Structure, Function, and Endothelium-Derived Hyperpolarizing Factor in the Caudal Artery of the SHR and WKY Rat

Shaun L. Sandow, Narelle J. Bramich, Hari Priya Bandi, Nicole M. Rummery, Caryl E. Hill

Objective—To quantify structural and functional characteristics of the caudal artery from spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats with particular reference to endothelium-derived hyperpolarizing factor (EDHF).

Methods and Results—Ultrastructural studies showed that the number of myoendothelial gap junctions, smooth muscle cell (SMC) layers, and medial cross-sectional area were significantly greater in SHR than WKY. Intracellular dye labeling demonstrated hyperplasia of SMCs in SHR. Analysis of nerve-mediated excitatory junction potentials recorded in SMCs at the adventitial and luminal borders demonstrated decreased radial coupling of SMCs in SHR. In both SHR and WKY, in the presence of NO-nitro-L-arginine methyl ester and indomethacin, acetylcholine-elicited EDHF was abolished by charybdotoxin and apamin, while iberiotoxin had no effect, implicating the involvement of small and intermediate, but not large, calcium-activated potassium channels. EDHF was abolished by Gap-mimetic peptides, N18-glycyrrhetinic acid, and endothelial removal but not affected by the NO scavengers hydroxocobalamin and carboxy-PTIO.

Conclusions—Significant differences in SMC morphology and homocellular and heterocellular coupling exist between the caudal artery of SHR and WKY rats. In the caudal artery of SHR, significantly greater heterocellular coupling compensates for other structural changes in the media to maintain a functional role for EDHF. (Arterioscler Thromb Vase Biol. 2003;23:822-828.)

Key Words: arterial morphology ■ endothelium ■ endothelium-derived hyperpolarizing factor ■ gap junctions ■ smooth muscle

Heterocellular and homocellular coupling of arterial smooth muscle cells (SMCs) and endothelial cells (ECs) within the walls of blood vessels plays an integral role in the coordination of vasomotor activity. Furthermore, the variability in vasomotor responses reported in and between different vascular beds depends in part on differences in the incidence and characteristics of vascular gap junctions and their constituent connexin proteins. Thus, structural variation is an important determinant of functional variation within the vasculature. For example, myoendothelial gap junctions (MEGJs), which permit the electrical and chemical coupling of EC and SMC layers, are increasingly considered integral to the activity of endothelium-derivated hyperpolarizing factor (EDHF), one of three important endothelium-derived vasodilatory factors. The importance of EDHF varies between different vessels and generally increases as vessel size decreases. Indeed it has been shown that the prevalence of MEGJs in different segments of the mesenteric vascular bed of the rat corresponds to the participation of EDHF in vasodilatory responses previously reported in these vessels. Furthermore, in the femoral artery of the Wistar rat, which lacks EDHF, there are no MEGJs.

Interestingly, while the majority of studies have emphasized the greater importance of EDHF in smaller-sized resistance vessels, recent studies have demonstrated a significant role for EDHF in larger resistance arteries and in conduit vessels. While MEGJs have been implicated in EDHF activity in smaller vessels, it is not clear whether they may be important in larger vessels which have more layers of smooth muscle cells. Our preliminary studies showed that MEGJs are present in large arteries, such as the caudal artery. However, no studies have examined the role of MEGJs in EDHF function in such larger vessels, nor have they correlated the incidence of MEGJs with changes in EDHF, which might occur during hypertension.

In hypertension, remodeling of the vascular wall accompanies changes in blood pressure. This remodeling typically comprises a decrease in luminal diameter and, frequently, an increase in medial thickness. The underlying basis for the change in the media is heterogeneous, being variously attrib-
Arterial Structure, Function, and EDHF

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Methods

For detailed methods, please see http://atvb.ahajournals.org.

Results

Blood Pressure Recordings

Systolic blood pressure of SHR was significantly greater than that of WKY animals (P<0.05; 188±6.4 and 121±2.6 mm Hg, n=10, for SHR and WKY, respectively).

Arterial and Smooth Muscle Cell Morphology

The number of layers of SMCs and the medial cross sectional area was significantly larger in SHR than in WKY, while the circumference as measured at the level of the internal elastic lamina (IEL) was significantly smaller (P<0.05; Table 1).

Following morphometric analysis of intracellular dye labeling with fluorescein-conjugated dextran, the surface areas of SMCs in SHR and WKY were found not to be significantly different (P>0.05; 782±56 μm², 18 cells, from 5 rats, and 757±41 μm², 23 cells, from 6 rats, for SHR and WKY, respectively), although the cells were significantly longer and thinner in the SHR than in the WKY (P<0.05; length, 171±6.7 μm and 138±4.4 μm; width, 8.1±0.4 μm and 9.8±0.5 μm; Figure 1; compare 1B with 1D; for SHR and WKY, respectively).

With lucifer yellow, dye was commonly seen to spread among adjacent SMCs in arteries from both SHR and WKY (Figure 1, compare 1A with 1C, 1E, and 1F; for SHR and
There were significantly more MEGJs in the SHR compared with WKY ($P<0.05$; Table 1). Two types of MEGJs were found: those between projections of ECs and SMCs (Figure 3A-C) and those between the projections of ECs and the surface of SMCs (Figure 3D-F). Of the total of 32 MEGJs found in both SHR and WKY, 59% were found on projections of both ECs and SMCs, while the remainder were found on projections originating from ECs only. No differences in the proportion of these two types of MEGJ were found between SHR and WKY.

The proximity of MEGJs and gap junctions between ECs was examined in SHR and WKY. In both cases, MEGJs were

### Incidence of MEGJs

There were significantly more MEGJs in the SHR compared with WKY ($P<0.05$; Table 1). Two types of MEGJs were

**TABLE 2. Characteristics of EJPs Recorded From SMCs of the Caudal Arteries of 11- to 13-Week-Old Male SHR and WKY Rats**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Location</th>
<th>No. of Animals</th>
<th>No. of Cells</th>
<th>Amplitude, mV</th>
<th>Rise Time, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Adventitial</td>
<td>7</td>
<td>19</td>
<td>19±1*</td>
<td>24±3</td>
</tr>
<tr>
<td></td>
<td>Luminal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Adventitial</td>
<td>6</td>
<td>17</td>
<td>21±3†</td>
<td>20±5§</td>
</tr>
<tr>
<td></td>
<td>Luminal</td>
<td>6</td>
<td>6</td>
<td>10±3†</td>
<td>50±6§</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>20</td>
<td>15±1§</td>
<td>32±6∥</td>
</tr>
<tr>
<td></td>
<td>Luminal</td>
<td>6</td>
<td>6</td>
<td>13±3</td>
<td>50±5∥</td>
</tr>
</tbody>
</table>

Results are mean±SEM.

A, Data for all adventitial SMCs. B, Data for those preparations in which adventitial and luminal SMCs were impaled.

Significantly different values are denoted by paired symbols *, †, ‡, §, ‖ ($P<0.05$).
within 2 μm of endothelial gap junctions (Figure 3C), and no significant difference was found for the distance between the MEGJs and endothelial gap junctions in the two groups (P > 0.05; 1.62 ± 0.18 μm, n = 19 for SHR and 1.68 ± 0.35 μm, n = 12, for WKY).

The majority of MEGJs had single plaques or regions of pentalaminar membrane structure (Figure 3A through 3C, for example). Five of the 20 MEGJs from SHR and 1 of the 12 MEGJs from WKY, however, had multiple plaques (Figure 3D through 3F, for example). The longitudinal distance over which MEGJs with single plaques were seen in vessels from SHR and WKY was determined by comparing the number of transverse sections in which each plaque appeared. The number of sections in which individual plaques appeared was not significantly different between SHR and WKY (P > 0.05; 3.4 ± 0.52 sections, n = 15 for SHR and 3.4 ± 0.48 sections, n = 11, for WKY). The width of single plaques was measured from high-magnification micrographs and found not to differ between SHR and WKY (P > 0.05; 135 ± 16 nm, n = 15 for SHR and 125 ± 18 nm, n = 11 for WKY). The MEGJs with multiple plaques were found in more sections than MEGJs with single plaques (6.3 ± 0.6, n = 4 for SHR, and 4 for the single MEGJ in WKY). The remaining MEGJ in the SHR series was found at the end of a series and therefore was not included in the present analysis, as the pentalaminar region may have continued outside the 50 sections examined.

EDHF Activity in Caudal Arteries
In the presence of L-NAME and indomethacin, ACh produced a concentration-dependent relaxation in caudal arteries from both SHR and WKY, which was not significantly different between the two strains (n = 5 and 6, respectively; Figure 4A). The combined addition of charybdotoxin and apamin abolished the EDHF relaxation in both SHR and WKY (n = 4, for each) and uncovered a contraction that was not significantly different between the two strains. 18β-Glycyrrhetinic acid significantly reduced the EDHF relaxation in SHR by 63% (remaining vasodilation was 10 ± 4.6% of phenylephrine-induced constriction, n = 4) and by 100% in the WKY (n = 3). Recirculation of Krebs’ solution containing the Gap-mimetic peptide combination (43 Gap26, 40 Gap27, and 37,43 Gap27), L-NAME, and indomethacin, however, significantly reduced the EDHF relaxation by 95.8 ± 4.3% in the SHR and by 79.3 ± 9.4% in the WKY (remaining vasodilation was 1.8 ± 1.8% and 7 ± 3% of phenylephrine-induced constriction, n = 4 for each; Figure II, available at http://atvb.ahajournals.org). Control data showed that the EDHF relaxation (31.8 ± 3.6%, n = 7) was not altered after 60 minutes of recirculation of the Krebs’ solution (109.4 ± 7.2%, n = 7, of the initial response to ACh). In the presence of indomethacin and the Gap-mimetic peptide combination, but in the absence of L-NAME, ACh did elicit a relaxation (82.4 ± 4.4%; n = 5, WKY data only; Figure II, available at http://atvb.ahajournals.org), that was abolished by subsequent incubation in L-NAME.
In both SHR and WKY, the addition of charybdotoxin (60 nmol/L) and apamin (0.5 μmol/L; n=8, for each) prevented the hyperpolarization following application of ACh. However, in all 8 SHR animals and in 4 of 8 WKY animals, ACh now produced a depolarization (7.1 ± 1 mV hyperpolarization and 3 ± 1 mV depolarization, 10 ± 2 mV hyperpolarization and 2 ± 1 mV depolarization, before and after addition of drug, respectively, for SHR and WKY, Figure 4Cb). Removal of the endothelium abolished both the hyperpolarization and the depolarization in SHR and WKY (n=3, for each). In both SHR and WKY, iberiotoxin (100 nmol/L) had no effect on the hyperpolarization induced by ACh (4.2 ± 1 mV and 4.2 ± 1 mV; 13 ± 3 mV and 13 ± 4 mV; before and after addition of drug, respectively, for SHR and WKY, respectively; n=3, for each, Figure 4D), while the addition of 18β-glycyrrhetinic acid (20 μmol/L) abolished the hyperpolarization to ACh (5 ± 1 mV and 0 ± 0 mV; 12 ± 2 mV and 0 ± 0 mV; before and after addition of drug, respectively, for SHR and WKY, respectively, in both SHR and WKY; n=3, for each). In the presence of L-NAME and indomethacin, the addition of hydroxocobalamin (100 μmol/L), had no effect on the hyperpolarization elicited by ACh (8 ± 1 mV and 8 ± 2 mV and 9 ± 2 mV and 8 ± 3 mV, before and after addition of hydroxocobalamin, for SHR and WKY, respectively).

### Discussion

Significant differences have been found in the present study between the media of the caudal artery of hypertensive SHR and normotensive WKY rats. We found a significantly larger medial cross-sectional area in SHR due to an additional two layers of SMCs. In contrast to the concept that eutrophic remodeling predominates in arteries of SHR, the present study demonstrates that hypertrophic remodeling occurs in the caudal artery of the SHR, consistent with the concept that the type of remodeling may vary according to the model examined and the vascular bed under investigation. Furthermore, our intracellular dye labeling studies have shed light on the basis for the hypertrophic remodeling seen in the caudal artery of the SHR. We have found that SMCs in SHR were not significantly different in size, although they varied in shape from those in WKY. Given the increased number of SMC layers and medial area in the SHR, these results suggest that hypertrophy in the caudal artery results from hyperplasia of SMCs. Since previous studies in other vessels of SHR have variously described both SMC hypertrophy and hyperplasia, we conclude that considerable heterogeneity also exists in the basis for the hypertrophic remodeling in different vascular beds, even within the same model of hypertension.

Our data demonstrate clearly that electrical and chemical coupling occurs between SMCs in the media of the caudal artery in both SHR and WKY rats. Dye transfer is seen within groups of SMCs and pentalaminar gap junctions are present between cells in adjacent layers of the media. However, our electrophysiological studies suggest that electrical coupling is reduced between SMCs in SHR compared with WKY. In WKY rats, differences in the time course of neurally evoked EJPs were observed between SMCs, near the sites of neurotransmitter release at the adventitial border, and at the luminal border, several cells away from these sites. However the
amplitude of EJPs was not significantly attenuated, consistent with the properties of an electrically short cable. Previous studies in the caudal artery of Wistar rats did show a significant decrease in amplitude of EJPs in luminal versus adventitial cells, although the reason for this discrepancy is not known. In contrast to the data from WKY rats, we found a significant attenuation of the amplitude, in addition to slowing of the time course, of neurally evoked EJPs in the SMCs on the luminal border compared with the adventitial border in SHR. The observed 25% increase in the number of SMC layers in the caudal artery of SHR would result in an increase in the conduction distance for EJPs from the adventitial to the luminal border. However, the changes seen in the properties of EJPs between the adventitia and lumen were larger than would be expected solely from the increase in the number of SMC layers in the wall of the SHR, suggesting that coupling between SMCs in SHR has been reduced. Despite the attenuation in the amplitude of EJPs in SHR, there was no difference in the properties of EJPs recorded in the luminal SMCs in SHR compared with WKY. This occurred because the EJPs in adventitial SMCs of SHR were significantly larger and faster than those in WKY, in accord with previous studies in mesenteric arteries from SHR and WKY rats. These differences presumably relate to the approximate doubling in the density of the sympathetic innervation found in the caudal artery of SHR compared with WKY.

In general, the role of EDHF is recognized to be more important in smaller resistance vessels, while NO is more important in larger vessels. Interestingly, however, in the present study we have described a significant EDHF-mediated hyperpolarization and relaxation in a large resistance artery of both hypertensive and normotensive rats. We found that the maximum hyperpolarizing response to ACh in SHR was only 28% less than that in WKY. This appeared to be due to the presence of an ACh-induced depolarization and contraction in this strain. On the other hand, EDHF induced similar-sized relaxations in the two strains. After blockade of EDHF in WKY, an ACh-induced contraction was also found, although this did not appear to result from depolarization like that in SHR. We confirmed that the hyperpolarizations and relaxations in both SHR and WKY were due to EDHF by demonstrating their abolition after application of charybdotoxin and apamin, universally accepted blockers of EDHF-mediated responses or by removal of the endothelium. The lack of effect of iberiotoxin on the hyperpolarization indicated that it did not result from the opening of large conductance calcium-activated potassium channels which have been shown to be activated by epoxyeicosatrienoic acid, a putative EDHF candidate. The involvement of intercellular gap junctions in the EDHF response was confirmed by the abolition of the hyperpolarization in both strains and the marked attenuation and abolition of the relaxation in the SHR and WKY, respectively, by 18β-glycyrrhetinic acid. The involvement of intercellular gap junctions in the EDHF response was further confirmed by inhibition of the relaxation in both strains by Gap-mimetic peptides. These latter peptides had no effect on ACh-induced relaxation due to NO. Recent studies have demonstrated that endothelium-dependent relaxation attributed to EDHF, may in part be due to L-NAME-insensitive (non–NO synthase) basal NO. However, the relaxation due to this latter component, as with EDHF, varies between vascular beds, being absent in the femoral artery of the mature Wistar rat, for example. We found no evidence for such an L-NAME insensitive NO component following the use of the two different NO scavengers.

In the caudal artery of both SHR and WKY, MEGJs were found connecting the endothelium and smooth muscle. Serial section electron microscopy showed that the incidence of MEGJs was significantly greater in SHR than in WKY, without an apparent difference in the morphology or size of the plaques. This greater degree of heterocellular coupling in the SHR may serve to offset the observed changes in the number of SMCs and their coupling in the media of the caudal artery of these rats, thus maintaining EDHF-mediated relaxation.

Implicit in discussions of cell coupling and blood vessel function is that SMCs are equally well coupled throughout the media, in both the radial and longitudinal directions. Contrary to this view, our results suggest that the coupling of SMCs in the radial direction is different from the longitudinal coupling of SMCs in both SHR and WKY. Intracellular injection of Lucifer yellow into SMCs at the adventitial or luminal borders, spread into adjacent cells in a radial, but not longitudinal direction. These data are consistent with previous studies in which Lucifer yellow did not spread in a longitudinal direction between SMCs in arterioles containing only a single layer of medial SMCs. While dye coupling indicates that electrical coupling can occur, a lack of electrical coupling cannot be inferred from a lack of dye coupling. Thus, in the present study, the selective movement of Lucifer yellow in the radial axis of the caudal artery in both SHR and WKY suggests that there may be interesting differences in the connexin makeup of the gap junctions involved in radial compared with longitudinal cell coupling and hence an asymmetry in the communication pathways within the media.

In conclusion, we have shown that significant anatomical and functional differences are found in the caudal artery of SHR and WKY rats. In the SHR, these differences are characterized by hyperplasia of SMCs and a reduction in SMC coupling, but an increased incidence of MEGJs which provide the potential for increased heterocellular coupling between the two cellular layers. Physiologically, these structural changes result in the maintenance of EDHF activity, which is shown to be dependent on intercellular coupling via gap junctions.

Acknowledgments

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References


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Online Methods

Protocols in the present study were approved by the Animal Experimentation Ethics Committee of the Australian National University, under guidelines issued by the National Health and Medical Research Council of Australia.

Animals, blood pressure measurements, choice of tissue and reagents

For comparative studies with SHR (inbred for >70 generations), normotensive WKY rats (inbred for >53 generations) were chosen, as these represented the best available genetically comparable strain (see1). Hypertensive and normotensive animal models were maintained through selection of appropriate breeding animals after monitoring blood pressure. These rats were originally from stock derived from the National Institutes of Health, Bethesda, MD. Age matched 11 to 13wk old male rats were used for all experiments. Systolic blood pressure was recorded in conscious rats that had been acclimatized to the apparatus according to standard procedures, using the tail cuff method (HyperRat, SDR Clinical), as previously described.2 For all other measurements, rats were deeply anaesthetized with intraperitoneal ketamine and rompun (44 and 8mg/kg, respectively). Unless otherwise stated all chemicals were obtained from Sigma.

Gap mimetic peptides were synthesized by the Biomolecular Resource Facility of the John Curtin School of Medical Research, Australian National University, to a
purity of >95%. Peptide sequences were; 43Gap26, VCYDKSFPISHVR; 40Gap27, SRPTEKNVFIV; and 37,43Gap27, SRPTEKTIFII.  

Morphometric analysis of caudal arteries

SHR and WKY rats were perfused at 60mmHg of constant pressure with 3% glutaraldehyde and 1% paraformaldehyde in 0.1µmol/L sodium cacodylate with 2mmol/L CaCl₂.6H₂O, 0.1mol/L sucrose, 10mmol/L betaine, pH 7.35 at 25°C and small segments of the distal one-third of caudal arteries processed for electron microscopy using standard procedures. The same constant perfusion pressure was chosen for both strains to minimize differences in the morphological parameters which might arise due to different fixation pressures. For maximum vasodilation, sodium nitrite (1.5mmol/L) was present in the pre-perfusion clearance solution. Under these conditions no significant difference has been shown in medial cross sectional area between a contracted or dilated state.

Vascular characteristics were determined from digitized electron microscopic montages (x2,500) of individual transverse arterial sections from each of three different SHR and WKY rats, and vessel circumference determined at the level of the internal elastic lamina (IEL). Medial cross sectional area was determined using the MCID imaging system (Imaging Research, Canada). The number of medial SMC layers was determined by averaging the number of SMC profiles ≥5µm in length, from the outer edge of the IEL to the inner edge of the external elastic lamina along four linear plots 90° apart.
Serial section electron microscopy

Serial, dark silver to gold transverse arterial sections were collected on Formvar (0.3% in ethylene dichloride with grey to silver interference colors) and carbon (~5nm) coated slot grids. Methods for serial section analysis of MEGJs were similar to those as previously described,5 whereby all MEGJs were identified and photographed at x20,000 to x40,000 through 5µm of serial sections from 3 different SHR and WKY rats. The number of MEGJs per EC in SHR and WKY was determined using the appropriate vessel circumference to calculate luminal surface area and the values for EC sizes published previously7 (that is, 265±11.3µm² and 384±16.4µm², for SHR and WKY ECs, respectively).

Selected gap junctions between adjacent ECs or between adjacent SMCs were photographed through serial sections at x20,000 to x40,000.

Morphology and electrical coupling of smooth muscle cells

Intracellular recordings were made from caudal artery segments pinned flat in a shallow recording chamber. Preparations were continuously superfused with physiological saline,8 gassed with 95% O₂, 5% CO₂, and membrane potential recordings made using conventional techniques with fine glass microelectrodes (resistance: 120-240MΩ) filled with 0.5mol/L KCl as previously described.8 Successful impalements of cells were characterised by a sharp decrease in potential of 20 to 40mV, followed by a progressively slower decrease to a resting value of around -60mV.

In order to assess SMC coupling, perivascular nerves, which are located at the medial/adventitial border, were stimulated with bipolar electrodes placed on either side of the vessel (0.1ms pulse duration, 100mA). Intracellular recordings were made
from SMCs immediately below the surface of the adventitia, near the nerve terminals, by advancing electrodes gently from the adventitial surface (Figure 1A,C,F, for example). Recordings were also made from SMCs immediately below the luminal surface (Figure 1E, for example), by removing side branches from the artery and advancing electrodes through the hole and the exposed endothelium. Successful impalements were characterized as having a rapid decrease in membrane potential upon advancement of the electrode. Dye filled electrodes were used to determine the position and nature of the recorded cells (see below).

Excitatory junction potentials (EJPs) were evoked by a single neural stimulus (0.1 ms pulse width), with stimulus strength being increased until the maximum response was evoked and these potentials were used for all measurements. In some experiments (in both SHR and WKY), action potentials were evoked but these responses were not included in the analysis. When a ‘peaky’ response was evoked (e.g. Figure 2Aa), the stimulus strength was gradually decreased to ensure that the EJP gradually reduced in amplitude and did not involve an active response. If this were the case, measurements were taken from the maximal EJP. The amplitude, rise time (10 to 90% of the peak amplitude) and time constant of decay of EJPs were measured from both the SHR and WKY rats.

In order to examine SMC morphology, cells were labelled intracellularly with electrodes whose tips were filled with 10% FITC-dextran (3000 MW) or 3% lucifer yellow and backfilled with 0.5mol/L KCl or LiCl. Preparations were fixed in 4% paraformaldehyde in PBS, mounted in glycerol and photographed with a Nikon Coolpix 950 digital camera on a compound microscope at 1600x1200 pixels. Measurements of cellular area, length and width were made using the MCID imaging system.
EDHF activity in caudal arteries

EDHF-mediated relaxation in caudal arteries was assessed using a Mulvany-Halpern style myograph\textsuperscript{11} in a similar manner to Tare et al.\textsuperscript{12} Briefly, 1 mm long arterial segments were mounted between two 40 \( \mu \)m wires and allowed to equilibrate for 30 minutes prior to incremental stretching to an equivalent tension of 80 mmHg. Preparations, continuously superfused with physiological saline,\textsuperscript{9} gassed with 95\% O\textsubscript{2}, 5 CO\textsubscript{2}, were preconstricted with phenylephrine (2.5 to 6\( \mu \)mol/L) to approximately 60\% of maximal constriction, equivalent to 0.85g of tension. Tissues were equilibrated for 15min prior to subsequent addition of drugs. Endothelial integrity of each preparation was assessed by initial application of ACh (1\( \mu \)mol/L). A relaxation of \(<40\%\) of the maximal constriction to phenylephrine was considered to indicate endothelial damage and such preparations were discarded. Individual preparations were used for each drug treatment. Tension was recorded using a MacLab chart recorder, with results being expressed as \% relaxation of phenylephrine-induced constriction.

The degree and characteristics of EDHF-mediated relaxation were assessed by the cumulative application of ACh in the presence of the nitric oxide synthase inhibitor, \( N_\text{w} \)-nitro-L-arginine methyl ester hydrochloride (L-NAME; 100\( \mu \)mol/L) and the prostaglandin inhibitor indomethacin (10\( \mu \)mol/L). EDHF mediated relaxation in response to a submaximal concentration of ACh (1\( \mu \)mol/L) was also assessed in the presence of L-NAME, indomethacin and the nitric oxide scavengers\textsuperscript{13,14} hydroxocobalamin (100\( \mu \)mol/L). and, separately, with carboxy-PTIO (100\( \mu \)mol/L). The effects of charybdotoxin (60n\text{mol}/L) and apamin (0.5\( \mu \)mol/L), blockers of large/intermediate and small conductance \( K_{\text{Ca}} \) channels, respectively, were examined
in the presence of L-NAME and indomethacin with a single dose of ACh (1µmol/L).
The effects of the putative gap junction antagonists 18β-glycyrrhetinic acid (20µmol/L) and Gap-mimetic peptides (43Gap26, 40Gap27 and 37,43Gap27; 100µmol/L each, in combination3), were also examined in the presence of L-NAME and indomethacin with a single dose of ACh (1µmol/L). Gap peptides were dissolved directly into the Krebs’ solution and recirculated for 60min before testing responses to ACh. Control experiments were conducted in which preparations were tested with ACh (1µmol/L), before and after recirculation for 60 min with physiological saline. Upon completion of experiments with Gap-mimetic peptides, the recirculation bath was cleaned thoroughly with 0.5mol/L HCl, as the peptides were found to have residual effects without this treatment. In order to examine the specificity of the Cx-mimetic peptides for gap junctions and EDHF, vasodilatory responses to ACh were examined in the presence of indomethacin, followed by incubation in indomethacin and the Gap-mimetic peptide combination (WKY only) and the patency of these responses to L-NAME was subsequently examined.

EDHF-mediated hyperpolarization was determined by impaling adventitial SMCs with fine microelectrodes as described above. Cumulative concentration-response curves to ACh were constructed, following incubation of preparations for at least 30min in L-NAME (100µmol/L) and indomethacin (10µmol/L). A 5min equilibration period followed application of each concentration of ACh. To ensure that no desensitization of responses occurred, single high concentrations of ACh were also tested. In order to confirm the involvement of EDHF, the effect of ionophoretically applied ACh (1mol/L, 200nA, 10s) was tested in the presence of charybdotoxin (60nmol/L) and apamin (0.5µmol/L) and in preparations in which the endothelium had been removed. The role of epoxyeicosatrienoic acid, gap junctions and L-NAME
insensitive nitric oxide in the EDHF response to ACh was also examined by addition of iberiotoxin (100nmol/L), 18β-glycyrrhetinic acid and hydroxocobalamin (100µmol/L), respectively.

**Statistical analysis**

Results are expressed as mean ± standard error of the mean (SEM). Data were fitted with curves using non-linear regression analysis (Graphpad Prism). Statistical significance was tested using a paired or unpaired t-tests. A P value of <0.05 was considered significant.
References:


Figure I. Gap junctions between endothelial cells (ec) and between smooth muscle cells (smc) in the caudal artery. Gap junctions between SMCs were very small, usually appearing in only a single section of a series on adjacent projections between SMCs (A,B; arrowheads). Gap junctions between ECs (C; arrowhead) were larger than those between SMCs. iel, internal elastic lamina. Scale bar = 1µm for A and 250nm for B and C.
Figure II. Endothelium-dependent relaxation in response to ACh (1μmol/L) in the caudal artery of the WKY rat (expressed as % of phenylephrine (PE)-induced constriction). In the presence of L-NAME (100μmol/L) and indomethacin (Indo; 10μmol/L), EDHF was present (A,B) and was significantly attenuated by addition of the Gap-mimetic peptide combination $^{43}$Gap26, $^{40}$Gap27 and $^{37,43}$Gap27 (100μmol/L each; A,C). In the absence of L-NAME and in the presence of Indo and the Gap-mimetic peptide combination, a nitric oxide (NO) mediated relaxation was present (A,D), that was significantly attenuated by subsequent incubation with L-NAME (A). Data are expressed as the mean ± standard error of the mean.