Aged Spontaneously Hypertensive Rats Exhibit a Selective Loss of EDHF-Mediated Relaxation in the Renal Artery

Eckhart Büssemaker, Rüdiger Popp, Beate Fisslthaler, Christiana M. Larson, Ingrid Fleming, Rudi Busse, Ralf P. Brandes

Abstract—Endothelium-dependent relaxation is frequently attenuated in hypertension. We hypothesized that the contribution of the endothelium-derived hyperpolarizing factor (EDHF) to the acetylcholine (ACh)-induced, endothelium-dependent relaxation is attenuated with aging in the renal artery of spontaneously hypertensive rats (SHR) compared with age-matched Wistar-Kyoto (WKY) rats. ACh-induced, NO-mediated relaxation was identical in young (8-week-old) WKY and SHR, whereas EDHF-mediated relaxations (assessed in the presence of Nω-nitro-L-arginine and diclofenac) were much more pronounced in SHR than WKY. KCl-induced relaxations were more pronounced in vessels from young WKY rats than from young SHR. The cytochrome P450 inhibitor sulfaphenazole significantly inhibited EDHF-mediated relaxation in vessels from young SHR but not WKY. Vessels from old (22 months) SHR exhibited a slightly reduced NO-mediated relaxation but a complete loss of EDHF-mediated responses. In contrast, aging did not affect EDHF-mediated responses in WKY. Moreover, ACh-induced hyperpolarization and resting membrane potential were decreased in old SHR but not in WKY. KCl-induced relaxation increased with age in WKY, whereas no response to KCl was recorded in arteries from aged SHR. In vessels from old WKY but not old SHR, mRNA expression of the Na-K-ATPase subunit α2 increased by 2-fold compared with young animals. These data indicate that the increase in EDHF responses in renal arteries from aged WKY can be attributed to the release of K⁺ ions from the endothelium, whereas increased EDHF responses in renal arteries from young SHR can be attributed to a sulfaphenazole-sensitive cytochrome P450-dependent EDHF. (Hypertension. 2003;42:562-568.)

Key Words: endothelium • nitric oxide • acetylcholine • endothelium-derived factors • animal models of hypertension • renal artery

Three different endothelium-derived vasodilators, prostacyclin, nitric oxide (NO), and the endothelium-derived hyperpolarizing factor (EDHF), play an important role in the control of local vascular tone.¹ In hypertension, endothelium-dependent relaxation is attenuated (a phenomenon referred to as endothelial dysfunction) and contributes to the increased peripheral resistance.²-³ Moreover, treatment of hypertension improves endothelium-dependent relaxation by increasing the NO-mediated and the EDHF-mediated relaxation.⁴⁻⁵

Endothelial dysfunction in hypertension has been linked to a decrease in NO bioavailability reflecting the impaired generation of NO and/or the enhanced scavenging and inactivation of NO by oxygen-derived free radicals.⁶⁻⁷ The mechanisms leading to the attenuation of EDHF-mediated relaxations in hypertension are poorly understood but are reportedly unrelated to enhanced vascular oxidative stress.⁸ Part of the problem in addressing the factors affecting EDHF-mediated responses is that more than one EDHF may exist and that different hyperpolarizing mechanisms dominate in different arteries. There is a general consensus that EDHF-mediated effects are exquisitely sensitive to the combination of the K⁺ channel inhibitors charybdotoxin and apamin and that the EDHF-mediated relaxation involves the hyperpolarization of endothelial cells, the subsequent hyperpolarization of smooth muscle cells, and the closure of voltage-dependent Ca²⁺ channels.⁹ To date, three main mechanisms for EDHF-mediated hyperpolarization have been proposed. First, K⁺ ions, released from the endothelium through Ca²⁺ -dependent K⁺ channels, can activate inwardly rectifying K⁺ channels¹⁰ or the Na-K-ATPase¹⁰ on vascular smooth muscle cells to evoke hyperpolarization.¹¹ Alternatively, smooth muscle hyperpolarization may occur as a consequence of the spread of the endothelial cell hyperpolarization through myo-endothelial gap junctions.¹² Finally, EDHF responses in some vessels are dependent on the activation of a cytochrome P450 epoxygenase and the generation of epoxyeicosatrienoic acids (EETs)¹³.

The contribution of EDHF to endothelium-dependent relaxation varies between vascular beds and species. Whereas in large conduit vessels, such as the aorta, endothelium-dependent responses are selectively mediated by NO, EDHF is the predominant endothelium-dependent vasodilator in...
Resistance vessels. In intermediate-sized vessels, such as coronary or the renal arteries, EDHF has been proposed to act as a backup system to maintain endothelial function in situations associated with a decreased bioactivity of NO. In the present study, we studied young (8 weeks) and old (22 months) spontaneously hypertensive rats (SHR) and age-matched Wistar-Kyoto (WKY) rats to assess the effects of hypertension on the EDHF-mediated hyperpolarization and relaxation of renal arteries.

### Methods

An expanded Methods section can be found in an online supplement available at [http://www.hypertensionaha.org](http://www.hypertensionaha.org).

### Organ Chamber Experiments

Experiments were performed in phenylephrine-precontracted renal artery rings in the presence of diclofenac (10 μmol/L), as described. Cumulative concentration-relaxation curves were obtained to either acetylcholine (ACh, 1 nmol/L to 10 μmol/L) or KCl (4 to 10 μmol/L). EDHF-mediated responses were defined as that portion of the endothelium-dependent relaxation that remained in the presence of N^\textsuperscript{-}nitro-L-arginine (L-NA, 300 μmol/L) and diclofenac. A table containing the values for maximal relaxation and half maximal effective concentration is provided in the online supplement.

### Membrane Potential Recordings

The membrane potential of renal arteries was recorded with the use of sharp microelectrodes, as described.

### Real-Time PCR Analysis

Total RNA was isolated. After reverse transcription, the rat α1, α2, α3, and γ subunits of the Na-K-ATPase were amplified by means of quantitative real-time PCR.

### Results

Endothelium-dependent relaxation to ACh was identical in renal artery rings from young SHR and WKY rats. Aging was associated with a significant rightward shift in the concentration-relaxation curve to ACh in both strains, although a more pronounced effect was detected in vessels from SHR (Figure 1A). Similar results were obtained when the experiments were performed in the presence of KCl (40 mmol/L) to prevent ACh-induced EDHF-mediated relaxations (Figure 1B).

EDHF-mediated relaxations, recorded in the combined presence of L-NA and diclofenac, were more pronounced in renal artery rings from young SHR than from young WKY rats. In vessels from older animals, however, the situation was reversed. Although the dose-response curve of EDHF-mediated relaxations of renal arteries from WKY rats was only slightly shifted to the right, almost no EDHF-mediated relaxation could be detected in arteries from SHR (Figure 1C).

The resting membrane potential of renal artery smooth muscle cells was identical in vessels from young and old WKY as well as from young SHR but was significantly depolarized in vessels from old SHR. ACh-induced, EDHF-mediated hyperpolarizations were comparable in renal artery rings from young WKY rats and SHR. In accordance with the results of the organ chamber experiments, EDHF-mediated hyperpolarizations were not markedly altered in vessels from old WKY rats but were significantly reduced in renal arteries from old SHR rats (Figure 2).

To study the role of K^\textsuperscript{+} ions and the Na-K-ATPase in EDHF-mediated responses, concentration-relaxation curves to KCl were performed, and the effect of the Na-K-ATPase inhibitor ouabain was determined. A low concentration of ouabain (50 μmol/L) had no effect on the EDHF-mediated relaxation of vessels from young animals, whereas 500 μmol/L ouabain, which induced a marked depolarization of the vessel (data not shown), almost completely inhibited EDHF-mediated relaxations (Figures 3A and 3B). KCl relaxed renal artery rings from young WKY, an effect that was sensitive to low concentrations of ouabain. In contrast, KCl had a negligible effect on the tone of rings from young SHR (Figures 3C and 3D). Accordingly, KCl-induced hyperpolarizations were significantly more pronounced in renal arteries from young WKY rats than from young SHR (Figure 3E). Ouabain (500 μmol/L) significantly inhibited the hyperpolarizations induced by ACh and KCl (Figure 3F).

Figure 1. Contribution of NO and EDHF to endothelium-dependent relaxation of renal arteries from young and old SHR and WKY rats. Concentration-relaxation curves to acetylcholine in renal artery rings from young (8 weeks, left panel) and old (22 months, right panel) SHR (○) and WKY rats (□) preconstricted with phenylephrine in the presence of diclofenac (10 μmol/L). Experiments were performed A, in the absence of additional inhibitors to study the combined endothelium-dependent relaxation to ACh; B, in the presence of KCl (40 mmol/L) to assess NO-mediated relaxation; and C, in the presence of L-NA (300 μmol/L) to assess EDHF-mediated relaxations. Results are mean±SEM; analysis was performed with 2-way ANOVA for repeated measurements.
pathways were studied by using specific inhibitors. A moderate concentration of catalase (1200 U/mL) had no effect on EDHF-mediated relaxation in vessels from either strain (data not shown). Blockade of gap junctions, using specific connexin-blocking peptides (GAP peptide) according to combinations and concentrations reported recently by others, failed to attenuate the differences in the EDHF-mediated response between the two strains (data not shown). Specific inhibition of cytochrome P450 CYP 2C epoxygenases using sulfaphenazole (10 μmol/L) selectively attenuated EDHF-mediated relaxations in renal artery rings from SHR (Figure 4A). In the presence of sulfaphenazole, EDHF-mediated responses in young SHR and WKY were virtually superimposable. In contrast, 17-ODYA (10 μmol/L), which preferentially blocks CYP 4A isoforms, had no effect on the relaxation of rings from either strain (Figure 4B). The non–isoform-selective CYP-inhibitor miconazole (3 μmol/L), which is also known to block potassium channels, attenuated relaxations in vessels from WKY as well as SHR (Figure 4C).

In contrast to the observations made using vessels from young rats, a low concentration of ouabain (50 μmol/L) inhibited ACh-induced EDHF-mediated relaxations in renal arteries from 22-month-old animals (Figures 5A and 5B). Compared with arteries from young WKY rats, the hyperpolarization and relaxation elicited by KCl increased on aging (see Figures 3C and 3E). In contrast, in renal artery rings from old SHR, KCl failed to elicit either hyperpolarization or relaxation (Figures 3C and 5D). Relaxation in response to the K⁺-channel openers 1-EBIO and cromakalim were identical and concentrations reported recently by others, failed to attenuate the differences in the EDHF-mediated relaxation in vessels from either strain (data not shown). Specific inhibition of cytochrome P450 CYP 2C epoxygenases using sulfaphenazole (10 μmol/L) selectively attenuated EDHF-mediated relaxations in renal artery rings from SHR (Figure 4A). In the presence of sulfaphenazole, EDHF-mediated responses in young SHR and WKY were virtually superimposable. In contrast, 17-ODYA (10 μmol/L), which preferentially blocks CYP 4A isoforms, had no effect on the relaxation of rings from either strain (Figure 4B). The non–isoform-selective CYP-inhibitor miconazole (3 μmol/L), which is also known to block potassium channels, attenuated relaxations in vessels from WKY as well as SHR (Figure 4C).

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With the use of real-time RT-PCR, the expression of mRNA encoding the α₁, α₂, and γ subunits of the Na-K-ATPase was assessed in renal arteries from WKY rats and SHR. In arteries from young animals, there were no clear strain-dependent differences in Na-K-ATPase subunit expression. Aging was associated with a marked increase in the expression of the α₁-subunit in arteries from both strains. In contrast, the expression of the α₂-subunit was exclusively increased in arteries from WKY rats. No differences in the expression of the γ-subunit in vessels from young or old rats of either strain were observed (Figure 6). The α₁-subunit, which was expressed at high levels in the rat brain, was not detectable in the renal artery (data not shown).
In the present study, we observed pronounced differences in EDHF-mediated and KCl-induced responses in renal arteries from young and old WKY rats and SHR. Although EDHF-mediated responses were prominent in young SHR and were partially inhibited by the cytochrome P450 inhibitor sulfaphenazole, they were lost during aging. In contrast, in arteries from WKY rats, EDHF-mediated responses, which were initially resistant to inhibition of the Na-K-ATPase, became ouabain-sensitive with age. A phenomenon that was associated with an increase in the expression of the \( \alpha_2 \) subunit of the Na-K-ATPase.

Aging has previously been linked to the attenuation endothelium-dependent relaxation in arteries from SHR, and this phenomenon has been attributed to both a decrease in the bioavailability of NO as well as a decrease in the generation of an EDHF.4,5,23–25 Although NO-mediated responses are relatively easy to evaluate, the main difficulty in investigating alterations in EDHF-mediated relaxations is related to the fact that different types of EDHF appear to exist and indeed may act in parallel. Currently, the only feature shared by all of the EDHFs described is the exquisite sensitivity of responses to the combination of charybdotoxin and apamin. This is thought to reflect the importance of the opening of Ca\(^{2+}\)-dependent K\(^+\) channels in endothelial cells.26 The efflux of K\(^+\) ions through Ca\(^{2+}\)-dependent K\(^+\) channels has been suggested to result in a sufficient increase in the subendothelial K\(^+\) concentration to activate inwardly rectifying K\(^+\) channels and/or the Na-K-ATPase in smooth muscle cells.10,27–29 There are, however, other means of transferring hyperpolarization from one cell type to another. For example, hyperpolarizing factor(s) such as EETs could be released from endothelial cells. Although it was initially suggested that EETs may act as diffusible hyperpolarizing factors, these lipid mediators may rather influence the magnitude of the endothelial cell hyperpolarization; which is the initial step in the generation of EDHF-mediated responses.9 Endothelial cell hyperpolarization could also spread electrotonically from endothelial to vascular smooth muscle cells through myo-endothelial gap junctions,20 thereby inducing an endothelium-
driven hyperpolarization of smooth muscle cells. Gap junctional communication as well as the activation of inwardly rectifying K⁺ channels and the Na-K-ATPase have all been shown to simultaneously contribute to the EDHF phenomenon in isolated rat vessels. However, only the activation of inwardly rectifying K⁺ channels and the Na-K-ATPase are dependent on an increase in the subendothelial concentration of K⁺ ions and can therefore be mimicked by the direct application of KCl.

A comparison of the responses of renal arteries from young and old SHR and WKY rats revealed that only vessels from the latter strain responded to exogenously applied KCl with a pronounced hyperpolarization and relaxation. Thus, it appears that the EDHF phenomenon in renal arteries from SHR is unrelated to the activation of the Na-K-ATPase or the inwardly rectifying K⁺ channels. Although such observations may indirectly imply a potential role for gap junctions in the regulation of Na-K-ATPase subunit expression. It would also be important to confirm the results of the RT-PCR experiments at the protein level, since the activity of the Na-K-ATPase is largely determined by posttranscriptional mechanisms, including subcellular translocation and phosphorylation. Unfortunately, expression of Na-K-ATPase protein in isolated arteries was too low to be assessed by Western blot analysis, and commercially available antibodies failed to label specific proteins in preparations obtained with the use of different extraction methods, although all of the antibodies tested detected the Na-K-ATPase in extracts of rat brain and human umbilical vein endothelial cells (authors’ unpublished observations, 2002).

Our observation that the expression of the α₁ subunit of the Na-K-ATPase increases with age in arteries from both SHR and WKY rats is in agreement with two previous studies using skeletal muscle from SHR. Although the altered expression of the α₁ subunit could potentially influence membrane potential, the resting membrane potential was not altered in vessels from old WKY rats and was even decreased.

Figure 6. mRNA expression of the Na-K-ATPase subunits α₁, α₂, γ, and γ in rat femoral arteries from SHR and WKY. Top, Results of quantitative real-time RT-PCR demonstrating expression of mRNA encoding α₁, α₂, and γ subunits of the Na-K-ATPase subunits in femoral arteries from young and old WKY rats and SHR. Na-K-ATPase signals were normalized to elongation factor 2 (EF2) signal and are expressed relative to the level observed in young rats of the respective strain. Results are mean±SEM, n=5 in each group, *P<0.05 old vs young. Analysis was performed with the 2-tailed t test. Bottom, Representative ethidium bromide–stained DNA gel showing Na-K-ATPase subunit expression in samples of rat renal artery (s), rat brain as positive control (pc). A negative control (nc) consisting of water and mRNA but without reverse transcriptase was included in each experiment.
in arteries from SHR. Neither of these effects appear to be compatible with the enhanced activation of the Na-K-ATPase.

In light of the marked KCl-induced relaxation and hyperpolarization as well as the ouabain insensitivity of the ACh-induced relaxation, it could be speculated that the EDHF-mediated responses observed in arteries from young WKY can be attributed to the activation of the inwardly rectifying K⁺ channels. However, a concentration of barium far exceeding that required to specifically block this channel11 was needed to attenuate EDHF-mediated relaxations in arteries from young WKY rats (Christiana M. Larson, unpublished observations, 2003).

In conclusion, our results demonstrate that the mechanisms underlying EDHF-mediated responses in renal arteries from SHR and WKY rats are distinct. The age-induced attenuation of EDHF-mediated responses in SHR is most probably the consequence of the loss of a K⁺ ion-independent pathway, involving cytochrome P450 epoxygenases, whereas the increase in EDHF-mediated responses in arteries from WKY rats appears to be the consequence of an increase in the expression of the α₅ subunit of the Na-K-ATPase.

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