2 months (young-adult), 4 months (mature), and 24 months (senescent). Animals were kept on standard rat chow (Panlab, Barcelona, Spain) with free access to tap water, in temperature- and humidity-controlled animal quarters under a 12 h light-dark cycle. All experiments were performed in compliance with the indications of the *Guide to the Care and Use of Experimental Animals*, routinely used at our laboratory.

The animals were treated intraperitoneally once daily for 1 week either with CyA, at a dose of 10 mg/kg b.wt per day (CyA groups), or with the CyA vehicle—olive oil—(controls) for the same period. The rats were weighed daily and the volumes of the solutions administered were adjusted to between 0.20 and 0.30 ml, depending on their body weights.

Eleven hours after the last injection, the animals were anaesthetized with sodium pentobarbital (50 mg/kg b.wt, i.p.) and keep at a constant temperature of 37.0 ± 0.5°C. A routine tracheotomy and laparotomy were performed and the bile duct was cannulated for bile collection. After collecting two 15 min baseline bile samples for bile flow determination, the carotid artery was catheterized and one blood sample was taken, centrifugated, and the plasma stored until analysis. After this, the animals were exsanguinated and the liver was quickly washed in situ with ice-cold isotonic saline, removed, and weighed. Small pieces weighing 0.5 g were harvested from the liver and immediately frozen in liquid nitrogen. Experiments were carried out in a matched fashion and planned so that they could be initiated for all animals at the same time daily—0900 h—to avoid variations due to the circadian rhythm.

Tissue preparation and biochemical analysis

For the preparation of liver homogenates (1 g of tissue plus 10 ml homogenization buffer), the frozen pieces were thawed on ice and then homogenized. Total glutathione and glutathione disulfide concentrations in liver homogenates were evaluated by the enzymatic recycling procedure using glutathione reductase and 5,5′-dithio-bis (2-nitrobenzoic acid), according to Griffith [34]. The GSSG/GSH molar ratio was calculated as (GSSG/GSH) × 100 [35]. TBARS were determined in liver homogenates according to Ohkawa et al. [36], employing an extinction coefficient of 1.56×10^5 $l \cdot mol^{-1} \cdot cm^{-1}$ in the calculation of the resulting concentration of malon-

Table 1. Effects of Aging and Treatment with Cyclosporin A on Biliary and Plasma Indicators of Hepatic Dysfunction and Toxicity

	Age (months)				ANOVA	
	1	2	4	24		
Bile flow (μl/min/g liver)						
Control	1.86 ± 0.09	1.65 ± 0.05[a]	1.68 ± 0.05	1.64 ± 0.11	Ag:	$p < .05$
Cyclosporin	1.52 ± 0.18*	1.33 ± 0.07*	1.39 ± 0.06*	1.09 ± 0.07*[ABC]	T:	$p < .05$
					Ag-T:	n.s.
Plasma bile acids (μmol/l)						
Control	6.07 ± 0.68	4.35 ± 0.69	6.89 ± 1.12	8.22 ± 1.83	Ag:	n.s.
Cyclosporin	18.0 ± 3.2*	15.8 ± 3.5*	18.9 ± 2.3*	32.6 ± 10.1*	T:	$p < .05$
					Ag-T:	n.s.
Plasma bilirubin (mg/dl)						
Control	0.014 ± 0.004	0.011 ± 0.003	0.013 ± 0.004	0.020 ± 0.006	Ag:	$p < .05$
Cyclosporin	0.115 ± 0.024*	0.090 ± 0.004*	0.092 ± 0.022*	0.207 ± 0.015*[ABC]	T:	$p < .05$
					Ag-T:	$p < .05$
Plasma AST (IU/l)						
Control	82 ± 4	57 ± 7	61 ± 3	81 ± 7	Ag:	$p < .05$
Cyclosporin	82 ± 5	49 ± 6[A]	58 ± 7	111 ± 12*[ABC]	T:	n.s.
					Ag-T:	n.s.
Plasma ALT (IU/l)						
Control	35 ± 2	33 ± 3	21 ± 6	14 ± 4[ab]	Ag:	$p < .05$
Cyclosporin	41 ± 7	31 ± 2[A]	25 ± 8[A]	27 ± 3*[A]	T:	n.s.
					Ag-T:	n.s.

Values are means ± SEM for six to eight rats in each group. * Significantly different from control rats of the same age ($p < .05$); *a, b,* or *c*: significantly different from 1, 2, or 4 month old in control rats, and *A, B,* or *C*: significantly different from 1, 2, or 4 month old in rats treated with CyA, respectively ($p < .05$). ANOVA: Ag = effect of age; T = effect of CyA treatment; Ag-T = interaction of age and treatment; n.s. = not significant.

dialdehyde equivalents; the results were expressed as nmol per g of liver. Hepatic superoxide dismutase (EC 1.15.1.1, SOD) was assayed according to Misra and Fridovich [37] at 30°C. Catalase (EC 1.11.1.6, CAT) activity was determined by measuring the exponential disappearance of H_2O_2 at 240 nm; one unit of activity is equal to the μmol of H_2O_2 degraded.min^{-1}, as described by Aebi [38]. Glutathione peroxidase (EC 1.11.1.19, GPx) activity was assayed according to Flohe and Gunsler [39]. Hepatic γ-glutamylcysteine synthetase (EC 6.3.2.2, GCS) was determined as described by Sekura and Meister [40]. Glutathione S-transferase (EC 2.5.1.18, GST) activity was determined spectrophotometrically at 340 nm following formation of 1-chloro-2,4-dinitrobenzene-GSH conjugated [41]. Protein concentrations were assayed by the method of Lowry using bovine serum albumin as standard. Bile flow was determined gravimetrically, assuming a bile density of 1.0 g/ml. Total bile acid and bilirubin concentrations in plasma and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) plasma activities were evaluated as reported previously [42,43].

Statistical analysis

Results are expressed as means ± standard error of means (SEM) for all data. The effects of aging and CyA treatment on all parameters studied were tested for significance by two-way analysis of variance (ANOVA) followed by the Fisher PLSD test; *p* values of less than .05 were considered to be statistically significant.

RESULTS

Effects of CyA treatment on the main hepatic function indicators

With the aim of determining if our experimental protocol reflects the hepatotoxicity previously described in human under CyA therapy, the effects of CyA treatment on several selected indicators of cholestasis and hepatic dysfunction—bile flow, plasma bile acid, and bilirubin levels, and AST and ALT activities (Table 1)—were measured. The cholestatic effect induced by CyA in man and rat [4,12–17] was also observed in our experimental model because bile flow was significantly reduced in all treated animals; this effect was significantly higher in the oldest rats because bile flow was reduced by 18, 19, 17, and 34% in the 1, 2, 4, and 24 month old rats, respectively, with respect to their controls. Similarly, CyA treatment altered the most sensitive biochemical plasma indicators of hepatic dysfunction: increased the plasma concentration of bile acid and bilirubin in all treated rats; the CyA-induced increases were, however, higher in the senescent rats (297 and 935%, respectively) than in 1 month old treated rats (197 and 721%, respectively) (Table 1). In addition, plasma AST and ALT activities

Table 2. Effects of Aging and Treatment with Cyclosporin A on Rat Liver Concentrations of Total Glutathione and Glutathione Disulfide and on the Hepatic Activity of γ-glutamylcysteine Synthetase (GCS) and Glutathione-S-transferase (GST)

	Age (months)				ANOVA	
	1	2	4	24		
Total glutathione (μmol/g liver)						
Control	2.64 ± 0.18	3.76 ± 0.16^{a}	4.84 ± 0.15^{ab}	4.17 ± 0.24^{ac}	Ag:	$p < .05$
Cyclosporin	1.89 ± 0.18*	2.16 ± 0.16*	4.63 ± 0.25^{AB}	2.91 ± 0.33*^{ABC}	T:	$p < .05$
					Ag-T:	$p < .05$
Glutathione disulfide (nmol/g liver)						
Control	45 ± 9	51 ± 3	93 ± 9^{ab}	108 ± 22^{ab}	Ag:	$p < .05$
Cyclosporin	39 ± 3	42 ± 11	127 ± 26^{AB}	138 ± 18^{AB}	T:	n.s.
					Ag-T:	$p < .05$
GCS (nmol/min/mg protein)						
Control	30.4 ± 1.3	31.1 ± 1.5	39.3 ± 2.5^{ab}	32.9 ± 3.9	Ag:	$p < .05$
Cyclosporin	28.9 ± 1.4	34.1 ± 1.8	36.8 ± 2.1^{A}	27.3 ± 1.5^{C}	T:	n.s.
					Ag-T:	n.s.
GST (nmol/min/mg protein)						
Control	387 ± 16	352 ± 10	453 ± 10^{ab}	362 ± 21^{c}	Ag:	$p < .05$
Cyclosporin	342 ± 14*	315 ± 13	494 ± 37^{AB}	334 ± 16^{C}	T:	n.s.
					Ag-T:	n.s.

Values are means ± SEM for six to eight rats in each group. * Significantly different from control rats of the same age ($p < .05$); *a, b,* or *c*: significantly different from 1, 2, or 4 month old in control rats, and *A, B,* or *C*: significantly different from 1, 2, or 4 month old in rats treated with CyA, respectively ($p < .05$). ANOVA: Ag = effect of age; T = effect of CyA treatment; Ag-T = interaction of age and treatment; n.s. = not significant.

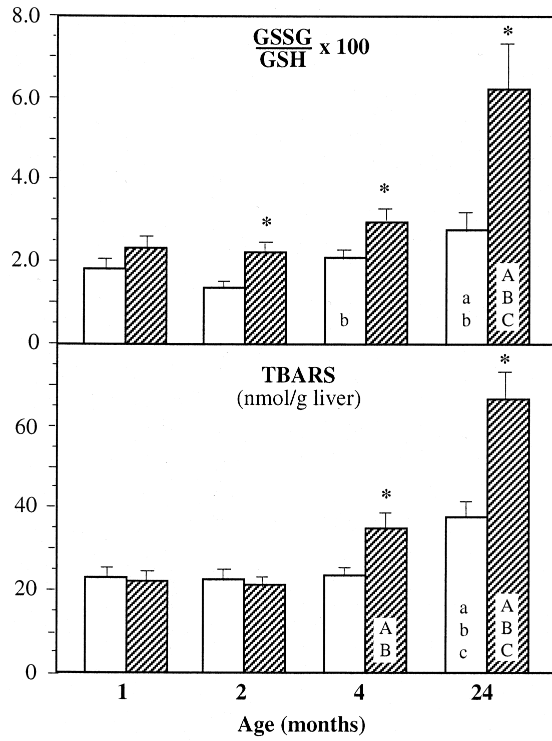
were increased by CyA treatment only in the senescent rats. The effects of age were found to be significant in all the above parameters, except for bile acid; the effects of treatment were significant for bile flow, bile acid, and bilirubin, and a significant interaction of age and treatment was only found for plasma bilirubin levels. These results confirm that CyA treatment for 1 week induces a moderate degree of cholestasis and hepatotoxicity in the rat.

Age-dependent changes and effects of cyclosporin A treatment on liver glutathione, the glutathione redox state, and lipid peroxidation

The liver concentrations of total glutathione and GSSG in the control and CyA-treated rats of different ages are shown in Table 2. In untreated rats, total and reduced glutathione increased with age until maturity, peaked at 4 months, and decreased in 24 month old rats, although the values were still higher than those of the young and young-adult rats. The time course of GSSG levels also showed a progressive increase with age, being 140% higher in the senescent than in the youngest rats. When CyA was administered for one week to 1, 2, and 24 month old rats, the liver contents of total glutathione decreased by about 30, 40, and 30%, respectively, but were unaffected in 4 month old rats. Although CyA reduced GSH and total glutathione, GSSG concentrations tended to increase, although not significantly, in both mature (+37%) and senescent (+28%) rats. The effects of age, CyA treatment and the interaction of age and treatment were found to be significant for total glutathione concentrations (Table 2).

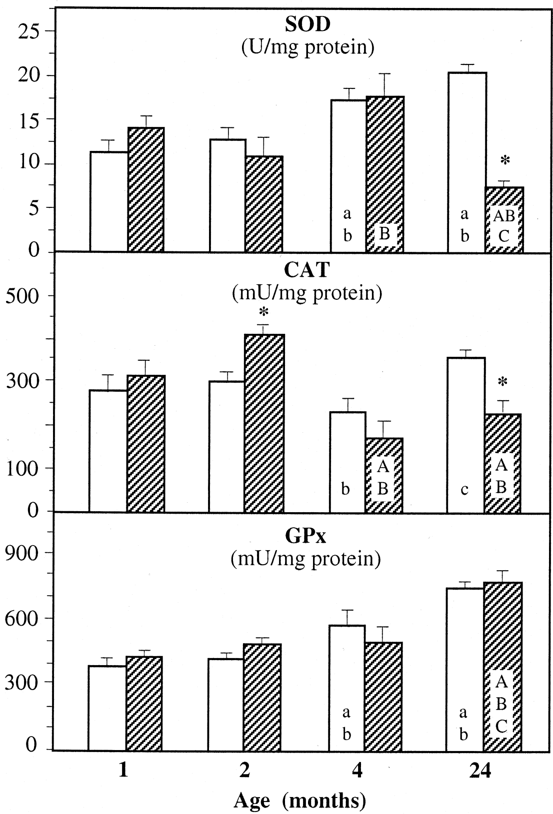
The variations with age of the values calculated for the GSSG/GSH molar ratio and TBARS levels in the livers of control rats, and the effect of CyA treatment on these parameters indicative of oxidative status and lipid peroxidation are depicted in Fig. 1. The GSSG/GSH ratio showed a progressive increase between 2 and 24 months of age in the control rat group, the values being about 50 and 100% higher in senescent than 1 and 2 month old animals, respectively. When rats were treated with CyA, the drug in 2, 4, and 24 month old animals significantly affected the GSSG/GSH ratio. The intensity of this effect was age-dependent, the values being increased by 23 and 36% at 2 and 4 months old, respectively, and by more than 100% in senescent rats. Although the effects of age and treatment were found to be significant, there was no significant interaction between both effects.

Regarding TBARS levels, no significant changes were detected in the period from 1 to 4 months in control rats, but these values were significantly increased (by about 60%) in senescent rats as compared with the youngest rats. TBARS levels in the livers of CyA-treated rats were significantly increased in mature and senescent rats (+45 and +75%, respectively) in comparison with the corresponding controls, but remained unaffected when the drug was given to 1 and 2 month old rats. The effects of age and treatment on liver TBARS levels, and the interaction between both factors were found to be significant.



ANOVA	GSSG/GSH	TBARS
Ag	$p < .05$	$p < .05$
T	$p < .05$	$p < .05$
Ag-T	n.s.	$p < .05$

Fig. 1. Effects of aging and treatment with cyclosporin A on the liver GSSG/GSH ratio and TBARS concentrations. Values are means ± SEM from six to eight animals. Control (▭) and CyA-treated (▨) rats. *Significantly different from control rats of the same age ($p < .05$); *a, b,* or *c*: significantly different from 1, 2, or 4 month old in control rats, and *A, B,* or *C*: significantly different from 1, 2 or 4 month old in rats treated with CyA, respectively ($p < .05$). ANOVA: Ag = effect of age; T = effect of CyA treatment; Ag-T = interaction of age and treatment.



ANOVA	SOD	CAT	GPx
Ag	$p < .05$	$p < .05$	$p < .05$
T	$p < .05$	n.s.	n.s.
Age-T	$p < .05$	$p < .05$	n.s.

Fig. 2. Effects of aging and treatment with cyclosporin A on the hepatic activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Values are means ± SEM from six to eight animals. Control (▭) and CyA-treated (▨) rats. *Significantly different from control rats of the same age ($p < .05$); *a, b,* or *c*: significantly different from 1, 2, or 4 month old in control rats, and *A, B,* or *C*: significantly different from 1, 2, or 4 month old in rats treated with CyA, respectively ($p < .05$). ANOVA: Ag = effect of age; T = effect of CyA treatment; Ag-T = interaction of age and treatment.

Age-dependent changes and effects of cyclosporin A treatment on antioxidant enzymes

The effects of aging and CyA treatment on the activities of liver SOD, CAT, and GPx were also assessed. The activities of GCS and GST—two enzymes that support the primary antioxidant enzymes by either supplying antioxidant substrates such as GSH, as is the case of GCS, or by reducing equivalents, as is the case of GST—were also evaluated.

Figure 2 illustrates the activity of SOD, CAT, and GPx in the control and CyA-treated rats. In untreated rats, SOD activity increased with age, mainly between the groups of animals of 1 and 4 months of age (+51%). When CyA was given to the oldest rats, SOD activity was significantly reduced (−65%, as compared with its controls), but slight, insignificant changes were noted when the drug was given to the 1, 2, and 4 month old rats. Analysis of variance revealed significance for the effects of age, treatment, and the interaction between age and treatment. Regarding CAT activity in untreated rats, we observed a significant decrease (−30%) between the 2 and 4 month old rats, followed by an increase (+71%), maximum activity being reached at 24 months old. After CyA treatment, CAT activity was significantly increased (+35%) in young-adult rats and significantly reduced (−38%) in senescent treated rats in comparison with untreated animals of the same age. The effect of age

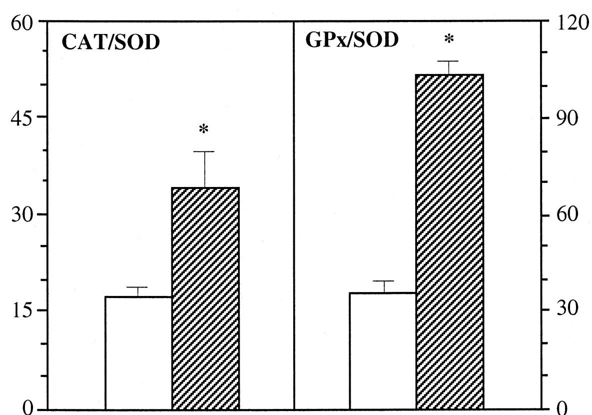


Fig. 3. Effects of treatment with cyclosporin A on hepatic catalase/superoxide dismutase (CAT/SOD) and glutathione peroxidase/superoxide dismutase (GPx/SOD) ratios in senescent rats. Values are means ± SEM from six to eight animals. Control (□) and CyA-treated (▨) rats. *Significantly different from control rats of the same age ($p < .05$).

and the interaction of age and treatment were found to be significant. Hepatic GPx activity and the GPx/SOD ratio (data not shown) increased with age, suggesting enhanced reliance on GPx in overall antioxidant defense. GPx activity was 1.6- and 2.0-fold higher, respectively, in 4 and 24 month old than in 1 month old rats. Treatment with CyA did not modify GPx activity in any of the age groups. Only the effect of age proved to be significant. A comparison of the enzyme activity ratios in the oldest control and CyA-treated rats is offered in Fig. 3. The CAT/SOD ratio, which was significantly reduced with aging (data not shown), was markedly increased (+82%) when senescent rats were treated with CyA. In the case of GPx/SOD, aging did not significantly alter the mean ratio values (data not shown), but CyA treatment markedly increased (+200%) this ratio in the oldest rats, as compared with the untreated senescent rats.

Age- and CyA-related changes in hepatic GCS and GST activities are shown in Table 2. The main noticeable events observed in the activity of both enzymes were a significant peak in their activity in mature control rats and a remarkable absence of the effects of CyA treatment in the activity of both enzymes, except for GST in 1 month old rats. The effect of age was significant for both enzymes.

In sum, even when the activity of antioxidant enzymes tended to increase, senescence was associated with a decrease in the liver content of reduced glutathione and an enhancement of GSSG and lipid peroxidation. After short-term CyA treatment, oxidative stress, lipoperoxidation, and glutathione depletion was higher, and increases in SOD and CAT activities lower in older than in younger rats.

DISCUSSION

The senescent liver has a number of characteristics consistent with oxidative injury and many studies have examined the effect of aging on the oxidative status in mammalian tissues. In rat liver, aging has been associated with enhanced ROS generation and oxidative stress [1,3,29,30,44]. The large increases in both GSSG and TBARS concentrations and in the GSSG/GSH molar ratio observed by us in the livers of 24 month old control rats, as compared with young rats, are in favor of a strong oxidative stress and enhanced ROS formation in senescent rats. Similar increases in liver TBARS have been previously reported [29,44–46]. Nevertheless, it has also been reported that liver TBARS contents decrease in female rats of different strains and that they are unaffected in old male rats and mice [3,31,47], suggesting that increased lipoperoxidation in the liver is not an inevitable consequence of aging.

Regarding antioxidant defenses, our data show that the hepatic concentration of total and reduced glutathione progressively increased from youth to maturity, peaking at 4 months, and thereafter declining in 24 month old rats. By contrast, GSSG content increased lineally and was 2.4-fold higher in the older than in the youngest rats. These changes in liver GSH levels are in agreement with previous studies [30,44,46], although some authors have found no changes with age [3,32,48]. Similar contradictory results have been reported for GSSG in the rat [3,27,45,48] and man [28,48]. Thus, the oxidative stress observed in our aged animals could be related not only to the marked increase in the GSSG/GSH molar ratio but also to the decline in GSH levels [49]. Increased generation of ROS and lipid peroxides has been reported following administration of GSH-depleting agents and after a reduction in liver GSH levels [35,46]. In contrast to liver GSH contents, antioxidant enzyme activities tended to increase with age. These results agree with those observed by other authors [30, 32,45]. The age-associated increases in antioxidant enzymes may be an attempt by the organism to counterbalance the decreases in the reducing power of the liver mediated by GSH. However, in senescent animals, the total antioxidant capacity of liver cells should not be sufficient to scavenge the ROS generated. Although according to the currently available literature it is unclear what causes such discrepancies, the inconsistent results obtained in different studies may reflect variations in species, strain, sex, and experimental design. In addition, chow diets that contain a variety of substances, often including fish products with unknown amounts of oxidized lipids, together with the use of non-pathogen-free animals [3] can also contribute to explaining such discrepancies in antioxidant hepatic defenses.

Regarding the effects of CyA treatment, our data clearly show a hepatotoxic/cholestatic effect because the drug alters three of the main sensitive liver function indicators, that is, bile flow and plasma levels of bile acids and bilirubin; these results are in agreement with those previously reported in this experimental model [12,13,42,43], and with other authors that have shown a CyA-induced cholestatic syndrome in man [4,13–17], having been consistently found that CyA therapy increases the plasma levels of bile acid and bilirubin in heart [15,50], kidney [16,51], and bone marrow [52] allograft recipients, and reduces bile flow [14,50] and the capacity of the liver to excrete endo- and xenobiotic into bile [13,14,50,53]. The largest increases in the plasma levels of bile acid and bilirubin induced by the treatment in the oldest rats, as well as the pronounced falls in bile flow observed in these animals suggest that the senescent rat liver is more susceptible to the hepatotoxic effect of CyA. On the other hand, previous studies have reported oxidative stress in the livers of 2 month old CyA-treated rats [24] and patients [11] and in microsomes from the livers or kidneys of humans [10], young rats [20], and rabbits [21] treated with CyA. Similar effects have been found in in vitro conditions using perfused rat liver [5] or rabbit kidney [22]. In agreement with these results, we observed that CyA sensitively affected the liver GSSG/GSH ratio in all rat groups. However, in addition, CyA treatment induced oxidative stress to a greater extent in aged than in young livers. On the one hand, this was because the CyA-induced increases in the GSSG/GSH ratio were significantly higher in the older (+112%) than in the younger (+25%) rats and, on the other, because liver TBARS concentrations, which were unchanged by treatment in 1 and 2 month old rats, significantly increased in 4 and, above all, in 24 month old rats. Similar negative effects of age on susceptibility to chemically induced hepatotoxicity and situations in which oxidative stress, ROS formation, and impaired cell antioxidant defense systems are involved, have been reported [1–3, 25,54].

The causes and mechanisms underlying age-related changes in CyA-induced oxidative stress cannot be fully identified from our results, but both increased ROS production and a decline in cellular antioxidant mechanisms may be important. In this regard, a rapid increase in oxidative stress has been found within hours of exposure of isolated hepatocytes to bile acids [55], and a similar finding has been observed in rats following bile duct ligation [56] and in patients with biliary obstruction [57]. CyA-induced cholestasis is due, at least in part, to inhibition of the hepatobiliary excretion of bile acids [4,7–9]; the cholestatic effect of CyA is higher in aged than in young rats, and plasma and liver bile acid levels are enhanced under CyA treatment [7,58]. Thus, accumulation of bile acids in the liver might lead to increased ROS production in CyA-treated senescent rats because bile acids reduce the activity of the electron transport chain in rat liver mitochondria [59], and it is well established that mitochondrial ROS production markedly increases when electron transport is impaired [60]. Uncoupling electron flow could also arise as a consequence of the accumulation of toxic CyA molecules in the liver due to decreased metabolism and elimination. CyA has been shown to alter microsomal NADH and NADPH oxidizing systems and mitochondrial cytochrome oxidase to a dramatic extent [61]. A higher accumulation of nonmetabolized CyA molecules in senescent liver should be considered because Kamataki et al. [62] have found that liver cytochrome P-450 levels decrease with age and become very low in male Wistar rats of 24 months of age, and bile secretion rates—and hence CyA elimination—are lower in aged than in younger rats [58].

Regarding antioxidant defenses, a higher GSH depletion was also present in senescent treated rats (−30% vs. −4% in 4 month old) and this would also lead to a higher susceptibility to the toxic effects of CyA [25,48], and to enhancing the susceptibility to mitochondrial dysfunction from oxidative stress [28,48]. Our data on antioxidant enzymes clearly show that SOD activity was not altered by CyA treatment in young, young-adult, or mature rats, whereas it was markedly inhibited when the drug was given to 24 month old rats. This coincides with previous observations from kidney transplant patients receiving CyA [11]. Similar changes were also observed for CAT after CyA treatment in senescent rats. Different authors [63] have proposed that the ratios between the activities of antioxidant enzymes could be considered as indices of the oxidative status; when we calculated the CAT/SOD activities and GPx/SOD ratios in 24 month old rats we observed that CyA treatment induced significant increases in both enzyme ratios, indicating that superoxide is not efficiently converted to hydrogen peroxide and then into water; that is, enzymes do not scavenge ROS efficiently.

The lower activity of SOD and CAT in aged rats could be a consequence of inhibitory effects due to excess of ROS generation [29,64,65]. SOD is inhibited by hydrogen peroxide [64], and CAT by an excess of superoxide radical [65], and CyA enhances formation of ROS, in turn [18,19]. A similar phenomenon has previously been reported to occur in isolated peroxisomes from rat liver [61] and in the rabbit kidney [22] after CyA treatment, as well as under conditions of oxidative stress induced by chronic ethanol ingestion [66] or liver parasitism [63]. Inhibition of the catalytic activities of proteins that express SOD and CAT activities could also be a consequence of the stronger CyA-induced liver GSH depletion in old animals. It has been shown that CyA

reduces the content of protein sulfhydryl groups and causes protein thiol oxidation in rat liver cells [18], probably due to the CyA-induced GSH depletion, a causative mechanism proposed for the inhibition observed in other enzymes [35]. CyA might also reduce the protein levels of antioxidant enzymes, because a marked inhibitory effect of the drug on the synthesis of protein in the rat liver has been demonstrated [4,61]. This hypothesis is reinforced by our previous data on liver protein contents [8] and is in keeping with the data obtained from a rat model of alcoholic liver injury [67], in which oxidative stress and enhanced TBARS formation were accompanied by decreased protein levels of SOD and CAT.

Importantly, the CyA-induced decreases in the enzymatic and nonenzymatic antioxidant defense systems in the oldest rats makes them more susceptible to oxidative damage, and compromise the antioxidant capacity of the liver to adequately scavenge the ROS generated during CyA therapy.

In sum, our data indicate that either glutathione depletion or disturbances in the glutathione redox status appear in all CyA-treated rats, but the marked toxic effect of short-term treatment is a relatively late age-associated event since lipoperoxidation appeared in 4 and 24, but not in 1 or 2 month old treated rats. In addition, we report the novel finding that the senescent liver is more susceptible to CyA-induced oxidative stress and lipoperoxidative injury, and that both enhanced GSH depletion and decreased antioxidant enzyme activities may contribute to this effect. If a higher susceptibility to CyA is also demonstrated in aged humans, treatments should include drugs with the capacity to inhibit ROS formation or to increase antioxidant defenses in order to avoid not only CyA-induced toxicity but also impairment in the detoxification of other drugs jointly administered with this immunosuppressor.

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ABBREVIATIONS

ALT—alanine aminotransferase
AST—aspartate aminotransferase
CAT—catalase
CyA—cyclosporin A
GCS—γ-glutamylcysteine synthetase
GPx—glutathione peroxidase
GSH—reduced glutathione
GSSG—glutathione disulfide
GST—glutathione S-transferase
ROS—reactive oxygen species
SOD—superoxide dismutase
TBARS—thiobarbituric acid-reactive substances