

In vivo expression and function of recombinant GTPCH I in the rabbit carotid artery

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Hynes, Sean O., Leslie A. Smith, Darcy M. Richardson, Imre Kovcsdi, Timothy O'Brien, and Zvonimir S. Katusic. In vivo expression and function of recombinant GTPCH I in the rabbit carotid artery. *Am J Physiol Heart Circ Physiol* 286: H570–H574, 2004. First published October 9, 2003; 10.1152/ajpheart.00669.2003.—Tetrahydrobiopterin (BH4) is an essential co-factor for endothelial nitric oxide synthase enzymatic activity. GTP cyclohydrolase I (GTPCH I) is the rate-limiting enzyme in BH4 synthesis. This study set out to test the hypothesis that in vivo gene transfer of GTPCH I to endothelial cells could increase bioavailability of BH4, enhance biosynthesis of nitric oxide and thereby enhance endothelium-dependent relaxations mediated by nitric oxide. In vivo gene transfer was carried out by adenovirus (Ad)-mediated delivery into rabbit carotid arteries. Each artery was transduced by 20-min intraluminal incubation of 10⁹ plaque-forming units of Ad-encoding GTPCH I (AdGTPCH) or β -galactosidase as a control. The rabbits were euthanized 72 h later, and vasomotor function of isolated arteries was assessed by isometric force recording. GTPCH I enzymatic activity, BH4, and oxidized biopterin levels were detected with the use of HPLC, and cGMP was measured with the use of radioimmunoassay. Expression of recombinant proteins was detected predominantly in endothelial cells. Both GTPCH I activity and BH4 levels were increased in arteries transduced with AdGTPCH. However, contraction to phenylephrine (10⁻⁵ to 10⁻⁹ M), endothelium-dependent relaxation to acetylcholine (10⁻⁵ to 10⁻⁹ M) and cGMP levels were not significantly affected by increased expression of GTPCH I. Our results suggest that expression of GTPCH I in vascular endothelium in vivo increases intracellular concentration of BH4. However, under physiological conditions, it appears that this increase does not affect nitric oxide production in endothelial cells of the carotid artery.

nitric oxide synthase; tetrahydrobiopterin; adenovirus

NITRIC OXIDE (NO) is a potent vasodilator and an important regulator of vascular homeostasis (4, 8, 16). Endothelial cells produce NO by the constitutively expressed enzyme endothelial NO synthase (eNOS) (5, 6). Tetrahydrobiopterin (BH4) is an essential cofactor required for optimal enzymatic activity of NOS and in particular eNOS. Although the exact mechanisms underlying the effects of BH4 on NO synthesis are not completely understood, existing evidence suggests that BH4 can function as both an allosteric and redox cofactor (2, 7, 15, 19, 23). Numerous studies have suggested that reduced availability of BH4 could contribute to pathogenesis of endothelial dysfunction, and these are reviewed elsewhere (9).

BH4 is produced from GTP in a series of enzymatic steps (7). The rate-limiting enzyme in this process is GTP cyclohydrolase I (GTPCH I). It appears that in cultured endothelial cells, intracellular concentrations of BH4 are subsaturating for eNOS enzymatic activity (20, 21). On the basis of this premise, it would appear that eNOS activity could be augmented through increased expression of GTPCH I. The direct delivery of sepiaterin, a precursor of biopterin, has proven beneficial in cases of endothelial dysfunction potentially acting through this mechanism (22). A recent study by Cai and colleagues (2) demonstrated that gene transfer of GTPCH I could be used to enhance BH4 production and increase eNOS activity in cultured endothelium. In the present study, we hypothesized that in vivo overexpression of GTPCH I in endothelial cells could increase bioavailability of BH4, stimulate biosynthesis of NO, and thereby enhance endothelium-dependent relaxations mediated by NO.

METHODS

Reagents. The following pharmacological agents were used: phenylephrine, acetylcholine, indomethacin, and papaverine hydrochloride (Sigma; St. Louis, MO). Reagents were dissolved in distilled water, and volumes of <0.15 ml were added to the organ chambers. Concentrations of all drugs are expressed as the final moles per liter concentration in distilled water. A modified Krebs solution was used in the present study with the following composition (in mM): 1.2 KH₂PO₄, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 25 NaHCO₃, 118.3 NaCl, and 11.1 dextrose. Protein levels throughout the study were determined with the use of a DC protein assay kit (Bio-Rad; Hercules, CA).

Viral vectors for GTPCH I and reporter gene. A recombinant adenovirus (Ad)-encoding human GTPCH I gene (AdGTPCH) driven by a cytomegalovirus promoter was generated as previously described (10). A recombinant adenoviral vector encoding the *Escherichia coli*- β galactosidase gene (AdLacZ) driven by the cytomegalovirus promoter was used as a control. The virus was purified with double cesium chloride gradient ultracentrifugation and was dialyzed against 10 mM Tris, 1.0 mM MgCl₂, 1.0 mM HEPES, and 10% glycerol for 4 h at 4°C. Viral titer was determined by plaque assay. Viral stocks were stored at -80°C.

Gene transfer into experimental animals. Two- to three-month-old male New Zealand White rabbits (2–3 kg) were obtained from Harlan (Indianapolis, IN). Housing facilities and all experimental protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic and are described elsewhere (11, 24).

Rabbits were anesthetized with ketamine (35 mg/kg), xylazine (5 mg/kg), and acepromazine (2.3 mg/kg) before surgery. A midline neck incision was made and the common carotid arteries were ex-

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posed. The arterial branches were cauterized, and heparin (100 U/kg) was administered intravenously. The artery was clamped, and after an arteriotomy was performed, an intraarterial catheter (24 GA) was introduced. An adenoviral load of 10^9 plaque-forming units (pfu) in 100 μ l of sterile PBS was then injected, the arteriotomy was sutured, and the virus was allowed to incubate in situ for 20 min. The clamps were then removed and the wound sutured. The rabbits were euthanized 72 h later, and tissues were harvested.

Analysis of vasomotor function. Isolated carotid arterial rings (3–5 mm) were connected to a force transducer for recording of isometric force and placed in organ baths filled with 25 ml of Krebs solution at 37°C (94% O₂-6% CO₂; pH 7.4). Isometric force was recorded continuously, and the rings were allowed to stabilize at 0.2 to 0.4 g for 1 h. Each ring was then gradually stretched to 3.0 g. All experiments were carried out in the presence of 10^{-5} M indomethacin to eliminate the influence of endogenous cyclooxygenase. The rings were incubated with indomethacin for 30 min before exposure to other reagents. Contractions to phenylephrine (10^{-5} to 10^{-9} M) were examined before exposure to acetylcholine. The relaxation responses to acetylcholine (10^{-5} to 10^{-9} M) were studied during contractions to a submaximal concentration of phenylephrine. Papaverine (3×10^{-4} M) was used to induce complete relaxation of the vessels.

Histochemical analysis. Expression of the β -galactosidase reporter gene was determined with the use of BluoGal and histochemical staining. After BluoGal staining, the arterial rings were frozen in optimum cutting temperature compound (Sakura; Torrance, CA). Five micrometer cross sections were cut onto slides. The sections were then counterstained with neutral red (11).

HPLC analyses. BH4 levels in carotid arteries were determined after differential oxidation in acid and base conditions by reverse-phase HPLC (3). GTPCH I activity was assessed as a function of neopterin production under standard conditions with GTP as a substrate. Results were normalized against tissue protein levels.

cGMP. Radioimmunoassay kits (Amersham; Buckinghamshire, UK) were used to perform the measurements. Rings were initially incubated in minimum essential medium supplemented with 0.1% bovine serum, 24 U penicillin, and 24 μ g streptomycin in a 5% CO₂ incubator for 30 min and were then incubated a further 30 min in minimum essential medium with 3-isobutyl-1-methylxanthine (10^{-4} M) to inhibit degradation of cyclic nucleotides by phosphodiesterases. The rings were then removed from the medium and immediately snap frozen with liquid N₂. After homogenization, cGMP levels were assessed with the use of a cGMP radioimmunoassay kit and normalized against tissue protein levels.

Statistical analyses. Results are represented as means \pm SE. Statistical analysis was performed by two-way repeated-measures ANOVA with a Bonferroni *t*-test to detect differences in multiple comparisons and by an unpaired *t*-test for comparisons between two groups or Mann-Whitney test where appropriate to the data. The tests applied are as stated in the figure legends. A *P* < 0.05 was considered significant.

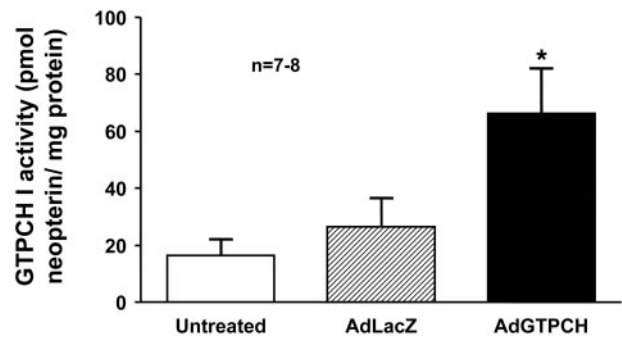


Fig. 2. GTPCH I activity levels in rabbit carotid arteries determined as a function of neopterin production are shown after transduction with 10^9 plaque-forming units (pfu) AdGTPCH. Levels of GTPCH I increased over twofold in AdGTPCH-treated arteries compared with controls. Results are shown as means \pm SE (*n* = 7 for untreated controls, *n* = 8 for both AdLacZ or AdGTPCH-treated arteries). **P* < 0.05.

RESULTS

Intraluminal delivery of recombinant proteins. Expression of the β -galactosidase reporter gene in AdLacZ-transduced arteries was confirmed with the use of histochemical analysis. Vessels from rabbits treated with 10^9 pfu of AdLacZ and harvested 3 days later showed that β -galactosidase activity was localized in the endothelial cells (Fig. 1A). In contrast, no staining was noted in the AdGTPCH-treated arteries (Fig. 1B).

Effect of recombinant proteins on GTPCH I activity and BH4. In AdGTPCH-transduced carotid arteries, basal levels of GTPCH I activity were significantly increased by more than twofold compared with both AdLacZ and untreated controls (*P* < 0.05; Fig. 2). In addition, levels of total biopterins (32.22 ± 7.6 vs. 11.79 ± 1.9 pmol/mg protein; *P* < 0.05) and BH4 (*P* < 0.05; Fig. 3A) were significantly increased by almost threefold in AdGTPCH-transduced arteries compared with AdLacZ-transduced arteries. Despite no significant difference noted in oxidized biopterin levels between the two treatment groups and untreated controls, a trend toward a significant increase was noted for AdGTPCH-transduced arteries (*P* < 0.1) (Fig. 3B). BH2, an oxidized form of BH4 can be an important factor in determining eNOS activity, because it may compete for the same binding site as BH4 in the eNOS molecule but will result in an inactive enzyme. Moreover, BH4, determined as a percentage of total biopterin (Fig. 3C), did not change significantly after AdGTPCH delivery. Collectively, these results demonstrate the successful expression and

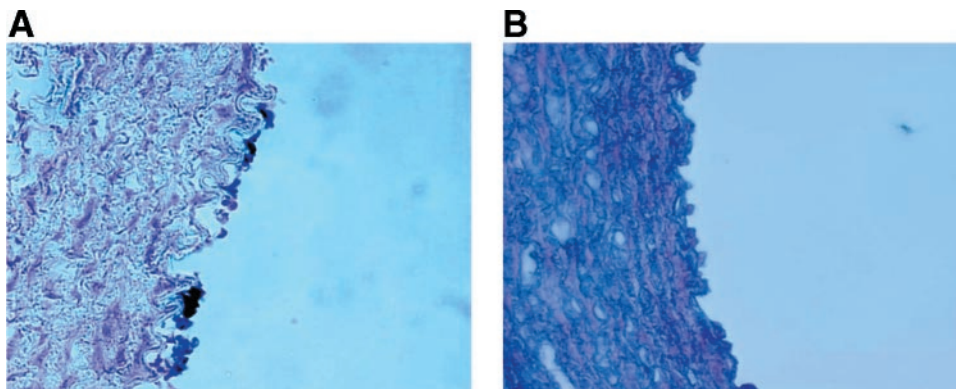
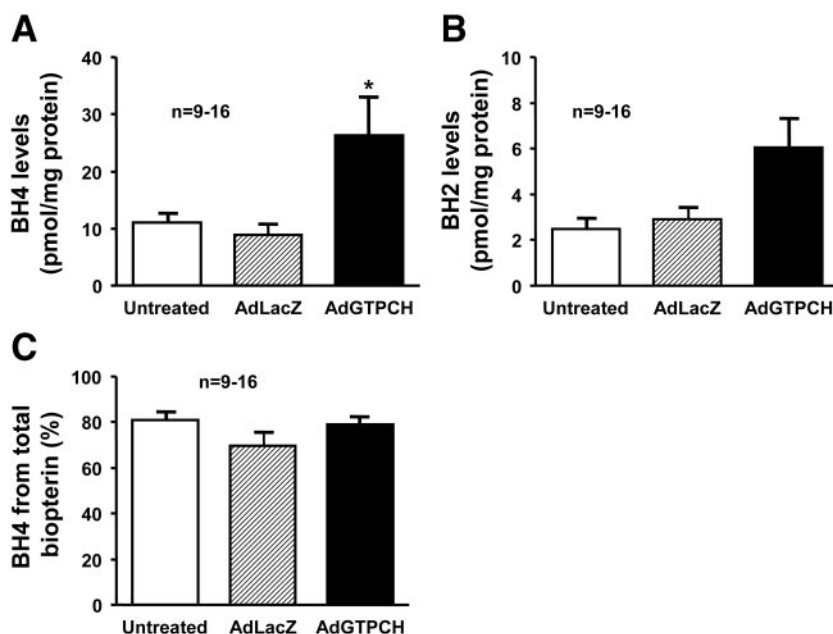


Fig. 1. Histological localization of β -galactosidase reporter gene expression in rabbit carotid arteries after exposure to adenovirus (Ad) vector encoding the *Escherichia coli*- β -galactosidase gene (AdLacZ) (A) and in control (B) arteries exposed to AdGTP cyclohydrolase (AdGTPCH). Blue staining demonstrates endothelium-specific gene transfer of β -galactosidase. Magnification, $\times 40$.

Fig. 3. The effect of GTPCH I gene delivery on bipterin levels in the rabbit carotid artery. Bar graphs show tetrahydrobiopterin (BH4) levels (A), oxidized biopterin levels (B), and BH4 (C) as a percentage of total biopterin in rabbit carotid arteries after transduction with 10^9 pfu AdGTPCH. Levels of BH4 increased over twofold compared with controls. Results are shown as means \pm SE ($n = 9$ for untreated controls, $n = 16$ for AdLacZ and AdGTPCH). * $P < 0.05$.



function of recombinant GTPCH I to increase BH4 production in vivo.

Effect of recombinant proteins on vasomotor function. Maximal contractions and sensitivity to phenylephrine were similar in arteries after delivery of AdGTPCH ($n = 9$ rings, 4 animals) or AdLacZ ($n = 7$ rings, 4 animals) (5.2 ± 0.50 vs. 6.6 ± 0.63 g; $P > 0.05$) (Fig. 4A). Furthermore, during submaximal contraction to phenylephrine, endothelium-dependent relaxations to acetylcholine (10^{-9} to 10^{-5} M) were not significantly affected in AdGTPCH-transduced carotid arteries compared with either AdLacZ-transduced arteries or untreated controls (Fig. 4B). The results from these functional assays suggest that eNOS activity was not affected by transduction of AdGTPCH because the relaxations induced using acetylcholine preceded by indomethacin are mediated by NO.

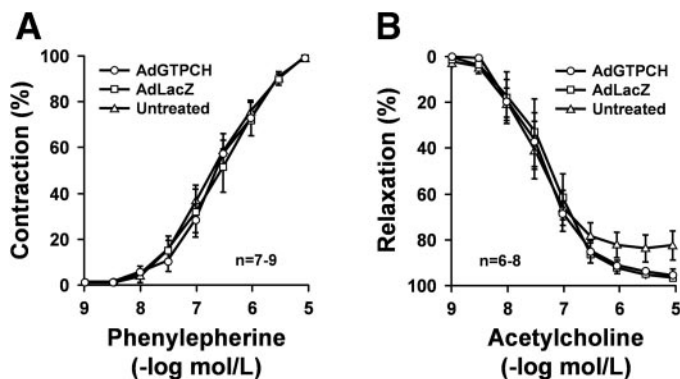


Fig. 4. A: concentration response curves to phenylephrine treatment. Data are shown as means \pm SE and are expressed as percentage of maximal contraction induced by phenylephrine (10^{-5} M; 100% = 5.69 ± 0.33 , 6.64 ± 0.63 , and 5.2 ± 0.50 g for untreated, AdLacZ, and AdGTPCH, respectively). B: concentration response curves for acetylcholine. Values are shown as means \pm SE and are expressed as the percentage of the maximal relaxation for papaverine (3×10^{-4} M; 100% = 3.75 ± 0.20 , 3.60 ± 0.34 , and 3.33 ± 0.29 g for untreated, AdLacZ, and AdGTPCH, respectively). $P > 0.05$ for repeated-measures two-way ANOVA for both sets of curves.

Effects of recombinant proteins on intracellular cGMP levels. In AdGTPCH-transduced arteries, mean basal levels of cGMP did not significantly differ from arteries transduced with AdLacZ ($n = 11$; Fig. 5), further indicating that eNOS activity did not increase in AdGTPCH-transduced arteries.

DISCUSSION

This is the first study to characterize the in vivo expression and function of recombinant GTPCH I in vascular endothelial cells. We report several novel findings. Intraluminal delivery of adenovirus-encoding GTPCH I into carotid artery resulted in increased enzymatic activity of GTPCH I, and a significantly higher intracellular level of BH4. In contrast, expression of the recombinant reporter gene β -galactosidase did not affect enzymatic activity of GTPCH I or BH4 level, ruling out the possibility that a replication-incompetent adenovirus may affect vascular expression and function of GTPCH I. Interestingly, the ratio between BH4 and oxidized biopterin remained the same in control, AdLacZ, and AdGTPCH-transduced arteries. This finding indicates that adenoviral-mediated gene delivery into endothelial cells does not affect BH4 metabolism

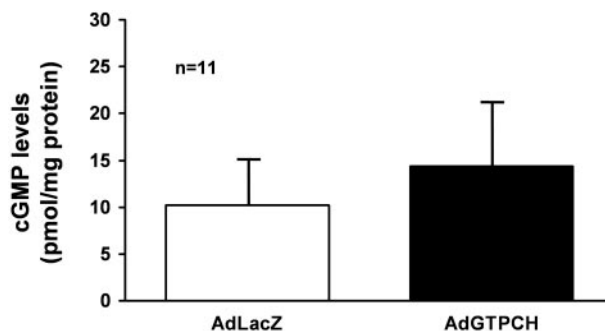


Fig. 5. Bar graph showing cGMP levels in rabbit carotid arteries after exposure to 10^9 pfu. No significant differences were noted between either AdGTPCH- or AdLacZ-treated arteries with the use of a t -test. Results are shown as means \pm SE ($n = 11$; $P > 0.05$).

or oxidation. Our study also provides evidence that in the carotid artery, endothelium-dependent relaxations mediated by NO are not affected by increased availability of BH4. Although indirect, this evidence refutes our hypothesis that subsaturating levels of BH4 for eNOS enzymatic activity are present in endothelium under physiological conditions.

In several previous studies (11, 24, 26), we demonstrated that intraluminal delivery of an adenovirus in the rabbit carotid artery resulted in expression of recombinant proteins in endothelial cells. Consistent with these findings, we detected expression of β -galactosidase in the endothelium (Fig. 1). We used a viral titer of 10^9 pfu per artery for both the adenovirus encoding the reporter gene and GTPCH I. This titer does not have a cytotoxic effect on endothelial cells, as illustrated by the fact that endothelium-dependent relaxations to acetylcholine were not affected in arteries transduced with β -galactosidase. Furthermore, contractions to phenylephrine were also not affected in arteries treated with 10^9 pfu of adenovirus, reinforcing our conclusion that in rabbit carotid artery, this viral titer is not cytotoxic. BH4 is one of the most potent naturally occurring reducing substances and may be oxidized with peroxynitrite (12, 14, 17). In vivo oxidation of BH4 has been proposed as a possible mechanism underlying the uncoupling of eNOS and endothelial dysfunction in experimental models of hypertension and hypercholesterolemia (13, 14). It is interesting and important to note that we did not detect any increase in BH4 oxidation in arteries exposed to AdLacZ. The lack of BH4 oxidation is further evidence to suggest that adenovirus (10^9 pfu) did not have a cytotoxic effect on endothelial cells. However, we provide evidence that 10^9 pfu of adenovirus-encoding GTPCH I is sufficient to increase local production of BH4 in the vascular wall. We observed an approximately two- to threefold increase in both GTPCH I enzymatic and biosynthesis of BH4 in arteries transduced with AdGTPCH.

In contrast to our findings, previous studies using cultured endothelial cells demonstrated that overexpression of GTPCH I resulted in an increased intracellular BH4 level, along with a subsequent increase in NO production (2). The stabilization of functional eNOS dimers by BH4 was demonstrated and suggested as a potential mechanism of increased NO biosynthesis. The discrepancy between in vitro and in vivo results is most likely because expression and enzymatic activity of GTPCH I are both very low (in some studies undetectable) in cultured endothelial cells. Indeed, a study by Rosenkranz-Weiss (21) demonstrated that BH4 levels are significantly higher in freshly isolated endothelial cells compared with cultured endothelium. The reason for reduction of GTPCH I expression and activity observed during culturing of endothelial cells is unclear. Nevertheless, this observation may explain the beneficial effect of BH4 supplementation in cultured endothelium (1, 2, 21).

In the present study, despite a successful expression of recombinant GTPCH I and subsequent increase in BH4 bioavailability, we did not observe any augmentation of endothelium-dependent relaxations mediated by the release of NO. Consistent with this observation, levels of cGMP, a secondary messenger for NO, did not change in arteries expressing recombinant GTPCH I. We did not directly measure NO and enzymatic activity of NOS; however, endothelium-dependent relaxations to acetylcholine and cGMP levels have traditionally served as very good indexes of NO production (25, 27). Our

findings suggest that in the rabbit carotid artery, eNOS is saturated with BH4 under physiological conditions.

In conclusion, the results of this study demonstrate that expression of recombinant GTPCH I in rabbit carotid artery results in increased enzymatic activity of GTPCH I and biosynthesis of BH4. Our findings suggest that in the vascular endothelium eNOS is saturated with BH4 under physiological conditions. In a similar manner, it has been demonstrated previously that L-arginine, the substrate for eNOS activity, may also prove saturating for recombinant eNOS activity (18). The relatively high basal levels of BH4, lack of viral toxicity, and an in vivo setting suggest that manipulation of vascular endothelium with adenovirus-mediated gene transfer may provide a useful model in studies designed to characterize mechanisms responsible for metabolism of BH4. Our results provide the basis for future studies designed to characterize the effects of GTPCH I gene transfer on endothelial dysfunction.

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DISCLOSURES

I. Kovesdi owns equity in GenVec, Inc., Gaithersburg, MD.

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