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Differences in the metabolic response to exogenous homocysteine in juvenile and adult rabbits

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Abstract

Homocysteine has recently received a lot of attention as an independent risk factor for atherosclerotic and thrombotic cardiovascular disease. Plasma homocysteine levels tend to rise with age, but are also greatly influenced by nutritional factors. Early reports suggested that there were differences in the metabolism of homocysteine in adult and immature animals. The current work tests the hypothesis that adult and juvenile animals respond differently to chronic administration of homocysteine. We have previously found that adult rabbits given homocysteine parenterally twice daily for seven weeks developed progressive folate deficiency and concurrently developed an impairment of homocysteine metabolism. We now report that juvenile rabbits do not develop folate deficiency with chronic homocysteine loading and do not have progressively higher trough levels of homocysteine, as do the adults. In addition, juvenile rabbits that have been chronically pre-treated with homocysteine exhibit a lower peak homocysteine level after a single dose than do juvenile rabbits that have never received homocysteine. This adaptation did not occur in the adult rabbits. In addition, adult homocysteine-treated rabbits had evidence of oxidative stress as evidenced by higher levels of malondialdehyde in liver tissue than adult controls. The homocysteine-treated juvenile rabbits had the same levels of malondialdehyde as the juvenile control rabbits. We conclude that the plasma elimination kinetics are altered in juvenile rabbits in response to homocysteine pre-treatment. The difference in metabolism of homocysteine may protect the juvenile rabbits from the damaging effects of homocysteine. Future studies are planned to elucidate the mechanism of this adaptive response.

Keywords: Homocysteine; Hyperhomocysteinemia; Lipid peroxidation; Folate metabolism

1. Introduction

Elevated plasma homocysteine is now recognized as an important risk factor for cardiovascular disease. Homocysteine is not normally present in the diet, but is produced as a normal metabolite of methionine. The metabolism of homocysteine is complex [1]. The metabolic cycle that interconverts homocysteine and methionine also produces S-adenosyl-methionine (SAM), a key intermediate in one-carbon metabolism. Homocysteine is converted to methionine by addition of a methyl group, a process called transmethylation. The methyl group can come from 5-methyltetrahydrofolate in a reaction that depends on the availability of the vitamins folate and B_{12}. Alternatively, betaine can serve as the methyl donor for formation of methionine from homocysteine. Betaine is a metabolite of choline, an intermediate in lipid metabolism. Homocysteine can be removed from the methionine cycle when it is converted to cystathionine by the transsulfuration pathway. The key enzyme in this pathway is cystathionine β-synthase, which requires vitamin B_6 as a cofactor. Cystathionine is then converted to cysteine and also glutathione, a key buffer of intracellular oxidation-reduction reactions. Thus, at least three major metabolic pathways could potentially have an impact on plasma homocysteine levels.

Hyperhomocysteinemia was initially linked to the development of atherosclerosis by McCully [2–4]. He noted that both genetic and non-genetic factors contribute to plasma homocysteine levels. The genetic factors include mutations in the enzymes that metabolize homocysteine. Deficiency of cystathionine β-synthase can cause severe hyperhomocysteinemia. This is also true of deficiencies of N5-methyltet-

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rahydrofolate methyl transferase and methylenetetrahydrofolate reductase, two of the enzymes in the cycle that converts homocysteine to methionine by addition of a methyl group. The level of homocysteine in such patients is often >100 μM and is classified as severe hyperhomocysteinemia. Normal levels are considered to be <16 μM [5]. Recent works suggest that even within the "normal" range those individuals with higher levels of homocysteine are at greater risk of cardiovascular disease and thrombosis [6].

It has been suggested that fasting levels of homocysteine do not always give a good indication of cardiovascular risk [7, 8]. Some individuals have "normal" levels of homocysteine in the fasting state, but develop abnormally high plasma levels of homocysteine following ingestion of a methionine load. These individuals can be identified with a methionine loading test (MLT). Graham et al. [9] studied patients with arterial occlusive disease (AOD) and compared them with controls. Patients with fasting homocysteine levels in the top fifth compared with the bottom four fifths of the population had a relative risk of AOD of 2.2. An additional 27% of AOD patients had normal fasting levels of homocysteine, but were identified as having abnormal methionine/homocysteine metabolism on the MLT. Thus, it appears that individuals with an increased risk of cardiovascular disease have an impaired ability to metabolize homocysteine formed from dietary methionine that is often, but not always, manifest as an elevated fasting level of homocysteine.

The non-genetic factors that influence homocysteine levels are related to nutritional status, gender and age, as well as drugs, toxins and renal function (reviewed in [10]. Elevated plasma homocysteine can result from deficiencies of vitamins B_{12}, B_6 and esepecially folate. In fact, one study showed that folate status is the major determinant of fasting plasma homocysteine levels in human subjects [11]. In addition to the levels of B-vitamins in the diet, the composition of dietary protein can influence homocysteine levels. Animal proteins contain three times the amount of methionine as plant proteins [12] and the ingestion of a methionine load results in a temporary increase in plasma homocysteine levels. Thus, a consistently high intake of animal proteins could potentially lead to a chronic elevation of plasma homocysteine.

Clinical and epidemiological studies have also correlated plasma homocysteine levels with gender and age [13–15]. The blood levels of homocysteine in premenopausal women averaged 2 μM less than those in men. With the onset of menopause women's homocysteine levels begin to increase. Men's levels progressively increase with age from puberty. Children had significantly lower levels of homocysteine than adults. In spite of these descriptive studies, the mechanisms linking homocysteine levels to age and gender are not understood.

There is some evidence that the relative activities of the different pathways involved in homocysteine metabolism differ in adult and juvenile animals. McCully demonstrated in guinea pigs and rats that the metabolism of homocysteine differs between adult and young animals [16]. The young animals were able to utilize exogenously administered homocysteine for growth, and never developed elevated plasma levels of homocysteine. However administration of exogenous homocysteine to adult animals resulted in an elevation of their plasma homocysteine levels. Studies of juvenile animals fed a methionine-deficient diet showed a decrease in growth rate and premature death, but homocysteine administration could ameliorate the effects of methionine deficiency in growing animals.

We hypothesized that juvenile animals might adapt better to an exogenous homocysteine load than would adult animals. We compared the rate of homocysteine clearance in juvenile and adult rabbits, half of which had been preconditioned by twice-daily injection of homocysteine for eight weeks. We have previously shown that adult rabbits injected chronically with homocysteine become folate deficient and exhibit a slower clearance of exogenous homocysteine than do controls [17]. By contrast, our current study found that juvenile rabbits that had been pre-treated with homocysteine did not become folate deficient, did not develop chronically elevated levels of homocysteine, and did not develop elevated levels of lipid peroxidation products. Thus, it appears that juvenile animals can modify their metabolic patterns to cope with a homocysteine load more effectively than can mature animals. If this adaptive or compensatory mechanism can be understood, it could lead to better management of human patients with elevated levels of plasma homocysteine or abnormal responses to a methionine load.

2. Methods and materials

2.1. Animal model

We used a rabbit model of hyperhomocysteinemia, similar to the protocol used by McCully in his experiments on homocysteine-induced atherosclerosis [18]. Six month old (juvenile, $n = 6$) and 12 month old (adult, $n = 6$) New Zealand White rabbits were obtained from a breeding facility (PSI Robinson Services, Clemmons, NC). Treatment of the rabbits was conducted in accordance with an Animal Research protocol approved by the Animal Use Committee. Their diet consisted of Prolab Rabbit 5P26 chow *ad libitum* and water (Purina Mills, St. Louis, MO.). An indwelling injection port (Vascular Access Ports, Access technologies, Skokie, IL.) was surgically placed under the skin, attached to the muscle layer and the catheter was inserted into the peritoneal cavity of each animal.

Control rabbits received injections of the diluent (5% dextrose/water) and experimental rabbits received 30 mg/kg DL-homocysteine (Sigma, St. Louis, MO) in 5% dextrose/water every twelve hours through an indwelling injection port into the peritoneal cavity. The homocysteine solution

was prepared fresh and sterile filtered before each dose. Blood was drawn approximately every two weeks to monitor the homocysteine levels and other blood chemistries.

After the pre-treatment period the clearance of a single 30 mg/kg ip dose of homocysteine was monitored in the four groups of rabbits. Blood samples were drawn into buffered 3.8% citrate anticoagulant before and after the injection of the homocysteine dose. Each sample was immediately spun at 2000 rpm for twenty minutes, and the plasma was kept frozen at −80°C until analysis.

The plasma homocysteine levels were plotted and fitted to the equation $C = C_0e^{-kt}$ using Excel (Microsoft Corp). Where C is the concentration at any given time, C_0 is the concentration at the first time point after dosing, k is the rate constant for elimination and t is the time in hours after dosing. Our earliest plasma level was taken one hour after dosing and thus the elimination curve does not reflect the early rapid redistribution phase of the clearance curve. Similar to what has been observed in human subjects [19], the elimination kinetics of plasma homocysteine were first-order (fit the exponential model) over a six hour period. The differences between levels achieved at various times after dosing were tested for statistical significance using a *t*-test corrected for multiple comparisons.

At the end of the clearance study, the rabbits were anesthetized, exsanguinated by cardiac puncture and the blood from each rabbit was collected into a syringe containing buffered citrate. The plasma was immediately frozen in aliquots and stored at −80°C until assayed. Tissue samples were frozen immediately in liquid nitrogen and stored at −80°C.

2.2. Blood chemistries

Total plasma homocysteine assays were performed in the Clinical Chemistry Laboratory of UNC Hospital (Chapel Hill, NC), using the IMX system (Abbott Labs, Oslo, Norway). Assays of aspartate amino transferase (AST), alanine amino transferase (ALT), calcium, glucose, Vitamin B12, and folate levels were performed in the Clinical Chemistry Laboratory, Durham VA Medical Center (Durham, NC).

2.3. Thiobarbituric acid reactive substances (TBARS) assay

The presence of the lipid peroxidation product malondialdehyde was assayed in lipids from liver samples as described [20]. The livers were extracted as described below, and one ml of lipid was put into a preweighed 13x100 screw top tube. 100 μL of 0.28M trichloroacetic acid was added and the tube was vortexed and spun at 4000g for fifteen minutes; 0.3 mL of the upper phase was added to a clean 13x100 tube. To this sample was added 1.5 mL Glycine HCl, 1.5 mL 0.04M thiobarbituric acid (TBA), and 1.7 mL distilled water. The tube was then placed in boiling water for fifteen minutes. The sample was cooled and 1 mL of top pink layer was put into a glass cuvette and the absorbance read at 532 nm. The assay was standardized by using 1,1,3,3-tetraethoxypropane (TEP) as a standard.

2.4. Glutathionine assay

Total glutathione levels were assayed by using a commercial assay kit (BIOXYTECH GSH-400, R&D Systems, Minneapolis, MN). Red Blood Cells (RBC) were extracted with two volumes of cold metaphosphoric acid and spun at 2000g for thirty minutes. The supernatant was removed and assayed for glutathione content. The liver samples were cut in small pieces, added to cold metaphosphoric acid (2.6 mg/1.69mls) and disrupted by sonication. The samples were then centrifuged at 3000g at 4°C for ten minutes and the supernatants used for the assay. The results were compared to a standard curve prepared from a fresh 0.5 mM glutathione solution.

2.5. Lipid extraction/fatty acid analysis

Fatty Acids were extracted from 1 g samples of frozen liver by using one ml of BHT chloroform and 9 mL methanol, followed by the addition of 10 mL of chloroform [21]. Tissue residue was removed, the upper phase was aspirated and discarded, lower phase was dried down and lipid weight determined. The lipid samples were subjected to separation over a silica column into non-polar and polar fractions. Further separation using 2% acetic acid in ethyl ether (PE, 98:2, HOAc) yielded the fatty acids from the non-polar fractions. Fatty acids were derivatized using a BF3/Methanol reagent, boiled in water for 30 min, dried down and re-dissolved in 200 mL of Iso-Octane. Analysis was performed by gas-liquid chromatography as described [22].

2.6. Protein assay

Protein was assayed with a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL), using bovine serum albumin to construct the standard curve.

3. Results

3.1. Blood chemistries

Homocysteine levels were obtained approximately every 2 weeks during the pre-treatment period for this study. Blood was drawn for measurement of homocysteine levels immediately before injection of the next dose, and thus represents the trough level. Levels for control and treated adults began to diverge after four weeks of treatment when the controls animals had levels of 14 ± 2.8 compared to 15.9 ± 3.6 μM in the treated animals. However, the difference did not reach statistical significance until the eight week of treatment. At this point the homocysteine-treated

Table 1
Selected Blood Chemistries[1]

	Juvenile Control	Juvenile HCys treated	Adult Control	Adult HCys treated
Homocysteine (μM)	12.7 ± 4.5	13.8 ± 4.9	12.3 ± 1.5	20.3 ± 9.9*
AST (U/L) (aspartate transaminase)	25.0 ± 2.5	19.0 ± 7.2	23.8 ± 10.3	22.6 ± 10.8
ALT (U/L) (alanine transaminase)	57.7 ± 15	55 ± 39	53.1 ± 25.4	51.7 ± 22.3
Calcium (mg/dl)	12.8 ± 0.3	12.9 ± 0.3	13.04 ± 0.99	11.75 ± 1.0
Glucose (mg/dl)	141.7 ± 8.9	171 ± 51	136 ± 49	106 ± 10.4
B12 (ng/ml)	37 ± 8.9	35.2 ± 4.8	65.1 ± 26.0	83.7 ± 11.2
Folate (ng/ml)	16 ± 5.3	26.7 ± 15.5	24.8 ± 6.0	16.2 ± 3.7*

[1] Serum samples were collected at the time of sacrifice and kept frozen at −80° C until the time of assays. Data are shown as the mean ± standard deviation.
* $p<0.05$ compared to age-matched controls

adult rabbits had trough levels of homocysteine of 20.3 ± 9.9 μM compared to 12.3 ± 1.7 μM for the control group (Table 1). By contrast, the trough levels of homocysteine were similar in the control and homocysteine-treated groups of juvenile rabbits throughout the treatment period. Homocysteine levels in the juveniles were also not different from the control adult rabbits (Table 1).

The plasma levels of several other analytes were also measured after eight weeks of homocysteine treatment (Table 1). AST and ALT levels were similar in the control and treated groups of both the adult and juvenile rabbits, indicating that the homocysteine treatment had not led to hepatic damage. Calcium, glucose, and vitamin B_{12} levels were also similar in control and treated animals.

After eight weeks of homocysteine administration the plasma folate levels were significantly lower in the adult HCys group than the adult control group (16.2 ng/mL ±3.7 vs. 24.8ng/mL ±6.0) in the adult rabbits (Table 1). However, the total plasma folate levels were not significantly different in the HCys and control groups of the juvenile rabbits. While not statistically significant, there was a tendency for the homocysteine-injected juvenile rabbits to have higher folate levels than the controls. We speculate that the depletion of folate in the adult HCys rabbits led to an impairment of homocysteine metabolism, thereby leading to the elevation of the plasma homocysteine levels.

3.2. Homocysteine clearance

The plasma clearance of a single dose of homocysteine was compared in rabbits that had been chronically administered homocysteine and those that had not previously received homocysteine. While the pre-dose level of homocysteine was higher in the adult HCys rabbits than in the controls, the peak level after dosing tended to be lower than in the controls (Fig. 1). This difference did not quite reach statistical significance in this study ($P = 0.06$). This suggests that there might be some degree of adaptation induced by chronic homocysteine exposure in the adult rabbits. However, the plasma homocysteine levels did not return to baseline as rapidly in the adult HCys rabbits as in the controls. The elimination rate constant (k) was 0.281 in the controls and 0.178 in the adult HCys animals. The elimination half-life was 2.47 hr in the adult controls and 3.89 hr in the adult HCys animals. This is consistent with the significant degree of folate deficiency that developed in the HCys rabbits over the course of the pre-treatment period. Folate deficiency would be expected to impair homocysteine metabolism by transmethylation to methionine.

Our findings were quite different in the juvenile rabbits than in the adults. In the juvenile rabbits there was a much greater difference in the peak level of homocysteine achieved in the HCys and control rabbits (Fig. 2). Because of the lower initial recovery of the dose in the plasma, the homocysteine level tended to return to baseline more quickly in the HCys-treated juveniles than in the juvenile

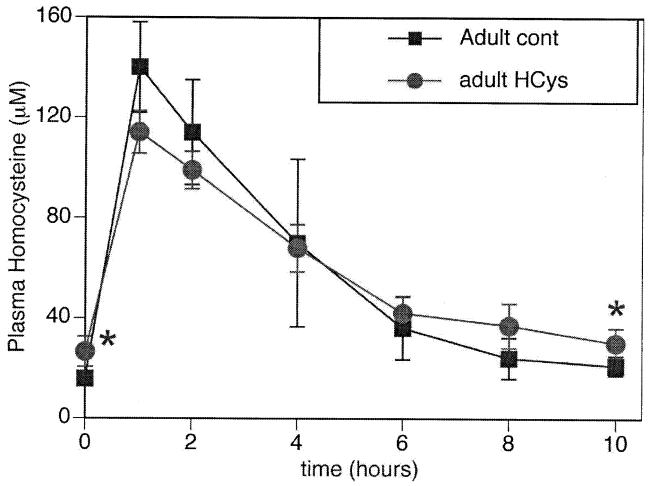

Fig. 1. Effect of homocysteine pre-treatment on the elimination of a dose of homocysteine. Adult New Zealand White rabbits were pre-treated with twice-daily administration of 30 mg/kg homocysteine (HCys) or buffer only (Control) for eight weeks. Each animal was then given a 30 mg/kg dose of homocysteine and serial plasma samples collected. The mean and standard deviation of the values are shown. The curves are from three animals each. Statistically significant differences from the control values ($P < 0.05$) are indicated by *. Note that the initial value for the HCys group was elevated. This value represents the trough value after the last pre-treatment dose.

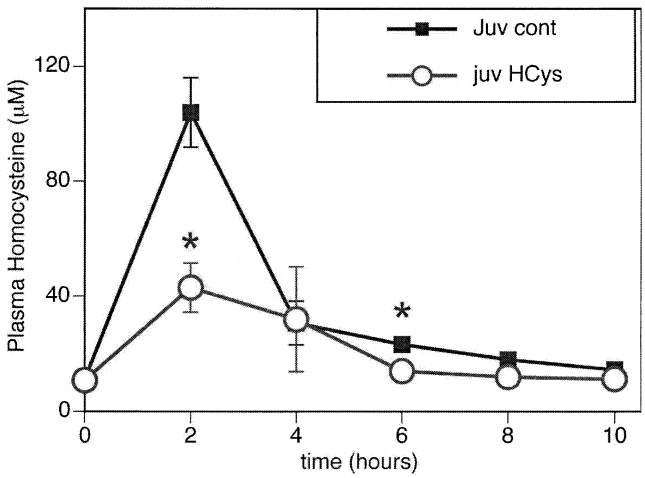

Fig. 2. Effect of homocysteine pre-treatment on the elimination of a dose of homocysteine. Juvenile (weanling) New Zealand White rabbits were pre-treated with twice-daily administration of 30 mg/kg homocysteine (HCys) or buffer only (Control) for eight weeks. Each animal was then given a 30 mg/kg dose of homocysteine and serial plasma samples collected. The mean and standard deviation of the values are shown. The curves are from three animals each. Statistically significant differences from control values ($P < 0.05$) are indicated by *.

controls. However, the elimination rate constants and elimination half lives were not significantly different in the juvenile homocysteine-treated rabbits (0.266 and 2.59 hr) and juvenile control rabbits (0.289 and 2.39 hr).

3.3. Lipid peroxides

Homocysteine has been hypothesized to cause cellular dysfunction contributing to the development of atherosclerosis by promoting oxidative stress [23]. Therefore, we hypothesized that administration of homocysteine would promote oxidation of polyunsaturated fatty acids to their lipid hydroperoxides. These hydroperoxides break down to form malondialdehyde, which is measured as thiobarbituratic acid-reactive substances (TBARS) and is used as an indicator of oxidation in vivo.

The amount of lipid peroxidation products in the liver extracts of the adult HCys rabbits was significantly higher than the adult control rabbits (Table 2). However, the levels of lipid peroxidation products in juvenile HCys rabbits were comparable to the juvenile control rabbits (Table 2). The lack of increase of lipid peroxidation products in the juvenile HCys rabbits seems to reflect an adaptation to homocysteine treatment that could be related to age.

Table 2
Lipid Peroxidation Products (TBARS) in Liver[1]

	Control	HCys-treated
Adult	1.72 ± 0.47	2.41 ± 0.46*
Juvenile	3.97 ± 2.25	1.83 ± 0.58

[1] Liver tissue was collected at the time of sacrifice, snap frozen in liquid nitrogen and kept at −80° C until the time of assay. Results are expressed as the mean ± standard deviation in mg thiobarbituric acid (TBARS) per kg liver tissue.

* $p<0.05$ compared to age-matched controls.

Table 3
Total Glutathione content in Liver[1]

	Control	HCys-treated
Adult	49 ± 7	52 ± 13
Juvenile	36 ± 5	42 ± 3*

[1] Liver tissue was collected at the time of sacrifice, snap frozen in liquid nitrogen and kept at −80° C until the time of assay. Results are expressed as the mean ± standard deviation in nmoles of glutathione per mg protein.

* $p<0.05$ compared to age-matched controls.

3.4. Fatty acid analysis

It is possible that homocysteine treatment might affect lipid metabolism, because one important pathway of homocysteine metabolism is transmethylation by betaine homocysteine methyl transferase. Betaine is derived from choline, a major precursor of the fatty acid-containing lipids, phosphatidylcholines. The fatty acid compositions of rabbit livers reflected the dietary fatty acid pattern, with the most abundant polyunsaturated fatty acid being linoleic acid. No significant differences in the fatty acid composition of livers from HCys and control rabbits of either age group were found (data not shown).

3.5. Glutathione analysis

Glutathione is both a potential metabolite of homocysteine (through the cystathionine synthase pathway) and an important intracellular antioxidant. Therefore, we expected its concentration to be altered by homocysteine treatment. Total glutathione content in liver from juvenile homocysteine-treated rabbits was significantly higher than controls (42 ± 3 vs. 36 ± 5 nmol/mg protein, $P < 0.05$). By contrast, liver glutathione levels were comparable in control and homocysteine-treated adult rabbits (49 ± 7 vs. 52 ± 13 nmol/mg protein) (Table 3).

4. Discussion

The elevation of plasma levels of homocysteine has been associated with an increased risk of atherosclerosis and thromboembolism. Therefore, it is of interest to evaluate the factors that play a role in determining the plasma homocysteine level. This is not a straightforward problem, since homocysteine is involved in several metabolic cycles. In addition, there is a continual flux of homocysteine between the tissues and plasma. It is not known whether the pool of homocysteine that is most directly related to cardiovascular disease is intravascular or intracellular. However, the

plasma level of homocysteine is at least a reflection of the pathophysiologically important pool.

In our study, we examined the plasma homocysteine levels achieved in response to a dose of homocysteine in adult and juvenile rabbits. A subset of each age group was chronically administered homocysteine. After eight weeks of injections we compared the clearance of a single test dose of homocysteine. We found two major differences in the elimination of homocysteine from plasma in the adult and juvenile rabbits.

First, the homocysteine-treated adults developed persistent elevation of plasma homocysteine levels over the course of the study as we have previously described [17]. Early in the study period each homocysteine-injected animal had completely cleared a dose of homocysteine before the subsequent dose was administered 12 hr later. By the end of the study, the adult animals no longer completely cleared each dose of homocysteine by the end of the 12-hr dosing interval and the elimination half-life was prolonged compared to controls. By contrast, the juvenile homocysteine-treated animals continued to clear each dose of homocysteine within a 12-hr period throughout the entire pretreatment period. The defect in homocysteine metabolism in the adult animals was associated with the progressive development of folate deficiency, even though the animals were fed a standard folate-replete diet. This is a novel observation, since we are not aware of previous reports suggesting that homocysteine loading can cause folate deficiency. In contrast to the adult animals, the juvenile rabbits did not become folate deficient in the face of chronic homocysteine administration.

We do not know why homocysteine administration led to folate deficiency in the adult, but not the juvenile rabbits. It has been suggested that oxidative stress may lead to inactivation of folate cofactors, resulting in folate deficiency [24]. The active form of the cofactor, methyl-tetrahydrofolate, is very susceptible to oxidation. Oxidative stress due to administration of homocysteine could lead to folate depletion that would then, in turn, impair homocysteine metabolism and exacerbate the hyperhomocysteinemia. Thus, folate deficiency could be both a cause and a consequence of hyperhomocsyteinemia. Any agent or condition that causes oxidative injury could tend to promote hyperhomocysteinemia. We speculate that the oxidative stress induced by homocysteine in the homocysteine-treated adult animals led to their folate deficiency. The juvenile animals might have better defenses against oxidative damage, which might have allowed them to maintain adequate folate stores. If our speculation proves to be true, it suggests that any of a number of apparently unrelated conditions that promote oxidant injury could potentially lead to hyperhomocysteinemia.

The second major difference between the handling of homocysteine in adult and juvenile animals was that the peak plasma levels achieved after a dose of homocysteine were significantly lower in the homocysteine-treated juvenile animals than in the homocysteine-treated adult animals or control animals. This finding is of interest because it suggests that juvenile individuals have the ability to metabolize or assimilate a homocysteine or methionine load in a fashion that is lost in adults. While we do not yet know the nature of the adaptive response in juvenile animals, it may be a major factor in the progressive rise in homocysteine levels that is associated with aging.

While the difference in folate status in the adult homocysteine-treated rabbits explains the longer elimination half-life in this group, it does not explain why the juvenile homocysteine-treated rabbits had much lower peak plasma levels of homocysteine in response to a standardized test dose. It is possible that these animals developed an enhanced ability to metabolize homocysteine. It is also possible that juvenile homocysteine-treated animals sequester homocysteine into the intracellular space much more efficiently than do the other groups of animals.

Our data suggest that impaired homocysteine elimination in the adult homocysteine-treated rabbits had potentially significant pathologic effects. The homocysteine-treated adult rabbits had elevated levels of lipid peroxidation products, as has previously been reported in a rat model of hyperhomocysteinemia [25]. The homocysteine-treated juvenile rabbits, even though they had been given equivalent doses of homocysteine over the homocysteine treatment period, did not have elevated levels of lipid peroxidation products.

It has been reported that glutathione levels tend to decline with age [26] while homocysteine levels tend to rise. Reduced glutathione is a major intracellular anti-oxidant. We hypothesized that glutathione levels would be higher in juvenile rabbits than in adults and that the levels might change in response to the oxidant damage induced by homocysteine. However, glutathione levels in liver from juvenile control rabbits were lower than in adult controls (on a per mg protein basis). The glutathione content of adult liver did not change with homocysteine loading, while liver glutathione increased significantly in homocysteine-treated juvenile rabbits.

Cysteine is the limiting amino acid in glutathione synthesis. About half of the cysteine in glutathione in human liver cells is derived from homocysteine by the transsulfuration pathway. This pathway converts homocysteine to cystathionine and subsequently cysteine. The fact that our homocysteine-treated juvenile rabbits developed higher glutathione levels than controls suggests that the activity of the cystathionine pathway can be induced in the juvenile rabbits, but not the adults. It has been reported that transsulfuration of homocysteine and glutathione synthesis are enhanced by pro-oxidants and reduced by anti-oxidants in cultured liver cells [27, 28]. Homocysteine appears to promote oxidant stress [25]. Thus, we hypothesize that pro-oxidant effects of homocysteine treatment promote enhanced glutathione synthesis in the juvenile rabbits. It is not clear why a similar effect was not observed in adults. How-

ever, there is a precedent for the induction of anti-oxidant enzymes in juvenile, but not adult animals. Juvenile rats exposed to hyperoxia increase their levels of pulmonary antioxidants [29], while adult animals do not [30]. The young animals survive in hyperoxic conditions while the adults develop pulmonary oxygen toxicity. Thus, our current data suggest that there may also be anti-oxidant pathways outside the lung that are inducible in juvenile, but not adult animals.

Differences in the plasma recovery and clearance of a dose of homocysteine in adults and juveniles could be due to any of several mechanisms and our current study does not distinguish among them. Since only about 2% of a homocysteine load is excreted unchanged in the urine [19], it is unlikely that differences in renal function contribute. Important factors could include the rate of redistribution of homocysteine between intravascular, extravascular and intracellular compartments; activity of key enzymes in the transmethylation and transsulfuration pathways; and utilization of methionine derived from homocysteine for growth and protein synthesis.

We conclude that juvenile rabbits adapt to chronic homocysteine loading in a manner that effectively decreases the plasma level achieved in response to a homocysteine dose. This adaptation is functionally significant, since it is associated with reduced oxidant stress, as indicated by lower TBARS and increased glutathione in the juveniles compared to similarly treated adults. Defining the mechanism of this adaptation may allow improved understanding and treatment of the progressive hyperhomocysteinemia that develops in humans with ageing.

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