The blood vessel endothelium produces several factors that regulate vascular tone. One factor, endothelium-derived relaxing factor (EDRF), is a labile mediator responsible for the vasodilator activity of such compounds as acetylcholine, thrombin, and bradykinin. This EDRF is not a prostaglandin but may be nitric oxide (NO) or a labile compound that degrades to NO. The relaxant activity of EDRF results from a stimulation of soluble guanylate cyclase of smooth muscle cells. Previous studies have shown that arachidonic acid also causes an endothelium-dependent relaxation. However, the factor involved in arachidonic acid-induced relaxations has not been well characterized and may differ from the EDRF released by acetylcholine. The identity of arachidonic acid–EDRF is also unknown.

Because other investigators have suggested that more than one EDRF may exist, we hypothesized that in rabbit aorta, relaxations to arachidonic acid are mediated by a factor that differs from the NO-like EDRF mediating acetylcholine-induced relaxations. The rabbit aorta was chosen because it does not relax to the prostaglandins prostacyclin and prostaglandin E2 (PGE2). Therefore, the vascular response to arachidonic acid can be studied without the compounding influence of these cyclooxygenase metabolites. To test our hypothesis, we investigated the ability of the rabbit aorta to relax in response to arachidonic acid and acetylcholine in vessels with and without endothelium.

The responses were also examined in intact vessels pretreated with indomethacin, a cyclooxygenase inhibitor; nor-dihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor; metyrapone, a cytochrome P₄₅₀ epoxygenase inhibitor; and N°-monomethyl-L-arginine (L-NMMA) and nitro-L-arginine (LNA), specific inhibitors of NO synthesis. Arachidonic acid metabolism by rabbit aorta in the presence or absence of indomethacin, NDGA, and metyrapone was determined. In an additional study, a bioassay system was used to investigate the release of a soluble, transferable vasodilator by either acetylcholine or arachidonic acid. Because the vascular relaxation mediated by the NO-like EDRF is associated with increased tissue cyclic guanosine monophosphate (GMP) production, we also determined the effect of acetylcholine and arachidonic acid on cyclic guanosine monophosphate in rabbit aorta.
GMP and cyclic adenosine monophosphate (AMP) concentrations in rabbit aorta.

Methods

Vascular Reactivity

Male New Zealand White (NZW) rabbits (4–6 weeks old) (Myrtle's Rabbitry, Thompson Station, Tenn.) were injected with a lethal dose of pentobarbital (120 mg/kg), and the thoracic aorta was removed and placed in Krebs-bicarbonate buffer (mM: NaCl 118, KCl 4, CaCl₂ 3.3, NaHCO₃ 24, KH₂PO₄ 1.2, MgSO₄ 1.2, and glucose 11). The tissue was carefully cleaned of adhering fat and connective tissue and cut into rings (3-mm thick), taking care not to damage the endothelium. Aortic rings were suspended in 15-mL tissue baths containing Krebs-bicarbonate buffer maintained at 37°C and continuously bubbled with a 95% O₂–5% CO₂ mixture. Isometric tension was measured with force-displacement transducers (Grass Instrument Co., Quincy, Mass.) and recorded with a Grass polygraph model 7D. Resting tension was adjusted to its length-tension maximum of 2 g, and vessels were allowed to equilibrate for 1 hour. Maximal contractions were produced by increasing the KCl concentration of the baths to 40 mM. After the vessels reached peak contraction, tissue baths were rinsed and vessels were allowed to return to resting tension. After the aortic rings had reproducible maximal responses to KCl, the tissue was contracted to approximately 50% of maximum with norepinephrine (10⁻⁷ M). When the contraction stabilized, cumulative concentration–response curves in response to arachidonic acid (10⁻⁷ to 10⁻⁴ M) or acetylcholine (10⁻⁴ to 10⁻³ M) were obtained. This procedure was repeated in vessels in which the endothelium had been removed by gently rubbing the intimal surface with a cotton-tipped swab. The responses to arachidonic acid and methacholine were also repeated in the presence of the following inhibitors: indomethacin (10⁻³ M), NDGA (5 x 10⁻³ M), metyrapone (10⁻⁴ M), L-NMMA (10⁻³ M), or LNA (3 x 10⁻³ M). Indomethacin, NDGA, or metyrapone were present for 10 minutes before the addition of norepinephrine. L-NMMA or LNA were given after norepinephrine reached its maximum concentration and 10 minutes before the addition of arachidonic acid or acetylcholine. In a separate experiment, vessels were pretreated with the thromboxane receptor antagonist SQ 29548 (10⁻³ M) for 10 minutes followed by contraction with norepinephrine (10⁻⁷ M). When the vessels reach maximum contraction, a cumulative concentration–response curve to arachidonic acid was obtained. The effect of arachidonic acid in vessels that were not precontracted was also determined.

Bioassay Experiments

A modification of the perfusion-superfusion bioassay method of Rubanyi et al¹² was used. A segment (1.0–1.5 cm) of rabbit aorta was removed, cleaned of adhering fat and connective tissue, and cannulated at both ends with a stainless steel cannula. This donor segment was then placed in an organ chamber filled with Krebs-bicarbonate buffer maintained at 37°C and continuously bubbled with a 95% O₂–5% CO₂ mixture. The segment was perfused in the direction of blood flow in vivo. It was perfused with buffer at constant flow (2 ml/min) by a roller pump (Rainin Instrument Co., Woburn, Mass.). A ring of rabbit thoracic aorta in which the endothelium was carefully removed was used as the detector or recipient ring. The ring was suspended below the organ chamber by means of steel hooks. One hook was attached to a Grass transducer to measure isometric tension, which was recorded on a Kipp and Zonen recorder. The detector ring, hooks, and force transducer could be moved freely, allowing the ring to receive the effluent from the rabbit aorta segment (donor vessel superfusion) or a stainless steel tube (direct superfusion). In some experiments, the endothelium was removed from the donor vessel, and the detector ring received effluent from a denuded donor vessel segment. The drugs tested could be added to the perfusate so they had access to the donor and detector segments or, distal to the donor segment, directly on the detector segment only. Throughout the experiment, atropine (10⁻⁵ M) was infused directly onto the detector ring to prevent activation of vascular smooth muscle muscarinic receptors. Resting tension in the detector ring was adjusted to 2 g and allowed to equilibrate for 1 hour during direct superfusion. The detector ring was then precontracted with norepinephrine (10⁻⁴ M) infused directly onto the detector ring. When the contraction reached maximum, acetylcholine (10⁻³ M) or arachidonic acid (10⁻⁴ M) was added to the perfusate, proximal to the steel tube or donor segment, and any changes in tension of the detector segment were measured. The approximate time delay between the distal end of the donor segment and the detector ring was less than 6 seconds.

Metabolism of [¹⁴C]Arachidonic Acid

Arachidonic acid metabolism by rabbit aorta was performed as previously described.¹³ Briefly, segments of aorta (200 mg wet weight) were placed in 5 ml N-2-hydroxethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (mM: HEPES 10, NaCl 150, KCl 5, CaCl₂ 2, MgCl₂ 1, glucose 6, pH 7.4) and treated with vehicle, indomethacin (10⁻⁵ M), NDGA (5 x 10⁻⁵ M), or metyrapone (10⁻⁴ M). After a 10-minute pretreatment at 37°C, vessels were preincubated with 10⁻⁵ M ¹⁴C arachidonic acid (0.05 μCi, 10⁻⁷ M, New England Nuclear, Boston, Mass., specific activity ≥200 mCi/mmol) and the calcium ionophore A23187 (2 x 10⁻⁵ M) for 15 minutes. After incubation, the HEPES buffer was removed, acidified to pH 2.0 with glacial acetic acid, and extracted using Bond-Elut C₁₈ octadecylsila columns, as previously described.¹³ The extracted metabolites were analyzed by reverse-phase high-performance liquid chromatography (HPLC) (Nucleosil C₁₈ Column, 5 μM, 4.6 x 250 mm, Phenomenex). Solvent A was water and solvent B was acetonitrile containing 0.1% glacial acetic acid. The program consisted of a 40-minute linear gradient from 50% solvent B in A to 100% solvent B. The flow rate was 1 ml/min. Radioactivity of the column eluate was monitored with a Ramona-D (Raytest) radioactivity detector.

Cyclic AMP and GMP Radioimmunoassays

Aortic tissue with endothelium was obtained from 1-month-old NZW rabbits and cut into strips of 3–5 mm widths. The strips were placed in HEPES buffer and preincubated with indomethacin (10⁻⁵ M) for 5 minutes,
followed by an additional 5-minute incubation with vehicle, arachidonic acid (10^{-4} M), acetylcholine (10^{-6} M), or isoproterenol (10^{-3} M). Isobutylmethylxanthine (10^{-4} M) was present throughout the entire incubation period of 10 minutes. The incubations were performed in HEPES buffer because HEPES buffer, unlike Krebs buffer, maintains a stable pH (7.4) if the tissue is not continuously bubbled with 95% O_2-5% CO_2 mixture. The aortic strips were removed, frozen in liquid nitrogen, added back to the original incubation buffer now containing 7% trichloroacetic acid, and homogenized using a Polytron homogenizer. A 0.5-ml aliquot of homogenate was removed for measurement of protein by the method of Lowry et al. The remaining homogenate was centrifuged at 3,000 g for 10 minutes, and 0.5-ml supernatant was washed three times with 10 vol water-saturated diethyl ether to remove the trichloroacetic acid. Residual ether was removed under a stream of nitrogen, and an aliquot of sample was then assayed for cyclic GMP and cyclic AMP. Cyclic GMP content was quantitated after acetylation with a cyclic GMP radioimmunoassay kit obtained from Advanced Magnetics, Inc. Tissue cyclic AMP levels were measured by a previously described radioimmunoassay technique using the acetylation method of Brooker et al. Cyclic AMP was obtained from New England Nuclear, and the cyclic AMP antibody was a gift from Dr. Gary Brooker of Georgetown University.

**Drugs**

The following drugs were used: norepinephrine, arachidonic acid (sodium salt), acetyl-β-methacholine chloride, indomethacin, NDGA, A23187, and isoproterenol were all from Sigma Chemical Co., St. Louis, Mo. Metyrapone was obtained from CIBA-GEIGY, Summit, N.J. L-NMMA was obtained from CalBiochem and LNA was from Serva. SQ 29548 was provided by S.J. Lucania of Bristol-Myers-Squibb, Princeton, N.J. Unless otherwise specified, drugs were dissolved in distilled water such that volumes of 0.05 ml were added to the tissue baths. Arachidonic acid was prepared in distilled water previously degassed with nitrogen. The stock solution and dilutions were made fresh for each experiment, kept on ice and under a nitrogen atmosphere. LNA was dissolved in Krebs-bicarbonate buffer. Indomethacin, NDGA, metyrapone, and SQ 29548 were diluted in ethanol and given in a volume that gave a final ethanol concentration of the bath or incubation buffer of less than 0.07%. Control studies indicated that the ethanol vehicle had no effect on basal tone or on the response of the vasoactive compounds.

**Statistical Analysis**

Data are expressed as the mean±SEM. Statistical evaluation of the vascular reactivity data was performed by using a one-way analysis of variance followed by the Sidak multiple comparison test when significant differences were present. A Student's t-test was used to calculate the cyclic GMP and cyclic AMP data. A value of p<0.05 was considered statistically significant.

**Results**

Arachidonic acid caused concentration-dependent relaxations in norepinephrine-precontracted intact aortic rings, but not in aortic rings with the endothelium removed (Figure 1). Pretreatment with indomethacin enhanced the relaxation response to arachidonic acid (maximal response, 31±2% versus 42±2% control versus indomethacin, p<0.01, Figure 1). However, indomethacin did not affect either the basal tension or norepinephrine-induced contractions. Acetylcholine also elicited concentration-dependent relaxations in norepinephrine-precontracted intact aortic rings, but not in aortic rings with the endothelium removed (Figure 2). In contrast to the results observed with arachidonic acid, indomethacin pretreatment had no effect on the relaxant response to acetylcholine (maximal response, 43±3% versus 41±3%, control versus indomethacin, NS, Figure 2). Because indomethacin potentiated the response to arachidonic acid, we investigated
the effect of the other inhibitors in the presence of indomethacin. When aortic rings were incubated with the lipoxygenase inhibitor NDGA, arachidonic acid failed to relax norepinephrine-precontracted vessels (Figure 3). NDGA also blocked the relaxations by acetylcholine (Figure 4). In contrast, the cytochrome P450 inhibitor metyrapone had no effect on arachidonic acid relaxation (Figure 3); however, metyrapone partially blocked the acetylcholine-induced relaxations. The NO inhibitors had varying effects on arachidonic acid- and acetylcholine-induced relaxations. No change was observed with L-NMMA on arachidonic acid-induced relaxations (maximal relaxation, 35.4±4.7% versus 35.3±3.3%, control versus L-NMMA, NS, Figure 5). L-NMMA elicited a 76% reduction in the maximal relaxation to acetylcholine (Figure 6). In contrast, LNA caused a 52% reduction in the maximal relaxation to arachidonic acid (maximal relaxation, 35.4±4.7% versus 16.8±1.4%, control versus LNA, p<0.05, Figure 5) and completely inhibited acetylcholine-induced relaxations (Figure 6). At concentrations of arachidonic acid less than 10^{-4} M, there was no inhibitory effect of LNA observed.

Because the ability of indomethacin to enhance arachidonic acid–induced relaxations may be due to inhibition of the synthesis of vasoconstrictor prostaglandins, control vessels were pretreated with the thromboxane receptor antagonist SQ 29548. No differences in the arachidonic acid–induced relaxation were observed between control and SQ 29548-treated vessels (maximal relaxation, 40±4% versus 34±4%, control versus SQ 29548).
29548, NS). However, in the concentration tested, SQ 29548 inhibited the thromboxane mimetic, U46619- and prostaglandin F\textsubscript{2\alpha} (PGF\textsubscript{2\alpha})-induced contraction (data not shown). Under basal tension, arachidonic acid elicited a concentration-related contraction of rabbit aorta (10\textsuperscript{-4} M, 11±1% of the maximal KCl contraction; 10\textsuperscript{-5} M, 16±3% of the maximal KCl contraction; p<0.05) that was completely inhibited by indomethacin.

In the bioassay experiments, neither acetylcholine nor arachidonic acid relaxed the detector ring when infused through the denuded donor vessel or steel tube. However, when acetylcholine or arachidonic acid was added to the perfusate proximal to the donor vessel segment, both compounds relaxed the detector ring (Figure 7). This indicated that both acetylcholine and arachidonic acid stimulate the release of a relaxant factor from a rabbit donor vessel with an intact endothelium. The relaxation observed with arachidonic acid differed from that seen with acetylcholine in that the response was more sustained and more difficult to wash out.

The effect of various inhibitors of cyclooxygenase, lipoxygenase, and cytochrome P\textsubscript{450} epoxygenase on the metabolism of [\textsuperscript{14}C]arachidonic acid was determined in aortic tissue (Figure 8). The control incubations indicated major radioactive metabolites comigrating with the prostaglandins and hydroxyeicosatetraenoic acids (HETEs). In addition, minor radioactive metabolites were apparent in the regions that corresponded to the dihydroxyeicosatetraenoic acids (DHETs), dihydroxyeicosatetraenoic acids (DiHETEs), and epoxyeicosatrienoic acids (EETs). The cyclooxygenase inhibitor indomethacin attenuated prostaglandin synthesis and slightly increased the HETEs. In contrast, the proposed lipoxygenase inhibitor NDGA suppressed the formation of all the radioactive arachidonic acid metabolites. Metyrapone, the cytochrome P\textsubscript{450} epoxygenase inhibitor, decreased DHETE-DHET and EET production but had little effect on prostaglandin and HETE synthesis.

Acetylcholine significantly increased the aortic content of cyclic GMP above control values, whereas arachidonic acid elicited a significant decrease in aortic

**Figure 7.** Representative tracings of three experiments show effect of acetylcholine and arachidonic acid on the release of a transferable vasodilator factor. An endothelium-intact donor vessel was perfused and the activity in the perfusate bioassayed on a norepinephrine (NE)-precontracted denuded rabbit aorta. Panel A: Effect of the drugs on direct superfusion (i.e., no donor vessel). Panel B: Effect of perfusion through the donor vessel.

**Figure 8.** Representative tracings show effects of various inhibitors on [\textsuperscript{14}C]arachidonic acid metabolism by rabbit aorta. Vessels were preincubated with vehicle, indomethacin (10\textsuperscript{-5} M), nordihydroguaiaretic acid (NDGA) (5×10\textsuperscript{-5} M), or metyrapone (10\textsuperscript{-4} M). After incubation, media was extracted and subjected to reverse-phase high-performance liquid chromatography as described in “Methods” section. Darkened bars on the x-axis indicate the fractions corresponding to the prostaglandins, dihydroxyeicosatetraenoic acid-DHETs, HETEs, and EETs. Migration of known standard eicosanoids are shown above the chromatogram. These standards indicate the relative retention times of the compounds and are not meant to identify the corresponding radioactive peaks. 6-Keto PGF\textsubscript{1α}, 6-ketoprostaglandin F\textsubscript{1α}; 14, 15-DHET, dihydroxyeicosatetraenoic acid; 15-HETE, hydroxyeicosatrienoic acid; 14, 15-EET, epoxyeicosatrienoic acid.
It is without effect on the rabbit aorta. Instead, our studies suggest that a non-cyclooxygenase product of arachidonic acid is involved in mediating the vasodilation. It should be noted that under basal tension (vessels not precontracted with norepinephrine), arachidonic acid did elicit a small cyclooxygenase-mediated contraction. Similar responses were reported by Singer and Peach. The significance of this effect is not known.

Because NDGA, but not metyrapone, blocked the response to arachidonic acid, it is possible that the relaxant factor released by arachidonic acid is a lipoxygenase product. Uotila et al reported that deendothelialized rat aorta incubated in the presence of soybean lipoxygenase relaxed to arachidonic acid, further supporting a relaxant role for a lipoxygenase metabolite of arachidonic acid. However, other studies in rabbit pulmonary artery and canine coronary artery have shown that endothelium-dependent arachidonic acid–induced relaxations were blocked by inhibitors of cytochrome P450.27 The dual cyclooxygenase and lipoxygenase inhibitor BW755C had no effect on arachidonic acid–induced relaxations in rabbit pulmonary artery. Thus, it is possible that the factor mediating arachidonic acid relaxation differs between both the species and vascular tissue used.

Alternatively, NDGA is reported to have nonspecific effects and may inactivate EDRF through a mechanism independent of its ability to block the lipoxygenase enzyme. Griffith et al showed that NDGA would not prevent the relaxation of rabbit thoracic aorta by acetylcholine unless used in a concentration that possessed antioxidant activity. Thus, it may be necessary to determine whether NDGA inhibits the production of EDRF by acetylcholine and arachidonic acid or if NDGA inactivates the factor after it has been released by these agents. A recent study by Minami and Toda found that specific 5-lipoxygenase inhibitors, with no antioxidant activity, partially inhibited the synthesis or release, or both, of EDRF and had no effect on EDRF after it had been released. Therefore, it may be interesting to test more specific inhibitors of lipoxygenase on arachidonic acid–induced relaxations.

It is surprising that the cytochrome P450 inhibitor metyrapone partially inhibited acetylcholine-induced relaxation but had no effect on arachidonic acid relaxations. These results, however, give further support to the possibility that the factor released by acetylcholine and arachidonic acid are different. They also suggest that at least part of the relaxant effect of acetylcholine is mediated by a cytochrome P450 product. Along these lines, EDRF is thought to be formed from arginine by NO synthetase. This enzyme, like cytochrome P450, requires NADPH as a cofactor, and NO synthetase catalyzes the hydroxylation of a guanidine nitrogen of arginine. It is possible that NO synthetase is a cytochrome P450-like enzyme that is sensitive to inhibition by metyrapone and related drugs. Alternatively, Forstermann et al showed that like the lipoxygenase inhibitors, the cytochrome P450 inhibitors possess nonspecific properties that could account for their blockade of EDRF.

To support our results with the effects of the various inhibitors or arachidonic acid– and acetylcholine-induced relaxations, experiments were performed to determine the effects of these inhibitors on the metabolism of arachidonic acid by rabbit aorta. We have
previously shown that rabbit aorta metabolizes arachidonic acid to prostaglandins and HETEs. The present study confirmed this observation. The earlier study indicated that indomethacin blocked 6-keto-PGF, but not 12- and 15-HETE production, whereas NDGA blocked 6-keto-PGF and 12- and 15-HETE production. In the present study, in concentrations and incubation times identical to those used for the vascular reactivity experiments, indomethacin blocked the prostaglandins, NDGA blocked both prostaglandins and HETEs, and metyrapone blocked the EETs. Since only NDGA (and not indomethacin or metyrapone) blocked arachidonic acid-induced relaxations, these results suggest that an unidentified lipoxygenase metabolite of arachidonic acid may mediate relaxation. The HETEs, and their precursors, the HPETEs, elicit vasoconstriction in some tissues. However, we have eliminated 12(S)- and 15(S)-HETE as possible mediators based on a previous study that showed no effect of these compounds on precontracted rabbit aorta.

Because both biological and chemical studies have identified the EDRF released by acetylcholine as NO, we examined the ability of specific inhibitors of NO synthase to alter the relaxations observed with acetylcholine and arachidonic acid in rabbit aorta. Our results with both L-NMMA and LNA confirm that the factor released by acetylcholine is NO or a NO-like derivative. However, it does not appear that arachidonic acid relaxations are mediated by NO since L-NMMA had no effect on relaxation and LNA elicited only a partial inhibition at the 10−4 M concentration of arachidonic acid.

The bioassay study indicated that both arachidonic acid and acetylcholine released a transferable factor that relaxed vascular smooth muscle. The fact that the responses to arachidonic acid were more sustained than those observed with acetylcholine suggest that different factors may be released. A more convincing argument for the existence of different factors are the results that showed acetylcholine elicited a twofold increase in aortic tissue cyclic GMP content, whereas arachidonic acid caused a significant decrease in cyclic GMP content as compared with control vessels. It is well established that acetylcholine and NO increase cyclic GMP content. However, conflicting reports exist regarding the ability of arachidonic acid and other fatty acids to increase cyclic GMP. Furchgott et al reported that the saturated fatty acid heptadecanoic acid increased cyclic GMP. Furchgott et al reported that the saturated fatty acid heptadecanoic acid increased cyclic GMP in rabbit aorta. Arachidonic acid was not tested. Using precontracted bovine intrapulmonary artery, Ignarro et al showed arachidonic acid elicited a 4.5-fold and twofold increase in cyclic GMP at 2 and 3 minutes, respectively. In contrast, Rapoport et al reported that arachidonic acid caused no change in rat thoracic aorta cyclic GMP content at 15 seconds, 1 minute, or 5 minutes. In our studies, arachidonic acid also failed to stimulate cyclic GMP content in rabbit aorta. Thus, under our experimental conditions, these results suggest that arachidonic acid relaxation is not only mediated by a different factor than that released by acetylcholine but also that the relaxation response is mediated via a different mechanism.

In summary, our findings suggest that the endothelium-dependent vasodilators acetylcholine and arachidonic acid act through different mediators to elicit vascular relaxation and thus support the concept that more than one EDRF may exist. Although the identity of the factor released by acetylcholine is believed to be NO or a NO-like compound, at the present time no information exists about the identity of the factor released by arachidonic acid. Our results from both the vascular reactivity and arachidonic acid metabolism studies suggest that the factor may be a lipoxygenase metabolite, but further experiments are required to confirm this conclusion. It is possible that the concentration of exogenously applied arachidonic acid is greater than what is measured under physiological conditions. Once the identity of the factor is determined, it will be important to correlate its synthesis with the concentration required to elicit vasoconstriction.
Arachidonic acid- and acetylcholine-induced relaxations of rabbit aorta.
S L Pfister and W B Campbell

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