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

Eckhart Büsse, 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 N\(^\text{\textsuperscript{\text{-}}\text{N}\}-\text{nitro-L-arginine}\) 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 \(\alpha\) was 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:III-III.)

Key Words: endothelium \(\bullet\) nitric oxide \(\bullet\) acetylcholine \(\bullet\) endothelium-derived factors
\(\bullet\) animal models of hypertension \(\bullet\) renal artery
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.

**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 μmol/L to 10 μmol/L) or KCl (4 to 10 mmol/L). EDHF-mediated responses were defined as that portion of the endothelium-dependent relaxation that remained in the presence of Nω-nitro-l-arginine (L-NA, 300 μmol/L) and diclofenac.

**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 WKY than from young SHR. 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⁺ 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).

To assess the mechanism(s) underlying the more pronounced but potassium-independent EDHF-mediated responses in renal arteries from young SHR, different EDHF
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,21,22 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 5C). In contrast, in renal artery rings from old WKY rats and SHR, C and D). Concentration-relaxation curves to KCl of endothelium-intact renal artery rings preconstricted with phenylephrine in the absence (C) and presence (D) of a low concentration of ouabain (50 μmol/L). E, Change in smooth muscle membrane potential (ΔMP) induced by bolus application of KCl (end concentration, 10 mmol/L) to renal artery rings from WKY rats (black bars) and SHR (white bars). F, Effect of ouabain (Oub, 50 μmol/L, shaded bars) on acetylcholine (ACh)- and KCl-induced hyperpolarization of renal artery smooth muscle from WKY rats. A through F, All experiments were performed in the continuous presence of diclofenac (10 μmol/L) and L-NA (300 μmol/L). Results are mean±SEM, *P<0.05. Analyses were performed with (A through D) 2-way ANOVA for repeated measurements; E, 2-tailed unpaired t test; F, 2-way ANOVA.

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 α2 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 Ca2+-dependent K+ channels in endothelial cells.26 The efflux of K+ ions through Ca2+-dependent K+ channels has been suggested to result in a sufficient increase in the subendothelial K+ concentration to activate inwardly rectifying K+ channels 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,30 thereby inducing an endothelium-
driven hyperpolarization of smooth muscle cells.\textsuperscript{31,32} Gap junctional communication as well as the activation of inwardly rectifying K\textsuperscript{+} channels and the Na-K-ATPase have all been shown to simultaneously contribute to the EDHF phenomenon in isolated rat vessels.\textsuperscript{18,33,34} However, only the activation of inwardly rectifying K\textsuperscript{+} channels and the Na-K-ATPase are dependent on an increase in the subendothelial concentration of K\textsuperscript{+} 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\textsuperscript{+} channels. Although such observations may indirectly imply a potential role for gap junctions in EDHF-mediated responses in these animals, it was not possible to selectively block EDHF-mediated relaxation in the renal artery of SHR using specific connexin blocking peptides (gap peptides). Moreover, EDHF-mediated responses in renal arteries from SHR were markedly less sensitive to the gap peptides than were vessels from WKY rats (Büssemaker, unpublished observations). Although cytochrome P450 epoxygenases play a crucial role in EDHF-mediated responses in some species, several studies,\textsuperscript{35,36} including one performed using rat renal arteries,\textsuperscript{37} have reported that the EDHF-mediated relaxation of vessels from WKY and Sprague-Dawley rats does not require the activation of a cytochrome P450 enzyme. Cytochrome P450 epoxygenases can, however, be induced by several pathways associated with the induction of hypertension. For example, dietary salt loading increases cytochrome P450 expression in the kidney,\textsuperscript{38} and EET production is reported to be higher in renal arteries from SHR than in the same vessels from WKY rats.\textsuperscript{39} In the present study, two cytochrome P450 inhibitors, the non–isoform-selective epoxygenase inhibitor miconazole and the selective 2C9 inhibitor sulfaphenazole, attenuated EDHF-mediated responses in arteries from SHR. In contrast, a cytochrome P450 inhibitor that preferentially blocks the \(\omega\)-hydroxylase was without effect. Miconazole, however, also attenuated the maximal EDHF-mediated relaxation in arteries from WKY rats, an effect that may be due to the nonspecific inhibition of K\textsuperscript{+} channels which has been reported as a side effect of some cytochrome P450 inhibitors.\textsuperscript{21,22}

Taken together, these observations suggest that a major difference in the EDHF-mediated responses recorded in renal arteries from young SHR and WKY rats can be attributed to the differential involvement of a cytochrome P450 epoxygenase that is sensitive to sulfaphenazole.

In contrast to the situation in SHR, the magnitude of EDHF-mediated responses in renal arteries from WKY rats were not affected by aging. There was, however, a marked age-dependent alteration in the sensitivity of the EDHF to a low concentration of the Na-K-ATPase inhibitor ouabain (50 \(\mu\)mol/L). Because much higher concentrations of ouabain are required to inhibit the \(\alpha_1\) subunit of the Na-K-ATPase, these data suggest that the age-dependent alteration in EDHF-mediated responses can be attributed to a change in the \(\alpha_2\) or \(\alpha_3\) subunits of the Na-K-ATPase.\textsuperscript{40} Indeed, using quantitative RT-PCR, we detected a significant, age-dependent increase in the expression of mRNA encoding the \(\alpha_2\) subunit in vessels from WKY rats. No such effect was detected in arteries from SHR. Although such observations correlate well with the functional studies, these data should be interpreted with caution, since limitations associated with tissue availability meant that Na-K-ATPase expression was assessed in femoral arteries. Thus, although renal and femoral arteries are similar in size, we cannot exclude that differences exist 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 meant that Na-K-ATPase expression was assessed in femoral arteries. Thus, although renal and femoral arteries are similar in size, we cannot exclude that differences exist 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.\textsuperscript{41,42} 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 \(\alpha_1\) 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.\textsuperscript{43,44} Although the altered expression of the \(\alpha_1\) subunit could potentially influence membrane potential, the resting membrane potential was not altered in vessels from old WKY rats and was even decreased...
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 channel was needed to attenuate EDHF-mediated relaxations in arteries from 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 aging-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 α₂ subunit of the Na-K-ATPase.

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References

8. Kaw S, Hecker M. Endothelium-derived hyperpolarizing factor, but not nitric oxide or prostacyclin release, is resistant to menadione-induced oxidative stress in the bovine coronary artery. Naunyn Schmiedebergs Arch Pharmacol. 1999;359:133–139.
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