Baseline blood flow and bradykinin-induced vasodilator responses in the human forearm are insensitive to the cytochrome P450 2C9 (CYP2C9) inhibitor sulphaphenazole

Jens PASSAUER*, Eckhart BÜSSEMAKER*, Grit LÄSSIG*, Frank PISTROSCH*, Joachim FAULER†, Peter GROSS* and Ingrid FLEMING‡

*Division of Nephrology, Carl Gustav Carus University Hospital, Technical University Dresden, Dresden, Germany, †Institute of Clinical Pharmacology, Technical University Dresden, Dresden, Germany, and ‡Institute for Cardiovascular Physiology, Johann Wolfgang Goethe-University, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany

ABSTRACT

A substantial portion of the vasodilator response elicited by bradykinin in the human forearm is unaffected by the combined inhibition of nitric oxide (NO) synthases and cyclo-oxygenases. The cytochrome P450 (CYP) 2C9 inhibitor sulphaphenazole was recently identified as a potent inhibitor of NO- and prostacyclin (PGI2)-independent relaxation in porcine coronary arteries. The aim of the present study was to determine the effect of sulphaphenazole on basal and bradykinin-induced NO/PGI2-independent changes in the forearm blood flow (FBF) of healthy subjects. Eleven healthy male volunteers participated in this placebo-controlled study. Test agents were infused into the brachial artery and FBF was measured by bilateral venous occlusion plethysmography. Sulphaphenazole (0.02–2 mg/min) alone did not affect basal blood flow. Inhibition of the NO synthases by N\textsubscript{G}-monomethyl-L-arginine (L-NMMA; 4 \mu mol/min) and cyclo-oxygenases by ibuprofen (1200 mg, orally) reduced FBF to 48 ± 7% in the absence and 50 ± 8% in the presence of sulphaphenazole (2 mg/min; \(P = \) not significant). After pretreatment with L-NMMA (16 \mu mol/min) and ibuprofen (1200 mg, orally), sulphaphenazole (6 mg/min) did not substantially inhibit bradykinin-induced vasodilation. We conclude that CYP2C9-derived metabolites (i) are not involved in the regulation of baseline blood flow, and (ii) do not mediate bradykinin-induced NO/PGI2-independent vasorelaxation in the human forearm. However, determining the contribution of this enzyme to regulation of blood flow in pathological conditions associated with endothelial dysfunction requires further studies.

INTRODUCTION

Humoral and haemodynamic stimuli modulate local blood flow by eliciting the production and release of vasoactive autacoids from the endothelium, such as nitric oxide (NO), prostacyclin (PGI2), endothelin-1, prostaglandin H\textsubscript{2} and superoxide anions. Of the vasodilator autacoids generated, most is known about the synthesis and actions of NO and PGI2 which, in addition to modulating local vascular tone, also exert significant effects on cell signalling and gene expression [1]. In several vascular beds, a substantial component of the vasodilator

Key words: bradykinin, cytochrome P450, endothelium-derived hyperpolarizing factor, forearm, sulphaphenazole, vasodilatation.

Abbreviations: BP, blood pressure; COX, cyclo-oxygenase; CYP, cytochrome P450; EDHF, endothelium-derived hyperpolarizing factor; EET, epoxyeicosatrienoic acid; FBF, forearm blood flow; I/C ratio, infused arm/control arm ratio; K\textsuperscript{+}\textsubscript{Ca} channel, Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel; l-NMMA, N\textsubscript{G}-monomethyl-l-arginine; NO, nitric oxide; NOS, NO synthase; PGI2, prostacyclin.

Correspondence: Dr Jens Passauer (e-mail passauer@rcs.urz.tu-dresden.de).
response observed in response to receptor-dependent agonists or increases in flow is insensitive to inhibitors of NO synthases (NOSs) or cyclo-oxygenases (COXs). These responses are thought to reflect the production of a third endothelium-derived vasodilator principle, the endothelium-derived hyperpolarizing factor (EDHF; for reviews see references [2,3]).

On the basis of the pharmacological characterization of NO/PGI2-independent relaxation of numerous vessels from different species, there appear to be several different EDHFs [4,5]. Of this family of hyperpolarizing factors, an EDHF produced by coronary arteries isolated from humans, pigs, dogs, cows and rats is critically dependent on the generation of epoxyeicosatrienoic acids (EETs) from arachidonic acid by a cytochrome P450 (CYP) epoxygenase expressed in endothelial cells [2]. Originally, EETs generated within endothelial cells were assumed to diffuse to the vascular smooth muscle and to directly activate $\text{Ca}^{++}$-dependent K$^+$ (K$^+$-Ca) channels on the muscle cells, thus inducing hyperpolarization and relaxation (for review, see [2]). However, EETs are lipophilic and current thinking tends towards the concept that EETs are responsible for initiating endothelial cell hyperpolarization, which is the initial step in the EDHF phenomenon [6]. Indeed, EDHF-mediated relaxation is brought about by the activation of K$^+$-Ca channels, and the down-regulation of CYP2C protein has been shown to abolish the bradykinin-induced endothelium-dependent hyperpolarization of coronary smooth muscle cells as well as the NO/PGI2-independent relaxation of these porcine coronary arteries [7].

Elucidating the role of a CYP-derived metabolite in NO/PGI2-independent diatilaion in vivo has been hampered by the fact that classical CYP inhibitors are not isoform specific [8] and directly affect K$^+$-Ca channels, which are thought to be the major target of EDHF [9], thus rendering the interpretation of experimental data difficult. The sulphonamide sulphaphenazole is an extremely selective inhibitor of CYP2C9 [10,11] and has been reported to markedly attenuate EDHF-mediated responses in the porcine coronary artery without directly affecting K$^+$-Ca channel activity [7]. Moreover, recent experiments using the porcine coronary artery have demonstrated that EDHF-mediated hyperpolarization and relaxation can be attenuated by sulphaphenazole [7,12].

The aim of the present study was therefore to determine whether or not a CYP2C9-dependent mechanism contributes to the NO/PGI2--independent regulation of forearm blood flow (FBF) in humans. To this end, we studied the effects of sulphaphenazole on baseline FBF, on baseline FBF in the presence of the NOS inhibitor N$^\omega$-monomethyl-L-arginine (L-NMMA) or the COX inhibitor ibuprofen, and on the bradykinin-induced increase in FBF in the presence of L-NMMA and ibuprofen.

### METHODS

#### Subjects

The study protocol was approved by the University of Dresden Ethics Committee. Eleven young healthy male volunteers were recruited and written informed consent was obtained before any investigation was started. Hypertension or underlying vascular disease was ruled out by physical examination of all subjects and those with known drug allergies were excluded. Cigarettes, alcohol and all caffeine-containing beverages were withheld for 12 h 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 FBF

FBF was measured simultaneously in both arms by venous occlusion plethysmography as described previously [13]. Pressure of the congesting cuffs of both upper arms was set at 40 mmHg. Mercury-in-silastic strain gauges were wrapped around the widest parts of the forearms and connected to a calibrated venous occlusion plethysmograph (Gutmann Medizinelektronik, Earsburg, Germany). The brachial artery of the non-dominant arm was cannulated with a 27-gauge steel needle (Coopers Needle Work, Birmingham, U.K.) for drug infusion. After cannulation of the brachial artery, saline was infused for 20 min to establish baseline conditions in each protocol. Individual measurements of FBF lasting 10 s were made every 15 s for 2.5 min during each dose of agent administered. The blood flow of the hands was excluded by a wrist cuff inflated to a suprasystolic pressure (220 mmHg) during each measurement period. Blood pressure (BP) was measured at baseline and at the end of each infusion period. All agents used were dissolved in physiological saline. During each protocol, the infusion rate was kept constant at 1 ml/min. At the end of each FBF measurement, BP was measured in the non-infused upper arm.

#### Experimental protocols

The study consisted of three different experimental protocols. At 1 h prior to each protocol, participants received 1200 mg of ibuprofen orally. This dose has been used previously for blockade of endothelial PGI2 generation [14].

**Protocol 1: influence of sulphaphenazole on baseline FBF**

$(n = 8 \text{ participants})$

Sulphaphenazole was infused at doses of 0.02, 0.2 and 2 mg/min and each dose was given over 10 min, after which FBF measurements were obtained. At the end of this protocol, a venous blood sample (10 ml) from an antecubital vein of the infused arm was drawn into
Protocol 2: influence of sulphaphenazole on baseline FBF in the presence of L-NMMA
Each participant \( (n=8) \) underwent two experimental sessions in a randomized order. L-NMMA (4 \( \mu \)mol/min) was infused over 10 min and, subsequently, sulphaphenazole (0.02, 0.2 and 2 \( \mu \)g/min; each dose for 10 min) or saline was co-infused. Short-term (6 min) infusions of L-NMMA at 4 \( \mu \)mol/min are known to elicit approx. 80 % of maximum L-NMMA-mediated vasoconstriction [15]. The total infusion period of L-NMMA in this part of the study was markedly longer (40 min). We did not use the recommended short-term dose of L-NMMA for complete inhibition of NOS (16 \( \mu \)mol/min; [15]) to reliably avoid systemic effects, which would be especially undesirable during this protocol.

Protocol 3: influence of sulphaphenazole on bradykinin-induced NO/PGI2-independent vasodilation
Each participant \( (n=10) \) underwent two experimental sessions in a randomized order. L-NMMA (16 \( \mu \)mol/min) was infused together with either sulphaphenazole (6 \( \mu \)g/min) or saline over 20 min and FBF was determined. Subsequently, the vasodilator response to increasing doses of bradykinin (20, 40 and 80 pmol/min, each given over 5 min) was measured. The doses of sulphaphenazole and L-NMMA were increased to account for the rise in FBF elicited by bradykinin. Furthermore, this dose of L-NMMA was shown to be maximally effective in reducing agonist-induced endothelium-dependent vasodilation [15].

Plasma concentrations of sulphaphenazole
Plasma concentrations of sulphaphenazole were assessed with a Jasco HPLC system (Großzimmern, Germany) using a ProntoSil 120-5-C18 H reverse-phase column (Bischoff, Germany). Plasma samples were deproteinized with trichloroacetic acid. After centrifugation the supernatant was directly injected into the HPLC system. The soluble phase consisted of acetonitrile/water (2:3, v/v) and 0.1 % trichloroacetic acid. The flow was 1 ml/min and sulphaphenazole was detected at a wavelength of 267 nm. There was a linear correlation \( (r^2 = 0.995) \) between 5 and 250 \( \mu \)mol/l sulphaphenazole. The day-to-day variation coefficient was 5.7 %. Blood was drawn from subjects at the end of protocol 1.

Drugs
Ibuprofen (Jenaprofen®) was obtained from Jenapharm (Jena, Germany); bradykinin, L-NMMA and the sodium acetate salt of sulphaphenazole were from Clinalfa (Lüefelfingen, Switzerland). All substances were dissolved in saline and infused at a constant rate of 1 ml/min.

Statistical analyses
FBF is expressed as the percentage change in the I/C (infused arm/control arm) ratio, which represents the ml of blood flow/100 ml of forearm volume per min. One FBF determination consisted of ten single FBF measurements. The final five blood flow recordings for each infusion step from both the infused and control arm were used to calculate mean FBF. Results are expressed as the mean percentage change of the I/C ratio from baseline \( \pm \) S.E.M. Statistical analysis was performed by use of two-way ANOVA for repeated measurements. Values of \( P < 0.05 \) were considered statistically significant.

RESULTS
All substances were tolerated well by all of the participants and there were no side effects of sulphaphenazole on either BP or heart rate (results not shown). Baseline characteristics of the subjects participating in each protocol are given in Table 1. The concentration of sulphaphenazole detected in samples of venous blood removed from subjects receiving 2 \( \mu \)g/min ranged from 50–180 \( \mu \)mol/l. We gave this apparently high dose to account for the considerable plasma protein binding of the drug, which is reported to be between 98 and 100 % [16]. To maintain the desired plasma concentrations we tripled the dose of sulphaphenazole during infusions of bradykinin. Comparable doses of sulphaphenazole have been shown to attenuate exercise-induced vasodilation in healthy volunteers [17].

Effect of sulphaphenazole on baseline FBF
Infusion of increasing concentrations of sulphaphenazole had no significant effect on resting FBF, the percentage changes in the I/C ratio were 2 \( \pm \) 10, 2 \( \pm \) 10 and 7 \( \pm \) 13 in response to 0.02, 0.2 and 2 \( \mu \)g/min respectively.
Effect of increasing doses of sulphaphenazole (0.02, 0.2 and 2 mg/min) on baseline FBF
No significant difference was observed as determined by ANOVA for repeated measurements. ($P = 0.39$; Figure 1). The concentration of sulphaphenazole in the venous outflow of the infused arm at the highest dose (2 mg/min) was $100 \pm 12 \mu$mol/l ($n = 8$).

Effect of sulphaphenazole on baseline FBF in the presence of L-NMMA
Infusion of L-NMMA (4 $\mu$mol/min) over 10 min decreased FBF by $34 \pm 3\%$. Co-infusion of either sulphaphenazole or saline resulted in a comparable decrease in FBF, the percentage changes in the I/C ratio were $43 \pm 5$, $48 \pm 5$ and $50 \pm 8\%$ in response to 0.02, 0.2 and 2 mg/min sulphaphenazole and $41 \pm 9$, $46 \pm 6$ and $48 \pm 7\%$ in response to saline respectively ($P = 0.66$; Figure 2). This indicates that the progressive decline of FBF in this protocol reflects a time-dependent effect of L-NMMA rather than a specific effect of sulphaphenazole. By comparing the effects of L-NMMA at 4 $\mu$mol/min with that of 16 $\mu$mol/min (protocol 3) in the same subjects ($n = 8$), we demonstrate that the lower dose exerted near-maximum inhibition of NOS after 20 min of infusion (16 $\mu$mol/min L-NMMA for 20 min, $-45 \pm 5\%$; 4 $\mu$mol/min L-NMMA for 20 min, $-46 \pm 6\%$; values are not significantly different as determined by paired Student’s $t$ test).

Effect of sulphaphenazole on bradykinin-induced NO/PGI$_2$-independent vasodilation
After pretreatment with L-NMMA (16 $\mu$mol/min) for 20 min, bradykinin elicited a concentration-dependent increase in FBF (Figure 3). When L-NMMA was infused together with sulphaphenazole for 20 min, co-infusion of bradykinin similarly increased FBF in a dose-dependent manner (Figure 3). Comparison of the changes of I/C ratio by ANOVA for repeated measurements revealed no significant difference between the results obtained in the absence and presence of sulphaphenazole: bradykinin + saline, $102 \pm 24$, $155 \pm 28$ and $261 \pm 45\%$; and bradykinin + sulphaphenazole, $83 \pm 15$, $150 \pm 40$ and $232 \pm 52\%$ ($P = 0.91$; Figure 3). No systemic effects of this concentration of the NOS inhibitor were apparent, as neither BP nor blood flow in the control arm differed from baseline values.

DISCUSSION
The results of the present study indicate that bradykinin-induced NO/PGI$_2$-independent vasodilation of the human forearm vasculature is not mediated by a mechanism sensitive to the CYP2C9 inhibitor sulphaphenazole. Thus an NO/PGI$_2$-independent vasodilator mechanism, comparable with the EDHF-mediated relaxation of porcine coronary arteries, is not involved in the regulation of FBF in healthy human subjects.
Figure 3  Effect of sulphaphenazole on bradykinin-induced NO-independent alterations in FBF

Bradykinin-induced (20, 40 and 80 pmol/min) changes in FBF were measured in ten subjects pretreated for 20 min with L-NMMA (16 µmol/min) plus saline or L-NMMA plus sulphaphenazole (SPZ; 6 mg/min). Infusions of L-NMMA and sulphaphenazole were continued throughout the entire experiment. No significant difference was observed as determined by ANOVA for repeated measurements.

To date, FBF measurements have helped to establish the crucial role played by NO in the regulation of vascular responses as well as to demonstrate the link between an apparent decrease in the bioavailability of endothelium-derived NO and cardiovascular diseases. Moreover, as a substantial vasodilator response can still be observed in the presence of NOS and COX inhibitors, this technique has also facilitated the identification of an NO/PGL2-independent component of the vasodilator response elicited by receptor-dependent agonists such as acetylcholine and bradykinin [18]. By analogy with experiments performed using isolated arteries, the residual vasodilator response has been attributed to an EDHF. One characteristic of EDHF-mediated responses is their sensitivity to the combination of charybotoxin and apamin, a toxin combination that inhibits K⁺Ca channel activity [6]. A similar sensitivity to the K⁺Ca channel toxins can be demonstrated in human vessels ex vivo [19], and the findings that the non-selective K⁺ channel blocker tetraethylammonium chloride [18] and a high intravascular concentration of K⁺ [20] attenuate the NO/PGL2-independent vasodilator response in the human forearm provide at least circumstantial evidence that a K⁺Ca channel-activating factor mediates NO/PGL2-independent vasodilation in the human forearm.

The results of the first experimental series (protocols 1 and 2) demonstrate that increasing doses of sulphaphenazole in the absence or presence of an NOS inhibitor do not affect resting FBF. Thus a CYP2C9-derived EDHF does not appear to contribute to baseline vascular tone in the forearm of healthy subjects. Such an observation is in accordance with the generally accepted view that the activation of CYP enzymes in endothelial cells is a Ca²⁺-dependent process, requiring the activation of phospholipase A2 to liberate the CYP2C substrate, arachidonic acid, from membrane phospholipids.

Our present finding that sulphaphenazole has no modulating effect on the vasodilator response to bradykinin in the healthy subjects is contrary to the recent observation [20] that miconazole, a non-isoform-selective CYP epoxygenase inhibitor, attenuated agonist-induced NO/PGL2-independent vasodilation in humans. One explanation for this discrepancy could be that isoforms other than CYP2C9 are involved in NO/PGL2-independent vasodilation in humans. Another explanation is pertinent to the fact that non-isoform-specific CYP inhibitors such as miconazole directly affect K⁺Ca channels which are the major targets of EDHF [9]. It is therefore conceivable that the observed effects of miconazole are derived from blockade of K⁺Ca channels instead of from blockade of CYP-dependent enzymes. Our results support the view that a CYP2C9-dependent EDHF does not play a major role in the regulation of the forearm vasculature in healthy subjects. Rather, in this vascular bed, the release of K⁺ from endothelial cells and a phenomenon involving gap junctional communication may be involved in mediating the endothelium-dependent hyperpolarization [6].

Although there was no effect of sulphaphenazole in healthy volunteers, it is possible that the sensitivity to this sulphonamide may be increased in subjects demonstrating a manifest endothelial dysfunction. Since NO interacts with haemoproteins, such as CYP, to inhibit enzyme activity, it follows that a decrease in the bioavailability of NO (e.g. in endothelial dysfunction) could be associated with an increase in CYP activity as well as EDHF-mediated responses [21]. Such a phenomenon has been described for bradykinin-induced changes in FBF in essential hypertensive patients [22,23] and in arterioles removed from patients with coronary artery disease, where vasodilation is mediated entirely by a mechanism sensitive to both CYP and K⁺Ca channel inhibitors [24]. In such a situation, the activation of
CYP2C9 may exert a dual effect on vascular reactivity, as the CYP2C epoxygenase critically involved in the generation of EDHF in large porcine coronary arteries generates reactive oxygen species in addition to metabolites of arachidonic acid, thus exacerbating oxidative stress within the vascular wall [25].

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