Original Contributions

Endothelium-Dependent Relaxation and Hyperpolarization in Aorta From Control and Renal Hypertensive Rats

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Endothelium-dependent relaxations are depressed in hypertension. In this study we investigated the possible involvement of endothelium-dependent smooth muscle hyperpolarization in this phenomenon. In isolated aortic segments from control rats, acetylcholine (10^{-8}-10^{-5} M) elicits relaxations after precontraction with norepinephrine (10^{-7} M), and acetylcholine or carbachol (10^{-5} M) induce smooth muscle hyperpolarization (10.6±0.9 mV). Both effects disappear after removal of the endothelium and are depressed by tetraethylammonium (3×10^{-3} M), a rather nonspecific blocker of K^+ channels, but not by glibenclamide (10^{-5} M), a potent blocker of the ATP-regulated K^+ channels, which has a marked effect on the relaxation induced by BRL 38227. The relaxation effect of acetylcholine is impaired in norepinephrine-contracted preparations from hypertensive rats but is not further depressed by tetraethylammonium. In aorta from hypertensive rats, hyperpolarization induced by carbachol was significantly reduced to a mean of only 21.8% of the values obtained in preparations from normotensive rats. From the relaxation–hyperpolarization relation obtained with BRL 38227 (opening K^+ channels), it is derived that the endothelium-dependent hyperpolarization (~10 mV) contributes for at least 20–30% of the maximal relaxation effect of acetylcholine on rat aorta. It is concluded that the diminished endothelium-dependent hyperpolarization may contribute to the depression of the endothelium-dependent relaxation in hypertension. (Circulation Research 1992;70:1–8)

Many vasodilating substances produce relaxation of vascular smooth muscle cells through the release of relaxing factors from the vascular endothelium. This has provoked extensive research on the nature of these relaxing factors, on the mechanisms by which they produce relaxation, and on their possible role in the regulation of the vascular tone in physiological and pathophysiological situations.1–3 It was found that the endothelial relaxing influence is importantly depressed in different models of hypertensive animals (renovascular, deoxycorticosterone acetate–salt, and genetic models), not only in the great conduit arteries4–10 but also at the level of the resistance vessels.11–13 Recent studies also revealed blunted endothelium-dependent relaxation responses in humans with hypertension.14,15 The cause of this impairment is not established.

Endothelium-dependent relaxations are mediated by an endothelium-derived relaxing factor (EDRF), which induces relaxation through increasing muscular cGMP levels, and also by an endothelium-derived hyperpolarizing factor (EDHF), which relaxes vascular smooth muscle cells through hyperpolarization as a result of opening of potassium channels.16–21

The present study was designed to investigate the endothelium-dependent hyperpolarization in smooth muscle cells of aorta from renal hypertensive rats.

Materials and Methods

Drugs

Acetylcholine chloride (ACh), norepinephrine bitartrate (NE), tetraethylammonium chloride (TEA), and carbamylcholine chloride (carbachol) were obtained from Sigma Chemical Co., St. Louis, Mo. BRL 38227 was a gift from Beecham Pharmaceuticals, Essex, UK, and glibenclamide was a gift from Hoechst, Belgium. All concentrations are expressed as final molar concentrations in the organ bath.
Induction of Renal Hypertension

Male Wistar rats weighing 150 g were anesthetized with 1% halothane in O₂. An incision was made on the left flank to provide access to the left renal artery, which was separated from the renal vein and cleaned from connective tissue. A U-shaped solid silver clip with an opening of 0.2 mm was applied on the exposed renal artery. The contralateral kidney remained untouched (two-kidney, one-clip renal hypertensive model). Hypertensive rats were selected on the basis of the systolic blood pressure values obtained in conscious rats by use of the tail-cuff method with a piezoelectric pneumatic pulse transducer. The heart was excised at death and dried at 105°C for 24 hours. The dry heart weight to body weight ratio (HW/BW×100) was calculated as a measure of the hypertrophy of the heart.

Electrophysiological Measurements

The thoracic aorta of renal hypertensive and control normotensive rats was isolated after killing and was dissected free of surrounding tissue. Great care was taken not to injure the endothelium. Aortic ring segments (5–7 mm) of normal and hypertensive rats were carefully slit along the longitudinal axis. The strips were pinned down, inside downward, on the bottom of an experimental chamber and continuously superfused with oxygenated Krebs-Ringer bicarbonate fluid containing (mM) NaCl 135, KCl 5, NaHCO₃ 20, glucose 10, CaCl₂ 2.5, MgSO₄·7H₂O 1.3, KH₂PO₄ 1.2, and EDTA 0.026 at 37°C. Transmembrane potentials were measured using conventional micro-electrodes, pulled with a David Kopf puller (model 750, David Kopf Instruments, Tujunga, Calif.) from filamented borosilicate glass (1 mm o.d., Hilgenberg, FRG), and filled with 3 M KCl. The electrical resistance of the microelectrodes, measured in normal Krebs-Ringer solution, ranged from 25 to 80 MΩ. The measured potential was followed on oscilloscope and traced with a pen recorder at low speed. Primary criteria for successful cell impalement were an extremely sharp voltage deflection on entering the cell, as judged visually on oscilloscope, and a sharp return to the baseline value on exit. Values of the membrane potential were taken as the difference of the stabilized potential and the zero potential on rapid withdrawal of the electrode from the cell. The effect of ACh and c-WL, and of BRL 38227, was examined in preparations from hypertensive and control rats in control conditions, after rubbing the endothelium, and in the presence of 3×10⁻³ M TEA or 10⁻³ M glibenclamide.

Tension Measurements

Ring segments were prepared and mounted in muscle chambers (20 ml) containing the Krebs-Ringer bicarbonate solution at 37°C, through which a mixture of 95% O₂–5% CO₂ was bubbled. To obtain solutions containing 80 mM K⁺, equimolar concentrations of NaCl were replaced by KCl. The rings were suspended under a tension of 0.5 g, and the isometric force of contraction was measured with a force-displacement transducer (UC-2 cell, Gould-Statham, Oxnard, Calif.). The preparations were equilibrated under tension for 1 hour before the start of the measurements. Preparations were precontracted by 10⁻⁴ M NE or 80 mM K⁺, and the relaxation curves that were obtained by addition of cumulative doses of ACh or BRL 38227 were registered in control conditions and in the presence of 3×10⁻³ M TEA or 10⁻³ M glibenclamide. For the construction of cumulative concentration-response curves, subsequent doses of ACh or BRL 38227 were added to the bath fluid. The maximal response attained at each concentration was used in the calculations.

Statistics

Results are expressed as mean±SEM. Statistical significance was evaluated using Student's t test for unpaired observations; n indicates the number of preparations obtained from different rats, unless otherwise indicated.

Results

Electrophysiological Experiments

In control preparations, a stable quiescent membrane potential of aortic smooth muscle was recorded with a mean value of −49.6±0.6 mV (n=30) under control conditions.

In a first series of experiments (n=6), the effect of addition of ACh and of c-WL on the membrane potential (Eₘ) was compared (Figure 1). Changing control superfusion solution for a solution containing 10⁻⁴ M ACh resulted in a peak hyperpolarization, which amounted to 10.5±2.2 mV and was followed by a more or less pronounced recovery of Eₘ. After washout of ACh, Eₘ returned to the control level. Addition of 10⁻⁴ M c-WL to the superfusate elicited a response (10.1±1.2 mV) analogous to that with ACh; the only significant difference was that peak hyperpolarization was reached somewhat more slowly (2.6±0.3 versus 1.6±0.3 minutes, p<0.01, n=6). In all other electrophysiological measure-
ments, the stable synthetic analogue of ACh was used. In 30 control preparations, carbachol transiently increased $E_m$ by a mean value of $10.6\pm0.9$ mV.

The effect of $10^{-5}$ M carbachol on $E_m$ was also assessed on aortic strips, which were first denuded of their endothelial cells. Resting $E_m$ values were significantly less negative ($-45.5\pm2.7$ mV, $n=8$, $p<0.05$) than those recorded in preparations with endothelium ($-49.6\pm2.7$ mV, $n=30$). Addition of $10^{-5}$ M carbachol to endothelium-denuded strips elicited no measurable changes in $E_m$ ($n=8$, $p<0.001$) (Figure 2).

The hyperpolarizing effect of $10^{-5}$ M carbachol was also measured after superfusing the aortic preparation for 30 minutes with the potassium channel inhibitor TEA ($3\times10^{-3}$ M). Under these conditions, a resting potential of $-48.7\pm1.8$ mV was recorded ($n=4$). In the presence of TEA, the hyperpolarizing effect elicited by $10^{-5}$ M carbachol was significantly reduced to $0.7\pm0.5$ mV ($n=4$, $p<0.01$) (Figure 2).

Glibenclamide ($10^{-5}$ M), the more specific blocker of ATP-dependent K+ ($K_{\text{ATP}}$) channels, did not have a significant influence on the hyperpolarization induced by $10^{-5}$ M carbachol (Figure 3). At this concentration, glibenclamide blocked completely the hyperpolarization induced by $0.5\times10^{-6}$ M BRL 38227, the active enantiomer of cromakalim, an activator of $K_{\text{ATP}}$ channels. This is illustrated in Figure 3, in which the effect of $10^{-5}$ M glibenclamide on the hyperpolarization produced by $10^{-5}$ M carbachol and $0.5\times10^{-6}$ M BRL 38227 was tested during a continuous cell impalement. From concentration–response curves constructed for BRL 38227, it could be derived that $10^{-7}$ M of the $K_{\text{ATP}}$ channel opener elicited a hyperpolarizing effect similar to that of $10^{-5}$ M carbachol.

$E_m$ was also measured in aortic strips from hypertensive rats 6 months after the induction of hypertension (HW/BW×100=0.092±0.004, $n=13$). In these preparations the resting $E_m$ was significantly less negative ($-42.4\pm1.2$ mV, $n=13$, $p<0.001$) than it was in strips from control normotensive rats ($-49.6\pm0.6$ mV, $n=30$) (HW/BW×100=0.058±0.001, $n=30$). Addition of carbachol to the superfusion solution elicited a mean hyperpolarization of $2.2\pm0.5$ mV ($n=13$), which was significantly ($p<0.01$) smaller than the value obtained in control strips ($10.6\pm0.9$ mV) (Figure 2). In some preparations, hyperpolarization was even completely absent. Figure 4 shows an experiment in which the effect of addition of $10^{-5}$ M carbachol and $10^{-6}$ M BRL 38227 was measured in a preparation from a hypertensive rat. $E_m$ did not change significantly after application of carbachol, but it increased in response to BRL 38227. In six preparations, $10^{-6}$ M BRL 38227 changed $E_m$ from a mean value of $-41.5\pm2.1$ to a mean value of $-69.6\pm2.6$ mV (in preparations from normotensive rats, $E_m$ changed from $-51.1\pm1.3$ to $-78.4\pm1.7$ mV).

**Figure 2.** Recordings showing changes in membrane potential ($E_m$) of rat aortic smooth muscle cells in response to carbachol ($10^{-5}$ M). Measurements were taken in preparations from normotensive rats in control conditions (control), after removal of the endothelium (rubbed), and after treatment with $3\times10^{-3}$ M tetraethylammonium (TEA) and in preparations from renal hypertensive rats (hypertension).

**Figure 3.** Original recordings showing membrane potential ($E_m$) changes of rat aortic smooth muscle cells in response to carbachol ($10^{-5}$ M) and BRL 38227 ($0.5\times10^{-6}$ M) in the absence and presence of glibenclamide ($10^{-5}$ M).
The relaxation effects of increasing doses of ACh (from $10^{-9}$ to $10^{-5}$ M) were compared on thoracic aorta from control rats (HW/BW $= 0.057 \pm 0.001$, $n = 3$ rats) and renal hypertensive rats (HW/BW $= 0.093 \pm 0.005$, $n = 3$ rats) that were precontracted with $10^{-7}$ M NE or 80 mM K'. The results obtained are summarized in Figure 5A. In aortic rings from control rats, it was found that the ACh-induced relaxation effect was much less pronounced in preparations precontracted with K' than it was in the NE-contracted rings. In aortic rings from hypertensive rats precontracted with NE, the ACh-induced relaxation was much smaller than it was in the rings from normotensive rats. In the K'-contracted rings, there was only a slight difference in ACh response between the aortic rings from the hypertensive and the normotensive rats.

Influence of TEA and glibenclamide on ACh-induced relaxations. The influence of preincubation with TEA ($3 \times 10^{-3}$ M for 60 minutes) on endothelium-dependent relaxations elicited by increasing doses of ACh (from $10^{-9}$ to $10^{-5}$ M) was examined on precontracted ($10^{-7}$ M NE) thoracic aorta of control rats (HW/BW $= 0.059 \pm 0.02$, $n = 5$ rats) and renal hypertensive rats (HW/BW $= 0.082 \pm 0.005$, $n = 5$ rats) 6 months after induction of hypertension. The results are summarized in Figure 5B. An original recording of such an experiment on a control preparation is shown in Figure 6. In aorta from control rats, the ACh-induced relaxation is very significantly impaired by treatment with TEA. On the other hand, the relaxation effect of ACh on aortic rings from hypertensive rats was not further inhibited by TEA. The influence of glibenclamide ($10^{-2}$ M for 10 minutes) was also assessed on ACh-induced relaxations on precontracted (NE) aorta of control rats. The results are depicted in Figure 7B, illustrating that the selective blocker of KATP channels does not influence the endothelium-dependent relaxation effect of ACh.

Experiments with BRL 38227. The results of the experiments performed with BRL 38227 are summarized in Figure 7. It shows the influence of TEA ($3 \times 10^{-3}$ M for 1 hour), a less specific potassium channel inhibitor, and of glibenclamid ($10^{-5}$ M for 10 minutes), a more specific blocker of ATP-dependent potassium channels, on BRL 38227-induced relaxations in NE-precontracted rings. It illustrates that glibenclamide potently blocks BRL 38227-induced relaxations (Figure 7A), while having no significant influence on the concentration–relaxation curve to ACh (Figure 7B). The relaxation effect elicited by BRL 38227 was also inhibited by TEA (Figure 7C), but apparently much less importantly than the relaxation effect of ACh (Figure 5B). In rings precontracted by 80 mM K', BRL 38227 was not able to induce any relaxing effect (Figure 7D).

The relaxation effects of increasing doses of ACh (from $10^{-9}$ to $10^{-5}$ M) and BRL 38227 (from $10^{-8}$ to $10^{-5}$ M) were also compared on thoracic aorta from control rats (HW/BW $= 0.059 \pm 0.002$, $n = 5$ rats) and from renal hypertensive rats (HW/BW $= 0.093 \pm 0.005$, $n = 3$ rats).
100 = 0.101 ± 0.006, n = 5 rats, 5 months after clipping) precontracted with 10^{-7} M NE. Both substances were tested on the same preparations. The results (Figure 8) demonstrate that the relaxation effects elicited by lower doses of BRL 38227 are significantly less pronounced in preparations from hypertensive rats than from control rats. The relaxation effects of high doses of BRL 38227 were, however, not depressed in hypertensive preparations and even more pronounced than in control preparations.

**Discussion**

The relaxation of precontracted vessel rings to substances that exert their effect through an endothelium-dependent mechanism is importantly impaired in isolated blood vessels from different models of hypertensive animals. Recent evidence also indicates blunted endothelium-dependent dilations in human forearm blood vessels of hypertensive patients. Several studies have shown that this depression of endothelium-dependent relaxation in experimental hypertension is related to the increased pressure applied on the vessel wall. It is only present in blood vessels under increased pressure, as shown in rats with constricted aorta in which the depression was observed in the high pressure part of the aorta and not in the normal pressure zone below the constriction. It is also a reversible phenomenon, since the relaxation response is restored after normalization of blood pressure. The exact mechanism for this decreased endothelium-dependent response in hypertension is, however, not firmly established.

It has been documented that the vascular endothelial cells produce several vasoactive factors, causing both vasoconstriction and vasodilation. It has been proposed that an increased production of endothelial contractile substances might be responsible for the depressed endothelium-dependent relaxations in aorta of spontaneously hypertensive rats. Such enhanced production of an endothelial contractile factor is, however, apparently not involved in the depressed endothelium-dependent responses of aorta from renal hypertensive rats.

Studies of the last years have also substantiated at least two different endothelium-mediated relaxing mechanisms: 1) stimulation of muscular guanylate cyclase induced by an EDRF, which apparently is identical or at least very related to nitric oxide, 2) hyperpolarization of the muscle cells through opening of K⁺ channels in the cell membrane induced by EDHF. The identity of EDHF and its relation to EDRF are still debated.

Since the original observations by Furchgott and Zawadzki, ACh is the substance most widely used for studying endothelium-dependent relaxations. This substance appears to release both EDRF and EDHF in various vessels. The present study confirms that muscarinic activation (either with carbachol or ACh) induces a partially transient hyperpolarization in smooth muscle cells from isolated rat aorta. The hyperpolarization is lost after removal of the endothelial cells, indicating the absence of a direct effect on rat aortic smooth muscle Eₚₑ.

Hyperpolarization of the cellular membrane is generally accepted to decrease Ca^{2+} influx by closing voltage-sensitive Ca^{2+} channels, thereby lowering intracellular calcium levels and relaxing the tissue. That rat aortic smooth muscle cells indeed relax in response to membrane hyperpolarization is illustrated by the potent inhibitory properties of BRL 38227, the active enantiomer of cromakalim, a well-known opener of K_{ATP} channels. The hyperpolarizing effect of BRL 38227 is blocked in the presence of glibenclamide, a substance accepted to specifically inhibit K_{ATP} channels. Although the hyperpolarizing effect of carbachol is not affected by glibenclamide, it disappears in the presence of TEA, a substance that is a less specific K⁺ channel blocker but, at the applied concentration, appears mainly to block
Ca²⁺-activated K⁺ channels. Also the relaxation effect of ACh was not influenced by glibenclamide (used in a concentration that profoundly influenced BRL 38227–induced relaxations), but it was markedly depressed in the presence of TEA. The inhibitory influence of TEA was much more pronounced on ACh-induced relaxation than on BRL 38227–induced relaxation. In contrast to the observations of Brayden on rabbit cerebral arteries, these results suggest that endothelium-dependent hyperpolarization of rat aortic smooth muscle relies on activation of K⁺ channels other than those of the ATP-sensitive type, perhaps Ca²⁺-dependent channels. This is consistent with the recent observations by Chen and Suzuki, who showed that the endothelium-dependent hyperpolarization by ACh on rat arteries has a Ca²⁺-dependent component.

Not only the profound influence of TEA but also the fact that the relaxation–response curves to ACh attain relaxation levels that are much larger in muscle precontracted by NE than by K⁺ indicate that Eₘ changes contribute importantly to endothelium-dependent relaxations. According to Chen and Suzuki, endothelium-dependent hyperpolarization contributes to ~20–40% of the total endothelium-dependent relaxation in rat arteries. From our observations, it appears that 10⁻⁵ M carbachol elicits a hyperpolarization similar to that elicited by 10⁻⁷ M BRL 38227, a substance that exclusively induces relaxation through hyperpolarization, as indicated by the lack of relaxation in preparations precontracted with 80 mM K⁺ (Figure 7D). This hyperpolarization elicits in our experimental conditions a mean relaxing effect of 25±4% of the induced tone (n=31). The contribution of hyperpolarization to the total relaxation after muscarinic activation thus can be estimated to be important. Since cGMP-induced lowering of intracellular calcium levels may occur much
more efficiently when Ca\(^{2+}\) entry into the cell is antagonized by hyperpolarization, it can be considered that the hyperpolarization may enhance and amplify the relaxing influence of EDRF. The hyperpolarizing effect of activation of muscarinic receptors must, however, not only be considered at the level of the smooth muscle cells; such an influence may also be effective at the level of the endothelium. It has indeed been shown that ACh also induces in the endothelial cells a Ca\(^{2+}\)-dependent K\(^+\) current, resulting in endothelial hyperpolarization.\(^{34,35}\) This mechanism is believed to trigger EDRF release. Therefore, the drastic blocking effect of TEA on endothelium-dependent relaxations and the smaller relaxation to ACh in 80 mM K\(^+\)-contracted preparations could possibly also be largely due to an inhibitory action on the activity of the endothelial cells.

**Hypertensive Rats**

The results obtained on the aorta from renal hypertensive rats confirm earlier observations\(^5\)–\(^9\) that endothelium-dependent relaxations to ACh are markedly depressed in preparations precontracted by NE. In these preparations, carbachol also showed much less influence on E\(_m\) than in the preparations from control rats: hyperpolarization effects of only 22% of those obtained in control preparations were observed. In contrast with the pronounced influence on preparations from normotensive rats contracted by NE, TEA had no influence on the already depressed relaxation to ACh in preparations from hypertensive rats. The relaxation in 80 mM K\(^+\)-contracted depolarized muscle of normotensive and hypertensive rats also showed only a small difference. These observations are consistent with the possibility of a smaller role of the membranous electrical component in the relaxation of hypertensive vessels.

The resting E\(_m\) of aortic smooth muscle cells from hypertensive rats consistently was less negative than that of the normotensive control preparations. Similar observations were also made on other preparations from hypertensive animals.\(^{36–39}\) The mechanisms responsible for the less negative resting E\(_m\) in our hypertensive preparations cannot be settled from the present experiments. As reported by others,\(^{10,18}\) we also found less negative resting E\(_m\)s in rubbed preparations. However, the difference in E\(_m\) between preparations from normotensive and hypertensive rats cannot exclusively be attributed to the absence of a possible basal release of hyperpolarizing factor from the vascular endothelium, since the mean resting E\(_m\) in the preparations from hypertensive rats was even less negative than that in rubbed aortic segments from control rats (which no longer responded to carbachol). The difference in E\(_m\) between normal and hypertensive preparations persisted after obtaining the maximal hyperpolarization response to BRL 38227, a substance that, by increasing K\(^+\) conductance, brings E\(_m\) much closer to the K\(^+\) equilibrium potential. This finding is consistent with the suggestion that the K\(^+\) equilibrium potential of the vascular smooth muscle cell is decreased by a lower intracellular K\(^+\) activity in hypertension.\(^{40}\) A lower intracellular K\(^+\) activity could also be responsible for the depressed endothelium-dependent hyperpolarization in hypertension and could also explain the shift to the right in the concentration–relaxation curve of BRL 38227 in rings from hypertensive rats (Figure 8).

However, a diminished liberation of EDHF by the endothelium can also not be excluded, although we found in previous bioassay experiments that the release of EDRF is not compromised in aorta from renal hypertensive rats.\(^8\)

We conclude that endothelium-dependent hyperpolarization is largely depressed in aorta from renal hypertensive rats and that this might contribute to the impaired endothelium-dependent relaxation in hypertension.

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**Key Words**: endothelium • hyperpolarization • hypertension • rat • aorta • potassium channels
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