Plasma homocysteine measurements after carotid artery manipulation and clamping in a rat CEA model

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Objective: The effect of a rat carotid endarterectomy (CEA) on homocysteine and the metabolic enzymes methylenetetrahydrofolate reductase (MTHFR) and cystathionine β-synthase (CBS) was studied.

Methods: Rats were placed into 7 groups: (1) no anesthesia (NA), (2) anesthesia only (AO), (3) skin opened and closed (O/C), (4) skin opened with exposure of the carotid artery and closed (O/E/C), (5) carotid isolated and clamped (CO), (6) open CEA, and (7) open femoral endarterectomy (FEA). End points included homocysteine, hepatic MTHFR, and CBS activity.

Results: Homocysteine in the NA, AO, O/C, O/E/C, and FEA were low and not different. CEA produced a 6-fold increase in homocysteine when compared with non-CEA groups. Specifically, CEA produced an increase in homocysteine versus the AO group at 2 weeks (11.3 ± 0.7 vs 2.1 ± 0.9 μmol/L; P < .001), 4 weeks (8.9 ± 0.7 vs 3.5 ± 0.9 μmol/L; P = .004) and 6 weeks (7.7 ± 0.9 vs 3.1 ± 1.5 μmol/L; P = .03). The CO group had increased homocysteine versus the O/C, O/E/C, and FEA, but was lower than the CEA group. CEA produced an increase in MTHFR and CBS versus the AO group.

Conclusions: CEA resulted in elevated levels of homocysteine; however, when broken down into its component parts, no elevation was observed except for a small increase with the CO procedure. Manipulation of the femoral artery did not raise homocysteine levels. The increase in homocysteine is possibly due to the combination of vessel wall damage and changes in cerebral blood flow dynamics. (J Vasc Surg 2004;40:796-803.)

Clinical Relevance: Our lab has identified increased plasma homocysteine, induced by diet modification, as a risk factor in the development of intimal hyperplasia. To date, the effect of an operative procedure on plasma homocysteine has not been evaluated. In this study, a carotid endarterectomy performed in the rat resulted in an elevation of plasma homocysteine. If, as we have previously shown, elevated plasma homocysteine produces and “exaggerated” intimal hyperplasia response after a vascular procedure, then the possibility that the operation itself can elevate homocysteine levels could be clinically important. A patient who presents with a mild increase in homocysteine could advance to a markedly elevated plasma level after undergoing a vascular operation. This elevation in plasma homocysteine could in turn result in the formation of clinically relevant intimal hyperplasia that may negate the effect of the operation.
ectomy (CEA), a subset of patients may develop an “exaggerated” intimal hyperplasia response due to an elevated homocysteine level.

In our rat studies using dietary modification to increase plasma homocysteine levels,\textsuperscript{21,22} we observed that increased plasma homocysteine combined with CEA resulted in medial smooth muscle migration and proliferation producing a significant increase in intimal hyperplasia and an increase in luminal stenosis.

We have not found any literature regarding the effect of an operative procedure alone on plasma homocysteine levels. However, with increasing evidence that homocysteine plays a role in atherosclerotic disease as well as postoperative intimal hyperplasia, the effect of an acute operation on homocysteine levels becomes important. The purpose of this study was to evaluate the effect of a vascular operative procedure on plasma homocysteine levels. In addition, if an increase was identified, then a breakdown of the operation into its component parts would be undertaken to evaluate which element was important in homocysteine elevation.

**METHODS**

**Animals.** Male Sprague-Dawley rats weighing \( \geq 450 \text{ g} \) (Harlan Sprague-Dawley, Madison, Wis) were maintained on standard lab chow (Teklad, Madison, Wis) and following either treatment or operation were euthanized 2 weeks later. Blood and liver tissue were removed for the measurement of the following end points: plasma homocysteine, red blood cell folate, and hepatic MTHFR and CBS enzyme activity. Groups were as follows: (1) no anesthesia (NA) \((n = 5)\), (2) anesthesia only (AO) \((2, 4, \text{and } 6 \text{ weeks}; n = 4, 3, 3, \text{respectively})\), (3) skin opened and closed \((O/C)\) \((n = 5)\), (4) skin opened with exposure of the carotid artery and closed \((O/E/C)\) \((n = 4)\), (5) carotid isolated and clamped \((CO)\) \((n = 5)\), (6) open CEA \((2, 4, \text{and } 6 \text{ weeks}; n = 5, 5, 5, \text{respectively})\), and (7) open femoral endarterectomy \((FEA)\) \((n = 4)\).

**Anesthesia.** For the NA group, the rats were exposed to CO\(_2\) until rendered unconscious and were then exsanguinated. The remainder of the groups that received anesthesia were given an ace promazine/ketamine (Fort Dodge, Fort Dodge, Iowa; Phoenix Sci, St Joe, Mo) mix of 0.75 mL/0.1 kg body weight intraperitoneal. Food and water were available \textit{ad libitum} throughout the experimental period.

**Surgery.** In the O/C group, following a brief exposure to isoflurane, rats were anesthetized and then prepped for surgery. Using sterile technique and a dissecting microscope (\( \times 40 \) SZ40 Olympus; Olympus America Inc, Melville, NY), a midline cervical incision was made and then closed. The O/E/C group had the superficial muscles divided and the dissection carried down to the level of the left carotid artery. The cervical nerves in the region of the artery were dissected free to preserve pharyngeal and esophageal functions and prevent postoperative respiratory compromise. Once the carotid was exposed, the wound was immediately closed. In the CO group the carotid was exposed and clamped using Rumel tourniquets for a period of 45 minutes (time required to complete an endarterectomy). In the CEA group the CEA surgery technique previously published was used.\textsuperscript{21,22} Briefly, after adequate carotid artery exposure, proximal and distal control at the bifurcation, approximately 1.5 cm apart, was obtained by using Rumel tourniquets fashioned from 3-0 silk suture and \( \# 50 \) polyethylene tubing. Using a Beaver corneal blade, an arteriotomy was made and extended to 6 mm in length with micro-Potts scissors. Using a 27-gauge needle, the intima was transversely scored across the vessel in 2 parallel lines, approximately 4 mm apart. The intima and inner medial layer was removed with microforceps. The arteriotomy was closed with a running 10-0 monofilament nylon suture with a tapered 3/8 circle needle beginning at the distal end. The distal Rumel tourniquet was slowly removed first to assess for suture line hemostasis followed by removal of the proximal Rumel tourniquet. Any suture line bleeding was gently tamponaded with a sterile cotton-tipped applicator until hemostasis was achieved. The endarterectomized carotid artery was assessed with a Doppler scan to confirm patency. The superficial muscle layer was closed with a running 3-0 Dexon suture and the skin closed with interrupted suture. In the FEA group, the femoral artery was exposed with a longitudinal groin incision and the proximal and distal control was obtained on the femoral artery using Rumel tourniquets. The artery was opened and the intima removed, and the artery was closed in an identical fashion to the CEA group described earlier. The soft tissue and skin were then closed. At 2 weeks post-surgery, the O/C, CO, CEA, and FEA operated rats were anesthetized and the carotid or femoral artery exposed. The abdomen was opened in the midline and the distal aorta and inferior vena cava exposed. Blood was drawn from the inferior vena cava for plasma homocysteine and folate analyses. A portion of the liver was removed for enzyme activity assays. (See the Table for a listing of groups.)

This study was approved by the VA Animal Care and Use Committee, and animal care complied with the National Research Council’s \textit{Guide for the Care and Use of Laboratory Animals}.

**Plasma homocysteine measurements.** All plasma homocysteine laboratory values were determined by the Clinical Pathology Department at the Central Arkansas Veterans Healthcare Facility, Little Rock, Ark. The plasma homocysteine level was measured by using a thiol-specific fluorogenic labeling reagent and the thiols were separated by a reverse-phase high-pressure liquid chromatography method using fluorescence detection.\textsuperscript{23}

**Serum folic acid measurements.** Serum folate analyses were determined by a \([^{125}\text{I}]\) rat folic acid RIA kit (Diagnostic Products Corporation, Los Angeles, Calif).

**Enzyme activity assays CBS.** Livers were immediately removed from the rats and frozen in liquid nitrogen until the assay was performed. CBS activity was assayed by modification of protocols by Mudd et al\textsuperscript{24} and Kraus.\textsuperscript{25} Briefly, liver tissue was homogenized in 0.05 mol/L KH\(_2\)PO\(_4\) (pH 7.5) and centrifuged at 10,000 \( \times \) g at 4°C and the supernatant was
Group designations

<table>
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<th>Anesthesia</th>
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<th>Procedure</th>
<th>Blood and tissue sampled</th>
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<td>3</td>
<td>FEA</td>
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</table>

NA, No anesthesia; AO, anesthetics only; O/C, open/closure of skin; O/E/C, open/expose carotid, close; CO, clamp only; CEA, carotid endarterectomy; FEA, femoral endarterectomy.

collected. The enzyme reaction mixture contained 0.1 mol/L Tris (pH 8.6), 1 mmol/L pyridoxal phosphate, 0.7 mg cellular protein extract, 0.5 mg/mL bovine serum albumin, 0.06 μCi 14C-serine, 10 mmol/L serine, 15 mmol/L L-homocysteine, and 1 nmol/L cystathionine. The reaction mixture was incubated for 3 hours at 37°C, during which time the 14C-serine was converted by CBS into 14C-cystathionine. The 14C-cystathionine was separated from 14C-serine by ascending paper chromatography using 2-propanol/formic acid/water (80:6:20) v/v as the mobile phase. The 14C-cystathionine was cut out and counted in scintillation fluid. We defined 1 unit of enzyme activity as that which formed one nmol of cystathionine per hour at 37°C. Enzyme specific activity was expressed as units/mg of cellular protein. Protein was determined using the BioRad protein reagent.

Methylenetetrahydrofolate reductase. The MTHFR activity assay was modified from the procedure by Mudd et al. The liver tissue was homogenized in 0.25 mol/L sucrose, centrifuged at 50,000 × g for 1 hour and the supernatant was collected. The enzyme reaction mixture contained the following: 0.18 mol/L KH₂PO₄ (pH 6.3), 3.6 mM menadione bisulfite, 1.4 mmol/L EDTA, 7.2 mmol/L ascorbic acid, 178 μmol/L flavin adenine dinucleotide and 420 μmol/L of 14C-5-methyltetrahydrofolate. The reaction mix was incubated for 1 hour at 37°C, and then terminated by the addition of 0.6 mol/L sodium acetate pH 4.5, 0.1 mol/L formaldehyde, and 0.4 mol/L dimesone (5,5 dimethyl-1,3-cyclohexanedi-one) in 50% ethanol. The samples were boiled, toluene was added, and 2 mL of the upper phase containing 14C-formaldehyde was removed for counting in scintillation fluid. The enzyme activity was expressed as nmol/h formed per mg of protein.

Statistics. Data was analyzed as means ± SE by analysis of variance and simple regression analysis, using the StatView for Windows Program, Version 5.0 (SAS Institute Inc. 1992-98).

RESULTS

Plasma homocysteine measured in rats receiving NA or AO was low and consistent with published data for a normal homocysteine value in a rat (Fig 1). Plasma homocysteine in rats 2 weeks after undergoing NA, AO, O/C, and O/E/C procedures were low and did not show any differences between groups (Fig 1). Rats undergoing FEA also had low plasma homocysteine levels measured at 2 weeks post-procedure (Fig 1). Rats undergoing a CEA procedure showed a nearly 6-fold increase in plasma homocysteine at 2 weeks post-CEA when compared to non-CEA groups (Fig 1). The CO rats exhibited an approximate doubling of plasma homocysteine versus the O/C, O/E/C, and FEA groups at P < .05 (4.1 ± 0.6 CO vs 0.9 ± 0.5, 1.8 ± 0.5, 2.0 ± 1.1, respectively). Plasma homocysteine levels in the CO group were elevated; however, they did not reach statistical significance compared with the AO and NA groups (P = .07). This small increase in the CO group was significantly less than the large increase seen in the CEA group (P < .0001). The increase in plasma homocysteine in the CEA group remained significant for up to 6 weeks post-CEA (Fig 1). At the 4- and 6-week post-CEA time points, plasma homocysteine concentrations in rats undergoing CEA increased 2 to 3 times compared with AO rats.

Red blood cell (RBC) folate analyses of CEA and AO rats demonstrated a positive correlation between plasma homocysteine concentrations and RBC folate at P = .005, r = .62 (Fig 2).

The CEA procedure produced an increase in MTHFR and CBS enzyme activity at 2, 4, and 6 weeks post-CEA as compared to enzyme levels measured in AO rats. This 4-fold increase in MTHFR (Fig 3) and nearly 50% increase in CBS activity (Fig 4) was observed at 2 weeks post-CEA and remained at these elevated values for the duration of the 6-week experimental protocol. Folate, a cofactor for MTHFR in the remethylation pathway of homocysteine to methionine, was positively correlated with MTHFR activity (P = .0003; r = .73) (Fig 5).

DISCUSSION

There are few reports examining plasma homocysteine levels after major vascular events and none describing the effect of a vascular procedure on these levels. We observed that, after a vascular surgical procedure to the rat carotid artery, measurable increases in plasma homocysteine were detected. This increase in plasma homocysteine seen at 2 weeks after a CEA was not duplicated after a femoral endarterectomy. Clamping the carotid artery alone produced a doubling of the homocysteine level versus other control groups except for NA and AO groups. While this small increase was observed, it was significantly less than the
increase in homocysteine levels observed in the CEA group. Perhaps significance was not quite reached versus the AO and NA groups due to the low numbers in each group (n = 5). No increase was observed when the components of the operative procedure were broken down into their respective parts and evaluated individually, except for the CO group. This study indicates that anesthetic alone, surgical dissection, and manipulation of the intima within the artery are not the prime etiological factors in producing the elevated homocysteine level. It appears that the carotid artery itself needs to be clamped and the intima removed to produce a significant 6-fold increase in plasma homocysteine. The relative contribution of these few factors is difficult to determine since it is obviously impossible to

Fig 1. Plasma homocysteine between groups over time. Homocysteine levels in no anesthesia (NA) compared with anesthesia only (AO) indicate low levels compatible with control rats and no differences between these groups. With the carotid endarterectomy (CEA) there was a significant increase in plasma homocysteine at P ≤ .01 compared with all groups, including NA, AO, skin incision groups open/close (OC) and open/expose carotid/close (O/E/C), rats that received a femoral endarterectomy (FEA), and a clamp-only (CO) group. The increase in homocysteine concentration that was observed with CEA remained at 4 and 6 weeks. The CO group had a significant increase over other non-CEA groups except for the NA and AO group. Means are ±SEM. *Indicates CEA group means P ≤ .05 versus all other treatment groups. †Indicates CO group means P ≤ .05 versus O/C, O/E/C, and FEA.

Fig 2. Positive correlation of plasma homocysteine and red blood cell folate. Regression analysis between plasma homocysteine and corresponding red blood cells indicate a significant correlation.

Fig 3. Hepatic MTHFR enzyme activity compared between carotid endarterectomy (CEA) operated and anesthesia only (AO). The stimulative response of the CEA injury produced an overall increase in nmol/h of enzyme activity for MTHFR, indicating an increase in remethylation in the homocysteine metabolism pathway. Means are ±SEM. *Indicates P < .05.

Fig 4. Hepatic cystathionine β-synthase (CBS) enzyme activity compared between carotid endarterectomy (CEA) operated and anesthesia only (AO). The stimulative response of the CEA injury produced an overall increase in U/mg of enzyme activity for CBS, indicating an increase in the transsulfuration pathway in homocysteine metabolism. Means are ±SEM. *Indicates P < .05.
remove the intima without applying a carotid clamp. However, it appears that a portion of the increase in homocysteine levels can be attributed to a carotid clamp. Clamping of the femoral artery, even with intima removal, does not produce an elevation in plasma homocysteine. In addition, the post-CEA elevation persisted for the duration of our observation period, a full 6-weeks post-CEA. The results lead us to speculate that perhaps cerebral-flow dynamic changes at time of the carotid occlusion coupled with vessel wall and intima disruption or trauma may be the etiological factor in the elevation in homocysteine concentrations following a CEA, since in the experimental design these were the only elements in the operative protocol that could independently be shown to increase plasma homocysteine levels. This would also explain the lack of increased homocysteine in the FEA group. The change in cerebral flow dynamics could be a trigger for the activation of a metabolic process that may progress through a number of steps that eventually lead to hyperhomocysteinemia. This trigger and process appear not to be transient in that increased homocysteine is observed as far as 6 weeks post-CEA. Obviously, this is pure speculation and the study did not involve an assessment of cerebral blood flow dynamics.

A few clinical studies have demonstrated an increase in plasma homocysteine levels in different clinical settings of either cerebral or myocardial flow changes or damage. Howard et al27 performed serial measurements in patients during the acute phase of a stroke. However, there was no evidence of stroke or symptoms of stroke with the increase in plasma homocysteine in our study. Howard et al27 observed a pattern of increasing homocysteine concentrations during the first 2 weeks, with levels peaking at 10 to 14 days (11.3 ± 0.5 vs 13.7 ± 0.7, P < .0001). Meiklejohn et al26 also measured plasma homocysteine concentrations in stroke patients within 4 days, with a second measurement between 68 to 270 days. Their results indicated no difference at the early measurement; however, measurements between 68 to 270 days were found to be significant above age-matched controls (women 8.1 vs 10.0 µmol/L; men 9.2 vs 10.2 µmol/L). This indicates that length of time post-trauma event may be a factor in determining plasma homocysteine levels. Egerton et al29 performed serial measurements of plasma homocysteine following an acute myocardial infarction. Measurements were performed on days 1, 3, and 7 and 3 weeks and 6 months after hospital admission. Their results indicate a significant peak increase in homocysteine concentration at day 7, which decreased by 21 days (18.7 ± 1.6 vs 16.8 ± 1.6 µmol/L, P < .05).

We have observed that intimal hyperplasia after CEA is increased in the setting of diet induced hyperhomocysteinemia.22 Damage to the carotid artery intima itself appears to result in an increase in homocysteine. Perhaps the increase in homocysteine in this study is part of a natural mechanism for repair of the arterial damage. While intimal hyperplasia can result in an untoward and unwanted clinical effect, the intimal hyperplasia response can be viewed as a mechanism by which the damaged and absent intima is replaced. The lack of this reparative response mechanism with FEA is unclear. Perhaps it is not observed because of the lack of synergistic effect of changes in cerebral blood flow.

Increased circulation of homocysteine has a profound effect on cell proliferation; studies of vascular smooth muscle cell culture systems show that homocysteine addition to the culture medium induces cell proliferation,30 TIMP-1, tPA, MMP,31 protein kinase C activation,32 nitric oxide synthesis,33 proto-oncogene expression, diacylglycerol activation32 and DNA synthesis shown by thymidine incorporation.34 Homocysteine stimulation of these factors can induce cell growth by contributing to smooth muscle migration and proliferation which are the hallmark of intimal hyperplasia and thus lead to restenosis.

In this study, the CEA may also be considered as a stimulating factor in altering folate levels and disrupting hepatic MTHFR and CBS activity. It is interesting to speculate that change in cerebral hemodynamics resulting from the occlusion of the carotid artery during the endarterectomy and/or intimal damage may have induced an increase of stress or inflammation and therefore an altered hepatic metabolic response. Systemic changes that can occur with acute stress or illnesses such as increases in hepatic proteins (ie, increased synthesis of C-reactive proteins and serum amyloid A35). The liver, as the primary site of homocysteine metabolism, may have responded to the increase in stress and altered its metabolism of homocysteine by shifting from remethylation to the transsulfuration route to remove excess homocysteine from the system.

To our knowledge, no other study has examined plasma homocysteine levels before and after surgery. However, there are numerous clinical studies of the B vitamins that are cofactors for homocysteine metabolism (folate and vitamins B6 and B12) decreasing plasma homocysteine concentrations. This would suggest a negative correlation.
of plasma homocysteine and folate levels, although, in our study, we observed a strong positive correlation of plasma folate and homocysteine. We can only conjecture that this observation may be due to the hepatic system attempting to compensate for the transitory increase in plasma homocysteine. Torres and co-workers were the first to indicate a connection between the effects with plasma homocysteine in the vasculature and the liver. They observed in human vascular smooth muscle cell cultures and hepatocyte cell cultures that homocysteine stimulated production of tissue inhibitor of metalloproteinases-1 (TIMP-1) and α1(1) procollagen expression, suggesting that elevated plasma homocysteine may participate in the pathogenesis of liver fibrosis as well as peripheral vascular disease. Heretofore, there are no studies examining the influence of stress as a factor, in response to surgical trauma on blood levels of homocysteine. However, there have been studies that have reported increased homocysteine levels in women placed in acute psychologically stressful situations. This study suggests that a CEA procedure can produce a significant and sustained increase with homocysteine. This increase in homocysteine has been shown to result from an increased intimal hyperplasia response, thus linking these 2 events. In addition, this study suggests that if hyperhomocysteinemia is an issue with the clinical care of a patient, it must be evaluated postoperatively and for some extended time after, and, if elevated, this treatment must continue in the postoperative period.

In conclusion, vascular intima manipulation in which cerebral blood flow change is a factor may induce a plasma homocysteine elevation resulting in an adaptive increase in activity of hepatic metabolic enzymes.

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REFERENCES