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ORIGINAL CONTRIBUTION

Aurélie Chanson
Edmond Rock
Jean-François Martin
Anne Liotard
Patrick Brachet

Preferential response of glutathione-related enzymes to folate-dependent changes in the redox state of rat liver

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A. Chanson · E. Rock · J.-F. Martin
A. Liotard · P. Brachet
Unité de Nutrition Humaine
Institut National de la Recherche Agronomique
Centre de Theix/Clermont-Ferrand
Saint-Genès Champanelle, France

P. Brachet, PhD (✉)
Stress Métabolique et Micronutriments
UMR 1019, INRA/Université d'Auvergne
Unité de Nutrition Humaine
Centre INRA de Theix
63122 Saint-Genès Champanelle, France
Tel.: +33-47/3624563
Fax: +33-47/3624638
E-Mail: brachet@clermont.inra.fr

Abbreviations: C diet: control diet, CAT: catalase, FA: folic acid, FD diet: folate-depleted diet, FS diet: folate-supplemented diet, CFD diet: completely FD diet, FRAP: ferric reducing ability of plasma, Hcy: homocysteine, HFS diet: high-level FS diet, H_2O_2: hydrogen peroxide, GPx: glutathione peroxidase, GR: glutathione reductase, GSH: reduced glutathione, GSS: GSH synthase, GSSG: oxidised glutathione, GST: glutathione S transferase, LFS diet: low-level FS diet, MFD: moderately FD diet, NTDs: neural tube defects, $O2^{\circ-}$: superoxide anion, PCA: principal component analysis, Prx: peroxiredoxin, RBC: red blood cells, ROS: reactive oxygen species, SOD: superoxide dismutase, TBARS: thiobarbituric acid reactive substances, tHcy: total Hcy

Abstract *Background* Oxidative stress likely constitutes an important contributing factor in the onset of degenerative diseases associated with folate deficiency. Direct, as well as homocysteine-linked, antioxidant properties of folate could explain its preventive effect on these pathologies. *Aim of the study* Our study aimed at determining the changes in the redox status of adult rats as a function of folate intake. *Methods* Adult male rats were pair-fed for 4 weeks with a semi-synthetic diet containing 0, 0.5, 1.5, 8 or 20 mg of folic acid/kg. Folate and homocysteine concentrations, redox status markers and antioxidant enzyme activities were measured in the plasma and/or liver of the rats. A principal component analysis of the overall data was performed to draw a general scheme of the changes observed between the conditions. *Results* Folate deficiency caused increased homocysteinemia and features of oxidative stress including reduced plasma antioxidant capacity together with increased lipid peroxidation in liver and heart. This was associated with an increase in the specific activity of several enzymes involved in liver glutathione metabolism (glutathione peroxidase, glutathione reductase and glutathione S-transferase), suggesting an adaptive tissue response to the oxidative stress induced by folate deficiency. In contrast, no such variation was observed for hepatic superoxide dismutase and catalase. *Conclusion* Despite no changes in hepatic levels of total glutathione, our findings indicate that glutathione-dependent antioxidant pathways could be particularly involved in the compensatory mechanism committed by liver to counteract the oxidative stress induced by folate deficiency. They also suggest that folate supplementation may not be associated with a better antioxidant protection of rats.

Key words folic acid – homocysteine – glutathione – anti-oxidant enzymes – oxidative stress – folate status

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Introduction

Oxidative stress is a major contributing factor in degenerative human diseases [1]. Folate deficiency can induce oxidative stress, as shown by increased tissue or plasma lipid peroxidation, in animal models [2–5]. One of the deleterious effects of folate deficiency is the decreased transfer of methyl groups from 5-methyltetrahydrofolate (5-mTHF) to homocysteine (Hcy) and, subsequently, the diminution of S-adenosylmethionine and methionine levels and the augmentation of S-adenosylhomocysteine and Hcy levels [6]. Elevated levels of plasma total Hcy (tHcy) may have a pro-oxidant effect and a role in the production of reactive oxygen species (ROS) that results in oxidative damage to tissues [7]. It has notably been associated with increased lipid and protein oxidation, and formation of hydrogen peroxide (H_2O_2), superoxide anion ($O2^{\circ -}$) and peroxynitrite [8, 9]. Peroxynitrite is a potent oxidant that is produced from the reaction between $O2^{\circ -}$ and the vasodilator nitric oxide (NO), thus also reducing bioavailability of endothelium-derived NO [10]. In addition, it may contribute to uncoupling of endothelial nitric oxide synthase (eNOS) during hyperhomocysteinemia yielding $O2^{\circ -}$ rather than NO production. Recent data indicate that hyperhomocysteinemia is also associated with elevated level of plasma asymmetrical dimethylarginine (ADMA) which can further contribute to reduced NO bioavailability and increased oxidative stress through inhibition of NOS and uncoupling of eNOS [11]. All the previous pro-oxidant mechanisms could be involved in the increased risk of age-associated degenerative diseases caused by folate deficiency and subsequent hyperhomocysteinemia [12].

Folate is currently under intense scrutiny because of its beneficial effect on the prevention of neural tube defects (NTDs) [13]. Numerous studies have also focused on the Hcy-lowering impact of folate and its possible protective effects against chronic diseases [14]. However, a beneficial effect of folate on endothelial function of patients has recently been suggested to occur through an Hcy-independent pathway [15, 16]. Direct antioxidant properties of folate have been observed in vitro [17–19] and in vivo in patients with hyperhomocysteinemia [20]. However, this hypothesis was not supported by the absence of changes in plasma pro- and antioxidant status in patients with coronary artery disease supplemented with folic acid [16].

By using a proteomic approach we recently showed that the abundance of the antioxidant enzymes glutathione peroxidase 1 (GPx 1) and, to a lesser extent, peroxiredoxin 6 (prx 6) was significantly increased in the liver of folate-deficient, 4 month-old rats [21]. We hypothesized from these data that many antioxidant enzymatic pathways – unrelated (superoxide dismutase, SOD; catalase, CAT) or related to hepatic glutathione homeostasis (GPx, glutathione reductase, GR; glutathione S-transferase, GST) – could be engaged in this tissue to counteract the oxidative stress induced by folate deficiency. Moreover, there have been, to our knowledge, no studies to test the effect of increasing intake of folic acid, from total depletion to high-level supplementation, on the plasma and tissue redox state in the adult rat. The present study was therefore designed to address the previous issues.

Materials and Methods

Diets, animals and tissue samplings

Pellets of semi-synthetic, L-amino acid-defined diets containing the antibiotic succinylsulfothiazole (1% w/w) and supplemented with 0, 0.5, 1.5, 8 or 20 mg folic acid/kg diet were prepared by the INRA - Unité de Préparation des Aliments Expérimentaux (Jouy en Josas, France), so that they only differed in their folic acid content. As previously reported [21], the diet supplemented with 1.5 mg folic acid/kg met the folate requirement of rats and was thus considered as the control (C) diet. The diets containing 0 and 0.5 mg folic acid/kg were denominated the completely and moderately folate-depleted (CFD and MFD) diets, respectively. The diets with 8 and 20 mg folic acid/kg were the low-level and high-level folate-supplemented (LFS and HFS) diets, respectively.

Male Wistar rats (n = 40, 4-month-old, starting weight, mean ± S.E.M.: 483 ± 15 g), obtained from the INRA - Unité Expérimentale de Nutrition Comparée (Theix, France), were raised and sacrificed as described in [21], however, with some modifications. They were housed individually in cages placed in a room maintained at 20–23°C, with an inverse 12-h dark/12-h light cycle. After a 3-week acclimation period where they were fed the C diet, the rats were randomly maintained on the C diet or assigned to one of the above-mentioned experimental diets and pair-fed for an experimental period of 4 weeks with free access to water. The rats were weighed three times a week and their food consumption was monitored daily. Blood and tissue samplings, hematocrit measurement, plasma preparation and sample storage were carried out as described previously [21]. For analysis of red blood cells (RBC) folate, blood aliquots were diluted with 10 volumes of 57 mM ascorbic acid, and then incubated during 1 h in a shaking water-bath at 37°C in order to hydrolyse RBC folylpolyglutamates by action of endogenous plasma conjugase [22]. This study was approved by the Ethical Committee of INRA – Theix Research Center.

Folate and homocysteine assays

Liver extracts for folate assay were prepared as described previously [21]. Plasma, RBC and liver folate concentrations were measured by a microbiological assay using *Lactobacillus casei* ATCC 7469 (*Lactobacillus rhamnosus*; Institut Pasteur, Paris, France) and free folic acid-casei medium (Difco Labs, Sparks, MD, USA) [21, 22]. Plasma tHcy concentrations were determined by HPLC and fluorometric detection (Waters, Guyancourt, France) following the procedure described in [23].

Oxidative stress markers

To estimate lipid peroxidation in tissues, heart and liver homogenates were prepared on ice (1 g wet tissue homogenised in 9 ml of 150 mM KCl using a Polytron homogeniser). Thiobarbituric acid-reactive substances (TBARS) concentrations were measured in homogenates after lipid peroxidation induced in vitro with $FeSO_4$ (2 μM) and ascorbic acid (50 μM) for 30 min at 37°C in an oxygen-free medium, using a standard of 1,1,3,3-tetraethoxypropane as previously described [24]. Plasma vitamin E (α-tocopherol) was analysed by HPLC-UV (Waters) as described previously [25], using α-tocopherol acetate (Sigma, Saint-Quentin-Fallavier, France) as internal standard and carrying out elution on a Nucleosil 250 × 4.6-mm C_{18} column (5-μm particle size; Interchim, Montlucon, France) with pure methanol as the mobile phase (flow rate : 2 ml/min). Ferric reducing ability of plasma (FRAP) was determined on 100 μL of plasma samples diluted twice with deionized water, as described in [26].

Assay of liver enzyme activities

Frozen pieces of liver (100 mg for CAT assay, 500 mg for the other enzyme assays) were homogenized in cold buffer (20 volumes of 50 mM of phosphate buffer pH 7 for CAT (EC 1.11.1.6) assay, 10 volumes of 100 mM of phosphate buffer pH 7.4 containing 0.13 mM of butylated hydroxytoluene and 1 mM of EDTA for the other enzymatic assays) using a Polytron homogeniser. Specific enzyme activities (expressed as U/mg protein) were measured in 1,000 g supernatants using a Cobas Mira analyser (Roche Diagnostics, Basel, Switzerland), except for CAT activity where an Uvikon 941 plus series spectrophotometer was used. CAT, GPx (EC 1.11.1.9), GR (EC 1.6.4.2) and GST (EC 2.5.1.18) were assayed as described in [27–29]. SOD (EC 1.15.1.1) activity was measured by using the RANSOD kit (Randox Laboratories, Montpellier, France) according to the supplier's instructions. The liver protein content was estimated by using the bicinchoninic acid procedure (Sigma) [30].

Liver glutathione determination

For total glutathione (GSH plus GSSG) measurement, frozen pieces of liver were homogenized in a solution of 0.2 M perchloric acid and 5 mM EDTA using a Polytron homogeniser. The homogenate was then centrifuged at 14,000 g for 15 min at 4°C. The supernatant was assessed for total glutathione content using a standard enzymatic recycling procedure and a Cobas Mira analyser, as previously reported [29].

Statistical analysis

Results were expressed as means ± S.E.M. One-way analysis of variance (ANOVA) was used to determine whether any differences between means of the five rat groups were greater than would be expected by chance. When ANOVA F test was significant, a Tukey post hoc test for pairwise comparisons was performed. Log transformation was applied in case of non-Gaussian distribution of data. Principal components analysis (PCA) was additionally used to globally estimate the changes in the different parameters measured in response to the various levels of dietary folate. This allowed to reduce the dimensionality of the data and to produce graphical representation of both the measured parameters (variables) and the rats (samples). Moreover, correlation between variables was assessed by linear regression analysis using the Pearson correlation coefficient. All statistical analyses were performed using statistical package XLSTAT version 7.5 (Addinsoft, Paris, France).

Results

Effect of folic acid intake on homocysteine and folate concentrations

Four-month old rats did not exhibit significantly altered weight, food intake and hematocrit after feeding either experimental diet for 4 weeks (not shown). As shown in Table 1, feeding increasing amounts of dietary folic acid (from 0 to 20 mg folic acid/kg diet) led to a gradually increase in the plasma, RBC and liver folate concentrations of rats. More exactly, both CFD and MFD rats had significantly decreased folate status, concomitantly with

Table 1 Homocysteine and folate status of rats fed increasing amounts of folic acid for 4 weeks

	Diet (mg folic acid/kg diet)				
	CFD (0)	MFD (0.5)	C (1.5)	LFS (8)	HFS (20)
Plasma Hcy, μM	37.6 ± 2.7[a]	20.0 ± 0.7[b]	14.7 ± 0.6[c]	12.4 ± 1.3[c]	14.4 ± 1.1[c]
Plasma folate, nM	4.6 ± 1.3[a]	24.3 ± 7.1[b]	35.6 ± 2.8[c]	169.8 ± 6.7[d]	222.9 ± 7.2[d]
RBC folate, nM	800 ± 41[a]	911 ± 95[a]	1239 ± 86[b]	1463 ± 117[b]	2377 ± 211[c]
Liver folate, nmol/g wet tissue	4.1 ± 0.5[a]	13.5 ± 0.9[b]	21.2 ± 1.5[c]	28.0 ± 1.8[d]	31.1 ± 0.9[d]

Results are given as mean ± SEM of 8 rats in each group. One-way analysis of variance (ANOVA) was used to determine significance of the differences between means in the 5 groups of folic acid intake. Values on the same line with a different superscript letter are significantly different at $P < 0.05$.

increased homocysteinemia as compared with the C rats, with the most dramatic changes for the CFD group. The MFD rats showed significantly higher plasma and liver folate concentrations and lower homocysteinemia than the CFD rats. The RBC folate status of the MFD rats was also higher than that of the CFD rats, but the difference was not significant. These parameters showed less variations in rats fed the LFS or HFS diet, except for RBC folate the level of which was strongly increased in HFS rats comparatively to C rats. Both FS groups displayed significantly increased plasma and liver folate concentrations when compared to the C group, however, without any variation at the level of homocysteine status. Moreover, the RBC folate concentration was not different between the LFS and the C rats.

Effect of folic acid intake on redox markers and antioxidant enzymes

Changes in FRAP value and plasma concentration of α-tocopherol occurred as a function of the dietary folate supply (Table 2). However, the differences were only significant between the HFS condition and the MFD or CFD condition for FRAP and between the HFS or the C condition and the CFD condition for α-tocopherol. This was concurrent with a significant decrease in the susceptibility to oxidation of heart and liver lipids, as evidenced by higher *ex vivo* induction of TBARS formation by ferrous ions in tissue homogenates of CFD rats when compared to rats of the all other groups. Additionally, measurement of enzyme activities in the liver homogenates indicated that folate deprivation (CFD condition) led to a significant elevation in the specific activity of antioxidant GPx as compared with the C, LFS and HFS conditions. GPx activities were similar in the latter three conditions, while specific activities of antioxidant SOD and CAT were steady in all groups. A trend for a global increase in the specific activities of two other enzymes involved in hepatic glutathione homeostasis, namely GR and GST, was also observed when the folic acid supply decreased from 8 to 0 mg/kg diet. This was not accompanied by variations in the levels of liver total glutathione. It is noteworthy that no significant differences were observed in biomarkers of plasma (FRAP, α-tocopherol) and tissue (liver GPx, GR, SOD and CAT activities, liver total glutathione levels)

Table 2 Redox markers and hepatic activities of glutathione homeostasis enzymes in rats fed increasing amounts of folic acid for 4 weeks

	Diet: mg folic acid/kg				
	CFD: 0	MFD: 0.5	C: 1.5	LFS: 8	HFS: 20
FRAP mM	185.7 ± 8.2[a]	195.0 ± 10.3[a]	218.6 ± 7.4[ab]	210.3 ± 11.1[ab]	237.1 ± 9.0[b]
Plasma α-tocopherol μM	15.7 ± 0.6[a]	21.7 ± 3.6[ab]	23.3 ± 2.0[b]	21.5 ± 2.3[ab]	24.1 ± 1.2[b]
Liver TBARS nmol/g wet tissue	201.8 ± 6.5[a]	151.6 ± 5.8[b]	166.9 ± 7.1[b]	152.0 ± 4.1[b]	177.1 ± 15.1[ab]
Heart TBARS nmol/g wet tissue	170.5 ± 15.8[a]	107.9 ± 5.6[b]	119.8 ± 7.0[b]	98.5 ± 4.2[b]	112.7 ± 10.4[b]
Liver GPx U/mg protein	0.97 ± 0.09[a]	0.80 ± 0.04[ab]	0.73 ± 0.01[b]	0.74 ± 0.03[b]	0.75 ± 0.03[b]
Liver GR mU/mg protein	28.8 ± 1.0[a]	24.0 ± 0.5[bc]	23.7 ± 1.1[bc]	20.2 ± 0.8[c]	24.8 ± 1.2[ab]
Liver GST U/mg protein	0.78 ± 0.02[a]	0.65 ± 0.03[bc]	0.71 ± 0.02[ab]	0.58 ± 0.02[c]	0.71 ± 0.04[ab]
Liver glutathione μmol/g wet tissue	6.4 ± 0.3	6.2 ± 0.3	6.0 ± 0.2	6.6 ± 0.4	7.2 ± 0.5
Liver SOD U/mg protein	11.4 ± 0.5	10.3 ± 0.8	10.8 ± 0.5	10.7 ± 0.6	10.0 ± 0.5
Liver CAT U/mg protein	341.1 ± 20.5	325.0 ± 13.4	344.8 ± 15.9	388.9 ± 13.6	337.7 ± 27.3

Results are given as mean ± SEM of 8 rats in each group. One-way analysis of variance (ANOVA) was used to determine significance of the differences between means in the 5 groups of folic acid intake. Values on the same line with a different superscript letter are significantly different at $P < 0.05$. No superscript letter means ANOVA F test was not significant.

Table 3 Correlation between plasma and tissue antioxidant enzymatic and non-enzymatic protection, and Hcy and folate status

	Plasma Hcy	Plasma folate	Liver folate
FRAP	r = −0.482 p = 0.002	r = 0.471 p = 0.002	
Plasma α-tocopherol	r = −0.532 p = 0.0004	r = 0.494 p = 0.001	
Liver TBARS	r = 0.451 p = 0.003		r = −0.496 p = 0.001
Heart TBARS	r = 0.625 p < 0.0001	r = −0.594 p < 0.0001	
Liver GPx	r = 0.565 p = 0.0001		r = −0.670 p < 0.0001
Liver GR	r = 0.595 p < 0.0001		r = −0.549 p = 0.0002
Liver GST	r = 0.422 p = 0.007		r = −0.399 p = 0.011
Liver SOD	r = 0.250 p = 0.120		r = −0.248 p = 0.124
Liver CAT	r = −0.348 p = 0.028		r = 0.173 p = 0.285

Correlations were performed with log-transformed or raw data from the 5 groups of rats (CFD, MFD, C, LFS, HFS), and assessed using the Pearson correlation coefficient (r). The correlation was considered significant when the *P*-value corresponding to the r value was <0.05.

antioxidant protection between the C rats and both groups of FS rats.

Direct correlation between antioxidant enzymes and non-enzymatic protection, and homocysteine and folate status

As shown in Table 3, a significant negative correlation was found between tHcy and plasma value of FRAP and α-tocopherol, concomitantly with a significant positive correlation between each of the latter two variables and plasma folate. This was concurrent with a significant positive correlation between liver and heart TBARS and plasma tHcy, and a significant negative correlation between the same TBARS parameters and liver and plasma folate. Moreover, a strong positive correlation was found between plasma tHcy and liver specific activities of GPx, GR and, to a lower extent, GST. The latter enzyme parameters were, on the contrary, negatively correlated with liver folate, the weaker association being again observed for GST. No association was observed between either tHcy or liver folate and specific activity of liver SOD. Finally, despite a significant negative correlation between tHcy and liver CAT activity, no association was found between the latter and liver folate.

Principal components analysis (PCA) of the data

As described above, a number of liver, heart and plasma parameters varied as a function of folic acid intake in the rats. In order to identify patterns in these variables and highlight their similarities or differences, we performed a principal components analysis (PCA) on data presented in Tables 1 and 2. As shown in Fig. 1, the first two PC's (PC1 and PC2) represented 68 % of the total variance of the data. The rats fed diets containing folic acid (i. e., the MFD, C, LFS and HFS diets) were clearly discriminated from the rats fed the CFD diet along PC1. This difference relied on the decreases in TBARs (heart and liver) and Hcy (plasma) concentrations, and GPx and GR (liver) specific activity, simultaneously with increases in fo-

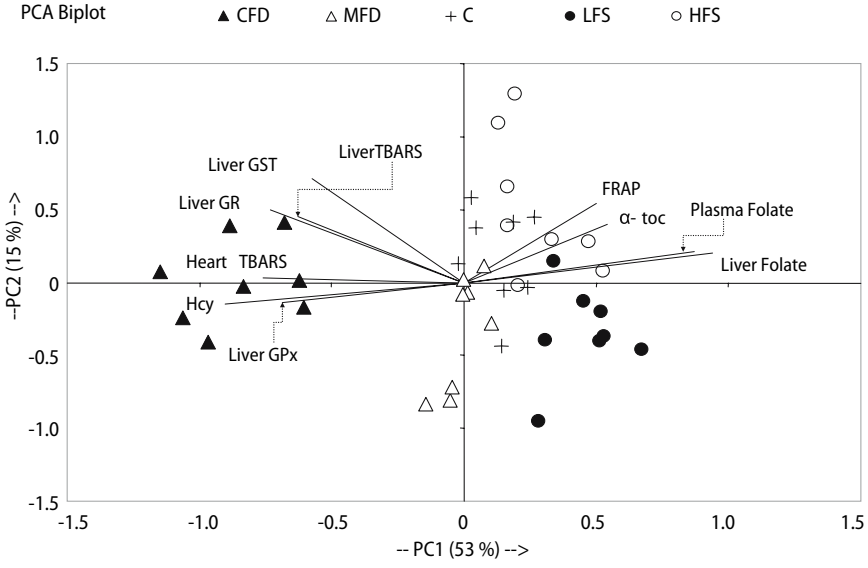

Fig. 1 Principal components analysis (PCA) of the data on Hcy, folate and redox status, and hepatic enzymes of glutathione homeostasis in the 5 groups of rats with increasing intakes of folic acid. PCA was used to produce a graphical representation of both variables (Hcy, folate, redox and enzyme parameters, except for SOD and CAT) and samples (rats fed either the CFD, MFD, C, LFS or HFS diet). PC1 and PC2 stand for the first and second principal components, respectively.

late (plasma and liver), and FRAP and α-tocopherol (plasma) concentrations.

Discussion

Variations in folate status are considered as having an important health impact because (i) folate deficiency is a recognised risk factor for age-associated degenerative diseases, and (ii) high folate intake can reduce the incidence of NTDs. However, information is still insufficient to understand the metabolic consequences of such large variations in folate status. We have previously shown that folate deficiency increased the abundance of the enzymes GPx 1 and, to a lesser extent, Prx 6 in the liver of 4-month-old rats, suggesting that induction of antioxidant systems could take place as an adaptive response to the oxidative stress caused by folate deficiency [21]. Our present results show that (i) a stepwise decrease in folate intake gives rise to an impaired folate and Hcy status concomitantly with an increase in several markers of oxidative stress, (ii) several liver glutathione-dependent antioxidant enzymes, unlike SOD and CAT, are significantly enhanced, seemingly as a compensatory response to the oxidative stress induced by folate deficiency, and (iii) despite significant correlation between plasma folate status and several markers of antioxidant systems, folate supplementation does not appear to improve significantly the level of antioxidant protection of adult rats.

In agreement with previous studies in adult or weaning rats [3, 21, 31], 4-month-old rats fed the CFD diet exhibited features of insufficient folate status, including substantially decreased plasma, RBC and liver folate concentrations, as well as increased plasma Hcy concentration, as compared with rats fed the C diet. Our work additionally shows that pair-feeding with the MFD diet allows to create a moderate folate deficiency in adult rats. This could constitute a study model of the metabolic impact of marginal folate deficiency, which is highly frequent in aging humans [12]. As compared to the C diet, the LFS and HFS diets caused an increase in plasma and liver folate status without significant decrease in homocysteinemia, suggesting that intake of supplemental folic acid for 4 weeks is not beneficial to normally-fed rats in term of Hcy-lowering impact. This is coherent with results obtained in previous studies in weaning or adult rats [3, 32, 33]. It also suggests that possible beneficial effects of supplemental folic acid, if any, could occur through Hcy-independent mechanisms.

In the present study, changes in the redox state of rats in response to large variations of dietary folate intake were assessed at the levels of tissue oxidative stress (TBARS as an index of lipid peroxidation), and plasma (FRAP and α-tocopherol) and liver (GPx, SOD and CAT activities and total glutathione concentration) antioxidant markers. GSH acts as a cellular redox buffer and is the major hepatic antioxidant [34]. It also serves as a cofactor for the antioxidant enzymes of the GPx family which degrade H_2O_2 and alkyl hydroperoxides, generating GSSG [35]. SOD are metalloenzymes that catalyse the conversion of $O2^{\cdot-}$ to H_2O_2 which immediately becomes a substrate for CAT and other H_2O_2-degrading enzymes such as GPx and Prx [36]. Regarding vitamin E, its antioxidant action has been ascribed to its ability to chemically act as a lipid-based free radical chain-breaking molecule [37]. Although controversial, association between vitamin E intake or plasma level of α-tocopherol, the predominant form of vitamin E in blood, and resistance to several chronic diseases involving lipid oxidation has been suggested by many studies [38]. With the hydrophilic antioxidant molecules contributing to more than 75% of the FRAP value [26], the FRAP and the α-tocopherol assays altogether provide a good estimate of the total antioxidant capacity of plasma. Additionally, measurement of increased *ex vivo* formation of TBARS induced by ferrous ions in tissue homogenates is considered to reflect an increased susceptibility of tissue lipids to peroxidation [39]. Taken together, all the previous biomarkers constitute a satisfactory indicator of the redox status. As shown by results from raw data analysis, PCA and correlation, we found, in association with decreased folate status and increased homocysteinemia, an increase in *ex vivo* formation of TBARS in heart and liver tissues, an elevation in liver GPx activity, and a decrease in FRAP and plasma α-tocopherol levels. Overall, the data indicate that the antioxidant protection of adult rats was perturbed after 4 weeks of dietary folate deficiency, as a result of an oxidative stress. Such an oxidative stress has previously been reported in folate-deficient and/or hyperhomocysteinemic patients [16, 20] or rats [2–5]. Additionally, the presently observed enhanced activity of liver GPx in FD rats agrees with our previous findings from proteomics [21] on the abundance of GPx 1, the predominant cytosolic form of GPx in rat liver [35]. Likewise, increased amount or activity of anti-antioxidant systems was shown in the brain of FD, normal or ApoE-deficient mice [40], and in the kidney and plasma of FD rats [5]. Moreover, plasma tHcy concentrations ≥ 20 μM in human subjects were associated with an increased activity of circulating SOD and GPx [41].

No variation was observed for the hepatic level of total glutathione between FD and C rats. Since hepatic activity of GPx was increased, we assumed that other enzymatic pathways of glutathione metabolism,

namely GST and GR, were also modified during folate deficiency. GST catalyses GSH conjugation to various electrophilic endogenous (e.g., lipid hydroperoxides during oxidative stress) and foreign compounds, resulting in detoxification of such molecules and in a net loss of glutathione [34, 42]. GR allows the regeneration of GSH from GSSG produced by GPx during H_2O_2 degradation [34]. Correlation analysis showed that decreased folate status and increased homocysteinemia were associated with increased hepatic activities of GR and, to a lower extent, GST. Increased hepatic GR activity in CFD rats may allow the maintenance of a normal GSH/GSSG ratio under conditions of increased tissue activity of GPx, thus providing a steady antioxidant potential of the liver. Augmented liver GST activity in CFD rats suggests an increased tissue loss of glutathione through excretion of GSH conjugates. Since the total hepatic level of glutathione was unchanged, GSH synthesis could have been increased to compensate for its potentially higher rate of excretion following GST conjugation. Increased GSH synthesis in the liver of FD rats could be due to increased glutamate-cysteine ligase and/or GSH synthase (GSS) activity, as well as increased cysteine availability [29, 34]. Increased GSS activity has been recently reported in the brain of both normal and ApoE−/− mice maintained on a folate- and vitamin E-deficient diet [43]. Moreover, cysteine formation could have been augmented through the Hcy-dependent transsulfuration pathway in the liver of FD rats. The transsulfuration pathway has indeed been shown to play an important role in the maintenance of the intracellular glutathione pool, especially in conditions of oxidative stress [44].

Unlike the activity of enzymes involved in glutathione homeostasis, hepatic activities of SOD and CAT were unaffected by any variations in folate intake (Table 2). This might suggest the occurrence of a glutathione-specific antioxidant mechanism in liver in response to oxidative stress induced by folate deficiency. This hypothesis is consistent with previous observation that only folate deficiency, and not vitamin E deficiency, could induce a compensatory increase in brain glutathione levels in both normal and ApoE−/− mice [45]. However, a weak, but significant, negative correlation was found between plasma tHcy and liver CAT activity (Table 3), that might suggest a trend for the latter to be sensitive to Hcy. Further studies are required to better evaluate this correlation. Moreover, we did not observe any variation in plasma NO levels between the different groups of rats (data not shown) by assaying nitrite + nitrate concentrations, as previously described [46]. This indicates that, under our conditions, folate deficiency or supplementation had neither a deleterious nor a beneficial effect on NO bioavailability, respectively. It can be further speculated that increased activity of endothelial GPx might compensate for the adverse effects of Hcy generated by folate deficiency on NO bioavailability. Weiss et al. [47] showed that, during moderate hyperhomocysteinemia, overexpression of GPx–1 protects mice or endothelial cells from endothelial dysfunction likely through reduction of oxidative damage. Additionally, increased GPx activity has been reported in different tissues of folate-deficient rats, namely the plasma and kidney [5] or the liver [our study], as well as in the plasma of moderately hyperhomocyteinemic humans [41].

Folate might have direct antioxidant properties [17–19]. Nevertheless, in the present study, we did not find any evidence for such an effect of supplemental folic acid in vivo, since none of the markers of plasma and tissue antioxidant protection measured was changed between the C and FS groups of rats. As shown by PCA, the only dietary folate group with a redox profile clearly discriminated from that of the others was the CFD group, showing that, unlike folate overload, severe folate deficiency can cause important changes in the redox state of animals. Our data, in addition to other studies [2–4, 48], suggest that elevated plasma tHcy concentration could contribute to these changes.

In conclusion, the present study shows that folate deficiency induced alteration in the redox state of adult rats leading to oxidative stress. Glutathione-dependent enzymatic mechanisms appear to be especially involved in the adaptive antioxidant response of liver to such an alteration. Further investigations will aim to document how these mechanisms are regulated and operate during folate deficiency. Moreover, studies need also to be designed to determine the mechanisms involved in the protective effects of high folate intake in healthy individuals. Our study shows that these mechanisms may be unrelated to increased antioxidant potential.

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