Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats

Karine Vidal, Denis Breuillé, Patrick Serrant, Philippe Denis, Francoise Glomot, Fabienne Bechereau, Isabelle Papet

▶ **To cite this version:**

Karine Vidal, Denis Breuillé, Patrick Serrant, Philippe Denis, Francoise Glomot, et al.. Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 2014, 53 (3), pp.963 - 971. 10.1007/s00394-013-0600-0 . hal-02634675

HAL Id: hal-02634675
https://hal.inrae.fr/hal-02634675v1

Submitted on 27 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats

Karine Vidal,[1] Denis Breuillé,[1] Patrick Serrant,[1] Philippe Denis,[2,3] Françoise Glomot,[2,3] Fabienne Béchereau,[2,3] Isabelle Papet[2,3,*]

[1] Department of Nutrition and Health, Nestlé Research Center, CH-10000 Lausanne 26, Switzerland, [2] INRA, UMR 1019, UNH, CRNH Auvergne, F-63000 CLERMONT-FERRAND and [3] Clermont Université, Université d'Auvergne, Unité de Nutrition Humaine, BP 10448, F-63000 CLERMONT-FERRAND

*Corresponding author: Dr Isabelle Papet, Unité de Nutrition Humaine, INRA Theix, 63122 Saint-Genès-Champanelle, France. Telephone: +33 4 73 62 42 01. Fax: +33 4 73 62 47 55. E-mail: Isabelle.Papet@clermont.inra.fr.

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

Abstract

Purpose Healthy ageing is associated with higher levels of glutathione. The study aimed to determine whether long-term dietary fortification with cysteine increases cysteine and glutathione pools thus, alleviating age-associated low-grade inflammation and resulting in global physiological benefits.

Methods The effect of a 14-wk dietary fortification with cysteine was studied in non-inflamed (NI, healthy at baseline) and in spontaneously age-related low-grade inflamed (LGI, prefrail at baseline) 21-mo-old rats. 57 NI rats and 14 LGI rats received cysteine-supplemented diet (4.0 g/kg of free cysteine added to the standard diet containing 2.8 g/kg cysteine). 56 NI rats and 16 LGI rats received a control alanine-supplemented diet.

Results Cysteine fortification in NI rats increased free cysteine ($P < 0.0001$) and glutathione ($P < 0.03$) in the liver and the small intestine. In LGI rats, cysteine fortification increased total non-protein cysteine ($P < 0.0007$) and free cysteine ($P < 0.03$) in plasma, and free cysteine ($P < 0.02$) and glutathione ($P < 0.01$) in liver. Food intake decreased over time in alanine-fed rats ($r^2 = 0.73$, $P = 0.0002$), whereas it was constant in cysteine-fed rats ($r^2 = 0.02$, $P = 0.68$). Cysteine fortification did not affect inflammatory markers, mortality, body weight loss, or tissue masses.

Conclusion Doubling the dietary intake of cysteine in old rats increased cysteine and glutathione pools in selected tissues. Additionally, it alleviated the age-related decline in food intake. Further validation of these effects in the elderly population suffering from age-related anorexia would suggest a useful therapeutic approach to the problem.

Key words Ageing rats . Dietary cysteine . Frailty . Low-grade inflammation

Abbreviations

LBP	lipopolysaccharide binding protein
LGI	low-grade inflamed
NAC	N-acetyl cysteine
NI	non-inflamed
sTNFR-1	soluble tumor necrosis factor-alpha receptor-1

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

Introduction

As the number of older individuals around the world is increasing dramatically, defining optimal nutrition for healthy ageing, i.e. a delay in the development of chronic disease [1], is an important challenge. Ageing *per se* and/or age-related diseases are associated with a progressive rise in oxidative stress [2] ,which plays a role in the expansion of homeosteny, notably low-grade inflammation and sarcopenia [3]. The main intra-cellular anti-oxidant is glutathione and imbalances in glutathione levels *per se* are thought to play a role in the ageing process [4]. Intra-cellular glutathione concentration decreases with age in various tissues in ageing rats [5]. Along the same lines, higher levels of glutathione are associated with better physical and mental health in elderly [6, 7]. Thus, dietary treatment aiming at increasing anti-oxidant pools, such as glutathione levels, could promote a healthy ageing.

Glutathione is a tripeptide composed of glutamic acid, cysteine and glycine. Intra-cellular glutathione levels are regulated by a complex series of mechanisms and cysteine is the rate limiting substrate for glutathione synthesis [8]. Cysteine is obtained from dietary proteins, breakdown of body proteins and glutathione. It can also be endogenously synthesised from methionine and serine through the transsulfuration pathway. When the endogenous disposal of cysteine is insufficient regarding its metabolic utilizations, it becomes an indispensable amino acid [9]. Indeed, increasing cysteine content of the diet of septic rats allows restoration of the liver glutathione pool [10] and limits body weight loss and muscle wasting [11]. In spite of the fact that interventional studies with the pharmacological molecule N-acetyl-cysteine (NAC) exhibited potential beneficial targeted effects for elderly [12-15], health-promoting effects of long-term dietary fortification with cysteine itself remain unevaluated.

The primary objective of the study was to determine whether long-term cysteine fortification would increase cysteine and glutathione levels in healthy (*i.e.* non-inflamed) old rats and consequently prevent age-associated development of inflammation and other adverse outcomes (*i.e.* towards a prefrail/frail status). The secondary objective was to investigate whether cysteine fortification is able to improve the prefrail status in elderly rats with low-grade inflammation. Biochemical targets were cysteine, glutathione, and inflammatory markers; physiological outcomes were mortality, food intake, and body, tissue and organ weights.

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

Material and methods

Animals

The experiments were conducted in accordance with the French National Research Council's Guidelines for the Care and Use of Laboratory Animals. Wistar male rats were bred in our conventional (non-specific-pathogen-free) animal facility (Unité Expérimentale de Nutrition Comparée, INRA, Theix, France). They were maintained in collective cages (3 to 4 per cage) under controlled conditions (temperature 21°C, relative humidity 55%, 12-h dark period starting at 20:00) with free access to water and standard diet until the experiment. The standard diet (A04 pellets from Scientific Animal Food and Engineering (SAFE), Villemoisson-sur-Orge, France) was composed of 16% protein, 3% fat, 60% carbohydrate, 12% water, fibers, vitamins, and minerals.

Experimental design

The effect of a 14-wk dietary fortification with cysteine was studied in non-inflamed and low-grade inflamed 21-mo old rats (Fig. 1).

During the pre-experimental period, plasma concentrations of fibrinogen and α_2-macroglobulin were measured at the ages of 18, 19 and 20.5 mo. Rats were weighed at the beginning and at the end of the pre-experimental period. Apparently ill rats and/or rats that exhibited pathological values of acute phase proteins (i.e. values predictive of short-term mortality according to our previous observations [16]), were excluded.

At the age of 21 mo rats were distributed into 2 sets based on hierarchical clustering using Ward distance on the following variables: α_2-macroglobulin, fibrinogen, body weight and body weight change during the pre-experimental period (XLSTAT, version 7.5 software (Addinsoft, Paris, France)). *A posteriori*, non-inflamed (NI, *i.e.* healthy at baseline) rats were distinguished from low-grade inflamed (LGI, *i.e.* prefrail at baseline) rats using an α_2-macroglobulin plasma threshold of 82 mg/L, which corresponds to the mean plasma level + 2 SD from adult rats [16]. At baseline, LGI rats did not exhibit any apparent pathologies or pathological levels of α_2-macroglobulin. They were considered as prefrail because their health status was expected to deteriorate more with ageing than the NI rats. Group names (numbers of rats) were as follows: NI-Ala (n = 56) and NI-Cys (n = 57) for NI rats receiving alanine-supplemented (Ala) and cysteine-supplemented (Cys) diets, respectively; and LGI-Ala (n = 16) and LGI-Cys (n = 14) for LGI rats receiving Ala and Cys diets, respectively. Cys and Ala diets were prepared by supplementation of the standard diet A04 with amino acids and distributed as pellets. Cys diet contained 6.8 g/kg diet i.e. 2.8 g/kg from dietary proteins plus 4 g/kg as free L-cysteine (Sigma). Cysteine level has been chosen taking into account the efficient level (11 g/kg diet) in septic rats [11] and the fact that age-associated inflammation is much lower than in sepsis. The selected cysteine level was in the same range as NAC treatment performed in rats [17]. Ala diet contained 2.8 g/kg diet of cysteine from dietary proteins and 2.9 g/kg diet of free L-alanine (Jerafrance) to make it iso-nitrogenous to Cys diet. Rats were maintained in collective cages (2 to 3 per cage) and received the experimental diets *ad libitum* for 14 wk. Food intake was recorded weekly per cage, and individual daily food intake was calculated according to the number of rats per cage. When a death or a moribund condition occurred in a cage, the corresponding data was eliminated. Blood was sampled from the lateral tail vein following 2, 6, and 13 wk of supplementation. Plasma was isolated and aliquots were stored at -80°C. After 14 wk of supplementation (24 month of age), rats were killed under

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

general anaesthesia induced by intra-peritoneal injection of pentobarbital (6 mg/100 g body weight, 0.1 mL/100 g body weight, Sanofi, Libourne, France) by exsanguinations through the abdominal aorta. Aliquots of plasma were collected and stored at -80°C. Posterior leg skeletal muscles, liver, small intestine, colon, and kidneys were rapidly isolated. Small intestine and colon were flushed with ice-cold NaCl (9 g/L). The isolated tissues and organs were blotted dry and weighed. Liver and small intestine were frozen in liquid nitrogen and stored at -80°C until cysteine and glutathione analyses.

Biochemical analyses

Plasma acute phase proteins, such as α_2-macroglobulin (CV 18%), fibrinogen (CV 2 %), albumin (CV 15%) and LPS-binding protein (LBP, lower detection: 1.56 ng/ml) and serum soluble tumor necrosis factor-alpha receptor-1 (sTNFR-1, lower detection: 15.6 pg/ml, CV 5%) were measured as previously described [16]. Total glutathione (reduced plus oxidized) was quantified with a spectrophotometer using a standard enzymatic recycling procedure and 5,5'-dithio-bis-2-nitrobenzoic acid as oxidant (CV 1%) [18]. Total non-protein cysteine (cysteine plus cystine and cysteine bound to proteins through disulfide bridges) was measured with a colorimeter in plasma or tissue homogenates treated with dithiothreitol before deproteinization (CV 8%) [19, 20]. Free cysteine (cysteine plus cystine) was quantified with the same method but dithiothreitol treatment was performed after protein precipitation. The difference between total non-protein cysteine and free cysteine provided the protein bound cysteine (cys-prot) value.

Statistical analysis

Survival curves were generated up to 13 wk of dietary treatment by the Kaplan-Meyer method and compared by log-rank test. Other data are given as means ± SEM. Linear regression was used to assess the relationship between mean food intake and time. Since rats were housed in collective cages and NI and LGI subgroups were made *a posteriori*, for this analysis NI and LGI subgroups were combined. The Mann-Whitney U test was used to analyze effects of cysteine fortification: i) NI-Cys plus LGI-Cys *vs* NI-Ala plus LGI-Ala, ii) NI-Cys vs NI-Ala and iii) LGI-Cys *vs* LGI-Ala. Differences between NI-Ala and LGI-Ala, and between NI-Cys and LGI-Cys were also analyzed for studying the effect of basal low-grade inflammation using the Mann-Whitney U test. The Wilcoxon's signed rank test was used to compare paired-data obtained at different time-points within a group. These non-parametric tests were used since variances were not homogenous for most of the variables. P values $\leq$ 0.05 were considered significant. Statistical analyses were performed using StatView for Windows, version 5 software (SAS Institute, Cary, NC).

Results

Pre-experimental characteristics of the aged rats

Baseline plasma concentrations of α_2-macroglobulin and fibrinogen, body weight and body weight change from NI-Cys and LGI-Cys rats were not significantly different from those of respective Ala groups (Table 1).

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

According to the experimental design, baseline concentrations of α_2-macroglobulin were significantly higher in LGI rats than in NI rats, being 471 % higher in LGI-Ala rats and 436 % higher in LGI-Cys rats than in the corresponding NI groups. In the same way, baseline concentrations in fibrinogen were 27 % higher in LGI-Ala rats and 39 % higher in LGI-Cys rats than in the corresponding NI groups. Body weight and body weight change during the pre-experimental period were not significantly different between LGI-Ala and LGI-Cys groups. LGI-Ala rats were lighter than NI-Ala rats, reflecting that LGI-Ala had lost body weight during the pre-experimental period.

Mortality during dietary treatments

During the experimental period, mortality rate was 12, 9, 31 and 43 % in NI-Ala, NI-Cys, LGI-Ala and LGI-Cys groups, respectively. As expected, comparison of the survival curves generated by Kaplan-Meyer method (not shown) indicated that mortality was higher in LGI-Ala and LGI-Cys rats (prefrail at baseline) than in the corresponding NI groups (healthy at baseline) (Logrank test = 2.79, $P = 0.095$ and Logrank test = 4.95, $P = 0.026$, respectively). Mortality was not significantly different between NI-Ala and NI-Cys rats (Logrank test = 0.134, $P = 0.71$), neither between LGI-Cys and LGI-Ala rats (Logrank test = 0.035, $P = 0.85$). All the results described below were obtained from survivors (i.e. NI-Ala, $n = 49$, NI-Cys, $n = 52$, LGI-Ala, $n = 11$ and LGI-Cys, $n = 8$).

Food intake, body weight, and organ weights

Food intake of rats fed with Ala diet (NI-Ala plus LGI-Ala rats) decreased over time ($P = 0.0002$), whereas it was constant in rats fed with Cys diet (NI-Cys plus LGI-Cys) (Fig. 2). From wk 6 to wk 13 the food intake was 3 to 16 % higher in Cys-fed rats than in Ala-fed rats ($P < 0.0001$ to 0.03).

Final body weight was not significantly different between NI-Ala and NI-Cys rats ($P = 0.22$), neither between LGI-Ala and LGI-Cys rats ($P = 0.87$) (Table 2). Final body weight of LGI-Ala and LGI-Cys rats (prefrail at baseline) was respectively 11 and 15 % lower than in the corresponding NI groups (healthy at baseline).

Absolute and relative weights of liver were respectively 10 and 8 % higher in NI-Cys rats than in NI-Ala rats, but were not significantly different between LGI-Ala and LGI-Cys rats (Table 2). Liver relative weight in LGI-Ala and LGI-Cys rats was respectively 23 and 20 % higher in than in corresponding NI groups. Absolute and relative weights of small intestine and colon were not significantly different between NI-Ala and NI-Cys rats, neither between LGI-Ala and LGI-Cys rats. Relative weight of small intestine, and absolute and relative weight of colon were respectively 30, 12, and 27 % higher in LGI-Ala rats than in NI-Ala rats.

Cysteine fortification had no significant effect on kidney or skeletal muscle weights whatever the basal inflammatory status of the rats (not shown). Kidney weight in LGI-Ala and LGI-Cys rats was respectively 34 and 26 % higher than in the corresponding NI groups. Differences between LGI groups and the corresponding NI groups were not significant for all studied skeletal muscles, excepted for EDL which was 10 % lighter in LGI-Cys rats than in NI-Cys rats ($P = 0.043$).

Cysteine concentrations in plasma and splanchnic tissues

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

Plasma total non-protein cysteine concentration was not significantly different between NI-Ala and NI-Cys rats (P = 0.47), but was 40 % higher in LGI-Cys rats than in LGI-Ala rats (Fig. 3A). This variation reveals a corrective effect of dietary cysteine supplementation in rats being prefrail at baseline, since plasma total non-protein cysteine concentration was 21% lower in LGI-Ala rats than in NI-Ala rats. Plasma free cysteine (*i.e.* cysteine plus cystine) concentration in LGI-Cys rats was 50 % higher than in LGI-Ala rats and 23 % higher than in NI-Cys rats. Cysteine fortification did not significantly modify the amount of cysteine bound to plasma proteins through disulfide bridges (cys-prot). Nevertheless, this parameter was 34 % lower in LGI-Ala rats than in NI-Ala rats.

Total non-protein cysteine and cys-prot concentrations in liver and small intestine were not significantly affected by cysteine fortification, neither by the basal inflammatory status (Fig. 4A and 5A). In the liver, free cysteine concentration was significantly higher in the Cys-supplemented rats than in the Ala rats: 16 and 44 % higher in NI-Cys and LGI-Cys rats than in the corresponding Ala groups. In addition, free cysteine concentration in the liver was 16 % lower in LGI-Ala rats than in NI-Ala rats. In the small intestine, free cysteine concentration was 24 % higher in NI-Cys rats than in NI-Ala rats.

Splanchnic tissues glutathione concentrations and liver γ-glutamylcysteine synthetase activity

Liver glutathione concentration was significantly higher in Cys-supplemented rats than in the Ala rats: 15 and 20 % higher in NI-Cys and LGI-Cys rats than in the corresponding Ala groups. It was lower in rats being prefrail at baseline than in rats being healthy at baseline: 12 and 8 % lower in LGI-Ala and LGI-Cys rats than in the corresponding NI groups (Fig. 4B). Liver γ-glutamylcysteine synthetase activity was significantly lower in Cys-supplemented rats that in the Ala rats: 32 and 68 % lower in NI-Cys and LGI-Cys rats than in the corresponding Ala groups (Fig. 4C). This enzyme activity was 45 % higher in LGI-Ala rats than in NI-Ala rats.

Small intestine glutathione concentration was 3 % higher in NI-Cys rats than in NI-Ala rats and 5 % higher in LGI-Ala rats than in NI-Ala rats (Fig. 5).

Acute phase proteins and sTNFR-1 responses to cysteine fortification

At any time points of the kinetic (wk -2, wk 2, wk 6 and wk 13), plasma α_2-macroglobulin and fibrinogen concentrations were not significantly different between NI-Ala and NI-Cys rats and neither between LGI-Ala and LGI-Cys rats (P = 0.09 to 0.94) (Fig. 6). As expected, α_2-macroglobulin and fibrinogen were significantly higher in LGI-Ala and LGI-Cys rats (prefrail at baseline) than in the corresponding NI groups (healthy at baseline). Time-dependent increases in α_2-macroglobulin and fibrinogen concentrations were observed in all groups: i) final α_2-macroglobulin concentration was significantly higher than its basal value by 582, 670, 488 and 680 % in NI-Ala, NI-Cys, LGI-Ala and LGI-Cys rats, respectively, and ii) final fibrinogen concentration was significantly higher than its basal value by 36, 53, 48 and 32 % in NI-Ala, NI-Cys, LGI-Ala and LGI-Cys rats, respectively.

Plasma LBP and sTNFR-1 concentrations were quantified at wk -2, wk 6 and wk 13 (data not shown). At any time points, no significant difference in levels of LBP or sTNFR-I were observed between NI-Ala and NI-Cys rats and neither between LGI-Ala and LGI-Cys rats (P = 0.07 to 0.80). Time-dependent increases in LBP

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

and sTNFR-1 concentrations were observed in all groups: i) final LBP concentration was higher than its basal value by 114, 59, 222 and 167 % in NI-Ala, NI-Cys, LGI-Ala and LGI-Cys rats, respectively, ii) final sTNFR-1 concentration was higher than its basal value by 102, 79, 153, and 191 % in NI-Ala, NI-Cys, LGI-Ala and LGI-Cys rats, respectively.

Concentration of plasma albumin quantified at euthanasia was not significantly different between NI-Ala and NI-Cys rats and neither between LGI-Ala and LGI-Cys rats (data not shown). As expected, it was lower in rats being prefrail at baseline than in rats being healthy at baseline: 31 and 30 % lower in LGI-Ala and LGI-Cys rats than in respective NI groups. A strong correlation was observed between plasma albumin and plasma cys-prot concentrations ($r^2 = 0.277$, $P < 0.0001$).

Discussion

To our knowledge, this is the first study to determine whether long-term (14 wk) cysteine fortification (6.8 vs. 2.8 g/kg) can exert beneficial effects in NI (*i.e.* healthy at baseline) and LGI (*i.e.* prefrail at baseline) old rats. This is also the first study reporting that cysteine supplementation in the diet increased the spontaneous food intake of old rats.

In NI (*i.e.* healthy at baseline) rats, cysteine fortification was efficient to increase free cysteine and glutathione pools in liver and small intestine. In agreement with the idea that ageing is a cysteine deficient syndrome [21], these increases could result from a correction of age-induced decrease in cysteine and glutathione pools. One cannot rule out an accumulation above normal ranges. However, such an accumulation is rather unlikely for cysteine due to the strong regulation of free cysteine pool by oxidation and degradation, mainly by cysteine dioxygenase [22]. The increase of glutathione pool in liver is consistent with the fact that NAC supplementation (3 g/kg diet) was efficient to reverse the age-associated decrease in liver glutathione in old rats [17]. Similarly, in a elderly cohort, supplementation with NAC and glycine for 3 wk restored the concentration and the rate of synthesis of blood glutathione [15]. In the present study, the observed increase in liver glutathione pool could directly result from an increase in free cysteine since availability of cysteine is a rate limiting factor for glutathione synthesis [23]. The finding of reduced γ-glutamylcysteine synthetase activity with cysteine fortification further ruled out the possibility of an increase in its activity as the underlying cause for increased glutathione levels. The most logical explanation for this reduction in enzyme activity is the feedback inhibition exerted by glutathione itself [4]. In a previous study, heat treatment plus NAC also resulted in a similar inhibition of γ-glutamylcysteine synthetase activity [24]. Following increased glutathione concentration in the liver, a subsequent increase in the hepatic efflux rate and a more favourable uptake of glutathione by extra-hepatic tissues are expected. This would result in better protection against pathological oxidative challenges in aged rats, but this remains untested. Regardless of the mechanism, cysteine fortification-induced increases in free cysteine and glutathione pools in liver and small intestine did not affect the kinetics of inflammatory markers in rats that were healthy at baseline. This is in contrast to the reported effects of NAC in other models [12, 25, 26]. Further, cysteine fortification-induced effects on cysteine and glutathione pools did not affect final organ, muscle or body weights (except increase in liver weight), or mortality rate in rats that were healthy at baseline. This lack of an effect on body weight rejects the potential relationship between dietary cysteine and obesity [27]. The absence of exercise in the experimental protocol may explain the lack of effect on the skeletal muscle weight. Indeed, NAC supplementation plus physical activity have been reported to improve muscular performance,

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

especially in frail elderly subjects with poor plasma arginine levels [12]. Altogether, the present results support that a deficiency in cysteine or glutathione is not a key player in the age-associated development of inflammation in rats that are healthy at baseline. It rather reflects the development of a subclinical pathology as attested by the increased levels of inflammatory markers reached at the end of the experiment. It has already been stressed that the distinction between ageing *per se* and age-associated pathology is almost impossible [28].

In LGI (*i.e.* prefrail at baseline) rats, the major effects of cysteine fortification were similar to those observed in NI rats that were healthy at baseline *i.e.* an increase in plasma free and total non-protein cysteine pools, an increase in free cysteine and glutathione pools along with a decrease in the activity of γ-glutamylcysteine synthetase in the liver. These effects of cysteine fortification are considered beneficial since most of these parameters were altered in low-grade inflamed rats compared to healthy ones (*i.e.* in LGI-Ala vs. NI-Ala groups). Nevertheless, cysteine fortification did not alleviate the progression of inflammation in rats that showed low-grade inflammation at baseline. The mortality rate of LGI rats was higher than that for NI rats, and LGI survivors exhibited a lower body weight, higher intestine and kidneys weights than the NI survivors. At the end of the experiment, the inflammatory markers of LGI rats reached pathological levels [16]. These observations confirm that basal low-grade inflammation is a predictive factor of frailty and increased mortality [16, 29-31]. Altogether, cysteine fortification did not stop the progression from prefrailty to frailty and the basal low-grade inflammation seems to be a likely consequence of nascent pathologies.

The most unexpected result of our experiment was that cysteine fortification alleviated the age-associated decrease in food intake. Our data clearly show that the progressive age-related decrease of food intake observed in ageing rats was suppressed when the animals were supplemented with cysteine. This beneficial effect could be of great importance since decreased food intake has been reported as a robust factor in predicting mortality in hospitalized patients, being even superior to weight loss [32]. While further validation of this effect would be prudent, published data does indeed, support such an effect. In a model of LPS-induced anorexia in mice, it has been shown that food intake is higher when brain glutathione is higher [33]. Brain anti-oxidative defences, notably glutathione, are known to decrease with age [34]. It is possible that the increased availability of cysteine in old rats receiving cysteine fortification allowed a better transport of cysteine into the brain and/or a higher synthesis of glutathione in this organ. Indeed, the transport of cysteine into the brain has been shown to be an important determinant of brain glutathione [35]. In addition, NAC supplementation led to an enhancement of glutathione concentration in brain [14]. These data suggest that the maintenance of food intake in our Cys-supplemented rats is due to by an increase in brain glutathione. Unfortunately, this hypothesis cannot be confirmed since no brain sampling was performed in the present study. Other mechanisms cannot be ruled out, notably those related to pro-inflammatory cytokines playing a role in anorexia [36]. Such cytokines have been shown to be decreased in brain of aged rats receiving NAC along with α-tocopherol and α-lipoic acid [37].

In conclusion, long-term cysteine fortification, leading to the doubling of its dietary supply, appears to be safe (mortality and organ weights unchanged) and efficient in increasing the size of cysteine and glutathione pools in rats that were either healthy or prefrail at baseline. Unfortunately, it did not counteract the development of inflammation in rats that were non-inflamed at baseline nor did it stop the progression of inflammation in rats that showed low-grade inflammation at baseline. Age-associated inflammation seems to be rather a marker of subclinical and clinical pathology than a consequence of a possible cysteine/glutathione deficiency. The novel finding of the study, which needs to be confirmed, was that cysteine fortification inhibited the age-associated anorexia.

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

Acknowledgments The authors would like to acknowledge Marie Perrot for help in the statistical analysis, Jacques Vuichoud for determination of amino acids in the standard diet, the Staff of the Unité Expérimentale de Nutrition Comparée for animal care, and Viral Vishnuprasad Brahmbhatt (Nestlé Research Center) for improving the manuscript. This work was supported by Institut National de la Recherche Agronomique (INRA), France and Nestlé, Switzerland.

Conflict of interest K. Vidal, D. Breuillé and P. Serrant are employees of Nestec Ltd. K. Vidal, D. Breuillé, and I. Papet are co-inventors on a patent related to the present study. P. Denis, F. Glomot and F. Béchereau, no conflict of interest.

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

References

1. Fries JF (1983) The compression of morbidity. Milbank Memorial Fund Quarterly-Health and Society 61:397-419
2. Beckman KB, Ames BN (1998) The free radical theory of aging matures. Physiol Rev 78:547-581
3. Meng SJ, Yu LJ (2010) Oxidative Stress, Molecular Inflammation and Sarcopenia. Int J Mol Sci 11:1509-1526
4. Ballatori N, Krance SM, Notenboom S, Shi S, Tieu K, Hammond CL (2009) Glutathione dysregulation and the etiology and progression of human diseases. Biol Chem 390:191-214
5. Mosoni L, Breuillé D, Buffière C, Obled C, Patureau Mirand P (2004) Age-related changes in glutathione availability and skeletal muscle carbonyl content in healthy rats. Exp Gerontol 39:203-210
6. Lang CA, Naryshkin S, Schneider DL, Mills BJ, Lindeman RD (1992) Low blood glutathione levels in healthy aging adults. J Lab Clin Med 120:720-725
7. Julius M, Lang CA, Gleiberman L, Harburg E, DiFranceisco W, Schork A (1994) Glutathione and morbidity in a community-based sample of elderly. J Clin Epidemiol 47:1021-1026
8. Maher P (2005) The effects of stress and aging on glutathione metabolism. Ageing Res Rev 4:288-314
9. Obled C, Papet I, Breuillé D (2004) Sulfur-containing amino acids and glutathione in diseases. In: Cynober LA (ed) Metabolic and Therapeutic Aspects of Amino Acids in Clinical Nutrition. CRC Press, p 667-687
10. Obled C, Papet I, Breuille D (2002) Metabolic bases of amino acid requirements in acute diseases. Current Opinion in Clinical Nutrition and Metabolic Care 5:189-197
11. Breuillé D, Béchereau F, Buffière C, Denis P, Pouyet C, Obled C (2006) Beneficial effect of amino acid supplementation, especially cysteine, on body nitrogen economy in septic rats. Clin Nutr 25:634-642
12. Hauer K, Hildebrandt W, Sehl Y, Edler L, Oster P, Dröge W (2003) Improvement in muscular performance and decrease in tumor necrosis factor level in old age after antioxidant treatment. J Mol Med 81:118-125
13. Arranz L, Fernández C, Rodríguez A, Ribera JM, De la Fuente M (2008) The glutathione precursor N-acetylcysteine improves immune function in postmenopausal women. Free Radical Biol Med 45:1252-1262
14. Kanwar SS, Nehru B (2007) Modulatory effects of N-acetylcysteine on cerebral cortex and cerebellum regions of ageing rat brain. Nutr Hosp 22:95-100
15. Sekhar RV, Patel SG, Guthikonda AP, Reid M, Balasubramanyam A, Taffet GE, Jahoor F (2011) Deficient synthesis of glutathione underlies oxidative stress in aging and can be corrected by dietary cysteine and glycine supplementation. Am J Clin Nutr 94:847-853
16. Mayot G, Vidal K, Martin JF, Breuillé D, Blum S, Obled C, Papet I (2007) Prognostic values of alpha2-macroglobulin, fibrinogen and albumin in regards to mortality and frailty in old rats. Exp Gerontol 42:498-505
17. Grattagliano I, Portincasa P, Cocco T, Moschetta A, Di Paola M, Palmieri VO, Palasciano G (2004) Effect of dietary restriction and N-acetylcysteine supplementation on intestinal mucosa and liver mitochondrial redox status and function in aged rats. Exp Gerontol 39:1323-1332

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

18. Malmezat T, Breuillé D, Pouyet C, Patureau Mirand P, Obled C (1998) Metabolism of cysteine is modified during the acute phase of sepsis in rats. J Nutr 128:97-105
19. Gaitonde MK (1967) A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. Biochem J 104:627-633.
20. Malloy MH, Rassin DK, Gaull GE (1981) A method for measurement of free and bound plasma cyst(e)ine. Anal Biochem 113:407-415
21. Dröge W (2005) Oxidative stress and ageing: is ageing a cysteine deficiency syndrome? Phil Trans R Soc B 360:2355-2372
22. Stipanuk MH, Ueki I, Dominy JE, Simmons CR, Hirschberger LL (2009) Cysteine dioxygenase: a robust system for regulation of cellular cysteine levels. Amino Acids 37:55-63
23. Griffith OW (1999) Biologic and pharmacologic regulation of mammalian glutathione synthesis. Free Radical Biol Med 27:922-935
24. Morrison JP, Coleman MC, Aunan ES, Walsh SA, Spitz DR, Kregel KC (2005) Thiol supplementation in aged animals alters antioxidant enzyme activity after heat stress. J Appl Physiol 99:2271-2277
25. Hack V, Breitkreutz R, Kinscherf R, Röhrer H, Bärtsch P, Taut F, Benner A, Dröge W (1998) The redox state as a correlate of senescence and wasting and as a target for therapeutic intervention. Blood 92:59-67
26. Kim CJ, Kovacs-Nolan J, Yang C, Archbold T, Fan MZ, Mine Y (2009) L-cysteine supplementation attenuates local inflammation and restores gut homeostasis in a porcine model of colitis. Biochim Biophys Acta 1790:1161-1169
27. Elshorbagy AK, Smith AD, Kozich V, Refsum H (2012) Cysteine and obesity. Obesity 20:473-481
28. Ferrucci L (2008) The Baltimore Longitudinal Study of Aging (BLSA): A 50-Year-Long Journey and Plans for the Future. Journals of Gerontology Series a-Biological Sciences and Medical Sciences 63:1416-1419
29. Cohen HJ, Harris T, Pieper CF (2003) Coagulation and activation of inflammatory pathways in the development of functional decline and mortality in the elderly. Am J Med 114:180-187
30. Ferrucci L, Penninx BWJH, Volpato S, Harris TB, Bandeen-Roche K, Balfour J, Leveille SG, Fried LP, Guralnick JM (2002) Change in muscle strength explains accelerated decline of physical function in older women with high interleukin-6 serum levels. J Am Geriatr Soc 50:1947-1954
31. Harris TB, Ferrucci L, Tracy RP, Corti MC, Wacholder S, Ettinger WHJ, Heimovitz H, Cohen HJ, Wallace R (1999) Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly. Am J Med 106:506-512
32. Landi F, Laviano A, Cruz-Jentoft AJ (2010) The Anorexia of Aging: Is It a Geriatric Syndrome? J Am Med Dir Assoc 11:153-156
33. Hernadfalvi N, Langhans W, von Meyenburg C, Onteniente B, Richard D, Arsenijevic D (2007) Role for glutathione in the hyposensitivity of LPS-pretreated mice to LPS anorexia. Eur Cytokine Netw 18:86-92
34. Chen TS, Richie JP, Jr., Lang CA (1989) The effect of aging on glutathione and cysteine levels in different regions of the mouse brain. Proc Soc Exp Biol Med 190:399-402

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

35. Aoyama K, Suh SW, Hamby AM, Liu J, Chan WY, Chen Y, Swanson RA (2006) Neuronal glutathione deficiency and age-dependent neurodegeneration in the EAAC1 deficient mouse. Nat Neurosci 9:119-126

36. Gautron L, Layé S (2009) Neurobiology of Inflammation-Associated Anorexia. Frontiers in Neuroscience 3:59

37. Thakurta IG, Chattopadhyay M, Ghosh A, Chakrabarti S (2012) Dietary supplementation with N-acetyl cysteine, α-tocopherol and α-lipoic acid reduces the extent of oxidative stress and proinflammatory state in aged rat brain. Biogerontology 13:479-488

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

Figure captions

Fig.1. Experimental design.

Fig. 2. Effect of cysteine fortification on food intake in ageing rats.
Ala: combined NI-Ala plus LGI-Ala groups, Cys: combined NI-Cys plus LGI-Cys groups.

Fig. 3. Effect of cysteine fortification on plasma non-protein cysteine concentration in old rats being non-inflamed or low-grade inflamed at baseline.
Bars are means ± SEM. Panel A, upper SEM stand for total cysteine, lower SEM for free cysteine or protein-linked cysteine. Symbols on the bars illustrate significant difference for free cysteine or protein-linked cysteine, symbols above the bars for total (free plus protein-linked) cysteine. *, ** different from corresponding NI group $P < 0.04$ and $P < 0.0007$, respectively. † different from corresponding Ala group $P < 0.006$.

Fig. 4. Effect of cysteine fortification on liver non-protein cysteine and glutathione concentrations and on γ-glutamylcysteine synthetase (γ-GCS) activity in old rats being non-inflamed or low-grade inflamed at baseline.
Bars are means ± SEM. Panel A, upper SEM stand for total cysteine, lower SEM for free cysteine or protein-linked cysteine. Symbols on the bars illustrate significant difference for free cysteine. * different from corresponding NI group $P < 0.05$. †, †† different from corresponding Ala group $P < 0.02$ and $P < 0.0004$, respectively.

Fig. 5. Effect of cysteine fortification on small intestine non-protein cysteine and glutathione concentrations in old rats being non-inflamed or low-grade inflamed at baseline.
Bars are means ± SEM. Panel A, upper SEM stand for total cysteine, lower SEM for free cysteine or protein-linked cysteine. Symbols on the bars illustrate significant difference for free cysteine. * different from corresponding NI group $P < 0.04$. †, †† different from corresponding Ala group $P < 0.03$ and $P < 0.0001$, respectively.

Fig. 6. Effect of cysteine fortification on plasma acute phase protein kinetics in ageing rats being non-inflamed or low-grade inflamed at baseline.
Bars are means ± SEM. $^{a, b, c, d}$ labelled bars within a group without a common letter differ, $P < 0.05$. * different from the same time point of the corresponding NI group $P < 0.03$.

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

Table 1. Plasma acute phase protein concentrations, body weight and body weight change before dietary supplementation[$]

Group	NI-Ala (n = 56)	NI-Cys (n = 57)	LGI-Ala (n = 16)	LGI-Cys (n = 14)
α_2-macroglobulin at wk -2, *mg/L*	35 ± 3	33 ± 2	202 ± 22***	177 ± 25***
Fibrinogen at wk -2, g*/L*	3.07 ± 0.10	2.90 ± 0.08	3.91 ± 0.20**	4.04 ± 0.18**
Body weight at wk 0, *g*	664 ± 11	659 ± 9	612 ± 13*	646 ± 25
Pre-experimental body weight change, *%/13 wk*	0.15 ± 0.80	1.54 ± 0.57	-2.53 ± 0.93*	-0.74 ± 1.39

[$] Data are means ± SEM. *, **, *** different from corresponding NI group $P < 0.02$, $P < 0.001$, $P < 0.0001$, respectively

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

Table 2. Effects of cysteine fortification on body and organ weights in old rats being non-inflamed or low-grade inflamed at baseline[$]

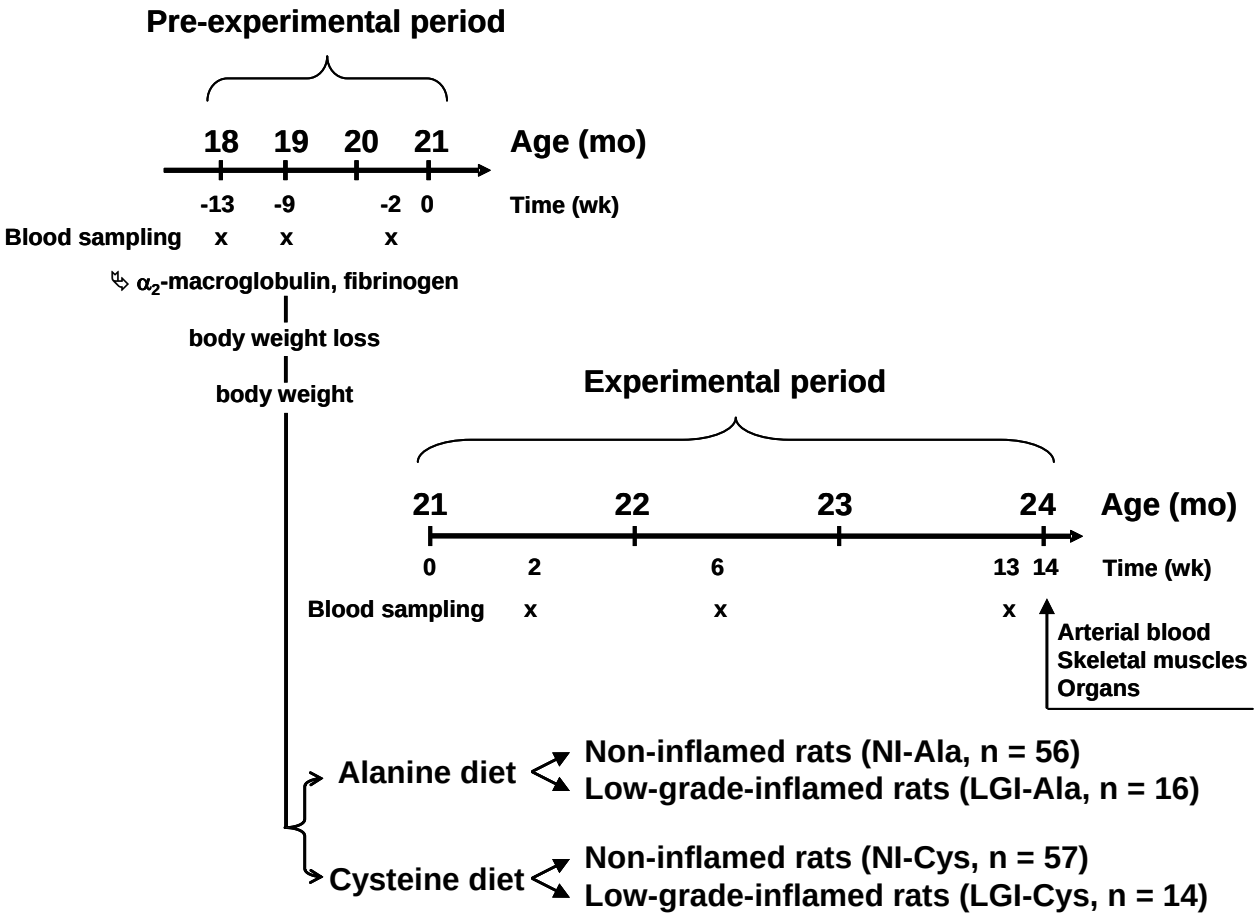
Group	NI-Ala (n = 49)	NI-Cys (n = 52)	LGI-Ala (n = 11)	LGI-Cys (n = 8)
Final body weight (BW), *g*	609 ± 15	627 ± 11	539 ± 19**	533 ± 46*
Liver, *g*	18.1 ± 0.6	19.9 ± 0.5†	19.6 ± 1.0	20.0 ± 1.3
Liver, *% BW*	2.96 ± 0.05	3.19 ± 0.06†	3.63 ± 0.12***	3.83 ± 0.19**
Small intestine, *g*	11.2 ± 0.3	12.0 ± 0.2	12.6 ± 0.7	11.2 ± 0.8
Small intestine, *% BW*	1.84 ± 0.03	1.93 ± 0.04	2.39 ± 0.22***	2.13 ± 0.10
Colon, *g*	2.62 ± 0.07	2.76 ± 0.06	2.94 ± 0.09*	2.53 ± 0.23
Colon, *% BW*	0.434 ± 0.008	0.444 ± 0.011	0.551 ± 0.024***	0.486 ± 0.038

[$] Data are means ± SEM. † different from NI-Ala $P < 0.03$. *, **, *** different from corresponding NI group $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

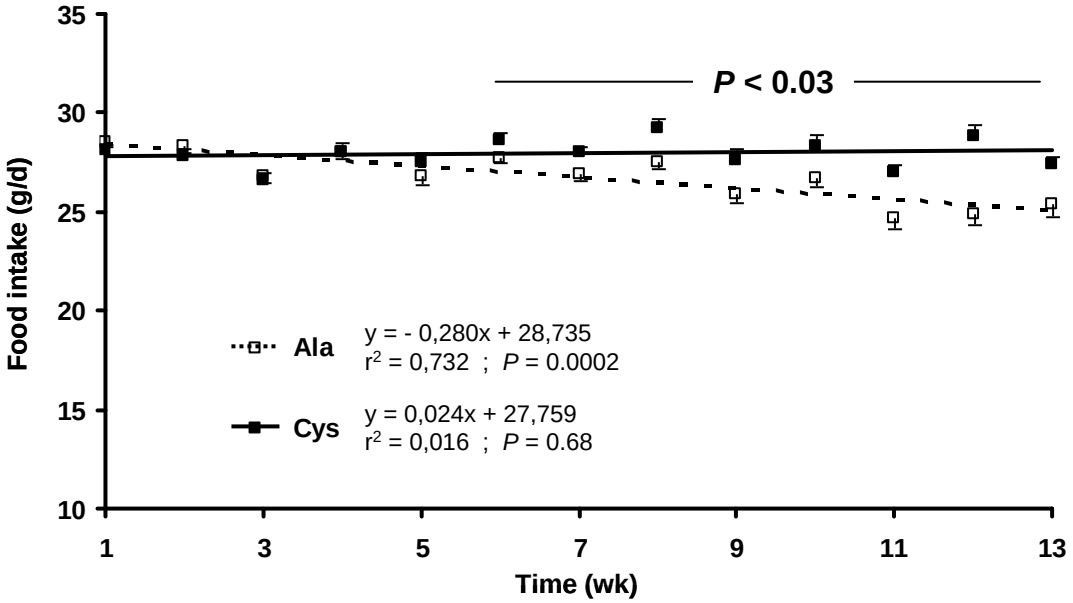
Fig. 1. Experimental design.



Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

Fig. 2. Effect of cysteine fortification on food intake in ageing rats.

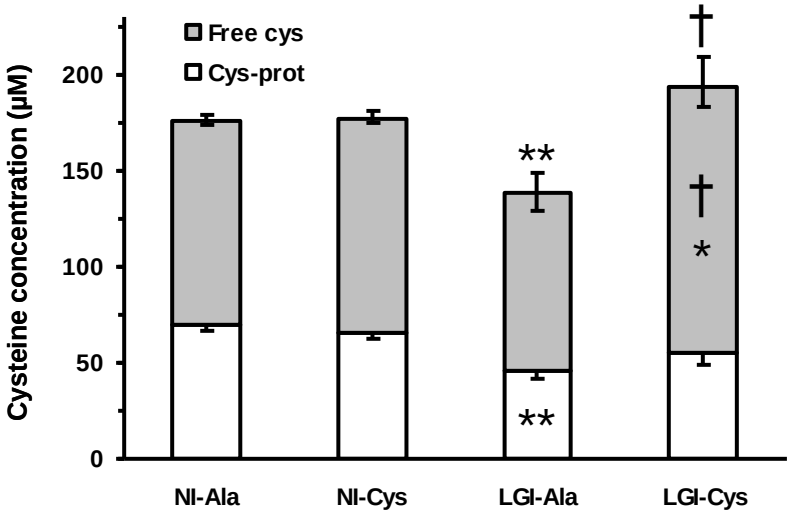


Ala: combined NI-Ala plus LGI-Ala groups, Cys: combined NI-Cys plus LGI-Cys groups.

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in
ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

Fig. 3. Effect of cysteine fortification on plasma non-protein cysteine concentration in old rats being non-inflamed or low-grade inflamed at baseline.

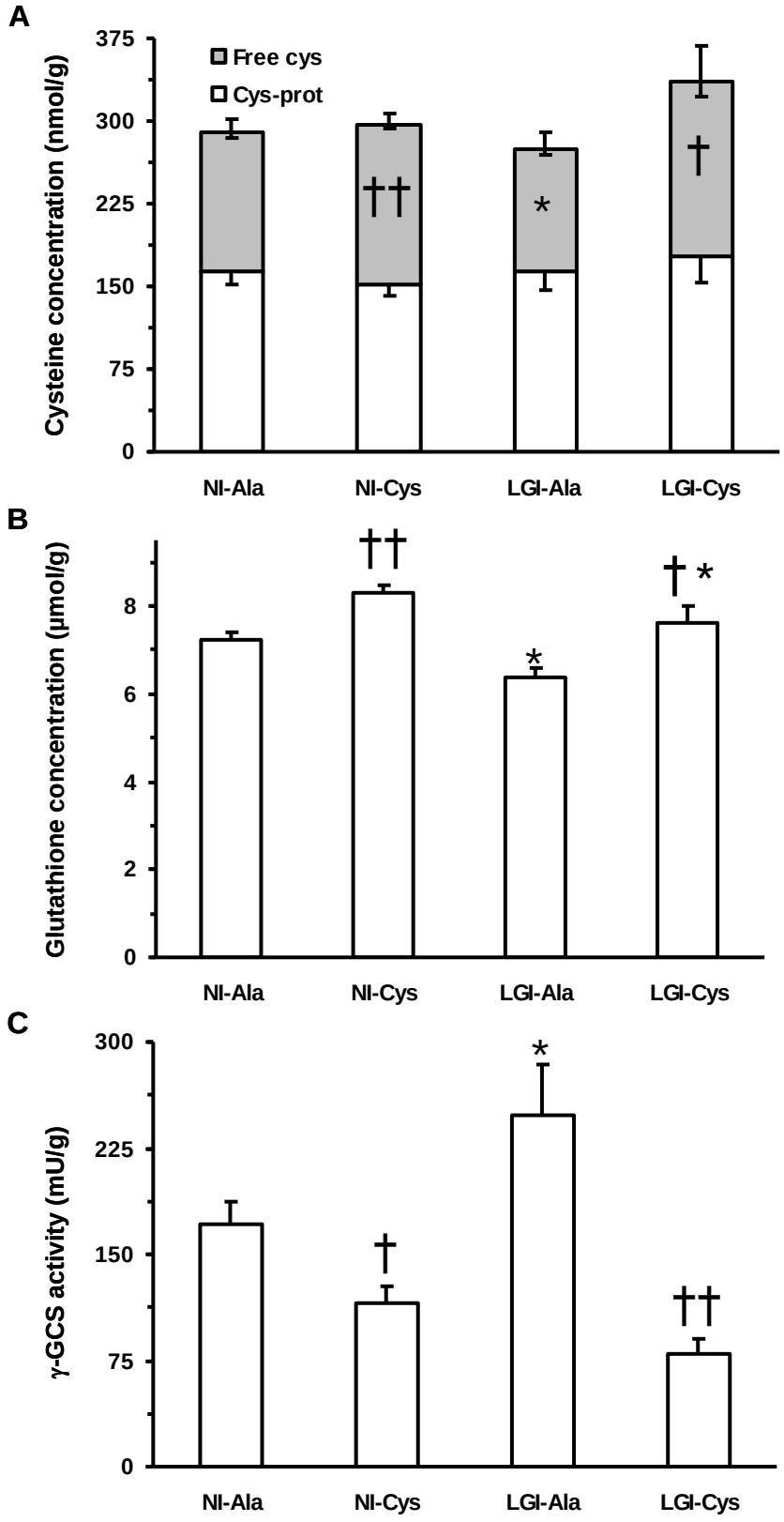


Bars are means ± SEM. Panel A, upper SEM stand for total cysteine, lower SEM for free cysteine or protein-linked cysteine. Symbols on the bars illustrate significant difference for free cysteine or protein-linked cysteine, symbols above the bars for total (free plus protein-linked) cysteine. *, ** different from corresponding NI group $P < 0.04$ and $P < 0.0007$, respectively. † different from corresponding Ala group $P < 0.006$.

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

Fig. 4. Effect of cysteine fortification on liver non-protein cysteine and glutathione concentrations and on γ-glutamylcysteine synthetase (γ-GCS) activity in old rats being non-inflamed or low-grade inflamed at baseline.

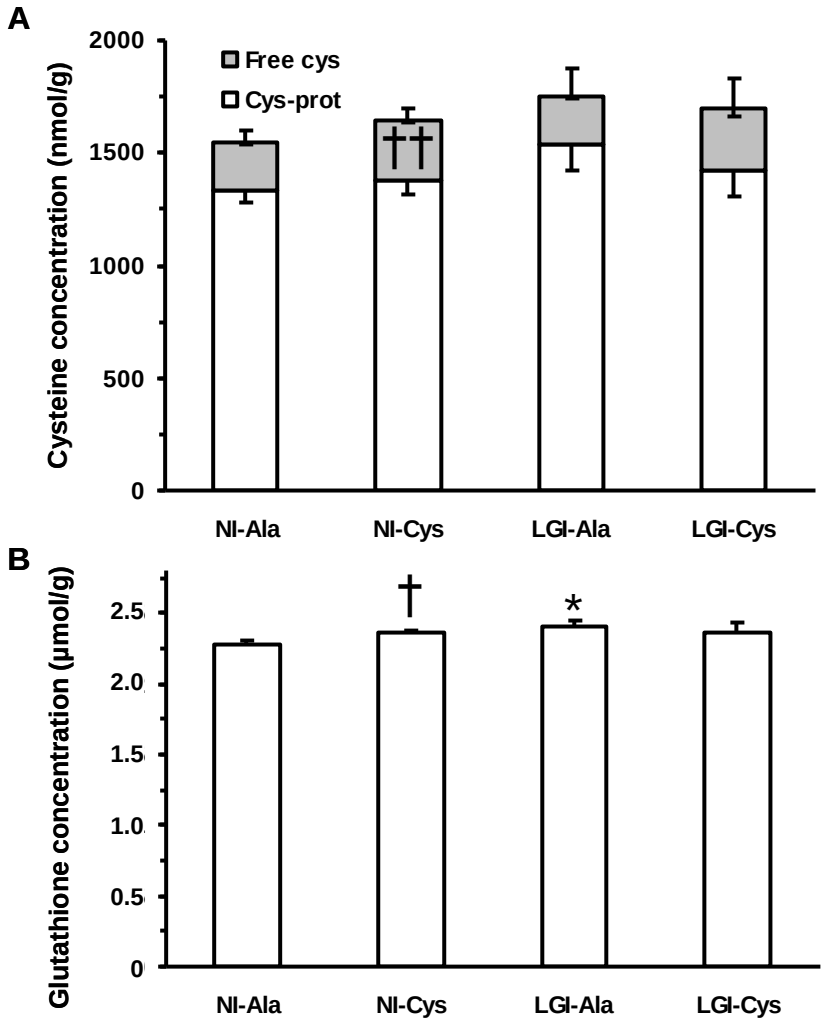


Bars are means ± SEM. Panel A, upper SEM stand for total cysteine, lower SEM for free cysteine or protein-linked cysteine. Symbols on the bars illustrate significant difference for free cysteine. * different from corresponding NI group $P < 0.05$. †, †† different from corresponding Ala group $P < 0.02$ and $P < 0.0004$, respectively.

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

Fig. 5. Effect of cysteine fortification on small intestine non-protein cysteine and glutathione concentrations in old rats being non-inflamed or low-grade inflamed at baseline.

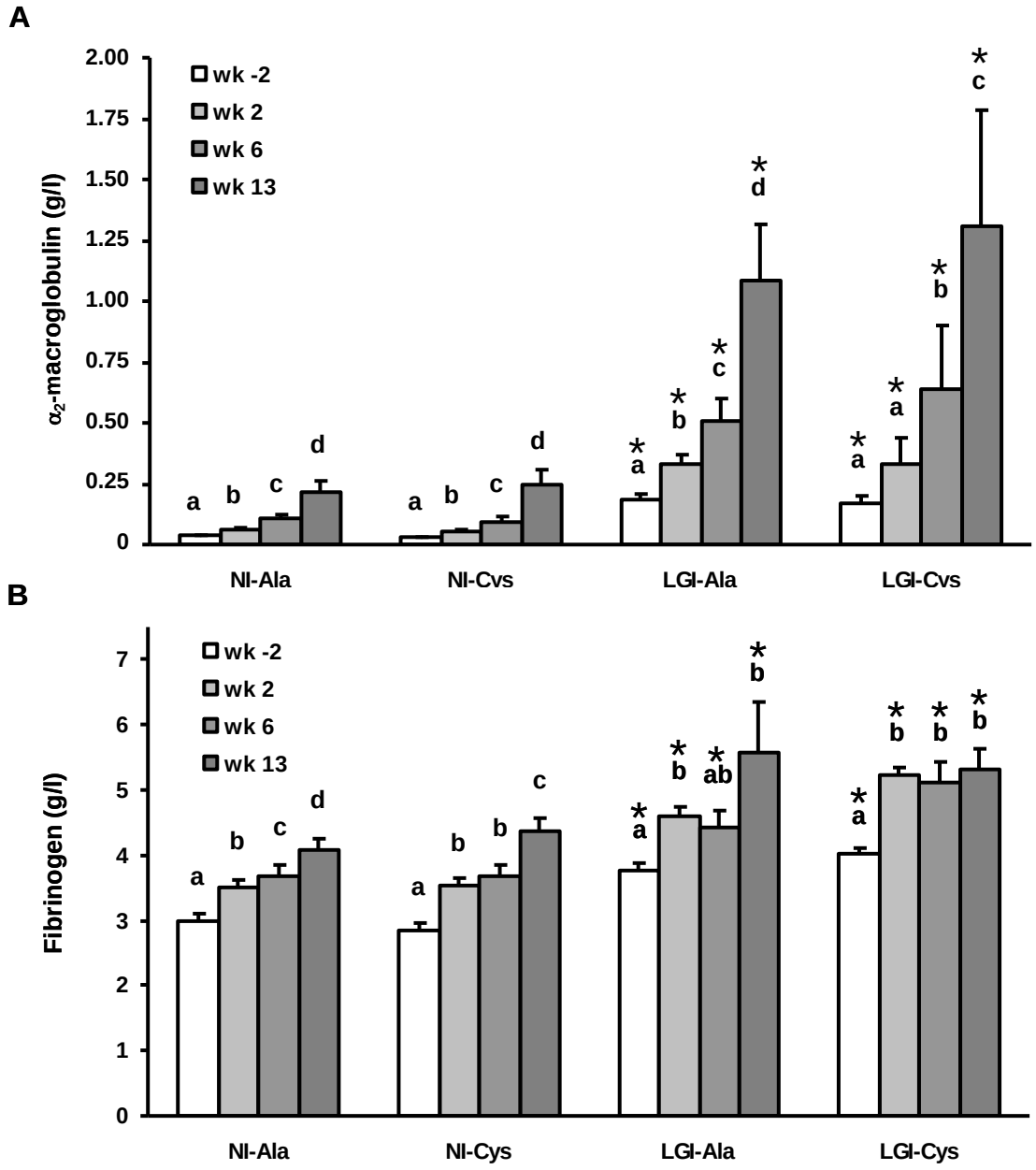


Bars are means ± SEM. Panel A, upper SEM stand for total cysteine, lower SEM for free cysteine or protein-linked cysteine. Symbols on the bars illustrate significant difference for free cysteine. * different from corresponding NI group $P < 0.04$. †, †† different from corresponding Ala group $P < 0.03$ and $P < 0.0001$, respectively.

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0

Fig. 6. Effect of cysteine fortification on plasma acute phase protein kinetics in ageing rats being non-inflamed or low-grade inflamed at baseline.



Bars are means ± SEM. [a, b, c, d] labelled bars within a group without a common letter differ, $P < 0.05$. * different from the same time point of the corresponding NI group $P < 0.03$.

Version postprint

Comment citer ce document :
Vidal, K., Breuille, D., Serrant, P., Denis, P., Glomot, F., Bechereau, F., Papet, I. (2014).
Long-term cysteine fortification impacts cysteine/glutathione homeostasis and food intake in ageing rats. European Journal of Nutrition, 53 (3), 963 - 971. , DOI : 10.1007/s00394-013-0600-0