Intimal Hyperplasia Following Carotid Endarterectomy in an Insulin-Resistant Rat Model

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Hyperhomocysteinemia, a known risk factor for cardiovascular disease, results in an elevation of intimal hyperplasia (IH) following a carotid endarterectomy (CEA) in a rat model. An exaggerated IH response following CEA has been observed in rats with dietary induced hyperhomocysteinemia. Type 2 diabetics often present with hyperhomocysteinemia and are at higher risk for developing vascular blockage following surgical procedures. To determine if insulin resistance increases IH risks following endarterectomy, the 3 goals of this study were: (1) to establish plasma homocysteine concentrations in dietary induced insulin-resistant rats and their controls, (2) to investigate whether a positive correlation of IH and plasma homocysteine response occurs following CEA in the insulin-resistant rat model, and (3) if so, to attempt to decrease IH by supplementation with folic acid, a known enzymatic cofactor in the homocysteine metabolic pathway. To achieve these aims, male rats (275 to 300 g) were fed 1 of 4 diets for a 4-month period: (1) high-fat sucrose (HFS), (2) low-fat complex carbohydrate (LFCC), (3) HFS + 25 mg/kg folic acid (HFS+F), or (4) LFCC + 25 mg/kg folic acid (LFCC+F). At the end of the 4-month period the rats underwent an open (non-balloon) unilateral CEA. Two weeks post-endarterectomy, blood, liver and carotid tissue were removed to measure plasma insulin, folic acid, and homocysteine, 2 key enzymes of homocysteine metabolism—methylenetetrahydrofolate reductase (MTHFR) and cystathionine β-synthase (CBS)—and percent lumenal stenosis (IH%). Computer-assisted morphometric analysis was used to measure the percentage of IH in the carotid artery. Plasma homocysteine was significantly higher in the HFS group when compared with the LFCC group (11.3 ± 1.3 μmol/L *v* 7.4 ± 0.6 μmol/L, *P* = .008) as was post-endarterectomy IH producing lumenal stenosis (30.7% ± 4.2% *v* 14.0% ± 4.3%, *P* = .008). Plasma insulin in the HFS group was higher than the LFCC (control) group and was significant (36.3 ± 3.0 μU/mL *v* 21.1 ± 0.8 μU/mL, *P* = .0004). Folic acid supplementation in the HFS group resulted in reductions of plasma homocysteine (HFS *v* HFS+F, 11.3 ± 1.3 μmol/L *v* 7.95 ± 1.0 μmol/L, *P* = .02) and post-endarterectomy IH (HFS *v* HFS+F, 30.7% ± 4.2 % *v* 10.4% ± 1.6%, *P* = .0001). The control or LFCC group was not statistically different from the HFS+F group in homocysteine or IH. Folate supplementation did not decrease insulin concentrations in the HFS+F group compared to the LFCC group. We conclude that the HFS diet produced an insulin-resistant state with an elevated plasma homocysteine and an exaggerated IH response following carotid endarterectomy in this rat model. Dietary folate supplementation reduced plasma homocysteine concentrations in the HFS diet, which implicates hyperhomocysteinemia as an etiologic factor in the development of post-CEA IH in this insulin-resistant rat model.

INSULIN RESISTANCE, the disease process in patients with type 2 diabetes, has been shown to be a risk factor for the development of atherosclerosis.[1,2] The mechanisms responsible for this are unclear; however, diabetes is associated with hormonal and vascular abnormalities that promote smooth muscle cell proliferation.[3] Smooth muscle cell proliferation and migration have also been shown to be an integral process in the development of atherosclerosis as well as intimal hyperplasia (IH).[4,5] A high-fat, sucrose (HFS) diet, when compared to a low-fat complex carbohydrate (LFCC) diet produces hyperinsulinemia and hyperhomocysteinemia in a rat model.[6] In previous studies,[7,8] male Fisher 344 and male Sprague-Dawley rats fed the HFS diet were nondiabetic and insulin-resistant, with high fasting insulin concentrations and normal fasting glucose concentrations.[8] This rat model also manifests other features of the "insulin resistance syndrome," including obesity, hypertension, hypertriglyceridema, and enhanced clotting.[7]

Carotid endarterectomy (CEA) is a commonly performed vascular operation that has proven to be very efficacious in preventing strokes. While CEAs can be performed with low morbidity and mortality it can be complicated with the development of postoperative IH. It is this IH that can result in restenosis and the development of thrombosis or embolic events that lead to transient ischemic attacks or stroke.[9-11]

Our laboratory has been interested in the development of post-CEA IH and has developed and established a hyperhomocysteinemia dietary model in combination with a rat CEA with arteriotomy, open endarterectomy, and primary closure that produces significant postoperative IH. This diet/surgery model has allowed us to identify etiologic factors involved in the development of post-CEA IH. One such factor is hyperhomocysteinemia.[12,13]

In initial studies, plasma homocysteine was elevated by the addition of dietary homocystine. Rats with elevated plasma homocysteine concentrations developed markedly increased IH after endarterectomy compared with rats fed lab chow diets.[12]

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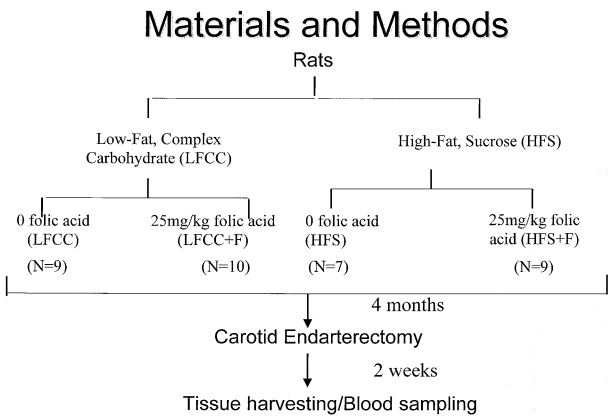

Fig 1. Description of experimental design of dietary groups with CEA. Diets were fed for 4 months before CEA was performed. The rats were then fed an additional 2 weeks before euthanasia and collection of blood and tissues.

In further studies, supplementary dietary folic acid in the homocystine diets resulted in decreased plasma homocysteine and subsequent reductions in the level of IH following CEA.[14] Thus isolating increased homocysteine as an etiological factor in post-CEA IH development in this model. Our previous work in this area was based on elevations of plasma homocysteine by dietary supplementation with homocystine.

In the current study, rats were fed a HFS diet to produce a physiologic increase in plasma homocysteine. Our hypothesis was that rats fed a HFS diet would develop insulin-resistance, hyperhomocysteinemia and increased levels of IH following CEA. In addition, folic acid supplementation would result in decreased concentrations of plasma homocysteine and post-CEA IH. If correct, this would isolate hyperhomocysteinemia as an independent risk factor for post-CEA IH in this insulin-resistant rat model.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (275 to 300 g) were randomly numbered and assigned to 1 of 4 diet groups that varied in the amount of fat and type of carbohydrate added. The 2 basic diets were the HFS (experimental) group, and a low-fat complex carbohydrate (LFCC) diet that served as the control. Folic acid was then supplemented to one group each of the 2 basic diets (Fig 1). The diets and number of rats in each diet group were as follows: (1) HFS (n = 7), (2) LFCC (n = 9), (3) HFS + 25 mg/kg folic acid (HFS+F) (n = 9), and (4) LFCC + 25 mg/kg folic acid (LFCC+F) (n = 10).

Treatments

Diet composition consisted of percentages of calories in protein, fat, and carbohydrates of 21%, 6%, and 73%, respectively, for the LFCC and 21%, 39.5%, and 39.5% for the HFS diets. The pelleted diets (Dyets Inc, Bethlem, PA) were prepared with vitamin-free casein as the source of protein and contain a standard mineral mix. The added folic acid consisted of 25 mg/kg folic acid per kilogram of diet. Based on a daily consumption of 17 g/d, the rats received 0.425 mg/d folic acid in the LFCC+F and HFS+F diet groups and 0.034 mg/d in the LFCC and HFS diet groups. The rats were allowed water and food ad libitum and were continued on their respective diet for 4 months prior to undergoing CEA. Following the CEA, the rats were continued on their preoperative diet regimen for 2 weeks until they were killed (Fig 1).

CEA Operation

The CEA was performed in an open fashion with suture closure of the arteriotomy as previously described.[12] Blood samples were obtained immediately prior to euthanasia. Tissue removal was performed following perfusion with saline and 10% formalin as previously described.[15]

Homocysteine Measurement

All plasma homocysteine laboratory values were measured using a thiol-specific fluorogenic labeling reagent and the thiols were separated by a reverse-phase high-pressure liquid chromatography method using fluorescence detection.[21]

Insulin/Folic Acid Measurement

Insulin was determined by coat-a-count [^{125}I] rat insulin radioimmunoassay (RIA) kit (Diagnostic Products Corp, Los Angeles, CA). Folic acid measurements were determined by [^{125}I] rat folic acid RIA kit (Diagnostic Products Corp).

Morphometric Analysis

After harvesting, the common carotid artery was processed, paraffin-blocked, sectioned, and elastin-stained with Verhoeff's and Van Gieson's stain. Multiple sections were taken at intervals of 3 μm each continuing along the distance of the continuous 10-0 nylon suture arteriotomy closure to standardize the region of sectioning. The elastin-stained slides were photographed using a Kodak DC 120 Zoom digital camera (Eastman Kodak, Rochester, NY). Images of the carotid sections were then downloaded to a computer and the lumenal areas of the carotids were analyzed using the National Institutes of Health (Bethesda, MD) ImageJ Software program, Version 0.99i. This software package allowed us to delineate the inner area of IH and thus obtain an accurate measurement of the cross-sectional area of the vessel lumen. The characteristic pattern of IH was used to identify the demarcation between the outer limits of IH and the medial/adventitial layers. The difference between the 2 areas (outer area of IH minus the actual lumen) was determined as the absolute area of IH. Because the arterial cross section had individual variations of shape, the values were expressed as a ratio of the absolute area of IH to the outer limit of IH, and were reported as percent lumenal stenosis. This ratio represents the proportion of the lumen area occupied by IH and allowed for comparison of the arterial cross sections of varying size.[17] All sectioning and measurements were made in a blinded fashion by 2 independent investigators.

Enzyme Activity Assays

Cystathionine β-synthase (CBS). Livers were immediately removed from the rats, prior to infusion with formaline, and frozen in liquid nitrogen until the assay was performed. CBS activity was assayed by as previously described.[13]

Methylene tetrahydrofolate reductase (MTHFR). The MTHFR activity assay was determined as previously described.[13]

Statistical Methods

Values are reported as means ± SE. A 1-way analysis of variance (ANOVA) was used to compare plasma homocysteine and IH% means among the HFS, LFCC, HFS+F, and LFCC+F treatment groups. If significance at the 5% level was observed among these treatment groups, a Fisher's protected least significant difference (LSD) post hoc test one *P* value was calculated for contrasts of interest. Additionally, the 95% confidence interval (CI) on the mean difference ± SE between

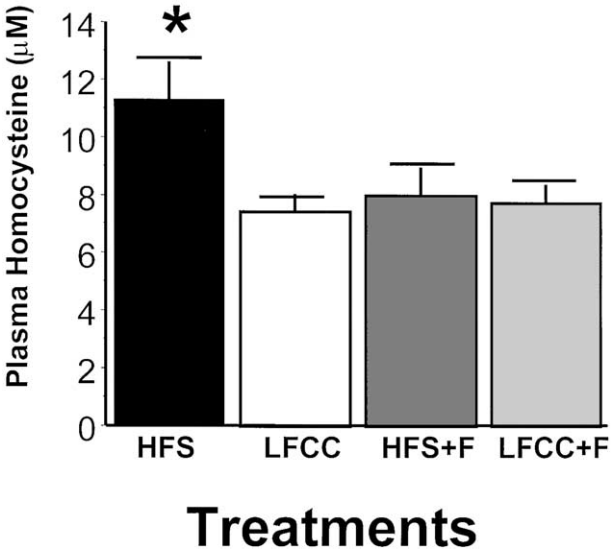

Fig 2. Plasma homocysteine concentrations among treatment groups. The HFS diet group had increased plasma homocysteine concentrations *v* the LFCC at *P* = .008. The addition of folic acid to the HFS group (HFS+F) significantly reduced plasma homocysteine *v* HFS at *P* = .02. Means ± SEM are reported. **P* < .05 *v* all groups.

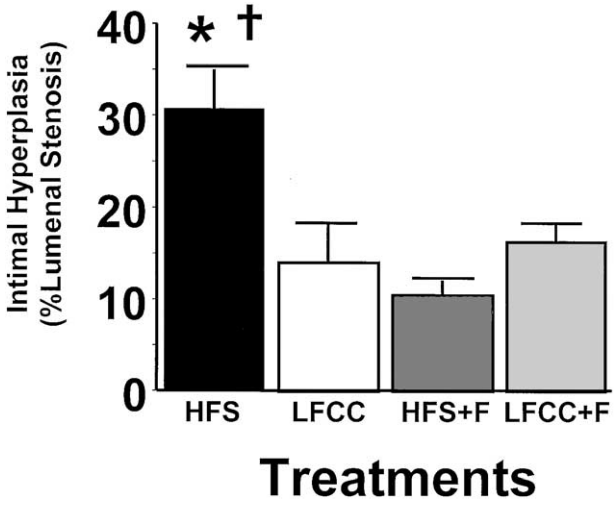

Fig 3. Intimal hyperplasia or percent lumenal stenosis among treatment groups. The HFS group had increased IH *v* the LFCC group at *P* = .001 and *v* the HFS+F group at *P* = .0001. Folic acid supplementation significantly decreased IH in the HFS+F diet group. Means ± SEM are reported. *Significance *v* LFCC, and †significance *v* HFS+F at *P* < .05.

the group means that were significant is reported. In this study, the HFS versus LFCC, HFS versus HFS+F, and LFCC versus LFCC+F were primary contrasts of interest. A linear regression model was fit to measure the relationship between percent IH/plasma homocysteine and IH/plasma folic acid. The *P* value for a significant linear effect and *R* value were calculated. These analyses were performed using the Stat View program (SAS Institute, Cary, NC) version 5.0.

RESULTS

Mean plasma homocysteine concentrations were significantly different among the diet groups ($f = 3.597$, $df = 3,27$; $P = .0263$). Mean plasma homocysteine was significantly increased in the HFS diet group in comparison to the LFCC diet group (11.3 ± 1.3 μmol/L *v* 7.4 ± 0.6 μmol/L, Fisher's LSD $P = .008$; 95% CI, 3.886 ± 2.8) (Fig 2). The addition of folic acid to the HFS diet (HFS+F group) decreased homocysteine concentrations to those of the LFCC diet group (7.95 ± 1.0 μmol/L *v* 7.4 ± 0.6 μmol/L, Fisher's LSD $P = .67$) and produced a significant reduction in plasma homocysteine concentrations when compared with the HFS diet (7.95 ± 1.0 μmol/L *v* 11.3 ± .3 μmol/L, Fisher's LSD $P = .017$; 95% CI, 3.35 ± 2.694) (Fig 2). The addition of folic acid to the LFCC diet (LFCC+F group) produced no significant difference in plasma homocysteine concentrations when compared with the LFCC diet group (7.7 ± 0.6 μmol/L *v* 7.4 ± 0.6 μmol/L, Fisher's LSD $P = 0.81$).

Mean IH% was significantly different among the diet groups ($f = 6.885$, $df = 3,31$; $P = .0011$). Mean IH% was significantly increased in the HFS diet group in comparison to the LFCC diet group (30.7% ±4.2 % *v* 14.0% ± 4.3% IH, Fisher's LSD $P = .008$; 95% CI, 16.776 ± 9.689). With folic acid supplementation, the HFS+F diet group showed two thirds less IH in comparison to the HFS diet group (10.4% ± 1.6 % *v* 30.7% ± 4.2%, Fisher's LSD $P = .0001$; 95% CI, 20.31 ± 9.689). IH was low and not significantly different in the LFCC diet group versus the LFCC+F diet group, as shown in Fig 3 (14.0% ± 4.3% *v* 16.2% ± 2.2%, Fisher's LSD $P = .60$). The degree of IH in the HFS+F diet group was not significantly different than that seen in the LFCC diet group (10.4% ± 1.6 % *v* 14.0% ± 4.3%, Fisher's LSD $P = .43$). We also tested for the effects of plasma homocysteine concentrations and IH for a linear trend, which had a significant, direct relationship based on regression analysis ($R = .42$, $P = .02$) (Fig 4).

Mean plasma insulin levels were significantly different among the diet groups ($f = 28.352$; $df = 3,25$; $P < .0001$).

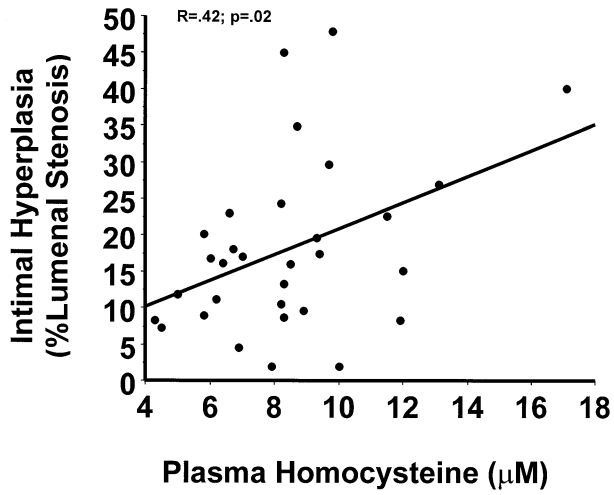

Fig 4. Regression analysis of intimal hyperplasia *v* plasma homocysteine. A strong positive correlation was observed between IH and plasma homocysteine, *P* = .02.

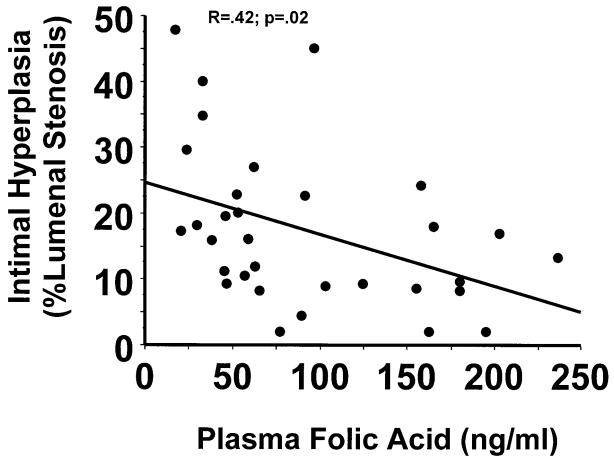

Fig 5. Regression analysis of intimal hyperplasia *v* plasma folic acid. A strong negative correlation was observed between IH and plasma folic acid concentrations at *P* = .02.

Mean plasma insulin was increased in the HFS diet group in comparison with the LFCC diet group (36.3 ± 3.0 μU/mL *v* 21.0 ± 0.8 μU/mL, Fisher's LSD $P = .0004$; 95% CI, 15.243 ± 7.709). The HFS+F diet group showed a significant increase in insulin concentrations when compared with the LFCC+F diet group (47.0 ± 3.9 μU/mL *v* 17.2 ± 1.8 μU/mL, Fisher's LSD $P < .0001$; 95% CI, −10.7 ± 7.709) and was not different from the HFS diet group. Addition of folic acid to the LFCC diet (LFCC+F diet group) did not change insulin concentrations (21.0 ± 0.8 μU/mL *v* 17.2 ± 1.8 μU/mL, $P = .30$).

Mean folic acid levels were significantly different among the diet groups ($f = 9.126$, $df = 3,28$; $P = .0002$). Folic acid supplementation to the HFS+F and the LFCC+F diets resulted in significant increases in plasma folic acid levels when compared with the HFS and LFCC diet (135.3 ± 20.0 μmol/L *v* 31.0 ± 5.6 μmol/L, $P = .0002$; 128.8 ± 22.0 μmol/L *v* 61.6 ± 8.0 μmol/L, $P = .006$, respectively). A significant, negative correlation was demonstrated between plasma folic acid and IH using logarithmic regression analysis ($R = .042$, $P = .02$) (Fig 5).

Body weights of the rats taken immediately before CEA indicated that the HFS diet produced higher body weights than the LFCC controls at 499.7 ± 13.1 g versus 463.3 ± 10.3 g ($P = .03$). The addition of folic acid did not decrease body weights as the HFS+F versus HFS body weights were not different 496.4 ± 13.2 g versus 499.7 ± 13.1 g, respectively (Table 1). The homocysteine metabolic hepatic enzymes, MTHFR and CBS, were not significantly different between groups (Table 2).

DISCUSSION

Type 2 diabetics with insulin resistance can present with hyperhomocysteinemia.[18,19] Hyperhomocysteinemia, in addition to its role as a risk factor for atherosclerotic disease, has been shown to associate with post-CEA lumenal stenosis due to IH[12,13] and increased arterial intimal-media thickness.[17] Human studies show plasma homocysteine concentrations of 9 to 15 μmol/L (normal) and 15 to 30 μmol/L (moderate) can increase chances for developing premature cardiovascular disease.[20,21] These low levels may also be associated with increased IH levels after arterial manipulation. This study was designed to investigate if insulin-resistant rats show an increased IH following CEA. The relationship between this increased IH and plasma homocysteine concentrations in diet-induced insulin resistant rats and their control counterparts was also investigated. Attempts to block or decrease IH by folic acid supplementation were also undertaken.

Our previous findings indicate that homocysteine may increase IH development post-CEA in the rat.[12,14] Our previous dietary models involved feeding 4.5 g/kg DL-homocystine to induce hyperhomocysteinemia, to supraphysiological levels (>50 μmol/L).[13] This current study, instead, induced an endogenous level of homocysteine that is more typical of normal clinical levels (5 to 15 μmol/L).[20,21] Also previously,[14] 10 and 25 mg/kg folic acid was used to decrease homocystine-induced plasma homocysteine concentrations and decrease IH percentages compared to control levels. In this study we opted to use 25 mg/kg folic acid supplementation. This level is 12.5 times the normal folic acid level for the rat. We were mimicking the clinical situation of increased folic acid supplementation (5 mg/d), which is 12.5 times the recommended daily allowance. The increase in dietary folic acid was reflected in the increase in plasma concentrations for the HFS+F and LFCC+F groups of 77% and 52%, respectively. Of interest in this study is the insulin resistance model and whether hyperinsulinemia influences IH development following endarterectomy. The HFS-fed rats presented with typical measurements as shown in previous studies using the HFS diet.[6,22,23] We observed that the HFS rats had increased body weight (Table 1), hyperhomocysteinemia (Fig 2), hyperinsulinemia (Fig 4), increased levels of IH (Fig 3), and increased lipids (not reported) versus their LFCC controls. The addition of folic acid to the insulin resistant rats

Table 1. Body Weights of Rats Immediately Before CEA

Treatment	N	Body Weight (g)
HFS	7	499.7 ± 13.1*
LFCC	9	463.1 ± 9.2
HFS + F	9	496.4 ± 13.2†
LFCC + F	10	472.0 ± 9.8

NOTE. Values are means ± SEM.

*HFS *v* LFCC is significantly different using Fisher's LSD pairwise comparison at $P = .03$.

†HFS + F vs LFCC is significantly different using Fisher's LSD pairwise comparison at $P = .04$.

Table 2. Hepatic CBS and MTHFR Enzyme Activity

Treatment	N	CBS (U/mg protein)	MTHFR (nmol/h/0.5 mg protein)
HFS	7	360.6 ± 15.5	3.1 ± 0.4
LFCC	9	380.5 ± 16.5	3.3 ± 0.4
HFS + F	9	390.2 ± 23.9	3.0 ± 0.4
LFCC + F	10	379.3 ± 14.8	3.6 ± 0.5

NOTE. Values are means ± SEM. All correlations and pairwise comparisons were nonsignificant.

(HFS+F diet group) decreased plasma homocysteine concentrations (Fig 2) and lumenal stenosis or IH% post-CEA (Fig 3) compared to the HFS group. Thus HFS rats have increased plasma homocysteine that was correlated with an increased IH response. Both the increased homocysteine and increase in IH were attenuated with the addition of folic acid. However, folic acid supplementation in the HFS group did not influence plasma insulin concentrations (data not shown). This may indicate that insulin resistance per se does not increase plasma homocysteine concentrations that instead, it is the increase in dietary fat, which causes obesity, increased triglycerides and free fatty acids. Increased plasma homocysteine concentrations have been observed in obese hyperinsulinemic patients versus non-obese patients.[18,19] Previous work observed that changes in plasma homocysteine and hyperinsulinemia were significantly correlated with obesity in rats fed the HFS diet.[6] Recent studies[24] have shown that, in normal human subjects, plasma homocysteine concentrations were positively correlated with 2 clinical parameters commonly associated with elevated fat intakes: weight and body mass index. The same study found a negative association between blood levels of S-adenosylmethionine (SAM) and the intakes of fat and energy. Other more classical studies have demonstrated the antagonistic effects of fat and dietary methyl donors in chemical carcinogenesis and hepatotoxicity.[25] Our present results are consistent with these earlier observations and support the hypothesis that increased dietary fats and calories diminish the availability of SAM and thus increase plasma homocysteine concentrations. This may then lead to increased IH.

Fonseca et al[6] observed significant correlations with MTHFR and CBS, 2 key enzymes in homocysteine metabolism, to plasma homocysteine and insulin concentrations. However, in this study there were no significant correlations or pairwise comparisons noted with MTHFR or CBS. This lack of significance may have been due to folic acid supplementation. Folic acid supplementation did have a significant negative correlation ($P = .02$) with levels of IH (Fig 5). Folic acid supplementation, commonly taken to reduce plasma homocysteine concentrations, indicates in this study a significant correlation that can also reduce IH following endarterectomy. With reductions in both IH response and plasma homocysteine concentration by folic acid administration, isolates postoperative elevation of homocysteine in this insulin resistance model as an etiological factor in IH development.

Clinically, patients who are to undergo CEA and have insulin resistance may be at an increased risk of post-CEA restenosis due to IH, especially if they present with a mild to moderate increase in plasma homocysteine. The finding that increased homocysteine may be associated with this increase in restenosis lends itself to a simple cost effective treatment with folic acid.

These findings focus attention on the possibility that increases in plasma homocysteine seen with insulin resistance is associated with increased IH following CEA and that these variables may be decreased with increased folic acid supplementation. Further experimentation should be performed to evaluate the relation of increased body weight or obesity with corresponding plasma homocysteine concentrations.

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