Endothelial Mechanism in the Vascular Action of Hydralazine

ERIC G. SPOKAS, PH.D., GIANCARLO FOLCO, PH.D., JOHN QUILLEY, PH.D., PRAVEEN CHANDER, M.D., AND JOHN C. McGiff, M.D.

SUMMARY Relaxation of precontracted isolated chains of aortic rings with intact endothelium and in those with the endothelium removed was studied in response to various antihypertensive vasodilator drugs. Of the drugs tested — nitroprusside, nitroglycerin, prazosin, minoxidil, diazoxide and hydralazine — only the vascular relaxant effects of hydralazine were found to be dependent, in part, on the presence of intact endothelium. The endothelial component of the hydralazine response represented a major contribution to the net relaxant effect on the vascular smooth muscle, particularly at lower concentrations, 90 nM to 1 µM, which are also clinically relevant.

KEY WORDS • prostacyclin • endothelium • hydralazine • antihypertensive drugs • vasodilatation

Of the antihypertensive drugs classified as "vasodilators", hydralazine (1-hydrazinophthalazine) is one of the most widely used for the long-term management of hypertension. The primary site of action of hydralazine is generally accepted to be the vascular wall, as low doses administered arterially cause local vasodilation of gradual onset. Although the basis for the direct vasodilator effect of hydralazine has been studied extensively for three decades, the mechanism has not been established. Part of the difficulty associated with efforts to define the underlying biochemical changes has been the variable in vitro activity of the drug. In studies with isolated blood vessels, millimolar concentrations may be required to evoke relaxation, even when superimposed on a strong contraction induced by norepinephrine (NE).

The vascular endothelium has been shown by Furchgott and Zawadzki to be crucial for demonstration of relaxation of blood vessels in response to acetylcholine (ACh) and some other naturally occurring vasodilator substances. More to the point, the failure of ACh to cause relaxation of isolated blood vessels in earlier studies was an artifact arising from unintentional removal of the intima attendant to preparation of vascular rings or strips for tension recording. Besides resolving a long-standing controversy as to the anomalous vascular effect of ACh in vitro, the results from Furchgott and Zawadzki's laboratory suggested to us the possibility of an endothelial component to the vasodilator effect of hydralazine, which might explain the large doses required to relax isolated blood vessels. We report here the effects of the removal of endothelial cells on the mechanical responses of aortic rings to hydralazine and to other vasodilator/antihypertensive agents. The effectiveness of the denuding procedure designed to remove the endothelium was assessed functionally from the response to ACh as well as histologically, using light microscopy.

Methods

Transverse rings were obtained from the descending thoracic aorta of male New Zealand white rabbits. Endothelium was removed by apposition of the intimal surfaces and gentle rubbing between the fingers for approximately 30 seconds. Four to six rings were joined together with surgical silk (3.0) to form a chain and placed in a 15 ml glass organ bath containing Krebs' Ringer bicarbonate solution at 37°C, aerated with a mixture of 95% O2 and 5% CO2. For each experiment, four chains of rings from the same aorta, consisting of two rubbed and two unrubbed chains were studied. Basal tension (2 g) was applied following an equilibration period of at least 2 hours, and the changes in isotonic contraction were monitored with Model 356 Harvard transducers and a model 1244 Soltec recorder. Responses to various vasodilator agents were studied during enhancement of vascular tone with...
a submaximal concentration of NE (10 or 20 nM) added in the presence of 35 μM ascorbic acid.

ACh was tested during an initial contracture evoked by NE. The bath was rinsed, and the Krebs medium replaced: 30 minutes later, tone was again increased with NE. Hydralazine was then added in a cumulative fashion at intervals of 12–15 minutes. Subsequently, the tissues were rinsed three times, and 30–45 minutes later the relaxant response to ACh was retested during a third NE-induced contracture. In some experiments rabbits were pretreated with indomethacin, 5 mg/kg i.v., 30 minutes before sacrifice; indomethacin (10 μM) was also added to the Krebs medium in the organ bath.

In some experiments immunoreactive PGE₂ was measured by radioimmunoassay (RIA). RIA of the unextracted incubates was carried out in duplicate, the lower limit of sensitivity for this assay was 60 pg/ml. PGE₂ antiserum was obtained from Institut Pasteur Production, Paris, France. The cross-reactivities of the antiserum were 3.2% for PGE₁, 2% for 6-keto-PGF₁α, 0.2% for PGA₁, 0.15% for 13,14-dihydro-PGE₂, 0.11% for 13,14-dihydro-15-keto-PGE₂, and less than 0.1% for PGE₂, PGA₁, 13,14-dihydro-PGE₁, 13,14-dihydro-15-keto-PGE₁, and PGF₁α.

The effectiveness of the denuding procedure designed to remove the endothelium was assessed functionally from the response to ACh as well as histologically, using light microscopy. Chains of aortic rings were placed in petri dishes containing Krebs' buffer and separated into individual rings. Each ring was then opened with fine dissecting scissors, forming a strip which was pinned at the corners to dental wax. Control and experimental tissues, rubbed and unrubbed, were subjected to a silver-staining technique modified from that described by Poole et al., followed by formalin fixation and mounting on glass slides without embedding. Percent endothelium remaining was determined semiquantitatively by ent face single blind light microscopic examination of the entire intimal surface of the rings. The rubbing procedure resulted in almost complete loss of endothelial cells.

The composition (mM) of the Krebs buffer was: NaCl 118, KCl 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 24, glucose 9.6; pH was 7.4. The following drugs were used: hydralazine HCl (Apresoline, Ciba-Geigy, Summit, New Jersey), sodium nitroprusside (Nipride, Hoffmann-LaRoche, Nutley, New Jersey), nitroglycerin (Parke-Davis, Detroit, Michigan), minoxidil (Upjohn, Kalamazoo, Michigan), diazoxide (Schering, Bloomfield, New Jersey), prazosin HCl (Pfizer, New York, New York), 1-norepinephrine bitartrate (Levophed, Winthrop, New York, New York), BW 755C (Wellcome Research Laboratories, Beckenham, Kent, England), 1-ascorbic acid, acetylcholine chloride, indomethacin and nordihydroguaiaretic acid (NDGA) (Sigma Chemical Company, St. Louis, Missouri). Drug solutions were prepared on the day of the experiment, stored on ice, and added to the tissue bath in a volume of 120–180 μl. A stock solution of hydralazine HCl (crystalline form) was prepared in distilled water (1 mg base/ml) and diluted further with Krebs solution. A stock solution of diazoxide (43 mM) was prepared in NaOH (0.4 M) and subsequently diluted using Krebs buffer. Indomethacin was solubilized in 0.9% saline containing 0.1% NaCO₃ (final concentration indomethacin = 3.57 mg/ml, pH 8.5). This stock solution was used undiluted for intravenous pretreatment of the animals (5 mg/kg) 30 minutes before sacrifice and diluted to 10 μM indomethacin with Krebs medium for treatment in the tissue bath. A stock solution of 160 mM NDGA was prepared in concentrated sulfuric acid and diluted with Krebs solution, adjusting the pH to 7.9 with a small volume of NaOH (5 M).

Individual dose-response curves were linearized by plotting on semilog paper after probit transformation. For the probit analysis, responses greater than 99% loss of NE-induced tone were arbitrarily assigned a value of 99%. The data from each chain were used for the calculation of mean responses by fitting with individual regression lines between probits 3 and 7 (least squares method for log curves). Mean probits, which were thereby equally-weighted, were plotted at selected concentrations to obtain the curves shown here. In most experiments, three or four concentrations of hydralazine were tested on each chain. ID₅₀ and ID₉₀ values were obtained from regression lines fitted with log curves by the least square method. Geometric means are presented as the statistical comparisons were carried out on the log ID values. Students' unpaired t test was used for these comparisons.

Results

Responses to concentrations of NE in the range of 1 nM to 10 μM were examined in preliminary studies carried out on six rubbed and six unrubbed chains. Rubbing did not affect the NE dose-response curve, either in terms of slope, threshold concentration or magnitude of the peak response. ACh, tested during an initial contracture evoked by NE, invariably relaxed the unrubbed, but not the rubbed rings (fig. 1). Expressed as percent inhibition of NE-induced tone, relaxation in response to 200 nM ACh averaged 57% ± 4% in unrubbed chains, whereas in rubbed chains either no response or a small contraction (−2% ± 2%) occurred. At the completion of the experiment during the third NE contraction, ACh response was found to be somewhat reduced (mean inhibition of NE-induced tone = 41% ± 9%), but not significantly (p > 0.05) when compared to the initial response using a paired t test.

Isolated aortic chains with intact endothelium were very sensitive to hydralazine. From the cumulative dose-response relationships shown in figures 1 and 2, it is evident that concentrations below 300 nM were effective in antagonizing contractions induced by NE. The hydralazine response of unrubbed chains was characterized by a gradual loss of tone which, at low concentrations of hydralazine, was sometimes preceded by a latent period of several minutes. Rubbing caused a reduction in the sensitivity of the tissue to hydralazine as indicated by the movement of the dose-
response curve to the right, although the maximal relaxant effect was comparable in rubbed and unrubbed chains (fig. 2). The ID₅₀, defined as the concentration of hydralazine which caused 50% inhibition of NE-induced tone, was determined for individual experiments where two to five concentrations were tested. Higher ID₅₀ values were found in the de-endothelialized chains (geometric mean 1.405 μM rubbed, versus 0.507 μM unrubbed, p > 0.001). An increase in the threshold concentration for the hydralazine response, due to rubbing, was suggested by a significant increase in the ID₅₀ to 180 nM (rubbed) from 91 nM (unrubbed), p < 0.05. In contrast to these results with hydralazine, the rubbing procedure did not alter the response of NE-precontracted aortic chains to other vasodilator test drugs including sodium nitroprusside, nitroglycerin, minoxidil, diazoxide and prazosin (table 1).

Histologic examinations (carried out single blind) confirmed the effectiveness of the rubbing procedure in removing endothelium. Rubbed rings from chains which did not relax in response to ACh evidenced almost complete loss of endothelial cells (fig. 3 upper right). Estimates for percent remaining ranged from 0 to 1% of the total intimal surface (n = 3 chains, 12 individual strips). Estimates for percent endothelium remaining on unrubbed chains which relaxed ranged from 15% to 65% of the total intimal surface (fig. 3 lower left) (n = 3 chains, 12 individual strips). Further, studies of unrubbed control aortic rings not mounted for tension recording, but stained and fixed for microscopy directly after sectioning the aorta with fine scissors, indicated that a large fraction of endothelium, 10% to 40% (n = 19), was routinely lost through surgical isolation and/or sectioning (fig. 3 upper left).

In experiments designed to investigate the possible role of prostaglandins, unrubbed aortic chains were treated with the cyclooxygenase inhibitor, indomethacin. Cyclooxygenase inhibition was verified by RIA analysis of basal PGE₂ released into the incubate over a 1-hour period. Mean control levels were 104 ± 11 pg/ml (n = 4); after indomethacin treatment immunoreactive PGE₂ was not detected, i.e., < 60 pg/ml. The relaxant effects of hydralazine (60 and 600 nM) were...
TABLE 1. Relieving Action of Drugs on Chains of Rabbit Aortic Rings Having Either Intact Endothelium or Endothelium Damaged by Rubbing

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (μM)</th>
<th>Intact* (% inhibition)</th>
<th>Rubbed* (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nitroprusside</td>
<td>0.006</td>
<td>20 ± 3 (4)</td>
<td>24 ± 2 (4)</td>
</tr>
<tr>
<td></td>
<td>0.024</td>
<td>51 ± 6 (4)</td>
<td>64 ± 7 (4)</td>
</tr>
<tr>
<td></td>
<td>0.084</td>
<td>82 ± 7 (4)</td>
<td>89 ± 6 (4)</td>
</tr>
<tr>
<td>nitroglycerin</td>
<td>0.006</td>
<td>29 ± 3 (5)</td>
<td>30 ± 3 (5)</td>
</tr>
<tr>
<td></td>
<td>0.024</td>
<td>64 ± 6 (5)</td>
<td>68 ± 4 (5)</td>
</tr>
<tr>
<td></td>
<td>0.084</td>
<td>88 ± 5 (5)</td>
<td>89 ± 2 (5)</td>
</tr>
<tr>
<td>minoxidil</td>
<td>0.060</td>
<td>12 ± 4 (5)</td>
<td>12 ± 3 (5)</td>
</tr>
<tr>
<td></td>
<td>0.660</td>
<td>32 ± 10 (5)</td>
<td>32 ± 8 (5)</td>
</tr>
<tr>
<td></td>
<td>6.67</td>
<td>46 ± 15 (4)</td>
<td>43 ± 11 (4)</td>
</tr>
<tr>
<td>diazoxide</td>
<td>0.060</td>
<td>8 ± 2 (4)</td>
<td>6 ± 2 (4)</td>
</tr>
<tr>
<td></td>
<td>0.660</td>
<td>25 ± 4 (4)</td>
<td>22 ± 1 (4)</td>
</tr>
<tr>
<td></td>
<td>6.67</td>
<td>48 ± 6 (4)</td>
<td>45 ± 3 (4)</td>
</tr>
<tr>
<td>prazosin</td>
<td>0.060</td>
<td>80 ± 6 (4)</td>
<td>77 ± 6 (4)</td>
</tr>
</tbody>
</table>

*Relaxation is expressed as percent inhibition (mean ± SE) of tone induced by 10 or 20 nM norepinephrine and was measured 5 minutes (nitroprusside, nitroglycerin, prazosin) or 20 minutes (minoxidil, diazoxide) after addition to the bath. These intervals were the respective cycle times between additions. All additions were made during the sustained phase of the norepinephrine contracture and the concentrations given are cumulative. Number of chains is shown in parentheses.

not affected by indomethacin treatment. Indomethacin also did not influence the relaxant effect of ACh on unrubbed chains.

Responses to hydralazine were tested in the presence of BW 755C (n = 2) (100-400 μM) or NDGA (n = 2) 20 to 50 μM, agents reported to inhibit the lipoxygenase pathway for arachidonic acid metabolism.12,13 For these experiments, the effect of the inhibitor was studied on paired preparations from the same aorta (one rubbed chain and one with intact endothelium) by addition (BW 755C) after hydralazine had caused approximately 80% relaxation, or by pretreatment (NDGA) for at least 30 minutes before cumulative additions of hydralazine. The relaxant effect of hydralazine on unrubbed and rubbed chains was not reduced by treatment with these lipoxygenase inhibitors. ACh-induced relaxation, studied using a similar experimental design, also was not antagonized (n = 5).

Discussion

We conclude that the rabbit aortic preparation is sensitive to hydralazine if care is taken to preserve the endothelium. Our results contrast with those reported in a number of previous in vitro studies of rabbit aorta.3-6 The high degree of responsiveness of aortic rings...
used in our study, even when rubbed, derives from several factors: 1) the use of chains of aortic rings rather than individual rings or spiral strips; 2) recording isotonic rather than isometric contractions; 2) precontraction of the aortic rings with NE rather than potassium. It is noteworthy that at concentrations thought to be attained in the plasma of patients receiving hydralazine for hypertension (1 μM or less), the endothelial component of the hydralazine response constitutes a major fraction. It may be significant that the reduction of vascular tone produced by hydralazine in organ bath studies is not rapid in onset but characteristically requires 10 minutes or more to develop fully. A similar time course has been described for the vascular resistance changes induced by hydralazine in vivo, a further indication that our in vitro finding of endothelial dependence may have relevance to the local vascular mechanism activated in the intact animal.

The contribution of endothelial cells to the aortic relaxing action of hydralazine may be a more general phenomenon, similar to that reported for ACh, as several large arteries of the dog — aorta, pulmonary and renal arteries — demonstrated blunting of hydralazine-induced relaxation after removal of the endothelium (unpublished observations). Our results do not permit a statement as to the nature of the endothelial contribution to the relaxing action of hydralazine. It does not appear to be prosta glandin-mediated inhibition of cyclooxygenase with indomethacin did not interfere with the effect of the drug. Neither did BW 755C or NDGA, agents which have been described to inhibit lipoxygenases, as it should be noted that inhibitors of lipoxygenases have been reported to attenuate ACh-induced relaxation of the femoral artery, an effect that may depend on the blood vessel used, the effective concentration of the inhibitor and the sensitivity of the experimental preparation to agonists. A product of phospholipase activity such as an arachidonate metabolite has been suggested to account for the endothelial contribution to vasoactive agents.

There are two additional considerations that bear on the interpretation of our study. First, as active metabolites of hydralazine are known, it is possible that hydralazine is transformed to a more active material by the endothelium. This explanation, however, lacks support as none of the known active metabolites is more potent than the parent compound, although a metabolite may possess physicochemical properties that favor vascular relaxation under these experimental conditions, e.g., facilitated access to active sites in the vasculature.

The endothelial independence of minoxidil-induced aortic relaxation was unexpected because of chemical similarities of minoxidil and hydralazine as well as similarities in their circulatory effects and affinity for vascular tissue. However, the vascular smooth muscle components for both antihypertensive agents may share a common mechanism.

Finally, the endothelial component of the aortic response to hydralazine accounts for only a portion, albeit large at low doses, of the net relaxant effect of hydralazine on vascular smooth muscle. The parallel shift in the dose response curve for hydralazine suggests subtraction of a constant factor through loss of the endothelium. The possibility should be considered that elements of the vasa vasorum present in the vascular rings may also act as a source of this factor. The nature of this factor awaits definition.

Acknowledgments

We thank Dr. Sylvan Wallenstein, Assistant Professor of Public Health, College of Physicians and Surgeons, Columbia University, for assistance with statistical analysis. We are grateful to Patricia Hejny, Giuseppe Rossoni, and Kathy Pettillo for their technical assistance and to Donna Centi and Sallie McGiff for preparing the manuscript. The authors also thank Dr. Eugene Weiss of Pfizer Laboratories for supplying prazosin.

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Endothelial mechanism in the vascular action of hydralazine.
E G Spokas, G Folco, J Quilley, P Chander and J C McGiff

Hypertension. 1983;5:1107
doi: 10.1161/01.HYP.5.2_Pt_2.I107

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