Inhibition of Cytochrome P450 2C9 Improves Endothelium-Dependent, Nitric Oxide–Mediated Vasodilatation in Patients With Coronary Artery Disease

Stephan Fichtlscherer, MD; Stefanie Dimmeler, PhD; Susanne Breuer, MD; Rudi Busse, MD, PhD; Andreas M. Zeiher, MD; Ingrid Fleming, PhD

Background—Nitric oxide (NO)– and prostacyclin-independent vasodilatation in several vascular beds has been linked to the activation of cytochrome P450 (CYP) epoxygenases expressed in endothelial cells. However, these enzymes, which generate vasodilator epoxyeicosatrienoic acids, may also produce oxygen-derived free radicals, which attenuate the bioavailability of NO. Here, we studied the involvement of CYP 2C9 in modulating endothelium-dependent and -independent changes in forearm blood flow (FBF) in healthy volunteers and in patients with manifest coronary artery disease.

Methods and Results—The effects of sulfaphenazole, a selective inhibitor of CYP 2C9, on endothelium-dependent (acetylcholine) and endothelium-independent (sodium nitroprusside, SNP) FBF responses were measured by venous occlusion plethysmography in 5 healthy subjects and in 16 patients with angiographically documented stable coronary artery disease. Sulfaphenazole did not modify FBF responses to acetylcholine or SNP in healthy subjects. In contrast, sulfaphenazole markedly and dose-dependently enhanced the FBF response to acetylcholine without affecting the response to SNP. Vitamin C also increased the FBF response to acetylcholine, but this effect was further potentiated by sulfaphenazole. In the presence of N\textsuperscript{\textsubscript{\textordm{H}}}3-monomethyl-L-arginine, sulfaphenazole failed to significantly improve acetylcholine-induced vasodilatation. The oxidation of serum proteins was enhanced in patients with coronary artery disease, and this effect was significantly attenuated by sulfaphenazole.

Conclusions—The CYP 2C9 inhibitor sulfaphenazole enhances endothelium-dependent vasodilator responses in patients with manifest coronary artery disease. This effect seems to be related to an increase in the bioavailability of NO, probably as a consequence of an attenuated generation of reactive oxygen species by CYP 2C9 in endothelial cells. (Circulation. 2004;109:178-183.)

Key Words: blood flow ■ coronary artery disease ■ endothelium-derived factors ■ free radicals ■ nitric oxide

A substantial portion of the agonist- or fluid shear stress–induced vasodilatation of many vascular beds is resistant to combined inhibition of nitric oxide (NO) synthases (NOS) and cyclooxygenase. Because NO- and prostacyclin-independent relaxation is associated with vascular smooth muscle cell hyperpolarization, it has been attributed to the release of an endothelium-derived hyperpolarizing factor (EDHF; for review, see McGuire et al').

We have previously demonstrated that a cytochrome P450 epoxygenase (CYP 2C) expressed in the endothelium plays a crucial role in the generation of EDHF-mediated responses in porcine coronary arteries.2 More recently, CYP 2C9 has been identified in the endothelium of human mammary arteries and shown to generate 11,12-epoxyeicosatrienoic acid (11,12-EET). Preventing the generation of 11,12-EET by these arteries abolished the acetylcholine- and bradykinin-induced NO/prostaglandin I\textsubscript{1}–independent relaxation.3 A similar CYP 2C9–dependent vasodilator mechanism has also been reported in the skeletal muscle circulation of healthy subjects.4

In addition to generating vasodilator EETs, CYP 2C9 also generates significant amounts of oxygen-derived free radicals, which compromise endothelium-dependent vasodilatation by scavenging NO.5 This aspect is clearly of potential clinical relevance. Therefore, the aim of the present investigation was to determine the effects of the selective CYP 2C9 inhibitor sulfaphenazole6 on acetylcholine-induced vasodilatation in patients with coronary artery disease and impaired acetylcholine-induced vasodilatation. Moreover, we aimed to determine which CYP 2C9 product, ie, EETs or oxygen-derived free radicals, is the most relevant for the regulation of vascular tone in subjects with coronary artery disease.

Received March 6, 2003; de novo received June 17, 2003; revision received September 12, 2003; accepted September 18, 2003.

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Circulation is available at http://www.circulationaha.org DOI: 10.1161/01.CIR.0000105763.51286.7F

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TABLE 1. Characteristics of Healthy Volunteers (n=5)

<table>
<thead>
<tr>
<th>Details</th>
<th>Values</th>
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</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>32.4±1.2</td>
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<tr>
<td>Hypertension</td>
<td>0</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>0</td>
</tr>
<tr>
<td>Active smoker</td>
<td>0</td>
</tr>
<tr>
<td>History of coronary bypass</td>
<td>0</td>
</tr>
<tr>
<td>Left ventricular ejection fraction by echocardiography, %</td>
<td>69.0±1.9</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>165.2±11.7</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>48.0±4.6</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>95.6±9.3</td>
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</tbody>
</table>

None of the volunteers were taking any acute or chronic medication.

Methods

Patients
A total of 5 male healthy volunteers (nonsmokers) and 16 male patients with angiographically documented stable coronary artery disease were studied. Patients with inflammatory disease (C-reactive protein levels >0.3 mg/dL) or malignancy, elevated troponin T levels of >0.1 ng/mL, or ejection fraction <45% were excluded. Vasoreactive medications, including calcium channel blockers, angiotensin-converting enzyme inhibitors, and long-acting nitrates, were withheld at least 24 hours before the study. The study protocol was approved by the Ethics Committee of the Johann W. Goethe University, Frankfurt am Main, Germany, and all patients provided written informed consent.

Study Protocol
Venous occlusion plethysmography of the forearm for measurement of the forearm blood flow (FBF) was performed in the morning in a quiet and temperature-controlled (22°C) laboratory as described previously.8 To assess endothelium-dependent vasodilatation, acetylcholine (Ciba Vision GmbH) was infused intra-arterially in 5 patients at a constant dose of 25 mg/min; sodium nitroprusside (SNP, Schwarz Pharma) was infused intra-arterially in 5 patients at a rate of 8 μmol/min for 10 minutes as described previously.10 To assess endothelium-independent vasodilatation, sodium nitroprusside (SNP, Schwarz Pharma) was infused intra-arterially in increasing doses of 4 and 8 μg/min.

Assessment of Serum Protein Oxidation
In a separate protocol, blood samples were obtained from 7 patients and 5 healthy volunteers immediately before and after 1 and 2 hours of continuous infusion of sulfaphenazole (2 mg/min). Serum samples were defrosted, and carbonyl groups on serum proteins were converted to their dinitrophenylhydrazone derivatives using the OxyBlot protein oxidation detection kit (Intergen Co). The derivatized protein samples were then separated by SDS-PAGE and blotted onto nitrocellulose as described previously.11 Proteins were detected with an anti-dinitrophenylhydrazone antibody and a horseradish peroxidase–coupled secondary antibody and were visualized by enhanced chemiluminescence with a commercially available kit (Amersham).

Statistical Analysis
Data are expressed as mean±SEM. Differences in forearm vascular reactivity were examined by repeated-measures ANOVA, and probability values of P<0.05 were considered statistically significant.

TABLE 2. Patient Characteristics (n=16)

<table>
<thead>
<tr>
<th>Details</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>61.3±5.2</td>
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<tr>
<td>Hypertension, n (%)</td>
<td>12 (75)</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>3 (18.8)</td>
</tr>
<tr>
<td>Active smoker</td>
<td>2 (12.5)</td>
</tr>
<tr>
<td>History of coronary bypass</td>
<td>4 (25)</td>
</tr>
<tr>
<td>Left ventricular ejection fraction by echocardiography, %</td>
<td>57.2±2.5</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>164.8±6.3</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>41.8±1.5</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>96.6±5.5</td>
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<tr>
<td>Concurrent medication, n (%)</td>
<td>16 (100)</td>
</tr>
<tr>
<td>β-Blockers</td>
<td>16 (100)</td>
</tr>
<tr>
<td>Statin</td>
<td>16 (100)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>16 (100)</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>16 (100)</td>
</tr>
<tr>
<td>Ca channel blockers</td>
<td>1 (6.25)</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>4 (25)</td>
</tr>
<tr>
<td>Nitrates</td>
<td>3 (18.8)</td>
</tr>
<tr>
<td>Diuretics</td>
<td>3 (18.8)</td>
</tr>
</tbody>
</table>

ACE indicates angiotensin-converting enzyme.

Results
The clinical characteristics of the study population are summarized in Tables 1 and 2. Arterial blood pressure and heart rate did not differ between the measurements. The intra-arterial infusion of sulfaphenazole was well tolerated, and no side effects were detected.

Effect of Sulfaphenazole on Basal Blood Flow
Basal blood flow did not change in healthy subjects during infusion of sulfaphenazole; blood flow was 2.9±0.2 mL·min⁻¹·100 mL forearm tissue⁻¹ before and 3.0±0.2 mL·min⁻¹·100 mL forearm tissue⁻¹ during infusion of 2.0 mg/min sulfaphenazole (P=NS). In patients with coronary artery disease, a small but nonsignificant increase in baseline FBF was observed after the administration of sulfaphenazole. Blood flow was 2.8±0.3 mL·min⁻¹·100 mL forearm tissue⁻¹ during saline infusion versus 3.2±0.3 and 3.4±0.3 mL·min⁻¹·100 mL forearm tissue⁻¹ in the presence of sulfaphenazole (0.2 and 2.0 mg/min, respectively; P=NS).

Effect of Sulfaphenazole on Acetylcholine- and Sodium Nitroprusside–Induced Changes in FBF
As illustrated in Figure 1, acetylcholine- and SNP-induced FBF responses were identical before and during sulfaphenazole infusion (2 mg/min) in healthy volunteers. In contrast, in patients with coronary artery disease, the acetylcholine-induced increase in FBF was significantly and concentration-dependently enhanced by sulfaphenazole (Figure 2A). Infusion of sulfaphenazole did not affect the endothelium-independent increases in FBF elicited by SNP (Figure 2B).

Effect of Combined Infusion of L-NMMA and Sulfaphenazole on Acetylcholine-Induced Changes in FBF
In patients with coronary artery disease, coinfusion of L-NMMA and saline reduced the baseline FBF from 2.5±0.3
to $1.6\pm0.2\text{ mL} \cdot \text{min}^{-1} \cdot 100\text{ mL forearm tissue}^{-1}$. This was increased to $2.0\pm0.1\text{ mL} \cdot \text{min}^{-1} \cdot 100\text{ mL forearm tissue}^{-1}$ by the addition of sulfaphenazole (2.0 mg/min), but this effect did not reach statistical significance. The maximal acetylcholine-induced vasodilatation was reduced by L-NMMA, and the inclusion of sulfaphenazole slightly increased the vasodilator response (Figure 2C). However, this effect did not achieve statistical significance, and the maximum response was lower than that observed in the presence of saline.

**Comparison of the Effects of Sulfaphenazole and Vitamin C**

Because vitamin C is reported to improve NO bioavailability and thus endothelium-dependent relaxation, we compared the effects of vitamin C, which is thought to restore the function of the uncoupled endothelial NOS, with the effects of sulfaphenazole, which may prevent the intracellular generation of CYP-derived free radicals.

The infusion of vitamin C in patients with coronary artery disease did not alter baseline FBF, which was $2.7\pm0.3$ before versus $2.6\pm0.2\text{ mL} \cdot \text{min}^{-1} \cdot 100\text{ mL forearm tissue}^{-1}$ after addition of vitamin C to the infusion. FBF was also not
Serum Proteins

Effects of Sulfaphenazole on the Oxidation of Serum Proteins

To determine whether the apparent increase in the bioavailability of NO observed in patients with coronary artery disease after treatment with sulfaphenazole was because of a reduction in oxidative stress, we assessed the oxidation of serum proteins in sera from healthy volunteers and from patients with coronary artery disease before and during the infusion of sulfaphenazole for up to 2 hours. The oxidation of a protein of \( \approx 180 \) kDa was apparent only in patients with coronary artery disease but not in healthy individuals. Importantly, during a 2-hour infusion period with sulfaphenazole, the oxidation of this protein was significantly attenuated (Figure 3B). Sulfaphenazole did not significantly attenuate the oxidation of proteins detected in the serum from healthy individuals.

Discussion

The results of the present investigation demonstrate that the CYP 2C9 inhibitor sulfaphenazole improves the vasodilator responsiveness of the forearm circulation to acetylcholine in patients with manifest coronary artery disease but has no effect in healthy individuals. This effect seems to be related to an improvement in the bioavailability of NO, probably as a consequence of an attenuated generation of reactive oxygen species by CYP 2C in endothelial cells.

CYP 2C plays a crucial role in the agonist-induced, EDHF-mediated relaxation of porcine coronary arteries. However, clear data showing that this enzyme plays a role in the regulation of vascular function in human arteries have been lacking. Part of the problem in delineating a role for such enzyme systems lies in the fact that most of the CYP inhibitors commonly available are not isoform-specific and can have unselective effects on the function of the K⁺ channels that are central to the EDHF phenomenon. Sulfaphenazole is, however, a highly selective inhibitor of CYP 2C9,6 that does not affect K⁺ channel function per se.2

Although CYP 2C9 is present in the endothelium of skeletal muscle arterioles from healthy individuals, we observed no effect of the CYP 2C9 inhibitor on acetylcholine- or SNP-induced changes in FBF. This result was, however, not unexpected, because redundancy in autacoid production is such that one vasodilator system can compensate for the lack of another under normal conditions. There have been previous attempts to assess the role of cytochrome P450 products in NO-independent relaxation in healthy subjects. For example, miconazole has been reported to slightly attenuate the bradykinin-induced vasodilatation of the human forearm vasculature in patients treated with NOS and cyclooxygenase inhibitors.15 However, these effects were very small and may in fact be a consequence of the indirect effects of the CYP inhibitor used. Moreover, a recent study failed to detect any effect of sulfaphenazole in healthy subjects treated with L-NMMA and ibuprofen.16 Indeed, to date, it has been possible to demonstrate a significant effect of sulfaphenazole on exercise-induced vasodilatation in skeletal muscle only when individuals are pretreated with a NOS inhibitor.4 Such results suggest that the relative contribution of cytochrome P450-derived products to the regulation of vascular tone is highly dependent on the physiological situation (ie, resting conditions versus exercising) and the vascular bed studied. They further indicate that CYP 2C contributes little to the regulation of FBF in healthy nonexercising subjects in either the presence or absence of a functional NO-generating pathway.

Because high concentrations of NO are known to inhibit CYP enzymes and low concentrations of NO are reported to attenuate EDHF-mediated responses, we had speculated that a decrease in the bioavailability of NO might alleviate the
intrinsic inhibition of the EDHF synthase. Thus, EDHF could act as a backup system to at least partially maintain vasodilator responses in patients with a depressed NO bioavailability.18 Indeed, mammary arteries removed from patients undergoing coronary artery bypass surgery express CYP 2C9, and inhibiting the enzyme abolishes the acetylcholine- and bradykinin-induced EDHF-mediated relaxation of these vessels.3 The results of the present investigation, however, do not suggest that CYP 2C epoxygenase contributes to acetylcholine-induced vasodilatation of the forearm vasculature in patients with coronary artery disease. Indeed, instead of decreasing vasodilator responses to acetylcholine in patients treated with L-NMMA, sulfaphenazole increased blood flow. Because CYP 2C9 activity was apparently not associated with the generation of a vasodilator EET, this finding can probably be attributed to a decrease in CYP 2C–derived superoxide anion formation, which results in the reduced scavenging of residual NO generated by an incompletely inhibited NOS.

We previously observed that the CYP epoxygenase expressed in porcine coronary endothelial cells generates superoxide anions, which attenuate the bradykinin-induced, NO-dependent relaxation, an effect that can be reversed by sulfaphenazole.5 This observation led us to suggest that rather than contributing to the maintenance of a vasodilator response, an increase in CYP 2C9 activity could aggravate endothelial dysfunction. The oxygen-derived free radicals that are associated with endothelial dysfunction can potentially come from a number of sources, including endothelial NOS.19 Because vitamin C has been shown to decrease superoxide levels and increase NO production by preventing the oxidation of the NOS cofactor tetrahydrobiopterin,12,19 we compared the effect of a high concentration of vitamin C with that of sulfaphenazole. Although vitamin C clearly enhanced the vasodilator response to acetylcholine, in line with previously published observations,21 responses were still significantly enhanced by the addition of sulfaphenazole. Therefore, it seems that CYP 2C9 can also be considered a significant source of oxygen-derived free radicals in patients with coronary artery disease.

Because the production of CYP 2C–derived reactive oxygen species in isolated porcine coronary arteries can be suppressed by sulfaphenazole, we looked for an indirect marker of oxidative stress in patients with coronary artery disease.3 We therefore assessed the effects of sulfaphenazole on protein oxidation by monitoring the occurrence of carbonyl groups on serum proteins; this modification occurs in response to several types of oxidative stress. The results obtained, although they did not provide a direct measure of the generation of oxygen-derived free radicals by vascular cells, clearly indicate that the concentration of sulfaphenazole that markedly improved the vasodilator response to acetylcholine also attenuated the oxidation of serum proteins. Thus, as in the porcine coronary artery, sulfaphenazole seems to attenuate oxidative stress within the forearm vasculature of patients.

Our observation that sulfaphenazole increases NO-mediated vasodilatation in patients with coronary artery disease but does not affect agonist-induced, NO- and prostacyclin-independent vasodilatation of the forearm vascular in healthy volunteers16 may reflect a difference in CYP 2C expression. Although relatively little is known about the factors regulating CYP 2C expression in endothelial cells,22 a number of currently used cardiovascular drugs23–25 as well as chronic hypoxia and/or oxidative stress can affect CYP protein levels and enzyme activity.26–27 Thus, it is conceivable that in patients with coronary artery disease, an increase in CYP expression underlies the increase in O2•− production. However, it remains to be determined whether or not an increase in the expression of CYP 2C in the vascular endothelium correlates with the development of vascular disease.

In summary, inhibiting CYP 2C9 significantly improves NO-mediated vasodilatation at least in part by attenuating oxidative stress, suggesting that endothelial CYP2C9 contributes to the impaired systemic endothelial vasodilator function in patients with coronary artery disease.

Acknowledgments
The research described in this article was supported by the Deutsche Forschungsgemeinschaft (FI 830/2-1 to R.B. and SFB 553/C5 to A.M.Z.). The authors are indebted to Margia Müller-Ardogan, Christiane Mildner-Rihm, and Tanja-Maria Mareczek for expert technical assistance.

References


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_Circulation_. 2004;109:178-183; originally published online December 8, 2003; doi: 10.1161/01.CIR.0000105763.51286.7F
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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