Evidence for a basal release of a cytochrome-related endothelium-derived hyperpolarizing factor in the radial artery in humans

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Bellien, Jeremy, Robinson Joannides, Michele Iacob, Philippe Arnaud, and Christian Thuilleux. Evidence for a basal release of a cytochrome-related endothelium-derived hyperpolarizing factor in the radial artery in humans. Am J Physiol Heart Circ Physiol 290: H1347–H1352, 2006. First published December 9, 2005; doi:10.1152/ajpheart.01079.2005.—Whether a cytochrome P-450 (CYP)-related endothelium-derived hyperpolarizing factor (EDHF), acting through calcium-activated potassium (KCa) channels, interacts with nitric oxide (NO) to regulate the basal diameter of human peripheral conduit arteries is unexplored in vivo. Radial artery diameter (echo tracking) and blood flow (Doppler) were measured, after oral aspirin (500 mg), in eight healthy volunteers during local infusion for 8 min of tetraethylammonium chloride (TEA; 9 μmol/min), as KCa channel inhibitor, and fluconazole (0.4 μmol/min), as CYP inhibitor, alone and in combination with Nω-monomethyl-L-arginine (L-NMMA; 8 μmol/min), as endothelial NO synthase inhibitor. Endothelium-independent dilatation was assessed by using sodium nitroprusside (SNP). Radial diameter was unaffected by L-NMMA (0.4 ± 0.9%) and fluconazole (−1.6 ± 0.8%) but was decreased by TEA (−5.0 ± 1.0%), L-NMMA + fluconazole (−5.3 ± 0.5%), and L-NMMA + TEA (−9.9 ± 1.3%). These effects are still significant even when the concomitant decreases in blood flow induced by L-NMMA (−24 ± 4%), TEA (−21 ± 3%), L-NMMA + fluconazole (−26 ± 5%), and L-NMMA + TEA (−35 ± 4%) were taken as covariate into analysis. Conversely, fluconazole alone slightly but not significantly increased radial flow (13 ± 6%), L-NMMA alone or with TEA and with fluconazole enhanced radial artery dilatation to SNP, whereas TEA and fluconazole alone did not modify this response. These results confirm in humans the involvement of NO and KCa channels in the regulation of basal conduit artery diameter. Moreover, the synergistic effect of combined inhibition of NO synthesis and CYP on the decrease in radial diameter in the absence of such effect after L-NMMA and fluconazole alone unmasks the role of CYP in this regulation and shows the presence of an interaction between NO and a CYP-related EDHF to maintain peripheral conduit artery diameter in vivo. Furthermore, the higher vasoconstrictor effect of TEA compared with fluconazole suggests that different KCa channel-dependent hyperpolarizing mechanisms could exist in conduit arteries.

endothelium-derived factors; calcium-activated potassium channels; cytochrome P-450

THE ENDOTHELIUM plays a crucial role in controlling vascular tone and homeostasis through the release of vasoactive factors (6, 10, 33). Endothelium-derived vasodilating factors include nitric oxide (NO), prostacyclin (PGL2), and a not yet fully identified endothelium-derived hyperpolarizing factor (EDHF). EDHF is described as the non-NO and non-PGL2 factor that induces the hyperpolarization and the relaxation of the vascular smooth muscle cells through the activation of vascular calcium-activated potassium (KCa) channels (6, 10, 33). In humans, it was demonstrated, by using the forearm model and the local inhibition of vascular KCa channels, that an EDHF contributes to the regulation of the basal tone and the endothelium-mediated dilatation of resistance arteries (21, 25). In addition, we have recently demonstrated in vivo that these channels are involved in balance with NO in the regulation of radial artery diameter and mechanics, strongly supporting the hypothesis for a basal release of an EDHF in human peripheral conduit arteries (5). However, the EDHF involved at this level has not been characterized in vivo.

The chemical identity of EDHF varies depending on the species and vascular territories explored (6, 10, 33). Nonetheless, endothelial cytochrome P-450 (CYP) epoxygenases produce metabolites of arachidonic acid, the epoxyeicosatrienoic acids (EETs), that have been identified as EDHF in various vascular beds (3, 13, 14, 24). In human conduit arteries, ex vivo studies have demonstrated a role for CYP-related EDHF in the regulation of the endothelium-mediated vasodilatation (3, 17). At the arteriolar level, CYP inhibitors failed to modify the forearm vascular resistance at baseline and after endothelial agonist infusion in most of the studies (12, 16, 38, 39). Nevertheless, it was shown that a CYP-dependent vasodilator mechanism and NO interact to regulate the exercise-induced increase in skeletal muscle blood flow, suggesting a CYP-related EDHF release in these conditions (19). Whether a CYP-related EDHF interferes in vivo with basal NO synthesis to regulate the resting diameter of conduit arteries in humans is unknown.

The present study was thus designed to explore in vivo, at the level of the radial artery, the physiological role of a CYP-related EDHF and its relation with NO in the regulation of basal peripheral conduit artery diameter in humans.

METHODS

Subjects. Eight male volunteers (means ± SE, 23 ± 1 yr) were explored on 3 separate days with a 3- to 4-wk washout period between
both. All subjects were normotensive and nonhypertensive and were nonsmokers. They were deemed healthy on the basis of a medical history and complete medical examination with a normal electrocardiogram and routine laboratory tests. The forearm volume of each subject was measured by using the water displacement method to adjust the doses of the drugs to be infused. The protocol was approved by the relevant Consultative Committee for the Protection of Persons Engaged in Biomedical Research (CCPPRB de Haute-Normandie), and all participants gave written informed consent.

Instrumentation. Systemic arterial pressure and heart rate were measured by means of a brachial cuff oscillometric device (Dinamap, 8103, Critikon). Radial internal diameter, blood flow velocity, and digital arterial pressure were continuously measured using a high-definition echo-tracking device (NIUS 02, Asulab) coupled to a Doppler system (Doptek, Deltex) and a finger photoplethysmograph (Finapres system, Ohmeda) as previously described (5, 27, 28). This device allows the measurement of radial artery internal diameter with a resolution on the diameter evolution of \( r < 1 \mu \text{m} \). Radial artery flow was calculated from the measurement of blood velocity and diameter. Total blood viscosity was measured in each subject from arterial blood samples by using a cone-plate viscometer (Ex100 CTB, Brookfield, Stoughton, MA) (5, 27). From the individual values of radial artery internal diameter \( d \), blood flow \( Q \), and total blood viscosity \( \mu \), the mean arterial wall shear stress, the stimulus of the flow-mediated dilatation, was calculated before and after each inhibitor by assuming a Poiseuillean model as \( \tau = 4\mu Q/\pi d^4 \), where radius \( r = d/2 \) (4, 23).

Study protocol. During the days of exploration, the subjects received the NO synthase inhibitor \( N^\omega \)-monomethyl-L-arginine (l-NMMA; 8 \( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} \), Clinalfa), the KC\(_{a} \) channel inhibitor tetraethylammonium chloride (TEA; 9 \( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} \), Clinalfa), the combination of l-NMMA + TEA, the CYP inhibitor fluclonazole (0.4 \( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} \), Pfizer Holding), and the combination of l-NMMA + fluconazole, in a randomized partial block design taking into account for the long-lasting effect of TEA (Table 1) (23).

Measurements were performed while subjects were supine in a quiet, air-conditioned room, maintained at a constant temperature (22–24°C). A 27-gauge needle was inserted under local anesthesia (1% lidocaine) into the brachial artery of the nondominant arm, and 22–24°C. A 27-gauge needle was inserted under local anesthesia (1% lidocaine) into the brachial artery of the nondominant arm, and saline was infused at constant rate (1 ml/min). After instrumentation, administration of the inhibitors

| Table 1. Randomized partial block design used for the administration of the inhibitors |
|------------------|------------------|------------------|
| Subjects | Visit 1 | Visit 2 | Visit 3 |
| S1 | l-NMMA; TEA | l-NMMA + TEA | l-NMMA + fluconazole; fluconazole |
| S5 | l-NMMA; TEA | l-NMMA + TEA | Fluconazole; l-NMMA + fluconazole |
| S2 | l-NMMA; l-NMMA + TEA | TEA | l-NMMA + fluconazole; fluconazole |
| S6 | l-NMMA; l-NMMA + TEA | TEA | Fluconazole; l-NMMA + fluconazole |
| S3 | l-NMMA + TEA; TEA | l-NMMA + fluconazole; fluconazole |
| S7 | l-NMMA; l-NMMA + TEA | l-NMMA; TEA | Fluconazole; l-NMMA + fluconazole |
| S4 | TEA | l-NMMA; l-NMMA + TEA | l-NMMA + fluconazole; fluconazole |
| S8 | TEA | l-NMMA; l-NMMA + TEA | Fluconazole; l-NMMA + fluconazole |

l-NMMA, \( N^\omega \)-monomethyl-l-arginine; TEA, tetraethylammonium chloride.

Fig. 1. Radial artery blood flow variation expressed in percent change from baseline after infusion of \( N^\omega \)-monomethyl-l-arginine (l-NMMA), tetraethylammonium chloride (TEA), the combination of l-NMMA + TEA, fluconazole, and the combination of l-NMMA + fluconazole. * \( P < 0.05 \) vs. baseline; † \( P < 0.05 \) vs. TEA and vs. l-NMMA; ‡ \( P < 0.05 \) vs. other inhibitors.

Fig. 1. Radial artery blood flow variation expressed in percent change from baseline after infusion of \( N^\omega \)-monomethyl-l-arginine (l-NMMA), tetraethylammonium chloride (TEA), the combination of l-NMMA + TEA, fluconazole, and the combination of l-NMMA + fluconazole. * \( P < 0.05 \) vs. baseline; † \( P < 0.05 \) vs. TEA and vs. l-NMMA; ‡ \( P < 0.05 \) vs. other inhibitors.

oral aspirin (UPSA 500 mg, Laboratoire BMS) was given to inhibit prostacyclin production. After 30 min of resting, arterial pressure, heart rate, radial artery blood flow, and diameter were recorded at baseline for 5 min. Subjects then received sodium nitroprusside (SNP; 5, 10, and 20 \( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} \); 3 min each dose), a nitrovasodilator, to assess endothelium-independent dilatation (29). After a 30-min washout period and return to basal radial artery diameter and blood flow, an inhibitor was infused during 8 min with the continuous measurement of all parameters. At the end of inhibitor infusion, the same protocol of SNP administration was repeated. After a 1-h washout period and return to basal radial blood flow and diameter, another inhibitor was infused during 8 min followed by SNP administration.

Statistical analysis. Results are expressed as means \( \pm \) SE. The effect of the inhibitors on vascular parameters was tested by ANOVA with subject as factor. In addition, the effect of the inhibitors on radial artery diameter was tested with the concomitant variation of the radial artery flow or mean wall shear stress as covariate. The differences in the effect of the inhibitors on basal radial artery blood flow and diameter were tested by paired t-test adjusted for multiple comparisons. The effect of the inhibitors on radial artery diameter during SNP infusion was tested by repeated-measures ANOVA with subject as factor. A value of \( P < 0.05 \) was considered statistically significant.

RESULTS

None of the inhibitors affected arterial blood pressure or heart rate.

Fig. 1. Radial artery blood flow variation expressed in percent change from baseline after infusion of \( N^\omega \)-monomethyl-l-arginine (l-NMMA), tetraethylammonium chloride (TEA), the combination of l-NMMA + TEA, fluconazole, and the combination of l-NMMA + fluconazole. * \( P < 0.05 \) vs. baseline; † \( P < 0.05 \) vs. TEA and vs. l-NMMA; ‡ \( P < 0.05 \) vs. other inhibitors.

Basal radial artery blood flow was similar before l-NMMA (9.9 \( \pm \) 1.8 ml/min), TEA (9.6 \( \pm \) 1.0 ml/min), the combination of l-NMMA + TEA (9.3 \( \pm \) 1.3 ml/min), fluconazole (9.1 \( \pm \) 1.3 ml/min), and the combination of l-NMMA + fluconazole (9.3 \( \pm \) 1.2 ml/min). Radial artery blood flow (Fig. 1) was reduced by l-NMMA (−24 \( \pm \) 4%, \( P < 0.01 \)), TEA (−21 \( \pm \) 3%, \( P < 0.01 \)), the combination of l-NMMA + TEA (−35 \( \pm \) 4%, \( P < 0.001 \)), and the combination of l-NMMA + fluconazole (−26 \( \pm \) 5%, \( P < 0.001 \)). Conversely, radial blood flow was slightly but not significantly enhanced by fluconazole (13 \( \pm \) 6%, not significant [NS]). The decrease in radial artery blood flow was similar with TEA, l-NMMA, and
L-NMMA/TEA (2.66 ± 0.08 mm), the combination of L-NMMA + fluconazole (2.63 ± 0.11 mm), and the combination of L-NMMA + fluconazole (2.62 ± 0.06 mm). Radial artery diameter (Fig. 2) was not significantly modified by L-NMMA (0.4 ± 0.9%, NS) and fluconazole (1.6 ± 0.8%, NS) but was reduced by TEA (−5.0 ± 1.0%, P < 0.001), the combination of L-NMMA + TEA (−9.9 ± 1.3%, P < 0.001), and the combination of L-NMMA + fluconazole (−5.3 ± 0.5%, P < 0.001). The decrease in radial artery diameter was more pronounced with the combination of L-NMMA + TEA compared with TEA and the combination of L-NMMA + fluconazole (both P < 0.001). Radial artery mean wall shear stress was not significantly modified by TEA (from 4.1 ± 0.4 to 3.8 ± 0.5 dyn/cm², NS), the combination of L-NMMA + TEA (from 3.9 ± 0.5 to 3.6 ± 0.6 dyn/cm², NS), and the combination of L-NMMA + fluconazole (from 4.1 ± 0.5 to 3.7 ± 0.6 dyn/cm², NS) but was reduced by L-NMMA (from 3.9 ± 0.5 to 3.0 ± 0.5 dyn/cm², P < 0.05) and increased by fluconazole (from 4.0 ± 0.5 to 4.7 ± 0.7 dyn/cm², P < 0.05).

The decrease in radial artery diameter after TEA, the combination of L-NMMA + TEA, and the combination of L-NMMA + fluconazole remains significant even after changes in radial artery blood flow or mean wall shear stress were included as covariates into analysis (all P < 0.05).

SNP induced an increase in radial artery diameter in all cases (Fig. 3). L-NMMA alone and in combination with TEA and with fluconazole enhanced radial artery dilatation to SNP (all P < 0.05), whereas TEA and fluconazole alone did not modify significantly this response.

DISCUSSION

The present study, performed in humans, demonstrates that an endogenous CYP-related EDHF is released in peripheral conduit arteries and that there is an interaction between this EDHF and NO to regulate the basal diameter of these arteries. These results are observed in the absence of evidence for such release at the arteriolar level at baseline.
To explore the physiological role of a CYP-related EDHF and its relation with NO in the regulation of the basal diameter of the radial artery, a model of peripheral conduit artery (5, 22, 27–29, 36), all experiments were performed after administration of oral aspirin to ensure that prostacyclin plays no role in the responses observed (5, 18, 25). We used the nonspecific inhibitor of vascular KCa channels, TEA, as a pharmacological tool for the exploration of EDHF activity because, although different endothelium-dependent hyperpolarizing mechanisms exist, it was demonstrated that KCa channel activation is an absolute prerequisite in EDHF-mediated responses (5, 6, 10, 25). Indeed, EDHF can diffuse from the endothelium and activate the muscular large KCa channels or act, as an autocrine factor, on the endothelial small and intermediate KCa channels, inducing the release of K+ and/or transmission of electrical signal from the endothelium, to promote smooth muscle hyperpolarization (6, 10, 33). TEA was thus infused at the dose of 9 μmol/min to reach a calculated local concentration of 1 mM according to a mean basal radial artery flow of 10 ml/min measured in our population during the control period. This concentration has been shown to specifically inhibit single KCa channels in arterial smooth muscle cells (31). Furthermore, to determine whether an endothelial CYP-derived product is involved in the regulation of radial artery diameter, we administered fluconazole at the dose of 0.4 μmol/min to reach in the same manner a calculated local concentration of 40 μM, five times higher than the in vitro Ki of CYP 2C9 (30, 43). Indeed, CYP 2C9 isoform was found in humans arteries on the endothelium and identified as an EDHF synthase (3, 13, 14, 19, 24). Moreover, ex vivo experiments demonstrate, in human internal mammary arteries, that this CYP isoform produces 11,12-EET, which induces the endothelium-dependent hyperpolarization and relaxation of the vascular smooth muscle cells through the activation of muscular KCa channels (3). Furthermore, to evaluate the possible interaction between EDHF and NO, TEA and fluconazole were administrated alone and in combination with l-NMMA.

As expected, the administration of l-NMMA reduced radial artery blood flow, confirming the role of endothelium-derived NO in the regulation of the basal vascular resistance in humans (5, 21, 22, 25, 28). In addition, the administration of TEA reduced radial artery blood flow. This result is in accordance with a recent plethysmographic study (25) but contrasts with previous experiments (21, 40). As already stressed (25), these discrepancies could be related to the NO-clamp method used that could have attenuated the vasoconstrictor effect of TEA (21) and to differences in terms of population explored (40). Moreover, the dose of TEA of 6 μmol/min used in these negative studies (21, 40) could be insufficient to induce a significant decrease in flow compared with those of the present study and with the cumulative doses of TEA used by Inokuchi et al. (25). We were thus able to attest, with our methodology, to the involvement of vascular KCa channels in the regulation of basal vascular resistance, supporting the hypothesis for a role of a basal release of EDHF at the level of the peripheral resistance arteries in healthy subjects (25). Conversely, fluconazole induced a slight increase in radial artery blood flow, in accord with some experiments obtained by using the CYP inhibitor sulfaphenazole and measuring forearm blood flow by plethysmography (12, 39). The mechanism suggested to explain this effect was the suppression of the basal release of reactive oxygen species because it was demonstrated that CYP epoxygenases can also produce these factors promoting the inactivation of NO (15). In addition, in patients with coronary artery disease, sulfaphenazole enhances the NO-dependent dilatation of forearm resistance arteries in response to acetylcholine by decreasing the oxidative stress (12). Whether this mechanism exists in healthy subjects is uncertain and has not been questioned in the present study. Furthermore, this absence of decrease in radial blood flow after fluconazole does not exclude a role for an EDHF at the arteriolar level because such vasoconstriction is present after TEA but strongly suggests that the EDHF involved is not a CYP-related product.

Concerning the conduit arteries, the administration of l-NMMA did not modify the radial artery diameter, a finding in accord with previous in vivo noninvasive studies performed in the radial (5, 22, 28, 29, 36), brachial (11), and pulmonary arteries (9). In this context, l-NMMA enhanced the radial artery dilatation to SNP compared with baseline conditions. This hypersensitivity of the smooth muscle cells to exogenous NO after endogenous NO suppression, previously observed both in vitro (35) and in vivo (29), reflects the existence of a tonic release of NO in the radial artery wall and its suppression in our experimental conditions. Thus this hypersensitivity to SNP and the absence of decrease in resting diameter despite the concomitant decrease in flow after l-NMMA suggest the emergence of compensatory mechanisms after inhibition of NO synthesis to maintain radial diameter (29). Furthermore, TEA and, in a more marked manner, the combination of l-NMMA and TEA induced a significant decrease in radial artery diameter with no modification in mean wall shear stress and radial dilatation to SNP. Thus, in accord with our previous observations, these results demonstrate that vascular KCa channels are involved in the regulation of basal peripheral conduit artery diameter in humans and compensate for the loss of NO synthesis to maintain this diameter, strongly suggesting the presence of a basal release of EDHF at the level of peripheral conduit arteries (3, 4).

In this context, fluconazole alone did not significantly modify radial artery diameter. This result could argue against the presence of a basal release of a CYP-related EDHF involved in the control of the resting conduit artery diameter through the activation of KCa channels as previously observed in bovine and dog large coronary arteries (8, 34). However, this lack of effect of fluconazole compared with TEA alone could be related to the presence of an intracellular storage form of CYP-related EDHF. Indeed, EETs can be incorporated into phospholipid pools and liberated on cell activation independently of CYP activity (44). Furthermore, the associated increase in mean wall shear stress, due to the increase in the flow-to-diameter ratio, could have counterbalanced and masked a slight vasoconstrictor effect of fluconazole by promoting a flow-mediated dilatation of the radial artery. In contrast, when administrated in combination with l-NMMA, fluconazole induced a significant decrease in radial artery diameter, demonstrating a synergic effect of both inhibitors. This effect cannot be related to a flow-dependent mechanism because radial artery mean wall shear stress was not significantly affected by the combination of fluconazole and l-NMMA. In addition, the radial artery dilatation in response to gradual doses of SNP was not modified by the previous
infusion of fluconazole, arguing against a decrease in the smooth muscle cell sensitivity to vasodilators after CYP inhibition. Furthermore, a direct interaction between fluconazole and KCa channels, previously described with some CYP inhibitors (2), appears unlikely in the present study because fluconazole did not decrease radial blood flow or potentiate the vasoconstrictor effect of L-NMMA at the arteriolar level. Thus these results demonstrate for the first time in humans that a CYP-dependent vasodilator pathway is involved in the control of the basal diameter of peripheral conduit arteries. Moreover, the synergic effect of L-NMMA with both TEA and fluconazole, observed in the same subjects, gives evidence of the presence of an interaction between NO and a CYP-related EDHF to maintain the basal diameter of the radial artery, explaining the absence of vasoconstriction when L-NMMA is administrated alone. Whether, in turn, the absence of decrease in radial artery diameter after fluconazole alone could result from the increase in NO remains uncertain. Our results are consistent with previous in vitro data showing that NO can inhibit the major families of CYP enzymes, including CYP epoxygenases, and the formation of CYP derivatives, explaining why blockade of the formation of NO is usually required to uncover the influence of EDHF on vascular tone (1, 32).

Subsequently, it was demonstrated in ex vivo experiments that an increase in NO availability is associated with a decrease in the CYP-dependent dilatation of rat renal microvessels (42). In addition, an interaction between NO and a CYP-dependent vasodilator pathway has been previously described in vivo in resistance vessels in humans and in large coronary arteries in dogs during endothelium-dependent dilatation but not in basal conditions (19, 34). This interaction between NO and this CYP-related EDHF could participate in the control of arterial conductance and cardiovascular coupling in humans. In addition, this mechanism may play a crucial role in cardiovascular homeostasis, in particular, through the regulation of vascular resistance, angiogenesis, and inflammation (41) and may serve as a backup system when NO availability is impaired.

Furthermore, we observed a higher vasoconstrictor effect of TEA on radial artery diameter, alone and with L-NMMA, compared with that of fluconazole. This could be related to the suppression of a spontaneous activity of muscular KCa channels present in parallel with their EDHF-dependent activity, as observed at the arteriolar level, to oppose myogenic tone (26). However, such mechanisms have never been reported at the level of the conduit arteries. Conversely, although additional experiments are necessary to confirm this hypothesis, this higher effect of TEA suggests that different endothelium-dependent or -independent hyperpolarizing mechanisms mediated by KCa channels could exist in the radial artery as previously suggested in animals (7, 37).

In conclusion, this study, performed at the level of the radial artery in healthy subjects, gives evidence that a CYP-related EDHF is involved in vivo in the regulation of peripheral conduit artery diameter at rest but not in the control of the basal vascular resistance in humans. In addition, these results demonstrate the presence of an interaction between NO and this EDHF to maintain conduit artery diameter under basal conditions and suggest that different KCa channel-dependent hyperpolarizing mechanisms could exist at this level.

GRANTS

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