Moderately Elevated Plasma Homocysteine Impairs Functional Endothelial Recovery Following Denudation of Mouse Carotid Arteries

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Increased total plasma homocysteine is an independent risk factor for cardiovascular disease. This study was designed to determine whether it can impair endothelial function, by examining the recovery of acetylcholine-evoked relaxation following mechanical denudation of the endothelium in the arteries of cystathionine β-synthase knockout (CβS$^{+/-}$) mice. Heterozygous CβS$^{+/-}$ mice had total plasma homocysteine concentrations significantly higher (8.9 ± 1.1 μmol/L, n = 12) than strain-matched wild-types (4.6 ± 0.4 μmol/L, n = 5; P = .003). Left common carotid arteries were denuded of endothelium using a 250-μm polytetrafluoroethylene filament. After 10 days, when the endothelium had completely regrown, relaxation to acetylcholine was measured in precontracted segments of artery. Uninjured right carotid arteries from the same animals served as internal controls. Relaxation to acetylcholine was significantly attenuated in the injured arteries of the CβS$^{+/-}$ mice, compared to wild-types (P = .017); furthermore, there was a significant negative correlation between sensitivity to acetylcholine and total plasma homocysteine concentration measured in the same animal (r = −0.69, P < .003). These data suggest that even modest homocysteinemia has a deleterious effect on the function of healed endothelium in mouse arteries. This may account for its adverse influence on chronic cardiovascular disease.

AN ELEVATED LEVEL of plasma homocysteine, a sulfur-containing amino acid and byproduct of methionine metabolism, has long been suspected as a risk factor for the development of cardiovascular disease.[1] Retrospective epidemiological studies have provided the strongest evidence for such a link.[2] However, more rigorous prospective observational cohort studies have shown less consistent data.[3,4] At the current time it is not clear if elevated plasma homocysteine is a causal factor, or simply a marker reflecting some underlying disease process.[5,6]

A variety of experimental approaches have shown that homocysteine can cause deleterious effects on the endothelium.[7-17] For example, cultured endothelial cells become detached in the presence of 0.1 to 10 mmol/L homocysteine.[18] The effects of homocysteine on endothelial nitric oxide (NO) are well established; NO release is impaired by chronically elevated homocysteine, and this can attenuate both the endothelial-dependent vasoregulatory function and the anti-thrombotic action of NO.[19-22]

In humans, grossly elevated levels of total plasma homocysteine can occur in some genetic conditions and are often associated with premature and severe atherosclerosis.[23] Epidemiological studies show that a 50% increase above normal levels of about 10 μmol/L total plasma homocysteine results in a 20% to 30% increase in the incidence of cardiovascular events.[24,25] Furthermore, meta-analysis indicates that elevated plasma homocysteine has a similar effect on the risk of developing cardiovascular disease as other well-established risk factors, such as hypercholesterolemia.[2] As many as 10% of all cases of coronary heart disease in the developed world may be attributable to hyperhomocysteinemia.

Studies on the effects of mildly elevated homocysteine are less numerous and the relationship between mild hyperhomocysteinemia and the development and progression of cardiovascular disease is not clear. Most studies that demonstrate homocysteine-induced cell damage, or changes in endothelial-dependent responses, do so at supraphysiological concentrations of the amino acid, often greater than 100 μmol/L.[17,26] However, moderate increases in total plasma homocysteine (to 25 μmol/L), produced by feeding a folate-deficient diet to heterozygous cystathionine β-synthase knockout (CβS$^{+/-}$) mice,[27] significantly attenuate aortic endothelium-dependent relaxation.[28] Similarly, attenuation of flow-mediated vasodilatation is reported in elderly humans with mild (19 μmol/L) hyperhomocysteinemia.[8] In addition, a study by Eberhardt et al[16] has reported reduced endothelial-dependent relaxation in aortas taken from mildly hyperhomocysteinaemic CβS$^{+/-}$ mice with plasma homocysteine levels of 9.5 μmol/L.

Loss of endothelial integrity is considered to be an important factor in the early development of atherosclerotic lesions. Established lesions have been shown to suffer de-endothelialization, presumably as the result of increased shearing force on surface endothelium.[29] One question this raises is whether healed endothelium, especially in the presence of elevated levels of homocysteine, possesses functional responses similar to those of native endothelium. To provide an answer to this question we have used an in vivo method of denuding the endothelium from the left common carotid artery of mice which does not result in medial damage or intimal hyperplasia, and after which endothelial healing occurs over a period of about 8 days. We have also used a genetic model of homocysteinemia, the heterozygous CβS$^{+/-}$ mouse. We examined the effect of elevated plasma levels of homocysteine on both endothelial regrowth and the recovery of endothelial-dependent relaxation after denuding injury.

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MATERIALS AND METHODS

Drugs and Solutions

Diethylamine NONOate (DEA-NO) was obtained from CN Biosciences (Nottingham, UK). Other drugs were purchased from Sigma (Poole, Dorset, UK). All drugs were dissolved in phosphate-buffered saline. Stock solutions were diluted by addition to the fixed volume of the myograph bathing chamber and reached tissues by diffusion alone. Krebs-Henseleit solution comprised (in mmol/L): NaCl 119, KCl 4.7, MgSO 4.7, H_2O 1.17, KH_2PO_4 1.18, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 11, pH 7.4.

CβS$^{+/-}$ Mice

Mice with a heterozygous mutation of the CβS gene between exons 2 and 5, by sequence replacement with a part of the neomycin-resistance construct,[28] were obtained from the Jackson Laboratory (Bar Harbor, ME; strain C57BL/6J-Cbstm1Unc). Heterozygous CβS$^{+/-}$ knockout mice were bred with normal mice from the same background (C57BL/6J) to produce CβS$^{+/-}$ mutant and wild-type animals.

Pup mice (10 weeks old) were genotyped by polymerase chain reaction (PCR) on tail DNA. Primers used to identify the presence of the neomycin-resistance construct were as follows: forward, GAA GTG GAG CTA TCA GAG CA (melting temperature [T_m] 55.3°C); reverse, GAG GTC GAC GGT ATC GAT A (T_m 55.7°C). These produced a 300-basepair product. PCR was also used to identify the endogenous *CβS* gene, with primers as follows: forward, the same as for neomycin-resistance, GAA GTG GAG CTA TCA GAG CA (T_m 55.3°C); reverse, CGG ATG ACC TGC ATT CAT CT (T_m 60.8°C). These gave a 500-basepair product.

Male mice, fed standard rodent chow, were used throughout. The housing, care, and procedures on all animals were in accordance with UK Home Office rules and guidelines.

Endothelial Denudation and Artery Removal

Left common carotid arteries from 20- to 40-week-old male mice (body weight, 25 to 30 g) were denuded in vivo as previously described.[30] Briefly, mice were anesthetized by intraperitoneal injection of sodium pentobarbitone, 70 mg/kg body weight. The distal left external carotid artery was occluded with a weighted ligature. The denudation device, a polytetrafluoroethylene (PTFE) filament, was the 250-μm diameter sheath taken from PTFE-coated 100-μm copper wire (Labfacility, Feltham, Middlesex, UK). It was introduced through an incision in the left external carotid artery and advanced under rotation into the left common carotid artery down to the aortic arch and then withdrawn under rotation to the level of the carotid bifurcation. The insertion/withdrawal process was conducted 4 times in total before the filament was completely removed and the left external carotid artery ligated.

After 10 days of recovery, animals were terminated by an overdose of sodium pentobarbitone. A 200-μL blood sample was taken by abdominal aortic catheterization for later analysis of homocysteine concentration, and phosphate-buffered saline was perfused through the systemic circulation at a constant pressure of 100 mm Hg to flush out residual blood through the incised jugular veins. Carotid arteries were dissected free and stored in ice-cold modified Krebs-Henseleit buffer until use. Arteries containing thrombi were not used.

Small-Vessel Wire Myography

Active tension was recorded in arterial segments using the technique described by Mulvany and Halpern.[31] Segments of carotid artery, 2 mm long, were dissected free and cleaned of surrounding adventitia before being mounted onto two 25-μm tungsten wires in a Mulvany-Halpern myograph (Model 410A; Danish Myo Technology, Aarhus, Denmark). Artery segments were placed under isometric load, which was normalized for each artery, and maintained in a static bath in oxygenated (95% O_2, 5% CO_2) modified Krebs-Henseleit buffer at 37°C. Recordings of artery tension were made to computer via an analogue-to-digital converter (MacLab Chart, AD Instruments, Castle Hill, Australia). In all experiments the right (uninjured) carotid artery was used as a time-matched internal control. The contractile stimulants used in assessing relaxation responses were phenylephrine (3 μmol/L) and 5-hydroxytryptamine (3 μmol/L). At the beginning of most experiments a concentration-effect curve for phenylephrine was determined, with a maximum concentration of between 3 and 30 μmol/L depending on the response of the artery. Where phenylephrine responses remained weak, a single application of 3 μmol/L 5-hydroxytryptamine was added. Levels of tone were comparable in all tissues examined regardless of final concentration or agonist used.

Histology

In some animals arteries were fixed in situ by perfusion of 10% formaldehyde in phosphate-buffered saline at a constant pressure of 100 mm Hg throughout the systemic circulation. After removal, the arteries were cut open longitudinally and pinned out on silicone rubber sheets with the luminal surface uppermost. Mayer's hematoxylin was used to reveal the extent of endothelial coverage over the lumen, which was measured using a computerised image analysis system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD).

Artery segments were also examined in a similar fashion after myography, to assess the percentage of endothelial cover. In this case the artery segments were fixed in phosphate-buffered saline containing 10% formaldehyde, cut open longitudinally, and pinned out luminal surface uppermost.

Analysis of Plasma Homocysteine Concentration

Blood samples were anticoagulated with EDTA (final concentration, 5 mmol/L) and centrifuged at 2,000 $\times$ *g* for 10 minutes at 4°C. Plasma was aspirated, and the homocysteine content was analyzed by the Chemical Pathology Laboratory at Bristol Royal Infirmary using reverse-phase high-pressure liquid chromatography (HPLC) with fluorescence detection.

Data Analysis

Tension data were normalised and expressed as a percentage of the total range of relaxation measured in each vessel. The level of maximal relaxation was found either by applying the exogenous NO donor DEA-NO (3 μmol/L) at the end of an experiment, or by using the level of isometric force produced before the addition of contractile stimulant(s). Both methods were used as no significant difference was found between them ($P = .694$, n = 44). The sensitivity of pre-contracted arteries was determined by calculating pD_2 values ($-\log EC_{50}$) from concentration-effect curves in each experiment using a logistic equation.

Statistical Analysis

All data are expressed as mean $\pm$ SEM, and n denotes the number of experiments. Differences between groups of mean values were compared using a 2-tailed unpaired Student's *t* test which does not assume equal variance between samples. Regression analysis was performed using a standard (Pearson) correlation. Significance was accepted where $P < .05$.

RESULTS

Plasma Homocysteine Concentration

The total plasma homocysteine concentration was significantly higher in CβS$^{+/-}$ mice (8.9 $\pm$ 1.1 μmol/L; n = 12) than in wild-type mice (4.6 $\pm$ 0.4 μmol/L; n = 5; p = 0.003).

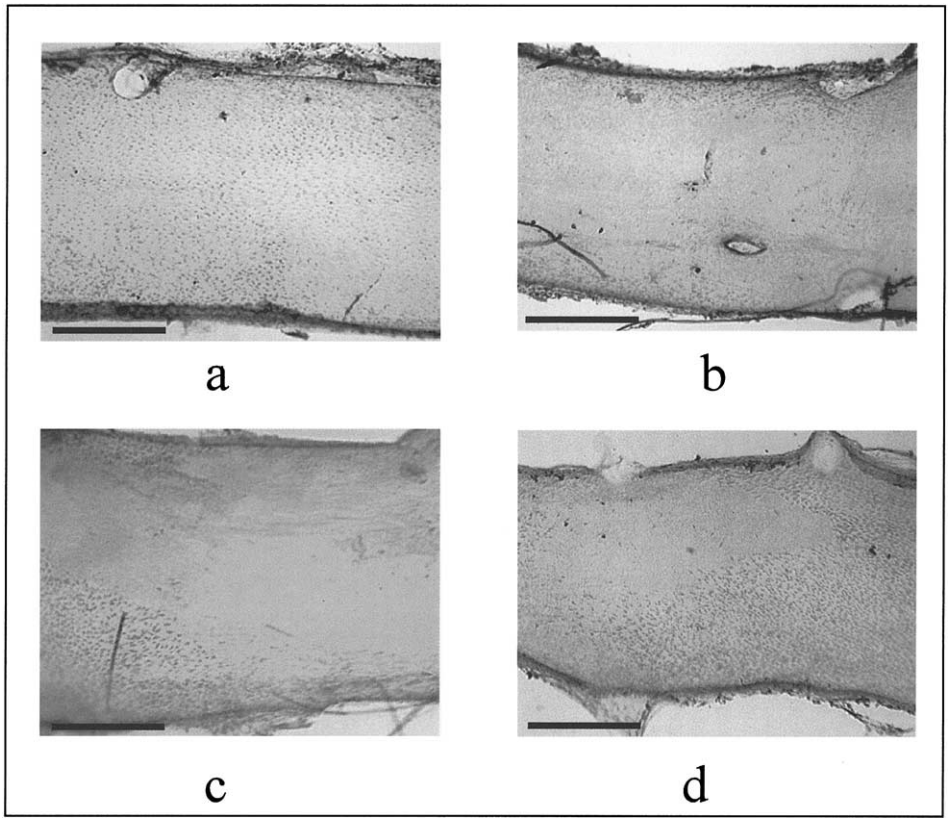

Fig 1. Extent of re-endothelialization in CβS$^{+/-}$ mice at different times following in vivo denudation injury. Bar = 500 μm. (a) Control artery. (b) Artery 1 day after injury. (c) Artery 3 days after injury. (d) Artery 5 days after injury.

Endothelial Denudation and Regrowth in Wild-Type Animals

Complete removal of the endothelium was verified 24 hours after injury by Mayer's hematoxylin staining of fixed arteries. Regrowing endothelium was first seen at day 3, and by day 5 the artery was 46% ± 20 % (n = 3) re-endothelialized (Fig 1). By day 8, complete re-endothelialization had occurred.

Relaxation Induced by Acetylcholine in Uninjured Carotid Arteries

The responses of uninjured pre-contracted CβS$^{+/-}$ mouse carotid arteries to acetylcholine were not significantly different in terms of the maximum level of relaxation (83.8% ± 7.4 %; n = 12) from those in normal mice (95.8% ± 1.8%; n = 5; P = .346).

There was no statistically significant difference in the sensitivity, expressed as the pD_2, of the response to acetylcholine of uninjured carotid arteries between CβS$^{+/-}$ (8.08 ± 8.53; n = 12) and wild-type mice (8.51 ± 9.31; n = 5; P = .108) (Fig 2).

Relaxation Induced by Acetylcholine in Injured Carotid Arteries

Injured arteries were examined on the wire myograph ten days after denudation. The maximum relaxations induced by acetylcholine in CβS$^{+/-}$ mice (76.8% ± 6.2 %) were not significantly different from those in wild-types (93.4% ± 4.8 %; P = .078). However, the rightward shift in the concentration-effect curve for relaxation to acetylcholine in injured arteries of CβS$^{+/-}$ mice (pD_2 = 7.49 ± 8.05; n = 12) was significant (wild-type pD_2 = 8.13 ± 8.88; n = 5; P = .017) (Fig 3). We have previously shown that de-endothelializing injury to mouse arteries does not influence smooth muscle contractile properties, showing that the changes in relaxation in the current study are related to defects in the endothelium rather than defects in the smooth muscle.[32]

Endothelial Regrowth Following Denudation Injury

Ten days after the denudation injury, the artery segments used in the myograph experiments were examined histologically to assess the degree of re-endothelialization that had occurred. Endothelial regrowth was essentially complete and there was no significant difference in the degree of re-endothelialization between CβS$^{+/-}$ (96.5% ± 1.1 %; n = 12) and wild-type mice (98.8% ± 1.2 %; n = 5; P = .191).

Relationship Between Plasma Homocysteine and Endothelium-Dependent Relaxation

To reduce the confounding effects of inter-animal variability, the acetylcholine response of each injured artery was normalised by dividing its pD_2 value by the pD_2 for the contralateral, nondenuded artery in the same animal. Comparison of the pD_2 ratios with the total plasma homocysteine in each animal revealed a significant association between these 2 variables (Pearson's r = −0.693; P = .012) (Fig 4).

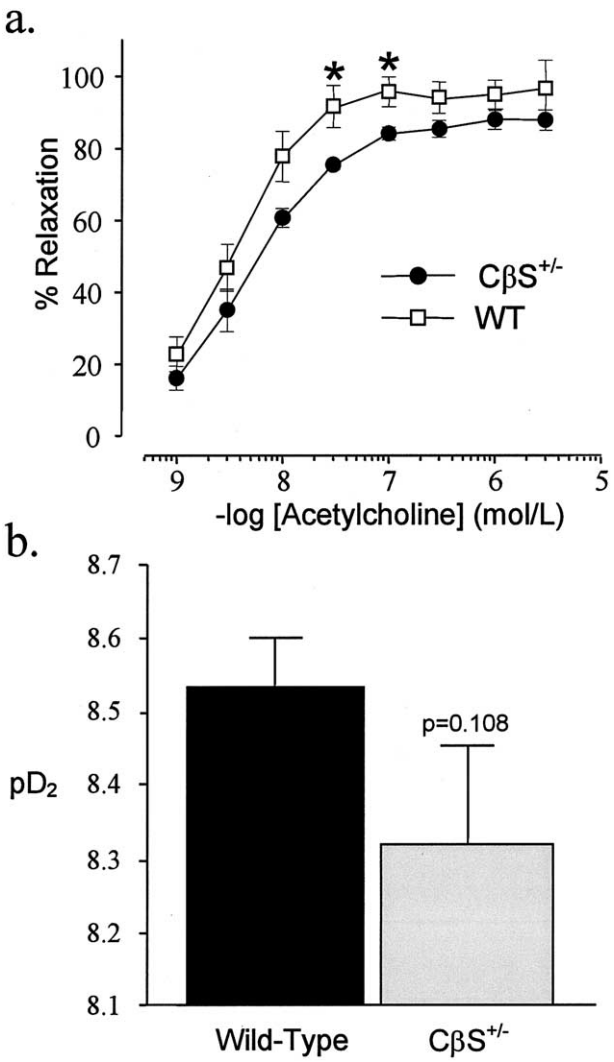

Fig 2. (a) Concentration-response curves to acetylcholine in nondenuded arteries obtained from CβS$^{+/-}$ mice (●) and normal mice (■). *P < .05. (b) Comparison of pD$_2$ values for acetylcholine responses at day 10 in injured arteries from normal mice (black; n = 12) and CβS$^{+/-}$ mice (grey; n = 5).

DISCUSSION

The aim of this study was to examine the influence of homocysteinemia on endothelial function in nondenuded arteries, and in arteries with endothelium that had healed after mechanical denudation. In this study we have shown that relatively small increases in total plasma homocysteine are associated with a decrease in the endothelial-dependent relaxation to acetylcholine of mouse carotid arteries, and that the effect is homocysteine concentration-dependent.

Plasma Homocysteine in CβS$^{+/-}$ and Wild-Type Mice

Mean levels of total plasma homocysteine have been reported to increase from 6.1 μmol/L in normal mice to 13.5 μmol/L in CβS$^{+/-}$ mice,[27] a value close to the lower limit for mild hyperhomocysteinemia in humans.[33] In the present study, CβS$^{+/-}$ mice had lower total plasma homocysteine levels than those reported by Watanabe et al,[27] but our results are in keeping with these results reported by other investigators using the same murine model.[16,28]

Effects of Homocysteine on Normal Arteries

There is some evidence to support the view that small elevations of homocysteine can affect endothelial function in humans. For example, Chambers and colleagues[11,34,35] and Bellamy et al[10] described reduced flow-mediated vasodilatation related to increased concentrations of total plasma homocysteine caused by methionine loading in humans. However, the ability of mild elevations of homocysteine to affect endothelium-dependent flow in humans has been disputed.[36,37] Even when more substantial rises in total plasma homocysteine were produced, flow altered dilatation was not observed in young

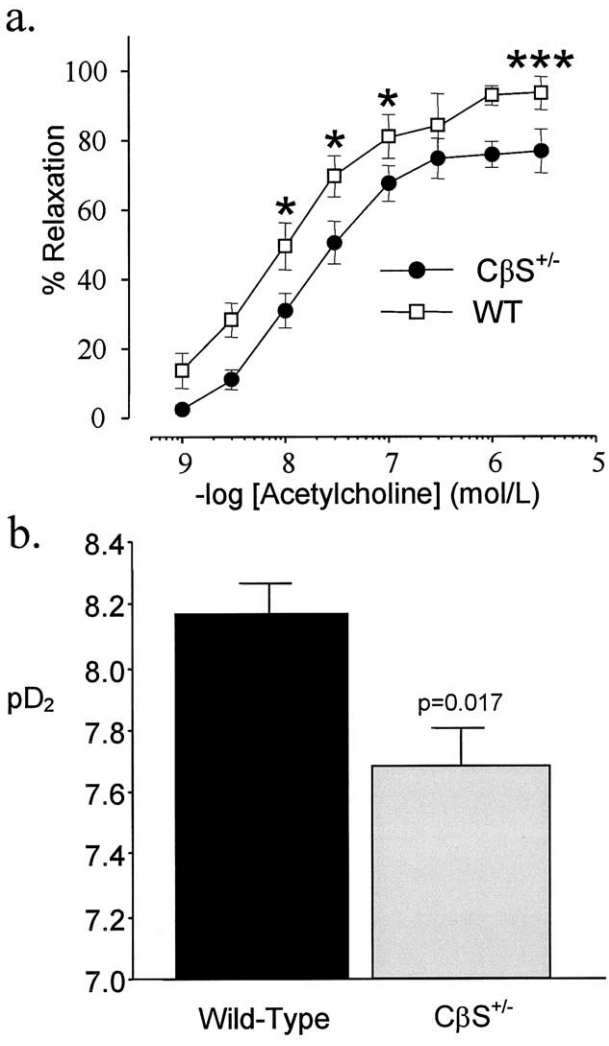

Fig 3. (a) Concentration-response curves to acetylcholine in reendothelialized arteries obtained from CβS$^{+/-}$ mice (●) and normal mice (□). *P < .05; ***P < .01. (b) Comparison of pD$_2$ values for acetylcholine responses at day 10 in injured arteries from CβS$^{+/-}$ mice (black; n = 12) and normal mice (grey; n = 13).

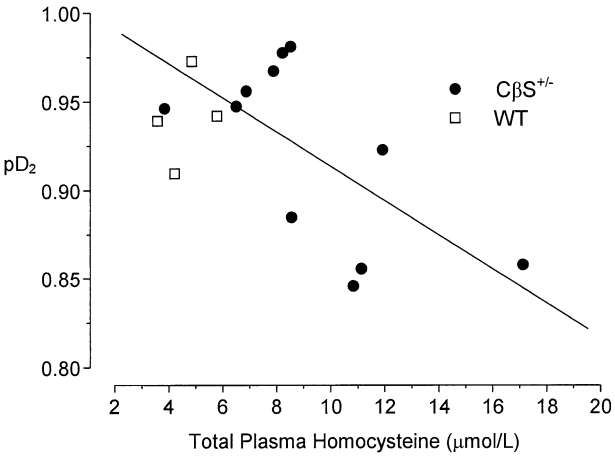

Fig 4. Plot of ratio of normalized pD_2 ratio values (from nondenuded and injured arteries) from each animal against concentration of total plasma homocysteine found in that animal at the time of injury. A significant correlation (Pearson's $r = -0.693$; $P = .012$) was found between the 2 variables.

healthy adults.[38] This uncertainty about the importance of small increases in plasma homocysteine is compounded by epidemiological studies where heterozygosity for a CβS mutation,[39,40] or homozygous mutation of the methyltetrahydrofolate reductase gene (which can increase total plasma homocysteine by up to 25%),[41] do not reveal a significant association with increased risk of cardiovascular disease.

In the present study we found that an increase in total plasma homocysteine from 4.6 μmol/L to 8.9 μmol/L had no effect on the endothelial-dependent vasorelaxation of normal, nondenuded arteries. The increase in total plasma homocysteine in our C$\beta S^{+/-}$ mice was similar to that reported by Eberhardt et al,[16] and who showed a significant attenuation of endothelial-dependent vasorelaxation of aortic rings. In that study, precontracted aortic rings from 10- to 20-week-old C$\beta S^{+/-}$ mice were 5-fold less sensitive to acetylcholine-induced relaxation than in age-matched wild-type mice. In intact carotid arteries we observed a change in mean pD_2 value from 8.51 in normal mice to 8.08 in C$\beta S^{+/-}$ mice, representing a 2.7-fold decrease in sensitivity. The difference between the 2 studies may reflect tissue-specific differences in sensitivity to acetylcholine, an idea supported by studies in these arteries in normal mice.[42]

Effects of Homocysteine on Injured Arteries

Studies in baboons have suggested that concentrations of homocysteine above 100 μmol/L can result in patchy endothelial desquamation,[43] and causes the detachment of endothelial layers in cell culture.[18] It is not known whether elevated total plasma homocysteine causes endothelial desquamation, which is then repaired only for another region to become subsequently desquamated, or whether there is a general depression of endothelial healing that gradually results in sheet desquamation in regions of high endothelial turnover. We hypothesized that, in addition to its possible cytotoxic effects, homocysteine may have a more noticeable effect on cellular function in arteries, particularly in those areas that have been denuded and where endothelial healing has occurred.

To test this hypothesis, we examined acetylcholine-induced vasorelaxation in artery segments that had previously been denuded by gentle mechanical injury with a very delicate PTFE filament. Unlike nondenuded arteries, the re-endothelialized arteries of C$\beta S^{+/-}$ mutant mice showed significant attenuation of responsiveness to acetylcholine compared to those from normal mice. Although maximum vasorelaxation was not affected, the sensitivity of arteries to acetylcholine was significantly lower, and this difference is not caused by an altered rate of regrowth or by the presence of endothelial cells themselves.

We noticed a significant inverse relationship between total plasma homocysteine concentration and sensitivity to acetylcholine in re-endothelialized arteries. This suggests that homocysteine interferes with the ability of endothelial cells to recover full functionality after regrowth. It may be the case that cells undergoing proliferation are more sensitive to the effects of elevated total plasma homocysteine and the increase in oxidative stress that is believed to be associated with it.[17,44,45]

In summary, we have shown significant attenuation by mild hyperhomocysteinemia of endothelial-dependent vasorelaxation in previously denuded mouse common carotid arteries. This effect of homocysteine is concentration-dependent, implying that chronic moderate increases in plasma homocysteine can have a deleterious effect on endothelial cell function.

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