Endothelial Dysfunction of Coronary Resistance Arteries Is Improved by Tetrahydrobiopterin in Atherosclerosis

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Background—Tetrahydrobiopterin (BH4), an essential cofactor for the synthesis of NO, improves endothelial dysfunction after ischemia/reperfusion. Therefore, we hypothesized that reduction of BH4 is involved in the attenuation of endothelium-dependent vasodilation in atherosclerosis, and we investigated the effect of alterations of the BH4 level on the vasodilatory potential of coronary resistance vessels from humans and pigs with atherosclerosis.

Methods and Results—Coronary arterioles were obtained from patients undergoing CABG (atherosclerosis group) or valve replacement (control group) and from pigs fed either a standard diet (control group) or atherogenic diet (atherosclerosis group). After isolation, vessels were cannulated, pressurized, and placed on the stage of an inverted microscope. Dose-response curves were investigated in response to the endothelium-dependent agonists histamine, serotonin, and acetylcholine (for pigs, substance P) and to the endothelium-independent agonist sodium nitroprusside (SNP) under control conditions and before and after incubation of the vessels with sepiapterin (substrate for BH4 synthesis). In vessels from patients and from animals with atherosclerosis, compared with vessels from the control groups, there was a significant (P < 0.05) reduction of vasodilation to all tested endothelium-dependent agonists but not to SNP. After application of sepiapterin, the responses to the endothelium-dependent agonists but not to SNP were significantly improved in vessels from the atherosclerosis groups. Sepiapterin did not influence vascular reactivity in the control groups.

Conclusions—Atherosclerosis severely compromises endothelial function of coronary resistance arteries. Administration of sepiapterin leads to a significant improvement of endothelium-dependent vasodilatation to different agonists in vessels from humans and pigs with atherosclerosis. Therefore, we conclude that a reduced availability of BH4 is involved in the development of endothelial dysfunction in atherosclerosis. (Circulation. 2000;102:2172-2179.)

Key Words: nitric oxide • tetrahydrobiopterin • vessels • atherosclerosis

Endothelial cells influence vascular tone by synthesis and release of a number of vasoactive substances. Among endothelium-derived vasorelaxing factors, NO has been shown to be of unequivocal importance in the regulation of coronary blood flow.1,2 In the presence of coronary risk factors and early in the development of atherosclerosis, there are functional alterations in the coronary microcirculation leading to an increase of coronary resistance.3–5 These functional alterations are generally described as endothelial dysfunction, characterized by an impaired vasodilation to endothelium-dependent agonists and caused by a reduced availability of NO.3,5–7 The underlying reason for the reduction of NO under these conditions has not yet been clarified. A lack of L-arginine, the substrate of NO synthesis, and impaired expression of NO oxide synthase have been discussed as possible reasons, but the experimental findings are controversial.8–10 In addition, atherosclerosis-induced increased production of oxygen-derived free radicals, such as superoxide anions, which easily react with NO and decrease the half-life of NO, may play a role.11 Recent investigations demonstrated a close relationship between the availability of the NO synthase cofactor, tetrahydrobiopterin (BH4), and NO synthesis in both endothelial and vascular smooth muscle cells.12,13 In endothelial cells, tetrahydrobiopterin is synthesized from GTP via a de novo pathway by the rate-limiting enzyme GTP-cyclohydrolase I. Alternatively, the synthesis of tetrahydrobiopterin can occur via a so-called salvage pathway with sepiapterin as a substrate. The precise role of tetrahydrobiopterin in the synthesis of NO has not been completely understood, but it is likely acting as both an allosteric and a redox cofactor in the formation of NO.14,15 In addition, tetrahydrobiopterin stabilizes NO synthase,16 and under in vitro conditions, lack of tetrahydrobiopterin causes NO synthase to generate superoxide anions instead of NO.17,18 Substitution of sepiapterin, the endogenous substrate of tetrahydrobiopterin synthesis via the salvage pathway, im-

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proves endothelial function in coronary resistance arteries after ischemia/reperfusion,19 and substitution of tetrahydrobipterin increases endothelium-dependent vasodilation in the brachial artery of individuals with hypercholesterolemia.20 On the basis of these observations, it seems likely that a reduced availability of tetrahydrobiopterin leading to an imbalance of the intracellular levels of NO on one side and toxic radicals on the other side is involved in the development of endothelial dysfunction in atherosclerosis. Therefore, we hypothesized that substitution of tetrahydrobiopterin will improve the endothelial dysfunction of atherosclerotic coronary arterioles. To test this hypothesis, we compared the responses of coronary arterioles from humans with and without atherosclerosis with various endothelium-dependent and endothelium-independent agonists before and after application of sepiapterin. Because of the difficulty of investigating true control vessels in humans, we additionally tested the influence of sepiapterin on the vasodilatory responses of vessels obtained from pigs fed either a standard diet or a cholesterol-rich diet for 4 months.

Methods

General Preparation

Animal Experiments

Göttinger minipigs (Fa Ellegaard; Dalmose, Denmark) were, starting at the age of 6 weeks, placed into 2 groups and fed either a standard diet (SDS) or a cholesterol-enriched atherogenic diet containing SDS plus cholesterol and egg yolk powder for 4 months. On the day of euthanasia, the pigs were sedated with azaperone (Stresnil 227, 4 to 8 mg/kg IM), anesthetized with 10% ketamine (10 mg/kg IM and then 15 mg · kg⁻¹ · h⁻¹ IV) and midazolam (Dormicum 227, 15 mg and then 0.1 mg/kg IV), intubated, and ventilated with room air. After a left thoracotomy, the pericardium was incised, and the heart was exposed. Heparin was administered (1000 IU/kg IV), and the heart was removed and placed immediately in cold (4°C) saline. From all hearts, segments of right coronary artery and small pieces of myocardium (for examination of small arteries and arterioles) were fixed in a glutaraldehyde-formaldehyde solution and embedded in methacrylate for morphological examination by light microscopy.

Human Tissue

Right atrial appendages were obtained from patients receiving CABG, valve replacement, or repair of cardiac defects. Before surgery, the presence of either severe 3-vessel disease (atherosclerosis group) or no significant coronary artery disease (control group) had been determined by coronary angiography. Immediately after removal, the tissue was placed in cold (4°C) saline.

In Vitro Measurements of Coronary Arteriolar Diameters

Coronary arterioles <100 μm in diameter were dissected carefully from the myocardial tissue at 4°C and were transferred to an acrylic resin vessel chamber containing physiological salt solution and albumin at pH 7.4. Both ends of each arteriole were cannulated with a glass micropipette with an external tip of ~40 μm and secured with 11-0 ophthalmic suture. Blood in the vessels was flushed out at low pressure (20 cm H₂O), and the other end of the microvessel was secured to a second pipette.

After the vessels were cannulated, the chamber was transferred to the stage of an inverted microscope (IM35, Carl Zeiss; objective×40, numerical aperture 0.75), which was fitted with a Cohu TV camera and video micrometer (Texas A&M Microcirculation Research Institute). Arterioles were pressurized to 60 cm H₂O by adjusting the height of a reservoir connected to each micropipette. By setting both reservoirs to the same height, the vessels were
pressurized without flow. Leaks were detected by closing off the system to the reservoirs and examining for a decline in intraluminal pressure. Vessels with leaks were excluded from further study. Internal diameters were recorded continuously during steady-state conditions after each intervention. The microvessels were set to their in situ length and were bathed in physiological salt solution and albumin with the temperature maintained at 36°C to 37°C by use of an external heat exchanger. All of the arterioles developed spontaneous tone of 10% to 20% of maximal diameter and were further preconstricted with endothelin (10^{-2} to 10^{-9} mol/L) to 25% to 30% of maximal diameter.

Isolated Microvessel Protocol
After the arterioles had been allowed to equilibrate in the bath and were preconstricted, dose-response curves to the endothelium-independent vasodilator sodium nitroprusside (10^{-9} to 10^{-3} mol/L) and to the endothelium-dependent vasodilators histamine (10^{-9} to 10^{-5} mol/L), serotonin (10^{-9} to 10^{-5} mol/L), and acetylcholine (10^{-9} to 10^{-5} mol/L) were measured during steady-state conditions. In pigs, substance P (10^{-10} to 10^{-6} mol/L) was used instead of acetylcholine. Thereafter, the microvessels were incubated with sepiapterin (1 μmol/L) before and during repetition of the dose-response measurements with all drugs.

Sepiapterin (Research Biochemical Intl) was dissolved in dimethyl sulfoxide. Sepiapterin stock solution (2 mL) was added to the bath to obtain a final concentration of 1 μmol/L. Administration of 2 μmol/L dimethyl sulfoxide alone to the bath had no vasoactive effect. The dose of sepiapterin was chosen on the basis of data from the literature and our previous experiments.13,17,19,20 Because there was usually a small nonsignificant vasodilation immediately after the application of sepiapterin, we incubated the vessels with sepiapterin for 15 minutes, thereby allowing them to reequilibrate before we repeated the dose-response studies.

Data Analysis
Measurements of microvascular diameters during interventions were expressed as percent dilation, with 100% representing the maximal baseline diameter before vessels were pressurized from the diameter elicited after preconstriction with endothelin-1. Statistical comparisons of dose-response curves to different interventions and baseline diameters before each intervention were made by use of 2-way ANOVA with repeated measures, followed by the Bonferroni test to detect individual differences. All statistics were computed with the use of Statview 4.1 on a Macintosh 8100 computer. A probability level of 95% was used in all studies as the criterion of statistical significance. All data were described as mean±SEM.

Results
Animal Experiments
Basic Data
There was a significant (P<0.05) difference in total cholesterol levels between atherosclerotic and control pigs (atherosclerotic pigs, total cholesterol 93.3 mg/dL and triglycerides 43.5 mg/dL; control pigs, total cholesterol 56.3 mg/dL and triglycerides 38.8 mg/dL).

Histological examination of coronary artery sections and samples of myocardial tissue from all animals by light microscopy showed no significant difference between the 2 groups. However, there was the development of some tissue fibrosis in the group treated with the cholesterol-rich diet.

Pharmacological Studies
In vessels from animals treated with the standard diet (control group, n=7), there was near maximal vasodilation to all tested agonists. After application of sepiapterin, there was no alteration of the effects of the different agonists (Figure 1).

In vessels from animals treated with the cholesterol-rich diet (atherosclerotic group, n=9), vasodilation to histamine and substance P was significantly reduced, and serotonin caused vasoconstriction instead of vasodilation, whereas the
The effect of sodium nitroprusside was unaltered compared with the control condition (57±9%, 65±8%, −100±10%, and 92±8% relaxation for histamine, substance P, serotonin, and sodium nitroprusside, respectively; Figure 2). After application of sepiapterin, the vasodilatory effects of histamine and substance P were significantly improved, and vasoconstriction to serotonin was significantly attenuated. Sodium nitroprusside induced maximal vasodilation without a significant difference to the baseline value (93±9%, 100±10%, −62±18%, and 100±8% relaxation for histamine, substance P, serotonin, and sodium nitroprusside, respectively; Figure 2).

Experiments in Human Vessels

Basic Data

In total, 16 vessels were investigated. Ten vessels were obtained from right atrial appendages from patients receiving CABG surgery for severe coronary atherosclerosis. Six vessels were obtained from patients receiving surgery for mitral valve replacement (3 patients), aortic valve replacement (2 patients), or ventricular septum repair (1 patient). The latter group of patients was considered the “control” group because by preoperative coronary angiography, the presence of significant coronary artery disease (stenoses >50% in diameter) had been excluded. In addition, in the control group, the incidence of coronary risk factors was very low (no patient with hypercholesterolemia, diabetes, or nicotine abuse and 2 patients with hypertension).

Pharmacological Studies

In vessels from patients without atherosclerosis (control group), there was near maximal vasodilation to sodium nitroprusside and histamine (101±2% and 98±6% relaxation, respectively). Serotonin caused some vasodilation (22±6%), and acetylcholine caused predominantly vasoconstriction (−53±30%). After application of sepiapterin, there was no alteration of the responses to the different agonists (Figure 3).

In vessels from humans with coronary artery disease, there was a significant reduction of vasodilatation to histamine and serotonin and increased vasoconstriction to acetylcholine. The effect of sodium nitroprusside was unaltered in vessels from patients with coronary artery disease compared with vessels from patients without significant atherosclerosis (68±11%, 4±23%, −86±20%, and 99±2% relaxation for histamine, serotonin, acetylcholine, and sodium nitroprusside, respectively; Figure 4). After application of sepiapterin, the effects of histamine and acetylcholine were significantly improved, and the response to serotonin was increased; however, most likely because of the large standard error, the improvement was not statistically significant compared with baseline values. Sepiapterin did not influence vasodilation to sodium nitroprusside (100±6%, 28±23%, −39±21%, and 100±3% relaxation for histamine, serotonin, acetylcholine, and sodium nitroprusside, respectively; Figure 4).

Discussion

Summary

The results of the present study demonstrate that in coronary resistance arteries from humans and pigs, atherosclerosis specifically induces an impairment of endothelial function, as shown by an attenuated response of the vessels to different endothelium-dependent agonists. Atherosclerosis does not induce alteration of smooth muscle cell function, demonstrated by an unaltered effect of an endothelium-independent
agonist. These findings have previously been reported in several investigations³,⁵ and have in the present study been extended to isolated human vessels. The very new finding of our investigation is that atherosclerosis-induced endothelial dysfunction can be significantly improved via an increase of tetrahydrobiopterin levels through exogenous application of sepiapterin, a substrate for tetrahydrobiopterin synthesis. Incubation with sepiapterin significantly increased vasodilation to histamine and substance P and decreased constriction to serotonin in porcine vessels. In human vessels, incubation with sepiapterin significantly increased the vasodilation to histamine and decreased the constriction to acetylcholine. The effect of sodium nitroprusside, an endothelium-independent vasodilator, was not influenced by sepiapterin in either species.

Critique of Experimental Methods
In the present study, our hypothesis was tested in 2 species. Because of the species dependence of different endothelium-dependent agonists, the interpretation of the results of studies investigating the regulation of vascular tone is often difficult, and the significance for human pathophysiology remains unclear. Because impairment of coronary blood flow due to atherosclerosis is a very important clinical issue, it is of great significance to perform experiments in human vessels. However, there are only few data available from experiments in isolated perfused human vessels³¹; this situation is most likely due to the difficult preparation and the difficulty in obtaining true control vessels. In the present study, we used as relative controls the vessels from patients without significant atherosclerosis, ie, vessels from patients undergoing open heart surgery for reasons other than atherosclerosis, such as valve replacement or repair of cardiac defects, in whom the presence of significant atherosclerosis had preoperatively been excluded via coronary angiography. To further diminish the risk of endothelial dysfunction of our control vessels, we chose tissue from patients without severe coronary risk factors. However, we still cannot exclude the existence of endothelial dysfunction and would even expect that there was some alteration of endothelial function in our control vessels due to the influence of an altered profile of pressure and flow, which influences endothelial and smooth muscle cell function.²²,²³ Nevertheless, we propose that endothelial function of our control vessels was better than endothelial function of vessels from patients with atherosclerosis, as demonstrated by a significantly better response of the control vessels to all tested endothelium-dependent agonists compared with vessels from atherosclerotic individuals without differences in the response to the endothelium-independent agonist.

We additionally tested our hypothesis in an established animal model of atherosclerosis.³ Göttinger minipigs treated with or without an atherogenic diet for 4 months showed significant differences in cholesterol levels and in the response to different endothelial agonists. However, because the time for induction of atherosclerosis is much shorter in this experimental model than in humans, there is also a limitation of the animal model.

Choice of Agonists
In pigs, we investigated the effects of histamine, serotonin, and substance P. All 3 agonists are well-established endothelial vasodilators in this species, exerting their effect via the release of NO.³,¹⁹ In control vessels, all 3 agonists caused maximal vasodilation, demonstrating regular endothelial
function. In vessels from atherosclerotic animals, vasodilatation to histamine and substance P was significantly reduced, and serotonin induced vasoconstriction instead of vasodilation. This confirms data from several investigations demonstrating impaired vasodilatation and augmented vasoconstriction to different endothelium-dependent vasoactive factors of arteries and arterioles in atherosclerosis and specifically in hypercholesterolemia.\textsuperscript{3,4,24–27} Inasmuch as the effect of sodium nitroprusside was unaltered, our data indicate that endothelial function and agonist-induced release of NO were severely depressed in these vessels, whereas smooth muscle responsiveness was unaltered.

Application of sepiapterin for 15 minutes restored the effects of histamine and substance P and significantly diminished vasoconstriction to serotonin in atherosclerotic vessels. To investigate whether sepiapterin specifically improves the endothelial function of vessels with atherosclerosis, we compared the effect of sepiapterin on the endothelial function of atherosclerotic vessels versus nonatherosclerotic vessels and on smooth muscle cell function. Sepiapterin did not influence the effect of either agonist in control vessels and did not alter the effect of sodium nitroprusside in either group of vessels. These results indicate that by increasing tetrahydrobiopterin levels with sepiapterin, endothelial dysfunction can be acutely improved. An increase of both endogenous tetrahydrobiopterin and NO synthesis through application of sepiapterin has previously been demonstrated by other authors.\textsuperscript{12,13,28}

Our choice of agonists for the investigation of endothelial function in human vessels was based on pilot experiments in which we determined the NO dependence of the different agonists by pretreatment with N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthesis. L-NAME nearly completely inhibited the dilatory response of human arterioles to histamine, induced constriction to serotonin, and significantly increased vasoconstriction to acetylcholine. In contrast, the response to sodium nitroprusside was not significantly altered. Therefore, in human arterioles, the vasodilatory effects of histamine and serotonin are mediated mainly via the release of NO, and NO seems to weaken acetylcholine-induced vasoconstriction (Figure 5).

In humans, we examined the influence of acetylcholine instead of substance P. Acetylcholine is widely used as a standard substance to test endothelial function in both human coronary arteries and arterioles.\textsuperscript{5,10} Under physiological conditions, acetylcholine causes vasodilatation of coronary vessels. However, in vessels with atherosclerosis or in the presence of coronary risk factors, acetylcholine has been shown to induce paradoxical vasoconstriction.\textsuperscript{29} In contrast, the effect of acetylcholine on porcine vessels is at least partially endothelium independent.\textsuperscript{30}

In our experiments, acetylcholine induced vasoconstriction both in control vessels and in atherosclerotic vessels. This could either indicate endothelial dysfunction of the control vessels or indicate that acetylcholine is a vasoconstrictor of human arterioles with a diameter <100 \( \mu \)m. Interestingly, histamine caused maximal vasodilatation in control vessels. Because in human vessels the effect of histamine is largely mediated via the release of NO, this indicates grossly normal endothelial function of our control vessels. Serotonin caused some vasodilatation in the control vessels. Interestingly, there was a great standard deviation of the effects of both acetylcholine and serotonin. We can only speculate that this may be due to a heterogeneity in the distribution of receptor subtypes and/or indicate early endothelial dysfunction of some of the control vessels.

In atherosclerotic vessels, vasodilatation to histamine was significantly diminished, vasodilatation to serotonin was blunted, and vasoconstriction to acetylcholine was greatly increased, whereas the response to sodium nitroprusside was unaltered. From these observations, we propose that vessels from patients with severe atherosclerosis demonstrated endothelial dysfunction.

Incubation with sepiapterin restored vasodilatation to histamine and significantly attenuated vasoconstriction to acetylcholine. The effect of serotonin was improved; however, probably because of the large standard deviation, the difference was not significant. As in porcine vessels, sepiapterin did not influence the effect of sodium nitroprusside.

Taken together, sepiapterin improved the effects of all tested endothelium-dependent agonists both in porcine and in human coronary vessels from species with atherosclerosis.
Physiological and Pathophysiological Implications

In a variety of investigations, the phenomenon of endothelial dysfunction has been observed early in the development of atherosclerosis. In the present study, we demonstrated for the first time the presence of endothelial dysfunction in isolated human coronary resistance vessels from patients with severe atherosclerosis. So far, the underlying reasons and the pathophysiological significance of endothelial dysfunction remain unclear.

Under physiological conditions, there is a balance between the endothelial production of NO and oxygen-derived free radicals. In the presence of vascular risk factors or atherosclerosis, there is a shift of this balance toward the production of vasotoxic oxygen-derived free radicals. Biochemically, NO synthase consists of a flavin-containing reductase domain, a heme-containing oxygenase domain, and a regulatory calmodulin-binding sequence. In addition to calcium/calmodulin, NO oxide synthase requires tetrahydrobiopterin as a cofactor. Tetrahydrobiopterin has been proposed to be the key molecule in the control of the generation of both NO and superoxide. Under conditions in which tetrahydrobiopterin is depleted, NO generates superoxide anions instead of NO. Therefore, we propose that in atherosclerosis, reduced availability of tetrahydrobiopterin leads to an increased production of superoxide anions via NO synthase. This is supported by the observation that substitution of tetrahydrobiopterin improves endothelial dysfunction via increasing the production of NO in hypercholesterolemic subjects and in animals after ischemia/reperfusion and that endothelial cell damage can be prevented by pretreatment with sepiapterin via increased intracellular levels of tetrahydrobiopterin. Additionally, it is confirmed by a recent study showing an improvement of coronary flow responses in humans with coronary artery disease after the application of tetrahydrobiopterin. Finally, it is in agreement with the results of the present study that endothelial dysfunction in atherosclerosis is improved by the application of sepiapterin.

The underlying reason for the decreased availability of tetrahydrobiopterin has not been clarified in the present investigation. Under physiological conditions, GTP-cyclohydrolase I is the rate-limiting enzyme in the production of tetrahydrobiopterin. Generation of tetrahydrobiopterin from sepiapterin is independent from GTP-cyclohydrolase I. Therefore, it can be speculated that decreased expression of GTP-cyclohydrolase I may be involved in the pathology of decreased tetrahydrobiopterin generation in atherosclerosis. This needs to be clarified in further investigations.

Another possible explanation for the reduced availability of tetrahydrobiopterin in atherosclerosis is an influence of toxic radicals, which induce an alteration in cellular redox state, on the biochemistry of tetrahydrobiopterin. Toxic radicals may interact with the role of tetrahydrobiopterin as a redox agent in the synthesis of NO, affect the biosynthesis of tetrahydrobiopterin via depletions of NADPH and/or prevent the recycling of tetrahydrobiopterin, which is supposed to occur via flavin nucleotides.

Conclusions

The results of the present investigation demonstrate that reduced vasodilation of coronary resistance arteries with atherosclerosis to endothelium-dependent agonists can be improved by the substitution of sepiapterin. We conclude that an altered bioavailability of tetrahydrobiopterin is involved in the pathophysiology of endothelial dysfunction in atherosclerosis.

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