Impaired EDHF-mediated vasodilation and function of endothelial Ca$^{2+}$-activated K$^+$ channels in uremic rats

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Impaired EDHF-mediated vasodilation and function of endothelial Ca$^{2+}$-activated K$^+$ channels in uremic rats.

Background. Chronic renal failure (CRF) is associated with increased cardiovascular morbidity, abnormal arterial tone, and endothelial dysfunction. Ca$^{2+}$-activated K$^+$-channels (K$_{Ca}$) are important regulators of endothelial function by controlling endothelial hyperpolarization and thus endothelium-derived hyperpolarizing factor (EDHF)-mediated vasodilations. Here we tested the hypothesis whether an altered function of endothelial K$_{Ca}$ and diminished EDHF-mediated vasodilation contribute to the endothelial dysfunction in the rat remnant kidney model of chronic renal failure.

Methods. Functional expression of endothelial K$_{Ca}$ currents and endothelium-dependent vasodilations in rat carotid arteries were assessed by using patch-clamp techniques, single-cell reverse transcription-polymerase chain reaction (RT-PCR), and a pressure myograph 8 weeks after either subtotal 5/6 nephrectomy or sham-operation.

Results. Acetylcholine (ACh)-induced EDHF-mediated vasodilations were present in sham-operated rats, but almost absent in both normotensive 5/6 nephrectomy rats and hypertensive 5/6 nephrectomy rats. In experiments without blocking nitric oxide/prostacyclin synthesis, endothelium-dependent vasodilation to ACh was significantly reduced in both normotensive 5/6 nephrectomy rats and hypertensive 5/6 nephrectomy rats. In sham-operated rats, 1-ethyl-2-benzimidazolinone (1-EBIO), a selective opener of endothelial small and intermediate K$_{Ca}$, induced a substantial EDHF-mediated vasodilation, which was greatly reduced in hypertensive 5/6 nephrectomy rats and in normotensive 5/6 nephrectomy rats. In patch-clamp experiments, mean K$_{Ca}$ currents were significantly reduced in endothelial cells from hypertensive 5/6 nephrectomy rats and normotensive 5/6 nephrectomy rats when compared to sham-operated rats. Concordantly, single-cell reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed a greatly reduced frequency of endothelial cells expressing the K$_{Ca}$ genes, SKCa3 and IKCa1, in 5/6 nephrectomy rats compared to sham-operated rats.

Conclusion. Experimental CRF leads to a loss of EDHF-type vasodilation which was caused at least in part by an impaired functional expression of endothelial hyperpolarizing K$_{Ca}$. The loss of EDHF-type vasodilation may contribute to endothelial dysfunction and abnormal arterial tone in CRF.

Patients with end-stage renal disease (ESRD) exhibit increased cardiovascular morbidity due to an increased risk of arteriosclerosis, abnormal regulation of arterial tone, and hypertension [1, 2]. There is growing evidence that chronic renal failure (CRF) is associated with an impaired endothelial function and thus defective endothelium-dependent vasodilation [3–6]. Endothelial dysfunction is an early feature of vascular complications in several diseases [7–10] such as hypertension, diabetes, hypercholesterolemia, and coronary heart disease, although the exact mechanism leading to endothelial dysfunction is not clear. In CRF, several pathogenic factors like excessive generation of reactive oxygen species [1, 11, 12], hypertension [1], dyslipidemia with increased serum levels of cytotoxic low-density lipoprotein (LDL) [11], competitive inhibition of endothelial nitric oxide by increased production of endogenous nitric oxide inhibitors such as asymmetrical dimethylarginine (ADMA) [13] have been proposed to cause endothelial dysfunction. Also a diminished nitric oxide availability due to nitric oxide inactivation by reactive oxygen species may result in endothelial dysfunction [11, 14]. In addition to an impaired nitric oxide production or nitric oxide availability recent studies suggest that also the third vasodilatory system, the endothelium-derived hyperpolarization factor (EDHF)-mediated vasodilation might be disturbed in CRF [17, 18], which may contribute to the impaired endothelium-dependent vasodilation in patients with CRF.

The EDHF-vasodilatory system is considered of major importance in the regulation of vascular tone. For instance, the EDHF system can compensate the loss of the nitric oxide system as concluded from the conserved...
endothelium-dependent vasodilation and mild hypertension in endothelial nitric oxide synthase (eNOS)+/− mice [19, 20]. Moreover, several studies suggested that the EDHF system becomes as important as the nitric oxide system or even more important when vessel size decreases [21]. Therefore, it seems that the EDHF system plays a significant role in the regulation of resistance arteries and thus the regulation of blood pressure.

Regarding the cellular mechanisms of EDHF-mediated vasodilations, endothelial Ca2+-activated K+ channels (KCa) play a pivotal role [16, 22–24] by mediating endothelial hyperpolarization and, especially, KCa of small conductance (SKCa) encoded by the SKCa gene SKCa3 (SK3 and KCa 2.3) and of intermediate conductance (IKCa) encoded by the IKCa gene IKCa1 (IK1, KCNN4, and KCa3.1) have been shown to be the predominant hyperpolarizing KCa channels expressed in human and rat endothelium [23, 25, 26]. Their activation following agonist stimulation resulting in endothelial hyperpolarization is considered a prerequisite for the initiation of EDHF-type vasodilations [16] and, concordantly, selective blockade of SKCa and IKCa channels suppresses EDHF-mediated vasodilation in most vascular beds from different species, including humans [16, 23]. The signal transduction pathways of EDHF-signaling beyond endothelial hyperpolarization are incompletely characterized [16]. Results of a large number of studies suggest that endothelial hyperpolarization may be directly propagated via gap junctions to the smooth muscle cells [16, 27]. Alternatively, K+ efflux from the endothelium through endothelial SKCa and IKCa could stimulate inwardly rectifying K+ channel and sodium/potassium adenosine triphosphatases (Na/K ATPases) in vascular smooth muscle cells (VSMCs) leading to VSMC hyperpolarization and thus vasodilation [28]. Other proposed EDHFs such as epoxyeicosatrienonic acids (EETs) may play a modulatory role [16, 29].

In the present study we tested the hypothesis that a decreased functional expression of endothelial KCa channels in CRF leads to an impairment of EDHF-type vasodilation and is part of the endothelial dysfunction in CRF. In rats with CRF due to 5/6 nephrectomy, we observed a severely impaired EDHF-mediated vasodilation, reduced expression of the endothelial KCa genes SKCa3 and IKCa1, and reduced KCa functions in carotid arteries from normotensive and hypertensive 5/6 nephrectomy rats.

**METHODS**

**Animal studies and treatment protocols**

Animal studies were approved by the local Animal Care and Use Committee. Sprague-Dawley rats (350 to 450 g) were subjected to 5/6 nephrectomy (N = 17) or sham operation (N = 7) as described previously [30]. The group of 5/6 nephrectomy rats was divided into two subgroups from which one (N = 6) was subjected to an antihypertensive triple drug treatment (a combination of reserpine 5 mg/L, hydralazine 80 mg/L, and hydrochlorothiazide 25 mg/L, in the drinking water) starting from day 1 after surgery. The other subgroup of 5/6 nephrectomy rats did not receive antihypertensive therapy. Systolic blood pressure was monitored weekly in the awake animals by the tail-cuff method. At the end of week 8, urine was collected for 24 hours and water consumption was determined in metabolic cages. All rats were sacrificed 8 weeks after surgery and left and right carotid artery, heart, and kidneys were excised. For evaluation of renal insufficiency in the 5/6 nephrectomy groups, levels of urea and creatinine were determined in urine and/or serum samples from all rats.

**Patch-clamp experiments and single-cell reverse transcription-polymerase chain reaction (RT-PCR) experiments in endothelial cells of carotid artery**

Single-cell RT-PCR analysis of KCa gene expression in endothelial cells of carotid artery in situ, whole-cell patch-clamp experiments, and data analysis were performed as described previously [23, 25]. In brief, vessel slices of freshly dissected carotid artery were mounted on holding pipette and preincubated with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) without Ca2+ and Mg2+ for up to 15 minutes followed by wash out. For in situ harvesting of endothelial cells, a single endothelial cell was selectively fixed with the patch pipette and mechanically detached from the vessel wall. After RT of mRNA from single endothelial cell sample, a “multiplex” single-cell RT-PCR was performed using intron-spanning primer pairs for the small- and intermediate-conductance KCa genes, rSKCa3 and rIKCa1, and endothelial nitric oxide synthase (reNOS) as endothelial cell marker. Sequences of primer pairs are stated elsewhere [23, 25]. Membrane currents in electrically isolated endothelial cells were recorded with an EPC-9 (Heka; Lamprecht, Pfalz, Germany) patch-clamp amplifier using voltage ramps (duration 1000 milliseconds) from −100 mV to +100 mV. For activation of KCa currents, endothelial cells were dialyzed with a KCl pipette solution containing 3 μmol/L [Ca2+]free.

**Pressure myograph experiments**

Pressure myograph experiments were performed as described previously [23, 25]. In brief, carotid artery segments of 3 to 4 mm in length were cannulated with micropipettes in an experimental chamber mounted on the stage of a Zeiss Axiovert 100 (Oberkochen, Germany). Vessel diameter was continuously monitored with a video camera. The bath and perfusion solution contained (in mmol/L): 145.0 NaCl, 1.2 NaH2PO4, 4.7 KCl,
1.2 MgSO₄, 2.0 CaCl₂, 5.0 glucose, 2.0 pyruvate, and 3.0 3-[N-morpholine] propane sulfonic acid (MOPS) buffer along with 1 g/100 mL bovine serum albumin (BSA) (pH 7.4) at 37°C. To block nitric oxide and prostacyclin synthesis the NOS inhibitor N⁰,N⁰-dimethylarginine (L-NNA) (100 μmol/L) and the cyclooxygenase inhibitor indomethacin (10 μmol/L) were added to both the bath and perfusion solution. Carotid arteries were pressurized to 80 mm Hg with a pressure myograph system (J.P. Trading P100, Aarhus, Denmark) and continuously perfused at a flow rate of 0.6 mL/min. After an initial equilibration period for 40 minutes, carotid arteries were preconstricted with 1 μmol/L phenylephrine in the bath solution. After development of stable tone, nitric oxide- and prostacyclin-dependent and independent vasodilatory responses were determined by perfusion with increasing concentrations of acetylcholine (ACh) (1 nmol/L to 10 μmol/L). Diameter changes are expressed as percentage of the maximal vasodilation measured in response to 1 μmol/L sodium nitroprusside (SNP). The selective SKCa and IKCa opener, 1-ethyl-2-benzimidazolinone (1-EBIO) (100 μmol/L) was made as 1000-fold stock solution in dimethyl sulfoxide (DMSO) and was applied intraluminally. 1-EBIO was obtained from Tocris (Köln, Germany); all other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA)

Statistical analysis

Data are given as mean ± SE. If appropriate, Student t test or χ² analyses were used to assess differences between groups. P values of P < 0.05 were considered significant.

RESULTS

Renal insufficiency and systolic blood pressure in 5/6 nephrectomy rats

Data from laboratory tests and general characteristics of both groups of 5/6 nephrectomy rats and of sham-operated controls are summarized in Table 1. Untreated 5/6 nephrectomy rats developed severe hypertension, whereas hypertension was prevented by the antihypertensive therapy in 5/6 nephrectomy rats. In hypertensive 5/6 nephrectomy rats and in normotensive 5/6 nephrectomy rats, serum concentrations of creatinine and urea were significantly increased and urine concentration of creatinine was greatly reduced when compared to sham-operated controls. Urine volume and fluid intake were significantly increased in both hypertensive 5/6 nephrectomy and normotensive 5/6 nephrectomy rats. Consequently, creatinine clearance was greatly reduced in both 5/6 nephrectomy groups which thus documents renal insufficiency in these rats. In both 5/6 nephrectomy groups, the wet weight of the remnant kidney was significantly higher than healthy
kneys of controls. Wet heart weight was significantly enhanced only in the untreated hypertensive 5/6 nephrectomy rats presumably as a consequence of hypertension, as heart weight was not significantly different in the antihypertensive-treated normotensive 5/6 nephrectomy rats.

**Impaired endothelium-dependent vasodilation in normotensive and hypertensive 5/6 nephrectomy rats**

In carotid arteries, endothelium-dependent vasodilation induced by ACh (1 nmol/L to 10 μmol/L) in the absence of NOS and cyclooxygenase inhibitors was significantly reduced in hypertensive 5/6 nephrectomy rats when compared to sham-operated controls (Fig. 1A). In the presence of NOS and cyclooxygenase inhibitors, ACh induced a significant EDHF-mediated vasodilation in the sham-operated controls, whereas in hypertensive 5/6 nephrectomy, EDHF-mediated vasodilation was almost absent (Fig. 1B). To rule out that the reduced “total” endothelium-dependent vasodilations and the loss of EDHF-mediated vasodilation was solely a consequence of the development of hypertension in these uremic rats, we also tested endothelium-dependent vasodilatory responses in the normotensive 5/6 nephrectomy rats. Similar to hypertensive 5/6 nephrectomy, “total” endothelium-dependent vasodilation was significantly reduced and EDHF-mediated vasodilation was severely impaired in the normotensive 5/6 nephrectomy rats. This indicates that defective endothelium-dependent vasodilation in 5/6 nephrectomy rats appears to be related to uremia and can not be attributed solely to endothelial damage caused by hypertension in this model.

Endothelium-independent responses to phenylephrine and SNP were similar in pressurized nonendothelium-denuded carotid arteries from hypertensive 5/6 nephrectomy rats, normotensive 5/6 nephrectomy rats, and sham-operated controls (data not shown), which indicates normal vascular smooth muscle function in these uremic rats.

**Impaired 1-EBIO–mediated vasodilation in normotensive and hypertensive 5/6 nephrectomy rats**

EDHF-mediated vasodilations in carotid arteries of healthy rats and in other vascular beds of rats and other species are closely related to the Ca$^{2+}$-dependent opening of endothelial SK$\text{Ca}$ and IK$\text{Ca}$ channels leading to endothelial hyperpolarization and thus vasodilation [16, 22–24]. Consequently, EDHF-type vasodilations in carotid arteries can also be mimicked by selective SK$\text{Ca}$ and IK$\text{Ca}$ openers such as 1-EBIO [25, 31]. On the other hand, EDHF-type vasodilation is abolished after selective inhibition of endothelial SK$\text{Ca}$ by apamin and IK$\text{Ca}$ channels by TRAM-34 [32], as previously shown by us [23].

To test whether the impaired EDHF-type vasodilation observed in normotensive 5/6 nephrectomy and hypertensive 5/6 nephrectomy rats was caused by endothelial K$\text{Ca}$ dysfunction, we studied vasodilatory effects of the selective SK$\text{Ca}$ and IK$\text{Ca}$ openers 1-EBIO in normotensive 5/6 nephrectomy rats, hypertensive 5/6 nephrectomy rats, and sham-operated controls. Intraluminal application of 1-EBIO induced a substantial vasodilation of approximately 20% in sham-operated controls (Fig. 2), whereas 1-EBIO-induced vasodilation was greatly reduced in both normotensive 5/6 nephrectomy and hypertensive 5/6 nephrectomy rats (Fig. 2).

These data from pressure myograph measurements strongly indicate that EDHF-mediated vasodilation is severely impaired in this model of CRF.
Alterations in \( K_\text{Ca} \) function and expression in endothelial cells of normotensive and hypertensive 5/6 nephrectomy rats

The finding of an impaired EDHF-mediated vasodilation and especially of endothelial hyperpolarization-mediated vasodilation induced by 1-EBIO might be indicative of a decreased functional expression of the endothelial \( K_\text{Ca} \) channels in hypertensive 5/6 nephrectomy rats and normotensive 5/6 nephrectomy rats. To test this hypothesis, we performed whole-cell patch-clamp experiments in endothelial cells from hypertensive 5/6 nephrectomy rats, normotensive 5/6 nephrectomy rats, and sham-operated controls to determine the function of \( SK_\text{Ca} \) and \( IK_\text{Ca} \) channels. To fully activate \( SK_\text{Ca} \) and \( IK_\text{Ca} \) channels by Ca\(^{2+}\) in whole-cell patch-clamp experiments, single endothelial cells were dialyzed with a pipette solution containing 3 \( \mu \text{mol/L} \) free Ca\(^{2+}\) [23], which is sufficient for maximal channel activation (EC\(_{50}\) 800 to 500 \( \text{mmol/L} \) Ca\(^{2+}\)) [26, 33–35]. The electrophysiologic and pharmacologic properties of \( SK_\text{Ca} \) and \( IK_\text{Ca} \) channels in endothelial cells of rat carotid arteries were described by us in more detail previously [23, 26].

The whole-cell patch-clamp experiments revealed that in endothelial cells from hypertensive 5/6 nephrectomy rats and normotensive 5/6 nephrectomy rats, composite \( SK_\text{Ca} \) and \( IK_\text{Ca} \) currents were significantly reduced by \( \approx 50\% \) when compared to those in endothelial cells from sham-operated controls. Original traces and mean composite \( SK_\text{Ca}/IK_\text{Ca} \) currents normalized to cell capacitance are shown in Figure 3.

Subsequent to patch-clamp experiments, we used “multiplex” single-cell RT-PCR [22, 26] to determine whether the decreased currents through \( SK_\text{Ca} \) and \( IK_\text{Ca} \) channels in endothelial cells from hypertensive 5/6 nephrectomy rats correlated with alterations in mRNA levels for the respective \( K_\text{Ca} \) genes \( SKCa3 \) [33] and \( IKCa1 \) [34, 35].

As shown in Figure 4, we detected \( SKCa3 \) and \( IKCa1 \) mRNA in almost every endothelial cell sample (\( SKCa3 \) 100%, 18/18 endothelial cell samples; \( IKCa1 \) 94%, 17/18 endothelial cell samples). In endothelial cells from hypertensive 5/6 nephrectomy rats, the frequency of endothelial cell samples containing \( SKCa3 \) and \( IKCa1 \) mRNAs was greatly reduced (\( SKCa3 \) 45%, 9/20 endothelial cell samples) (\( P = 0.09, \chi^2 \) analyses) (\( IKCa1 \) 25%, 5/20
DISCUSSION

Using the 5/6 nephrectomy rat model of CRF, we provide evidence that the EDHF-vasodilatory system is impaired in CRF. This is based on the observation that EDHF-mediated vasodilation induced by agonist stimulation or pharmacologically is severely reduced in uremic rats. With respect to the underlying cellular mechanism, we show that function and mRNA expression of endothelial KCa channel, which are a prerequisite for the initiation of the EDHF response [16], are reduced in uremic rats. Therefore, the loss of endothelial KCa channel functions and the subsequently diminished EDHF-mediated vasodilation could be a component of endothelial dysfunction in CRF.

Endothelial dysfunction is a general characteristic of patients with ESRD, but the underlying pathophysiologic mechanisms are poorly understood [1–5]. In addition, it is not clear whether this endothelial dysfunction is a direct consequence of an accumulation of uremic toxins or secondary to hypertension in these patients. In recent years, it has been postulated that endothelial dysfunction and thus defective regulation of vascular tone might be caused by either a disturbed nitric oxide production due to decreased expression levels of the endothelial NOS isoform [36] or by diminished nitric oxide availability due to nitric oxide scavenging and inactivation by increased levels of reactive oxygen species [2, 11, 12]. Also an increase in the level of endogenous nitric oxide inhibitors has been proposed to hamper endothelial nitric oxide production in CRF [13]. In the present study we found that ACh-induced endothelium-dependent vasodilation in the absence of nitric oxide/prostacyclin inhibitors was significantly reduced in uremic rats which may indicate diminished nitric oxide production or availability in these uremic rats.

The inhibition of nitric oxide/prostacyclin synthesis resulted in a reduced ACh-induced vasodilator response in healthy rats, but in an almost complete loss of ACh-induced vasodilations in uremic rats. This finding may also imply that the putative impairment of nitric oxide- or prostacyclin-mediated vasodilations is not compensated by the third vasodilatory system, the EDHF system which have been proposed to act as an important backup system when nitric oxide production is impaired [19, 37]. An alternative interpretation of the data might be that impairment of “total” ACh-induced endothelium-dependent vasodilation was caused by a loss of the EDHF system alone. This alternative interpretation assumes that EDHF system contributes to “total” endothelium-dependent vasodilation even in the presence of the other two vasodilating factors. Noteworthy, in healthy rats, selective blockers of endothelial hyperpolarizing KCa channels [23, 32], which are prerequisite for the generation of the EDHF response [16, 22–28], reduce “total” endothelium-dependent vasodilation to a level similar to that observed in uremic rats [23].

Despite the large number of studies investigating the nitric oxide system in CRF, only little attention has been given to the EDHF system so far. However, data from some studies already indicated that the EDHF-vasodilatory system may play a role in endothelial dysfunction observed in human or experimental CRF [17, 18]. In the present study we show that the ACh-induced EDHF-mediated vasodilation is severely impaired in carotid arteries of uremic rats. Moreover, a vasodilatory...
response to endothelial hyperpolarization elicited by direct pharmacologic opening of endothelial SKCa and IKCa channels was greatly reduced in the uremic rats. Since activation of these endothelial SKCa and IKCa channels is also of crucial importance in the initiation of the EDHF signal following agonist stimulation [16, 22–28], the defective EDHF-mediated vasodilation as well as the impaired endothelial hyperpolarization-mediated vasodilation might thus be caused by a loss of these hyperpolarizing KCa channels in the endothelium. This interpretation was further supported by the results of our electrophysiologic studies which demonstrate a significant reduction of SKCa and IKCa currents in endothelial cells from uremic rats. This impaired function of SKCa and IKCa channels might be explained by a reduced mRNA expression of the respective KCa genes SKCa3 and IKCa1 as revealed by our single-cell RT-PCR experiments. However, the mechanism by which CRF may lead to a down-regulation of endothelial expression of SKCa3 and IKCa1 remains to be clarified. Interestingly, a similar loss of endothelial SKCa and IKCa channels as result of a decreased SKCa3 and IKCa1 gene expression and consequently a lack of EDHF-mediated vasodilation have been shown to occur also in the regenerated endothelium after balloon catheter angioplasty [25]. Thus it is tempting to speculate that a defective functional expression of endothelial SKCa and IKCa channels might represent a more general feature of endothelial dysfunction although the underlying pathogenic process or cardiovascular disease states may be different.

Hypertension has been reported to cause endothelial dysfunction and more recently also EDHF-mediated vasodilation in a model of experimental hypertension [38]. To rule out that the defective EDHF-mediated vasodilation was caused by hypertension in uremic rats, we determined EDHF-mediated vasodilation in normotensive uremic rats subjected to antihypertensive therapy. We found that the antihypertensive treatment did not prevent the loss of functional IKCa and SKCa expression as well as the impairment of EDHF-mediated vasodilation. Correspondingly, the endothelial hyperpolarization-mediated vasodilation induced by direct pharmacological opening of IKCa and SKCa channels was similarly impaired in normotensive uremic rats. Thus these findings indicate that the impaired EDHF-mediated vasodilation in carotid arteries of 5/6 nephrectomy rats appears to be related to uremia but not solely to the development of hypertension.

CONCLUSION

The present study revealed an impaired EDHF-mediated vasodilation and endothelium hyperpolarization-mediated vasodilation in a rat model of CRF which was presumably caused by a loss of functional IKCa and SKCa expression in the endothelium. An improvement of endothelial EDHF function could be beneficial in preventing endothelial dysfunction and thus abnormal regulation vascular tone in patient with ESRD.

ACKNOWLEDGMENT

This work was supported by the Deutsche Forschungsgemeinschaft (FOR-341, Ho-1103/5-4, GRK-276/2, and GRK 865).

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