CCR2-Mediated Antiinflammatory Effects of Endothelial Tetrahydrobiopterin Inhibit Vascular Injury–Induced Accelerated Atherosclerosis

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Background—Vascular injury results in loss of endothelial nitric oxide (NO), production of reactive oxygen species (ROS), and the initiation of an inflammatory response. Both NO and ROS modulate inflammation through redox-sensitive pathways. Tetrahydrobiopterin (BH4) is an essential cofactor for endothelial nitric oxide synthase (eNOS) that regulates enzymatic synthesis of either nitric oxide or ROS. We hypothesized that endothelial BH4 is an important regulator of inflammation and vascular remodeling.

Methods and Results—Endothelium-targeted overexpression of GTP cyclohydrolase 1 (GCH), the rate limiting enzyme in BH4 synthesis, increased levels of tetrahydrobiopterin (BH4), reduced endothelial superoxide, improved eNOS coupling, and reduced vein graft atherosclerosis in transgenic GCH/ApoE-KO mice compared to ApoE-KO controls. Immunohistochemistry using anti–MAC-3 and MAC-1 antibody staining revealed a marked reduction in vein graft macrophage content, as did RT-PCR expression of macrophage marker CD68 mRNA levels in GCH/ApoE-KO mice. When we investigated the potential mediators of this reduction, we discovered that mRNA and protein levels of MCP-1 (CCL2) but not RANTES (CCL5) were significantly reduced in GCH/ApoE-KO aortic tissue. Consistent with this finding we found a decrease in CCR2-mediated, but not CCR5-mediated, chemotaxis in vascular tissue and plasma samples from GCH/ApoE-KO animals.

Conclusion—Increased endothelial BH4 reduces vein graft neointimal hyperplasia and atherosclerosis through a reduction in vascular inflammation. These findings highlight the importance of MCP-1/CCR2 signaling in the response to vascular injury and identify novel pathways linking endothelial BH4 to inflammation and vascular remodeling. (Circulation. 2008;118[suppl 1]:S71–S77.)

Key Words: antioxidants ■ atherosclerosis ■ inflammation ■ endothelium ■ remodeling

The endothelium regulates vascular homeostasis through effects on platelet function, blood pressure, vasomotor tone, and vascular remodeling. Dysfunction of the endothelium is a hallmark of vascular disease states and contributes to disease pathogenesis in atherosclerosis. A common feature of endothelial damage and denudation is loss of nitric oxide (NO) bioavailability, increased reactive oxygen species (ROS), and the initiation of an inflammatory response. It is now understood that these processes are not separate entities, rather they interact in a cascade response to vascular injury. In the vasculature, endothelial nitric oxide synthase (eNOS) is critical to these signaling pathways.1

In the healthy vasculature eNOS produces NO conferring antithrombotic, antimigratory, antiproliferative, antiinflammatory, and antioxidant effects.2 eNOS is regulated by the availability of the NOS cofactor tetrahydrobiopterin (BH4). In vascular disease states, where both oxidative stress and inflammation are ubiquitous, BH4 may be depleted directly through altered pterin metabolism or oxidative degradation.3 When BH4 bioavailability is limited, enzymatic reduction of molecular oxygen by eNOS is no longer coupled to L-arginine oxidation, resulting in generation of superoxide rather than NO.4 In keeping with evidence that ROS activate inflammation through downstream signaling pathways,5 BH4 is a potentially important factor for reducing both oxidative stress and inflammation induced vascular remodeling after vascular injury.

Many pathways with key roles in atherosclerosis and vascular remodeling are regulated by NO and ROS signaling.6 Whereas NO reduces expression of proinflammatory signaling pathways, such as through inhibition of NF-KB, conversely ROS may activate NF-KB and AP-1–mediated...
inflammatory pathways. Among the effectors of these activated pathways are chemokines; chemoattractant cytokines which recruit circulating monocytes to sites of inflammation or infection. Recent evidence has established a fundamental role for chemokines (CKs) in atherosclerosis. Of the 4 subclasses of CKs, the CC-CK class is the largest, and most are potent activators for monocyte and macrophage migration. Broad spectrum CC-CK inhibition reduces monocyte recruitment and vascular remodeling in both native atherosclerosis and vein graft accelerated atherosclerosis. Furthermore, within this class, CCR2 has been identified as a major contributor to atherosclerosis. Targeted deletion of CCR2 and deletion of its principal ligand MCP-1 (CCL2) reduces atherosclerosis in mice models. In models of vascular injury, anti–MCP-1 gene therapy reduced neointimal hyperplasia after perivascular injury, balloon injury, stent injury, and vein bypass grafting.

We hypothesized that increasing endothelial BH4 would have salutary effects on vascular inflammation and remodeling after vascular injury. We quantified the response to venous bypass grafting in the ApoE-KO mouse, in comparison with matched transgenic animals with constitutively increased endothelial BH4. Furthermore, we investigated the effect of increased endothelial BH4 levels on macrophage recruitment, chemokine expression, and chemokine signaling.

**Methods**

**Mice and Vein Graft Procedure**

All animal procedures were carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. Mice were maintained in temperature-controlled (20°C to 22°C) individually-ventilated cages with a 12-hour light-dark cycle. Sterile water and standard chow diet were available ad libitum. Mice overexpressing human GTP cyclohydrolase I (GCH) targeted to the vascular endothelium under the Tie-2 promoter (GCH-Tg), were crossed onto C57BL/6J ApoE-KO mice (Jackson Laboratories, Bar Harbor, Me) background. Eighteen to 22-week-old male mice underwent carotid interposition bypass grafting using the vena cava from isogenic littermate female donor mice as described previously. Vena cava from ApoE-KO donor mice were grafted into ApoE-KO recipients and GCH/ApoE-KO donor vena cava were grafted into GCH/ApoE-KO recipients. Briefly, the right common carotid artery was isolated and mobilized from the thoracic inlet to the bifurcation, divided at its midpoint, and cuffs placed over the ends. The artery was inverted over the cuffs and ligated with 8-0 silk sutures. The supradiaphragmatic vena cava from a littermate donor mouse was harvested and grafted as an interposition graft by sleeving the vein over the 2 ends of the carotid artery, and ligating with 8-0 silk sutures. Vigorous pulsation in the conduit vessel confirmed successful engraftment. The total operating time for the procedure was 40 minutes. In total 74 mice underwent successful operative procedures.

**Lipid and Lipoprotein Analysis**

Total plasma cholesterol and triglyceride concentrations were measured using enzymatic assay (Roche) on a Cobas Mira Plus automated analyzer (Roche).

**Tissue Preparation, Histology, and Lesion Quantification**

Grafts were harvested at 28 (n=8 to 12/treatment group) or 56 days (n=5 to 8/treatment group) post surgery and snap frozen in OCT or fixed in paraformaldehyde for processing and analysis as described previously. Briefly, grafts were sectioned for 150 μm from the midpoint, collecting 5 μm sections. Three representative sections, separated by 50 μm were stained with Masson-Goldner for analysis. Vessel wall area was determined by subtracting the luminal area from total vessel area. Image analysis was performed using Image Pro Plus software (Media Cybernetics).

**Immunohistochemical Staining**

Immunohistochemistry and analysis was performed using anti–Mac-3 (Clone M3/84, Pharmingen) and anti–Mac-1 (Clone M1/70, Pharmingen) monoclonal antibodies as described previously.

**Bioppterin Measurements**

Total bioppterins (BH4, BH2, and bioppterin) and BH4 (Total bioppterin–BH2+bioppterin) were measured using high-precision liquid chromatography (HPLC) analysis with fluorescent detection after differential iodine oxidation of tissue extracts or plasma in either acidic or alkaline conditions, respectively, as described previously.

**Lucigenin-Enhanced Chemiluminescence Detection of Superoxide**

Total aortic superoxide was measured by lucigenin-enhanced chemiluminescence as described previously. Briefly, aortas were harvested flushed with Krebs-HEPES buffer, and divided into halves. Vessels were gassed with 95% oxygen/5% carbon dioxide in warmed Krebs HEPES buffer for 30 minutes at 37°C. Chemiluminescence was measured in an FB12 luminometer (Berthold Detection Systems) using 5 μmol/l lucigenin under basal conditions, after addition of NADPH (300 μmol/l), NADPH oxidase inhibitor diphenyleiouione (DPI; 10 μmol/l), and NOS inhibitor L-NAME (1 mmol/l). After measuring baseline readings for 4 minutes, samples were equilibrated and dark adapted for 5 minutes, and chemiluminescence recorded in 5-minute intervals. Recordings were performed blinded to the samples identity. Results were expressed as counts per second per milligram of tissue dry weight.

**Oxidative Fluorescent Microtopography**

Superoxide was detected in the layers of the vessel wall using fluorescent probe dihydroethidium as described previously. Briefly, fresh sections of descending thoracic aorta or midsection of vein graft were frozen in OCT compound. Cryosections (30 μm) were incubated with Krebs-HEPES buffer for 30 minutes at 37°C with or without 1 mmol/l L-NMMA (Sigma), followed by 5 minutes dark incubation with 2 μmol/l dihydroethidium (DHE; Molecular Probes). Images were obtained on a confocal microscope (Bio-Rad MRC-1024 laser; filter settings: excitation filter 488 nm; emission filter 550 nm). Endothelial 2-hydroxyethidium fluorescence (intensity×area) was measured from high power (60× magn) images of the luminal side of the internal elastic lamina. Medial 2-hydroxyethidium fluorescence (intensity×area) was measured from high power (60× magn) images between the internal and external elastic lamina. Analysis was performed blind to the samples identity using Image Pro Plus software (Media Cybernetics). Mean fluorescence was calculated from 4 separate high power fields from each quadrant to produce n=1.

**Quantitative Real-Time PCR**

Total RNA extracted from snap frozen vein grafts freshly harvested 28 days after surgery were used to measure CD68, MCP1, and RANTES mRNA expression. Total vein graft RNA (50 ng) was used in RT-PCR (Qiagen Quantitect SYBR Green RT-PCR kit, Qiagen) using the following primers for various mRNA transcripts (Genbank) (5′-3′): (X68273) CD68: forward, TTG GGA ACT ACA CAC GTG GGC; reverse, CGG ATT TGA ATT TGG CCT GTTG; (M19681) MCP1: forward, TCT CTC ACC CAT AGC AGC AAG TGG; reverse, TCA GCC GGC AAC TGT GA; (M136530) RANTES: forward, GAG TAT TTC TAC ACC AGC AGC AAG TG; reverse, TGA CAA ACA CGA CTT CAA GAT TG. Quantitative fluorescent real-time RT-PCR analysis was used to compare relative quantities of mRNA using the Rotor-Gene system (Corbett Research Ltd). Standards were prepared using 10-fold dilutions of atherosclerotic plaque total RNA. Relative quantities of PCR product in each sample were measured.
against the standard curve and normalized using human hypoxanthine phosphoribosyltransferase (HPRT) as the optimal control (determined as the optimal control from a range of housekeeping genes as described previously): forward, GCT CGA GAT GTC ATG AAG GAG AT; reverse, AAA GAA CTT ATA GCC CCC CTT GA. Samples were processed in triplicate with a reverse transcriptase negative control reaction for each. Quantification was performed using proprietary software to generate standard curves, expressing relative quantities of PCR products in the sample in arbitrary units relative to the standard curve. Mean values were calculated from triplicates to produce a=1.

Chemokine Enzyme Linked Immunosorbent Assay
Chemokines MCP-1 and RANTES were measured in snap-frozen aortas and plasma from experimental animals. Levels of MCP-1 and RANTES were measured by sandwich ELISA using commercial kits (Quantikine; R&D Systems) according to the manufacturer’s instructions.

Chemotaxis Assay
We quantified CCR2 and CCR5 directed cell migration of plasma or homogenized aorta using transwell membranes (ChemoTx, 6.0-mm diameter, 8-μm pore size, Receptor Technologies) as described previously. Briefly, 293 cells were grown to 50% confluence in Dulbecco modified Eagles media (DMEM)/H11005. Cells were processed in triplicate with a reverse transcriptase negative control reaction for each. Quantification was performed using proprietary software to generate standard curves, expressing relative quantities of PCR products in the sample in arbitrary units relative to the standard curve. Mean values were calculated from triplicates to produce a=1.

Statistical Analysis
Data are presented as mean±SEM. Data were subjected to the Kolmogorov-Smirnov test to determine distribution. Groups were compared using the Mann–Whitney U test for nonparametric data or the Student t test for parametric data. When comparing multiple groups data were analyzed by analysis of variance (ANOVA) with Newman–Keuls post test for parametric data or Kruskal–Wallis test with Dunns post test for nonparametric data. A value of P<0.05 was considered statistically significant.

Results
Effect of GTPCH Overexpression on Bioperin Levels in Vascular Tissue
We first aimed to confirm that increased endothelial GCH expression resulted in increased levels of BH4 in vascular tissues. BH4 levels in GCH/ApoE-KO mice were significantly increased by ~4-fold in aorta and vena cava and ~8-fold in vein grafts compared to those observed in ApoE-KO control mice (Figure 1). BH4 levels were approximately 60% to 80% of total biopterin levels in GCH/ApoE-KO mice compared with 15% to 50% in Apo-E-KO, mice suggesting increased oxidative degradation of BH4 in ApoE-KO mice. Importantly, plasma BH4 levels were not different in GCH/ApoE-KO compared to ApoE-KO mice, demonstrating that endothelial-specific GCH overexpression does not have general systemic effects on BH4 levels (Figure 1). Total cholesterol, triglyceride levels, and body weights were similar in both GCH/ApoE-KO and ApoE-KO mice (Table).

Effect of Increased BH4 on Vein Graft Atherosclerosis
Next we investigated the effect of increased endothelial BH4 in GCH/ApoE-KO mice on vein graft atherosclerosis, by quantifying vein graft wall area at 28 and 56 days postoperatively. Mean vessel wall area was significantly reduced in GCH/ApoE-KO mice at both 28 (69%, P=0.01, Figure 2) and 56 days (53%, P=0.04, Figure 2), compared with ApoE-KO controls. These findings suggest that increased vascular BH4 levels reduce neointimal hyperplasia and atherosclerosis after vein grafting.

Effect of Increased BH4 Levels on Superoxide and eNOS Coupling
We next sought to evaluate the effects of increased endothelial BH4 on superoxide levels in ApoE-KO versus GCH/ApoE-KO mice. Total vascular superoxide production, quantified by lucigebin-enhanced chemiluminescence, was reduced by more than 60% in GCH/ApoE-KO mouse aorta compared with ApoE-KO (Figure 3A). NADPH stimulation led to a dramatic increase in total chemiluminescence that was similar in both groups, likely indicating activation of endothelial and vascular smooth muscle NADPH oxidases. This increase was abolished by the NADPH oxidase inhibitor diphenylene iodonium. Finally, we assessed the proportion of superoxide production attributable to uncoupled NOS. NOS inhibition by L-NAME significantly decreased chemiluminescence in ApoE-KO aorta, and increased chemiluminescence in GCH/ApoE-KO aorta indicating the production of superoxide and nitric oxide respectively from NOS in these animals under basal conditions.

We next investigated the effects of increased BH4 levels specifically on endothelial superoxide production in GCH/ApoE-KO and ApoE-KO mice. We measured endothelial superoxide production using dihydroethidium oxidative fluorescence microtopography. Whereas medial vessel wall fluorescence was not different between groups (Table).
To investigate the hypothesis that increased endothelial BH4 attenuated lesion macrophage content through effects on CCR2 ligands and CCR2-mediated chemotaxis, we quantified levels of MCP-1 (CCL2) and RANTES (CCL5), in both plasma and aortic homogenates. There was no difference in the concentration of either MCP-1 or RANTES in the plasma of GCH/ApoE-KO compared with ApoE-KO mice (Figure 5A). However, MCP-1 levels were significantly reduced in aortic homogenates of GCH/ApoE-KO animals compared to ApoE-KO (Figure 5A). In contrast, RANTES levels in aortic homogenates were similar in GCH/ApoE-KO and ApoE-KO animals (Figure 5A).

We next performed quantitative RT-PCR for MCP-1 on total RNA extracted from vein grafts of experimental animals. In keeping with the ELISA findings, MCP-1 mRNA levels were significantly reduced in GCH/ApoE-KO mice compared with ApoE-KO control (Figure 5B). No difference was detected in mRNA levels of RANTES, indicating the effect of BH4 on inflammation was not attributable to a generalized decrease in all chemokines (Figure 5C).

**Effect of Increased Endothelial BH4 on CCR2- and CCR5-Mediated Chemotaxis**

To investigate the effects of increased endothelial BH4 on chemotactic activity mediated by CCR2 and CCR5, we quantified migration of cells transfected with either CCR2 or CCR5 in response to plasma or aortic extracts. Both plasma and aorta (Figure 6) from GCH/ApoE-KO mice induced significantly lower levels of CCR2-mediated cell migration compared with ApoE-KO animals. In contrast, CCR5-mediated cell migration was not significantly different between groups, suggesting a specific role for endothelial BH4 in regulation of CCR2 ligands and CCR2-mediated chemotaxis.

**Discussion**

In this study, we used vein bypass grafting in hypercholesterolemic ApoE-KO mice with or without increased levels of endothelial BH4, to test the effect of endothelial BH4 on inflammation and vascular remodeling. Endothelial overexpression of the rate limiting enzyme GCH led to increased endothelial BH4 levels in vascular tissue and markedly reduced vein graft atherosclerosis, endothelial superoxide, eNOS uncoupling, and vein graft macrophage content at both 28 and 56 days after surgery. This effect of BH4 on vein graft...
Atherosclerosis was accompanied by reduced expression of MCP-1 in vein grafts and reduced CCR2-mediated chemotactic activity in both plasma and aortas, whereas CCR5-mediated chemotaxis was unaffected. Our results suggest that augmenting endothelial BH4 could be a valid strategy to reduce local chemokine expression and monocyte recruitment and hence reduce accelerated atherosclerosis after vascular injury such as vein graft surgery.

Venous bypass grafting increases ROS production that has been implicated as a mediator of inflammation, smooth muscle cell migration, and proliferation leading to vein graft neointimal hyperplasia and accelerated atherosclerosis.20,21 We have shown previously that reducing superoxide levels after vascular injury can positively alter vein graft remodeling.20,22 We now show that one specific redox mechanism, the availability of BH4 in the vascular endothelium, is sufficient to modulate vein graft inflammation and atherosclerosis, and that the mechanism of this altered remodeling is, at least in part, attributable to reduced CCR2-mediated chemotaxis. Our data indicate that CC-CKs acting on the CCR2 receptor, including MCP-1, appear to be important in mediating the effects of increased endothelial BH4, in contrast to a generalized reduction in chemokine activity as evidenced by our
has been suggested by only one previous study, in a model of redox-sensitive transcription factors, such as NF-kinases and phosphoinositide-3-kinases (PI-3-K) as well as production pathways such as mitogen-activated protein (MAP) cytokine-receptor activity, despite no detectable difference in MCP-1 levels.

Interestingly, we found that plasma from GCH/ApoE-KO mice had significantly lower CCR2-mediated chemotactic activity, despite no detectable difference in MCP-1 levels.

RANTES/CCR5 data. Further studies investigating the effect of BH4 on other CKs are warranted.

BH4 is an important regulator of eNOS function and ROS production in the vascular endothelium. Although several studies have now shown salutary effects of BH4 on endothelial function and eNOS regulation (reviewed in Alp et al24), a potential role for BH4 in modulating vascular inflammation has been suggested by only one previous study, in a model of transplant rejection.25 ROS have been implicated in initiating inflammatory responses through activation of signal transduction pathways such as mitogen-activated protein (MAP) kinases and phosphoinositide-3-kinases (PI-3-K) as well as redox-sensitive transcription factors, such as NF-KB and AP-1 leading to enhanced gene expression of proinflammatory mediators (reviewed in Kunsch et al5). Among the multiple gene products regulated by these transcription factors involved in atherosclerosis is the CC-KC MCP-1.26 Because MCP-1 is a potent mediator of monocyte and macrophage migration, known to be a major contributor to atherosclerosis, we investigated the hypothesis that the reduced macrophage content after vascular injury in vessels from GCH/ApoE-KO mice was the result of reduced MCP-1. MCP-1 mRNA levels were indeed reduced in GCH/ApoE-KO mice as were MCP-1 protein levels in aortas of GCH/ApoE-KO.

Figure 5. MCP-1 content in vascular tissues of GCH/ApoE-KO and ApoE-KO mice. A, ELISA used to detect MCP-1 levels in experimental animals revealed a significant reduction in aorta (white bars) but not plasma (black bars) of GCH/ApoE-KO mice. No difference could be detected in control RANTES levels.

The ex vivo chemotaxis assay detects the bioactivity of all CC-CCKs active at the CCR2 receptor, including MCP-2, MCP-3, MCP-4, MCP-5, and eotaxin in addition to MCP-1.27 Thus, it is possible that chronic inflammation induced by the vein graft procedure, visualized by MAC-1+ve and MAC-3+ve staining in vein grafts at 28 days, leads to increased systemic levels of these or other CC-CCKs that are detected in the ex vivo bioassay. Furthermore, the CCR2 and CCR5 chemotaxis bioassays were performed in transfected 293 cells, whereas MCP-1 expression and protein levels were measured more specifically by RT-PCR and ELISA, respectively. In vivo, MCP-1 protein may undergo modification, such as limited N-terminal proteolysis, leading to discordance between protein levels and functional chemotactic activity. Indeed, N-terminal deletion leading to loss of CCR5 signaling has been reported for the CC-CK RANTES.28

In conclusion, we have demonstrated that increased endothelial BH4 synthesis in vivo, reduces the inflammatory response to vein bypass surgery through a pathway leading to MCP-1/CCR2. These findings highlight the importance of redox state in the response to vascular injury and raise the prospect of BH4 as a rational therapeutic target for reducing vein graft accelerated atherosclerosis.

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Disclosures

None.

References


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