Guanosine Triphosphate Cyclohydrolase I Expression and Enzymatic Activity Are Present in Caveolae of Endothelial Cells

Timothy E. Peterson, Livius V. d’Uscio, Sheng Cao, Xiao-Li Wang, Zvonimir S. Katusic

Abstract—Tetrahydrobiopterin is an essential cofactor required for the synthesis of NO. GTP cyclohydrolase I (GTPCH I) is the rate-limiting enzyme for tetrahydrobiopterin production in endothelial cells, yet little is known about the subcellular localization of this enzyme. In this study, we demonstrated that GTPCH I is localized to caveolar membrane microdomains along with caveolin-1 and endothelial NO synthase. GTPCH I activity was detected in isolated caveolar membranes from cultured endothelial cells. Confocal and electron microscopy analyses confirmed GTPCH I colocalization with caveolin-1. Consistent with in vitro studies, GTPCH I activity was evident in isolated caveolar microdomains from lung homogenates of wild-type mice. Importantly, a 2-fold increase in GTPCH I activity was detected in the aortas of caveolin-1–deficient mice, suggesting that caveolin-1 may be involved in the control of GTPCH I enzymatic activity. Indeed, overexpression of caveolin-1 inhibits GTPCH I activity, and tetrahydrobiopterin biosynthesis is activated by the disruption of caveolae structure. These studies demonstrate that GTPCH I is targeted to caveolae microdomains in vascular endothelial cells, and tetrahydrobiopterin production occurs in close proximity to endothelial NO synthase. In addition, our findings provide new insights into the regulation of GTPCH I activity by the caveolar coat protein, caveolin-1. (Hypertension. 2009;53:189-195.)

Key Words: GTP-cyclohydrolase I ■ tetrahydrobiopterin ■ endothelium ■ caveolin-1 ■ nitric oxide

The production of the vasodilator NO by endothelial NO synthase (eNOS) is critical for the maintenance of normal vasomotor function.1 Tetrahydrobiopterin (BH4) is an essential cofactor required for activity of eNOS.2 We and others have shown that the vascular endothelium is a major source of BH4 in the arterial wall.3–5 BH4 is synthesized from GTP in a 3-step process that is initiated by the enzyme GTP cyclohydrolase I (GTPCH I).6 The molecular mechanisms regulating GTPCH I activity in the vascular endothelium are not well understood. GTPCH I activity has been demonstrated to be increased by cytokines, hydrogen peroxide, protein kinase C, and fluid shear stress imposed on endothelium by circulating blood.7–10 Several groups have also demonstrated that protein-protein interactions can influence GTPCH I activity in vitro.11,12 Most notably, the N-terminal sequence of GTPCH I was shown to bind several membrane-associated proteins, suggesting that GTPCH I may be involved in membrane trafficking.12

It is now established that eNOS activity is regulated at the posttranscriptional level by the protein caveolin-1, an important structural protein associated with plasma membrane microdomains called caveolae.13–16 In endothelial cells, caveolae are flask-like shape invaginations of the plasma membrane and associated vesicles that provide a platform for many signaling complexes.17,18 The role of caveolin-1 in the control of GTPCH I function and BH4 synthesis has not been studied in vitro or in vivo. Because proper eNOS function depends on both GTPCH I activity and its subcellular localization to caveolae,19 we hypothesized that GTPCH I localizes in caveolae and is regulated by caveolin-1.

Materials and Methods

Experimental Animals

Male caveolin-1–deficient mice (Cav1–/–) and eNOS-deficient mice (B6.129P2-Nos1tm1Unc/J; eNOS–/–), as well as strain-matched wild-type mice B6129SF2/J and C57BL/6J, respectively, were obtained from the Jackson Laboratories (Bar Harbor, ME). Lungs of GTPCH I-transgenic mice were provided by Dr Alex Chen (Michigan State University, East Lansing, Mich) with permission of Dr Keith M. Channon (University of Oxford, Oxford, United Kingdom). Mice were maintained on standard chow with free access to drinking water. Housing facilities and all of the experimental protocols were approved by the Mayo Clinic Institutional Animal Care and Use Committee and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were euthanized by overdose of pentobarbital (60 mg/kg IP), and whole aortas and lungs were carefully harvested and dissected free from connective tissue.

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From the Departments of Anesthesiology and Molecular Pharmacology and Experimental Therapeutics (T.E.P., L.V.d., Z.S.K.) and Divisions of Gastroenterology (S.C.) and Cardiovascular Diseases (X.-L.W.), Department of Internal Medicine, Mayo Clinic College of Medicine, Rochester, Minn.

The first 2 authors contributed equally to this work.

Correspondence to Zvonimir S. Katusic or Livius V. d’Uscio, Departments of Anesthesiology and Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. E-mail Katusic.Zvonimir@mayo.edu or dUscio.Livius@mayo.edu

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Cell Culture and Adenoviral Overexpression Techniques

Human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex and were passaged in endothelial growth medium-2 (Cambrex). All of the experiments were performed using HUVECs between passages 3 and 7. A recombinant adenovirus encoding the human GTPCH I gene (Ad-GTPCH I) driven by a cytomegalovirus promoter and at a multiplicity of infection (MOI) of 100 was used to overexpress GTPCH I to a level that was detectable by Western blot analysis. In separate studies, adenoviral encoding human caveolin-1 (Ad-Cav1; Vector Biolabs) at an MOI of 30 was used to overexpress caveolin-1. HUVECs were infected for 12 hours in serum-free medium (EBM-2) and were then fed with growth medium for 48 hours before analysis. A recombinant adenoviral vector with a deletion of ΔE1 (Ad-ΔE1) was used as a control.

Western Blot Analysis

Cells were washed twice in cold PBS and flash frozen in 200 μL of lysis buffer in an ethanol/dry ice bath followed by scraping and a 5-second sonication to achieve a homogeneous solution. Twenty to 30 μg of total protein were separated by 12% SDS-PAGE, transferred to nitrocellulose membrane, and stained with Ponceau-S stain. Membranes were probed using primary antibodies against GTPCH I (Sigma) to ensure equal protein loading. For Western blot analysis, membranes were incubated for 45 minutes. Cells were then incubated for 5 minutes with an adenovirus encoding hemagglutinin-tagged GTPCH I (a gift from Dr Keith Channon) at an MOI of 20 in EBM-2 for 6 hours. Cells were then fed growth medium for 24 hours before analysis. After treatment, cells were fixed with methanol for 15 minutes at 4°C and blocked with 10% normal goat serum for 30 minutes. Slides were then incubated for 1 hour with a mixture of mouse anticaveolin-1 and rabbit anti-hemagglutinin antibody (Sigma). A secondary antibody mixture of goat antimouse fluorescein isothiocyanate and goat anti-rabbit Texas Red (Invitrogen) was added and incubated for 45 minutes. Cells were then incubated for 5 minutes with 10 μg/mL of Hoechst 33258 (Sigma) to stain for nuclei. Cover slips were mounted using Prolong Gold mounting medium (Invitrogen), and the cells were visualized using a Zeiss LSM 510 laser scanning confocal microscope.

Immunogold Labeling of Mouse Aorta by Electron Microscopy

Isolated aortic ring segments from wild-type mice were fixed in 4% formaldehyde plus 1% glutaraldehyde overnight, dehydrated in a series of ethanol from 35% to absolute while progressively lowering the temperature to −20°C, embedded in LR white resin, and polymerized at 55°C. Thin sections were mounted on nickel grids and labeled for GTPCH I and double-labeled for caveolin-1. GTPCH I was labeled by blocking free aldehydes with 1% glycine and blocking with PBS containing Tween-20 and 2% normal goat serum. A rabbit polyclonal antibody specific for mouse GTPCH I was diluted 1:2 in PBS containing Tween-20 and 2% normal goat serum, and grids were incubated at room temperature for 2 hours. Grids were rinsed extensively in PBS containing Tween-20 and incubated in a goat antirabbit secondary antibody conjugated to 10-nm gold beads (Amersham) for 1 hour at room temperature. Caveolin-1 was labeled by blocking in glycine and PBS containing Tween-20 and 2% normal goat serum similar to GTPCH I labeling. Mouse anticaveolin-1 was diluted 1:5 in PBS containing Tween-20 and 2% normal goat serum and incubated for 2 hours at room temperature. After rinsing in PBS containing Tween-20, the sections were incubated in goat antimouse secondary antibody conjugated to 5-nm gold beads (Amersham). When double labeling was completed, the sections were stained in lead and uranyl for transmission electron microscopy.

Measurements of Biopterin Levels and GTP-Cyclohydrolase I Activity

BH4 and 7,8-dihydrobiopterin levels, as well as GTPCH I enzymatic activity, were determined in fresh aortas using a reverse-phase high-performance liquid chromatography method, as described previously.

Statistical Analysis

Data are expressed as means±SEM, and “n” indicates the number of animals from which tissues were harvested. Single values were compared by 1-way ANOVA with Bonferroni correction for multiple comparisons. For simple comparisons between 2 groups, an unpaired Student t test was used where appropriate. A value of P<0.05 was considered significant.

Results

In Vitro Analysis of GTPCH I Subcellular Localization

To track GTPCH I protein localization in vitro, an adenoviral construct encoding human GTPCH I was used in HUVECs at an MOI of 100 (Figure 1A). Initial experiments demonstrated that GTPCH I protein expression was equally partitioned to both Triton X-100 soluble and Triton X-100 insoluble fractions, suggesting that a significant fraction of GTPCH I protein is associated with cellular membranes, along with caveolin-1 and eNOS (Figure 1A). Subsequent studies using sucrose gradient ultracentrifugation demonstrated that both GTPCH I protein expression and activity were localized to caveolar microdomains, as well as noncaveolar-associated membrane fractions (Figure 1B). Confocal microscopic analysis of HUVECs infected with an adenovirus encoding a hemagglutinin-tagged GTPCH I demonstrated that GTPCH I staining was localized to perinuclear regions of the cell (Figure 1C) as reported previously; however, there was also...
a considerable amount of colocalization of GTPCH I with caveolin-1 at the cell membrane (Figure 1C).

**In Vivo Analysis of GTPCH I Subcellular Localization**

To determine whether similar results could be obtained in vivo, initial studies using electron microscopy were performed to determine whether GTPCH I and caveolin-1 might be localized in similar subcellular compartments to each other in endothelial cells from wild-type mouse aorta. Indeed, a substantial amount of GTPCH I labeling was found both in the cytoplasm and at the plasma membrane of mouse aortic endothelial cells. Dual labeling for both caveolin-1 and GTPCH I showed that the 2 proteins were often associated in close proximity with each other (Figure 2A). Analysis of membrane-associated proteins using detergent-free sucrose density gradient ultracentrifugation demonstrated that GTPCH I activity is concentrated in the caveolae-rich fraction of the wild-type mouse lung (Figure 2B).

To further investigate the functional significance of GTPCH I localization to caveolar microdomains, we measured GTPCH I protein expression and activity in the aortas of Cav1+/−/−/− mice, which lack any morphological caveolar structures.23 Western blot analysis demonstrated similar levels of GTPCH I protein expression in the aorta from wild-type and Cav1+/−/−/− mice (Figure 3A), whereas high-performance liquid chromatography analysis revealed a significant increase in GTPCH I activity in the aorta and lung from Cav1+/−/−/− mice when compared with wild-type controls (Figure 3B). In contrast, GTPCH I activity was unaltered in other organs, such as the brain and liver of Cav1+/−/−/− mice (Table). Along with the increases in GTPCH I activity, we also observed an increase in BH₄ levels from the aorta of Cav1+/−/−/− mice compared with wild-type controls, whereas oxidative products of BH₄ and 7,8-dihydrobiopterin levels were unchanged (data not shown), indicating that the selective increase in BH₄ levels was because of the increased de novo biosynthesis of BH₄ via GTPCH I (Figure 3C). Furthermore, we could not detect a difference in BH₄ levels in the aortas of eNOS−/− mice (Figure 3D). Together, these data demonstrate that...
caveolin-1 may act as a functional inhibitor of GTPCH I activity in vivo.

Interaction Between Caveolin-1 and GTPCH I Activity

Ad-Cav1–transduced cells showed increased Cav1 protein expression, as detected by Western blot analysis (Figure 4A). Interestingly, enzymatic activity of GTPCH I was reduced by ≈50% in HUVECs transduced with Ad-Cav1 as compared with the control Ad-ΔE1 vector (Figure 4B).

Western blot analysis of membrane fractions of the wild-type mouse lung demonstrated that GTPCH I was present in the caveolae-rich fraction (fraction 2; Figure 5A, top), again confirming that GTPCH I was targeted to the cholesterol-rich, low buoyant density caveolae-rich fraction of the membrane. In contrast, GTPCH I activity was significantly reduced in fraction 2 of Cav1<sup>−/−</sup> mice (Figure 5A, bottom). To further demonstrate that GTPCH I activity is localized in cholesterol-enriched membrane microdomains, isolated mouse aortas were treated in vitro with methyl-β-cyclodextrin (β-CD) that binds cholesterol and cause reversible disassembly of caveolae. Indeed,

Table. Enzymatic Activity of GTPCH I in Wild-Type and Cav1<sup>−/−</sup> Mice

<table>
<thead>
<tr>
<th>Organ</th>
<th>WT</th>
<th>Cav1&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>15.5±0.9</td>
<td>25.2±1.6*</td>
</tr>
<tr>
<td>Brain</td>
<td>0.6±0.3</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>250.2±30.1</td>
<td>202.3±18.8</td>
</tr>
</tbody>
</table>

WT indicates wild-type (B6129SF2/J). Data are means±SEMs (n=4) and are expressed in picomoles of neopterin per milligram of protein.
*P<0.05 vs WT.

Caveolar Activity of GTPCH I in GTPCH I Transgenic Mice

Because overexpression of GTPCH I in endothelium has well-established vascular protective effects, we performed experiments on transgenic mice with endothelial-targeted overexpression of GTPCH I. Consistent with findings on cultured endothelial cells, we found that GTPCH I activity was significantly increased in caveolar-rich fractions (and noncaveolar membrane fractions) of GTPCH I transgenic mice as compared with wild-type mice (Figure 5B).

Discussion

The results of this study reveal several novel findings regarding the regulation of BH₄ synthesis in endothelial cells. First, we demonstrate that GTPCH I protein expression and enzymatic activity are localized in both caveolar and noncaveolar membrane compartments. Second, enzymatic activity of GTPCH I and BH₄ levels were increased in the aorta of Cav1<sup>−/−</sup> mice. Third, caveolin-1 overexpression inhibits GTPCH I activity, and BH₄ biosynthesis is activated by the disruption of caveolae structure. Fourth, overexpression of GTPCH I increased GTPCH I activity in caveolar microdomains of endothelial cells. Given that optimal NO synthesis occurs in caveolar microdomains, we propose that the close spatial localization of eNOS and GTPCH I may help to ensure proper NO synthesis in endothelial cells. In addition, it is likely that caveolar localization contributes to the well-established vascular protective effect of GTPCH I in experimental models of diabetes mellitus and hypercholesterolemia, as well as in pulmonary and systemic hypertension.24–27
Although GTPCH I is known to be critical for the synthesis of BH₄ in multiple cell types, there is little information about the subcellular localization of this enzyme in endothelial cells. Using an adenoviral construct to overexpress human GTPCH I in endothelial cells, we were able to demonstrate the subcellular localization of this enzyme in endothelial cells. Western blot analysis with anti-GTPCH I antibody was performed to determine the localization of GTPCH I in gradient fractions. Please note that GTPCH I was fractionated to the low buoyant density fraction in wild-type mice (top), and this was almost eliminated in fraction 2 of Cav1⁻/⁻ mice (bottom). Effect of cholesterol depletion on BH₄ levels. Isolated mouse aortas of wild-type (WT; B6129SF2/J) and Cav1⁻/⁻ mice were exposed in vitro with β-CD (10 mmol/L) for 1 hour at 37°C, and BH₄ levels were then determined. Bar graph showing BH₄ levels after treatment without or with β-CD. Results are means±SEMs (n=6). *P<0.05 vs untreated wild-type mice.

Figure 5. A. Density gradient fractions of lung lysates from strain-matched wild-type (B6129SF2/J) and Cav1⁻/⁻ mice were performed that separate caveolar microdomains from other cellular constituents. Western blot analysis with anti-GTPCH I antibody was performed to determine the localization of GTPCH I in gradient fractions. Please note that GTPCH I was fractionated to the low buoyant density fraction in wild-type mice (top), and this was almost eliminated in fraction 2 of Cav1⁻/⁻ mice (bottom). B. Effect of cholesterol depletion on BH₄ levels. Isolated mouse aortas of wild-type (WT; B6129SF2/J) and Cav1⁻/⁻ mice were exposed in vitro with β-CD (10 mmol/L) for 1 hour at 37°C, and BH₄ levels were then determined. Bar graph showing BH₄ levels after treatment without or with β-CD. Results are means±SEMs (n=6). *P<0.05 vs untreated wild-type mice.

Figure 6. Enzymatic activity of GTPCH I in caveolar (A) and in noncaveolar (B) membranes after density gradient fractions of lung lysates from wild-type (C57BL/6J) and GTPCH I transgenic (GTPCH-tg) mice. Results are means±SEMs (n=3). *P<0.05 vs wild-type mice.

aortas obtained from wild-type and Cav1⁻/⁻ mice. These observations are consistent with our conclusion regarding the caveolar localization of GTPCH I. Whether trafficking²⁸ of GTPCH I or BH₄ biosynthesis occurs in caveolae remains to be determined in future studies.

Because caveolin-1 has been reported to be an important regulator of eNOS activity²⁹ and eNOS activity is enhanced in Cav1⁻/⁻ mice,²³,³⁰ we determined basal GTPCH I activity in the aortas of Cav1⁻/⁻ mice. Surprisingly, we found that there was a 2-fold increase in GTPCH I activity in aortas of Cav1⁻/⁻ mice when compared with wild-type mice. We also observed a selective increase in BH₄ levels from the aorta of Cav1⁻/⁻ mice, suggesting that this increase was caused by the increased de novo biosynthesis of BH₄ via GTPCH I. In contrast, GTPCH I activity was unchanged in the liver or brain of Cav1⁻/⁻ mice when compared with wild-type controls, suggesting that caveolin-1 may exert an inhibitory effect on basal GTPCH I activity in vivo, in an organ-specific manner. Alternatively, this observation could also be explained by differential regulation of BH₄ biosynthesis between large conduit and small resistance arteries. Indeed, it is generally accepted that NO (and possibly BH₄) plays more prominent functional role in the control of large conduit arteries.³¹ The inhibitory effect of caveolin-1 on GTPCH I activity was further demonstrated by the fact that transduction of HUVECs with Ad-Cav1 resulted in significant suppression of GTPCH I activity. On the other hand, treatment with the cholesterol-binding drug β-CD, which prevents formation of functional caveolae by depletion of cholesterol,³² increased BH₄ biosynthesis in the aorta of wild-type mice. Of note, β-CD treatment did not further increase BH₄ levels in the aorta of Cav1⁻/⁻ mice, reinforcing our conclusion that caveolin-1 has a negative regulatory effect on the enzymatic activity of GTPCH I.

Over the past decade, studies from several groups have reported the beneficial effects of increasing endothelial BH₄ levels in various models of vascular disease. Indeed, a number of investigations have demonstrated that supplementation with BH₄ can prevent endothelial dysfunction. Accordingly, acute and chronic supplementation of BH₄ in experimental models of oxidative stress and in patients with cardiovascular disease improved endothelium-dependent relaxations and increased eNOS activity.⁵,³³–³⁶ In the present
study, GTPCH I enzymatic activity was increased in the low buoyant density membrane fraction of transgenic mice with endothelial-targeted overexpression of GTPCH I. This observation is important, because several recent studies demonstrated that endothelial overexpression of GTPCH I reduces superoxide anion production and preserves NO release, suggesting that endothelial dysfunction can be restored by increasing the local concentration of BH$_4$. Relevant to interpretation of our results, previous studies have demonstrated that caveolar microdomains are sensitive to oxidative and nitrosative stress. Whether GTPCH I localization in the caveolar membrane is critical for the protection of caveolae against oxidative stress in vivo remains to be determined.

**Perspectives**

Results of the present study have several important implications for the understanding of vascular endothelial function. In addition to the well-established role of BH$_4$ in the activity of eNOS, our findings underscore the importance of cellular localization of GTPCH I, a critical enzyme responsible for the biosynthesis of BH$_4$. Colocalization of GTPCH I and eNOS in caveolae is most likely designed to provide the optimal local concentration of BH$_4$ required for the biosynthesis of endothelial NO. Because elevated concentration of superoxide anion and subsequent formation of peroxynitrite are some of the most important mechanisms underlying endothelial dysfunction, the relevance of BH$_4$ in the preservation of caveolar architecture and function should be investigated in the future studies.

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**Disclosures**

None.

**References**


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