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FASTING-INDUCED DEPLETION OF GLUTATHIONE IN THE AGING MOUSE

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Abstract—Previous results indicate that aging is associated with a general deficiency of glutathione (GSH). As fasting is also known to lower hepatic GSH levels, we investigated the combined effects of aging and 24 hr of food deprivation on liver, kidney and blood GSH and cyst(e)ine levels in C57BL/6N mice of ages 6 (young), 12 (mature) and 24 (old) months. No age-related differences in baseline hepatic GSH were observed in these mice, consistent with previous findings where the deficiency in liver is not apparent until about 29 months of age. By 6 hr of fasting, an age-related reduction in hepatic GSH was evident, culminating in a 4-fold greater decrease during maturation, and a 5-fold greater decrease during aging ($P < 0.001$) compared to young animals. Liver weight also declined, decreasing total liver GSH content by 24% in young, 44% in mature, and 56% in old mice. Renal GSH and hepatic cyst(e)ine concentrations were unaffected by fasting. In young and mature mice, depletion of hepatic GSH was accompanied by a concomitant increase in blood GSH and kidney cyst(e)ine levels after 6 hr of fasting, suggesting enhancement of hepatic GSH efflux. However, in old animals, GSH depletion was associated with decreased blood GSH and kidney cyst(e)ine. Altogether, these results suggest that the stress of fasting reveals aging changes in hepatic GSH homeostasis occurring well before the GSH deficiency of aging is observed. These aging changes are likely due to decreased GSH turnover resulting from impaired biosynthesis.

Survival depends, in part, on the ability of an organism to withstand repeated challenge by a variety of environmental and endogenous toxins. As an organism ages, its ability to withstand these insults diminishes [1]. The aging process, therefore, represents the biochemical changes that occur from maturity to senescence, rendering an organism more vulnerable to disease and toxicity, and leading eventually to death. Indeed, the incidence of many diseases such as cancer and atherosclerosis, the major causes of death in the U.S., increases exponentially during aging [2].

One biochemical compound that plays an important role in both detoxification and longevity is glutathione (GSH§). The protective capacity of this ubiquitous tripeptide (γ-Glu-Cys-Gly) is due to the reactive sulfhydryl group of cysteine (Cys), which can bind electrophilic sites on xenobiotics and endogenous toxins. The resulting conjugate, being highly water soluble, is excreted through the kidney [3]. GSH is also capable of neutralizing free radicals generated by oxidative stress [4, 5] and radiation [6]. Scavenging of free radicals by GSH can occur either nonenzymatically or in conjunction with GSH peroxidase [7, 8].

Besides detoxification, numerous other essential cellular functions are ascribed to GSH [9, 10]. GSH mediates cellular redox reactions, maintains sulfhydryl groups and protein disulfide bonds, upholds membrane integrity and immune function, facilitates transport of amino acids, affects metabolic fuel sources through gluconeogenesis, and assists in the synthesis of DNA, proteins and prostaglandins. Depletion of GSH has crucial effects on cell survival, and sustained low GSH levels are associated with cell damage and increased susceptibility to toxicity [11].

Our previous results and those of others have demonstrated that aging is characterized by a deficiency of GSH. This GSH deficiency was observed in all tissues of a number of senescent organisms including the adult mosquito (*Aedes aegypti*) [12], adult housefly (*Musca domestica*) [13], C57BL/6N mouse [14–17], F344 and Lobund-Wistar rats [18, 19] and in blood [20] and lens tissue [21, 22] from elderly human subjects. Based on the important functional roles of GSH in health maintenance and survival, we proposed that a GSH deficiency represents a key factor in the aging process and may underlie a number of the changes that occur during senescence.

Support for this proposal lies in the demonstration that nutritional correction of the GSH deficiency extends life span [23]. Biosynthesis of GSH is limited by the availability of Cys either from the diet or derived from methionine through the hepatic cystathionine pathway [24]. In our aging mosquito model, we observed that Cys levels were depleted during aging and that the deficiency of both Cys and GSH during aging was corrected by feeding a Cys precursor [25]. Moreover, when this precursor was fed to adult mosquitos throughout their entire life span, a 38% prolongation of average life span was achieved [23]. The role of GSH in longevity is further

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§ Abbreviations: GSH, glutathione; GSSG, glutathione disulfide; MPA, metaphosphoric acid; and APAP, acetaminophen.

supported in studies of mice and rats in which caloric restriction of the diet, the only accepted means of prolonging maximal life span in mammals, raised GSH levels in the blood and liver [18, 19].

Just as supplementation of the diet with GSH precursors results in increased GSH levels, deficiencies in the diet can lower GSH, through restriction of available Cys. A diet deficient in protein or the essential amino acid methionine prevents GSH synthesis [24]. Extreme dietary restriction, starvation, diminishes hepatic GSH levels by 30–40% in rat liver after 24 hr of food deprivation [26–28]. By the same mechanism, GSH levels fluctuate according to a diurnal rhythm, set by the timing of food consumption during the dark period [28–31].

To our knowledge, the effects of fasting on GSH, Cys and other parameters have not been investigated in aging animals. Considering the depleting effects of both aging and fasting on GSH, we hypothesized that fasting would deplete GSH and Cys concentrations in aged mice more severely than in younger mice. To test this hypothesis, we subjected mice of three age groups to a 24-hr fast, during which we measured GSH and cyst(e)ine levels, and organ weights, in liver, kidney and blood.

MATERIALS AND METHODS

Experimental protocol. Male C57BL/6NNIA mice, a standard well-characterized aging model, were obtained from the National Institutes of Aging colony at Charles River Breeding Laboratories (Kingston, NY). Three age groups were selected for study, based on previously reported life-span characteristics [15]: age 6 months (young), 12 months (mature adults), and 24 months (old). Mice were acclimated to a 12-hr light/dark cycle at 22° in our AAALAC-accredited research animal facility for at least 1 week prior to experimentation, which began at 8:00 a.m. All mice were clinically healthy and active upon receipt and free of gross pathology. Prior to investigation, they had free access to food (NIH-07) and water. On the days of experiments, mice were relocated to the laboratory, weighed and returned to their plastic cages with wood chip bedding, from which food but not water was removed. These mice were used as controls in other studies and were subjected to i.p. injections of 0.02 mL of 0.9% NaCl/g and motor activity measurements, which had no effect on GSH levels. At various time points after the removal of food (2, 6 or 24 hr), they were anesthetized with diethyl ether and killed by exsanguination by cardiac puncture. Whole blood was collected into syringes containing 100 μL of 0.05 M disodium EDTA (Sigma, St. Louis, MO). Liver and kidneys were excised quickly, trimmed, rinsed in ice-cold 0.9% (w/v) saline to remove blood, blotted dry and weighed.

Tissue processing. Whole blood was processed by the addition of 4 vol. of ice-cold 5% (w/v) metaphosphoric acid (MPA) (Mallinckrodt, Paris, KY). Liver and kidney samples were homogenized (10%, w/v) in 5% MPA using an all-glass Ten-Broeck homogenizer. Acid extracts of blood and tissues were obtained by centrifugation at 13,000 *g* for 3 min in an Eppendorf Microfuge and stored at −70°.

Table 1. Body and organ weights of mice of different ages

Age (months)	Body* (g)	Liver* (g)	Kidneys* (g)
6	32.25 ± 0.55†	1.56 ± 0.11	0.45 ± 0.01
12	38.85 ± 0.80	1.59 ± 0.06	0.41 ± 0.06
24	33.70 ± 0.71†	1.93 ± 0.18	0.54 ± 0.05

* Results are means ± SEM. For body weight, N = 43, 58 and 47 for age groups 6, 12 and 24 months, respectively. Organ weights are based on 4 mice per age group.

† Significantly different from 12-month value ($P < 0.001$).

Glutathione analysis. Total glutathione [GSH and glutathione disulfide (GSSG)] was assayed according to our modifications of the procedure of Tietze [32] and Owens and Belcher [33], with volumes appropriate for 96-well microtiter plates. Dilutions of tissue extracts in 100 mM NaH_2PO_4/5 mM disodium EDTA buffer, pH 7.5 (Fisher, Fair Lawn, NJ, and Sigma) (50 μL) were added to 50 μL of 2.5 mM 5,5′-dithiobis-(2-dinitrobenzoic acid) (DTNB) (Sigma); and 5 U glutathione oxidoreductase (EC 1.6.9.2, Sigma) (1 unit reduces 1.0 μmol of GSSG/min at pH 7.6 at 25°). The reaction was initiated by addition of 50 μL of 1.2 mM NADPH (Sigma) in buffer. The rate of color change was monitored in a Dynatech MR700 ELISA plate reader at 410 nm. This rate is proportional to the amount of total glutathione in the sample, established by authentic standards of GSH and GSSG (Sigma). Readings were corrected for blank reactions in the absence of substrate. All samples were analyzed in duplicate. Some of the samples were also analyzed for GSH and GSSG separately, using our HPLC-electrochemical detection method [34]. In all cases, GSSG levels were low (<5% of total), so that total glutathione levels primarily reflected GSH content.

Cyst(e)ine analysis. Cyst(e)ine (cysteine + cystine) was assayed according to the method of Gaitonde [35]. Briefly, samples were reduced in acidic medium with 10 mM dithiothreitol, and conjugates of ninhydrin were assayed spectrophotometrically at 560 nm.

Statistical analysis. Data are expressed as means ± SEM. Differences between groups were considered significant at $P < 0.05$ by Student's *t*-test or by a two-way ANOVA with Scheffe's post-hoc test [36].

RESULTS

Body and organ weights. Body and organ weights of mice of different age groups are presented in Table 1. Initial body weights increased from 6 to 12 months of age due to growth, and decreased from 12 to 24 months of age during aging ($P < 0.001$). No significant differences were observed in liver and kidney weights between the three age groups. This was in contrast to our previous observations that

Table 2. Time course of fasting-induced depletion of liver weight

Age (months)	Time (hr)	Liver weight* (g)	Liver/Body weight* (× 100)
6	2	1.56 ± 0.11 (4)	4.40 ± 0.21 (4)
	6	1.48 ± 0.05 (6)	4.78 ± 0.13 (6)
	24	1.29 ± 0.06† (4)	3.68 ± 0.15† (4)
12	2	1.59 ± 0.06 (4)	4.96 ± 0.12 (4)
	6	1.65 ± 0.10 (9)	4.37 ± 0.17 (10)
	24	1.35 ± 0.06† (4)	3.99 ± 0.09† (4)
24	2	1.93 ± 0.18 (4)	6.05 ± 0.65 (4)
	6	1.48 ± 0.10† (7)	4.61 ± 0.21 (7)
	24	1.45 ± 0.09 (3)	4.55 ± 0.42† (4)

* Results are means ± SEM (N).
† Significantly different from 2-hr value in the same age group ($P < 0.04$).

liver and kidney weights increase from 6 to 12 months of age [17, 37], and may be a function of the small number of animals and the large SEM per group in the current study. Fasting for 24 hr lowered liver weight ($P < 0.04$) in animals of all ages, expressed either as grams total liver weight or as liver weight per 100 grams of body weight (Table 2). Liver weight decreased 17% in young, 15% in mature, and 25% in old mice after 24 hr. The extent of weight loss did not differ statistically between age groups. There were no changes in kidney weight attributable to fasting.

Effects of fasting on GSH. The response of hepatic GSH levels to fasting is summarized in Fig. 1. Fasting lowered GSH concentration ($P < 0.001$), the extent of which depended upon the age of the animals (Fig. 1a). The effect of food restriction was significant during the first 6-hr period in adult mice, where the GSH content of 12-month-old mice decreased 14% ($P < 0.04$) and of 24-month-old mice, 29% ($P < 0.03$). Further declines occurred between 6 and 24 hr, culminating in total decrements of 34% in the 12-month-old ($P < 0.002$) and 49% in the 24-month-old animals ($P < 0.004$). While decreases in GSH concentrations in the 6-month-old mice were observed after 6 hr (9%) and 24 hr (14%), these differences were not statistically significant.

Since fasting reduced liver weight as well as GSH concentration, we examined the effects of age and fasting on total GSH content per liver (summarized in Fig. 1b). Total hepatic GSH levels were decreased significantly in mice of all ages ($P < 0.001$), and the depletion thus expressed was more extensive than when the data were expressed on a gram tissue basis. By 24 hr, young animals displayed a total GSH loss of 24% ($P < 0.003$), mature of 44% ($P < 0.001$) and old of 56% ($P < 0.004$) from original levels.

Renal and whole blood GSH levels during fasting are presented in Fig. 2. In contrast to liver, baseline renal and whole blood GSH in old mice were significantly lower than in young and mature mice ($P < 0.001$). Fasting had no consistent effect on GSH levels in the kidney. Blood GSH levels in young and mature mice were elevated at 6 hr after food deprivation, an effect that was significant only in the 12-month-olds ($P < 0.04$).

When GSH concentrations in fed and 24-hr fasted

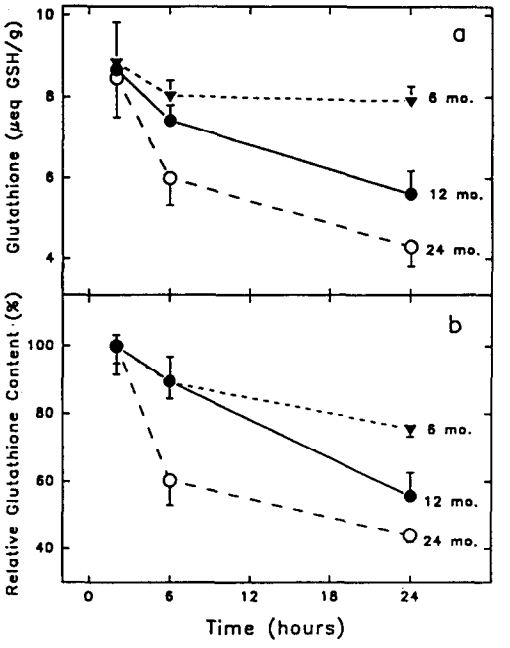

Fig. 1. Aging-dependent depletion of mouse hepatic GSH during fasting. Total GSH (GSH + GSSG) was determined in 10% liver homogenates from mice fasted for 2, 6 or 24 hr. Each point and bar represent the mean ± SEM of 4–9 mice. (a) GSH is expressed as μeq GSH/g liver; (b) GSH values are μeq GSH/total liver, expressed as a percentage of 2-hr values. One hundred per cent values for 6-, 12- and 24-month mice were 13.4 ± 0.69, 13.7 ± 0.45 and 15.9 ± 1.35 μeq GSH/liver, respectively.

mice were considered as a function of age (Fig. 3), the differential effects of fasting in aging were underscored. Before fasting, no differences were observed in hepatic GSH between mice of the three age groups. However, after the animals were fasted, age-related decreases in hepatic GSH became apparent. Maturation from 6 to 12 months resulted in a 29% loss of GSH in fasted mice ($P < 0.007$), and aging from 12 to 24 months resulted in a further 24% depletion ($P < 0.001$). This was in contrast to

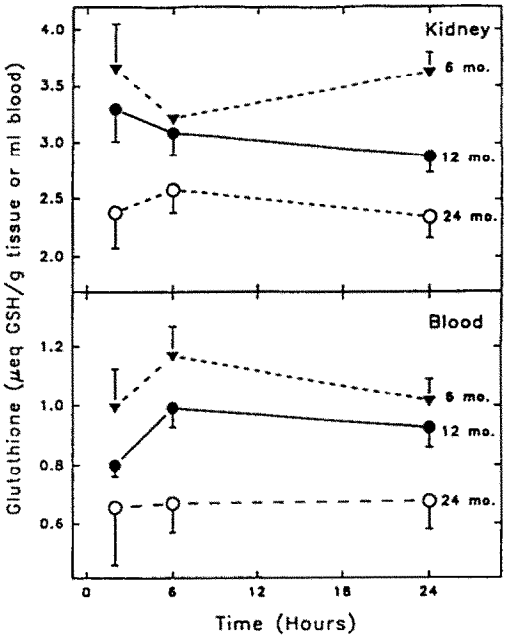

Fig. 2. GSH levels in mouse kidney and blood during fasting. Total GSH was determined in 10% kidney homogenates and 20% whole blood homogenates of mice starved for 2, 6 and 24 hr. Each point and bar represent the mean ± SEM of 4–10 mice.

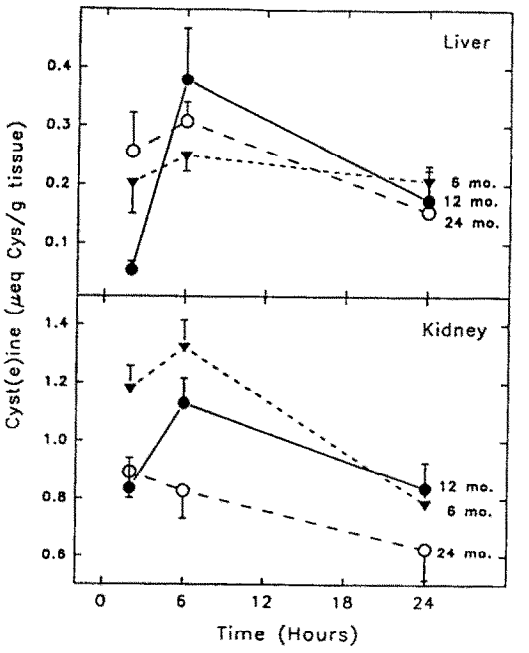

Fig. 4. Cyst(e)ine levels in mouse liver and kidney during fasting. Cyst(e)ine was determined in 10% liver and kidney homogenates of mice starved for 2, 6 and 24 hr. Each point and bar represent the mean ± SEM of 4–10 mice.

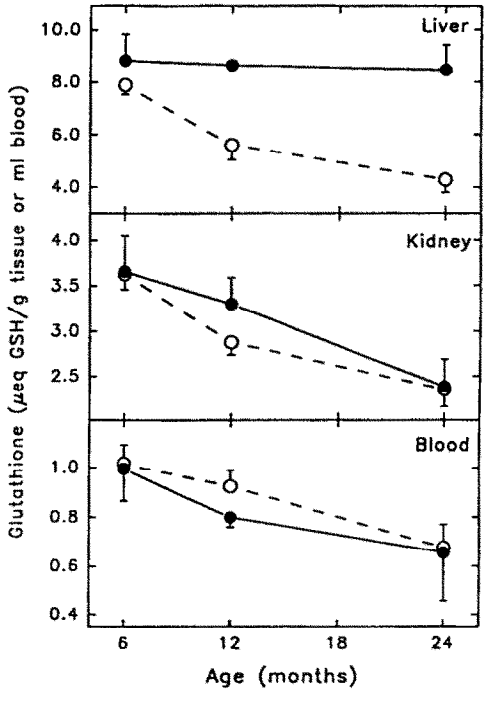

Fig. 3. Age profile of GSH content in liver, kidney and blood of fed and fasted mice. The values for the 6-, 12- and 24-month-old mice in the fed state are represented by the solid line, and for the 6-, 12- and 24-month-old mice in the fasted state by the dashed line. Each point and bar represent the mean ± SEM of 4 mice.

the kidney and blood, where aging from 12 to 24 months alone resulted in decreases of 12 and 17% in GSH levels, respectively, whereas fasting had no additional effect.

Effects of fasting on cyst(e)ine. Age-specific responses of hepatic and renal cyst(e)ine concentrations to fasting are shown in Fig. 4. Cyst(e)ine content of the liver exhibited no statistically significant changes attributable either to age or fasting. However, in 12-month-old mice, an apparent increase in liver Cys from a low baseline level was observed after 6 hr, the importance of which is unknown. Initial Cys levels in the kidneys of mature and old mice were 25% lower than in young mice ($P < 0.03$). The three age groups each displayed different renal cyst(e)ine profiles in response to food deprivation. In young mice, cyst(e)ine concentrations increased 12% after 6 hr ($P < 0.0001$), and subsequently decreased to 66% of initial levels after 24 hr ($P < 0.004$). In mature mice, cyst(e)ine levels increased 35% after 6 hr ($P < 0.05$), and returned to baseline values by 24 hr. Old mice displayed declining cyst(e)ine concentrations throughout the 24-hr time period, ending with a level 70% of that initially observed ($P < 0.0001$).

DISCUSSION

We report for the first time that aged mice have a more severe hepatic response to 24 hr of fasting than do younger mice. Fasting decreased both liver weight and GSH, as observed previously in young animals [28, 38, 39]. Age affected the extent of this

hepatic GSH depletion, so that the fasting-induced loss of GSH was 4-fold greater in mature and 5-fold greater in old mice when compared with young animals. Thus, increased susceptibility to fasting-induced GSH depletion occurred during both the maturational (6–12 months) and aging (12–24 months) periods of the mouse life span. When the cumulative effect of liver weight loss and GSH depletion was taken into account, decreases in total liver GSH capacity were 24% in young, 44% in mature, and 56% in old mice. This latter representation of GSH content may be a more appropriate index than GSH concentration for study of fasting effects, as water is the primary constituent of hepatic weight loss during food deprivation [40].

These findings impact upon the nature of the GSH deficiency of aging, which, in liver, is generally not observed until the average life expectancy of 29 months for C57BL/6N mice is approached [15, 17]. Indeed, in our data, no changes in hepatic GSH levels were observed between the 24-month-old mice and their younger counterparts in the fed state. However, after as little as 6 hr of food deprivation, and even more so after 24 hr, the age-related decrements in liver GSH levels became apparent. Thus, the stress of a 24-hr fast revealed the presence of changes in hepatic GSH status well before the aging changes in steady-state levels were observed. Age-related differences in GSH homeostasis, evident as early as 12 months of age, may thus contribute to the pathologies and changes observed in aging. This lends support to the underlying hypothesis that a GSH deficiency plays a major role in the etiology of aging.

Our data on C57BL/6N mice confirmed previous studies performed in other younger animals in which starvation caused a decrease in hepatic GSH [26–28, 39, 41–44] and liver weight [39, 41–43]. Six-month-old mice of this strain appear more resistant to fasting than other strains of mice [28, 41], as liver weight and GSH concentration were not decreased significantly after 24 hr of this treatment. However, when the combined effect of these two parameters was examined, fasting did result in a significantly lowered value of total hepatic GSH capacity ($P < 0.003$). While this protocol was not designed to study circadian fluctuations of hepatic GSH, we observed in adult C57BL/6N mice significant decreases in GSH and liver weight within the first half of a circadian time span, similar to those previously reported [31]. Thus, the extent of the GSH response to a diurnal rhythm of food restriction may also be age dependent.

Food deprivation has been shown to stimulate changes in hepatic GSH turnover in young animals [42–44]. Turnover of GSH is the sum of two opposing processes: formation and utilization [45]. Formation occurs by synthesis from dietary precursors or amino acids degraded from protein or peptides, and by reduction of GSSG or mixed protein disulfides. Utilization consists primarily of extrahepatic efflux, and, to a lesser extent, conjugation, oxidation and degradation. GSH turnover is increased in young animals deprived of food [42, 43], due specifically to an enhanced rate of GSH efflux from the liver in an attempt to maintain optimal GSH levels in extrahepatic tissues [30, 43, 46]. Increased efflux signals an increase in GSH synthesis by relieving the feedback inhibition mechanism of the biosynthetic enzyme, γ-glutamylcysteine synthetase [47], even during periods of starvation [44].

Our findings suggest that turnover of hepatic GSH is decreased during aging due to a progressive age-dependent impairment of biosynthetic capacity. Young mice responded to food deprivation by maintaining hepatic GSH levels, presumably in the presence of accelerated hepatic efflux as suggested by the observed increases in blood GSH and renal Cys levels after 6 hr. In contrast, mature and old mice experienced significant decreases in hepatic GSH, while extrahepatic GSH levels were maintained. Thus, older mice were unable to synthesize sufficient levels of GSH in the liver to compensate for increased rates of efflux during starvation. A biosynthetic impairment during aging was also suggested in earlier studies in which hepatic GSH was depleted by acetaminophen (APAP) [17] or ethanol [48]. In these studies, mature and senescent mice experienced only a partial recovery of hepatic GSH levels, whereas GSH concentrations in young mice returned to normal levels within a 24-hr recovery period. In an aging mosquito model, a decrease in GSH biosynthesis was responsible for the deficiency during aging [49]. Likewise, an impaired biosynthetic capacity accounted for decreased GSH levels in the ocular lens of elderly humans [22]. Finally, while age-related decreases in GSH turnover have been reported previously [50–52], they pertain to growth of young animals and not to aging.

Renal cyst(e)ine concentrations support the suggestion of an aging-related decrease in the capacity for GSH turnover. The kidney, which contains high levels of γ-glutamyl transpeptidase, is primarily responsible for the degradation of plasma GSH and the subsequent recirculation of Cys to the liver [53]. Thus, renal Cys levels are likely to reflect the hepatic efflux and interorgan turnover of GSH. Our observation of elevated renal Cys levels in young and mature mice after 6 hr of fasting suggests an increased rate of efflux of hepatic GSH, as seen by other investigators [43, 44]. In old mice, however, Cys levels declined throughout the 24-hr period, reflecting rapid depletion of hepatic GSH with a subsequent decrease in the rate of hepatic efflux.

Enhanced susceptibility to toxic challenge is an established consequence of depletion of hepatic GSH levels by a variety of pharmacologic agents, such as diethyl maleate and buthionine sulfoximine [54]. Depletion of GSH by the aging process likewise results in reduced detoxification capacity for APAP in the mosquito and mouse [17, 55]. However, mature mice as well as old mice displayed a state of enhanced vulnerability, evidenced by an inability to replenish hepatic GSH after depletion by APAP. This finding of impaired recoverability in mature mice prior to the appearance of the GSH deficiency of aging, together with the present results, suggest that a decrease in GSH turnover may also be an important factor in regulating susceptibility to toxic damage. Little is known of how the rate of hepatic GSH turnover affects vulnerability to chronic toxicity

and disease, and our results point to the need for further study of this phenomenon.

Overnight fasting of laboratory animals is commonly used to enhance absorption of test compounds and to minimize variation in organ weights [40, 41]. As fasting depletes hepatic GSH more extensively in mature and old mice compared with young, a greater degree of xenobiotic toxicity may result in older animals due specifically to starvation effects. Thus, careful consideration must be given to the practice of overnight fasting in toxicity studies in aging animals.

In conclusion, we suggest that during maturation and aging, there occurs a decline in the rate of hepatic GSH turnover, which is the biochemical antecedent of and a likely cause of the GSH deficiency of aging. This modification in the rate of turnover limits the ability of GSH to participate in the myriad of biochemical functions essential for physiological health and survival. Further study of the origin, nature and consequence of the alteration of GSH turnover is indicated.

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