Nitric oxide– and EDHF-mediated arteriolar tone in uremia is unaffected by selective inhibition of vascular cytochrome P450 2C9

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Nitric oxide– and EDHF-mediated arteriolar tone in uremia is unaffected by selective inhibition of vascular cytochrome P450 2C9.

**Background.** Uremia is a state of endothelial dysfunction as demonstrated by a reduced agonist-induced endothelium-dependent vasodilatation. Recent studies suggest that an endothelial cytochrome P450 (CYP) epoxygenase (CYP 2C9) can modulate endothelium-dependent vasodilatation in two different ways: (1) by the production of epoxyeicosatrienoic acids (EETs), which elicit hyperpolarization and relaxation; and (2) by the release of oxygen-derived free radicals, which compromise the bioavailability of nitric oxide. We therefore determined whether one of these pathways is involved in endothelial dysfunction of uremia.

**Methods.** Using venous occlusion plethysmography, we measured forearm blood flow (FBF) in response to the intrabrachial infusion of acetylcholine (ACh; endothelium-dependent vasodilator; 1, 5, 10, 50, 100, and 300 nmol/min) and sodium nitroprusside (SNP; endothelium-independent vasodilator; 2.5, 5, and 10 μg/min) in 10 stable patients on hemodialysis (HD) and 9 healthy control subjects. In HD patients, ACh infusions were repeated together with sulfaphenazole (SPZ, 6 mg/min), a highly selective inhibitor of CYP 2C9 with and without concomitant blockade of the nitric oxide synthase (NOS) by N\textsuperscript{\text{G}}-monomethyl L-arginine (L-NMMA, 16 μmol/min).

**Results.** Endothelium-dependent vasodilatation to ACh was reduced in HD compared to control subjects (P = 0.002), indicating endothelial dysfunction in the patients examined. Endothelium-independent vascular responses to SNP were attenuated in HD, but not significantly different to control. SPZ failed to modulate both baseline FBF and ACh-induced vasodilatation in HD. Furthermore, SPZ had no effect on baseline FBF and ACh-mediated vasodilatation in the presence of L-NMMA in HD.

**Key words:** nitric oxide, endothelium-derived hyperpolarizing factor, sulfaphenazole, plethysmography.

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**Conclusion.** Our results do not support a major role for CYP 2C9-derived products in the regulation of arteriolar tone in early endothelial dysfunction of uremic subjects.

Endothelial dysfunction, a term that refers to attenuated agonist-induced endothelium-dependent vasodilation, is regarded as an early sign of atherosclerosis [1], and has been shown to be a significant predictor of future cardiovascular events [2, 3]. Uremia is associated with excessive cardiovascular morbidity and mortality [4], and impaired endothelial function has been demonstrated in uremic patients [5–7]. Understanding the mechanisms underlying uremia-associated endothelial dysfunction may be important for future therapeutic interventions.

Agonist-induced, endothelium-dependent vasodilatation is in part mediated by the generation of nitric oxide (NO) and prostacyclin (PGI\textsubscript{2}). A substantial portion of this response is, however, resistant to the combined blockade of the nitric oxide synthases (NOS) and cyclooxygenases. Because NO/PGI\textsubscript{2}-independent vasodilation is associated with hyperpolarization of vascular smooth muscle cells, it has been attributed to the release of an endothelium-derived hyperpolarizing factor (EDHF). The term EDHF does not represent a single hyperpolarizing factor that is a distinct chemical identity, but seems to encompass a number of mechanisms linked to smooth muscle hyperpolarization and relaxation [8]. Although a CYP 2C epoxygenase is the source of vascular smooth cells hyperpolarizing epoxyeicosatrienoic acids (EETs) in porcine coronary arteries [9] and human mammary arteries [10], this enzyme is also able to generate reactive oxygen species, which interfere with the bioavailability of NO and, thus, interfere with NO-mediated vasodilatation and the expression of redox-sensitive genes [11]. The latter mechanism appears to dominate in...
situations of endothelial dysfunction since a considerable improvement in the acetylcholine-induced NO-dependent vasodilatation of the forearm vasculature was recorded in patients with coronary heart disease when CYP 2C9 was inhibited [12].

Uremia is a state of endothelial dysfunction, as shown by a reduced agonist-induced endothelium-dependent vasodilatation [7, 13]. The aim of the present study was, therefore, to determine whether a CYP 2C9 product (either 11,12 EET or oxygen-derived free radicals) has a modulating effect on endothelium-dependent vasodilatation in patients on hemodialysis.

**METHODS**

### Subjects

The study protocol was approved by the University of Dresden ethics committee. Ten stable, nondiabetic male patients on regular hemodialysis (HD), and 9 healthy volunteers matched for age, gender, height, weight, and smoking habits were recruited, and written informed consent was obtained before any investigation was started. All HD patients enrolled were on a transplant waiting list. Physical examination, ECG, exercise testing, and laboratory screening were performed regularly in this context and revealed no evidence of clinically relevant atherosclerotic disease. HD patients had mild hypertension requiring drug treatment (beta-blockers $N = 4$, ACE inhibitors $N = 4$, calcium antagonists $N = 2$). Antihypertensive medication was withdrawn 48 hours before the start of the study. HD patients were studied in the morning during their short dialysis-free interval. Their actual weight did not exceed the estimated dry weight by more than 2 kg. In healthy control subjects, hypertension or underlying vascular disease was ruled out by physical examination. Cigarettes, alcohol, and all caffeine-containing beverages were withheld for 12 hours before the study. All investigations were performed in a quiet room kept at a constant temperature of between 22°C and 24°C. Each subject was supine, with both forearms resting slightly above heart level.

**Measurement of forearm blood flow (FBF)**

FBF was measured simultaneously in both arms by venous occlusion plethysmography as described previously [7]. In HD patients, only the arm not bearing the arteriovenous fistula was available for FBF recordings. Pressure of the congesting cuffs of both upper arms was set at 40 mm Hg. Mercury-in-silastic strain gauges were wrapped around the widest parts of the forearms and connected to a calibrated venous occlusion plethysmograph (Gutmann Medizinelektronik, Eurasburg, Germany). The brachial artery of the nondominant arm (or the arm without arteriovenous fistula, respectively) was cannulated with a 27-G steel needle (Coopers Needle Work, Birmingham, UK) for drug infusion. After cannulation of the brachial artery, saline was infused for 20 minutes to establish baseline conditions in each protocol. Individual measurements of FBF lasting 10 seconds were made every 15 seconds for 2.5 minutes during each dose of agent administered. The blood flow of the hands was excluded by a wrist cuff inflated to a suprasystolic pressure (220 mm Hg) during each measurement period. Blood pressure was measured at baseline and at the end of each infusion period. During each protocol, the infusion rate was kept constant at 1 mL/minute. Blood pressure was measured supine before arterial cannulation on the infusion arm using a Dinamap Pro 200 (Criticon, Tampa, Mexico). At the end of each FBF determination, blood pressure was measured at the left artery dorsalis pedis.

**Experimental protocols**

The study consisted of 2 different experimental protocols. One hour before each protocol participants received 1200 mg ibuprofen orally. This dose has been used previously to inhibit endothelial PGI₂ production [14].

**Protocol 1: Influence of sulfaphenazole on baseline FBF and on acetylcholine-induced endothelium-dependent vasodilatation (HD, $N = 10$; control, $N = 9$)**

Acetylcholine was infused at increasing doses of 1, 5, 10, 50, 100, and 300 nmol/min, and FBF measurements were obtained at the end of each dosing period (5 minutes). We used this wide dose range of acetylcholine because dose-dependent differences in the generation of nitric oxide and EDHF in response to the drug have been observed [15]. After a resting period of 30 minutes to reestablish baseline FBF, sulfaphenazole was infused at 2 mg/min over 10 minutes and FBF measurements were obtained. Sulfaphenazole is a highly specific inhibitor of CYP 2C9, and inhibits enzyme activity and reactive oxygen species generation by a microsomal preparation from CYP 2C9 overexpressing cells with an IC₅₀ of approximately 2 μmol/L [16]. We have previously shown that this dose of sulfaphenazone results in plasma concentrations of 50 to 180 μmol/L in the forearm under baseline conditions [17]. We gave this apparently high dose to account for the considerable protein binding of the drug, which is reported to be between 98% and 100%. Comparable doses have been used to attenuate exercise-induced vasodilatation in healthy subjects [18], and to improve endothelium-dependent vasodilatation in patients with coronary heart disease [12]. In HD patients, sulfaphenazole was increased to 6 mg/min, and continued during subsequent infusion of acetylcholine as described above. The dose of sulfaphenazone was increased to maintain the desired plasma concentration during infusion of acetylcholine.
Protocol 2: Influence of sulfaphenazole on baseline FBF and on acetylcholine-induced endothelium-dependent vasodilatation in the presence of N"-monomethyl-L-arginine (L-NMMA; HD, N = 7)

L-NMMA (16 μmol/minute) was infused over 10 minutes. This dose has been shown to mediate maximal inhibition of both baseline and stimulated vascular nitric oxide synthesis in the forearm [15]. After FBF measurements, acetylcholine was added as described in protocol 1. After a 30-minute washout-period of saline infusion, L-NMMA (16 μmol/minute) was started again and FBF was measured after 10 minutes. Thereafter, sulfaphenazole (2 mg/minute) was coinfused for 10 minutes, and FBF recordings were repeated. L-NMMA (16 μmol/minute) and sulfaphenazole (6 mg/min) were continued, and acetylcholine was added as shown before.

In a third experimental session (performed in all participants on a separate day) we tested endothelium-independent vasodilatation using graded infusions of sodium nitroprusside at 2.5, 5, and 10 μg/min. Assessment of endothelium-independent vasodilatation was completed by determination of peak FBF after 5 minutes of forearm ischemia in a further experimental session.

Drugs

Ibuprofen (Jenaprofen®) was obtained from Jenapharm (Jena, Germany); L-NMMA and the sodium acetate salt of sulfaphenazole were from Clinalfa (Läufelfingen, Switzerland); acetylcholine (Miochol E®) was obtained from Ciba Vision (Germering, Germany); and sodium nitroprusside (nipruss®) was from Schwarz Pharma (Monheim, Germany). All agents used were dissolved in physiologic saline except for sodium nitroprusside, which was dissolved in glucose 5%, avoiding exposure to light.

Statistics

FBF is expressed either as an absolute value (mL per dL of forearm tissue per minute) or the difference (Δ FBF) between the absolute FBF during drug infusion and the FBF measured before drug infusion. One FBF determination consisted of 10 single FBF measurements. The final 5 blood flow recordings for each infusion step were used to calculate mean FBF. Results are presented as mean ± SEM. Comparison of group characteristics and vascular responses to sulfaphenazole at rest were performed using the Student t test. Dose-response curves to acetylcholine and sodium nitroprusside were analyzed by two-way analysis of variance (ANOVA) for repeated measurements. Values of P < 0.05 were considered statistically significant.

RESULTS

Arterial puncture was performed without complications in all subjects. Substances were tolerated well by all participants. Blood pressure and heart rate remained stable in each individual during all experimental sessions (data not shown). FBF in the noninfused arm (which could be assessed in control subjects only) was constant during each experimental protocol (data not shown).

Baseline characteristics of the subjects participating in each group are given in Table 1.

Effect of sulfaphenazole on baseline FBF

Infusion of sulfaphenazole into the resting forearm vascular bed resulted in a small but insignificant increase in FBF in both HD (from 3.4 ± 0.7 to 3.8 ± 0.7 mL/dL × min; P = 0.65) and control (from 1.8 ± 0.2 to 2.0 ± 0.3; P = 0.30).

Endothelium-dependent and endothelium-independent vasodilation

Baseline FBF before infusion of acetylcholine was 3.8 ± 0.5 mL/dL × min in HD and 2.5 ± 0.4 in control. Endothelium-dependent vasodilatation in response to increasing doses of acetylcholine was significantly reduced in HD compared to control (P = 0.002 by ANOVA), as outlined in Figure 1A.

Baseline FBF before infusion of sodium nitroprusside was 2.9 ± 0.4 mL/dL × min in HD and 3.0 ± 0.4 in control. Endothelium-independent vasodilatation mediated by this drug tended to be lower in HD; however, this difference did not reach statistical significance (P = 0.14 by ANOVA). Results are illustrated in Figure 1B. Application of 5 minutes of forearm ischemia increased FBF from 3.0 ± 0.4 to 16.1 ± 1.3 in HD, and from 3.0 ± 0.7 to 17.7 ± 1.6 mL/dL × min in control. These results were statistically not different (P = 0.51 by ANOVA).

Effect of sulfaphenazole on acetylcholine-induced endothelium-dependent vasodilatation in HD

Baseline FBF was identical before infusion of acetylcholine alone or acetylcholine in combination with

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HD (N = 10)</th>
<th>Control (N = 9)</th>
<th>P value</th>
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<tr>
<td>Age years</td>
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<td>67 ± 2</td>
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</tr>
<tr>
<td>Time on HD years</td>
<td>6.6 ± 1.5</td>
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Values represent mean ± SEM.

Table 1. Baseline characteristics of hemodialysis patients (HD) and control subjects
sulfaphenazole, being $3.8 \pm 0.5$ mL/dL × min. Coinfusion of sulfaphenazole had no modulating effect on acetylcholine-mediated vascular responses in HD ($P = 0.55$ by ANOVA), as shown in Figure 2.

**Effect of sulfaphenazole on baseline FBF in the presence of L-NMMA in HD**

Baseline FBF in the presence of L-NMMA was $1.7 \pm 0.3$ mL/dL × min. Infusion of sulfaphenazole had no measurable effect on FBF ($1.7 \pm 0.3; P = 0.77$).

**Effect of sulfaphenazole on acetylcholine-induced endothelium-dependent vasodilatation in the presence of L-NMMA in HD**

Before infusion of acetylcholine, baseline FBF was $2.3 \pm 0.3$ mL/dL × min in the presence of L-NMMA alone, and $1.7 \pm 0.3$ in the presence of both L-NMMA and sulfaphenazole. Sulfaphenazole had no modulating effect on acetylcholine-induced NO/PGI$_2$-resistant endothelium-dependent vasodilatation ($P = 0.60$ by ANOVA), as demonstrated in Figure 3.

**DISCUSSION**

This study was conducted to test the hypothesis that in uremic patients an endothelial CYP 2C9-dependent enzyme acts as a significant source of oxygen-derived free radicals (compromising the bioavailability of vascular NO) and/or 11,12-EET (which may act as an EDHF). We found that in HD patients with proven endothelial dysfunction, the inhibition of CYP 2C9 by sulfaphenazole had no effect on both baseline FBF and on acetylcholine-mediated endothelium-dependent vasodilatation.
Previous studies have demonstrated that in the forearm circulation of healthy humans, CYP 2C9–dependent products are not involved in the regulation of vascular tone. This was primarily shown by the fact that sulfaphenazole, a highly specific inhibitor of CYP 2C9, had no effect on baseline FBF [17]. In addition, sulfaphenazole did not modulate bradykinin-induced NO/PGI\(_2\)-independent NO-dependent vasodilation [12] in control subjects. It is conceivable, however, that the sensitivity to this sulfonamide is increased in patients demonstrating a manifest endothelial dysfunction. Nitric oxide interacts with hemoproteins, such as CYP, to inhibit enzyme activity. It follows that a decrease in the bioavailability of NO (as in endothelial dysfunction) could be associated with an increase in CYP activity.

Our results demonstrate that sulfaphenazole does not significantly affect resting FBF in either the absence or presence of L-NMMA. We conclude that CYP 2C9–derived products do not contribute to baseline arterial tone in the forearm in HD patients. This observation is not too surprising as it is generally accepted that the antioxidant vitamin C improves endothelium-dependent vasodilatation, indicating that this pathway is not prominent in the forearm vasculature of HD patients.

**CONCLUSION**

Activation of CYP 2C9 is not involved in early arteriolar endothelial dysfunction of uremic subjects. Our observations may indicate that blood pressure regulation does not critically depend on endothelial expression of CYP 2C9 in this setting. We cannot rule out that such a pathway plays a role in conduit vessels and/or later in the process of atherogenesis in uremia. These questions may be subject to further studies.

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**REFERENCES**


