Hemoglobin Inhibits Endothelium-Dependent Relaxation to Acetylcholine in Human Coronary Arteries In Vivo

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Background. The endothelium can regulate vascular tone by releasing both endothelium-derived relaxing factor (EDRF) or nitric oxide and contracting factors. To date, there has only been circumstantial evidence to indicate EDRF activity in vivo in human coronary arteries. Using human hemoglobin as a specific inhibitor, the hypothesis that acetylcholine-induced coronary vasodilation is due to EDRF release was tested.

Methods and Results. We studied the response of normal coronary arteries to acetylcholine (an endothelium-dependent vasodilator) and isosorbide dinitrate (an endothelium-independent vasodilator) in seven patients. The specificity of any vasodilator response was assessed by the infusion of reduced free human hemoglobin. Hemoglobin 10⁻⁴ M infusion alone had no effect on coronary artery diameter. Drugs were infused into the coronary artery, and the diameter changes were assessed by quantitative angiography. Acetylcholine 10⁻⁴ M increased left anterior descending coronary artery diameter from 2.30±0.12 mm to 2.79±0.20 mm (mean±SEM, n=7, p<0.01). Hemoglobin both in the concentration of 10⁻⁴ M and 10⁻³ M reversed this vasodilator effect, causing constriction to 2.11±0.18 mm (p<0.001 compared with acetylcholine 10⁻⁴ M) and 2.29±0.14 mm (p<0.05 compared with acetylcholine 10⁻³ M). Isosorbide dinitrate in the presence of hemoglobin caused dilatation of the coronary artery in all cases to 3.04±0.24 mm (p<0.001 compared with acetylcholine 10⁻³ M and hemoglobin 10⁻⁴ M).

Conclusions. Using a specific inhibitor of nitric oxide, reduced free hemoglobin, we have demonstrated that basal EDRF release does not appear to play an important role in the maintenance of human epicardial coronary artery diameter in vivo but is responsible for the acetylcholine-induced dilatation.

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KEY WORDS • coronary arteries, human • vasodilation, coronary • endothelium-derived relaxing factor

The vascular endothelium is capable of releasing both endothelium-derived relaxing factor (EDRF)¹ and contracting factors.²,³ EDRF is released by a number of endogenous hormones and physico-chemical stimuli, including acetylcholine.¹ Acetylcholine stimulates endothelium-dependent relaxation in coronary arteries from the dog,¹,⁴ monkey,⁵ rabbit,⁶ and human.⁷ EDRF has been chemically identified as nitric oxide⁸ or a nitric oxide–containing substance such as S-nitrosocysteine.⁹ Endothelium-dependent vascular relaxation and the release of EDRF in vitro is well established. However, to date there has only been circumstantial evidence to indicate its release in vivo in human epicardial coronary arteries, suggested by the vasodilator effects of acetylcholine¹⁰,¹¹ and substance P¹² infused into angiographically normal human coronary arteries. In one study, the infusion of methylene blue potentiated human epicardial coronary artery constriction with acetylcholine in vivo,¹³ suggesting that acetylcholine releases EDRF. The mechanism of methylene blue inhibition of EDRF dilator effects was thought to be due to a direct inhibition of guanylate cyclase, thereby preventing the rise in smooth muscle cGMP, which results in relaxation. Recent data suggest that this inhibition by methylene blue is not due to the inhibition of guanylate cyclase but may be due to the generation of superoxide free radicals, which then inactivate EDRF.¹⁴,¹⁵ Recent studies in humans have confirmed that endothelium-derived nitric oxide has a regulatory action at the level of resistance vessels of the human forearm.¹⁶ This study used the specific inhibitor of the synthesis of endothelium-derived nitric oxide, N⁵-monomethyl-L-arginine. We report for the first time an inhibition of the acetylcholine-induced vasodilation by reduced free hemoglobin, a known inhibitor of EDRF¹⁷,¹⁸ and nitric oxide,¹⁹ which indicates that EDRF can be released by acetylcholine and may therefore have an important regulatory role in this vascular bed. Infusion of N⁵-monomethyl-L-arginine was not

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attempted because this substance has a long half-life in vivo, and the possibility of a prolonged inhibition of endothelium-derived nitric oxide was thought to be potentially hazardous.

Methods

Study Population

Seven patients (six women and one man; mean age±SD, 51±11 years) were recruited for the study. Patients selected for the study were undergoing coronary angiography for clinical indications. All patients had angiographically normal coronary arteries and were selected after a diagnostic coronary angiogram. Patients had valvular disease or atrial septal defects. The study protocol was approved by the Royal Brompton National Heart and Lung Hospital’s Ethical Committee, and all patients gave written informed consent for the study. Routine premedication was withheld before cardiac catheterization, and all medications were stopped at least 24 hours before the study. None of the patients had a history of any other disease process, and in particular none of them complained of atypical chest pain.

Human Coronary Artery In Vivo Study Protocol

After routine cardiac catheterization, full standard heparinization (100 units/kg body wt) was given before the study. A standard guiding catheter (8F, Cordis [UK] Ltd.) was placed in the left coronary ostium. A coronary infusion catheter (2F, Cordis [UK] Ltd.) was advanced through the guiding catheter into the proximal portion of the left anterior descending coronary artery for administration of vasoactive agents. Drugs or 5% dextrose was infused continuously into the left anterior descending coronary artery through the infusion catheter. Infusions were of 3-minute duration, and infusion rates were 1.5 ml/min or less. Solutions were infused with constant infusion syringes (Trionic IPS, Vickers) to achieve estimated final blood concentrations in the coronary artery, based on the assumption that blood flow in the left anterior ascending coronary artery is 80 ml/min. At the end of each infusion, coronary arteriography was performed in single-plane views with the use of nonionic contrast medium (iohexol). Throughout each infusion, heart rate, systemic arterial pressure, and the ECG were monitored continuously. Intracoronary infusions and vasoactive agents were administered in the following sequence: 1) control (5% dextrose in water) for 3 minutes, 2) hemoglobin 10^{-5} M for 3 minutes, 3) acetylcholine 10^{-7} M for 3 minutes, 4) acetylcholine 10^{-7} M with hemoglobin 10^{-4} M for 3 minutes, 5) acetylcholine 10^{-7} M with hemoglobin 10^{-5} M for 3 minutes, 6) 1,000 µg isosorbide dinitrate administered into the left anterior descending coronary artery at the end of the last infusion.

To rule out the possibility of tachyphylaxis to acetylcholine, six patients were given intracoronary control infusion followed by acetylcholine 10^{-7} M, and angiograms were performed at 3 and 9 minutes after the start of the acetylcholine infusion.

Human and Rabbit Coronary Artery In Vitro Study Protocol

Parallel studies were conducted in vitro using human and rabbit coronary arteries to test the agents used in vivo.

Proximal left anterior descending coronary arteries were excised from male New Zealand White rabbits (weight, 2–3 kg) and from coronary vessels from normal human donor hearts obtained from the transplant program at the Royal Brompton National Heart and Lung Hospitals. The vessels were trimmed of adhering fat and connective tissue as previously described. Transverse rings of coronary artery were cut with surgical blades. In some of the rings, endothelium was removed by gentle rubbing. The rings were mounted in glass 10-ml organ baths at 37°C. The bathing fluid was Krebs solution containing (mM): NaCl 118, KCl 4.8, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 24, glucose 11, and Na2 EDTA 0.03. It was continuously gassed with 95% O2-5% CO2. Two stainless steel hooks were used to mount each ring. The lower hook was attached to the base of the muscle chamber, the upper to a strain gauge. All preparations were equilibrated for 90 minutes, and basal tension was set to 1 g before drug additions. The records show the point at which the drug addition was made.

Quantitative Coronary Angiography

Selective left coronary arteriography was performed by the injection of 7–8 ml of nonionic, low-osmolar radiographic contrast medium (iohexol) containing 350 mg of iodine per milliliter at a rate of ~5 ml per second. Single-plane coronary arteriograms were recorded at 50 frames per second on a Siemens cineradiographic system, with a dose rate of 20 µR per frame at the input face of a 17-cm field, with a resultant spatial resolution on the cinefilm of 3.8 line pairs per millimeter.

Calibration and end-diastolic cineframes were then videodigitized using an IGE CAP35 cineprojector and Kontron Cardio 500 Image processing workstation. Each frame was stored on a 512²-pixel matrix with digitization to a depth of eight bits with a resultant 256 shades of gray per pixel. The calibration images for each patient were analyzed to correct for geometric magnification and to determine the distance in millimeters for each pixel. This ranged from 0.15 to 0.20 mm/pixel, (mean, 0.18). Calibration and diameter measurements of the coronary artery segments were performed using the interactive program of the micro Mipron interpreter software installed on the Cardio 500 workstation.

Both the calibration and vessel diameter measurements were performed by an independent observer who was unaware of the infusion sequence or protocol.

Drugs

Human hemoglobin from donors negative for hepatitis B surface antigen and human immunodeficiency viral antibodies 1 and 2 tested for by enzyme-linked immunosorbent assay was supplied by Sigma Chemical Company. It contained a mixture of oxyhemoglobin and methemoglobin and was prepared as previously described. The following procedures were performed in the department of hematology. A 20-mM excess of reducing agent, sodium dithionite (Na2S2O4), was added to a 1-mM solution of twice-crystallized human hemoglobin. Sodium dithionite was removed by dialysis using 2 l of distilled water for 1½ hours in the dark. This procedure was repeated three times, and on each occasion, the dialysate was changed. Purity of the hemoglobin solutions was determined by electrophore-
sis and always contained less than 5% methemoglobin. Sterility during the procedure was assured by the use of sterile, pyrogen-free water and containers. These were deoxygenated by gassing with oxygen-free nitrogen. The final solution was filtered through 0.45- and 0.2-μm filters (Flow Laboratories) and filtered again immediately before use in the patients. The solutions were stored at −80°C for no longer than 2 weeks. Samples of the solutions used in the patients were immediately subjected to repeat electrophoresis (for methemoglobin), and these solutions always contained less than 5% methemoglobin. The solutions were assessed for pyrogens using rabbit pyrogen tests as recommended in the British Pharmacopoeia (1988, volume II, appendix XIV K, test for pyrogens; A183–A184). The tests for pyrogens were negative for all solutions tested. Sterility was confirmed by submission of samples from the final stage of preparation to microbiological analysis. The solution was also immediately tested for sterility after the infusion procedures. Acetylcholine chloride was from Coopervision; isosorbide dinitrate was from Schwarz.

Statistical Analysis

Results are expressed as coronary artery diameter in millimeters ± SEM; results for the in vitro study are expressed as percentage relaxation and reversal of relaxation ± SEM. Comparisons are made using ANOVA for repeated measures and a paired Student’s t test, respectively. Statistical significance was assumed at the 0.05 probability level.

Results

In Vivo Study

Acetylcholine 10⁻⁷ M significantly increased the left anterior descending coronary artery diameter from control: 2.30±0.12 mm to 2.79±0.20 mm (p<0.01). Hemoglobin, both in a concentration of 10⁻⁴ M and 10⁻⁵ M, completely reversed this vasodilator effect to 2.11±0.18 mm (p<0.001 compared with acetylcholine 10⁻⁷ M) and 2.29±0.14 mm (p<0.05 compared with acetylcholine 10⁻⁷ M). Injection of isosorbide dinitrate caused dilation of the left anterior descending coronary artery in all cases in the presence of hemoglobin to 3.04±0.24 mm (p<0.001 compared with acetylcholine 10⁻⁷ M and hemoglobin 10⁻⁴ M). Hemoglobin (10⁻³ M) alone had no effect on coronary artery diameter (Figure 1). No evidence for tachyphylaxis to acetylcholine infusion was found. Acetylcholine 10⁻⁷ M significantly increased coronary artery diameter from control: 2.35±0.11 mm to 2.97±0.11 at 3 minutes and 2.90±0.17 at 9 minutes from the start of the infusion (p=NS between 3 and 9 minutes of acetylcholine 10⁻⁷ M; p<0.01 and 0.05 compared with control and acetylcholine 10⁻⁷ M at 3 and 9 minutes, respectively).

Heart rate, systemic arterial pressure, and ECG did not change in any patient. There were no reported sensations, and none of the subjects was aware of the infusions. Hemoglobin, white cell count, platelet count, urea and electrolytes, prothrombin time, kaolin, and cephalin clotting time were no different after the study.

In Vitro Study

Acetylcholine 10⁻⁷ M relaxed KCl-preconstricted human epicardial coronary arteries by 56±6% (n=4, p<0.05). Hemoglobin 1.25x10⁻⁴ M reversed the relaxation effect of acetylcholine to 103±1% (p<0.05). Acetylcholine 10⁻⁷ M relaxed KCl-preconstricted rabbit epicardial coronary arteries by 52±7% (n=4, p<0.01). Hemoglobin 1.25x10⁻⁴ M reversed the relaxation effect of acetylcholine to 109±6% (p<0.05). Hemoglobin did not have a significant effect on tone either in resting or preconstricted human and rabbit coronary arteries. There is, therefore, no evidence in vitro in either preparation of a direct constrictor effect of hemoglobin. Representative tension recordings are shown in Figure 2.

Discussion

It has been demonstrated in the human coronary artery in vivo that acetylcholine can elicit a vasodilator response, and this can be reversed by a specific inhibitor of nitric oxide: reduced free hemoglobin. This provides the most convincing evidence to date that acetylcholine can release EDRF from human coronary endothelium in vivo.

Acetylcholine has been identified as a coronary vasodilator in patients with angiographically normal coronary arteries. The specificity of this vasodilation has not been fully tested. Indeed, some authors claim that acetylcholine causes epicardial coronary artery constriction. However, the doses of acetylcholine in one study were higher than used previously and in this study. Other workers have used bolus injections that do not achieve steady-state drug concentration in the coronary artery. Also, it has recently been claimed that risk factors for coronary artery disease positively correlate with an abnormal vasoconstrictor response to acetylcholine in angiographically normal coronary arteries. We confirm that at a concentration of 10⁻⁷ M, acetylcholine significantly increased epicardial coronary diameter.

The concept of using hemoglobin solutions as blood substitutes has been recognized for many years, but renal toxicity limited its development; however, it was
Hemoglobin binds avidly to nitric oxide. Hemoglobin may be combining with nitric oxide at the luminal endothelial cell surface, and this may create a "sink" for intimately released nitric oxide. An alternative explanation is that hemoglobin is penetrating the endothelial cell surface either by endocytosis or transcytosis. Immunocytochemical procedures have been used to localize albumin transport through the capillary endothelium of the murine myocardium. It appears to pass through the endothelium via plasmalemmal vesicles and appears in the pericapillary spaces less than 15 seconds after the beginning of its perfusion. Albumin has a molecular weight of approximately 69,000 and tetrameric hemoglobin a molecular weight of approximately 64,000. Both are globular proteins and have similar molecular radii. The possibility exists that tetrameric hemoglobin may follow a similar transcytotic vesicular pathway through the coronary vascular endothelium in vivo. Although the hemoglobin used in this study was shown electrochemically to be the tetrameric form, there is evidence in vivo that the tetramer, composed of two polypeptide chains, dissociates into two peptide chain dimers, which would, theoretically, enable an even faster endothelial transit time. This, of course, cannot be shown to be the case in this study. It is also noteworthy that the permeability of the renal glomeruli for hemoglobin is even greater than that for albumin and molecules of similar molecular weight. Hemoglobin may therefore follow a similar pathway and would then be available at an appropriate site to inhibit released nitric oxide in the subendothelial space. Myoglobin (molecular weight, 17,800) has been shown to penetrate the endothelium within 30 seconds.

Hemoglobin solutions have been demonstrated to have coronary vasoconstrictor activity in the isolated, buffer-perfused rabbit heart. This vasoconstrictor activity was considerably reduced by the purification of the hemoglobin. It is reassuring that in our study, when hemoglobin alone was infused, it did not appear to have a direct vasoconstrictor effect. However, it did abolish the acetylcholine-induced endothelium-dependent vasodilation. This would argue against an important basal EDRF effect in the epicardial coronary arteries in humans and also against a direct vasoconstrictor effect of the reduced hemoglobin used in our study. A coronary vasoconstrictor effect of human stroma-free hemoglobin has been identified in isolated rabbit hearts perfused with Krebs-Henseleit solution. More importantly, in this study, the vasoconstrictor effect was preserved when the rabbit heart was perfused with whole blood at a constant coronary flow rate. In buffer-perfused hearts, stroma-free hemoglobin in concentrations of 5–200 mg/dl produced dose-related increases of coronary perfusion pressure. In blood-perfused hearts, increasing plasma hemoglobin to 1.6±0.1 g/dl (2.5×10⁻⁴ M) without changing total hemoglobin or arterial oxygen content increased coronary perfusion pressure by 36±13 mm Hg. It was concluded from this study that stroma-free hemoglobin solutions exert a coronary vasoconstrictor effect that is unrelated to oxygen delivery and may involve the inhibition of some tonic dilator activity (e.g., EDRF–nitric oxide). There is evidence that hemoglobin possesses direct smooth muscle constrictor activity in isolated cerebral arteries from certain species and that it may release a vasoconstrictor.
prostaglandin in monkey and dog cerebral arteries and pig coronary arteries. There was no evidence that the reduced free human hemoglobin used in this study had any direct constrictor activity in human or rabbit coronary arteries either in vitro or in vivo.

Increasing blood flow has been shown to result in endothelium-dependent relaxation of animal epicardial coronary arteries. There are studies that suggest that this mechanism exists in angiographically normal coronary arteries in humans by demonstration of proximal increases in coronary artery diameter to the pharmacological agents papaverine, adenosine, and acetylcholine. Flow-induced dilation was shown to be defective in patients with coronary artery irregularities as a result of coronary atherosclerosis. Coronary vascular resistance was not measured in this study. It is therefore theoretically possible that the epicardial coronary artery diameter increases are due to a flow-mediated effect of acetylcholine by a decrease in coronary vascular resistance (microvascular endothelial EDRF release), resulting in the release of EDRF from the epicardial coronary artery endothelium. If the mechanism of acetylcholine-induced increase in epicardial coronary artery diameter is flow-mediated, it still does not detract from the fact that this acetylcholines-induced (presumed to be due to nitric oxide released from the endothelium) epicardial coronary artery dilation effect has been reversed by an infusion of hemoglobin, thus implying that at whatever level (epicardial or microvascular) nitric oxide release is being stimulated by acetylcholine, its effect is being reversed by the infused hemoglobin.

Direct evidence that endothelium-dependent dilation is present in humans in vivo was first shown in the large dorsal veins of the hands of normal subjects. Subsequently, specific inhibition of endothelium-derived nitric oxide has been achieved in the forearm of healthy human volunteers. Infusion of N\textsuperscript{G}-monomethyl-L-arginine attenuated the increase in flow induced by acetylcholine, and the effect of N\textsuperscript{G}-monomethyl-L-arginine could be partly reversed by L-arginine. N\textsuperscript{G}-monomethyl-L-arginine appears to cause substantial vasoconstriction that can last for 45–60 minutes unless L-arginine is used partially to reverse the effect. Such a long-lasting effect could theoretically be dangerous in the coronary vascular bed. Reduced hemoglobin appears to have a short-acting, easily reversible effect.

This study provides strong evidence that basal nitric oxide release does not play an important role in the maintenance of epicardial coronary artery diameter in vivo. This, of course, does not rule out an important role for basal nitric oxide in the microvascular coronary bed. It also strongly suggests that endothelium-derived nitric oxide is responsible for the acetylcholine-induced dilation in human coronary arteries in vivo. Reduced free hemoglobin appears to be an effective inhibitor of endothelium-derived nitric oxide in vivo and has been shown to be safe at the concentrations used in these studies.

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