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Decreased Synthetic Capacity Underlies the Age-Associated Decline in Glutathione Content in Fisher 344 Rats

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ABSTRACT

Although it is well documented that the concentration of glutathione (GSH), the most abundant intracellular free thiol and an important antioxidant, declines with age in many tissues of different animal species, the underlying mechanism is not well understood. In a previous study, we showed that the expression of the glutamate cysteine ligase genes was down-regulated with age, accompanied by a decline in GSH content in the liver, kidney, and lung of Fisher 344 rats. The aim of this study was to examine the age-associated changes in the activities of three other enzymes, which also play important roles in GSH biosynthesis, to further explore the mechanism underlying the age-associated decline in GSH content in Fisher 344 rats. The results showed for the first time that the activity and gene expression of glutathione synthase, which catalyzes the second reaction in *de novo* GSH synthesis, were also decreased with increased age in the lung and kidney, but not in the liver or heart. No age-associated change in the activity of either γ-glutamyltranspeptidase or glutathione reductase was observed in any of the organs examined. The results further indicate that decreased GSH synthetic capacity is responsible for the age-associated decline in GSH content in Fisher 344 rats. *Antioxid. Redox Signal.* 5, 529–536.

INTRODUCTION

THE MECHANISM UNDERLYING AGING, an inevitable biological process that affects most living organisms, and the age-related diseases is still an area of significant controversy. A large body of evidence indicates that oxidative damage of macromolecules increases while the antioxidant defense capacity including the concentration of glutathione (GSH), the most abundant intracellular nonprotein thiol, decreases with age and also in some age-related diseases. Therefore, it has been suggested that the decreased cellular antioxidant defense capacity permits the observed increase in oxidative damage, which, in turn, contributes to the aging process and the pathogenesis of many age-related diseases. Consequently, uncovering the mechanism underlying the decrease in the GSH content during the aging process will be very important, not only for understanding the aging process, but perhaps for revealing the etiology of age-related diseases as well.

There are several mechanisms by which cells maintain their intracellular GSH content, including GSH redox cycling, direct uptake, and *de novo* synthesis. GSH redox cycling, catalyzed by glutathione reductase (GR), prevents the loss of GSH in the form of glutathione disulfide (GSSG) that is generated during the reduction of various oxidants with GSH, by reducing GSSG back to GSH. A few types of cells, such as Hep G2 and intestinal mucosa, can directly take up intact GSH from the surrounding extracellular fluid (2, 39). Most cells, however, depend on *de novo* synthesis to maintain their intracellular GSH content under both normal and oxidative stress conditions. *De novo* GSH synthesis is a two-step process. The first step is catalyzed by glutamate cysteine ligase (GCL), whereas the second step is catalyzed by glutathione synthase (GS). Although GCL is considered as the rate-limiting enzyme in *de novo* GSH synthesis, increased evidence indicates that GS may also play an important role in maintaining GSH homeostasis under certain conditions (3, 4, 7, 20, 33, 34). In-

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dividuals with inherited one recessive, mutant allele for GS have a lower GSH level in their erythrocytes and show a variety of disorders, including metabolic acidosis, hemolytic anemia, 5-oxoprolinuria, and mental retardation (3, 4, 33, 34). In addition to GCL, the availability of cysteine is considered to be another rate-limiting factor controlling *de novo* GSH synthesis (21). Although other sulfur-containing amino acids such as methionine can be used in the cystathionine pathway to produce cysteine in some tissues/organs such as liver, the cysteine required for GSH synthesis is actually obtained by most cells through degradation of circulating GSH, catalyzed by a membrane bound enzyme, γ-glutamyltranspeptidase (GGT). Therefore, GGT also plays an important role in maintaining intracellular GSH homeostasis for most cells (11, 16, 18, 30).

In a previous study, we showed that GCL activity, as well as the expression of both GCL catalytic and regulatory subunits, was decreased with age in several visceral organs of Fisher 344 rats, accompanied by a decrease in GSH content (14). To explore further the potential mechanism underlying the age-associated decline in GSH content, we examined the age-associated changes in the activities of GS, GR, and GGT, three other enzymes that also play important roles in GSH metabolism. The results showed that GS activity and mRNA content were decreased in the lung and kidney but not in the liver or heart of old rats as compared with young rats. No age-associated change in the activity of GGT or GR was observed in any of the organs examined. The results suggest that GS may play an important role in maintaining GSH homeostasis for certain organs, and further support the hypothesis that decreased synthetic capacity underlies the age-associated decline in GSH content in Fisher 344 rats.

MATERIALS AND METHODS

Animals

Young (3 months), adult (12 months), and aged (24 months) Fisher 344 male rats were purchased from National Institute of Aging (NIA, Harlan Sprague Dawley, Indianapolis, IN, U.S.A) and were acclimated for 1 week in our animal facilities before initiation of our experiments. The animals were maintained on a 12-h light/dark cycle at 22°C, and water and food were provided *ad libitum*. The rats were killed by intraperitoneal injection of phenobarbital, followed by decapitation, between 10:00 a.m. and 12:00 p.m. Visceral organs were removed and immediately frozen in liquid nitrogen. Samples for GSH measurement were washed twice in cold phosphate-buffered saline (PBS) and sonicated immediately in 10% perchloric acid 2 m*M* EDTA solution. All procedures involving animals were approved by the Institutional Animal Care and Use Committees at the University of Alabama at Birmingham.

Reagents

TRIzol reagent, an RNA isolation solution, was from Life Science Technologies (Grand Island, NY, U.S.A.). QuickHyb solution and salmon sperm DNA were from Stratagene (La Jolla, CA, U.S.A.). γ-Glutamylcysteine was purchased from Bachem (Torrance, CA, U.S.A.). Microcon-10 columns were from Amicon Co. (Beverly, MA, U.S.A.). All HPLC solvents were Baker Analyzed HPLC-grade reagents from VWR Scientific (San Diego, CA, U.S.A.). All chemicals used were at least analytical grade.

Measurement of GSH, GSSG, and cysteine content in rat tissues

The concentrations of tissue GSH, GSSG, and cysteine were determined using a well established HPLC method as described previously (17). In brief, the tissues were cut and washed with cold PBS twice, then sonicated in 10% perchloric acid/2 m*M* EDTA containing 7.5 nmol of γ-glutamyl-glutamic acid, which was used as an internal standard. After centrifugation, 450 µl of supernatant was derivatized and the products were analyzed by HPLC. Standards were run under the same conditions, and the peak areas were measured. GSH, GSSG, and cysteine contents were calculated based on the internal standard and standard curves.

Measurement of GS activity

The activity of GS was measured by an HPLC assay as described previously (7). In brief, the tissues were homogenized in 0.25 *M* sucrose buffer containing 2 µg/ml leupeptin and aprotinin, as well as 50 µg/ml phenylmethylsulfonyl fluoride. The homogenates were then centrifuged at 3,000 *g* for 10 min, and the supernatant was centrifuged at 10,000 *g* for 20 min and then at 105,000 *g* for 30 min at 4°C. To remove endogenous inhibitors such as GSH, acceptors, and amino acids, the supernatants were centrifuged in Microcon-10 (Amicon) columns for 20–30 min at 4°C at 12,000 rpm and washed twice with 0.3 ml of lysis solution (0.1 *M* Tris-HCl, pH 8.2, 150 m*M* KCl, 20 m*M* $MgCl_2$, and 2 m*M* EDTA). GS activity was measured using the reaction mixture containing 5 m*M* dithiothreitol, 10 m*M* ATP, 0.1 *M* Tris-HCl, pH 8.2, 150 m*M* KCl, 20 m*M* $MgCl_2$, 2 m*M* EDTA, 3 m*M* γ-glutamylcysteine, 20 m*M* glycine, and 0.04 mg/ml acivicin. After incubation at 37°C for 30 min, the concentration of product GSH was measured by HPLC. The baseline was measured for each sample by adding enzyme after addition of the stop solution and was subtracted. The results were calculated using standard curves generated with a known amount of GSH. Protein concentration was determined by the bicinchoninic acid method (37), and GS activity was reported as nanomoles of product formed per milligram of protein per minute.

Measurement of GGT activity

GGT activity was measured as described before (8). In brief, the tissues were washed and sonicated in 1 × PBS buffer. GGT activity was measured using the fluorescent substrate γ-glutamyl-7-amino-4-methylcoumarin. The specificity of this assay was confirmed using acivicin, a specific inhibitor of GGT. The results were expressed as nanomoles of product formed per milligram of protein per minute.

Analysis of GR activity

GR activity was determined by a spectrophotometry assay as described by Sies and Akerboom (35). In brief, the cytosol

was collected after ultracentrifugation, and the enzyme activity was measured by continually monitoring NADPH consumption at 340 nm. The activity was expressed as nanomoles of product formed per milligram of protein per minute.

Northern hybridization analysis of GS mRNA

Tissues were briefly washed with cold PBS, and total RNA was extracted with TRIzol Reagent (Life Science Technologies) according to the protocol provided by the manufacturer. GS mRNA content was determined by northern hybridization analysis as described previously (7). After hybridization, the membranes were scanned and the radioactivity was quantified by electronic autoradiography using an InstantImager (Packard Instrument Co., Meriden, CT, U.S.A.).

Statistics

Data were expressed as means ± SEM and evaluated by one-way ANOVA. Statistical significance was determined by Fisher LSD test. $p < 0.05$ was considered significant.

RESULTS

GSH content was decreased with increased age in several visceral organs

GSH, GSSG, and cysteine concentrations in several visceral organs of young (3 months), adult (12 months), and old (24 months) rats were measured by HPLC. It was found that the GSH content in liver, kidney, and lung was significantly decreased with increased age, whereas there was no age-associated change in GSH content in heart. GSSG content also showed an age-dependent decline in the liver, heart, and kidney. However, the differences were not significant except in the liver, in which a significant decrease was observed in 12-month-old rats as compared with 3-month-old rats. The total GSH content was decreased by 33 and 40% in the liver, 20 and 23% in the kidney of 12- and 24-month-old rats, respectively, and 30% in the lung of 24-month-old rats, as compared with 3-month old rats (Table 1). There was no significant age-associated change in either ratios of GSSG to GSH or the cysteine content in any tissues/organs examined (data not shown).

GS activity was decreased with increased age in the lung and kidney

To explore further the potential mechanism underlying the age-associated decline in GSH content in these organs, we examined age-related change in GS activity. It was found that GS activity was significantly decreased with increased age in the lung and kidney. However, no age-associated change in GS activity was observed in the liver or heart. GS activity decreased by 70 and 60% in the lung and by 39 and 40% in the kidney of 12- and 24-month-old rats, respectively, as compared with 3-month-old rats (Fig. 1). These results further suggest that decreased GSH synthetic capacity underlies the age-associated decline in GSH content in Fisher 344 rats.

GS mRNA content was decreased with age in rat lung and kidney

The mechanism underlying the age-associated decrease in GS activity in the lung and kidney was further examined by northern hybridization analysis to determine whether the age-dependent decrease in GS activity was due to a down-regulation of GS gene expression. It was found that the amount of GS mRNA was significantly decreased in both lung and kidney of 24-month-old rats as compared with 3-month-old rats (Fig. 2). These data suggest that the age-dependent decrease in GS activity was due, at least in part, to a down-regulation of GS gene expression.

GGT or GR activity did not change with age in rat tissues

In addition to GCL and GS, GGT and GR also play important roles in maintaining intracellular GSH homeostasis. Therefore, we examined the activities of these two enzymes for age-associated changes to elucidate further the potential mechanism underlying the age-associated decline in GSH content in these

TABLE 1. AGE-ASSOCIATED CHANGE IN THE GSH CONTENT IN THE TISSUES OF FISHER 344 RATS

Tissues	*Age*	*GSH*	*GSSG*	*Total*	*GSSG/GSH+GSSG*
Liver	3 months	22.26 ± 2.71 (5)	3.01 ± 0.58 (5)	28.27 ± 3.51 (5)	0.105 ± 0.011 (5)
	12 months	15.48 ± 2.10 (5)*	1.77 ± 0.21 (5)	19.03 ± 2.50 (5)*	0.094 ± 0.003 (5)
	24 months	13.57 ± 1.55 (5)*	1.71 ± 0.07 (5)*	16.99 ± 1.47 (5)*	0.103 ± 0.009 (5)
Heart	3 months	1.99 ± 0.28 (5)	0.71 ± 0.17 (5)	3.41 ± 0.55 (5)	0.203 ± 0.020 (5)
	12 months	2.25 ± 0.49 (5)	0.62 ± 0.04 (5)	3.49 ± 0.55 (5)	0.193 ± 0.028 (5)
	24 months	3.07 ± 0.55 (5)	0.54 ± 0.03 (5)	4.15 ± 0.56 (5)	0.138 ± 0.021 (5)
Lung	3 months	6.30 ± 1.08 (5)	0.92 ± 0.17 (5)	8.14 ± 1.26 (5)	0.116 ± 0.020 (5)
	12 months	3.27 ± 0.48 (5)*	2.15 ± 0.66 (5)	7.87 ± 1.26 (5)	0.254 ± 0.044 (5)
	24 months	3.52 ± 0.53(5)*	1.09 ± 0.30 (5)	5.70 ± 0.11 (5)*	0.189 ± 0.051 (5)
Kidney	3 months	7.71 ± 0.33 (5)	0.31 ± 0.03 (5)	8.32 ± 0.37 (5)	0.037 ± 0.002 (5)
	12 months	6.09 ± 0.21 (5)*	0.26 ± 0.03 (5)	6.62 ± 0.25 (5)*	0.040 ± 0.003 (5)
	24 months	5.88 ± 0.75 (5)*	0.25 ± 0.05 (5)	6.37 ± 0.78 (5)*	0.039 ± 0.007 (5)

GSH content is expressed in nmol/mg of protein ± SEM (n).
*Significantly different from 3-month-old group ($p < 0.05$).

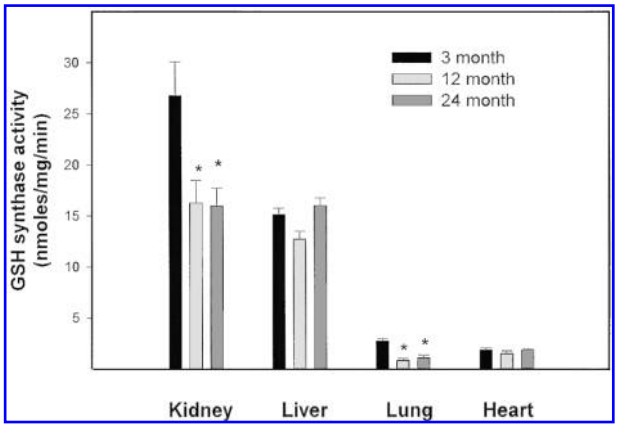

FIG. 1. Age-associated decline in GS activity in Fisher 344 rat tissues. GS activity was determined using an HPLC assay as described in Materials and Methods. Values represent means ± SEM of five samples. *Significantly different from 3-month-old rats ($p < 0.05$).

organs. The results showed that there was no significant age-associated change in GGT activity in any of the organs examined, although a slight, but not significant ($p = 0.073$, $n = 8$), decrease was observed in the kidney. GR activity, on the other hand, was increased in the lung tissue during the maturation period (3-month-old rats versus 12-month-old rats) and then decreased slightly, but not significantly, during the aging process. There was no significant age-associated change in GR activity in either kidney or liver (Fig. 3). These data indicated that neither the decreased availability of cysteine nor decreased GR activity was responsible for the age-associated decline in GSH content in Fisher 344 rats.

DISCUSSION

Generally, a decrease in intracellular GSH content can be caused by (a) increased consumption, due to exposure to massive oxidants or decreased GR activity, which leads to a reduction of redox cycling of oxidized glutathione and therefore an increased loss of glutathione in GSSG form, and/or (b) decreased GSH synthesis, due to decreased availability of substrate and/or the activities of the enzymes involved in this process. Although many studies have shown that GSH content decreases with age, the underlying mechanism has not been completely elucidated. In previous studies, we showed that the age-associated decline in GSH content in several visceral organs as well as in the brain of Fisher 344 rats was accompanied by a decline in GCL gene expression and enzyme activity (13, 14). In this study, we further demonstrated that GS activity and gene expression were also decreased with increased age in the kidney and lung, whereas the activity of GR or GGT was not changed in any of the organs examined during the aging process. The results further suggest that decreased GSH

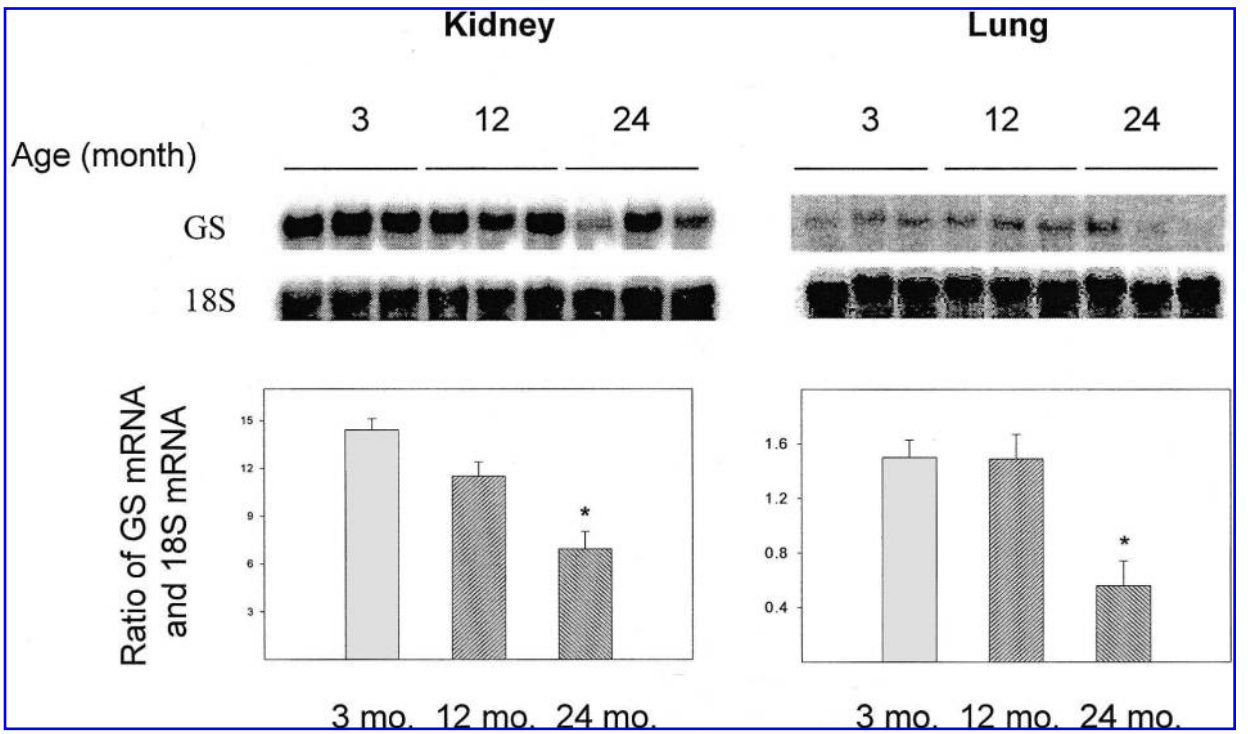

FIG. 2. Age-associated decline in GS mRNA content in the kidney and lung of Fisher 344 rats. GS mRNA content in the kidney and lung was determined by northern hybridization as described in Materials and Methods. Hybridization with an 18S cDNA probe was performed to show even sample loading and to normalize GS signal. Radioactivity was quantified by an InstantImager after hybridization. Values represent means ± SEM of three samples. *Significantly different from 3-month-old rats ($p < 0.05$).

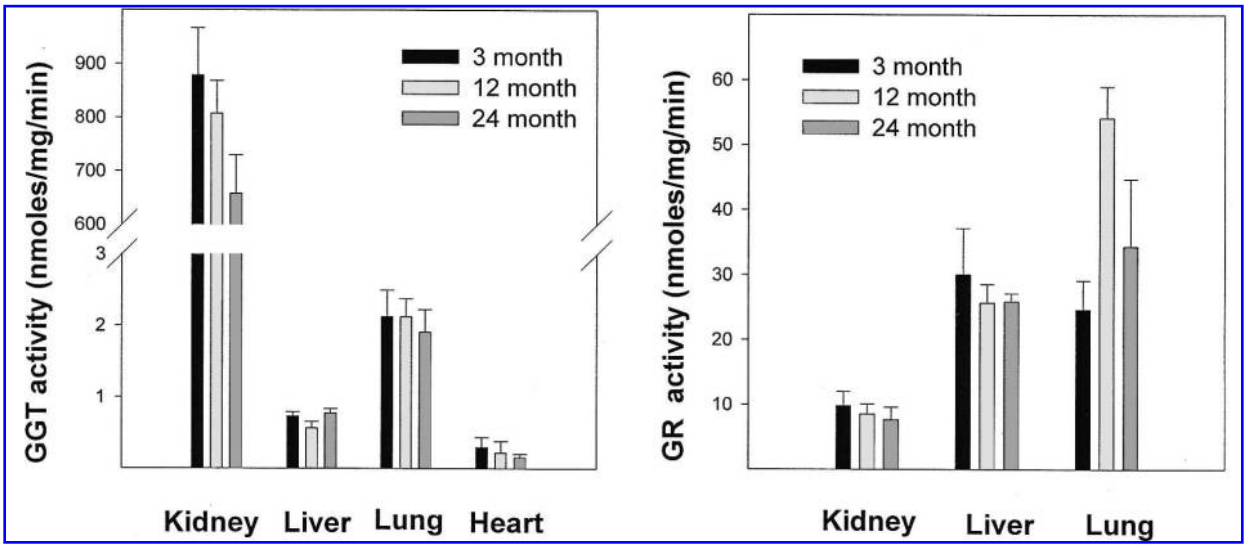

FIG. 3. GGT or GR activity was not significantly decreased with age in rat tissues. GGT and GR activities were determined as described in Materials and Methods. Values represent means ± SEM of five to eight samples.

synthetic capacity is responsible, at least in part, for the age-associated decline in GSH content in Fisher 344 rats.

Exposure to high concentrations of oxidants or electrophiles can lead to a rapid depletion of GSH due to increased consumption of GSH in glutathione peroxidase- and glutathione transferase-catalyzed reactions. However, a long-term low-level oxidative stress, which is most likely the case in the aging process, usually increases rather than decreases intracellular GSH as it can induce the expression of the enzymes involved in GSH synthesis (15, 17, 19, 23, 24). Nevertheless, whether increased oxidative stress is responsible for the age-associated decline in GSH content remains controversial. In the present study, no age-dependent increase in GSSG content, an indicator of oxidative stress, was observed in any organs that experienced an age-associated decline in GSH content. In fact, the GSSG content in the liver, heart, and kidney showed a trend of decrease with age although the differences were not statistically significant. Consistent with our observation, other studies have also showed that decreased GSH content in aged animals was not accompanied by an increase in GSSG content (5, 26, 29, 45). Nevertheless, the results from this study do not support a role of increased oxidative stress in the age-associated decline in GSH content in Fisher 344 rats.

GSH content may also decline when the activity of GR decreases even though the generation of reactive oxygen species is not increased. Although it has been reported that the GR activity decreased with age in some organs/tissues (10, 22, 40), clearly this is not a universal phenomenon as other studies have indicated that GR activity did not change, or even increased, with age (12, 13, 31, 32). In this study, we did not observe any age-dependent decline in GR activity in any of the organs examined, suggesting that the age-associated decline in GSH content in Fisher 344 rats is not due to decreased GR activity. The mechanism underlying tissue/organ and species-specific change in GR activity during the aging process remains to be further explored.

Another potential mechanism underlying the decrease in GSH content in aged animals is a reduction in the rate of GSH synthesis. The availability of the substrate cysteine is considered to be one of the rate-limiting factors controlling GSH synthesis. Cysteine is toxic to cells and cystine has a very low solubility in the plasma. Circulating GSH, therefore, serves as an important source of cysteine for cells that express GGT, a membrane-bound enzyme with its catalytic site toward the outside of cells. Although a few studies have shown that the age-dependent decrease in GSH content was associated with a decrease in the uptake of cysteine in some tissues/organs, many other studies indicated that there was no age-associated change in cysteine concentration and/or GGT activity (5, 13, 25, 26, 45). The results from this study suggest that the age-associated decrease in GSH content in Fisher 344 rats does not arise from cysteine deficiency as neither GGT activity nor cysteine concentration changed with age in any of tissues/organs examined.

De novo GSH synthesis is a two-step reaction catalyzed by GCL and GS, sequentially. Although it has been well documented that GCL has the predominant role in maintaining intracellular GSH homeostasis under both physiological and pathological conditions (6, 13–15), increased evidence has shown that GS activity can be regulated (38, 43) and may also play an important role in regulating intracellular GSH level under certain conditions (3, 4, 7, 20, 33, 34). An age-associated decrease in GS activity has been reported in the lens of human and monkeys, accompanied by a decline in GSH content (27, 28). In this study, we showed that GS activity and gene expression were also decreased with age in the lung and kidney of Fisher 344 rats. However, no age-associated change in GS activity was observed in the liver and heart, nor in the brain (13), suggesting that down-regulation of GS gene expression may contribute to the age-associated decline in GSH content in certain tissues/organs, but it is not a universal mechanism. The mechanism underlying the disassociated regulation of GS

gene expression between different tissues/organs during the aging process remains to be further explored.

Although the gene structure of mammal GS was revealed in the early 1990s, little is known about the regulation of GS gene expression under either physiological or pathological conditions. Recent studies have shown that agents that induced GCL gene expression, such as diethyl maleate, buthionine sulfoximine, and *tert*-butylhydroquinone, also increased GS gene expression (9, 46). Sequence analysis revealed that there are multiple transcription factor activator protein 1 (AP-1) binding sites in the promoters of both rat GS and GCL genes, and increased AP-1 binding is associated with the induction of both GS and GCL by *tert*-butylhydroquinone (46). Therefore, it was proposed that GS may be regulated together with GCL and that AP-1 is responsible for the induction of GS by oxidants (46). In this study, however, we found that the age-dependent down-regulation of GS gene expression in Fisher 344 rats was independent of that of GCL as the expression of GCL was significantly decreased, whereas GS activity was not changed with age in the liver and brain (13). Interestingly, it was reported that aging is associated with decreased AP-1 binding activity in various tissues and cells (1, 36, 41, 42). Whether the down-regulation of GS gene expression in the lung and kidney of old rats is due to decreased AP-1 activity remains to be further explored.

It was also noticed that whereas GS activity decreased in the lung and kidney of 12-month-old rats as compared with 3-month-old rats, no decrease in GS mRNA content was detected until 24 months of age. The mechanism underlying such a dissociated regulation of GS mRNA and activity is not clear at this moment. Xiang and Oliver (44) reported that jasmonic acid increased GS mRNA but not GSH content in plants, suggesting that the expression of this gene is controlled at both transcriptional and posttranscriptional levels. It is possible that the rate of transcription of the GS gene is notchanged but the translation machinery starts to decline in the middle of age, which leads to disassociated regulation of GS mRNA and activity during the aging process. Further investigation is needed to answer this question.

Conclusion

In this study, we demonstrated for the first time that the activity and gene expression of GS were decreased with age in the kidney and lung of Fisher 344 rats. No age-associated change in either GR or GGT activity was observed in any of the organs examined. The results from this study suggest that down-regulation of GS gene expression may contribute to the age-associated decline in GSH content in certain organs/tissues. The data also further support our previous notion that decreased synthesis underlies the age-associated decline in GSH content in Fisher 344 rats.

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ABBREVIATIONS

AP-1, activator protein 1; GCL, glutamate cysteine ligase; GGT, γ-glutamyltranspeptidase; GR, glutathione reductase; GS, glutathione synthase; GSH, glutathione; GSSG, glutathione disulfide; PBS, phosphate-buffered saline.

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