Title

Studies on the physiology and chemistry of endothelium-derived relaxing factor and nitric oxide

Permalink

https://escholarship.org/uc/item/4b73x4xp

Author

Guerra, Ricardo,

Publication Date

1990

Peer reviewed|Thesis/dissertation

Studies on the Physiology and Chemistry of Endothelium-Derived Relaxing Factor and Nitric Oxide

Ricardo Guerra, Jr.

Submitted in fulfillment of the requirements for the M.D. Degree with Thesis.

University of California, San Francisco
April 17, 1990

<u>Thesis Advisors:</u>
Dr. J.I.E. Hoffman, M.D.
Dr. J. P. Kane, M.D., Ph.D.
Dr. P.R. Myers, Ph.D., M.D.

Dedication

This work is dedicated to my mentors and my parents -
what they have taught me, I will carry with me always.

Abstract

In 1980, Furchgott first reported that relaxation of arteries by acetylcholine required the presence of endothelium. Subsequent work by Furchgott and many others has lead to a model of control of vascular tone partly mediated by the release of a labile, non-prostanoid factor or factors collectively referred to as endothelium-derived relaxing factor (EDRF). The release of EDRF has been shown to be stimulated by receptor-mediated agents (eg., acetylcholine, ADP, serotonin, thrombin) and non-pharmacologic stimuli (eg., flow induced sheer stress). Activation of soluble guanylate cyclase and the subsequent rise in cyclic GMP levels within smooth muscle cells is likely responsible for the relaxing action of EDRF. In addition to its vasodilator effects, EDRF also inhibits platelet aggregation, induces disaggregation of aggregated platelets, and inhibits platelet adhesion to vascular endothelium. It is likely that the endothelium also provides a mechanism to protect the vessel wall from pathologic constrictor stimuli. It is conceivable that loss or dysfunction of this protective function of the endothelium would make a vessel vulnerable to unwanted constrictor effects, leading to vasospasm and/or thrombosis.

Recent studies have demonstrated that endothelium-dependent vascular responses are abnormal in atherosclerosis. We tested the hypotheses that the abnormal vascular reactivity is due to decreased production or release of endothelium-derived relaxing factor (EDRF) by atherosclerotic vessels and that atherosclerotic vessels are less sensitive to the relaxing effects of EDRF due to an intimal barrier. EDRF release was quantified by assessing the response of two separate bioassay techniques. We found that atherosclerotic vessels released significantly less EDRF than normal vessels in response to both receptor and non-receptor mediated stimuli. We also compared the sensitivities of normal and atherosclerotic vessels to the relaxing effects of both EDRF and nitric oxide, a putative EDRF, applied to the luminal surface of these vessels. Atherosclerotic vessels, compared to normal vessels, were equally sensitive to nitric oxide, but were actually more sensitive to EDRF derived from normal rabbit aortae. This suggests that there may be one or more substances besides nitric oxide that are responsible for the relaxing effects of EDRF. We conclude that decreased EDRF production is the primary mechanism resulting in abnormal vascular responses in atherosclerosis.

Recently, Palmer et al. and Ignarro et al. have suggested that nitric oxide (NO) is at least one of the compounds responsible for the activity of EDRF. Critical to the conclusion that EDRF is, in fact, nitric oxide, is the ability to account for all of EDRF's vasodilator

activity. We set out to test the hypothesis that nitric oxide is released from cultured bovine aortic endothelial cells in quantities sufficient to account for EDRF-induced vascular relaxation. These studies employed a chemiluminescence technique to quantify the amount of nitric oxide released by cultured endothelium. The amount of nitric oxide actually released was compared to the amount required to cause equivalent relaxations of detector vessels. We found that the cultured endothelial cells released far less nitric oxide than would be required to explain bioassay detector ring relaxations. One intriguing possibility that might explain this discrepancy is that EDRF might actually be a nitric oxide containing compound that is much more potent than nitric oxide itself.

To test the hypothesis that EDRF is a highly potent nitrosocompound, we compared the half-lives, nitric oxide content and bioactivity of EDRF, standard solutions of nitric oxide, and a model compound, S-nitrosocysteine. These experiments demonstrated that S-nitrosocysteine bears greater resemblance to EDRF than nitric oxide. We therefore conclude, at least for cultured bovine aortic endothelial cells, authentic nitric oxide is likely not the endothelium-derived relaxing factor. It is conceivable that the relaxing agent released by these cells is actually a potent nitrosocompound such as S-nitrosocysteine.

To test the hypothesis that the decreased bioactivity of EDRF from atherosclerotic vessels observed in the first set of experiements is due to impaired nitrosocompound release, we examined EDRF and nitric oxide release from atherosclerotic vessels using the same bioassay and chemiluminescence methods employed above. These preliminary studies showed that nitric oxide release from atherosclerotic vessels is actually increased even in the face of markedly impaired bioactivity of released relaxing factors. This might be explained by postulating a defect in a final step in the synthesis of EDRF such that nitric oxide is not incorporated into a more potent nitrosocompound such as S-nitrosocysteine. Release of a less potent relaxing factor might account for the abnormalities in endothelium-dependent vascular relaxation observed in atherosclerosis. It is also interesting to speculate that the increased nitric oxide release noted from atherosclerotic vessels might be due to a loss of a feedback inhibition on the biosynthetic pathway which generates the normal nitrosocompound EDRF.

Table of Contents

List of Tables

List of Illustrations

Section 1

Introduction and Overview

In 1980, Furchgott and Zawadski (1) first reported that relaxation of arteries by acetylcholine required the presence of endothelium. Subsequent work by Furchgott and many others (eg. 2-4) has yielded substantial insight into how the endothelium modulates vascular tone. A variety of agonists (eg. serotonin, ADP, acetylcholine, thrombin) interact with receptors on the surface of the endothelial cells which line a vessel's lumen. Several vasoactive substances may be released by these cells in response to agonist stimulation, diffuse to the underlying smooth muscle, and prompt constriction or relaxation. These vasoactive substances include prostacyclin (5-7), prostanoid and non-prostanoid constrictor factors (8-11), and one or more non-prostanoid relaxing factors, collectively referred to as endothelium-derived relaxing factor (EDRF; 1, 12-13). The endothelium releases EDRF in response to both physical (14-16) and pharmacologic (eg., acetylcholine, serotonin, ADP, thrombin, 12-13) stimuli. EDRF then diffuses to the adjacent vascular smooth muscle and produces vascular relaxation. Activation of soluble guanylate cyclase and the subsequent rise in cyclic GMP levels within smooth muscle cells is likely responsible for the relaxing action of EDRF (17-19).

A great deal of experimental evidence strongly suggests that EDRF plays a very important role in modulation of vessel tone in normal (13-16) and pathophysiologic (20-23) conditions. It is likely that the endothelium also provides a mechanism to protect the vessel wall from pathologic constrictor stimuli. For instance, in addition to its vasodilator effects, EDRF also inhibits platelet aggregation, induces disaggregation of aggregated platelets, and inhibits platelet adhesion to vascular endothelium. As with vascular relaxation, these effects of EDRF are mediated through stimulation of the soluble guanylate cyclase and the consequent elevation of cyclic guanosine 3',5'-monophosphate within platelets. The release of vasoconstrictors such as serotonin and thromboxane from aggregating platelets has been postulated to be the underlying mechanism of coronary vasospasm (35, 36) occurring at locations of atherosclerotic plaque formation. It is conceivable that loss or dysfunction of this protective function of the endothelium would make a vessel vulnerable to unwanted constrictor effects.

It is clear that knowledge of EDRF's chemical structure, the biosynthetic pathway leading to its generation, and its interaction with vessel wall elements and other vasoactive

substances is important in understanding vascular abnormalities observed in pathologic states such as atherosclerosis, hypertension, and diabetes. However, the first step in this process, identification of EDRF, has been complicated by its short half-life (24-26) and the lack of specific pharmacologic inhibitors of biosynthesis. Also, several studies suggest that the humoral substance(s) released by endothelium in response to different stimuli, both pharmacologic and mechanical, may vary between species and between vascular beds (27, 28). Recently, however, Palmer et al. (29) and Ignarro et al. (30) have suggested that nitric oxide (NO) is at least one of the compounds responsible for the activity of EDRF. They and others have shown that NO is synthesized by vascular endothelium from the terminal guanidino nitrogen atom(s) of the amino acid L-arginine. (54, 55) This suggests the existence of an enzyme-based pathway in which L-arginine is the endogenous precursor for the synthesis of NO. The discovery of the release of NO by vascular endothelial cells, the biosynthetic pathway leading to its generation, and its interaction with other vasoactive substances opens up new avenues for research into the physiology and pathophysiology of the vessel wall.

The purpose of the studies presented within this thesis is to examine endothelium-dependent vascular relaxation responses and EDRF release in atherosclerosis and to gain further information regarding the chemical identity of EDRF.

The first series of experiments examined possible mechanisms for abnormal endothelium-dependent vascular relaxation in atherosclerosis. These studies demonstrate that a defect in the synthesis or release of EDRF rather than a barrier to the diffusion of EDRF is likely responsible for these abnormalities. Additionally, differences in the relaxing effects of nitric oxide and naturally occurring EDRF on normal and atherosclerotic vessels were observed. This finding suggests that nitric oxide may not be the sole or even the actual EDRF. Critical to the conclusion that EDRF is, in fact, nitric oxide, is the ability to account for all of EDRF's vasodilator activity. Accordingly, we set out to test the hypothesis that nitric oxide is released from cultured bovine aortic endothelial cells in quantities sufficient to account for EDRF-induced vascular relaxation. These studies employed a chemiluminescence technique to quantify the amount of nitric oxide released by cultured endothelium. The amount of nitric oxide actually released was compared to the amount required to cause equivalent relaxations of detector vessels. We found that the cultured endothelial cells released far less nitric oxide than would be required to explain bioassay detector ring relaxations. One intriguing possibility that might explain this discrepancy is

that EDRF might actually be a nitric oxide containing compound that is much more potent than nitric oxide itself.

The possibility that EDRF might be a nitrosocompound lead to a third set of experiments in which the half-lives, nitric oxide content and bioactivity of EDRF, standard solutions of nitric oxide, and a model compound, S-nitrosocysteine were compared. These experiments demonstrated that S-nitrosocysteine bears greater resemblance to EDRF than nitric oxide. We therefore conclude, at least for cultured bovine aortic endothelial cells, authentic nitric oxide is likely not the endothelium-derived relaxing factor. It is conceivable that the relaxing agent released by these cells is actually a potent nitrosocompound such as S-nitrosocysteine.

In the final group of experiments, nitric oxide release from atherosclerotic vessels was examined using the chemiluminescence technique. These preliminary studies showed that nitric oxide release from atherosclerotic vessels is actually increased even in the face of markedly impaired bioactivity of released relaxing factors. This might be explained by postulating a defect in a final step in the synthesis of EDRF such that nitric oxide is not incorporated into a more potent nitrosocompound such as S-nitrosocysteine. Release of a less potent relaxing factor might account for the abnormalities in endothelium-dependent vascular relaxation observed in atherosclerosis. It is also interesting to speculate that the increased nitric oxide release noted from atherosclerotic vessels might be due to a loss of a feedback inhibition on the biosynthetic pathway which generates the normal nitrosocompound EDRF.

Introduction and Overview References

1. Furchgott RF, Zawadski JV: The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288:373-376, 1980.

2. Furchgott RF: Role of the Endothelium in Responses of Vascular Smooth Muscle. Circ Res 53:557-573, 1983.

3. Vanhoutte PM, Rimele TJ: Role of the endothelium in the control of vascular tone. J Phyiol 78:681-686, 1983.

4. Busse R, Trogisch G, Bassenge E: The role of the endothelium in the control of vascular tone. Basic Res Cardiol 80:475-490, 1985.

5. Weksler BB, Eldor A, Falcone D, et al: Prostaglandins and vascular endothelium, in Herman AG, Vanhoutte PM, Denolin H, Goossens A (eds): Cardiovascular Pharmacology of the Prostaglandins. New York, Raven Press, pp 137-148, 1982.

6. Frangos JA, Eskin SG, McIntire LV, Ives CL: Flow Effects on Prostacyclin Production by Cultured Human Endothelial Cells. Science 227:1477-1479,1985.

7. Pohl U, Busse R, Kuon E, Bassenge E: Pulsatile Perfusion Stimulates the Release of Endothelial Autocoids. Journal of Applied Cardiology 1:215-235, 1986.

8. Hickey KA, Rubanyi G, Paul RJ, Highsmith RF: Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. Am J. Physio. 248:C550-C556, 1985.

9. Rubanyi G, Vanhoutte PM: Hypoxia Releases a Vasoconstrictor Substance from the Canine Vascular Endothelium. J. Physiol 364:45-56, 1985.

10. Luscher TF, and Vanhoutte PM: Endothelium-Dependent Contraction to Acetylcholine in the Aorta of the Spontaneously Hypertensive Rat. Hypertension 8:344-348, 1986.

11. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Yazaki Y, Goto K, Masaki T: A Novel Potent Vasocontrictor Peptide Produced by Vascular Endothelial Cells. Nature 332:411-415, 1988.

12. Grifith TM, Edwards DH, Lewis MJ, Newby AC, Henderson AH: The nature of endothelium-derived relaxant factor. Nature 308:645-647. 1984.

13. DeMey JG, Claeys M, Vanhoutte PM: Endothelium dependent inhibitory effects of acetylcholine, adenosine triphosphate, thrombin, and arachdonic acid in the canine femoral artery. J Pharacol Exp Ther 222:166-173, 1982.

14. Pohl U, Holtz J, Busse R, Bassenge E: Crucial role of the endothelium in the vasodilator response to increased flow in vivo. Hypertension 8: 37-44, 1986.

15. Holtz J, Forstermann U, Pohl U, Giesler M, Bassenge E: Flow-dependent, endothelium-mediated dilation of epicardial coronary arteries in conscious dogs: Effects of cyclooxygenase inhibition. J Cardiovascular Pharmacol 6: 1161-1169, 1984.

16. Rubanyi GM, Romero JC, Vanhoutte PM: Flow-induced release of endothelium-derived relaxing factor. Am J Physiol 250:H145-H149, 1986.

17. Rapoport RM, Darznin MB, Murad F: Endothelium-Dependent relaxation in Rat Aorta may be mediated through cyclic GMP-dependent protein phosphorylation. Nature 306:174-176, 1983.

18. Ignarro LJ, Byrns RE, Wood KS: Endothelium-Dependent Modulation of cGMP Levels and Intrinsic Smooth Muscle Tone in Isolated Bovine Intrapulmonary Artery and Vein. Circ Res 60:82-92, 1987.

19. Murad F: Cyclic Guanosine Monophosphate as a mediator of vasodilation. J Clin Invest 78:1-5, 1986.

20. Cohen RA, Shepherd JT, Vanhoutte PM: Inhibitory Role of the endothelium in the response of isolated coronary arteries to platelets. Science 221: 273-274, 1983.

21. Houston DS, Shepherd JT, Vanhoutte PM: Aggregating platelets cause direct contraction and endothelium-dependent relaxation of isolated canine coronary arteries: Role of serotnonin, thromboxane A2, and adenine nucleotides. J Clin Invest 78:539-544, 1986.

22. Radomski MW, Palmer RMJ, Moncada S: The anti-aggregating properties of vascular endothelium: Interactions between prostacyclin and nitric oxide. Br J Pharmac 92: 639-646, 1987.

23. Shimokawa H, Aarhus L, Vanhoutte PM: Porcine coronary arteries with regenerated endothelium have a reduced endothelium-dependent responsiveness to aggregating platelets and serotonin. Circ Res 61:256-270, 1987.

24. Griffith TM, Hughes ED, Lewis MJ, Newby AC, Henderson AH: The nature of endothelium-derived relaxing factor. Nature 308:645-647, 1984.

25. Cocks TM, Angus JA, Campbell JH, Campbell GR: Release and properties of endothelium-derived relaxing factor from endothelial cells in culture. J Cell Physiol 123:310-320, 1985.

26. Gryglewski RJ, Moncada S, Palmer RMJ: Bioassay of prostacyclin and endothelium-derived relaxing factor from porcine aortic endothelial cells. Br J Pharm 87:685-694, 1986.

27. Forstermann U, Trogisch G, Busse R: Species-Dependent Differences in the Nature of Endothelium-derived Vascular Relaxing Factor. Eur J Pharmac 106:639-643, 1984.

28. Rubanyi GM, Vanhoutte PM: Nature of Endothelium-Derived Relaxing Factor: Are There Two Relaxing Mediators? Circ Res 61 Suppl II:II61-II67, 1987.

29. Palmer RMJ, Rerrige AG, Moncada S: Nitric oxide release accounts for the biological activity of the endothelium-derived relaxing factor. Nature 327:524-526, 1987.

30. Ignarro LJ, Byrns RE, Buga GM, Wood KS: Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. Circ Res 61:866-879, 1987.

31. DeMay JG, Vanhoutte PM: Heterogenous behavior of the canine arterial and venous wall. Circ Res. 51:439-447, 1982.

32. Frieman PC, Mitchel GG, Heistad DD, Armstrong ML Harrison DG: Atherosclerosis impairs endothelium-dependent vascular relaxation to acetylcholine and thrombin in primates. Circ Res 58:783-789, 1986.

33. Verbeuren TJ, Jordaens FH, Zonnekeyn LL, Van Hove CE, Coene MC, Herman AG: Effect of hypercholesterolemia on vascular reactivity in the rabbit. I. Endothelium-dependent and endothelium-independent contractions and relaxations in isolated arteries of control and hypercholesterolemic rabbits. Circ Res 58:552-564, 1986.

34. Jayakody L, Seraratne A, Thomson A, Kappagoda T: Endothelium-dependent relaxaion in experiemntal atherosclerosis in the rabbit. Circ Res 60:251-264, 1987.

35. Shimokawa H, Tomoike H, Naybeama S, Yamamoto H, Araki H, Nakamura M: Coronary Artery Spasm Induced in Atherosclerotic Miniature Swine. Science 221: 487 - 491, 1983.

36. Yamamoto Y, Tomoike H, Egashira K, Kobayashi T, Kawasaki T, Nakamura M: Pathogenesis of coronary spasm in miniture swine with regional intimal thickening after balloon denudation. Circ Res 60:113-121, 1987.

37. Schroeder JS, Bolen JL, Quint RA, Clark DA, Hayden WG, Higgins CB, Wexler L: Provocation of Coronary Spasm with Ergonovine Maleate. Am J Cardiol 40: 487, 1977.

38. Waters DD, Szlachic J, Bonan R, Miller DD, Dawue F, Theroux P: Comparative Sensitivity of Exercise, Cold Pressor and Ergonovine Testing in Provoking Attacks of Variant Angina in Patients with Active Disease. Circ 67: 310-315, 1983.

39. Maseri A, L'Abbate A, Barold G, Chierchia S, Marzilli M, Ballestra AM, Severij S, Parodi O, Biagini A, Distante A, Pesula A: Coronary vasospasm as a possible cause of myocardial infarction, a conclusion derived from the study of preinfarction angina. New Eng J Med 229: 1271-1277, 1978.

40. Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander W, Ganz P: Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. New Engl J Med 315:1046-1051, 1986.

41. FontJin A, Sabadell AJ, Ronco RJ: Homogeneous chemiluminescent measurement of nitric oxide with ozone. Implications for continuous selective monitoring of gaseous air pollutants. Anal Chem 42:575-579, 1970.

42. Greenburg S, Rubanyi GM: Ouabain Enhances Basal Release of Endothelium-Derived Relaxing Factor (EDRF) from Canine Femoral Artery (Abstract). Fed Proc 2(4):A316, 1988.

43. Singer HA, Saye JA, Peach MJ: Effects of cytochrome P-450 inhibitors on endothelium-dependent relaxation in rabbit aorta. Blood Vessels 21:223-230, 1984.

44. Pinto A, Abraham NG, Mullane KM: Arachidonic acid-induced endothelium-dependent relaxation of canine coronary arteries: Contribution of a cytochrome P-450-dependent pathway. J Pharm Exp Ther 240:856-863, 1987.

45. Clark DL, Linden J: Modulation of guaunylate cyclase by lipoxygenase inhibitors. Hypertension 8:947-950, 1986.

46. Cannon PJ: Eicosanoids and the blood vessel wall. Circ 70:523-528, 1984.

47. Marcus AJ: The eicosanoids in biology and medicine. J Lipid Res 25:1511-1516, 1984.

48. Long CJ, Shikano K, Berkowitz BA: Anion exchange resins discriminate between nitric oxide and EDRF. Eur J Pharmac 142:317-318, 1987.

49. Shikano K, Ohlstein EH, Berkowitz BA: Differential selectivity of endothelium-derived relaxing factor and nitric oxide in smooth muscle. Br J Pharmac 92:483-485, 1987.

50. Vanhoutte PM: The end of the quest?: Nature 327:459-460, 1987.

51. Murray JJ, Fridovich I, Makhoul RG, Hagen P: Stabilization and partial characterization of endothelium-derived relaxing factor from cultured bovine aortic endothelial cells. Biochem Biophys Res Comm 141:689-696, 1986.

52. Gryglewski RJ, Palmer RMJ, Moncada S: Superoxide anion is involved in the breakdown of endothelium-derived relaxing factor. Nature 320:454-456, 1986.

53. Rubanyi G, Vanhoutte PM: Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. Am J Physiol 250:H822-H827, 1986.

54. Moncada S, Palmer RM, Higgs EA: The discovery of nitric oxide as the endogenous nitrovasodilator. Hypertension, 12(4):365-72, Oct 1988.

55. Sakuma I, Stuehr DJ, Gross SS, Nathan C, Levi R: Identification of arginine as a precursor of endothelium-derived relaxing factor. Proceedings of the National Academy of Sciences of the United 85(22):8664-7, 1988.

Section 2

Mechanisms of Abnormal Endothelium-Dependent Vascular Relaxation in Atherosclerosis

Introduction

Several recent studies have shown that endothelium-dependent vascular relaxation is impaired in human and animal models of atherosclerosis. Responses to acetylcholine, thrombin and other agents are markedly abnormal in atherosclerotic monkeys and rabbits (1-6). Similarly, enhanced constriction to serotonin and histamine is observed in porcine coronary arteries with focal atherosclerotic lesions (7-9). Human coronary arteries with atherosclerosis are more susceptible to spasm induced by ergonovine (10,11) and may be predisposed to spontaneous vascular spasm (10-12). Recently, Ludmer et al. (13) showed that acetylcholine caused constriction of mildly atherosclerotic coronary arteries of patients but caused dilation of normal coronary arteries.

There are three possible explanations for abnormal endothelium-dependent modulation of vascular smooth muscle tone in atherosclerosis. First, production or release of endothelium-derived relaxing factor (EDRF) may be decreased in atherosclerotic vessels. This abnormality may be secondary to alterations of membrane receptors or dysfunction of the pathways leading to EDRF synthesis and release. Second, atherosclerotic vessels may be less sensitive to EDRF. This may be due to the thickened intima or increased lipids in the vessel wall of atherosclerotic vessels acting as a barrier to diffusion of EDRF from the endothelium to the underlying vascular smooth muscle. Alternatively, EDRF may be inactivated by oxygen radicals released by inflammatory cells present in the atherosclerotic vessel wall (14,15). Third, the endothelium of atherosclerotic vessels may release a constrictor factor which may diminish or negate the effect of concomitantly released EDRF.

In the present study, release of EDRF was examined using two independent bioassay procedures. The capacity for EDRF to diffuse through the intimal surface of normal and atherosclerotic rabbit thoracic aortae was also examined. For the latter experiments, two sources of EDRF were used. The first was EDRF released from the endothelial cells of normal rabbit aortae. Since Palmer et al. (16), have recently shown that nitric oxide is possibly one of the non-prostanoid, endothelium-derived vasodilator factors, a nitric oxide solution was used as a second source of EDRF.

Finally, we examined constriction of normal and atherosclerotic vessels to acetylcholine. Some of these studies were performed in the presence of inhibitors of EDRF to unmask the potential effect of a concomitantly released constrictor factor. To test the hypothesis that greater degrees of atherosclerosis might result in greater release of constrictor factors, an 18 month cholesterol-fed rabbit group was also studied.

Methods

Source of Normal and Atherosclerotic Vessels

Atherosclerosis was induced in two groups of New Zealand white rabbits. One group was fed a 1% cholesterol diet for 8-10 weeks. A second group was fed a 1% cholesterol diet for 4 months and subsequently a 0.5% cholesterol diet for 2 months. Control rabbits were maintained on standard rabbit chow. The mean serum cholesterol at the end of 8-10 weeks was 2841 ± 134 mg/dl in the short-term group, 1634 ± 80 mg/dl in the long-term group, and 56 ± 2 mg/dl in the control group.

On the day of the study, rabbits were killed by an overdose of sodium pentobarbital. The thoracic aortae were isolated and excised.

Isolated Ring Preparation

These experiments were performed to examine the effect of atherosclerosis on endothelium-dependent vascular relaxation in isolated vessels. Segments of the descending thoracic aorta (just distal to the aortic arch to the level of the diaphragm) from normal and atherosclerotic rabbits were cut into 5-mm ring segments and suspended in a vertically oriented organ bath in 25 ml of Kreb's buffer (composition in mM: NaCl, 118.3; KCl, 4.7; CaCl, 2.5; $MgSO_4$, 1.2; KH_2PO_4 1.2, $NaHCO_3$, 25; glucose, 11.1; pH 7.40) aerated with 95% O_2, 5% CO_2, and maintained at 37 degrees centigrade. Tension was measured with a linear force transducer (Grass FTO3c) and recorded on an oscillographic recorder.

Over a period of one hour, the resting tension of the vascular ring was gradually increased until the optimal tension for generating force during isometric contraction was reached. At each tension, the vessel was exposed to KCl (100 mM) and the tension recorded. After each KCl dose, the baths were washed with fresh buffer. The resting tension was increased until the responses to KCl increased no further. The vessels were left at this optimal tension for the remainder of the study.

Following preconstriction with PGF2α (0.1 to 1μM), cumulative concentration-response curves were obtained to acetylcholine (1nm to 100 μM) and the calcium ionophore, A23187 (0.03 μM to 3 μM). The amount of relaxation was calculated as the percent decrease in the active tension due to PGF2α. Constriction to acetylcholine was compared in normal and atherosclerotic rings studied in the quiescent (non-preconstricted) state. In some experiments quiescent rings were incubated with either methylene blue (10 μM) or LY83583 (10 μM) for at least one-half hour. These agents negate the effects of EDRF on vascular smooth muscle and thus should unmask the effect of any concomitantly released constrictor factor.

Detection of EDRF Production by a Cascade Bioassay Preparation

EDRF Donor Segments

In these experiments, EDRF release from normal and atherosclerotic vessels was simultaneously examined. Thoracic aorta segments from normal and short term atherosclerotic rabbits were cannulated with stainless steel cannulas, taking care not to damage the endothelium. These vessels were mounted in parallel in an organ bath containing Kreb's buffer solution of the same millimolar concentration as described above with indomethacin (1 μM), phentolamine (3 x 10^{-6} M), and propanolol (1 μM) added. This solution was aerated with 95% O_2, 5% CO_2, and maintained at 37°. The donor vessels were perfused in the direction of their normal flow with the same solution from a separate reservoir at 4 ml/min by a roller pump.

Three to five mm segments from the proximal thoracic aorta from normal rabbits were used as bioassay rings for this preparation. These rings were denuded of endothelium by gently rubbing the intimal surface with the tip of a closed hemostat and suspended below the outflow ports of the organ bath chamber by means of steel stirrups, one of which was attached to a Grass FT03c force transducer. Adequate denudation was confirmed by the absence of relaxation to acetylcholine or calcium ionophore, A23187, applied directly to the rings. Changes in isometric tension generated by the detector vessels were recorded on an oscillographic chart recording device. The organ chamber could be moved freely, thus allowing the detector vessel to be superfused by effluent from either of the mounted vessel segments or a direct channel through a steel tube placed in the organ chamber.

At the beginning of the experiment, the detector vessel was stretched to its optimal tension for constriction to KCl as described above for the isolated ring preparation.

Boluses of increasing concentrations of the receptor-mediated agonist, acetylcholine, or the non-receptor mediated agonist, calcium ionophore, A23187, were injected proximal to the donor vessel and the effluent allowed to superfuse the detector vessel that had been preconstricted with PGF2a. Throughout all experiments, atropine (final concentrations: 100μM) was infused directly into the detector vessel to prevent activation of muscarinic receptors on the vascular smooth muscle of the detector vessel. Care was taken not to expose cannulated vessel segments to this antagonist. The amount of relaxation was calculated as a percent decrease in the active tension generated to PGF2α.

Detection of EDRF Production by Activation of Guanylate Cyclase

The effects of EDRF are though to be related to the activation of smooth muscle guanylate cyclase, followed by a rise in intracellular cGMP (18, 19). It has recently been shown that cultured human umbilical vein endothelial cells contain guanylate cyclase but not functional muscarinic receptors (17). Therefore, the amount of EDRF released from normal and atherosclerotic aortas was quantified by measuring the increase in cGMP levels in cultured endothelial cells incubated for a brief period within these vessels.

Preparation of cultured human umbilical vein endothelial cells

Freshly removed human umbilical cords were cleaned and rinsed free of blood with phosphate-buffered saline, pH 7.4 (0.14 M NaCl, 0.003 M KCl, 0.0012 M KH_2PO_4, 0.008 M Na_2HPO_4, and 0.01 g/liter phenol red). Cords were clamped and veins filled with phosphate-buffered saline containing 0.1% Type I collagenase. Enzymatic digestion was continued for 6 minutes at room temperature. The vein was next drained and rinsed with approximately 50 ml of modified Medium-199. The recovered cells were then subcultured on Cytodex 3 microcarrier beads and incubated in 10% fetal calf serum culture medium for 48 hours. These cells do not express functional muscarinic receptors. Thus,

when the cells alone are exposed to acetylcholine, intracellular cGMP levels do not change (Table 1). However, when incubated within the lumina of segments of rabbit aortae in the presence of 1-methyl-3-isobutylxanthine, a cyclic nucleotide phosphodiesterase inhibitor, and acetylcholine, changes in the concentration of cyclic GMP within the cells directly reflect the stimulatory action of EDRF released from the donor vessel endothelium.

Vessel Preparation

To perform these studies the thoracic aortae of both normal and atherosclerotic rabbits were removed as described above, taking care to preserve the endothelium. The normal and atherosclerotic segment were each cannulated with stainless steel cannulae and mounted in parallel in an organ bath filled with oxygenated Kreb's buffer.

Guanylate Cyclase Activation EDRF Bioassay Preparation

Human umbilical vein endothelial cells cultured on Cytodex 3 microcarrier beads were resuspended to approximately 20 million cells/4 ml of Hanks/HEPES buffer. This suspension was divided into aliquots of 200 µl which were gently rocked at 37 degrees. Immediately prior to incubation within the lumina of the EDRF donor vessels, the cyclic nucleotide phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine, was added to the aliquot to achieve a final concentration of 1 mM. Successive aliquots were incubated within the donor vessels for ten minutes under control conditions and in the presence of 10 µM acetylcholine, a receptor-mediated agonist. Parallel incubations were carried out with cell/bead suspensions in test tubes. After the incubations, the cells were recovered by passing the collected suspension through a filter column. Intracellular cGMP was assayed according to the protocol described by Brotherton (17). Briefly, trichloroacetic acid (final concentration: 10%) was added to the column to lyse the cells and release cellular cGMP. Tritiated cGMP was next added to monitor recovery of cGMP during purification by sequential Dowex/Alumina chromatography. Radioimmunoassay was used to measure the

amount of cGMP in each sample. Data were corrected for recovery of cGMP and the amount of protein in the cell/bead suspension.

EDRF Sensitivity Studies

Serial Perfusion Bioassay Preparation

The specific purpose of this preparation was to examine the sensitivity of normal and atherosclerotic vessels to EDRF applied only to the luminal surface of detector vessels. Two to three centimeter segments of thoracic aorta from either normal or atherosclerotic animals were denuded by passing a wooden applicator stick through the vessel lumen and gently rolling the aorta. These vessels were then cannulated and mounted in a specially designed perfusion bath (Figure 1). This preparation permitted the denuded detector vessels to be perfused in series distal to an endothelium-intact aortic segment from a normal rabbit (EDRF donor vessel). Drugs could be infused proximal (Port 1) or distal (Port 2) to the EDRF donor vessel. Tension was recorded near the center segment of the denuded detector vessel by means of two small wire hooks pierced through the vessel walls, one of which was attached to a Grass FT03c force transducer. Changes in isometric tension generated by the detector vessels were recorded on an oscillographic chart recording device. Resting tension was adjusted to approximately five grams. The effectiveness of endothelial denudation of the detector segment was tested by direct application of A23187 (10^{-5} M, 0.2 cc) to the detector vessel via Port 2. No response to this agent as evidence of adequate denudation.

The perfusion delay to the detector vessel from the donor vessel was approximately 1 second for EDRF and 6 seconds for nitric oxide solution infused through Port 2. To prolong the effects of both EDRF released from the donor vessel and nitric oxide, the

Kreb's buffer was aerated with 20% O_2, 5% CO_2, 75% N_2. We examined cumulative relaxation responses to EDRF (stimulation of donor vessels with A23187; 0.2 cc bolus through port 1, 10^{-8} to 10^{-4} M) and nitric oxide (infused through port 2, 5.4×10^{-7} to 4.9×10^{-5} M). Before each concentration-response curve, the detector vessels were preconstricted with $PGF_{2\alpha}$ (2×10^{-6} M). Between each concentration-response curve, the detector vessels were allowed to equilibrate for one hour. The amount of relaxation was calculated as the percent decrease of the active tension generated in response to $PGF_{2\alpha}$.

Drugs Used

The following agents were used: acetylcholine chloride, atropine sulfate, indomethacin, the calcium ionophore, A23187, propanolol, phentolamine (all purchased from Sigma), $PGF_{2\alpha}$ (Upjohn), and nitric oxide gas (Matheson) and the guanylate cyclase inhibitor, LY83583 (Lilly Pharmaceuticals). All these drugs were prepared in the Kreb's solution except for indomethacin which was prepared as a 10^{-3} M stock solution in $NaCO_3$. A 1% nitric oxide solution was prepared according to the method of Palmer et al. (16). Briefly, we injected 250 μl of nitric oxide gas into a 25 cc gas tight syringe filled with distilled water which had been deoxygenated by bubbling with helium for one hour.

Histological studies

At the completion of an experiment, each vascular segment was preserved in 2.5% glutaraldehyde in 0.1 sodium cacodylate buffer, pH 7.2). EDRF donor vessels were examined by scanning electron microscopy to confirm the presence of endothelium. Segments of detector vessels from the serial perfusion bioassay preparation were stained with hemotoxylin and eosin and examined for the presence of atherosclerosis by light microscopy.

Tissue Cholesterol Content

Total and free cholesterol levels of bioassay EDRF donor vessels and serial perfusion detector vessels were measured. The arterial segments were first homogenized and extracted two times with chloroform:methanol (2:1, vol/vol) (18). The cholesterol content of the lipid extracts was next measured before and after saponification with mild ethanolic alkali at 100° C (19). Cholesterol was measured as the free sterol with a gas chromatograph, using cholestane as an internal standard. Cholesteryl ester content was calculated as the difference of total and free cholesterol fractions.

Data analysis and statistical analysis

Data are presented as the mean ± the standard error of the mean. Relaxation responses to acetylcholine, A23187, EDRF, and nitric oxide are expressed as the percent relaxation from the amount of preconstriction produced by $PGF_{2\alpha}$. Comparisons of plasma and tissue cholesterol levels and vessel responses to each agonist were made between normal and atherosclerotic groups using unpaired student t-tests. A p-value less than 0.05 was considered significant.

Experimental Protocols

Isolated Ring Preparation

Normal and atherosclerotic isolated rings were preconstricted with $PGF_{2\alpha}$ (10^{-7} to 10^{-6} M). Cumulative concentration-response curves were obtained for acetylcholine (10^{-9} to 10^{-4} M) and the calcium ionophore, A23187 (10^{-13} to 3×10^{-6} M). The amount of relaxation was calculated as the percentage decrease in the active tension due to $PGF_{2\alpha}$.

EDRF Production

Cascade Perfusion Bioassay

To examine EDRF production by control and atherosclerotic vessels, a bolus of the receptor-mediated agonist, acetylcholine (0.2 cc of 10^{-9} to 10^{-4} M), or the non-receptor mediated agonist, the calcium ionophore, A23187 (0.2 cc of 10^{-16} to 10^{-4} M), was injected proximal to a donor vessel and the effluent allowed to superfuse the detector vessel that had been preconstricted with $PGF_{2\alpha}$. Throughout all experiments, atropine (10^{-4} M) was infused directly onto the detector vessel to prevent activation of muscarinic receptors on the detector vessel's vascular smooth muscle. The cannulated vessel segments were never exposed to this antagonist. The amount of relaxation was calculated as the percentage decrease in the active tension due to $PGF_{2\alpha}$.

RESULTS

Isolated Rings

The optimal resting tension was similar for normal and short and long term atherosclerotic vessels (4.7 $\pm$ 0.2, 5.0 $\pm$ 0.2, and 5.0 $\pm$ 3 grams respectively). Additionally, the preconstriction by $PGF_{2\alpha}$ was similar between normal, short, and long term atherosclerotic vessels (3.8 $\pm$ 0.4, 4.6 $\pm$ 0.4, and 3.2 $\pm$ 0.5 grams respectively).

Responses to receptor and non-receptor mediated agonists

Relaxations of isolated vessels to the receptor mediated agonist, acetylcholine, were markedly impaired by both short and long term atherosclerosis (Figure 2). The peak relaxation of atherosclerotic isolated vessels was about one third that observed in normal vessels. Relaxation to acetylcholine was markedly impaired by the presence of either the guanylate cyclase inhibitor, LY83583, or methylene blue (Figure 2). Similarly, responses

to the non-receptor mediated agonist, A23187, were also significantly attenuated by atherosclerosis (figure 3).

Studies in non-preconstricted (quiescent) vessels

Acetylcholine produced similar constrictions of normal and long term atherosclerotic vessels studied in the quiescent state (Figure 4). These responses were augmented by methylene blue in normal, but not atherosclerotic vessels. Constrictions to acetylcholine were not altered in either group by the addition of LY83583.

EDRF Release

Cascade Perfusion Bioassay

Injection of acetylcholine and A23187 through the lumina of donor vessels stimulates the release of EDRF which, in these experiments, was quantified by relaxation of detector rings. When injected through atherosclerotic donor vessels, acetylcholine elicited a peak relaxation of detector vessel which was about half that observed when injected through normal vessels (Figure 5). A23187 differed slightly from acetylcholine in that this agent induced a biphasic pattern of EDRF release from normal vessels but not atherosclerotic vessels. Very low concentrations of A23187 induced release of small amounts of EDRF resulting in a stable detector vessel response while concentrations 10^{-6} μM and above eliciting steadily increasing amounts. This early EDRF release response was absent in atherosclerotic vessels. Like acetylcholine, A23187 stimulated less release of EDRF from atherosclerotic vessels than normal (Figure 5).

Since relaxation of detector vessels in these experiments reflects the release of EDRF by donor vessels, these studies provide evidence for decreased release of EDRF by atherosclerotic vessels in response to both receptor and non-receptor mediated stimuli.

Guanylate Cyclase Activation EDRF Bioassay

Exposure to sodium nitroprusside (100 μM) but not acetylcholine (10 μM) increased cGMP levels in human umbilical vein endothelial cells studied in test tubes. When human umbilical vein endothelial cells were incubated within either normal or atherosclerotic aortae without acetylcholine, baseline levels of intracellular cGMP were not different. After stimulation of donor vessels with 10 μM acetylcholine, cGMP levels within human umbilical vein endothelial cells incubated within atherosclerotic vessel segments were approximately half that of cells incubated within normal vessels (Table 1).

EDRF Sensitivity

Serial Perfusion Bioassay

Application of EDRF released from donor vessels stimulated by A23187 to the luminal surface of atherosclerotic detector vessels resulted in significantly greater relaxation than when administered to the luminal surface of normal vessels (Figure 6). Nitric oxide solutions were infused from a gas tight syringe through teflon tubing into port 2 of the preparation. Application of nitric oxide solution to the luminal surface of atherosclerotic detector vessels caused similar concentration-dependent relaxation of atherosclerotic and normal detector vessels (Figure 6). Infusion of deoxygenated water alone into detector vessel perfusate did not change preconstricted tone.

Histological studies and Tissue Cholesterol Content

Short term group

Atherosclerotic lesions were not observed in vessels from the control group. Histological examination showed a mild degree of atherosclerosis in the short term cholesterol-fed group (Figure 7). Examination of detector vessels revealed accumulations of lipid-laden

cells resting on the subendothelial layers. Occasional flat or slightly raised plaques containing lipid-laden cells and extracellular lipid were also seen.

Long term group

In the long term cholesterol-fed group, the degree of intimal thickening was striking. Histological examination revealed diffuse intimal thickening ranging from 2 to 4 times the thickness of the media (Figure 7b). Areas of intimal fibrosis were present and both lipid-laden macrophages and inflammatory cells were present within the intima.

Tissue Cholesterol Content

The tissue contents of cholesterol ester and total cholesterol were respectively 0.87 ±0.11 and 1.34 ± 0.14 mg/g in normals, 1.62 ± 0.18 and 5.38 ± 1.27 in the short term cholesterol fed group, versus 20.77 ± 2.55 and 21.40 ± 2.42 mg/g in the long term cholesterol fed group. Thus, cholesterol short and long term feeding markedly increased the vessel content at both esterified and free cholesterol ($p < 0.05$ for short term and 0.001 for long term).

Discussion

In these experiments, bioactivity of EDRF released by atherosclerotic vessels in response to both receptor and non-receptor mediated agonists was found to be less than that of normal vessels. The sensitivity of normal and atherosclerotic vessels to EDRF and nitric oxide, a possible EDRF, was also compared. Normal and atherosclerotic vessels were similarly sensitive to nitric oxide. Surprisingly, atherosclerotic vessels exhibited increased sensitivity to EDRF obtained from normal donor vessels. We also found no evidence for the concomitant production of an endothelium-derived constrictor factor during stimulation of EDRF release by acetylcholine. Thus these experiments suggest that the predominant

abnormality of endothelium dependent vascular relaxation in atherosclerosis is related to decreased or abnormal release of EDRF rather than altered sensitivity to EDRF.

The vasodilator action of many agents is mediated by the release of a labile, non-prostanoid substance(s) referred to as EDRF (19, 20). The relaxation produced by EDRF is likely caused by activation of soluble guanylate cyclase and the subsequent rise in cyclic GMP levels within smooth muscle cells (21,22). Identification of the chemical nature of EDRF has been complicated by its short half-life and the lack of specific pharmacologic inhibitors. Also, there is evidence that the humoral substance(s) released by endothelium in response to different stimuli, both pharmacologic and mechanical, may vary between species and between vascular beds (21, 24, 25). Recently, Palmer et al. (16) suggested that nitric oxide is at least one of the compounds responsible for the activity of EDRF. Because of these considerations, we examined sensitivity of atherosclerotic vessels to both nitric oxide and EDRF from normal rabbit aortae. The advantage of employing a nitric oxide solution is the ability to administer precise amounts of this relaxing agent to detector vessels. Administering EDRF from a donor vessel has the advantage of allowing assessment of the effects of potential vasodilator substances other than nitric oxide released by donor vessel endothelium.

These studies show that neither EDRF nor nitric oxide are degraded or bound by cellular or chemical elements within the cell wall in early atherosclerosis. It is unlikely that the increased distance from endothelium to smooth muscle alone contributes to abnormal vascular reactivity since endothelium-dependent responses in vessels from monkeys fed an atherogenic diet for 18 months followed by a normal diet for another 18 months were normal in spite of significantly thickened intimal layers (25). The regressed lesions in that study no longer contained active inflammatory cells. It is possible that oxygen radicals released by inflammatory cells in an active lesion may destroy EDRF in transit, thus forming a functional barrier to EDRF.

In addition to examining EDRF production using a cascade perfusion bioassay, we studied the activation of cGMP within human umbilical vein endothelial cells by EDRF released from normal and atherosclerotic vessels. These experiments, like the cascade bioassay study, provide evidence for impaired release of EDRF in response to acetylcholine from atherosclerotic vessels. In contrast to the cascade bioassay studies, these studies examined an autocrine function of EDRF, the capacity to activate guanylate cyclase within the endothelium. The precise function of cGMP within the endothelium has yet to be defined, however, these studies clearly show that cGMP accumulation occurs within endothelial cells as a result of stimulation by EDRF. Furthermore, this autocrine function of the endothelium is abnormal in the setting of diet induced atherosclerosis. These findings, taken together with the results of the cascade perfusion bioassay experiments, strongly support the conclusion that EDRF production is abnormal in atherosclerotic vessels.

Impaired release of EDRF from atherosclerotic vessels might be due to a defect in the receptor mechanisms leading to activation of the EDRF biosynthetic pathway. To rule out receptor abnormalities as the sole mechanism of decreased EDRF production, we also examined EDRF release in response to the calcium ionophore, A23187. This agent directly activates EDRF synthesis and release independent of receptor activation. Atherosclerotic vessels released less EDRF in response to stimulation with A23187 which indicates that one or more cellular events beyond the activation of receptors are impaired. The present experiments suggest that atherosclerosis alters the biosynthetic pathway of EDRF, resulting in decreased synthesis. Alternatively, EDRF may be inactivated or destroyed before leaving the endothelial cell, thus resulting in decreased release and a correspondingly smaller vasodilator effect.

The finding that endothelium-dependent vascular responses in atherosclerotic isolated vessels are abnormal are in agreement with numerous studies performed by other groups

(1-9, 27, 28). We have further demonstrated that decreased or abnormal release of EDRF by atherosclerotic vessels is likely to be the mechanism that underlies this abnormality. Our observation of impaired release of EDRF by atherosclerotic vessels is in agreement with a study performed by Sreeharan et al. (29) who demonstrated decreased EDRF release by atherosclerotic rabbit aortae to acetylcholine stimulation. The aforementioned study, however, did not differentiate between abnormalities of receptor mediated and non-receptor mediated release of EDRF. Verbeuren et al. (4) found no difference in EDRF release between atherosclerotic and normal vessels. However, that particular study examined EDRF production by the abdominal aorta. Since atherosclerosis in this animal model appears to be more severe in the proximal than in the distal aorta (4, 6, 29), it is possible that the endothelial cells of the abdominal aorta were not affected by the atherosclerotic process to the same degree as those located in more proximal segments. None of these previous studies have examined the sensitivity of atherosclerotic vessels to EDRF.

Endothelial cells may release potent vasoconstrictor factors, both under basal conditions and in response to either mechanical or humoral stimuli. Recently, a vasoconstrictor peptide, termed endothelin, has been characterized and factors that influence its synthesis and release defined (31). It is not known if its production is altered by atherosclerosis. Luscher et al. have shown that acetylcholine can stimulate the release of a cyclooxygenase derived vasoconstrictor from the endothelium of hypertensive rat aortas (32). It has been suggested that the superoxide anion may serve as an endothelium-derived constricting factor, released in response to several vasoactive agents (33). Excessive release of a vasoconstrictor substance from the vascular endothelium of atherosclerotic vessels in response to acetylcholine would serve to negate the effect of concomitantly released EDRF. This seems not likely the explanation for impaired endothelium-dependent relaxation in atherosclerosis, however, because atherosclerotic vessels studied in the quiescent state did

not constrict excessively in response to acetylcholine in the presence or absence of inhibition of EDRF's effect.

Summary and Implications

Recent evidence suggests that endothelium-dependent vascular relaxation is abnormal in humans, as well as animals, with atherosclerosis and that abnormalities of endothelial function may play a role in vasospastic syndromes. In the present experiments, we demonstrated that EDRF release in response to both receptor and non-receptor mediated stimuli is reduced or abnormal in atherosclerotic vessels. This abnormality may likely be the primary mechanism resulting in abnormal vascular responses in atherosclerosis. The results of this study may have important implications in understanding the pathophysiolgy of altered vascular reactivity in atherosclerosis and provide further information regarding the chemical identity of the endothelium-derived relaxing factor.

REFERENCES

1. Jayakody, R.L.; Senaratne M.P.J.; Thomson, A.B.R.; Kappagoda, C.T.: Cholesterol feeding impairs endothelium-dependent relaxation of rabbit aorta. Can. J. Physiol. Pharmacol. 63:1206-1209 (1985).

2. Freiman, P.C.; Mitchell, G.G; Heistad, D.D.; Armstrong, M.L.; Harrison, D.G.: Atherosclerosis impairs endothelium-dependent vascular relaxation to acetylcholine and thrombin in primates. Circ. Res. 58:783-789 (1986).

3. Habib, J.B.; Bossaller, C.; Well, S;. Williams, C.; Morrisett, J.D.; Henry, P.D.: Preservation of endothelium-dependent vascular relaxation in cholesterol-fed rabbits by treatment with the calcium blocker PN 200110. Circ. Res. 58:305-309 (1986).

4. Verbeuren, T.J.; Jordaens, F.H.; Zonnekeyn, L.L; Van Hove, C.E.; Coene, M-C; Herman, A.G.: Effect of hypercholesterolemia on vascular reactivity in the rabbit. I. Endothelium-dependent and endothelium-independent contractions and relaxations in isolated arteries of control and hypercholesterolemic rabbits. Circ. Res. 58:552-564 (1986).

5. Chappell, S.P.; Lewis, M.J.; Henderson, A.H: Effect of lipid feeding on endothelium dependent relaxation in rabbit aortic preparations. Cardiovasc. Res. 21:34-38 (1987).

6. Jayakody, L.; Senaratne, M; Thomson, A.; Kappagoda, T: Endothelium-dependent relaxation in experimental atherosclerosis in the rabbit. Circ. Res. 60:251-264 (1987).

7. Shimokawa, H.; Tomoike, H.; Nabeyama, S.; Yamamoto, H.; Araki, H.; Nakamura, M.: Coronary artery spasm induced in atherosclerotic miniature swine. Science 221:560-562 (1983).

8.   Kawachi, Y.; Tomoike, H.; Maruoka, Y.; Kibuchi, Y; Araki, H; Ishii, Y.;
     Tanaka, K.; Nakamura, M.: Selective hypercontraction caused by
     ergonovine in the canine coronary artery under conditions of induced
     atherosclerosis. Circulation 69:441-450 (1984).

9.   Yamamoto, Y.; Tomoike, H.; Egashira, K.; Kobayashi, T.; Kawasaki, T.;
     Nakamura, M.: Pathogenesis of coronary artery spasm in miniature swine
     with regional intimal thickening after balloon denudation. Circ. Res.
     60:113-121 (1987).

10. Schroeder, J.S.; Bolen, J.L.; Quint, R.A.; Clark, D.A.; Hayden, W.G.;
     Higgins, C.B.; Wexler, L.: Provocation of coronary spasm with
     ergonovine maleate. Am. J. Cardiol. 40:487-491 (1977).

11. Waters, D.D.; Szlachcic, J.; Bonan, R.; Miller, D.D.; Dauwe, F.;
     Theroux, P.: Comparative sensitivity of exercise, cold pressor and
     ergonovine testing in provoking attacks of variant angina in patients
     with active disease. Circulation 67:310-315 (1983).

12. Maseri, A.; L'Abbate, A.; Barold, G.; Chierchia, S.; Marzilli, M.;
     Ballestra, A. M.; Severij, S.; Parodi, O.; Biagini, A.; Distante, A.;
     Pesula, A.: Coronary vasospasm as a possible cause of myocardial
     infarction, a conclusion derived from the study of preinfarction angina.
     N. Engl. J. Med. 229:1271-1277 (1978).

13. Ludmer, P.L.; Selwyn, A.P.; Shook, T.L.; Wayne, R.R.; Mudge, G.H.;
     Alexander, R. W.; Ganz, P.: Paradoxical vasoconstriction induced by
     acetylcholine in atherosclerotic coronary arteries. N. Engl. J. Med.
     315:1046-1051 (1986).

14. Gryglewski, R.J.; Palmer, R. M. J.; Moncada, S.: Superoxide anion is
     involved in the breakdown of endothelium-derived vascular relaxing
     factor. Nature 320:454-456 (1986).

15. Rubanyi, G.M.; Vanhoutte, P.M.: Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. Am. J. Physiol. 250:H822-H827 (1986).

16. Palmer, R.M.J.; Ferrige, A.G.; Moncada, S.: Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327:524-526 (1987).

17. Brotherton, A.F.A.: Induction of prostacyclin biosynthesis is closely associated with increased guanosine 3',5'-cyclic monophosphate accumulation in cultured human endothelium. J. Clin. Invest. 78:1253-1260 (1987).

18. Folch, J.; Lees, M.; Stanley, G. H. Sloane: A simple method for the isolation of total lipids from animal tissues. J. Biol. Chem. 226:497-509 (1957).

19. Abell, L.L.; Levy, B.B.; Brodie, B.B.; Kendall, F.E.: A simplified method for the estimation of total cholesterol in serum. J. Biol. Chem. 195:357-366 (1952).

20. Furchgott, R.F.; Zawadzki, J.V.: The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288:373-376 (1980).

21. Griffith, T.M.; Edwards, D.H.; Lewis, M.J.; Newby, A.C.; Henderson, A.H.: The nature of endothelium-derived relaxing factor. Nature 308:645-647 (1984).

22. Murad, F.: Cyclic guanosine monophosphate as a mediator of vasodilation. J. Clin. Invest. 78:1-5 (1986).

23. Forstermann, U.; Mulsch, A.; Bohme, E.; Busse, R.: Stimulation of soluble guanylate cyclase by an acetylcholine-induced endothelium-

derived factor from rabbit and canine arteries. Circ. Res. 58:531-538 (1986).

24. De Mey, J.G.; Vanhoutte, P.M.: Heterogeneous behavior of the canine arterial and venous wall. Importance of the endothelium. Circ. Res. 51:439-447 (1982).

25. Forstermann, U.; Trogisch, G.; Busse, R.: Species-dependent differences in the nature of endothelium-derived vascular relaxing factor. Eur. J. Pharmacol. 106:639-643 (1984).

26. Harrison, D.G.; Armstrong, M.L.; Freiman, P.C.; Heistad, D.D.: Restoration of endothelium dependent relaxation by dietary treatment of atherosclerosis. J. Clin. Invest. 80:1808-1811 (1987).

27. Henry, P.D.; Yokoyama, M.: Supersensitivity of atherosclerotic rabbit aorta to ergonovine. Mediation by a serotonergic mechanism. J. Clin. Invest. 66:306-313 (1980).

28. Aksulu, H.E.; Cellek, S.; Turker, R.K.: Cholesterol feeding attenuates endothelium-dependent relaxation response to acetylcholine in the main pulmonary artery of chickens. Eur. J. Pharmacol. 129:397-400 (1986).

29. Sreeharan, N.; Jayakody, R.L.; Senaratne, M.P.J.; Thomson, A.B.R.; Kappagoda, C.T.: Endothelium-dependent relaxation and experimental atherosclerosis in the rabbit aorta. Can. J. Pharmacol. 64:1451-1453 (1986).

30. Rosenfeld, M.E.; Tsukada, T.; Gown, A.M.; Ross, R.: Fatty streak initiation in Watanabe heritable hyperlipemic and comparably hypercholesterolemic fat-fed rabbits. Arteriosclerosis 7:9-23 (1987).

31. Yanagisawa, M.; Kurihara, H.; Kimura, S.; Tomobe, Y.; Kobayashi, M.; Mitsui, Y.; Yazaki, Y.; Goto, K.; Maski, T.: A novel potent

vasoconstrictor peptide produced by vascular endothelial cells. Nature 332:411-415 (1988).

32. Luscher, T.F.; Vanhoutte, P.M.: Endothelium-dependent contractions to acetylcholine in the aorta of spontaneously hypertensive rat. Hypertension 8:344-348. (1985).

33. Vanhoutte, P.M.; Katusic, Z.S.: Endothelium-derived contracting factor: Endothelin and/or superoxide anion? Trends in Pharmacological Sciences 9:229-230 (1988).

34. Palmer, R.M.J.; Ashton, D.S.; Moncada, S.: Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 333:664-666 (1988).

35. Myers, P.R.; Guerra, R.; Harrison, D.G.: Release of NO and the EDRF from cultured bovine aortic endothelial cells. Am. J. Physiol.: Heart and Circulatory Physiology 256:H1030-H1037 (1989).

TABLE 1

Cyclic GMP Levels within Cultured Endothelial Cells (picograms/ml protein)

	Cells in Tubes	Cells in Vessels	
		Normal Vessel	AS Vessel
Control	3.7 ± 0.7 n=8	1.9 ± 0.6 n=6	2.6 + 0.5 n=8
10 μM Acetylcholine	4.2 ± 0.3[NS] n=8	9.7 ± 1.3 n=8	4.3 ± 0.3* n=8
100 μM Sodium Nitroprusside	34.5 ± 9.1 n=5		

AS = Atherosclerotic
NS = p>0.05 compared to Control cells in tubes
*p<0.05 AS vs. Normal after donor vessel stimulation by 10 μM Acetylcholine

FIGURES

Figure 1: Schematic diagram of the bioassay apparatus used to examine sensitivity of normal and atherosclerotic rabbit thoracic aortae to luminally applied EDRF and nitric oxide. A segment of normal rabbit aorta with intact endothelium was cannulated and mounted perfusion with oxygenated Kreb's buffer. An endothelium-denuded normal or atherosclerotic detector vessel was similarly cannulated and mounted in the chamber such that its lumen could be perfused in series from the EDRF donor vessel. Wire hooks were pierced through the detector vessel wall and were connected to a force transducer for continuous measurement of vessel tension. After preconstriction with $PGF_{2\alpha}$, the luminal surface of the detector vessel was exposed to EDRF released from the normal donor vessel (stimulated by injection of A23187 into Port 1) or to nitric oxide solution infused through Port 2.

Figure 2: Relaxation of thoracic aorta rings from normal rabbits (n=7), normal treated with methylene blue (n=5), control treated with the guanylate cyclase inhibitor LY83583 (n=6), short term cholesterol-fed rabbits (n=6), and long term cholesterol-fed rabbits (n=6) to acetylcholine. Isolated, endothelium-intact segments of rabbit aorta were mounted for measurement of tension development. Studies were performed after preconstriction of $PGF_{2\alpha}$ (0.1 to 1 μM) in the presence of indomethacin (1 μM). Data are presented as mean ± standard error.

Figure 3:   Relaxations to the calcium ionophore A23187 in normal (n=7)

short term atherosclerotic (n=6) and long term atherosclerotic

vessels (n=4). Studies were performed after preconstriction of

$PGF_{2\alpha}$ and in the presence of indomethacin (1 μM). Data are

presented as mean ± standard error.

Figure 4:   Constrictions of normal and atherosclerotic vessels studied in

the quiescent state to acetylcholine. Experiments were

performed in the presence and absence of the guanylate cyclase

inhibitors LY83583 and methylene blue (n=11 for control, 9 for

normal with LY83583, 8 for normal with methylene blue,

7 for AS, 8 for AS with LY83583, and 6 for AS with methylene

blue).

Figure 5:   EDRF release by normal (closed squares) and atherosclerotic

(open squares) rabbit aortas in response to the receptor

mediated agonist, acetylcholine [A], and the non-receptor

mediated agonist, A23187 [B]. Detector vessels denuded of

endothelium and mounted for measurement of tension development

were superfused with effluent from thoracic aortas of normal or

atherosclerotic rabbits. Studies were performed in the

presence of indomethacin (1 μM). Detector vessels were

preconstricted by $PGF_{2\alpha}$. Responses of detector vessels are

presented as mean ± standard error (*p<0.05 compared to normal

vessel responses). Acetylcholine caused the release of about

twice as much EDRF from normal (n=7) compared to

atherosclerotic (n=7) rabbit aortae. Similarly, A23187 caused

the release of greater amounts of EDRF from normal (n=7) compared to atherosclerotic (n=8) vessels.

Figure 6: Relaxation of endothelium-denuded normal (closed squares, n=6) and atherosclerotic (open squares; n=7) rabbit aorta segments in response to the luminal application of EDRF derived from a normal rabbit aorta [A] and nitric oxide solution [B]. An endothelium-denuded normal or atherosclerotic detector vessel was cannulated and mounted in an organ bath and perfused in series with an endothelium-intact donor vessel. Stainless steel wire stirrups pierced through the detector vessel wall and connected to a force transducer allowed continuous measurement of vessel tension. Detector vessels were preconstricted with $PGF_{2\alpha}$. The luminal surface of detector vessels was exposed to EDRF from donor vessels [Panel A] or nitric oxide solution [Panel B] and relaxation responses measured. Responses of detector vessels are presented as mean ± standard error (*p<0.05 compared to normal vessel responses).

Figure 7: A. Cross-section of a thoracic aorta from a rabbit fed a 1% cholesterol diet for 6 months (hematoxylin and eosin stain, 60X). The atherosclerotic lesion involves approximately 40% of the vessel circumference. Scattered lesions similar to these were observed throughout the thoracic aorta.

B. Cross-section of thoracic aorta, rabbits fed a 1% cholesterol diet four months followed by a 0.5% cholesterol diet for the subsequent 2 months (hematoxlyn and eosin stain, 40X). Marked atherosclerotic intimal proliferation is present

involving the entire circumference of the thoracic aorta.
Throughout all sections the intima was 2 to 4 times greater in
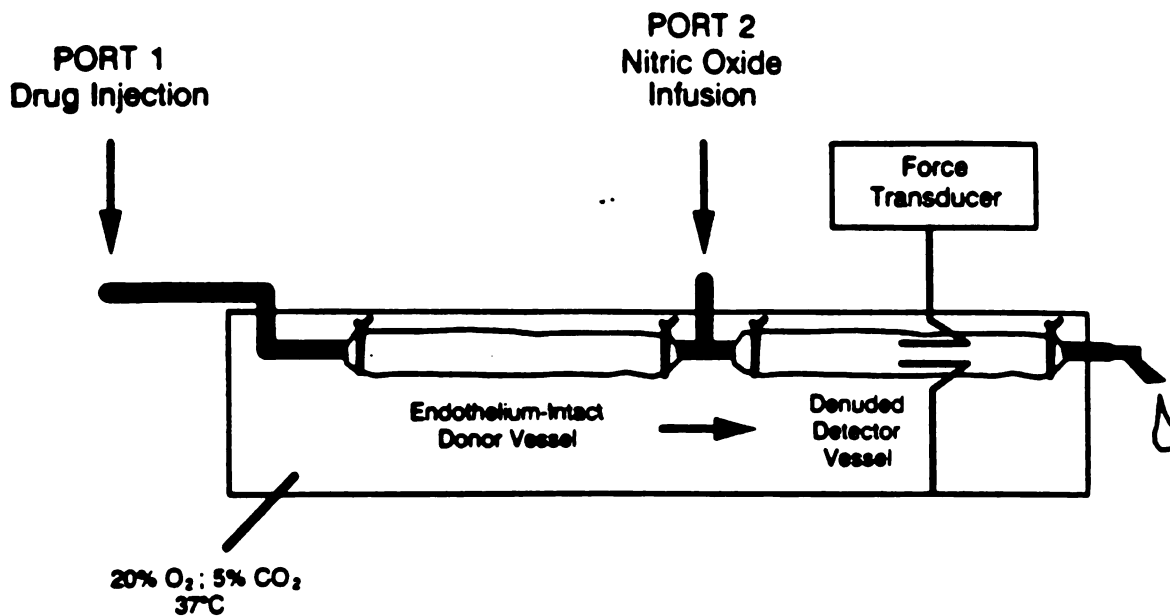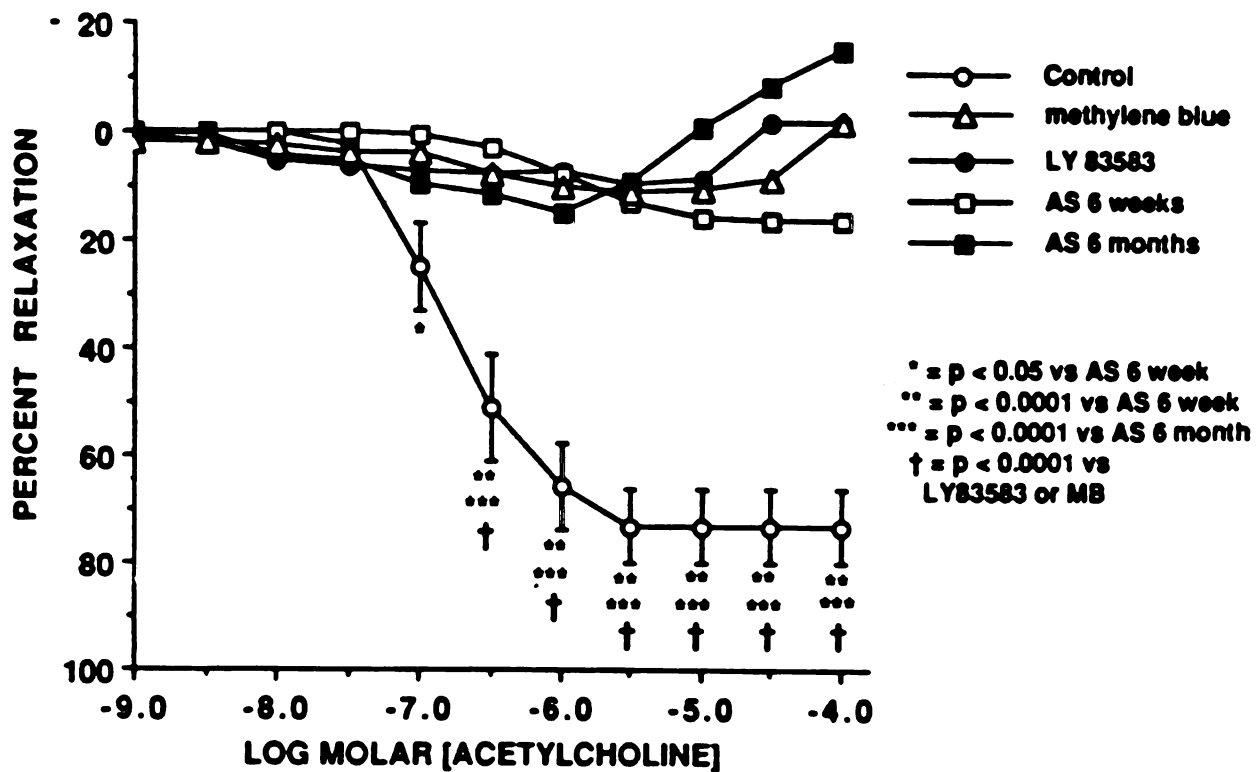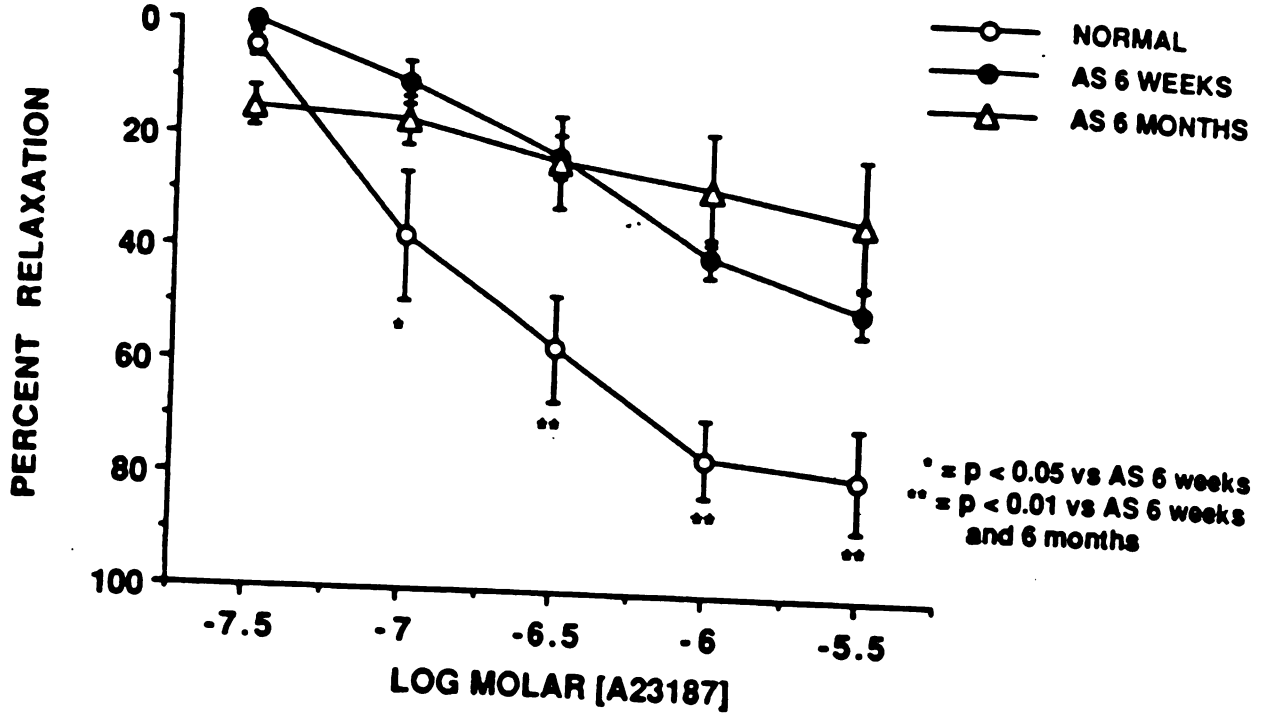thickness than the media.

PORT 1
Drug Injection

PORT 2
Nitric Oxide
Infusion

Force
Transducer

Endothelium-intact
Donor Vessel

Denuded
Detector
Vessel

20% O_2 : 5% CO_2
37°C

FIGURE 1

* = p < 0.05 vs AS 6 week
** = p < 0.0001 vs AS 6 week
*** = p < 0.0001 vs AS 6 month
† = p < 0.0001 vs
LY83583 or MB

Control
methylene blue
LY 83583
AS 6 weeks
AS 6 months
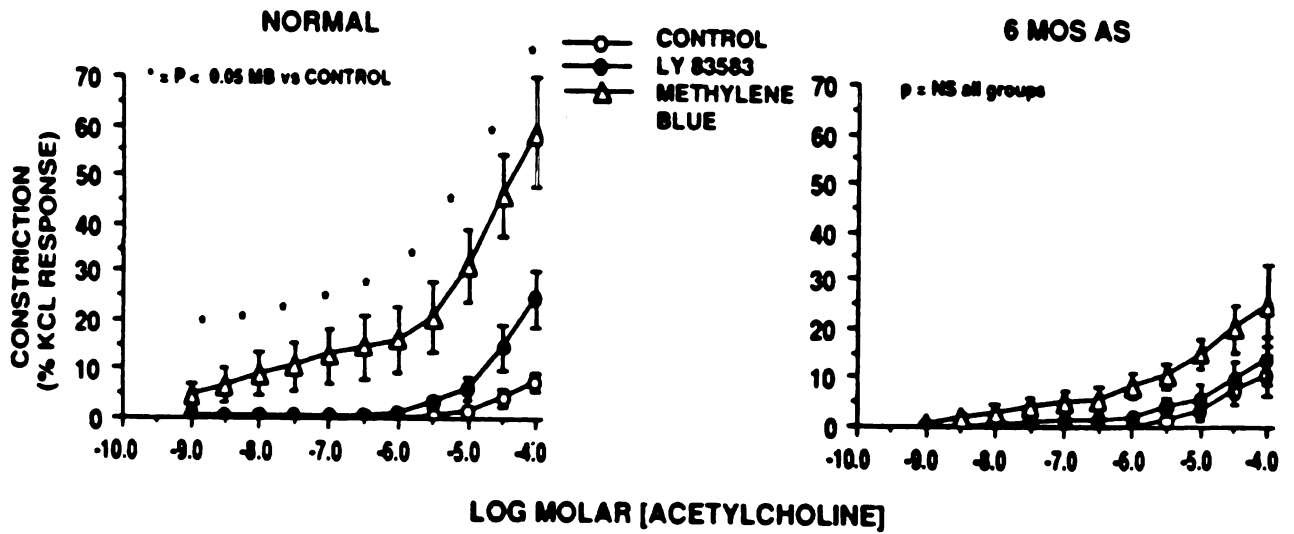
FIGURE 2
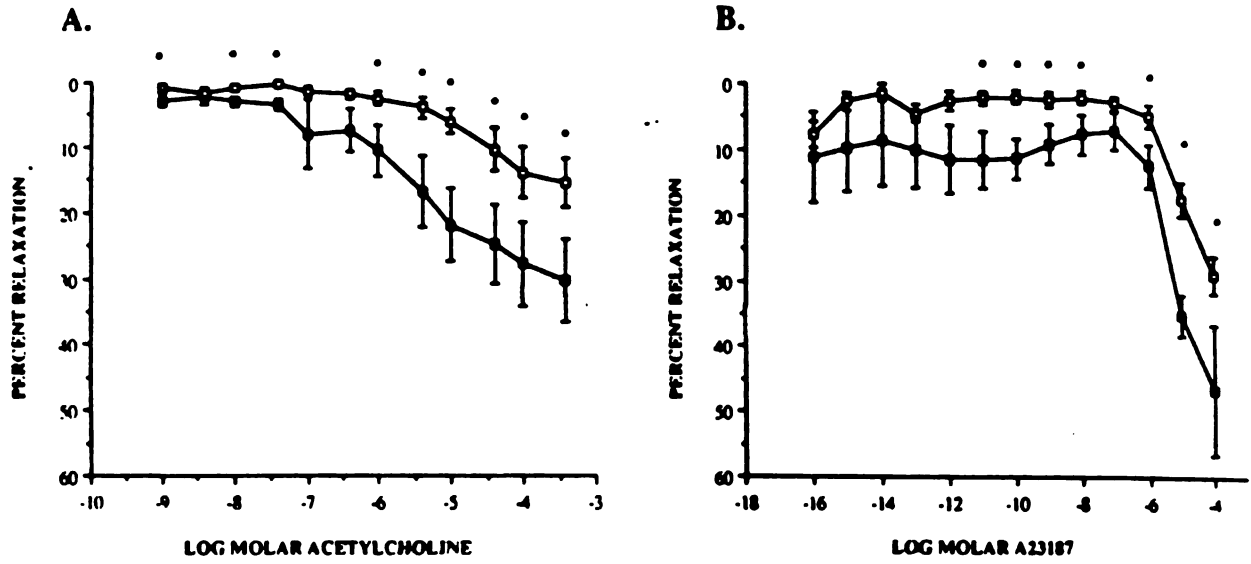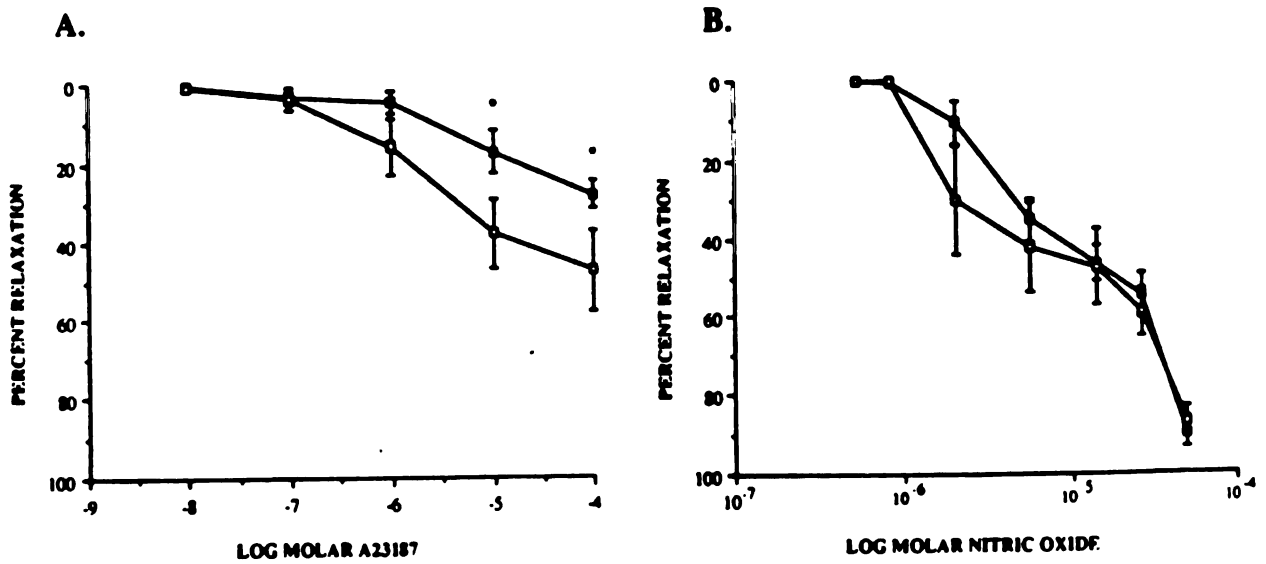
FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6

FIGURE 7

Section 3

Release of Nitric Oxide and EDRF from Cultured Bovine Aortic Endothelial Cells

The next group of studies presents information regarding the chemical identity of EDRF.

The Endothelium-Derived Relaxing Factor (EDRF) is a labile, non-prostanoid substance released both spontaneously and in response to a variety of neurohumoral stimuli (4, 6, 8). The short half-life of this substance has made chemical identification of this compound(s) difficult. Recently, evidence has been presented that EDRF is nitric oxide (7, 11, 21). Palmer and co-workers (21) have used a chemiluminescence technique to demonstrate that nitric oxide may be detected in the effluent of superfused endothelial cells in culture. Subsequent work has suggested that nitrite and nitric oxide produced in mammalian cells may be derived from arginine (10, 14, 20).

Despite these observations, there is evidence to suggest that EDRF and nitric oxide are not identical. The nitric oxide measurement technique used by Palmer and co-workers (21) involves a chemical process that can nonspecifically cleave nitric oxide from several nitroso compounds. This raises the possibility that nitric oxide observed in their study may have been derived from another, nitric oxide containing, compound. Also, several groups have reported discrepancies between chemical and biological characteristics of nitric oxide and EDRF. For example, anion exchange columns have been reported to bind naturally derived EDRF but to have no effect on nitric oxide (18). Furthermore, the effects of natural EDRF and nitric oxide on nonvascular smooth muscle have been reported to differ (1, 5, 23). Finally, there may be more than one EDRF (24) as non-prostanoid relaxing factors may not only differ between species and vascular beds, but also with respect to the various stimuli used to elicit the factor's release (15, 17, 24), both physical and pharmacological.

The ability to account for all of EDRF's vasorelaxant activity by nitric oxide simultaneously released from the same source is critical to the conclusion that EDRF is nitric oxide. The present study was undertaken to determine whether the amount of nitric oxide released from cultured endothelial cells could account for all of the vasodilator effects of EDRF. A bioassay technique coupled with a chemiluminescence method to monitor nitric oxide released from cultured bovine aortic endothelial cells was employed. The relaxing properties of standard quantities of authentic nitric oxide were compared with the amount of nitric oxide and the total relaxing activity of EDRF released from cultured endothelial cells.

METHODS

Cultured endothelial cells as a source of EDRF

Cultured bovine aortic endothelial cells were obtained as previously described. Characteristics of this cell line have been previously described in detail (9, 16). Cells from passage 7 to 10 were grown to confluence over three days on Cytodex 3 microcarrier beads in Medium 199 with Earle's salts and 20% fetal calf serum.

On the day of this study, ~ 100-500 x 106 cells were placed in an 8-ml holding chamber that was placed in a heated water bath maintained at 37 degrees C. The chamber was continuously perfused at a rate of 4 ml/min with Kreb's buffer of the following composition (in mM): NaCl, 118.3; KCl, 4.7; CaCl, 2.5; MgSO4, 1.2; KH2PO4 1.2, NaHCO3, 25; glucose, 11.1; pH 7.40 aerated with 95% O2, 5% CO2, and maintained at 37 degrees centigrade.

Preparation of bioassay vascular ring for detection for EDRF.

Bioassay vascular rings were prepared from 3-5 mm segments of porcine (n=13) or canine (n=3) proximal circumflex arteries. These rings were denuded of endothelium by gently rubbing the intimal surface with the tip of a closed hemostat and suspended below the outflow ports of the organ bath chamber by means of steel stirrups, one of which was attached to a Grass FT03c force transducer. Changes in isometric tension generated by the detector vessels were recorded on an oscillographic chart recording device. The rings were positioned such that they could be superfused via a direct perfusion channel or via effluent from the cultured endothelial cells. The transit time from the cultured cells to the bioassay detector ring was 3 seconds.

Over a period of one hour, the vascular ring was stretched to an optimal resting tension for generating force during isometric contraction (~1-2 grams). The vessels were left at this optimal tension for the remainder of the study. Each ring's reconstricted tension was obtained using 0.1-1 μM prostaglandin F2α.

Preparation of authentic nitric oxide

Twenty-five micoliter of authentic nitric oxide gas (Matheson) were dissolved in 25 ml of distilled water previously deoxygenated by vigorous bubbling for at least one hour with

helium. Since nitric oxide is highly soluble in water (7.38 ml/100ml at 1 atm., 0 C), this yielded a 45.5 μM stock solution of nitric oxide. The solution was maintained in a gas-tight syringe and remained stable (as assessed by assay with the chemiluminescence technique) for several hours.

Measurement of nitric oxide released from cultured endothelial cells by chemiluminescence

Nitric oxide was measured using a nitric oxide analyzer commercially produced by either Bendix (Model 810c: Lewistown, WV) or Dasibi (Model 2108; Glendale, CA). The device is schematically illustrated in Figure 1. Effluent from either cultured endothelial cells or the direct perfusion channel was allowed to pass through a degassing chamber. In this chamber, the effluent was exposed to a constant stream of inert nitrogen gas under a vacuum. Injected nitric oxide or nitric oxide released from cultured endothelial cells was carried by this stream into the nitric oxide analyzer. Within the analyzer, the gas was heated to 45 C and mixed with ozone in a reaction chamber. Ozone and nitric oxide spontaneously react to release light at 6500 - 8000 Å wavelength. The amount of light generated by this system is concentration dependent and is measured with a photomultiplier tube, thus allowing an accurate quantification of nitric oxide.

In some experiments, the device was modified such that the degassing chamber was replaced with a reflux chamber. Effluent from cultured cells was refluxed in a solution of 1% sodium iodide in glacial acetic acid. Exposure to this powerful reducing environment was employed to degrade nitric oxide containing compounds, releasing nitric oxide, which could then be measured by chemiluminescence, as described for the non-reflux experiments. This treatment also converts NO2 to nitric oxide as well as preserving nitric oxide.

Drugs

Bradykinin, the calcium ionophore, A23187, indomethacin, and hemoglobin were all obtained from Sigma Chemical (St. Louis, MO). Prostaglandin F2a was obtained from UpJohn (Chicago, IL). Bradykinin and PGF2a were dissolved in distilled water and subsequently diluted in controlled buffer solution to desired concentrations. Indomethacin was dissolved in 0.9 NaCl at a pH of 8. Hemoglobin was prepared according to the method of Martin and coworkers (19). Briefly, a 10^{-3}M solution of bovine hemoglobin was added to an equal volume of a 10^{-2}M solution of sodium dithionite. This was subsequently dialyzed in distilled water (60 x volume) bubbled with nitrogen for two

hours to remove the sodium dithionite. This solution was stored for no more than 24 hours before use. All other agents were prepared the morning of use.

Protocol

All studies were performed in the presence of indomethacin (1 μM). The bioassay vascular ring was initially superfused with the control solution through a direct channel. After reaching a stable preconstriction (3.5 ± 0.2 grams), the bioassay vascular ring was superfused with effluent from the bovine aortic endothelial cells, and the amount of relaxation was observed for 5-7 minutes (until a stable baseline was reached). The effluent from the bovine aortic endothelial cells was then directed to the nitric oxide analyzer and the released nitric oxide was monitored. The transit times from the bovine aortic endothelial cells to the detector vessel and to the degassing chamber of the nitric oxide analyzer were identical (3 seconds).

In six experiments (without acid reflux), the release of nitric oxide and EDRF in response to Bradykinin (0.01 μM) was subsequently examined. Bradykinin was infused for 10 minutes while the effluent from bovine aortic endothelial cells remained directed to the nitric oxide analyzer. After the nitric oxide signal reached a new stable level, the cell effluent was then directed to the bioassay vascular ring and relaxation recorded (Figure 5).

An identical protocol that examined the effect of bradykinin on EDRF and nitric oxide release was employed in nine experiments with reduction treatment of cell effluent. Subsequently, in five of these experiments and in one additional study, an identical protocol (with acid reflux) was followed examining EDRF and nitric oxide release in response to the calcium ionophore, A23187 (10 μM). In these latter experiments, A23187 was always administered at least 15 minutes after Bradykinin.

In seven experiments, the effect of hemoglobin on relaxations of the bioassay vascular ring was assessed. Hemoglobin (10μM), infused distal to the cultured cells, consistently reversed basal relaxations and relaxations to Bradykinin and the calcium ionophore, A23187.

At the conclusion of each study, preconstricted bioassay rings were exposed to increasing concentrations of nitric oxide infused in to the direct perfusion channel (Figure 3). The transit time from the point of infusion to the bioassay ring was identical to that of the perfusate passing from the cultured endothelial cells to the bioassay vessel.

Data Analysis

Data are presented as means ± SEM. The nitric oxide analyzers were calibrated daily using known concentrations of authentic nitric oxide. The amount of nitric oxide released from cultured endothelial cells in the presence or absence of acid reflux preprocessing was determined using the respective nitric oxide standard curve. The signal (in parts per million) obtained during infusions of nitric oxide standard solutions was compared with the nitric oxide signal obtained from cultured endothelial cells under either basal conditions or during stimulation with bradykinin or the calcium ionophore, A23187. Relaxation of the bioassay vascular rings was expressed as a percent of the preconstricted tension. The amount of nitric oxide necessary to account for equivalent relaxation of the bioassay ring was compared with the amount of nitric oxide produced by cultured endothelial cells using paired t-tests with an appropriate Bonferonni correction for multiple comparisons.

RESULTS

Effects of nitric oxide on bioassay detector vessels

Nitric oxide infused on the bioassay detector tissue produced concentration-dependent relaxation of the detector vessel with a peak relaxation of 100% of the preconstricted tension at 4μM and an ED50 of 0.5 μM (Figure 3).

Measurement of authentic nitric oxide by chemiluminescence

Nitric oxide standard curves were determined with and without acid reflux preprocessing (Figures 2 and 4). Under both conditions, nitric oxide could be detected at concentration substantially less than that needed to cause relaxation of the bioassay detector vessel. Acid reflux substantially increased the sensitivity of the nitric oxide detector (Figure 4).

Release of EDRF from cultured bovine aortic endothelial cells

Bovine aortic endothelial cells spontaneously released sufficient amounts of EDRF to relax the detector ring by 41 ± 8% of the preconstricted tension (Figures 5-7). Neither bradykinin nor A23187 relaxed the detector ring when directly applied to this tissue. When perfused through bovine aortic endothelial cells, 0.01 μM bradykinin stimulated the release of additional EDRF, sufficient with the basally released EDRF to produce relaxation of the

detector ring by 74 ± 7% (Figure 5 and 7). Similarly, A23187 (10 µM) relaxed the detector ring by 69 ± 13% (Figure 6 and 7).

Detection of nitric oxide from bovine aortic endothelial cells, comparison with nitric oxide standard curves, and nitric oxide effect on bioassay detector vessels

Without chemical reduction treatment, nitric oxide was not detected in the effluent of bovine aortic endothelial cells under basal conditions in four of six experiments. In the other two experiments, quantities of nitric oxide comparable to 0.1 and 0.4 µM, as calculated from standard curves of nitric oxide, were obtained in the acid reflux. During bradykinin infusion, quantities of nitric oxide were detected equivalent to 0.3 ± 0.06 µM (Figure 7).

With acid reflux preprocessing, nitric oxide was detected during the basal release of EDRF in amounts equivalent 0.12 ± 0.03 µM as calculated from the standard nitric oxide curves obtained during acid reflux. During 0.01 µM bradykinin infusion, the detected nitric oxide increased to 0.21 ± 0.05 µM (Figures 5 and 7). During infusion of the calcium ionophore, A23187, the amount of nitric oxide detected after acid reflux preprocessing was equivalent to 0.16 µM (Figure 6 and 7).

Comparison of nitric oxide detected with nitric oxide required to account for detector vessel relaxation

Figure 7 compares the relaxation of detector rings observed under basal conditions with the amount of nitric oxide released and the amount of nitric oxide necessary to account for the degree of relaxation. Under both basal conditions and during stimulation with bradykinin and A23187, the amount of nitric oxide detected was consistently 7-10-fold less than that required to account for the entire biological activity of EDRF.

The above discrepancy could not be accounted for by binding or degradation of nitric oxide released from bovine aortic endothelial cells by other substances co-released with nitric oxide. Additional authentic nitric oxide injected into the effluent of bovine aortic endothelial cells releasing EDRF either basally or in response to calcium ionophore, A23187, repeatedly produced a chemiluminescence signal, which was identical to that detected when nitric oxide was injected only into the control buffer solution (Figure 6).

DISCUSSION

In the present experiments, a chemiluminescence technique was used to quantify nitric oxide release from cultured endothelial cells during both spontaneous and agonist-stimulated EDRF release. In both instances, even after reflux of the cell effluent in a strong reducing environment, the amount of nitric oxide detected (compared with the amount detected when standard quantities were infused under identical conditions) was substantially less than that required to account for the entire vasorelaxant activity of EDRF released from bovine aortic endothelial cells.

On the basis of these studies, one can not conclude that EDRF released from bovine aortic endothelial cells, either spontaneously or in response to bradykinin or the calcium ionophore, A23187, is solely or principally nitric oxide. At least three possible explanations exist for the discrepancy between the amount of nitric oxide released and the amount of nitric oxide required to account for EDRF's biological activity.

First, it is conceivable that EDRF is a nitric oxide containing compound that is substantially more potent than authentic nitric oxide. This would also be compatible with observations of others suggesting substantial biological and chemical differences between nitric oxide and naturally derived EDRF (1, 5, 18, 23). It is possible that nitric oxide formed within endothelial cells reacts with other compounds to produce a potent vasodilator substance. Nitric oxide derived from pharmacological vasodilators is thought to react intracellularly with sulfhydryl groups (12, 13). Whether or not nitrosothiols or other other nitrocompounds can be released from intracellular sites either spontaneously or in response to physical or chemical stimuli has not been established. If EDRF is a nitric oxide containing or forming compound, it may require conversion to nitric oxide by the vascular smooth muscle before it exerts its vasodilatory effect. It is possible that such a process is specific for vascular smooth muscle and does not occur in non-vascular smooth muscle.

Second, it is also conceivable that other non-prostanoid vasodilators are co-released in addition to either nitric oxide or a nitric oxide containing or forming compound. The cumulative vasorelaxant activity of the these EDRF's would exceed that of the detected nitric oxide. This hypothesis is compatible with the evidence that there may be more than one EDRF (20, 21).

Finally, it is possible that other substances released simultaneously from endothelial cells may bind or destroy nitric oxide. This is unlikely because nitric oxide could be detected by

the chemiluminescence technique when injected into the effluent of cells that had been stimulated to release EDRF (Fig. 6).

Since all experiments were performed in the presence of indomethacin, it is unlikely that the vasorelaxing activity of the effluent from bovine aortic endothelial cells in the present study was induced by prostacyclin or other cycloxygenase products. Furthermore, desaturated hemoglobin consistently reversed relaxations to the effluent from cultured endothelial cells under basal conditions and during stimulation with either bradykinin or the calcium ionophore, A23187.

Relationship to previous research

These experiments are the first to compare the vasorelaxing activity of non-prostanoid EDRF with that of nitric oxide simultaneously released from bovine aortic endothelial cells. Previous work has shown that nitric oxide can be detected, after exposing effluent from cultured endothelial cells to a strong reducing environment stimulated to release EDRF by bradykinin (21). However, the relationship between the amount of nitric oxide released and the amount required to account for all of EDRF's vasorelaxing activity, however, was not precisely analyzed in prior reports.

The present experiments were performed using bovine aortic endothelial cells. There is substantial evidence that the nature of EDRF may vary depending on vascular bed and species. The present findings therefore, may not be applicable the endothelial cells obtained from other vessels or to intact vessels. These data do, however, show that for bovine aortic endothelial cells, the biological activity of EDRF released basally and in response to two agonists, bradykinin and the calcium ionophore, A23187, cannot be accounted for by nitric oxide release.

Use of acid reflux to detect nitric oxide

The chemical reduction process used in prior reports and in the present study is a very non-specific reduction reaction that serves to release nitric oxide from many nitric oxide - containing compounds (3). The benefit of this methodology in detecting nitric oxide is that nitric oxide in the presence of oxygen is converted spontaneously to NO2, which cannot be detected by the chemiluminescence technique. Thus the process is advantageous in recovering nitric oxide that has been converted to NO2. This undoubtedly accounts for the increased sensitivity of the chemiluminescence technique during acid reflux (Figure 4). A variety of other compounds, however, including nitrosothiols and nitrosamines can be

degraded to nitric oxide by reflux with acetic acid and sodium iodide (25). Thus the detection of nitric oxide after such preprocessing does not conclusively demonstrate that authentic nitric oxide is accountable for any or all of EDRF's effect.

In summary, the present studies show that the release of nitric oxide from cultured endothelial cells cannot account for EDRF's vasorelaxant activity. It is conceivable that EDRF is a nitroso-compound with biological activity substantially greater than that of authentic nitric oxide or that other non-prostanoid vasodilators are released with nitric oxide which represent a large portion of EDRF's biological activity.

Section 3 References

1. Berkowitz, BA, EH Olstein, and K Shikano. Progress on the characterization of endothelium-derived relaxing factor (Abstract). Pharmachologists 29:128, 1987.

2. Collins, P, SP Chappell, TM Griffith, MJ Lewis, and AH Henderson. Differences in basal endothelium-derived relaxing factor activity in different artery types. J Cardiovasc. Pharmacol. 8:1158-1162, 1986.

3. Cox, R. Determination of nitrate and nitrate at the parts per billion by chemical luminescence. Anal. Chem. 52:332-335, 1980.

4. De Mey, JG, and PM Vanhoutte. Role of the intima in cholinergic and purinergic relaxation of isolated canine femoral arteries. J.Physiol. Lond. 316:347-355, 1981.

5. Dusting, GJ, MA Read, and AG Stewart. Endothelium-derived relaxing factor released from cultured cells: Differentiation from nitric oxide. Clin. Exp. Pharmacol. Physiol. 15:83-92, 1988.

6. Furchgott, RF Role of endothelium in responses of vascular smooth muscle. Circ. Res. 53:557-573, 1983.

7. Furchgott, RF Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In: Mechanisms of Vasodilation, edited by PM Vanhoutte. New York: Raven, vol. IV. In Press.

8. Griffith, TM, DH Edwards, MJ Lewis, AC Newby, and AH Henderson. The nature of endothelium derived vascular relaxant factor. Nature Lond. 308:645-647, 1984.

9. Hadjiagopious, C, TL Kaduce, and AA Spector.Eicosapentaenoic acid utilization by bovine aortic endothelial cells: effects on prostacyclin production. Biochim. Biophys. Acta 875:369-381, 1986.

10. Hibbs, JB, Jr., RR Taintor, and Z Vavrin. Macrophage cytotoxicity: role of L-arginine deaminase and imino nitrogen oxidation to nitrate. Science Wash. DC.235:473-476, 1987.

11. Ignarro, LJ, RE Byrns, GH Buga, and KS Wood. Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. Circ. Res. 61:866-879, 1987.

12. Ignarro, LJ, H Lippton, JC Edwards, WH Baricos, AL Hyman, PJ Kadowitz, and CA Gruetter. Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrates, nitroprusside and nitric oxide: evidence for the involvement of S nitrosothiols and active intermediates. J Pharmacol. Exp. Ther. 218: 739-749,1981.

13. Ignarro, LJ, and CA Gruetter. Requirement of thiols for activation of coronary arterial guanylate cyclase by glyceryl trinitrate and sodium nitrate. Possible involvement of S nitrosothiols, Biochim. Biophys. Acta 631:221-231, 1980.

14. Iyengar, R, DJ Stuehr, and MA Marletta. Macrophage synthesis of nitrite, nitrite, and N-nitrosamines:precursors and role of the respiratory bust (Abstract). Proc. Natl. Acad. Sci. USA 84:6369, 1987.

15. Forstermann, U, G Trogisch, and R Busse. Species-dependent differences in the nature of endothelium-derived vascular relaxing factor. Eur. J. Pharmacol. 106:639-643, 1984.

16. Kaduce, TL, AA Spector, and RS Bar. Linoleic acid metabolism and prostaglandin production by cultures bovine pulmonary artery endothelial cells. Arteriosclerosis 2:380-389, 1982.

17. Kontos, HA, EP Wei, JT Povlishock, and CW Christman. Oxygen radicals mediate the cerebral arteriolar dilation from arachidonate and bradykinin in cats. Circ. Res. 55:295-303, 1984.

18. Long, CJ, K Shikano, and BA Berkowitz. Anion exchange resins discriminate between nitric oxide and EDRF. Eur. J Pharmacol. 142:317-318, 1987.

19. Martin, W, GM Villani, D Jothianandan, and RF Furchgott. Blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation of rabbit aorta by certain ferrous hemoproteins.J Pharmacol. Exp. Ther. 233: 679-685, 1984.

20. Palmer, RMJ, AG Ferrige, and S Moncada. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature Lond. 327:524-526, 1987.

21. Palmer, RMJ, AG Ferrige, and S Moncada. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature Lond.327: 524-526, 1987.

22. Rubanyi, GM, RR Lorenz, an PM Vanhoutte. Bioassay of endothelium-derived relaxing factor(s): inactivation by catecholamines. Am J. Physiol.249 (heart Circ. Physiol. 18): H95-H101, 1985.

23. Shikano, K, and BA Berkowitz. Endothelial derived relaxing factor is a selective relaxant to vascular smooth muscle. J Pharmacol. Exp. Ther. 243:65-70, 1987.

24. Rubanyi, GM, and PM Vanhoutte. Nature of endothelium-derived relaxing factor:ar there two relaxing mediators? Circ. Res. 61:II-161-II-167, 1987.

25. Walters, CL, CS Dyke, MJ Saxby, and R Walker. In: Environmental N-Nitroso Compounds Analysis and Formation, Lyon, France: IARC Scientific. 1976, vol. 14, p.191.
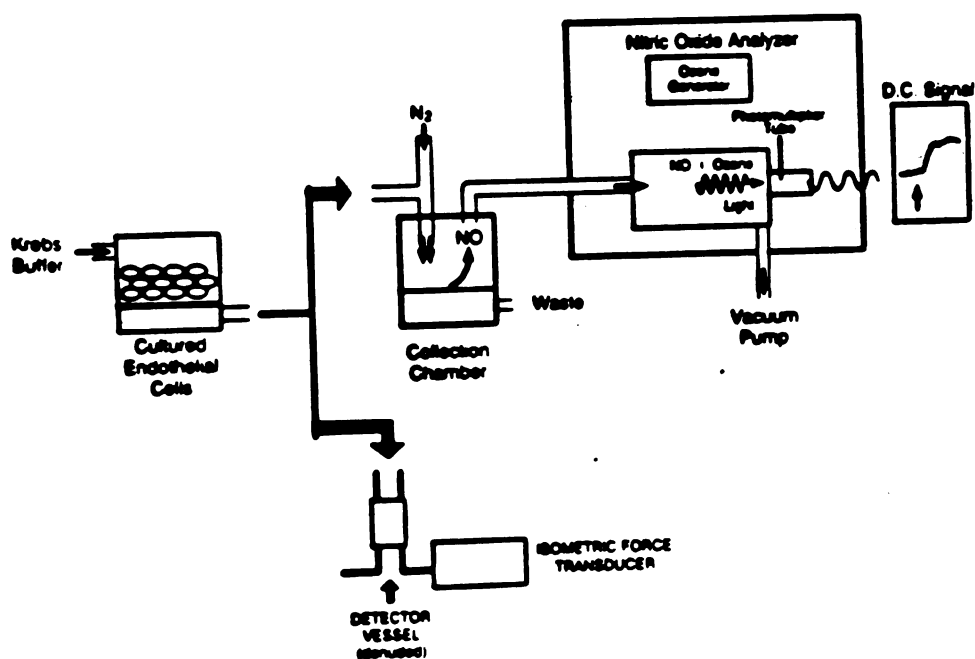
FIG. 1. Experimental preparation employed. Effluent from cultured endothelial cells either superfused a detector vessel or was diverted into a degassing chamber of the nitric oxide analyzer device (n = 6). In other experiments (n = 10), the degassing chamber was replaced with a reflux chamber containing a strong reducing environment of 1% sodium iodide in glacial acetic acid. Nitric oxide was carried into a reaction chamber by a stream of inert nitrogen gas under negative pressure. Here nitric oxide was exposed to ozone. This reaction released light detected with a photomultiplier tube that generated a DC signal proportional to the amount of nitric oxide present.
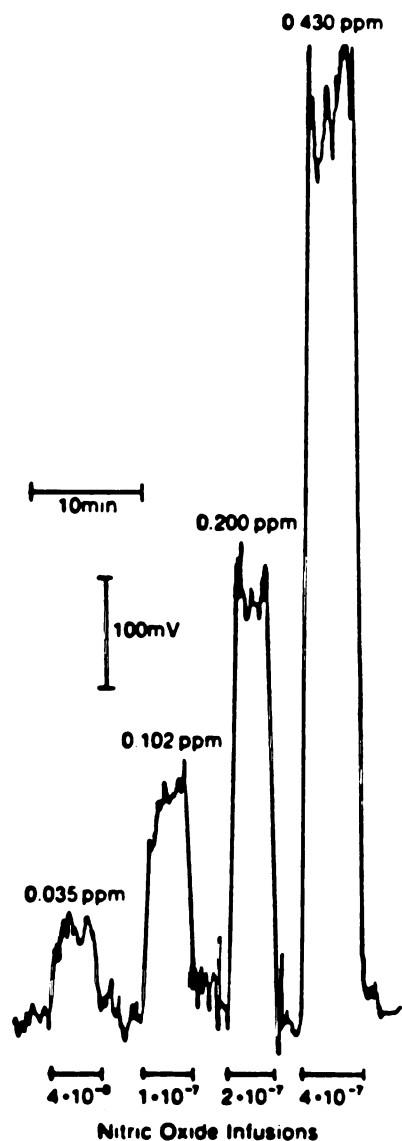
FIG. 2. Nitric oxide signals detected by chemiluminescence during 10-min infusions of known quantities of nitric oxide after reflux in glacial acetic acid and 1% sodium iodide. Similar measurements obtained during each study were used to quantify amount of nitric oxide released with cultured endothelial cells under basal conditions and during stimulation with either bradykinin or calcium ionophore A23187.
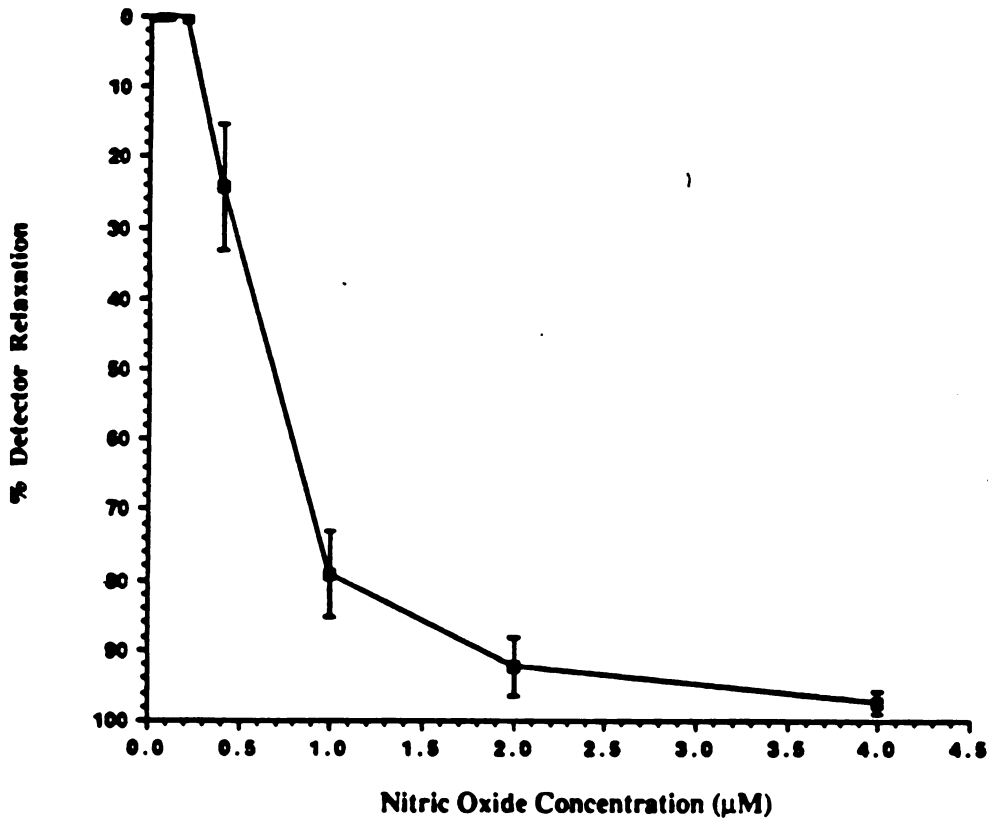
FIG. 3. Average relaxations of bioassay detector vessels to nitric oxide (n = 16). Bioassay detector vessels were preconstricted with prostaglandin $F_{2\alpha}$ (0.1–1 μM) and exposed to increasing concentrations of nitric oxide administered as a constant infusion. Transit time for nitric oxide infusion to detector vessel was 3 s, identical to that for EDRF exiting the holding chamber for cultured cells (see Fig. 1).
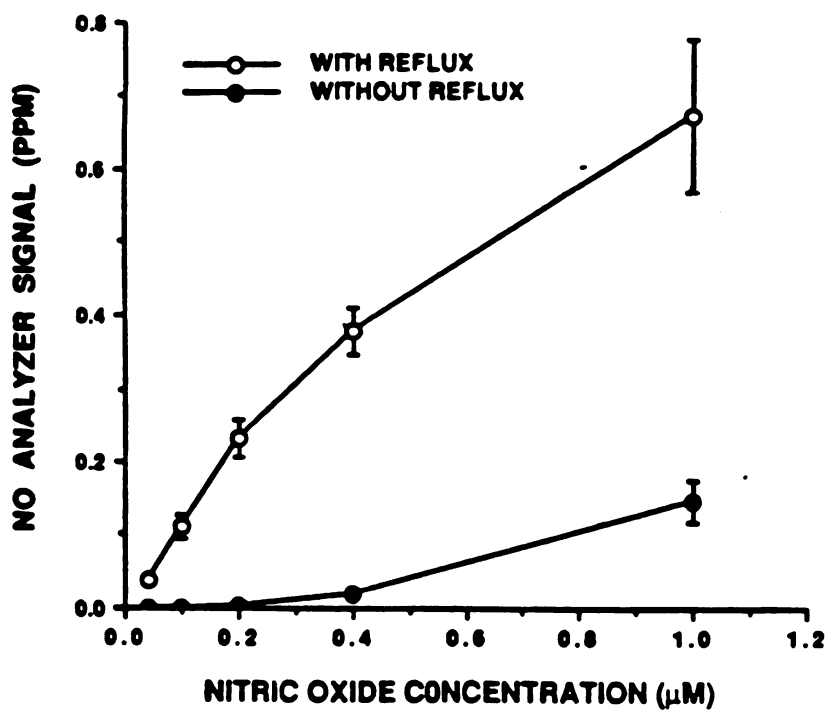
FIG. 4. Nitric oxide signals [in parts per million (PPM)] obtained in response to infusions of standard quantities of nitric oxide. Average signals obtained when samples were infused through a degassing chamber ($n = 6$) were compared with those obtained when sample was treated with a strong reducing environment of sodium iodide in glacial acetic acid reflux ($n = 10$). Acid reflux substantially increased the sensitivity of chemiluminescence detector, although both techniques permitted measurements of nitric oxide concentrations sufficient to cause relaxation of a bioassay detector vessel (<0.5 μM, see Fig. 3).
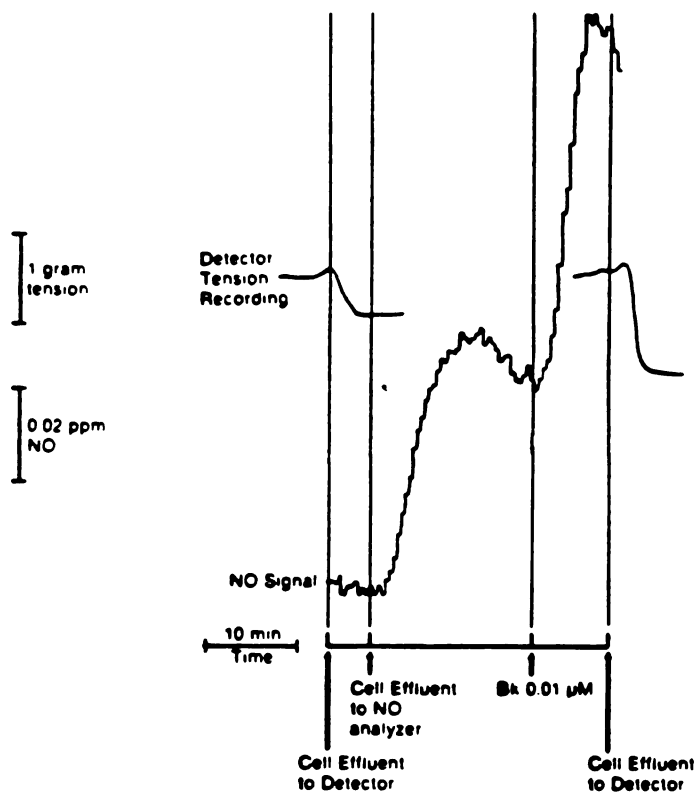
FIG. 5. Basal- and bradykinin (BK)-induced release of EDRF (bioassay ring responses) and nitric oxide release (chemiluminescence signal). Studies were performed in presence of indomethacin (1 μM). Effluent from cultured bovine aortic endothelial cells was first allowed to superfuse the bioassay ring under basal conditions. After a stable relaxation was observed, effluent was diverted away from bioassay ring to nitric oxide (NO) analyzer. Release of NO was monitored for ~10 min, and the cells were then exposed to bradykinin (0.01 μM). After the NO signal produced in response to bradykinin had stabilized, the cell effluent was diverted from NO analyzer to the detector ring.
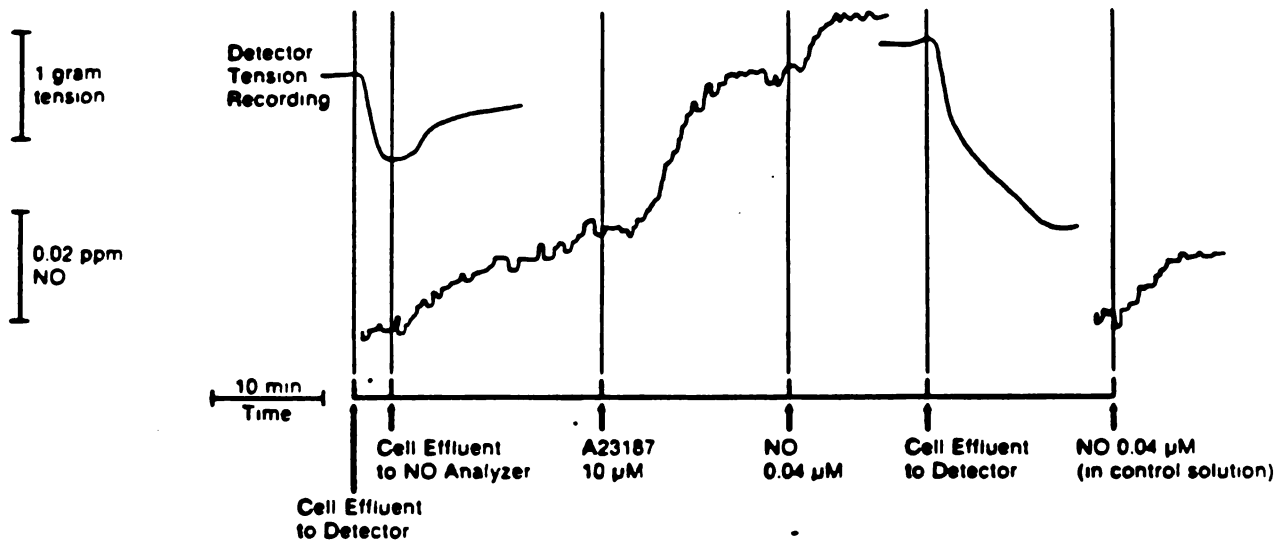
FIG. 6. Basal- and calcium ionophore A23187-induced release of EDRF (bioassay ring responses) and nitric oxide release (chemiluminescence signal). Studies were performed in presence of indomethacin (1 μM). Effluent from cul·ured bovine aortic endothelial cells was first allowed to perfuse bioassay ring under basal conditions. After a stable relaxation was observed, effluent was diverted away from bioassay ring to the nitric oxide (NO) analyzer. Release of NO was monitored ~10 min, and the cells were then exposed to the calcium ionophore A23187 (10 μM). NO signal produced by the cells in response to A23187 was then noted. Effluent was subsequently diverted from NO analyzer to the detector ring, and additional relaxation produced by EDRF released in response to the calcium ionophore A23187 was observed. Also note infusion of NO into effluent from cultured cells produced a signal identical to that observed when NO was infused through control solution alone.
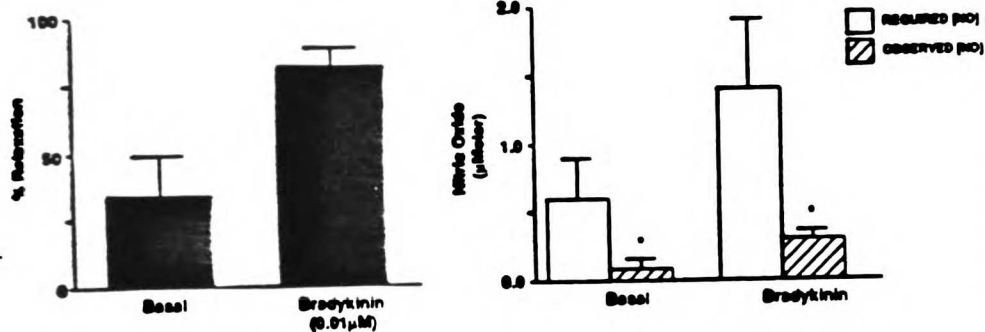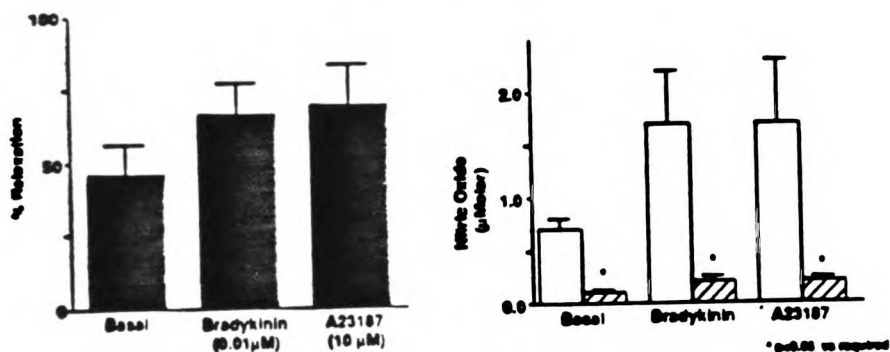
FIG. 7. *Top*: percent relaxation by basally released EDRF during agonist-stimulated release of EDRF compared with observed nitric oxide (NO) and NO required to account for observed vascular relaxation in absence of acid reflux. *Bottom*: percent relaxations by basally released EDRF and during stimulation with either bradykinin or calcium ionophore. These results are compared with the observed NO signals required to account for the observed detector ring relaxation determined during conditions of acid reflux. As in studies without acid reflux, the amount of NO required to account for EDRF-induced detector relaxation was 7-10-fold greater than NO observed.

Section 4

A Comparison of Chemical and Biological Properties of S-nitrosocysteine to Endothelium-Derived Relaxing Factor and Nitric Oxide

EDRF is a non-prostanoid, labile vasodilator released from the endothelium in response to mechanical and humoral stimuli (1-4). It has been suggested that EDRF and nitric oxide are the same compound because these substances share several biological characteristics (5,6). For example, both nitric oxide and EDRF have short half-lives in physiological fluids (5,7). Both produce vascular relaxation and inhibit platelet aggregation by activation of guanylate cyclase and both are inactivated by hemoglobin. And finally, Palmer, et al. have shown that nitric oxide can be detected in the effluent of cultured bovine aortic endothelial cells upon stimulation with bradykinin (8).

Studies of cultured bovine aortic endothelial cells from this laboratory using quantitative chemiluminescence techniques have shown that the amount of nitric oxide released under either basal condition or in response to bradykinin or the calcium ionophore, A23187, is insufficient to account for the vasorelaxant activities of EDRF (9). This discrepancy can only be explained by one of two possibilities. The first is that EDRF consists of multiple factors of which nitric oxide is only one. The second is that EDRF is a compound which contains nitric oxide in its structure but is substantially more potent than nitric oxide in terms of its vasorelaxant activity. This second possibility is conceivable because the methodology to detect nitric oxide (chemiluminescence coupled with chemical reduction of the endothelial cell effluent) does not distinguish between nitric oxide and several compounds which contain a nitric oxide moiety within their structure (10). A candidate for such a molecule is one of several nitrosothiols. It has been suggested that nitric oxide liberated from pharmacological nitrodilators reacts with thiol containing compounds within vascular smooth muscle to form an active vasodilator compound. While this has been shown to occur in vascular smooth muscle, this process has not been demonstrated in endothelial cells nor has it been acknowledged that endogenously formed nitric oxide may be converted to a nitrosothiol before or during release from the endothelium. This latter mechanism is feasible because NO_2 readily reacts with free cysteine to form S-nitrosocysteine. We now report that the biological activity of EDRF more closely resembles S-nitrosocysteine than nitric oxide.

S-nitrosocysteine was synthesized by the reaction of L-cysteine and nitrogen dioxide in cold methanol. Chromatographic separation by HPLC demonstrated near 100% conversion of cysteine to S-nitrosocysteine by this reaction (Figure 1).

The half-lives of S-nitrosocysteine, nitric oxide, and EDRF were similar, approximately 30 seconds each (figure 2).

Nitric oxide in physiologic solutions spontaneously degrades to nitrite. The chemiluminescence technique used in this and prior reports (8,9) requires reflux in a strong reducing environment to convert nitrite formed from nitric oxide back to nitric oxide. In addition, this process cleaves the nitric oxide moiety from a variety of nitroso-compounds (11). In the present studies, we found that reflux with sodium iodide stoichiometrically cleaved nitric oxide from S-nitrosocysteine (Figure 3).

The vasorelaxing activities of nitric oxide, S-nitrosocysteine, and EDRF were assessed using a bioassay preparation. S-nitrosocysteine was found to be approximately 80 times more potent than nitric oxide (Figure 4a). Relaxations to both S-nitrosocysteine and nitric oxide were consistently reversed by hemoglobin.

The vasorelaxing activities of nitric oxide, S-nitrosocysteine, and EDRF were compared as a function of the amount of nitric oxide from each compound using chemiluminescence. The relationship between relaxation produced by both basally released EDRF and that released after stimulation with the calcium ionophore, A23187, and the amount of recovered nitric oxide was quite similar to that observed with S-nitrosocysteine, but bore no similarity to that of nitric oxide (Figure 4b).

Several pharmacologic nitrodilators produce their effect by activating guanylate cyclase, a property shared with EDRF (12, 13, 14). It has been suggested that nitroglycerin releases nitric oxide which then (via unknown intermediates) reacts with thiol groups within vascular smooth muscle to form nitrosothiol compounds (15). The most abundant source of free sulfhydryl groups in mammalian tissue is cysteine (in either the free state or in peptide form) and the nitrosothiol formed by the reaction of cysteine and nitrite is S-nitrosocysteine (16). While the present experiments do not conclusively prove that EDRF is S-nitrosocysteine, the data clearly illustrate that the biological potency of EDRF more closely resembles S-nitrosocysteine than nitric oxide. We propose that endothelial cells are capable of synthesizing either S-nitrosocysteine or a related nitrosothiol as the EDRF. It is conceivable that EDRF may be composed of several different nitrosothiols, thus accounting

for subtle differences in half life and biological activity observed in different species and vascular beds. In additional studies we examined the potencies and half-lives of two additional nitrosothiols, S-nitrosoglutathione and S-nitrosomercaptoethanol. Both were found to be potent vasodilators with ED50's of approximately 10 nanomolar or less. The half life of both of these compounds, however, was substantially longer than EDRF from bovine aortic endothelial cells, exceeding two minutes (n=3 for both).

The incorporation of nitric oxide into a nitrosocompound, such as S-nitrosocysteine may have several important biological implications. When nitric oxide is incorporated into S-nitrosocysteine, its potency is substantially enhanced. The release of such a compound would permit more exquisite instantaneous control of vascular tone that the release of a less potent compound. The incorporation of nitric oxide into S-nitrosocysteine may be important in the control of EDRF secretion. It is conceivable that transmembrane transport relies on the association of nitric oxide with a "carrier molecule" such as an amino acid or other thiol compound. Furthermore, uptake of a nitrosthiol into the vascular smooth muscle may depend on a transport mechanism. Alternatively, the nitrosothiol may be degraded at the smooth muscle membrane to yield nitric oxide. This process may more efficiently deliver nitric oxide to the cytoplasm of the vascular smooth muscle.

Several pathologic processes are associated with abnormalities of endothelium-dependent vascular relaxation. These include acute hypertension (17), diabetes (18), ischemia with subsequent reperfusion (19), and atherosclerosis (20). Many of these processes are associated with the generation of oxygen free radicals within endothelial cells which may oxidize free sulfhydryl groups to the disulfide form. This may result in either an inability of the endothelium to release nitric oxide as a nitrosothiol, or result in the release of authentic nitric oxide, a compound substantially less potent than naturally occurring EDRF. In either instance, endothelium-dependent vascular relaxation would be impaired by the oxidation of sulfhydryl groups within endothelial cells.

REFERENCES

1. Griffith, T. M. Edwards, D. H., Lewis, M. J., Newby, A. C. & Henderson, A. H. *Nature* **308**, 645-647 (1984).

2. Furchgott, R. F. *Circ. Res.* **53**, 557-573 (1983).

3. Griffith, T. M., Edward, D. H., Lewis, M. J., Newby, A. C. & Henderson, A. H. *Nature* **308**, 645-647 (1984).

4. DeMey, J. G., Claeys, M. & Vanhoutte, P. *J. Pharmacol. Exp. Ther.* **222**, 166-173 (1982).

5. Ignarro, L. J., Byrns, R. E., Buga, G. H. & Wood, K. S. *Circ. Res.* **61**, 866-879 (1987).

6. Furchgott, R. F in *Mechanisms of Vasodilatation* (ed Vanhoutte, P. M.), Vol. IV, (Raven Press, New York) (In Press).

7. Hibbs, J. B., Taintor, R. R. & Vavrin, Z. *Science* **235**, 473-476 (1987).

8. Palmer, R. M., Ferrige, A. G. & Moncada, S. *Nature* **327**, 524-526 (1987).

9. Myers, P. R., Guerra R. Jr. & Harrison, D. G. *Am. J. Physiol.* (In Press).

10. Walters, C. L., Dyke, C. S., Saxby, M. J. & Walker, R. in *Environmental N-Nitroso Compounds Analysis and Formation.* IARC Scientific Publ. No. 14, Lyon, France, page 181 (1976).

11. Ignarro, L. J. & Gruetter, C. A. *Biochim. Biophys. Acta* **631**, 221-231 (1980).

12. Gruetter, C. A., D. Y. Gruetter, D. Y., Lyon, J. E., Kadowitz, P. J. & Ignarro, L. J. *J. Pharmacol. Exp. Ther.* **219**, 181-186 (1981).

13. Ignarro, L. J., Burke, T. M., Wood, K. S., Wolin, M. S. & Kadowitz, P. J. *J. Pharmacol. Exp. Ther.* **228**, 682-690 (1984).

14. Ignarro, L. J., Lippton, H., Edward, J. C., Baricos, W. H., Hyman, A. L., Kadowitz, P. J. & Gruetter, C. A. *J. Pharm. Exp. Ther.* **218**, 739-749 (1981).

15. Rapoport, R. M. & Murad, F. *Circ. Res.* **52**, 352-357 (1983).

16. Jocelyn, P. C. (ed.) in *Biochemistry of the SH Group* (Academic Press), 1972.

17. Wei, E. P., Kontos, H. A., Christman, C. W., De Witt, D. S. & Povlishock, J. T. *Circ. Res.* **57**, 781-787 (1985).

18. Pieper, G. M. & Gross, G. J. Am. J. Physiol. 24, H825-H833 (1988).

19. Vanbenthuysen, K. M., McMurtry, I. F. & Horowitz, L. D. *J. Clin. Invest.* **79**, 265-274 (1987).

20. Freiman, P. C., Mitchell, G. C., Heistad, D. D., Armstrong, M. L. & Harrison, D. G. *Circ. Res.* **58**, 783-789 (1986).

FIGURE LEGENDS

Figure 1: High pressure liquid chromatographic separation of S-nitrosocysteine from L-cysteine. S-nitrosocysteine was synthesized from the reaction of 1 mMole cysteine in methanol and 2 mMole nitrogen dioxide gas in cold methanol. The resulting 1 M S-nitrosocysteine solution was stable in methanol at -20°C in the dark. HPLC detection of cysteine and S-nitrosocysteine was accomplished using a C-18 reverse phase column with a mobile phase consisting of 20% methanol, 80% 50 mM sodium phosphate, pH 2.2 and 1 mM octane sulfonic acid. UV detection at 210 nM was employed.

Top: Cysteine before reaction with nitrogen dioxide. Bottom, after reaction with nitrogen dioxide. Peak A co-migrates with inorganic nitrous acid, peak B is S-nitrosocysteine. Inset - Spectrophometric analysis of peak B showing absorption characteristic of a nitrosothiol at 340 nM. In methanol or deoxygenated water this compound would not spontaneously yield nitric oxide, as assessed by chemiluminescence.

Figure 2: Half-lives of nitric oxide (n=6), S-nitrosocysteine (n=6), and EDRF (n=4) released from cultured bovine aortic endothelial cells in response to the calcium ionophore A23187. Vasorelaxation was determined using a 3-5 mm segment of porcine left circumflex coronary artery denuded of endothelium and preconstricted with 0.1 to 1 μM prostaglandin $F_{2\alpha}$. The vasorelaxant activity of each agent was determined using transit times of 3, 12, 20 and 30 seconds from the site of infusion or site of the endothelial cells to the detector ring . Transit times were varied by employing a series of

stopcocks with varying lengths of interposed tubing immersed in a 37° bath. The amount of relaxation of the detector vessel exhibited a negative linear relationship to the transit time ($r=-0.97 \pm 0.01$, -0.94 ± 0.03, and -0.93 ± 0.02 for EDRF, S-nitrosocysteine and nitric oxide respectively). The transit time required to produce 50% relaxation relative to the amount observed at zero delay was calculated from the regression equation of each of these relationships.

Figure 3: Nitric oxide measurements obtained using chemiluminescence during infusions of either nitric oxide or S-nitrosocysteine. Each compound was infused into a reaction chamber containing 1% sodium iodide in glacial acetic acid. Released nitric oxide was transported by a stream of nitrogen gas under vacuum to chemiluminescence nitric oxide detector similar to that previously described (8,9). Standard concentrations of nitric oxide (water solubility = 7 ml/100 ml at STP) were prepared daily by dissolving 25 μl of authentic nitric oxide in 25 ml distilled water previously deoxygenated with helium. This gave a stock solution of $4-10^{-5}$ M which was stable over 12 hours. Standard dilutions of S-nitrosocysteine were prepared in deoxygenated distilled water. S-nitrosocysteine was stoichiometrically degraded to nitric oxide by reflux preprocessing($n \geq 4$ for each concentration of nitric oxide and S-nitrosocysteine). The purity of nitric oxide solutions prepared in this manner was assessed by the relative nitric oxide signals obtained when these solutions were purged with a stream of nitrogen gas which was then directed to the nitric oxide analyzer

before and following addition of an excess of sodium iodide and glacial acetic acid (to convert nitrite in solution to nitric oxide gas). Solutions prepared in this manner were found to contain 97±1% nitric oxide and 3±1% nitrite.

Figure 4:   A. Concentration response relationships for vascular relaxation caused by nitric oxide and S-nitrosocysteine (n=4-10 for each concentration).

B. Vascular relaxation produced by nitric oxide, S-nitrosocysteine, and EDRF expressed as a function of the amount of nitric oxide detected by chemiluminescence during infusion of identical concentrations of each agent. All studies were performed in the presence of indomethacin (1 μM) and after preconstriction of the detector ring with $PGF_{2\alpha}$ (0.1-1.0 μM). EDRF derived from bovine aortic endothelial cells was released spontaneously (open squares) and after stimulation with 10 μM calcium ionophore A23187 (closed squares). The transit time from the site of infusion of nitric oxide or S-nitrosocysteine was identical to the transit time of EDRF from the cultured endothelial cells (3 seconds).
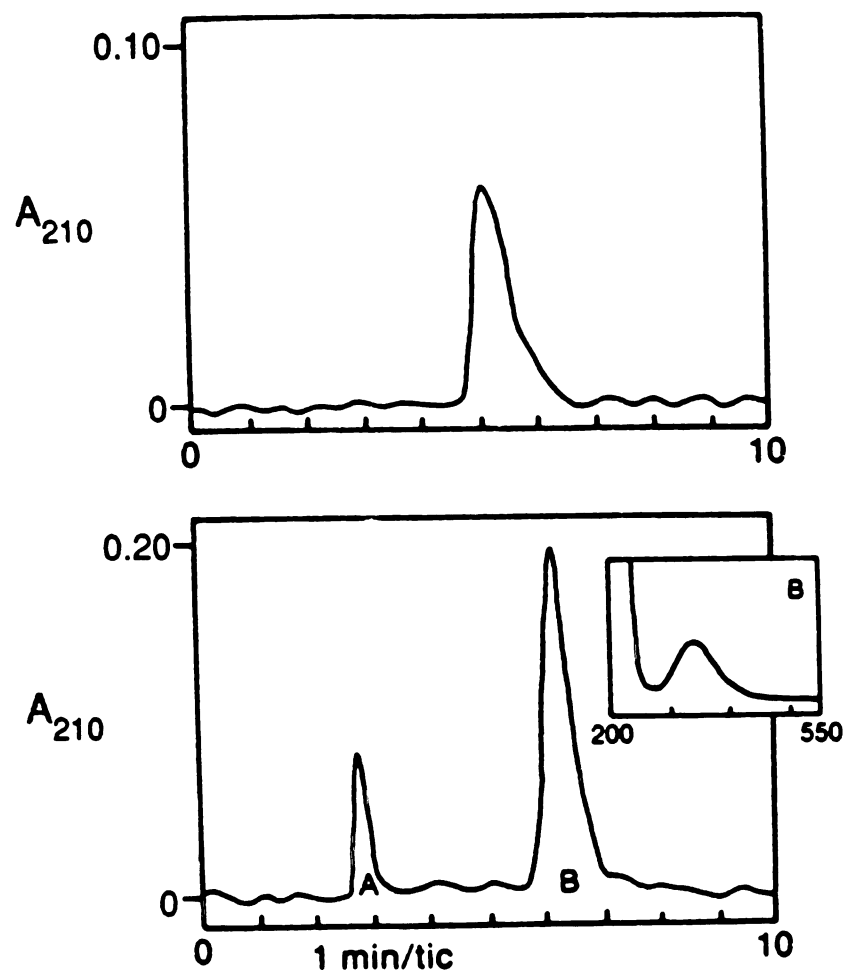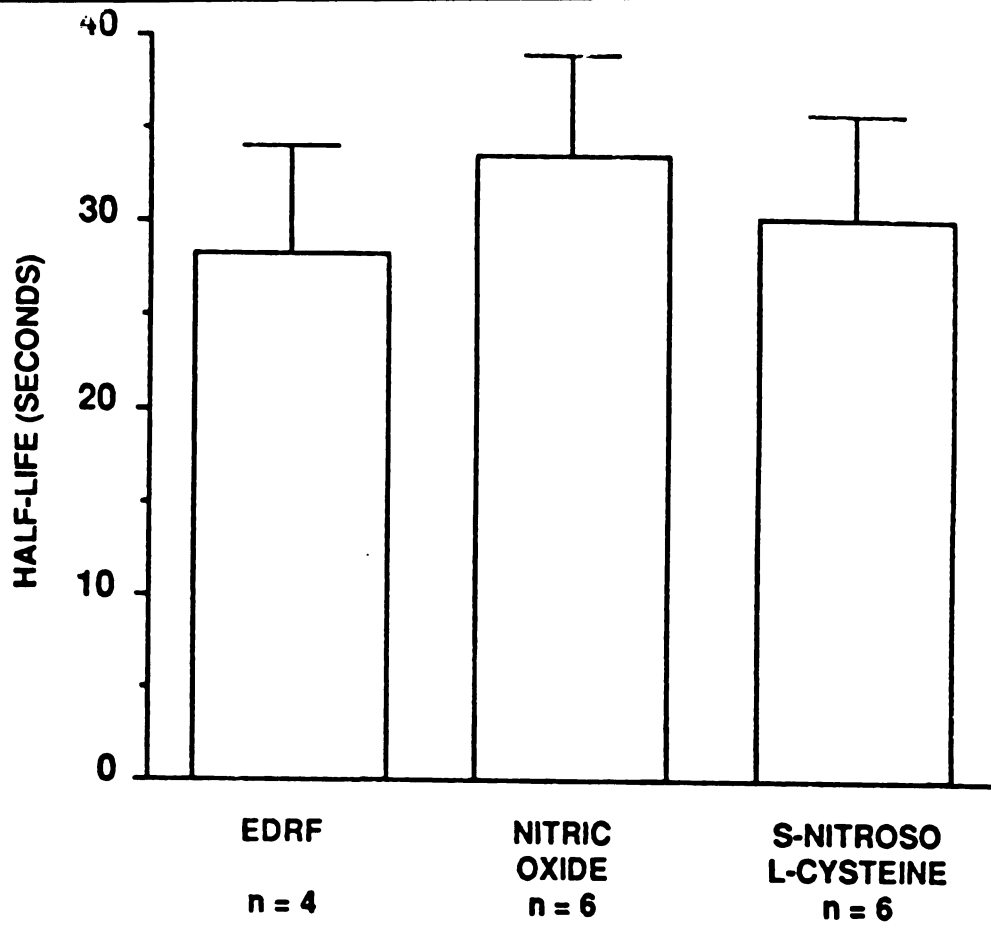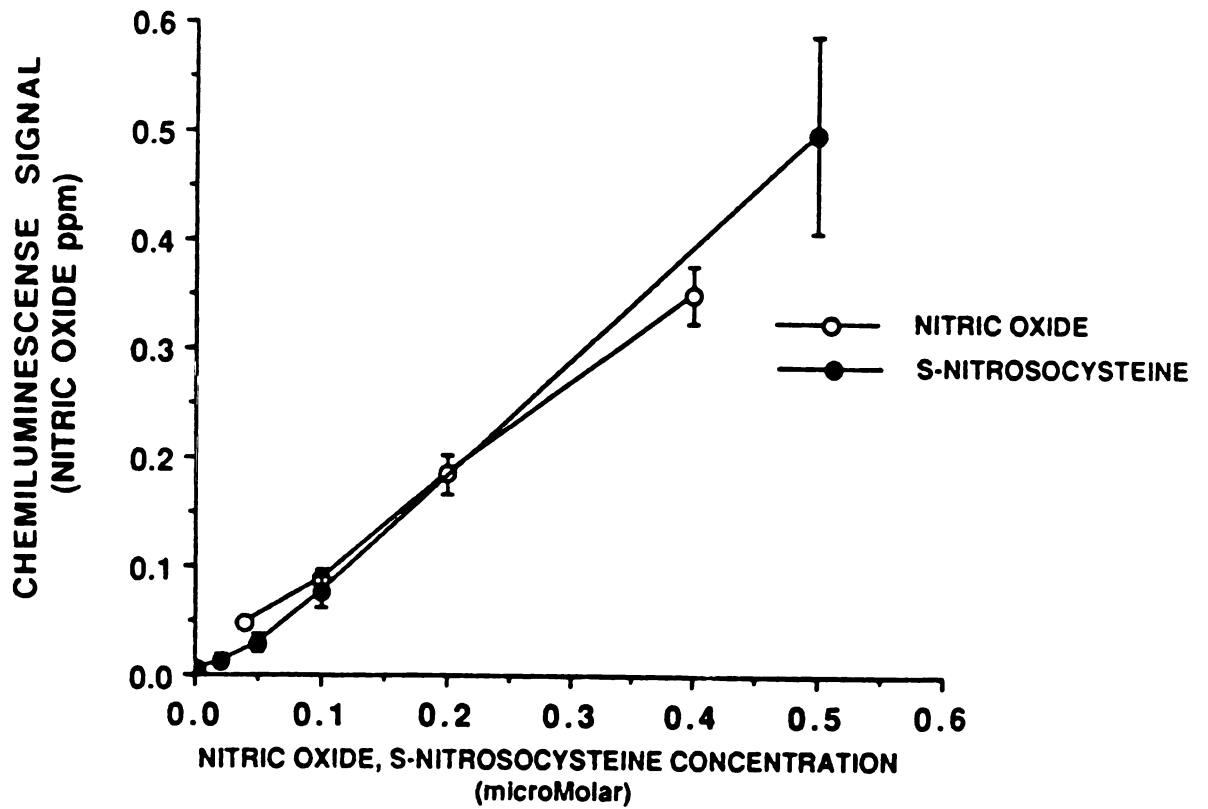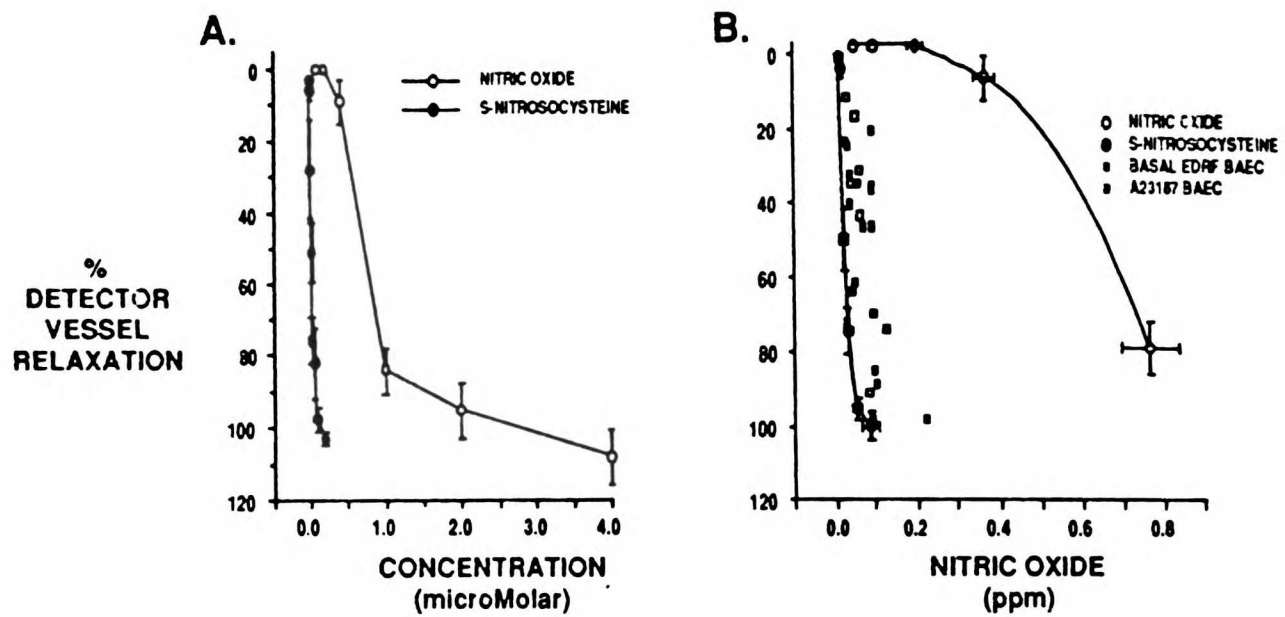
FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

Section 5

Endothelium-Derived Relaxing Factor and Nitric Oxide Release from Atherosclerotic Vessels

Atherosclerosis impairs endothelium-dependent vasodilation in both animal models (1-3) and human coronary arteries (4), and thereby may predispose to vasoconstriction and arterial spasm. Bioassay studies presented previously in this manuscript have suggested that the mechanism underlying the defect in endothelial function is based on a defect in EDRF synthesis or release. These studies, however, can not differentiate between the possibilities of decreased bioactivity due to decreased production (synthesis) of EDRF, normal synthesis but increased intra-endothelial destruction of EDRF, or synthesis of a defective, ie., less potent, relaxing factor. All three possibilities might account for the bioassay results.

The preliminary studies presented here were designed to examine release of EDRF and nitric oxide from normal and atherosclerotic vessels using bioassay and chemiluminescence techniques. Three conditions were studied: the basal release of EDRF and the release of EDRF upon stimulation with the agonist acetylcholine and the calcium ionophore, A23187. The results of further studies in our laboratory by Dr. Robert Minor are also presented in the various figures and Table and are examined in the Discussion section.

METHODS

Source of Normal and Atherosclerotic Vessels

Two groups of New Zealand white rabbits were used. One group was fed a 1% cholesterol diet for four months, followed by a 0.5% diet for two to five weeks (n=8). Age matched control rabbits were maintained on standard rabbit chow. The mean serum cholesterol in the long-term group was 1768 ± 244 mg/dl in the long-term group, and 38 ± 4 mg/dl in the control group.

On the day of the study, rabbits were killed by an overdose of sodium pentobarbitol. The thoracic aortae were isolated and excised.

EDRF Donor Segments

In these experiments, EDRF and nitric oxide release from normal and atherosclerotic vessels was simultaneously examined. Thoracic aorta segments from normal and atherosclerotic rabbit were cannulated with stainless steel cannulas taking care not to damage the endothelium. These vessels were mounted in parallel in an organ bath containing Kreb's buffer solution (composition in mM: NaCl, 118.3; KCl, 4.7; CaCl, 2.5; MgSO$_4$, 1.2; KH$_2$PO$_4$ 1.2, NaHCO$_3$, 25; glucose, 11.1; pH 7.40) aerated with 95% O2, 5% CO2, and maintained at 37 degrees centigrade. The donor vessels were perfused in the direction of their normal flow with the same solution from a separate reservoir at 4 ml/min by a roller pump.

Preparation of bioassay vascular ring for detection for EDRF.

Three to five mm segments from the proximal thoracic aorta from normal rabbits were used as bioassay rings for this preparation. These rings were denuded of endothelium by gently rubbing the intimal surface with the tip of a closed hemostat and suspended below the outflow ports of the organ bath chamber by means of steel stirrups, one of which was attached to a Grass FT03c force transducer. Adequate denudation was confirmed by the absence of relaxation to acetylcholine or calcium ionophore, A23187, applied directly to the rings. Changes in isometric tension generated by the detector vessels were recorded on an oscillographic chart recording device. The organ chamber containing the donor vessels could be moved freely, thus allowing the detector vessel to be superfused by effluent from either of the mounted vessel segments or a direct channel through a steel tube placed in the organ chamber (Figure 1).

Over a period of one hour, the vascular ring was stretched to an optimal resting tension for generating force during isometric contraction (~1-2 grams). The vessels were left at this optimal tension for the remainder of the study. Each ring's reconstricted tension was obtained using 0.1-1 μM prostaglandin F2α.

Measurement of nitric oxide released from donor vessels by chemiluminescence

Nitric oxide was measured using a nitric oxide analyzer commercially produced by either Bendix (Model 810c: Lewistown, WV) or Dasibi (Model 2108; Glendale, CA). The device is schematically illustrated in Figure 1. Effluent from either cultured endothelial cells or the direct perfusion channel was allowed to pass through a chamber in which effluent from donor vessels was refluxed in a solution of 1% sodium iodide in glacial acetic acid. Exposure to this powerful reducing environment was employed to degrade

nitric oxide containing compounds, thus releasing nitric oxide, which could then be measured by chemiluminescence. A constant stream of inert nitrogen gas under a vacuum carried any nitric oxide gas into the analyzer. Within the analyzer, the gas was heated to 45 C and mixed with ozone in a reaction chamber. Ozone and nitric oxide spontaneously react to release light at 6500 - 8000 Å wavelength. The amount of light generated by this system is concentration dependent and is measured with a photomultiplier tube, thus allowing an accurate quantification of nitric oxide (Figure 1).

Drugs

Acetylcholine, the calcium ionophore, A23187, and indomethacin were all obtained from Sigma Chemical (St. Louis, MO). Prostaglandin F2a was obtained from UpJohn (Chicago, IL). Drugs were dissolved in distilled water and subsequently diluted in controlled buffer solution to desired concentrations. Indomethacin was dissolved in 0.9 NaCl at a pH of 8. All agents were prepared immediately prior to use.

Protocol

All studies were performed in the presence of indomethacin (1 mM) to inhibit the production of vasoactive cyclooxygenase products. Measurements of EDRF and nitrosocompounds within the effluent of aortic segments were made following a ten minute equilibration period of superfusion with control physiologic buffer. Transit times from the aortic segment to the detector vessel and to the reflux chamber of the nitric oxide analyzer were identical (3 seconds). The physiologic buffer was infused at the rate of 4 ml/minute through both the direct superfusion channel and the aortic segments.

The bioassay detector vessel was initially superfused through the direct channel, bypassing the donor aortic segments. After a stable preconstriction to prostaglandin F2α was obtained, effluent from the rabbit aorta was directed onto the detector vessel, and the basal response observed (Figure 2). When the response reached a stable baseline, the effluent was directed into the reflux chamber of the nitric oxide analyzer. Nitric oxide recovered from the effluent was then measured by chemiluminescence.

The release of EDRF and nitrosocompounds in response to acetylcholine (1 mM) was then examined. During administration of acetylcholine, atropine (10 μM) was simultaneously infused onto the detector vessel to block smooth muscle muscarinic receptors. Acetylcholine was infused at a constant rate into the aortic segment while effluent from the aorta remained to the denuded bioassay detector vessel. After a stable relaxation was

recorded, the effluent was directed into the reflux chamber of the nitric oxide analyzer and nitric oxide measurements recorded.

An identical protocol was subsequently employed to examine the effects of the calcium ionophore, A23187, at 10 μM. An interval of at least 15 minutes was allowed following cessation of acetylcholine before infusions of A23187.

RESULTS

Effects of Atherosclerosis on EDRF Release

Detector vessels were preconstricted to a resting tension of 4.0 ± 0.2 grams for both normal and atherosclerotic vessel groups. Atherosclerosis markedly impaired EDRF vasodilator activity as measured in bioassay (Table 1, Figure 3). Under basal flow conditions, effluent from normal vessels consistently caused relaxation of detectors. In contrast, effluent from atherosclerotic vessels caused constriction. EDRF release elicited by the receptor-mediated agonist, acetylcholine (1 μM), from normal vessels was sufficient to cause additional relaxations of detectors, but the vasodilator activity of EDRF released from atherosclerotic vessels was dramatically impaired. Effluent from normal vessels stimulated with A23187 (10 μM) caused significant additional relaxation. However, atherosclerosis dramatically impaired the vasodilator activity of EDRF released by A23187.

Detection of Nitric Release by Chemiluminescence

Despite significant impairment of the vasodilator activity of EDRF by atherosclerosis, the quantity of nitric oxide recovered for the effluent of atherosclerotic vessels was significantly greater than from controls and also increased in response to agonists (Table 1, Figure 4).

DISCUSSION

The first important finding of this study was that atherosclerosis resulted in a decrease in vasodilator activity of EDRF as determined by a bioassay preparation. This finding confirms previous work (5). The second important finding was that the release of nitrosocompounds from atherosclerotic vessels was not reduced, but actually markedly increased in comparison to normal vessels even in the face of decreased bioactivity. Furthermore, the augmentation in nitric oxide release from atherosclerotic vessels after

agonist administration was substantially greater than that observed from normal vessels. These observations suggest synthetic pathway responsible for the production of nitric oxide itself by the endothelium is not impaired by atherosclerosis.

There are several possible explanations for these observations. First, it is possible that atherosclerosis somehow prevents incorporation of nitric oxide into a more potent nitrosylated compound or extracellular degredation of EDRF is accelerated. The technique used to detect nitric oxide by chemiluminescence in this study and others does not distinguish free nitric oxide from a variety of nitrosylated compounds (6, 7). We and others have found that one such co, mpound, S-nitrosocysteine, more closely resembles the EDRF than does nitric oxide (8, 9). It is conceivable that the efects of hypercholesterolemia and/or atherosclerosis could deplete essential EDRF substrates and therefore prevent the release of nitrosylated compounds which might be substantially more stable and potent than nitric oxide. A possible mechanism might involve oxidation of endothelial cell sulfhydryl groups to the disulfide form, rendering them unavailable to form nitrosothiols. This oxidation might occur due to overproduction of oxygen free radicals observed to occur in various disease states that are also associated with impaired vascular relaxation such as diabetes (10), acute hypertension (11) and reperfusion (12). Atherosclerosis might result in intracellular changes leading to increased free radical formation.

Second, it is possible that atherosclerosis accelerates the intracellular or extracellular degradation of EDRF. It is known that there are increased levels of superoxide anions and other free radicals within endothelial cells in various injury states. It is further known that both EDRF and nitric oxide are quickly inactivated by these radicals. As noted above, these reactive molecules might inactivate substrates or destroy final products in the EDRF pathway. It is interesting to speculate that either the inability to synthesize a potent vasodilator nitrosocompound or the accelerated intracellular degradation of a nitrosocompound EDRF might lead to a loss of feedback inhibition on a nitric oxide synthesizing enzyme or enzyme system. Without further knowledge of the actual biosynthetic pathway for the nitrosocompound EDRF, it is impossible to determine however, this hypothesis will certainly form the basis for further research.

There are several other less likely explanations for the increased release of a non-vasoactive nitrogen oxides as a result of cholesterol feeding. These possibilities have been examined in our laboratory and the results of these experiments are briefly summarized below (Dr. Robert Minor, University of Iowa, personal communications).

If multiple relaxing factors are released by the endothelium, one explanation for the present finding is that atherosclerosis impairs an EDRF unrelated to nitric oxide. Incubation of vessels in the presence of L-NMMA, an L-arginine analogue, virtually abolished the release of any detectable relaxing substance while blocking the synthesis of nitric oxide. This strongly suggests that a nitrosocompound is the major EDRF released by rabbit aorta. Thus, it is unlikely that the present findings could be explained by an abnormality in release of a non-nitrosocompound EDRF. The results of these studies are depicted in figure 5

Activated macrophages (13) and neutrophils (14) can produce nitric oxide and nitrite. Another explanation for our findings, therefore, is that atherosclerosis does indeed impair nitric oxide synthesis from the endothelium, and that the excess nitric oxide detected by chemiluminescence was derived from nitric oxide or nitrite released from intimal inflammatory cells. Intimal lesions in atherosclerotic aortae had extensive inflammatory cell infiltrates. However, microscopic studies of vessels from rabbits how where fed the cholesterol diet for only 2-4 weeks and thus made hypercholesterolemic had not yet developed. Corresponding transmission electron micrographs also revealed no intimal macrophage infiltrates in this group. Despite these histological findings, the amount of nitric oxide recovered from the effluent of hypercholesterolemic vessels was actually greater than that of atherosclerotic vessels. Therefore, increased nitric oxide release could not have been solely related to the presence of subendothelial macrophages. These findings are summarized in Table 1 and figure 3

Yet another explanation for the presented findings is that atherosclerosis increases the release of vasoconstrictors from the endothelium. Constricting factor release in response to acetylcholine or the calcium ionophore, A23187, could have reduced the apparent potency of EDRF released by these agonists, while not affecting nitric oxide signals. This seems unlikely because atherosclerotic rabbit aortae studied in a quiescent state do not constrict more than normal vessels in response to acetylcholine in the presence or absence of inhibition of EDRF. While aortae of cholesterol fed rabbits were observed to cause a small amount of constriction under basal conditions, this likely represented the effect of normally released constrictor factors by the endothelium, as L-NMMA also unmasked a similar basal constriction from normal vessels. The known constricting factors include the polypeptide, endothelin (15) and one or more products of the cyclooxygenase pathway (16). It is not known if hypercholesterolemia or atherosclerosis alters the release of endothelin. Enhanced release of vasoconstrictor prostanoids could not have accounted for

our observations because experiments were performed in the presence of indomethacin. The results from these experiments are shown in figure 5

Summary and Implications

Atherosclerosis results in increased synthesis of nitric oxide or related nitrosocompounds from the endothelium while dramatically reducing the vasodilator activity of EDRF. These observations cannot be explained by the impairment of an EDRF unrelated to nitric oxide, by the enhanced release of constricting factors by the endothelium, or by excess release of nitric oxide and nitrite by inflammatory cells. Impaired endothelium-dependent vasodilation may be due to absence of nitric oxide incorporation into a more potent nitrosocompound, perhaps by depletion of an essential EDRF substrate. Alternatively, accelerated intracellular or extracellular degradation of a nitrosocompound EDRF may occur which may result in the loss of an important nitric oxide synthesis pathway feedback inhibition resulting in overproduction of the less potent anti-platelet agent and vasodilator, nitric oxide. It is interesting to speculate that the loss of both vasodilator and anti-platelet aggregation functions might predispose affected vessel segments to the development of atherosclerosis, vasospasm, or thrombosis.

Section 5 References

1. Jayakody, RL, Senaratne MPJ, Thomson ABR, Kappagoda CT. 1985. Cholesterol Feeding impairs endothelial dependent relaxation of rabbit aorta. Can. J. Physiol. Pharmacol. 63:1206-1209.

2. Freiman, PC, Mitchell GC, Hiestad DD, Amstrong ML, Harrison DG. 1986. Atherosclerosis impairs endothelial dependent vascular relaxation to acetylcholine and thrombin in primates. Circ. Res. 58:783-789.

3. Harrison DG, Freiman PC, Armstrong ML, Marcus ML, Heistad DD. 1987. Alterations in vascular reactivity in atherosclerosis. Circ. Res. 61:II74-II80.

4. Forstermann U, Mugge A, Acheidu U, Haverich A, Frolich JC. 1988. Selective attenuation of endothelial mediated vasodilation: Atherosclerotic human coronary arteries. Circ. Res. 62:185-190

5. Guerra R, Brotherton AFA, Goodwin PJ, Clark CR, Armstrong ML, Harrison DG. 1990. Mechanisms of abnormal endothelium-dependent responses in atherosclerosis. Blood Vessels (In press).

6. Palmer RMJ, Ferrigi AG, Moncada S. 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature (London) 327:525-526.

7. Myers PR, Guerra R, Harrison DG. 1989. Release of nitric oxide and EDRF from cultured bovine aortic endothelial cells. Am. J. Physiol. 256(Heart Circ Physiol 25):H1030-H1037.

8. Myers PR, Guerra R, Bates JN, Harrison DG. 1989. Comparative studies on nitric oxide and nitrosothiols: Similarities between EDRF and S-nitroso-1-cysteine. FASEB J. (abst) 3:5336.

9: Rubanyi GN, Johns A, Harrison DG, Wilcox D. 1989. Evidence that EDRF may be identical with an S-nitrosothiol and not with free nitric oxide. Circ. 80(Suppl II): II281.

10: Mayhan WG. Impairment of endothelium-dependent dilatation of cerebral arterioles during diabetes mellitus. Am. J. Physiol. 1989. 256:H621-H625.

11: Wines PA, Schmitz JM, Pfister SL, Club Jr. FJ, Buja LM, Willerson JT, Campbell WB. Augmented vasoconstrictor responses to serotonin precede development of atherosclerosis in aorta of WHHL rabbit. 1989. Arteriosclerosis 9:195-202.

12: Vanthuysen KM, McMurty IF, Horowitz LD. Reperfusion after acute coronary occlusion in dogs impairs endothelium-dependent relaxation to acetylcholine and augments contractile reactivity in vitro. 1987. J. Clin. Invest. 79:265-274.

13: Marletta MA, Yoon PS, Iyengar R, leaf CD, Wishnok JS. 1988. Macrophage oxidation of L-arginine to nitrite and nitrate: Nitric oxide is an intermediate. Biochemistry 24:8706-8711.

14: Rimele TJ, Sturm RJ, Adams LM, Henry DE, Heaslip RJ, Weichman BN, Grimes D. 1988. Interaction of neutrophils with vascular smooth muscle: Identification of a neutrophil derived relaxing factor. J. Pharm. Exp. Ther. 245:102-11.

15: Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kabayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332:411-415.

16: Shirahase H, Usui H, Kurahashi K, Fujiwara M. 1987. Possible role of endothelial thomboxane A2 in the resting tone and contractile responses to acetylcholine and arachadonic acid in canine cerebral arteries. Cardiovasc. Pharmacol. 10:517-522.

TABLE 1

Aortic Effluent Vasorelaxant Activity and Recovery of Nitric Oxide

	EDRF (% Relaxation of Bioassay Detector Vessel)		
	Basal	Ach 1.0 μM	A23187 10 μM
Control	9 ± 3	28 ± 6	65 ± 5
Hypercholesterolemia	$-9 \pm 3^{*x}$	$11 \pm 4^{*}$	59 ± 6
Atherosclerosis	$-7 \pm 1^{*x}$	$4 \pm 2^{*}$	$33 \pm 6^{*+}$

Nitric Oxide
(Chemiluminescence Signal, parts per million)

	Basal	Ach 1.0 μM	A23187 10 μM
Control	$.011 \pm .001$	$.013 \pm .001$	$.020 \pm .003$
Hypercholesterolemia	$.028 \pm .004^{*}$	$.040 \pm .005^{*}$	$.051 \pm .007^{*}$
Atherosclerosis	$.023 \pm .003^{*}$	$.029 \pm .004^{*+}$	$.035 \pm .005^{*+}$

$^{*}p<0.05$ compared to identical treatment in control group.

$^{+}p\leq0.05$ compared to identical treatment in hypercholesterolemia group.

^{x} minus denotes constriction

Data expressed as mean $\pm$ S.E.M., n=10 for control, n=7 for hypercholesterolemia, n=8 for atherosclerosis.

Section 5 Figure Legends

Figure 1:

Experimental preparation used to quantify EDRF and nitric oxide release. Effluent from aortic segments first superfused a detector vessel for recording of bioassay responses. At the point of maximal response, effluent was immediately diverted into the collection chamber containing 1% NaI in glacial acetic acid under reflux at 55 degrees centigrade, a strong reducing environment. Recovered nitric oxide was transported in a stream of nitrogen under negative pressure into the nitric oxide analyzer and exposed to ozone. Light enmitied by the resultant chemiluminescence reaction was detected by a photomultiplier tube, generating a DC signal proportional to the amount of nitric oxide present. Transit times for vessel effluent to the detector vessel and the collection chamber were identical (3 seconds).

Figure 2:

Representative experimental recording from normal (A) and atherosclerotic (B) rabbit aortas. Control solution was initially superfused over the detector vessel and preconstricted tension was obtained. Effluent from the aortic segment was then directed to the ring detector (•) and basal responses were recorded. Effluent was then immediately diverted into the reflux chamber of the nitric oxide analyzer ( ), and nitric oxide signals were recorded as control solution was again superfused onto the detector. This protocol was repeated during administration of acetylcholine (1 μM) and subsequently the calcium ionophore, A23187 (10 μM). During administration of acetylcholine, atropine (10 μM) was directly superfused onto the detector.

Figure 3:

EDRF measured in bioassay (A). Responses of the bioassay detector ring to aortic effluent, measured as changes in preconstricted tension, were observed in the basal state and during constant infusions of either acetylcholine (1 μM) (with atropine 1 μM infused directly on the detector), or A23187 (10 μM). * = $p < 0.05$ compared to control; + = $p < 0.05$ compared to hypercholesterolemia. Data expressed as mean $\pm$ SEM.

Figure 4:

Nitric oxide measured by chemiluminescence. At the moment of maximal bioassay response, effluent from aortic segments was immediately diverted into an acid reflux chamber and nitric oxide content of the effluent was subsequently measured with the

chemiluminescence technique. * = p < 0.05 compared to control; + = p < 0.05 compared to hypercholesterolemia. Data expressed as mean ± SEM.

Figure 5:

Effect of L-NMMA on EDRF release from normal rabbit aorta. L-NMMA infusions abolished both basal and acetylcholine stimulated release of EDRF. The release of EDRF evoked by A23187 was also virtually abolished. Data expressed as mean ± SEM.
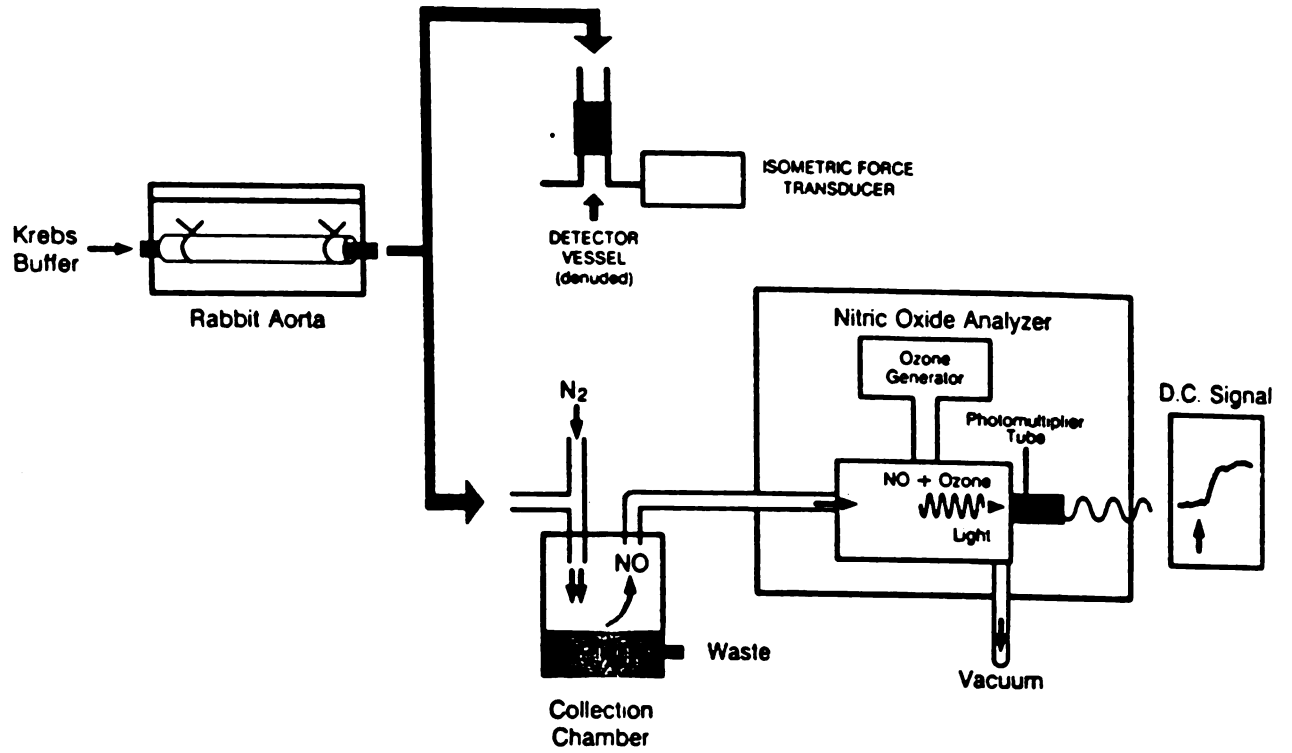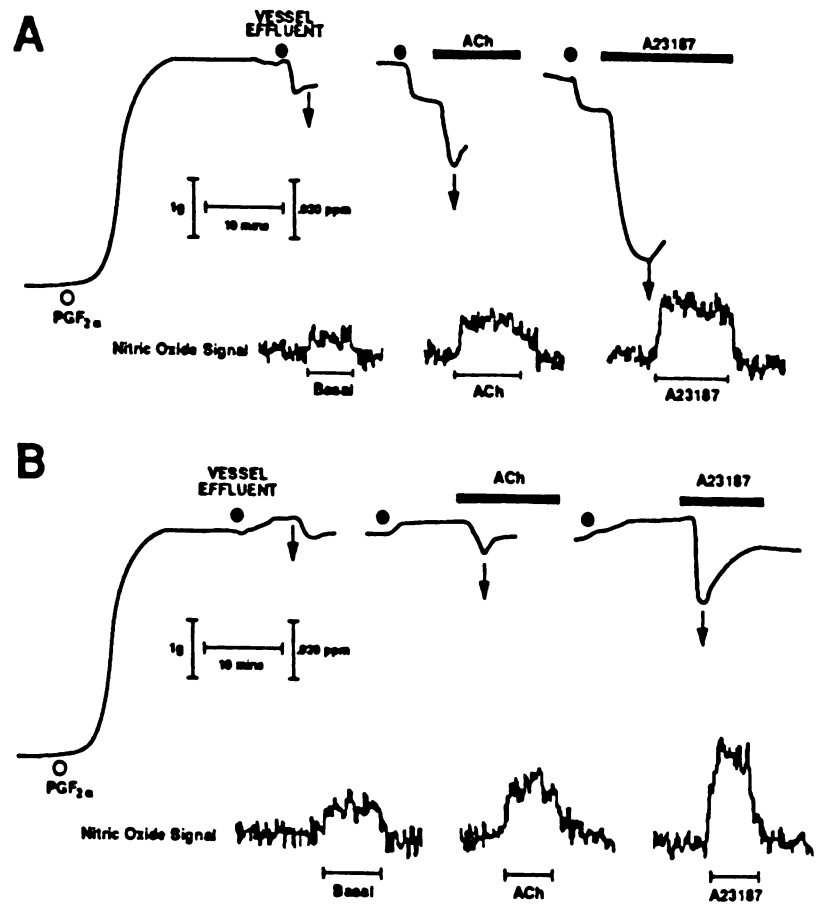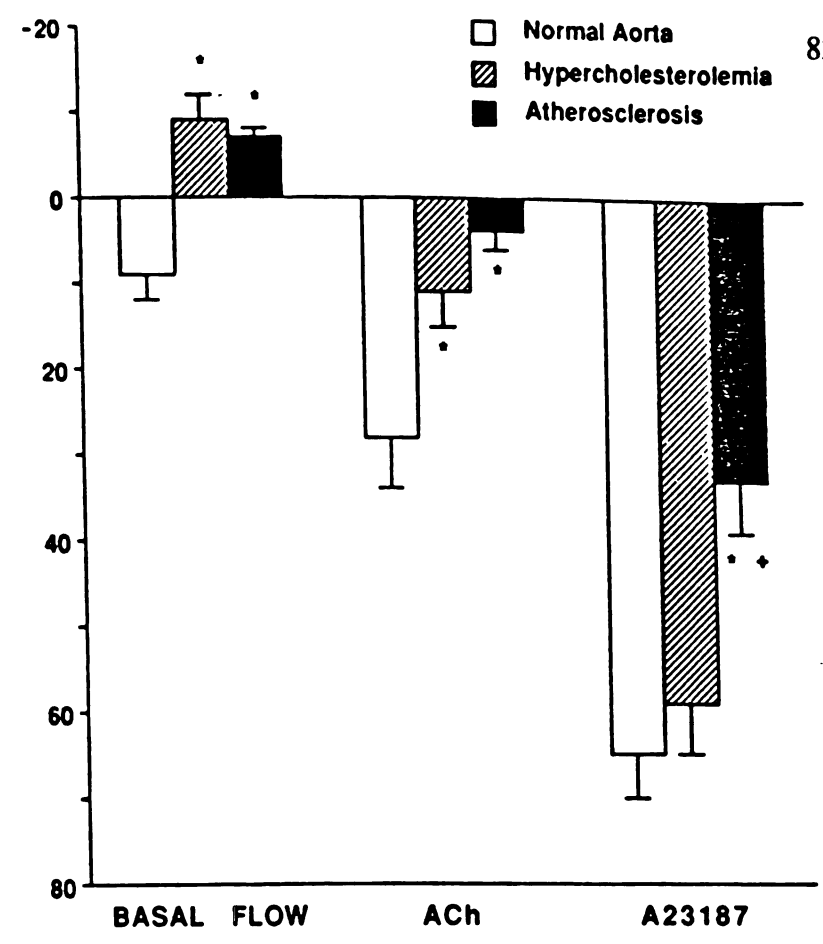
FIGURE 1

FIGURE 2

% RELAXATION

DETECTOR VESSEL

Figure 3

NITRIC OXIDE

CHEMILUMINESCENCE SIGNAL

(parts per million)
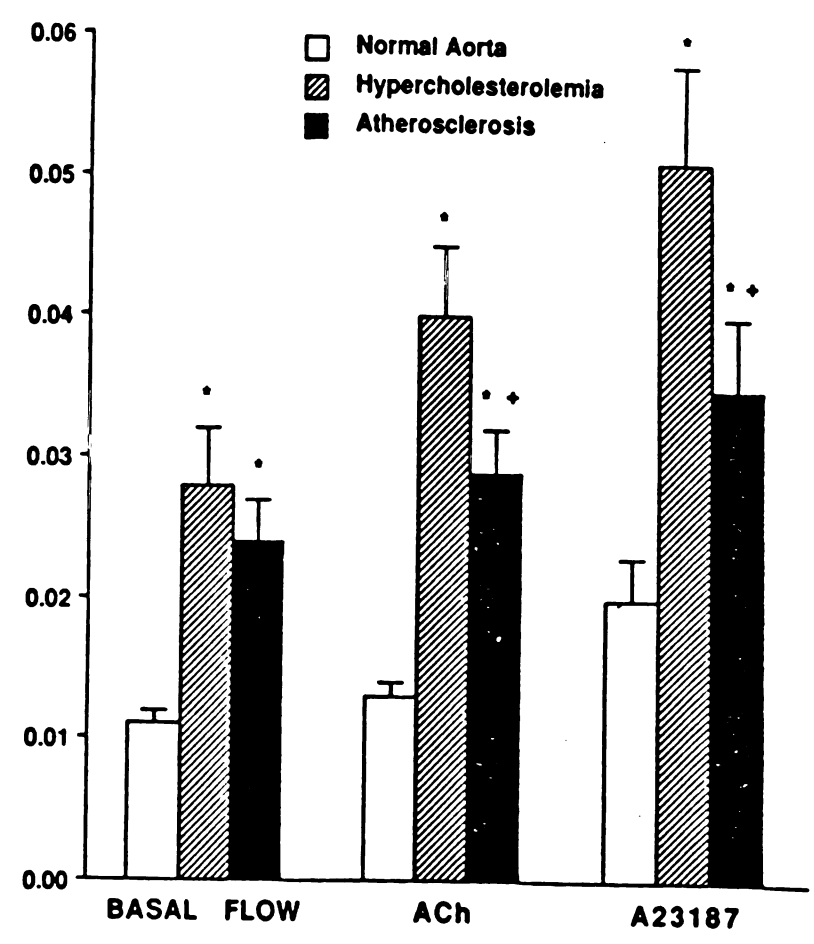
Figure 4
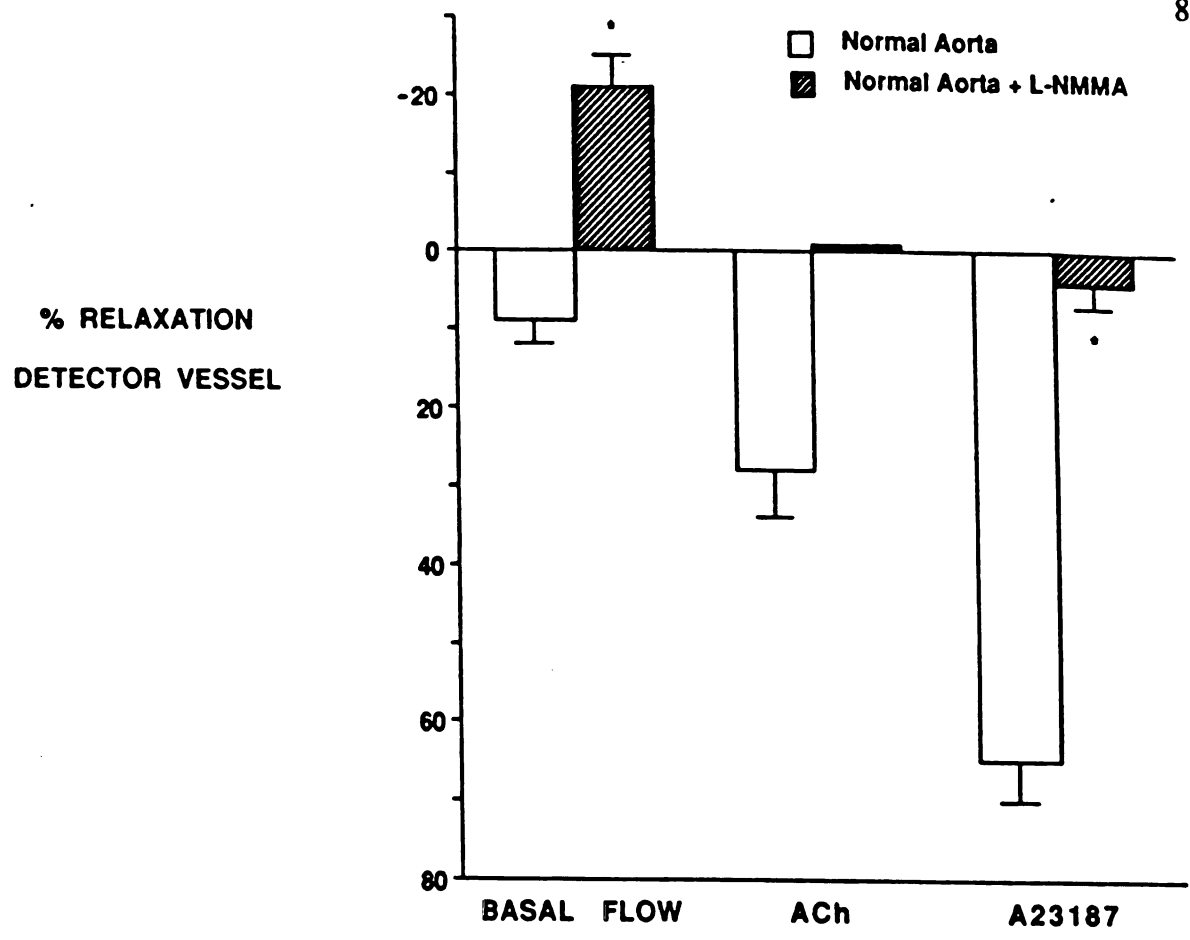
Figure 5

Section 6

Summary

The purpose of the studies presented within this thesis is to examine endothelium-dependent vascular relaxation responses and EDRF release in both health and disease and to gain further information regarding the chemical identity of EDRF. This section will summarize the finding of the various studies presented herein and discuss possible implications and further directions for research.

The first series of studies confirmed that endothelium-dependent vascular relaxation is abnormal in atherosclerosis. Further studies on possible mechanisms underlying this abnormality lead to the conclusion that there is some defect in synthesis or release of EDRF rather than a physical or functional barrier to the diffusion of EDRF formed by plaque lesions. In addition, the responses to natural EDRF from a normal vessel and a putative EDRF, nitric oxide, of atherosclerotic vessels differed. This suggested that the EDRF released from rabbit aortas was some compound or compounds other than nitric oxide. This observation is not consistent with the findings of Palmer et al. who reported that nitric oxide was likely the EDRF. In order to conclude that nitric oxide is identical to EDRF, however, one must be able to demonstrate that the amount of nitric oxide simultaneously released from the EDRF producing vessel can completely account for the observed relaxations of bioassay detector tissue. To test this hypothesis, nitric oxide and EDRF simultaneously released from cultured bovine aortic endothelial cells were compared. We found that the amount of nitric oxide released could not account for the observed detector vessel relaxations. Given that the chemiluminescence technique used in these studies can not differentiate between authentic nitric oxide and a nitric oxide containing compound that can chemically degrade and release nitric oxide, this finding again suggested that either multiple non-nitrosocompound factors are released or EDRF is a nitric oxide containing compound that is a great deal more potent than nitric oxide alone. These intriguing possibilities have important implications in understanding the mechanisms of the EDRF synthesis and release and may potentially explain abnormalities present in pathologic conditions. To test the hypothesis that EDRF consists of a potent nitric oxide containing compound, biological and chemical characteristics of a model compound, S-nitrosocysteine, were compared to those of natural EDRF. S-nitrosocysteine was found to more closely resemble EDRF than nitric oxide. This compound was found to be roughly 10 times more potent than nitric oxide. It is conceivable that the release of a nitrosothiol

compound similar to S-nitrosocysteine might account for the bioactivity of EDRF. The nitrosothiol experiments seem to give further support to the hypothesis that EDRF is a highly potent nitrosocompound. The synthesis and release of a nitrosocompound EDRF may potentially explain abnormalities present in pathologic conditions. For example, while the final effector mechanism of EDRF may well involve authentic nitric oxide, the "grafting" of nitric oxide to a "carrier molecule" might be of some importance in the synthesis, secretion, or end organ efficacy of EDRF. A defect in the formation of this nitric oxide containing compound could disrupt endothelium-dependent relaxation process at any step of the process. The effects of atherosclerosis on EDRF and nitric oxide release was reexamined using the chemiluminescence technique. The release of nitric oxide was found to be enhanced in atherosclerosis even though the bioactivity of the release factors was decreased. This result suggests possible mechanisms that might explain the impairment of vascular relaxation seen in atherosclerosis. Abnormal endothelium-dependent vasodilation may be due to prevention of nitric oxide incorporation into a more potent nitrosocompound, perhaps by depletion of essential EDRF substrate. Alternatively, accelerated intracellular or extracellular degradation of a nitrosocompound EDRF may occur which may result in the loss of an important nitric oxide synthesis pathway feedback inhibition resulting in overproduction of the less potent vasodilator, nitric oxide.

Future Directions

Recently, Bredt and Snyder (Proc. Nat. Acad. Sci. USA 87(2):682-685, 1990) have isolated and purified nitric oxide synthetase from rat cerebellum. This enzyme appears to be similar to other known NADPH oxidative enzymes, is monomeric with a molecular mass of about 200 kDa, and requires both calcium and calmodulin to function. While not yet demonstrated, it seems very likely that a similar, if not identical enzyme will be isolated from endothelial cells. With homogeneous preparations of purified enzyme from endothelium, it will be possible to clarify the precise mechanisms by which this enzyme catalyzes the reaction which produces nitric oxide from an arginine substrate. Also, purified extracts will enable a more definitive study of all substrates and cofactors required in this reaction will certainly enhance understanding of the pathogenesis of impaired release of nitrosocompound EDRF. This knowledge of the molecular basis of EDRF synthesis, will in turn, hopefully result in more effective therapeutic modalities for the management of diseases of endothelial dysfunction, including atherosclerosis.
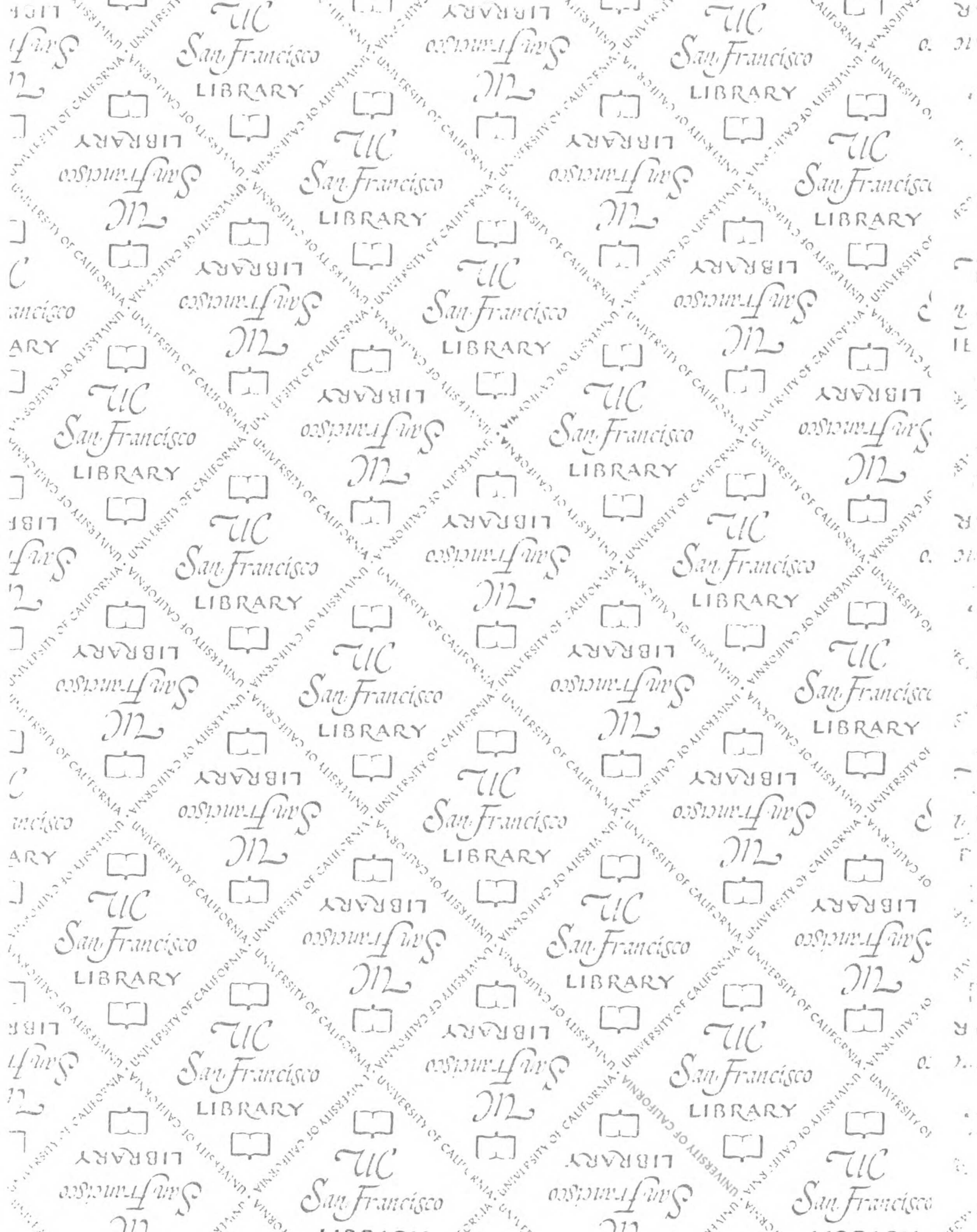
Acknowledgements