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Studies on the life prolonging effect of food restriction: glutathione levels and glyoxalase enzymes in rat liver

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Abstract

Cytosolic and mitochondrial levels of glutathione (GSH) as well as the activities of glyoxalase I (GI) and glyoxalase II (GII), GSH-dependent enzymes involved in the detoxification of 2-ketoaldehydes, were investigated in the liver of ad libitum (AL) fed and food restricted (FR) rat during aging. Both cytosolic and mitochondrial GSH level was lower in old than in adult AL fed rats. Food restriction did not prevent this decrease, but its extent was attenuated considering the cytosolic GSH. As regards the mitochondrial GSH, its content was higher in adult FR animals than in the age-matched AL fed ones. Thus, the subsequent age-dependent decrease of GSH, occurring also in FR animals, resulted in a thiol concentration not different from that observed in young and adult AL fed animals. Considering the enzymatic activities, cytosolic GI decreased in old rats irrespective of diet, whereas GII activity remained constant in all the experimental groups. The higher glutathione content found in both cellular compartments of old FR rats as compared to the old AL fed ones, could help to explain the life prolonging effect of FR treatment. Moreover, the observation that the activity of glyoxalases was not influenced by food restriction does not necessarily mean that the cells of diet-conditioned animals are scarcely protected against the toxic effect of methylglyoxal. Indeed, the production of this compound should be lower in

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FR animals as compared to AL fed ones, due to the lower level serum glucose concentration during the life span of the former with respect to the latter group.

Keywords: Aging; Food restriction; Liver; Glutathione; Glyoxalases

1. Introduction

A contribution to the understanding of the biology of aging may stem from studies on models in which the aging process is delayed and the lifespan is increased by specific treatments. Caloric restriction is still to date the only well-documented experimental method to delay aging and related consequences in rodents. Several studies have shown that the application of food restriction (FR) to rats was effective in recovering some age-dependent alterations (Goodrick et al., 1982; Maeda et al., 1985; Iwasaki et al., 1988; Laganiere et al., 1989a; Pieri et al., 1994a).

The possible involvement of mitochondria in the aging process is the object of considerable interest. Mitochondrial impairment resulting from oxidant-induced damage has been considered to play a critical, possibly causative role, in both the aging process and many age-related diseases (Linnane et al., 1989; Wallace et al., 1992). This hypothesis implies that the senescence-related loss of adaptability is, at least partially, due to the effect of oxidative stress as it has been demonstrated to occur in mitochondria of several tissues, including heart, kidney and liver (Nohl et al., 1978; Muscari et al., 1990; Sohal et al., 1990). However, the actual cell damage is directly determined by the rate of reactive oxygen species produced and by the efficiency of the defence mechanisms, which include the concentration of antioxidants and the expression of professional enzymes.

Glutathione (GSH) is a well-known tripeptide involved in a variety of reactions, including scavenging of free-radicals. The effect of aging and FR on cytosolic rat liver GSH and on most of the GSH-related enzymes has already been investigated (Laganiere et al., 1989b). However, there are no data concerning the GSH content in the mitochondrial matrix. In the present paper we investigated the effect of aging and FR on the cytosolic and mitochondrial GSH content as well as on the activity of glyoxalase I (GI) and glyoxalase II (GII), two glutathione-dependent enzymes not previously investigated, involved in the detoxification of 2-ketoaldehydes formed during oxidative stress (Mannervik et al., 1980).

2. Materials and methods

2.1. Animals

Female Wistar rats of the breeding colony were used. FR was applied by feeding the animals on an every-other-day schedule from the age of 3.5 months with the

same diet given to the AL control group. As previously reported in detail (Pieri et al., 1990), this treatment was able to prolong the mean, median and maximum life span of the animals. In the present experiment the survival curves of AL and FR fed animals were determined again in small groups of rats (15 each), to confirm the previously obtained results. The chow (Nossan, Italy) contained 41% carbohydrate, 21% protein and 6% fat. For the FR animals, food was provided in the morning hours and removed the following morning; the animals had free access to water and were kept in threes per cage in plastic cages. The young, adult and old AL controls were killed when they were 6, 11 and 24 months old. The FR rats were killed at 14 and 26 months of age after 24 h feeding. Since FR was applied starting from the age of 3.5 months, young (6 months old) FR animals were not taken into account to avoid the influence of metabolic adaptation to the new feeding regimen on the considered parameters. Each group consisted of ten animals.

2.2. Chemicals

All chemicals used were purchased from Boehringer Mannheim (GmSH, Germany) and Sigma (St. Louis, Mo) and were of the highest purity available. *S*-D-lactoylglutathione was synthesised and purified as described (Ball et al., 1979). Methylglyoxal was purified by fractional distillation and standardised by endpoint enzymatic assay with glyoxalase I (McLellan et al., 1992).

2.3. Preparation of mitochondrial and cytosolic fractions

The animals were killed by cervical dislocation after ether anaesthesia and exanguinated. The purification of mitochondria from rat liver was carried as follows: aliquots of liver tissue (4–5 g) were homogenised 1:10 (w/v) in ice-cold buffer, pH 7.5 containing 75 mM sucrose, 225 mM mannitol, 1 mM EDTA, 5 mM HEPES and 0.5 mg/ml fatty-acid free bovine serum albumin. The homogenate was centrifuged for 10 min. at $600 \times g$ at 4°C. Sediment was discarded and supernatant was centrifuged for 20 min. at $1200 \times g$ at 4°C, obtaining a mitochondrial pellet and a supernatant. This latter was centrifuged for 30 min. at $55\,000 \times g$ at 4°C and the supernatant (soluble cytosolic fraction) was divided into 1 ml aliquots. The mitochondrial pellet was washed two times (by careful resuspensions in ice-cold homogenisation buffer and centrifugation, 10 min. at $2\,800 \times g$ at 4°C) and purified mitochondria were carefully resuspended in 1 ml of ice-cold homogenisation buffer.

2.4. Glutathione determination

Aliquots of mitochondrial and cytosolic fractions were immediately deproteinized in ice-cold perchloric acid, centrifuged ($15\,000 \times g$ for 15 min. at 4°C) and supernatants stored at -80°C until used for glutathione determinations. Pellets were dissolved in 1 M sodium hydroxide and used for protein determinations (Lowry et al., 1951) using bovine serum albumin as standard. Perchloric acid was removed by mitochondrial and cytosolic deproteinized samples by neutralisation with potassium

carbonate. Total glutathione (GSH + 2GSSG) was measured by the glutathione reductase recycling assay at 412 nm in the presence of 5,5′-dithio-bis-nitrobenzoic acid (Akerboom et al., 1981). The amount of total glutathione is reported as nmol/mg of protein of the respective fractions.

2.5. Enzymatic assays

Samples were aliquots of cytosolic and mitochondrial fractions kept frozen at − 80°C until use. Mitochondrial pellets were dissolved in buffer containing 0.5% (v/v) Triton X-100. Determinations of enzymatic activities were carried out at a constant temperature of 20°C. GI was determined at 240 nm (Ekwall et al., 1970) using 1.0 mM GSH/methylglyoxal hemithioacetal as substrate in 100 mM sodium phosphate buffer, pH 6.8. The hemithioacetal is generated in situ by pre-incubation of methylglyoxal with GSH in sodium phosphate buffer at 37°C and this step is essential to avoid condition where the formation of the hemithioacetal is rate-limiting (Vander Jagt et al., 1975). GII was determined at 412 nm using 0.9 mM *S*-D-lactoylglutathione as substrate in 100 mM MOPS buffer, pH 7.2, containing 0.2 mM 5,5′-dithio-bis-nitrobenzoic acid (Principato et al., 1987). Enzyme activities are given in units/g wet weight, where one unit of activity represents the formation of 1 μmol of product/min. under assay conditions.

2.6. Statistical analysis

Results are reported as mean ± standard deviation (S.D.). The statistical significance between groups was assessed by computer assisted analysis of variance (ANOVA). The survival curves were estimated using the Kolmogorof-Smirnov 2-sample test (Mode et al., 1984).

3. Results and discussion

Present work was aimed to study the GSH content as well as the GI and GII activities of cytosolic and mitochondrial fractions of liver during aging. The influence of hypocaloric dietary treatment on the same parameters was also investigated. Wistar rats used in this study were female fed AL or FR, this latter accomplished by feeding laboratory chow on an every-other-day schedule basis (Pieri et al., 1990). In agreement with previous results (Goodrick et al., 1982; Pieri et al., 1990), it was confirmed that FR was able to prolong the life span of the animals by about 20% and the survival curves are significantly different ($P < 0.001$). Is noteworthy that more than 80% of FR-animals, as compared to 40% of controls, survived and were healthy up to 22 months (Fig. 1).

Cytosolic and mitochondrial glutathione contents of AL groups did not change when young and adult rats were compared, whereas a significant ($P < 0.01$) age-dependent decrease was evident in old animals (Figs. 2 and 3). The application of FR consistently affect the glutathione content of both cell fractions studied.

Cytosolic glutathione level is significantly ($P < 0.05$) higher in old FR animals with respect to the old AL fed counterparts. However, it has to be noted that the application of dietary restriction did not prevent the age-dependent decrease of cytosolic GSH content, but attenuated its decrement. Indeed, the cytoplasmic thiol content was lower than that observed in young and adult AL rats. A more pronounced effect of FR can be observed considering mitochondrial GSH. Indeed, the concentration of the thiol was higher ($P < 0.01$) both in adult and old-FR animals with respect to the age-matched AL fed rats. It is remarkable that no statistically significant difference was found comparing mitochondrial glutathione content of old FR animals with that of young and adult AL fed rats.

These results are of interest because the maintenance of high mitochondrial glutathione level with age may be an important factor contributing to the life prolonging effect of FR. In fact, mitochondrial GSH depletion may facilitate the onset of oxidative damage due to a decreased defence power. It is well known that the steady state level of oxidative stress depends on both prooxidant generation and antioxidant defences. It has been reported that the rate of superoxide radical and/or hydrogen peroxide generation by mitochondria increased as a result of aging in several rat organs (Nohl et al., 1978; Muscari et al., 1990; Sohal et al., 1990). Taken together, these data point to a shifting toward a prooxidant status during aging,

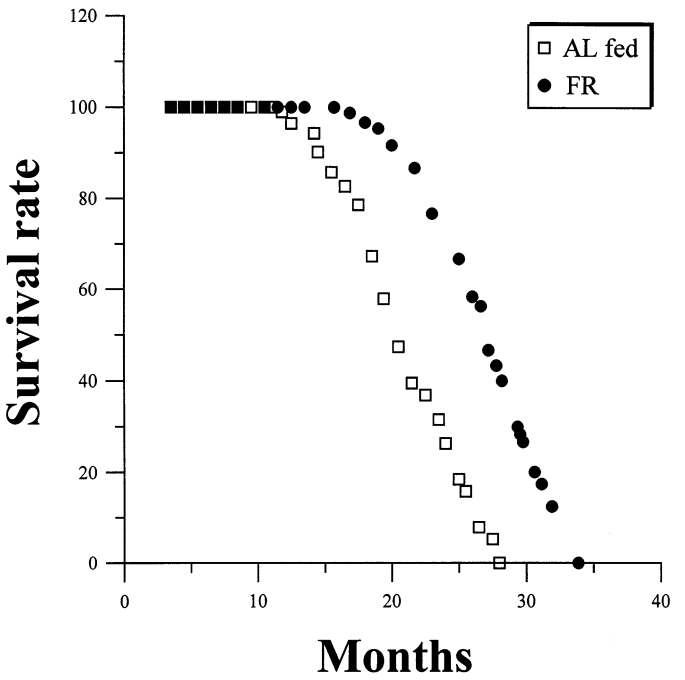

Fig. 1. Survival curves of AL fed and FR female Wistar rats. Each group consisted of 15 animals. Statistical comparisons were estimated using the Kolmogorof-Smirnov 2-sample test and the difference between the two dietary groups was statistically significant ($P < 0.001$).

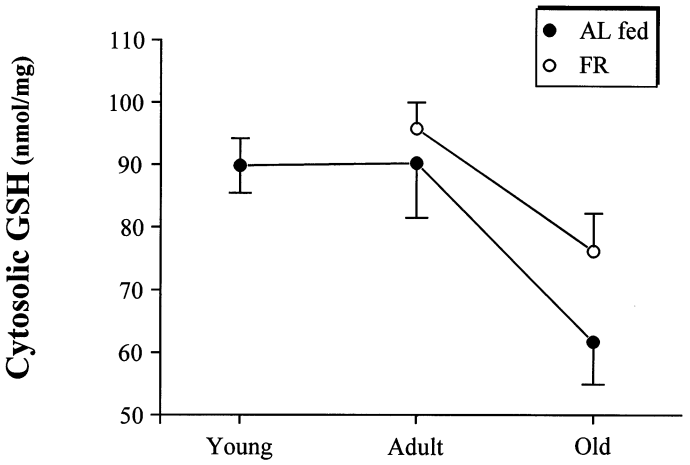

Fig. 2. Effects of age and food restriction on the concentration of total glutathione in the rat liver cytosolic fractions. Each point is the mean ± S.D. of ten independent experiments. Statistical comparisons have been made by ANOVA. The difference between adult and old animals was statistically significant ($P < 0.01$) in both groups as was the difference between old AL fed and old FR animals ($P < 0.05$).

which seems to be prevented by FR and it is likely that the impairment of mitochondrial function during aging could be linked to GSH loss. Support to this conclusion stems from previous studies performed on splenocytes, showing that FR was able to prevent the depolarization occurring in mitochondria of mitogen

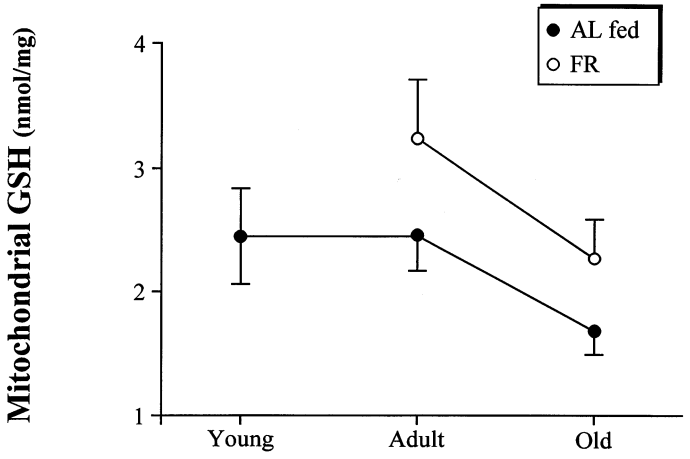

Fig. 3. Effects of age and food restriction on the concentration of total glutathione in the rat liver mitochondrial fractions. Each point is the mean ± S.D. of ten independent experiments. Statistical comparisons have been made by ANOVA. The difference between adult and old animals was statistically significant ($P < 0.01$) in both groups as was the difference between the age-matched AL fed and FR animals ($P < 0.01$).

stimulated cells from old animals (Pieri et al., 1993, 1994a). Moreover, the observation that this age-dependent impairment could also be prevented by the supplementation of GSH (Pieri et al., 1994b), strongly supports either that mitochondrial depolarization was due to peroxidative stress and that GSH plays a fundamental role in protecting mitochondria.

Two possible mechanisms may be responsible for the observed decrease of cellular GSH content during aging. One is the decrease of its synthesis, as found in rat splenocytes (Pieri et al., 1994b), the other is the excessive consumption of the thiol due to peroxidative stress. In the case of mitochondria, another mechanism may participate to GSH decrease. Indeed, if one refers the measured GSH concentration to the relative volume of mitochondrial matrix and takes into account its water content, the concentration of GSH is higher in mitochondria than in the cytosol (Wahllander et al., 1979), suggesting that a carried-mediated active transport is necessary to allow the uptake of GSH by mitochondria.

Probably, the driving force is the proton gradient, tightly linked to inner membrane integrity which depends on the level of lipid peroxidation. Since it has been shown that both the basal level as well as the induced mitochondrial membrane peroxidation are lower in FR animals than in the AL fed counterparts (Laganiere et al., 1987), it can be speculated that the active transport of GSH into the mitochondria is better preserved in FR animals than in AL fed ones during aging.

In addition, new perspectives come from *S*-D-lactoylglutathione (LSG) as a possible source of intramitochondrial glutathione (Principato et al., 1996). This thiolester seems to cross mitochondrial membranes and by means of mitochondrial GII (Talesa et al., 1988, 1989) releases GSH and D-lactic acid. A possible metabolic role of the ubiquitary glyoxalase system could also be the regulation of mitochondrial GSH levels through synthesis and hydrolysis of LSG. Cytosolic GI catalyses the formation of LSG from GSH and methylglyoxal, a side-product of glycolysis, or other α-ketoaldehydes that can be formed during lipid peroxidation (Mannervik et al., 1980). Cytosolic and mitochondrial GII compete for the hydrolysis of LSG to D-lactate and GSH. D-lactate is intramitochondrially transformed into pyruvate (Tubbs et al., 1961) and then to CO_2 and water (Giesecke et al., 1981). It is thus possible that LSG constitutes a source of mitochondrial GSH as well as of pyruvate.

In AL and FR old rats (Table 1) there is a decrease of GI activity but GII is fairly constant in both cytosol and mitochondrial fractions. A decreased GI activity can represent a harmful situation because the inactivation of methylglyoxal as well as of products of lipid peroxidation is less efficient. However, it has to be taken into account that substantial metabolic differences exist comparing FR and AL fed animals. Indeed, through the lifespan, the mean 24 h plasma glucose concentration of the FR group was lower than that of the AL-fed group of the same age (Masoro et al., 1992) and it is known that the rate of methylglyoxal formation depends on the serum glucose level (McLellan et al., 1994). Thus, it can be hypothesised that the low GI activity observed both in AL-fed and FR old animals might be sufficient to inactivate the low amount of methylglyoxal produced by diet conditioned animals but not the presumed high amount produced by normal feeding.

Table 1
Glyoxalase I and glyoxalase II activity levels in citosol and mitochondria from rat livers of Wistar rats, young, adult and old groups feed ad libitum or old food restriction group

Group	Enzyme activities (μmol/min per mg)	
	Glyoxalase I[a]	Glyoxalase II[a]
Rat liver cytosol		
Young (AL)	0.376 ± 0.027	0.092 ± 0.008
Adult (AL)	0.395 ± 0.071	0.104 ± 0.016
Adult (FR)	0.362 ± 0.027	0.107 ± 0.011
Old (AL)	0.287 ± 0.042[b]	0.114 ± 0.014
Old (FR)	0.250 ± 0.048[b]	0.108 ± 0.023
Rat liver mitochondria		
Young (AL)	ND	0.063 ± 0.016
Adult (AL)	ND	0.065 ± 0.006
Adult (FR)	ND	0.068 ± 0.021
Old (AL)	ND	0.087 ± 0.024
Old (FR)	ND	0.065 ± 0.007

Statistical comparison has been made by ANOVA.
ND, not detectable.
[a] mean $\pm$ S.D.
[b] $P < 0.01$ in respect to young-adult groups.

In conclusion our data show that old-FR rat liver cells seems to be better protected with respect to old-AL rats against oxidative stress, in agreement with other authors (Laganiere et al., 1989a). The high glutathione levels in the mitochondria of FR animals may contribute to protect their membranes from lipid peroxidation. This fact could be at the same time cause and effect of improved GSH uptake into mitochondria. In addition, our results may also contribute to explain those of Chung et al., (1992) in which mitochondrial DNA integrity was found to be protected from oxidative stress by FR treatment in rats.

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