Changes in Markers of Vascular Injury in Response to Transient Hyperhomocysteinemia

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The purpose of this study was to test whether transient increases in homocysteine would promote changes in markers of endothelial injury, cellular fibronectin (cFN), and soluble vascular cell adhesion molecule 1 (sVCAM-1). Homocysteine, cFN, and sVCAM-1 concentrations increased significantly in response to a methionine load by 6 hours in human subjects. However, no correlation was observed between homocysteine and cFN or sVCAM-1. To directly test whether homocysteine can injure endothelial cells, human umbilical vein endothelial cells (HUVEC) were incubated with increasing concentrations of homocysteine, plasma, or serum from hyperhomocysteinemic mice or from the methionine-loaded test subjects. cFN release was increased from endothelial cells cultured with plasma (but not serum) of hyperhomocysteinemic transgenic mice or from methionine-loaded human subjects. These data suggest that very high homocysteine concentrations can promote endothelial injury; however, this effect is likely mediated by secondary effects that include a factor(s) present in plasma that affects endothelial cells.

ELEVATED CIRCULATING homocysteine is an independent risk factor for peripheral vascular disease and coronary artery disease.[1-3] Homocysteine is a demethylated metabolite of the essential amino acid methionine. Elevated circulating homocysteine concentrations are associated with increased lipid peroxidation, dyslipidemia, and blunted endothelial-mediated vasorelaxation.[4-9] Furthermore, increased homocysteine has been postulated to affect the function of the vasculature and cause endothelial injury.[10,11] However, no study has demonstrated whether in vivo increases in homocysteine can affect markers of endothelial injury. We previously reported a strong association between homocysteine and a marker of endothelial injury, cellular fibronectin (cFN), in normal pregnant women and women with the pregnancy complication pre-eclampsia.[12] This relationship between cFN and homocysteine suggested a possible direct effect of homocysteine on the endothelium to induce injury.

In the present study we investigated whether transient increases in homocysteine in healthy individuals would increase circulating cFN and soluble vascular cell adhesion molecule 1 (sVCAM-1), and whether these changes in markers of endothelial injury would correlate with homocysteine concentrations. Furthermore, we tested whether homocysteine or serum or plasma from hyperhomocysteinemic subjects was capable of directly promoting endothelial injury in vitro.

MATERIALS AND METHODS

Subjects

Forty subjects, 12 men and 28 women, were recruited for a methionine loading study. The study was approved by the hospital institutional review board and all subjects gave informed consent. The average age of the men was 28.7 years (range, 21 to 42), and the average age of the women was 24.1 years (range, 19 to 44). There was no significant difference in body mass index (BMI: 24.1 ± 7.2 *v* 28.7 ± 6.6), multivitamin use (10/28 *v* 4/12), or cigarette use (4/28 *v* 3/12) between the women and men in the study, respectively. All subjects were healthy and were not known to have chronic hypertension, or renal or metabolic disease. Twelve additional subjects were recruited to serve as placebo controls for the methionine load.

Blood Samples

Plasma and serum samples were obtained after an overnight fast then 6 and 24 hours after a methionine load. All blood samples were collected from the women during the follicular phase of their menstrual cycle to standardize the effect of steroid hormones. Plasma was immediately prepared from blood anticoagulated with EDTA. Serum samples were collected in the absence of anticoagulants. Samples were aliquoted under sterile conditions and stored at −80°C until assayed.

Methionine Load

The methionine load consisted of an oral dose of L-methionine (0.1 g/kg body weight) (Ajinomoto, Teaneck, NJ) administered in orange juice. Subjects were then restricted to a low-methionine snack (bagels and an apple or pear) for the next 6 hours until after the next blood sample was collected. After the collection of the second blood sample, subjects were allowed to return to their normal diet, but fasted for 12 hours before the third and final blood sample was collected (24 hours post methionine load).

Homocysteine Determination

Total plasma homocysteine was analyzed according to the procedure of Jacobsen et al:[13] In brief, samples (100 μL) were mixed with 10 μL of water and 5 μL of n-amyl alcohol. Subsequently, 35 μL 1.43-mmol/L $NaBH_4$ in 0.1 mmol/L NaOH was added to reduce disulfide bonds, and, after vortexing, 35μL 1N HCl added. After mixing, the thiols were derivatized by incubating at 42°C for 12 minutes with 50 μL of 10-mmol/L monobromobimane (thiolyte; Calbiochem, San Diego, CA) in 4 mmol/L Na EDTA, pH 7. The samples were cooled to room temperature and then mixed with 50 μL of 1.5-mol/L perchloric

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acid. The samples were left at room temperature for 10 minutes and then microcentrifuged for 10 minutes. Twenty microliters of 2-mol/L Tris was carefully added to the tubes, which were then gently inverted to avoid disrupting the pellets. The samples were next microfuged for 4 minutes.

Aliquots (20 μL) were separated on a 4.6 $\times$ 150 mm (5-μm particle) Prodigy column (Phenomenex, Torrance, CA) maintained at 30°C. Eluant A was water:methanol:acetic acid (94.5:5.0:0.5 by volume). Eluant B was 100% methanol. Total flow rate was 1 mL/min and the solution gradient involved minor modification of the Jacobsen et al[13] method: 0 to 1 minute, 10% B; 1 to 6 minutes, 10% to 35% B; 6 to 7.3 minutes, 35% B; 7.3 to 8 minutes, 35% to 100% B; 8 to 9 minutes, 100% B; 9 to 10 minutes, 100% to 55% B; 10 to 11 minutes, 55% to 10% B; and 11 to 12 minutes, 10% B. The thiol derivatives were detected fluorimetrically with excitation at 390 nm and emission at 470 nm. The calibration curves for homocysteine were generated from external standards and were included at the beginning and end of each analytical set. The standard curve consisted of normal human plasma spiked with 0, 2.5, 5, 7.5, 10, 15, 20, and 25μmol/L L-homocystine. The coefficient of variation between assays was 7%.

Human cFN Quantitation

Plasma human cFN was determined for each sample by an enzyme-linked immunosorbent assay (ELISA) specific for the ED-A domain of human cFN. This ELISA was modified from that described by Freidman et.al.[14] Briefly, 96-well microtiter plates were coated overnight at room temperature with a monoclonal antibody specific to the ED-A domain of human cFN (Sigma, St Louis, MO; clone FN-3E2) diluted 1 to 100 in phosphate-buffered saline (PBS), 50 μL per well. After overnight incubation, the wells of the plate were incubated for 1 hour in a blocking buffer of PBS with 2% bovine serum albumin (BSA) and 0.05% Tween 20. After 1 hour, the wells were washed 3 times with a wash buffer of PBS and 0.05% Tween 20 and samples and standards added and incubated for 1 hour at room temperature. After incubation, all wells were washed 3 times with wash buffer and then 50 μL of a 1 to 500 dilution of the secondary antibody (polyclonal anti-plasma fibronectin, horseradish peroxidase–conjugated; Dako, Carpinteria, CA) was added to each well and incubated for 1 hour at room temperature. After incubation, all wells were washed 3 times with wash buffer and then 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (BioRad, Hercules, CA) was added to each well and the color reaction was allowed to develop for 5 minutes. The reaction was stopped by the addition of 100 μL of 1N sulfuric acid to each well, and then the absorbance was read at 45 0nm. Plasma concentrations of cFN were determined by calculating from the linear equation of the standard curve. The standard curve for the assay included 0, 12.5, 25, 50, 100, 250, 350, and 500 ng/mL of human cFN (Upstate Biotechnology, Lake Placid, NY). The standards were quantified by protein determination with a Pierce (Rockford, IL) bicinchonic acid (BCA) kit and by ELISA using kits from Adeza Biomedical (Sunnyvale, CA). Standards and samples were diluted in a PBS, 2% BSA buffer. The optimal dilution of each sample was predetermined to provide values in the middle of the standard curve, usually 1 to 50 or 1 to 100 dilutions. A quality control sample was assayed with each microtiter plate. The coefficient of variation between runs has been 7%. This assay is specific for human cFN and does not recognize bovine or murine cFN.

sVCAM-1 Quantitation

sVCAM-1 was determined by ELISA. The ELISA kit was purchased from Bio-Source (Camarillo, CA) and all samples were assayed according to the manufacturer's protocol. The sensitivity of the assay is 0.5 ng/mL and the coefficient of variation between runs has been 8%.

Endothelial Cell Culture

Endothelial cells from human umbilical veins from normal pregnancies (HUVEC) were prepared as previously described[15] and grown at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. Cells were used between passages 4 and 5. Cells were cultured in M-199 media with 20% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/mL of penicillin-streptomycin, 0.5 μg/mL of amphotericin B (fungizone), 1 ng/mL basic fibroblast growth factor, and 30 U/mL of heparin. Cell cultures were dispersed with 0.05% trypsin–0.53 mmol/L EDTA, plated at 1×10^5 cells per well in 24-well plates, and allowed to attach overnight. All analyses with the HUVEC were normalized to cell number. Culture wells were precoated with 0.2% gelatin.

HUVEC Incubation Experimental Design

Cells were made quiescent in serum-free M-199 media containing 0.05% BSA instead of FBS for 24 hours before experimental stimulation. All cell stimulation experiments were for 24 and 96 hours (media changed after 48 hours) unless noted otherwise. Cells were treated with increasing concentrations of L-homocysteine (12.5 to 500 μmol/L, made by reducing L-homocysteine thiolactone with 5N NaOH at 37°C and then neutralizing with 2N HCl and Tris buffer) as well as homocysteine in the presence of 2 μmol/L $FeCl_3$ or $CuCl_2$ as a source of excess free iron or copper to promote homocysteine oxidation. Cells were also cultured in the presence of 5% (by volume) plasma or 5% (by volume) serum from cystathionine beta-synthase (CBS)-deficient transgenic mice,[16] 5% (by volume) plasma from methionine-loaded mice, or 5% (by volume) plasma or serum from subjects who had undergone the methionine loading test.

Statistical Methods

Concentrations of homocysteine, sVCAM-1, and cFN from the methionine load study were analyzed by a 2-tailed paired *t* test. Changes in cFN in the endothelial cell cultures were analyzed by 1-way anlaysis of variance (ANOVA) with Bonferroni/Dunn post-hoc testing or 2-tailed paired *t* test as appropriate. Means and standard deviations are reported. Correlations were by standard regression analysis. The Statview 5.0 software package was utilized for these analyses (Abacus Concepts, Berkeley, CA). Statistical significance was accepted for $P < .05$.

Table 1. Markers of Endothelial Injury Are Increased in Human Plasma After a Methionine Load

	Methionine-Loaded Subjects (n = 40)			Placebo Control Subjects (n = 12)		
	Fasting	6 Hours Post	24 Hours Post	Fasting	6 Hours Post	24 Hours Post
Homocysteine (μmol/L)	9.4 ± 1.8	32.4 ± 9.0*	15.9 ± 5.5*	7.7 ± 1.8	7.5 ± 1.6	7.6 ± 2.3
cFN (μg/mL)	7.03 ± 3.7	8.01 ± 4.5†	7.05 ± 4	11.8 ± 5.5	11.2 ± 4.8	11.9 ± 5.7
sVCAM-1 (μg/mL)	0.70 ± 0.23	0.77 ± 0.23†	0.68 ± 0.23	0.65 ± 0.14	0.69 ± 0.12	0.62 ± 0.09

NOTE. Values are means ± SD.

*$P < .0001$, †$P < .005$ compared to the fasting sample.

RESULTS

Transient Hyperhomocysteinemia In Vivo Increases Markers of Endothelial Injury

There was a significant increase in plasma homocysteine at 6 and 24 hours in volunteers given a methionine load compared to the volunteer's fasted plasma samples. Homocysteine concentrations did not change in the placebo controls (Table 1). Similar to previous published reports, fasting plasma homocysteine was significantly higher in the samples from men compared to women (10.8 ± 1.9 μmol/L *v* 8.7 ± 1.4 μmol/L respectively, $P < .01$).[17,18]

Circulating cFN and sVCAM-1 increased significantly by 6 hours after the methionine load, and this increase coincided with the highest measured value of circulating homocysteine (Table 1). By 24 hours after the methionine load, the circulating concentrations of cFN and sVCAM-1 returned to pre-methionine load levels (Table 1). There was no significant change in cFN or sVCAM-1 across the 3 sampling times in the placebo controls (Table 1). While the changes in cFN and sVCAM-1 in the methionine-loaded subjects coincided with the changes in homocysteine, no direct correlation was observed between the increased homocysteine and cFN or sVCAM-1 ($r^2 = 0.002$ and $r^2 = 0.004$, respectively). However, there was a weak but significant positive association between the change in homocysteine between the first and second sample compared to the change in cFN between the first and second sample ($r^2 = 0.11$, $P = .03$), but this association did not extend to the change in sVCAM ($r^2 = .006$, $P = .62$)(no graph shown).

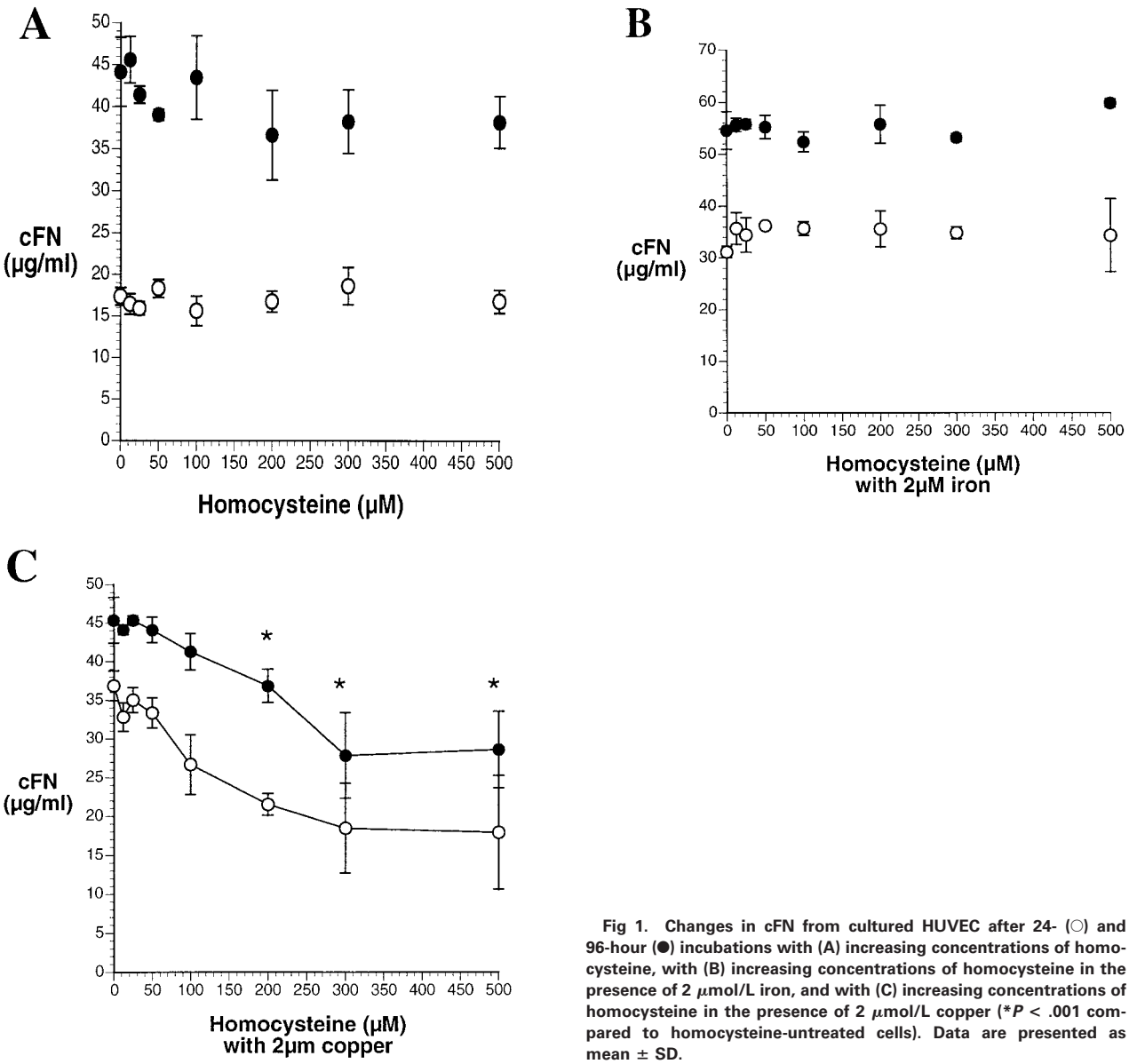

Fig 1. Changes in cFN from cultured HUVEC after 24- (○) and 96-hour (●) incubations with (A) increasing concentrations of homocysteine, with (B) increasing concentrations of homocysteine in the presence of 2 μmol/L iron, and with (C) increasing concentrations of homocysteine in the presence of 2 μmol/L copper (*$P < .001$ compared to homocysteine-untreated cells). Data are presented as mean ± SD.

Table 2. Plasma From CBS-Deficient Transgenic Mice Promotes cFN Release From HUVEC

Genotype (homocysteine [μmol/L])	cFN (μg/mL) 2-Hour Incubation	cFN (μg/mL) 24-Hour Incubation
Wild types, n = 3 (11.5 ± 2.5)	0.78 ± 0.24	8.88 ± 3.5
Heterozygotes, n = 4 (20.8 ± 6.0)	0.87 ± 0.32	7.22 ± 2.07
Knockouts, n = 3 (222.1 ± 35.9)	2.35 ± 0.38*	21.78 ± 5.56*

NOTE. Values are means ± SD.
*$P < .001$ compared to wild types.

Increased Homocysteine In Vitro Does Not Directly Affect Endothelial Cells

To test for possible direct effects of homocysteine on endothelial cells, HUVEC were cultured and treated with increasing concentrations of L-homocysteine for 24 and 96 hours. As shown in Fig 1A, increasing concentrations of homocysteine did not change cFN release.

Homocysteine is thought to exert a pro-oxidant effect in vivo and in vitro in the presence of transition metals such as copper or iron.[10,19] Therefore, we modified our experimental conditions to include 2 μmol/L copper chloride or ferric chloride as a source of copper or iron. Neither copper nor iron in the presence of increasing concentrations of homocysteine significantly increased the release of cFN (Fig 1B and C). However, increasing homocysteine in the presence of copper did significantly decrease cFN after 24 and 96 hours of incubation ($P <$ .001, compared to untreated cells) (Fig 1C). This decrease in cFN release after incubation with increasing homocysteine and 2 μmol/L copper was not the result of increased cell death as assessed by trypan blue exclusion (≥88% exclusion in all cases).

Plasma From Chronic or Acutely Hyperhomocysteinemic Mice Does Affect Endothelial Cells In Vitro

HUVEC were treated with 5% plasma from the three different genotypes of the hyperhomocysteinemic CBS-deficient transgenic mouse model[16] to examine effects of plasma from a setting in which homocysteine is chronically elevated. The mice have either one or both alleles for the CBS gene disrupted. Homocysteine concentrations averaged 11.5 μmol/L in wild-type, 20.8 μmol/L in heterozygous, and 222.1 μmol/L in knockout mice. Cells were incubated for 2 and 24 hours with the mouse plasma. cFN increased significantly but not proportionally with increasing plasma homocysteine concentration at both times (Table 2). Importantly, our cFN ELISA only recognizes human cFN and not mouse or bovine cFN, and therefore the changes observed in cFN are the result of changes in cFN release by the HUVEC. In a similar experiment we treated mice orally with methionine (100 mg/kg) to acutely increase plasma homocysteine concentration and collected plasma from them after 6 or 24 hours (plasma homocysteine: untreated mice, 11.2 ± 1.3 μmol/L; 6hours post methionine, 20.2 ± 12.3; and 24 hours post methionine, 12.2 ± 3.1 μmol/L). This plasma was then used to stimulate HUVEC as described above to assess possible differences between chronic and acute hyperhomocysteinemia. The results for this experiment are shown in Table 3. Similar to the results shown in Table 2, when HUVEC were treated for 24 hours with 5% plasma from mice that were made acutely hyperhomocysteinemic, cFN release was increased. However, this change was again not proportional to the increase in homocysteine ($r^2 = 0.09$, $P = .13$, data not shown).

We attempted to duplicate the above results using plasma or serum from the subjects who had undergone the methionine load. We initially chose serum for the assay to avoid confusion by cFN present in human plasma (cFN is removed largely with blood clotting). To make the experiments comparable to the mouse experiments we subsequently used plasma and assayed the diluted plasma for cFN prior to incubation. This value (usually <8% of final values) was subtracted from the concentration of cFN measured after the incubation period. HUVEC were cultured in the presence of 5% human plasma or serum from the 3 time points of the methionine load described above. Culture media was assayed for the presence of cFN after 2 and 24 hours. As shown in Table 4, there was no difference in cFN release from HUVEC after treatment with serum from subjects who had the methionine load. However, similar to the experiments with mouse plasma, there was a small but significant increase in cFN release when the cells were treated with plasma from the methionine-loaded subjects. Again, there was no correlation between plasma or serum homocysteine values and cFN after incubation ($r^2 = 0.06$, $P = .65$ and $r^2 = 0.01$, $P =$ .93, respectively, data not shown). None of the above treatments induced nonspecific cell death or cytotoxicity as assessed by trypan blue exclusion (≥85% dye exclusion in all cases).

Lastly, we used serum from the hyperhomocysteinemic CBS-deficient transgenic mice to test whether the differences in the results above were the result of differences between plasma and serum. Incubation of HUVEC with 5% serum from the CBS transgenic mice resulted in no significant differences in cFN release from the cells after 2 hours (wild-type [n = 4], 0.93 ± 0.2 μg/mL; heterozygote [n = 3], 0.98 ± 0.3 μg/mL; knockout [n = 3]. 0.86 ± 0.1 μg/mL, $P = .78$) or 24 hours (wild-type [n = 4], 17.3 ± 0.7 μg/mL; heterozygote [n = 3], 15.6 ± 0.4 μg/mL; knockout [n = 3], 17.9 ± 2.9 μg/mL, $P = .58$).

Table 3. Plasma From Acute Hyperhomocysteinemic Mice Promotes cFN Release From HUVEC

Treatment (homocysteine [μmol/L])	cFN (μg/mL) 2-Hour Incubation	cFN (μg/mL) 24-Hour Incubation
Untreated wild types, n = 3 (11.2 ± 1.3)	0.78 ± 0.24	8.88 ± 3.5
6-hour methionine-loaded mice, n = 3 (20.2 ± 12.3)	0.83 ± 0.27	11.9 ± 2.9*
24-hour methionine-loaded mice, n = 3 (12.2 ± 3.1)	0.51 ± 0.16	7.3 ± 1.1

NOTE. Values are means ± SD.
*$P < .05$ compared to wild types.

Table 4. Plasma From Methionine-Loaded Subjects Promotes cFN Release From HUVEC

Treatment (homocysteine [μmol/L])	Serum cFN (μg/mL)		Plasma cFN (μg/mL)	
	2-Hour Incubation	24-Hour Incubation	2-Hour Incubation	24-Hour Incubation
Fasting samples, n = 16 (10.1 ± 1.7)	0.54 ± 0.2	5.67 ± 2.7	0.34 ± 0.2	3.41 ± 0.5
6-hour post methionine load, n = 16 (34.1 ± 9.2)	0.55 ± 0.2	5.71 ± 3.0	0.38 ± 0.2*	3.58 ± 0.5
24 hours post methionine load, n = 16 (17.7 ± 6.7)	0.61 ± 0.3	5.77 ± 2.9	0.31 ± 0.1	3.64 ± 0.4

NOTE. Values are means ± SD.
*$P < .05$ compared to fasting samples.

DISCUSSION

Increased plasma homocysteine is associated with an increased risk of stroke, as well as coronary artery and peripheral vascular disease.[1-3] Furthermore, transiently increased homocysteine has been demonstrated to alter vascular function in vivo and therefore is postulated to promote vascular and endothelial injury.[20-22] One study has previously demonstrated that chronically hyperhomocysteinemic patients without vascular disease do not show evidence of changes in several markers of endothelial cell function (von Willebrand factor [vWF] propeptide, tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1), C-reactive protein, cFN, and thrombomodulin) compared to controls; however, there was a significant increase in vWF in the hyperhomocysteinemic patients.[23] However, no study has investigated whether acute increases in homocysteine in vivo can affect markers of endothelial injury. The acute increases in homocysteine are particularly interesting since this has most recently been associated with altered vascular function and therefore is most likely to demonstrate changes in markers of endothelial injury.[24,25] Therefore, this study set out to investigate whether increased homocysteine is capable of promoting endothelial injury in vivo or in vitro by monitoring changes in markers of endothelial injury. cFN and sVCAM-1 were chosen as reliable markers of endothelial injury. Fibronectin is a large multifunctional glycoprotein that is important in extracellular matrix organization, tissue remodeling, and wound healing.[22,26] cFN is an isoform of fibronectin that occurs through alternative splicing of its pre-mRNA, and is different from fibronectin by the inclusion of ED-A and/or ED-B domains.[27,28] cFN normally makes up less than1% to 2% of the total fibronectin present in the plasma, and is synthesized locally by endothelial cells, smooth muscle cells or fibroblasts in response to tissue injury.[28] Vascular cell adhesion molecule 1 (VCAM-1) is a member of the immunoglobulin superfamily of adhesion molecules and is expressed as both a membrane bound and soluble form (VCAM-1 and sVCAM-1, respectively) .[29-31] Like cFN, sVCAM-1 is also released by endothelial cells, smooth muscle cells, and fibroblasts in response to cytokine stimulation or vascular injury.

This study demonstrates that transiently increasing plasma homocysteine concentrations in vivo with a methionine load is associated with evidence of endothelial injury as indicated by increases in cFN and sVCAM-1. Both cFN and sVCAM-1 increased significantly by 6 hours after a methionine load, at a time when homocysteine concentrations had tripled. However, both cFN and sVCAM-1 returned to pre-methionine load levels by 24 hours after the load at a time when homocysteine concentrations had decreased but were still higher than the pre-load value. cFN concentrations were modestly elevated at baseline in the placebo controls, but not different from individuals receiving methionine. Nonetheless, cFN and VCAM concentrations did not change over time. We did not observe a direct correlation between homocysteine and cFN or sVCAM-1. However, the increase of homocysteine and cFN between the first and second sample correlated weakly ($r^2 = 0.11$). This correlation did not extend to the change in sVCAM. These data suggest that homocysteine can induce endothelial injury; however, the direct effect of homocysteine appears to be modest. This result conflicts with our previous finding of a significant correlation between cFN and homocysteine in late pregnancy samples from both pre-eclamptic and normal pregnant women.[12] At least two possibilities may explain this discrepancy. The first is that the association between cFN and homocysteine may exist only during pregnancy, possibly as a result of an increased sensitivity of the maternal vasculature to injury by homocysteine as we speculated previously.[12] The second is that homocysteine may not be directly responsible for injuring the endothelium, but rather induces another unidentified factor (cytokine, growth factor, altered cellular redox status, etc) that is responsible for causing the release of cFN and sVCAM-1.

To test the direct effect of increased homocysteine, we cultured HUVEC in the presence of increasing concentrations of homocysteine alone, or homocysteine in the presence of copper or iron (as a source of free transition metals to promote oxidative stress). In each case, these experimental conditions failed to significantly increase cFN release from the endothelial cells. Therefore, increased homocysteine alone does not appear to promote endothelial injury in vitro. This result is surprisingly contrary to that reported previously by Starkbaum and Harlan.[10] However, upon closer inspection the main injury effect stimulated by homocysteine in the presence of copper on endothelial cells reported by Starkbaum and Harlan occurred in bovine endothelial cells and was muted in human endothelial cells. These data suggest that bovine endothelial cells should not be assumed to behave the same as human endothelial cells. Furthermore, it was surprising that cFN concentrations were significantly decreased in the presence of increasing concentrations of homocysteine and copper, and this was not the result of nonspecific cell toxicity as assessed by trypan blue dye exclusion. One possible explanation for this result may be that the copper ions and free thiols from homocysteine catalyzed the polymerization of cFN, and therefore reduce the amount of antigen available for the ELISA. This process has been de-

scribed previously for plasma fibronectin in the presence of copper and dithiothreitol.[32]

To test for a less direct effect of homocysteine to increase cFN, we investigated whether plasma from hyperhomocysteinemic mice or humans might promote changes in cFN release in endothelial cells in vitro. When HUVEC were treated with plasma from a chronically hyperhomocysteinemic transgenic mouse model (CBS- deficient), the endothelial cells did demonstrate an increase in the release of cFN, and this increase was greater with higher plasma homocysteine concentrations. Furthermore, when endothelial cells were treated with plasma from mice that had been made acutely hyperhomocysteinemic via a methionine load or plasma from human subjects who had also undergone a methionine load, there was again a modest increase in the release of cFN. Interestingly, when we tested serum from methionine-loaded human subjects or mice, or serum from hyperhomocysteinemic CBS transgenic mice, we did not observe an increase in cFN. The finding that homocysteine alone, or with copper or in serum, does not increase cFN release makes a direct "toxic" effect of homocysteine on endothelial cells unlikely. Nonetheless a factor(s) present in plasma does increase cFN release. We propose that in hyperhomocysteinemic individuals the synthesis of this factor is likely enhanced in vivo, and this factor is lost/removed or inhibited during the serum clotting process. The identity of this factor is not immediately obvious given the myriad of factors that are removed or reduced during the clotting process as well as additional substances released by activated platelets.

Our findings support the concept that increased homocysteine can promote endothelial injury and this may account for part of the noted effects of increased homocysteine on vascular function. However, homocysteine does not appear to be acting directly on endothelial cells to promote this injury, and instead these data suggest that increased homocysteine is stimulating the production of another factor(s) that affects endothelial cells. Future studies should be directed toward identifying this homocysteine-induced mediator of endothelial injury.

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