Folate Depletion and Elevated Plasma Homocysteine Promote Oxidative Stress in Rat Livers[1]

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ABSTRACT This study was designed to determine whether nutritional folate depletion exerts hepatic oxidative stress in relation to elevated plasma homocysteine. To mimic various extents of folate depletion status in vivo, male Wistar rats were fed an amino acid–defined diet containing either 8 (control), 2, 0.5, or 0 mg folic acid/kg diet. After a 4-wk feeding period, the plasma and hepatic folate concentrations of the rats decreased significantly with each decrement of dietary folate. Folate depletion did not significantly affect two major liver antioxidants: reduced glutathione and α-tocopherol. Conversely, folate depletion decreased Cu-Zn superoxide dismutase and glutathione peroxidase activities, but had no effect on catalase activity in liver homogenates. Lipid peroxidation products, as measured by thiobarbituric acid–reactive substances, were significantly higher in livers of folate-depleted rats than in those of the controls. This occurrence of hepatic oxidative stress in folate-depleted rats was confirmed by demonstrating an increased susceptibility of livers of folate-depleted rats to lipid peroxidation induced by additional H_2O_2 or Fe^{2+} treatments compared with the controls. Decreasing dietary folate intake resulted in graded increases in plasma homocysteine concentrations of folate-depleted rats. Elevated plasma homocysteine and decreased plasma and hepatic folate concentrations in folate-depleted rats were all strongly and significantly correlated with increased liver lipid peroxidation ($|r| \geq 0.58$, $P < 0.0003$). These data demonstrate that folate depletion and elevated plasma homocysteine promote oxidative stress in rat livers. J. Nutr. 131: 33–38, 2001.

KEY WORDS: • *folate depletion* • *plasma homocysteine* • *oxidative stress* • *rats*

Low folate intake results in elevated plasma homocysteine concentrations in healthy men (Jacob et al. 1994), middle-aged adults (Shimakawa et al. 1997), elderly populations (Selhub et al. 1996) and postmenopausal women (Jacob et al. 1998). Folate depletion accompanied by elevated homocysteine concentrations is associated with the increased risk of neurological, cardiovascular disorders and carcinomas (Boushey et al. 1995, Hankey and Eikelboom 1999, Pietrzik and Bronstrup 1997, Selhub et al. 1996). It has been suggested that homocysteine-induced injury involves oxidative damage (Hultberg et al. 1997a, Loscalzo 1996, Olszewski and McCully 1993, Welch et al. 1997). In vitro studies have shown that homocysteine exerts its toxicity on endothelial cells by increasing H_2O_2 production (Starkebaum and Harlan 1986), affecting antioxidant defense systems (Blundell et al. 1996), and promoting lipid peroxidation (Jones et al. 1994). Folate deficiency–induced hyperhomocysteinemia exerts prothrombotic effects on platelets and macrophages, and is related to increased plasma lipid peroxidation in rats (Durand et al. 1996). In humans, decreased folate intake results in elevated plasma homocysteine concentrations and urinary malondialdehyde (lipid peroxidation measurement) (Jacob et al. 1998). These oxidative insults induced by folate depletion and elevated homocysteine appear to play a major role in the pathogenesis of cardiovascular diseases.

Previous studies have emphasized that folate depletion–induced hyperhomocysteinemia promotes vascular oxidative injury. Little is known about the possibility of folate depletion triggering oxidative stress in other localized tissues. In animals, the liver contains the most body folate storage and is susceptible to folate depletion (Clifford et al. 1990, Varela-Moreiras and Selhub 1992). Folate deficiency disturbs hepatic one-carbon metabolism (Balaghi et al. 1993) and causes impaired homocysteine catabolism in the liver of rats by decreasing cystathionine synthesis and inhibiting homocysteine remethylation (Miller et al. 1994). It has been reported that methyl donor/folate–deficient diets decrease hepatic antioxidant concentrations (Henning et al. 1997). However, whether inferior folate status and elevated homocysteine lead to hepatic oxidative damage remains to be determined.

In the present study, we investigated the effects of folate depletion on hepatic oxidative stress and its association with plasma homocysteine. To mimic various extents of folate depletion status, rats were fed an amino acid–defined diet containing either 8 (control), 2, 0.5 or 0 mg folic acid/kg diet. Folate and homocysteine status, hepatic antioxidant capability and lipid peroxidation in the liver were measured.

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MATERIALS AND METHODS

Experimental diets. A folate-deficient, L-amino acid–defined diet (Walzem and Clifford 1988) was specially formulated by Harlan Taklad (Madison, WI) and was supplemented with either 8, 2, 0.5 or 0 mg folic acid/kg diet. The diet containing 8 mg folic acid/kg was the folate control based on previous studies using an amino acid–based formula (Balaghi and Wagner 1995, Cravo et al. 1992, Miller et al. 1994, Varela-Moreiras and Selhub 1992, Walzem and Clifford 1988). Compared with the folate control diet (Control), the diets with descending folate concentrations (2, 0.5 or 0 mg folic acid/kg diet) were designated as moderate folate (MF),[3] low folate (LF) or no folate (NF) diets, respectively. All diets contained 1 g succinylsulfothiazole/100 g to suppress intestinal microfloral folate production.

Animals. The experimental protocols were approved by the Institutional Animal Care Committee of Fu-Jen University. Male weaning Wistar rats ($n = 28$) were obtained from the Animal Center of National Science Council (Taipei, Taiwan). Rats were housed in stainless steel wire cages in an air-conditioned room maintained at 25°C and 70% humidity with a 12-h dark/light cycle. After a 3-d acclimation period during which rats were fed a nonpurified diet, they were randomly assigned to the Control, MF, LF or NF diets. Access to food and tap water was available ad libitum. The rats were weighed twice a week and killed with diethyl ether at the end of wk 4. Blood and tissues were removed for further analysis.

Blood and liver folate assay. Blood and tissue samples for folate analysis were prepared according to Varela-Moreiras and Selhub (1992). Briefly, aliquots of plasma, serum or erythrocytes and weighed livers were added to an extraction solution containing 5 mmol 2-mercaptoethanol, 0.1 mol sodium ascorbate, 50 mmol HEPES, and 50 mmol 2-[*N*-cyclohexylamino]ethanesulfonic acid per L (pH 7.85). The mixture was heated in a boiling water bath for 10 min and cooled on ice. Liver samples were homogenized using a Polytron homogenizer (OMNI 2000, Waterburg, CT) and centrifuged at 20,000 × *g* for 10 min. Blood samples were centrifuged at 10,000 × *g* for 15 min. The supernatant extract was stored at −70°C in nitrogen for later analysis. After incubation of the thawed sample extracts with chicken pancreas conjugase (v/v 4:1) at 37°C for 6 h, a microbiologic assay was performed using cryoprotected *Lactobacillus casei* in 96-well microtiter plates (Horne and Patterson 1988). Absorbance was detected at 600 nm in an MRX model ELISA reader (Dynatech Laboratories, West Sussex, U.K.).

Plasma homocysteine assay. Plasma samples were prepared and total homocysteine concentrations were analyzed using HPLC according to Durand et al. (1996). Aliquots of fasting plasma samples were first mixed with 500 μmol *N*-acetylcysteine/L and then were treated with 10% (v/v) tri-*n*-butylphosphine in dimethylformamide for 30 min at 4°C. The mixture was precipitated with 0.6 mol cold perchloric acid/L containing 1 mmol EDTA/L and derivatized with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) (Sigma Chemical Co., St. Louis, MO) using a similar procedure, as described by Araki and Sako (1987). Homocysteine in samples was analyzed using an HPLC system consisting of a Shimadzu LC-10AT Liquid Chromatograph, a Shimadzu F1050 fluorescence detector (at excitation 385 nm, emission 515 nm) and an SIC Chromatocorder 12 integrator (Shimadzu, Kyoto, Japan). Separation was carried out using a reverse phase column (C18 ODS, 150 × 4.6 mm; Hyclone Laboratories, Logan, UT) protected by a guard column (45 × 4.6 mm, Hyclone Laboratories). Analysis was performed under isocratic conditions (0.1 mol acetate buffer/L with 2% methanol, pH 4.0) at a flow rate of 1.2 mL/min for 14 min. The concentration of plasma homocysteine was calculated by the integrator using SBD-homocysteine as external standard and *N*-acetylcysteine as internal standard.

Measurement of α-tocopherol. Tissue α-tocopherol was measured according to Huang and Shaw (1994). Briefly, liver tissues were homogenized in phosphate buffer (0.01 mol/L, pH 7.4) and saponified with absolute ethanol (containing 10 g pyrogallol/L) and saturated KOH in a 70°C water bath for 30 min. α-Tocopherol was extracted using *n*-hexane (containing 1.25 g butylated hydroxytoluene/L). The reverse phase HPLC analyses of α-tocopherol were performed with a Shimadzu LC-10AT pump with a C18 column (5 μm, 4 × 125 mm) protected by a 5-μm guard column (E. Merck, Darmstadt, Germany). Methanol was used as the mobile phase at a flow rate of 1.0 mL/min. The α-tocopherol was detected by a Shimadzu SPD-10A (UV/VIS) detector (292-nm wavelength), and concentrations of α-tocopherol (retention time 5.1 ± 0.3 min) were quantified using an EZChrom Chromatography Data Integration System (Scientific Software, San Ramon, CA).

Measurement of antioxidant enzyme activities and reduced glutathione (GSH) concentrations in rat livers. After rats were anesthetized, livers were removed, immediately frozen in liquid nitrogen and stored at −80°C until used for the following assays. The activity of Cu-Zn superoxide dismutase (SOD) was determined by monitoring the inhibition of the autoxidation of pyrogallol (Marklund and Marklund 1974). Liver homogenates were treated with a solution of ethanol and chloroform (25:15) to inactivate the manganese-dependent SOD (Paynter et al. 1979). At 25°C and 320 nm, the rate of pyrogallol oxidation was recorded with a Shimadzu UV 1201 spectrophotometer (Shimadzu). One unit of Cu-Zn SOD activity was defined as the amount of enzyme required to inhibit pyrogallol oxidation by 50%. Catalase activity was measured according to Aebi (1984). One unit of catalase activity was defined as the amount of enzyme required to decompose 1 μmol of hydrogen peroxide in 1 min. Total glutathione peroxidase (GPx) activity was determined by means of a coupled enzyme assay (Tappel 1977). After the addition of *t*-butylhydroperoxide, the conversion of NADPH to $NADP^+$ by glutathione reductase and generated oxidized glutathione was continuously monitored in a spectrophotometer at 340 nm for 2 min. One unit of GPx activity was expressed as the amount of enzyme catalyzing the oxidation of 1 nmol NADPH/min. GSH in liver was determined using a fluorometric method (Hissin and Hilf 1976). The protein content in the samples was determined according to Bradford (1976).

Lipid peroxidation. Lipid peroxidation was quantified by measuring thiobarbituric-reactive substances (TBARS) production as described by Fraga et al. (1988). The reaction reagents contained 3 g sodium dodecyl sulfate, 0.1 mol HCl, 10 g phosphotungstic acid and 0.7 g 2-thiobarbituric acid per L. The sample mixture was incubated for 45 min at 95°C, and TBARS were extracted in 2.5 mL of 1-butanol. After centrifugation at 1000 × *g* for 10 min, the fluorescence of the butanol layer was measured using an Hitachi F-3000 Fluorospectrophotometer (Hitachi, Japan) at 555 nm emission and 515 nm excitation. The TBARS values were expressed as nmol of malondialdehyde equivalents per g of tissue using a standard curve of 1,1,3,3-tetraethoxypropane. For H_2O_2- or Fe^{2+}-stimulated TBARS production, liver homogenate was incubated with 10 mmol H_2O_2/L or 50 μmol $FeSO_4$/L at 37°C for 60 min. Butylated hydroxytoluene (4 g/L) was added to terminate the reaction.

Statistical analyses. Data are presented as means ± SD. The effects of dietary folate intake on animal growth, folate status, hepatic antioxidant status and lipid peroxidation were analyzed by one-way ANOVA and Duncan's multiple range test using the General Linear Model of SAS Institute (Cary, NC). Differences were considered significant at $P < 0.05$. The Pearson correlation coefficients were used to measure the association among hepatic lipid peroxidation, plasma homocysteine and folate depletion variables.

RESULTS

Growth. The NF group consumed less food and had lower weight gains and liver weights than the control group (**Table 1**). The MF group also had lower total food intake and liver weight than controls, probably due to the lighter initial weight of the rats in this group. Feeding efficiencies and relative liver weight were not significantly different among the groups (Table 1). Hematocrit, hemoglobin concentration and serum L-alanine aminotransferase activity also did not differ among the groups (data not shown).

[3] Abbreviations used: GPx, glutathione peroxidase; GSH, reduced glutathione; LF, low folate; MF, moderate folate; NF, no folate; SBD-F, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate; SOD, superoxide dismutase; TBARS, thiobarbituric-reactive substances.

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TABLE 1

Growth of rats fed diets with various folate concentrations for 4 wk[1]

	Dietary folate,[2] *mg folic acid/kg diet*			
Growth parameter	Control (8)	MF (2)	LF (0.5)	NF (0)
Initial weight, *g*	112.2 ± 8.9[a]	97.1 ± 8.9[b]	103.5 ± 13.3[ab]	100.5 ± 9.1[ab]
Accumulated weight gain, *g*	220.1 ± 26.2[a]	195.5 ± 27.8[ab]	204.4 ± 10.7[a]	175.7 ± 20.5[b]
Total food intake, *g*	750.5 ± 44.1[a]	698.7 ± 20.5[b]	724.7 ± 23.4[ab]	649.7 ± 42.3[c]
Feed efficiency, *g gain/g feed*	0.29 ± 0.03	0.28 ± 0.04	0.28 ± 0.01	0.27 ± 0.02
Liver weight, *g*	10.57 ± 1.38[a]	9.56 ± 1.03[b]	10.75 ± 1.22[a]	8.84 ± 1.00[c]
g/100 g body	3.17 ± 0.14	3.27 ± 0.15	3.40 ± 0.41	3.20 ± 0.19

[1] Values are means ± SD, $n = 7$. Values in a row with different superscripts differ, $P < 0.05$.
[2] MF, LF and NF indicate moderate, low and no folate diets, respectively.

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Folate and homocysteine status. Plasma and liver folate concentrations decreased significantly with each decrement of folate intake (**Table 2**). Compared with the control group, the decreases in plasma folate concentrations of the MF, LF and NF groups were 9, 29 and 71%, respectively. Hepatic folate concentrations in the MF, LF and NF groups were 15, 49 and 90% lower, respectively, than in the control group. Red blood cell folate concentrations did not differ between the control and MF groups but were significantly lower in the LF and NF groups than in control and MF groups. At 4 wk, the increased plasma homocysteine concentrations were associated with the decreased level of folate in the diet. The NF group had the highest plasma homocysteine concentration, and the control group had the lowest. There was a significant negative correlation between plasma folate and homocysteine concentrations ($r = -0.90$, $P = 0.0001$) and between hepatic folate concentrations and plasma homocysteine concentration ($r = -0.92$, $P = 0.0001$).

Hepatic nonenzymatic antioxidants. At 4 wk after feeding folate-lowering diets, hepatic concentrations of the antioxidants α-tocopherol and GSH in livers were not significantly affected (data not shown).

Hepatic enzymatic defense system. As folate intake decreased, hepatic GPx activity decreased (**Table 3**). The NF group had significantly lower hepatic GPx activity than the control group. Cu-Zn SOD activity was significantly lower in the LF and NF groups compared with the control and MF groups. Catalase activity was not significantly altered by dietary folate.

Lipid peroxidation. Although the ex vivo assessment of TBARS in liver homogenates may not reflect the genuine in vivo oxidation, it provides a way to evaluate the relative peroxidative status of livers in the control and folate-depleted rats. TBARS production in unstimulated liver homogenates from the MF, LF and NF groups were significantly higher than that in the controls (**Table 4**). In the presence of 10 mmol H_2O_2/L or 50 μmol $FeSO_4$/L, liver homogenates from the MF, LF and NF groups had significantly greater TBARS concentrations than the control group. In liver from the NF group, Fe^{2+}-stimulated TBARS concentration was greater than that in livers of rats fed the LF and MF diets. To confirm that the highest lipid peroxidation observed in the NF group was not due to decreased food intake or lower body weight, TBARS concentrations in the liver homogenate of rats pair-fed control diet with the mean consumption of the NF group ($n = 4$) were measured. Pair-fed control rats had TBARS in liver homogenates of 37.91 ± 6.13 nmol/g, H_2O_2-stimulated concentrations of 73.30 ± 7.10 nmol/g and Fe^{2+}-stimulated concentrations of 460.59 ± 55.79 nmol/g. These values in pair-fed control rats were not significantly different from those of the control rats that consumed food on an ad libitum basis.

Correlations among plasma homocysteine, folate status and hepatic lipid peroxidation. There were strong and significant correlations among plasma homocysteine concentrations, folate-depletion status and hepatic lipid peroxidation variables in rat livers ($|r| \geq 0.58$, $P < 0.0003$) (**Table 5**). The Fe^{2+}-stimulated liver lipid peroxidation data had the strongest correlations with folate-depletion variables and elevated plasma homocysteine ($|r| \geq 0.79$, $P < 0.0001$).

DISCUSSION

During a 4-wk experimental period, rats fed an MF, LF or NF diet attained various degrees of folate depletion, characterized by lower plasma and liver folate concentrations than in rats fed a control diet. The magnitude of blood and liver folate

TABLE 2

Homocysteine and folate status of rats fed diets with various folate concentrations for 4 wk[1]

	Dietary folate,[2] *mg folic acid/kg diet*			
Homocysteine and folate status	Control (8)	MF (2)	LF (0.5)	NF (0)
Plasma homocysteine, *µmol/L*	11.86 ± 1.60[c]	15.54 ± 2.71[c]	20.11 ± 2.67[b]	26.39 ± 2.66[a]
Plasma folate, *nmol/L*	207.7 ± 11.7[a]	189.6 ± 13.7[b]	148.5 ± 7.5[c]	59.58 ± 9.9[d]
Red blood cell folate, *nmol/L*	1542 ± 128[a]	1530 ± 128[a]	769 ± 78[b]	626 ± 93[c]
Liver folate, *nmol/g*	21.55 ± 1.41[a]	18.17 ± 1.18[b]	10.85 ± 0.30[c]	2.08 ± 0.27[d]

[1] Values are means ± SD, $n = 7$. Values in a row with different superscripts differ, $P < 0.05$.
[2] MF, LF and NF indicate moderate, low and no folate diets, respectively.

TABLE 3

Activities of glutathione peroxidase, catalase and Cu-Zn superoxide dismutase in livers of rats fed diets with various folate concentrations for 4 wk[1]

	Dietary folate,[2] *mg folic acid/kg diet*			
Liver antioxidant enzymes	Control (8)	MF (2)	LF (0.5)	NF (0)
GPx,[3] *units/mg protein*	505.13 ± 52.61^{a}	469.06 ± 50.42^{ab}	462.93 ± 51.82^{ab}	434.55 ± 66.41^{b}
Catalase,[4] *units/mg protein*	153.62 ± 17.73	163.68 ± 19.52	155.34 ± 13.44	151.74 ± 9.30
Cu-Zn SOD,[5] *units/mg liver*	24.49 ± 1.21^{a}	23.71 ± 1.29^{a}	21.06 ± 0.75^{b}	22.23 ± 1.14^{b}

[1] Values are means ± SD, $n = 7$. Values in a row with different superscripts differ, $P < 0.05$.
[2] MF, LF and NF indicate moderate, low and no folate diets, respectively.
[3] One unit of glutathione peroxidase (GPx) activity was defined as the amount of enzyme catalyzing the oxidation of 1 nmol NADPH/min.
[4] One unit of catalase activity was arbitrarily defined as the amount of enzyme required to decompose 1 μmol H_2O_2/min.
[5] One unit of Cu-Zn superoxide dismutase (SOD) activity was arbitrarily defined as the amount of enzyme require to inhibit 50% autoxidation of pyrogallol.

depletion in response to decreased dietary folate intake in our study was comparable to the data published by Clifford et al. (1990). The inverse relationship between plasma homocysteine and folate intake found in the present study was also consistent with previous reports in humans (Jacob et al. 1994 and 1998, Selhub et al. 1996) and rats (Durand et al. 1996, Lin et al. 1989, Miller et al. 1994). Although rats fed an amino acid–defined diet without folate for 4 wk had reduced growth, consistent with previous reports (Balaghi et al. 1993, Clifford et al. 1990, Welzem and Clifford 1988), feed efficiency, relative liver weight and hematological variables among treated groups in the present study were not significantly different.

We found that two major hepatic antioxidants, α-tocopherol and GSH, were unaffected by 4 wk of folate depletion. Conversely, a previous study showed that after 15 mo, hepatic α-tocopherol concentrations were significantly lower in livers of rats fed methyl donor/folate–deficient diets (Henning et al. 1997). A methionine/choline/folate–deficient diet significantly decreased hepatic GSH concentrations (Henning et al. 1989). It appears that the duration and extent of folate depletion affect these antioxidant concentrations in liver. On the other hand, because folate deficiency was accompanied with elevated plasma homocysteine concentrations, homocysteine might play a role in tissue GSH concentrations. In in vitro studies, increasing homocysteine concentrations decreased intracellular GSH concentrations in endothelial cells (Hultberg et al. 1997b) or in an HeLa cell culture (Hultberg et al. 1997a). Folate-depleted human hepatoma HepG2 cells released homocysteine into media at a level twice that of controls, which was associated with a significant decrease in intracellular GSH concentrations (Cheng 1998). Our in vivo data, however, suggested that homeostasis of these hepatic antioxidants, including α-tocopherol and GSH, in folate-depleted rats was not disrupted during the 4-wk experimental period.

Folate depletion was associated with a compromised hepatic antioxidant enzymatic defense. Inferior folate status resulted in lower GPx and Cu-Zn SOD activities, whereas catalase was unaffected. The causes for this folate depletion–induced damage to hepatic antioxidant enzymes are unknown. Reduction in GPx activity may not be a consequence of limited reductant substrate (GSH), because decreased folate intake did not affect hepatic GSH pools. Food restriction was previously reported to elevate hepatic SOD and GPx activity (Xia et al. 1995), so it is not likely that the decrease of hepatic GPx and SOD activity in rats fed a NF diet can be ascribed to lower food intake and body weights. We postulate that the impairment of GPx and SOD activities in livers of folate-depleted rats may be related to elevated plasma homocysteine concentrations. It was reported by Nishio and Watanabe (1997) that homocysteine decreased the activity of purified bovine liver GPx in a time- and dose-dependent manner. Upchurch et al. (1997) found that homocysteine reduced GPx mRNA concentrations and enzymatic activity in bovine aortic

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TABLE 4

Thiobarbituric acid-reactive substances (TBARS) concentrations in the absence or presence of H_2O_2 or Fe^{2+} in liver homogenates from rats fed diets with various folate concentrations for 4 wk[1,2]

	Dietary folate,[3] *mg folic acid/kg diet*			
Condition	Control (8)	MF (2)	LF (0.5)	NF (0)
		malondialdehyde equivalents, nmol/g wet liver		
No stimulation	38.3 ± 2.2^{b}	69.9 ± 6.7^{a}	69.8 ± 2.3^{a}	70.3 ± 4.4^{a}
H_2O_2 stimulated	66.0 ± 3.1^{b}	118.2 ± 5.6^{a}	119.0 ± 6.9^{a}	120.3 ± 10.0^{a}
Fe^{2+} stimulated	470.6 ± 17.7^{c}	680.0 ± 36.4^{b}	689.4 ± 38.8^{b}	777.4 ± 40.6^{a}

[1] Values are means ± SD, $n = 7$. Values in a row with different superscripts differ, $P < 0.05$.
[2] TBARS were measured in liver homogenate in the absence (no stimulation) or in the presence of 10 mmol H_2O_2/L (H_2O_2 stimulated) or 50 μmol $FeSO_4$/L (Fe^{2+} stimulated).
[3] MF, LF and NF indicate moderate, low and no folate diets, respectively.

TABLE 5

Pearson correlation coefficients for relationships among plasma homocysteine, folate status and thiobarbituric acid-reactive substances (TBARS) in liver homogenate from rats fed diets varying in folate concentration for 4 wk[1]

	Plasma homocysteine	Plasma folate	Hepatic folate
		r (P)	
No stimulation	0.64 (0.0003)	−0.58 (0.001)	−0.67 (0.0001)
H_2O_2 stimulated	0.68 (0.0001)	−0.61 (0.0005)	−0.68 (0.0001)
Fe^{2+} stimulated	0.81 (0.0001)	−0.79 (0.0001)	−0.83 (0.0001)

[1] TBARS were measured in liver homogenate in the absence (no stimulation) or in the presence of 10 mmol H_2O_2/L (H_2O_2 stimulated) or 50 μmol $FeSO_4$/L (Fe^{2+} stimulated).

endothelial cells. By the cDNA microarray method, Outinen et al. (1999) demonstrated that homocysteine inhibited gene expression of GPx and SOD in human umbilical vein endothelial cells. Although the mechanism is unknown, hydrogen peroxide elicited by homocysteine (Starkebaum and Harlan 1986) was suggested to play a role on the inhibition of antioxidant enzymatic activities (Nishio and Watanabe 1997). Further studies are needed to investigate whether hepatic antioxidant enzymes in folate-depleted rats may be directly modified by homocysteine through oxidative pathways.

The present study provides evidence that inferior folate status was also associated with increased hepatic oxidative damage. Compared with the controls, livers of folate-depleted rats had greater TBARS concentrations, especially when H_2O_2 or Fe^{2+} was added to liver homogenates. The aggravated lipid oxidative damage in livers of folate-depleted rats may be ascribed to either a decreased hepatic antioxidant capability, probably due to the decreased hepatic antioxidant enzyme function (Table 3), or the presence of pro-oxidants, such as increased plasma homocysteine (Table 2). This postulation is supported by the observation that rats fed the NF diet had the highest plasma homocysteine concentrations and the lowest antioxidant enzymatic function in their livers and had significantly higher TBARS concentrations induced by Fe^{2+} treatment compared with rats fed the LF and MF diets (Table 4). The pro-oxidative activity of homocysteine in the presence of transition metal ions on oxidative damage was previously reported in human umbilical vein endothelial cells (Jones et al. 1994, Starkebaum and Harlan 1986). Hyperhomocysteinemia induced by nitrous oxide exposure in pigs was associated with elevations in heart iron stores and increased in vivo lipid peroxidation (Young et al. 1997). Consistently, our findings reveal that rats with the lowest folate content and the highest plasma homocysteine concentrations were more susceptible to lipid peroxidative damage in the livers under the additional oxidative stress of increased metal iron.

A unique feature of the present study was that it allowed study of the plasma homocysteine response to hepatic folate depletion and oxidative damage, whereas previous studies have focused on the vascular systems (Hankey and Eikelboom 1999, Welch and Loscalzo 1998). In our folate-depleted animal model, a strong negative correlation was found between plasma homocysteine and hepatic folate concentrations ($r = -0.92$, $P = 0.0001$), suggesting that plasma homocysteine is responsive to dietary folate depletion and may serve as a sensitive indicator of liver folate deficiency. Furthermore, elevated plasma homocysteine concentrations in folate-depleted rats were strongly and significantly correlated with increased liver lipid peroxidation in either the absence or presence of additional oxidative stress ($|r| \geq 0.58$, $P < 0.0003$). The data indicate that elevated plasma homocysteine concentrations certainly mirror the increased risk of liver oxidative damage in folate-depleted rats.

In summary, the present study demonstrated for the first time that folate depletion resulted in compromised hepatic antioxidant enzymatic defenses. Reduced folate intake and elevated plasma homocysteine concentrations elicited an increased susceptibility of livers of folate-depleted rats to lipid peroxidation in either the absence or presence of additional oxidative stress. Our data suggest that folate depletion and elevated plasma homocysteine promote oxidative stress in rat livers.

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