Involvement of cytochrome epoxygenase metabolites in cutaneous postocclusive hyperemia in humans

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FOLLOWING BRIEF ARTERIAL occlusion, marked vasodilation associated with a transient rise in blood flow to the postischemic tissues has been described since the 1950s. This transient increase in blood flow, called postocclusive reactive hyperemia (PORH) or reactive hyperemia, has been observed in most vascular beds (7, 30, 34, 36). It is used as a trigger for the study of flow-mediated dilation in conductance vessels such as the brachial or radial arteries (31). In such vessels, one of the mediators implicated in the response to a 5-min occlusive episode was initially suggested to be nitric oxide (NO), to a greater or lesser extent depending on whether the artery observed was, respectively, proximal or distal to the site of arterial occlusion (28). More recently, Bellien et al. showed that a cytochrome-related endothelium-derived hyperpolarizing factor (EDHF) was involved in the flow-mediated dilatation of the human radial artery (3). Moreover, the effect was synergistic with NO, suggesting a functional interaction between NO and cytochrome-related EDHF pathways.

In human skin, following the end of arterial occlusion an immediate and significant transient increase in cutaneous flux is observed that lasts a few minutes (15). Several mediators contribute to this PORH. Local anesthesia using lidocaine/prilocaine cream partially alters the PORH (22), suggesting that a local axon reflex involving sensory nerves is implicated. The implication of sensory nerves has been consistently described thereafter (14, 24). In contrast to skeletal muscle where both NO and cyclooxygenase (COX) metabolites play a role (18), the inhibition of NO production does not alter PORH in the skin of the forearm (36). Furthermore, PORH occurs through mechanisms that are not associated with a measurable increase in local NO concentrations (38). Interestingly, Medow et al. confirmed that the blockade of NO synthase exerts no effects on PORH but that the inhibition of COX un_masks the NO dependence of PORH (26). Results are conflicting concerning the implication of prostaglandins, but there is no strong evidence that prostanooids participate in PORH. One study showed that 1 g of acetylsalicylic acid decreased the PORH peak (6), while another group showed no effect of a 900-mg intravenous lysine acetylsalicylate injection (16) and a slight reduction in PORH duration without effect on the peak response following the ingestion of 1 g acetylsalicylic acid (1). Using the nonspecific COX inhibitor ketorolac (10 mM) and a cutaneous microdialysis fiber system, one group found a higher PORH peak following COX blockade (26), but no effect was found in a subsequent study (24).

Apart from NO and prostacyclins, the third major endothelial-dependent dilation mechanism involves the EDHF (17). The classical EDHF pathway initiates endothelial cell hyperpolarization through activation of endothelial small- and intermediate-conductance calcium-activated potassium channels, leading to vascular myocyte hyperpolarization through potassium or gap junctions (17). A second category of EDHF pathways does not require endothelial cell hyperpolarization and involves the endothelial release of various molecules, including arachidonic acid metabolites (17). Endothelial cells metabolize arachidonic acid via three distinct enzymatic pathways [COX, lipoygenase, and cytochrome P-450 (CYP)]. CYP epoxygenases add oxygen across the double bonds of arachidonic acid to produce the four regiiosomers cis-epoxides...
14,15-, 11,12, 8,9-, and 5,6-epoxyeicosatrienoic acids (EETs), each of these existing as two stereoisomers S,R and R,S. In human endothelial cells, these epoxyenes are CYP2C8/2C9 and CYP2J2 (37). In conductance arteries, EDHF produced by endothelial CYP epoxygenase accounts for two-thirds of the flow-mediated vasodilation during prolonged hyperemic stimulation (3, 19), whereas in forearm microcirculation, it contributes to resting microvascular dilator tone (29). Using the nonspecific large-conductance calcium-activated potassium channel blocker tetraethylammonium, Lorenzo and Minson showed that such channels play a major role in the EDHF component of PORH, in part independent of the axon reflex (24). However, the exact nature of the EDHF implicated in human skin PORH remains unknown.

The main objective of our study was to investigate the specific contribution of EETs in human skin PORH. Secondary objectives were to identify any potential interaction with the axon reflex and the NO pathways. We hypothesized that an inhibition of EETs production through CYP epoxygenase inhibition by fluconazole would decrease the PORH in the skin microcirculation, assessed using the recently described skin microdialysis technique coupled with laser speckle contrast imaging (LSCI) (13).

MATERIALS AND METHODS

Study Population

We enrolled eight healthy subjects recruited through the Clinical Research Center Volunteer database. Inclusion criteria were age 18 years or older and no significant medical history. For women of child-bearing age, the possibility of pregnancy was excluded by urine tests at the beginning of each study visit. For all subjects, noninclusion criteria included any allergies to local anesthetics, cigarette smoking, and any dermatological pathology affecting the arms. Grenoble Institutional Review Board (IRB no. 6705) approval was obtained, and each volunteer gave written informed consent before participation.

Experimental Set-Up

Subjects attended an initial enrollment visit where inclusion and noninclusion criteria were checked by a physician, and full information was given. Later (7 ± 3 days), they returned for the first experimental visit, then 14 ± 4 days later, for the second experimental visit. For women, experimental visits were performed during the menstrual phase.

On arrival at the laboratory between 8:00 and 9:00 A.M., subjects were placed in a temperature-controlled room (24 ± 1°C). After checking for visible veins, lidocaine/prilocaine cream (10 g) was applied to the ventral face of the right forearm for 40 min, with an occlusive transparent dressing placed over the cream (14). The subjects were supine for the whole duration of the experiments.

Microdialysis fiber insertion was performed as previously described (13). After the removal of lidocaine/prilocaine cream, we randomly chose four skin sites, 2–3 cm apart, on the ventral side of the right upper forearm, avoiding veins. Local anesthesia was performed using chlorhexidine gluconate soap (4% Hibiscrub; Regent Medical, Manchester, UK), followed by swabbing with chlorhexidine gluconate and benzalkonium chloride (Bisepite; Bayer, Gaillard, France). Two sterile fields were placed on the lateral and median face of the forearm. Next, sterile 21-gauge, 50-mm needles were inserted into the skin over a length of 1.5–2 cm, and a CMA 66 linear microdialysis catheter with a 1-cm long, 500-μm diameter, 20,000-Dalton cut-off membrane (CMA Microdialysis, Solna, Sweden), previously rinsed with sterile 0.9% NaCl, was immediately inserted through the needle. The fiber was immediately connected to a portable battery-driven syringe pump (CMA 107 Microdialysis Pump; CMA Microdialysis). Once closed, the pump started a 5-min flush sequence at 15 μl/min and then automatically decreased to the preset flow rate of 2 μl/min, as used previously (25). The insertion procedure was immediately repeated with four microdialysis fibers spaced at 2- to 3-cm intervals.

Fiber sites were randomly received to any of the four potential experimental combinations, i.e., placebo, fluconazole, placebo + lidocaine/prilocaine skin application, or fluconazole + lidocaine/prilocaine skin application in experiment 1; and placebo, fluconazole, Nω-monomethyl-L-arginine (L-NMMA), or fluconazole + L-NMMA in experiment 2. All drugs and syrings were blinded by an independent pharmacist working in a separate preparation room.

The arm was then placed on a vacuum cushion to decrease artifacts associated with arm movements, and cutaneous blood flow was measured with LSCI (PeriCam PSI System; Perimed, Järfälla, Sweden). The LSCI wavelength was 785 nm, and the laser head was placed 15 cm above the skin (with a resolution of ~6,944 pixels/cm²). The image size was 12 × 5–7 cm, and the acquisition rate was 3/5 during the whole procedure.

Two hours after lidocaine/prilocaine removal, blood flow was occluded for 5 min by inflating a cuff placed on the right upper arm to 50 mmHg above the volunteer’s systolic blood pressure. At the end of all experiments, 29 mM sodium nitroprusside (SNP) was perfused at all sites simultaneously for 15 min to obtain maximal cutaneous vascular conductance (CVCmax), as previously described for this protocol (13).

Blood pressure was recorded continuously throughout the experiment (Nexfin monitor; Bmeeye, Amsterdam, The Netherlands) on the controlateral hand.

Experiments

Protocol 1: EETs and sensory nerve inhibition. We investigated the effects of fluconazole (a CYP2C9 and -2C19 inhibitor used to block EETs production) and lidocaine/prilocaine cream (a Na⁺ channel blocker used to inhibit sensory nerve activity) alone and in combination on skin PORH. One site was perfused with 6.5 mM fluconazole for 100 min. At another site lidocaine/prilocaine cream was applied for 100 min. At a third site both fluconazole (6.5 mM) perfusion and lidocaine/prilocaine cream were used, and the fourth site was perfused with placebo only (0.9% NaCl).

Protocol 2: EETs and NO inhibition. In this experiment we investigated the effects of fluconazole and L-NMMA (a NO synthase inhibitor used to inhibit NO production) alone and in combination on skin PORH. One site was perfused with 6.5 mM fluconazole for 100 min. At another site L-NMMA was perfused for 100 min. At a third site both fluconazole (6.5 mM) and L-NMMA (10 mM) were perfused together, and the fourth site was perfused with placebo (0.9% NaCl). The choice of L-NMMA as an inhibitor of NO synthase was based on previous work on conductance arteries to facilitate comparison between studies (2).

Drugs

Fluconazole (2 mg/ml) was purchased from Panpharma (Beaucé, France). The choice of drug concentration was determined from a pilot study in our laboratory in which we observed the effect of the perfusion of fluconazole at 650 μM and 6.5 mM on skin PORH. We tested only two concentrations, since 6.5 mM is the highest concentration commercially available for human use. We performed 5 min PORH in four subjects and observed a systematic inhibition of PORH at 6.5 mM, with a lower nonsystematic inhibition at 650 μM.

L-NMMA (250 mg/vial) was purchased from Clinalfa Basic (Bachem Distribution Services, Weil am Rhein, Germany). L-NMMA (10 mM) systematically blunted the NO-dependent local thermal hyperemia plateau in a pilot study in four subjects. SNP (Nitrate) was purchased from Serb (Paris, France). One vial containing 50 mg of...
SNP was diluted in 8 ml of 0.9% NaCl solution (Aguettant, Lyon, France) to obtain 29 mM SNP. All solutions were prepared extemporaneously and discarded the same day. Lidocaine/prilocaine cream was purchased from Aguettant (5-g tubes containing 125 mg lidocaine and 125 mg prilocaine).

Data Analysis

Skin blood flux was averaged over four regions of interest area of 60 mm². For each region of interest, an additional region of interest was used as a quality control, but not used for data analysis. Data were digitized, stored, and analyzed off-line with signal-processing software (PimSoft 1.1.1; Perimed). The biological zero, recorded during the 5-min occlusion, was subtracted from all raw values, and data were subsequently expressed as cutaneous vascular conductance [CVC, perfusion units (PU)/mmHg], which is the flux in PU divided by the mean arterial pressure in millimeters mercury and as a percentage of CVCmax (29 mM SNP infusion). The area under the curve (AUC) for CVC expressed as PU s⁻¹ mmHg⁻¹ was also calculated.

CVC values were averaged over 4-min periods for the baseline and SNP and over 20 s for the peak PORH. The PORH AUC (total hyperemic response) was given by: raw PORH AUC over 6 min – (baseline CVC × 360 s).

Statistical Analysis

Quantitative data are expressed as mean and SD and were analyzed by one-way ANOVA for repeated measurements followed by Bonferroni’s post hoc test or by paired t-tests for paired analyses, with each subject serving as his/her own control. P values < 0.05 were considered statistically significant. Statistical analysis was performed using SPSS 13.0 for Windows (SPSS, Chicago, IL).

RESULTS

Study Population

Four Caucasian males and 4 females were enrolled in the study. Their mean age was 23 ± 3 yr, their body mass index was 23 ± 3 kg/m², and their systolic/diastolic arterial pressure was 123 ± 11/71 ± 4 mmHg. The mean pain scores on insertion of the microdialysis fibers assessed on a 10-point visual analogic scale were 1.5 ± 0.9 and 1.6 ± 0.7 for protocols 1 and 2, respectively. One volunteer presented a vasovagal malaise after fiber insertion during protocol 2 and spontaneously recovered within 10 min. Five days after protocol 2, another volunteer presented with a 2-mm-diameter noninflammatory papula at the entry site of the proximal fiber that spontaneously disappeared within 7 days.

EET and Sensory Nerve Inhibition

There was no significant difference between treatment sites in absolute CVCmax values obtained following 29 mM SNP infusion at the end of the experiment (placebo: 1.9 ± 0.4 PU/mmHg; fluniconazole: 1.9 ± 0.5; lidocaine: 2.1 ± 0.6; fluniconazole + lidocaine: 2 ± 0.7). We observed a nonsignificant trend toward lower baseline flux values at the lidocaine/prilocaine-treated site (12.8 ± 4%CVCmax) vs. placebo (14 ± 3%CVCmax), fluniconazole (16.1 ± 3%CVCmax), and fluniconazole + lidocaine/prilocaine (16.4 ± 4 sites).

The mean PORH peak at the placebo site was 66 ± 11%CVCmax. Compared with the placebo site, it was significantly lower at the fluniconazole (47 ± 10%CVCmax; P < 0.001), lidocaine (29 ± 10%CVCmax; P < 0.001), and fluniconazole + lidocaine (30 ± 10%CVCmax; P < 0.001) sites (ANOVA P < 0.001, Fig. 1). The PORH peak was also significantly lower at the combination site compared with the fluniconazole alone site (P = 0.003). The PORH AUC was lower than placebo (4,477 ± 2,410%CVCmax/s) at the fluniconazole (2,451 ± 1,417%CVCmax/s; P = 0.008), lidocaine (2,247 ± 1,851%CVCmax/s; P = 0.001), and fluniconazole + lidocaine (1,683 ± 1,015%CVCmax/s; P = 0.01) sites (ANOVA P < 0.001, Fig. 1).

In a post hoc analysis, we tested whether the effect of fluniconazole and lidocaine/prilocaine was different on PORH peak and AUC. Data were expressed as a percentage of placebo peak and AUC. Fluniconazole effect on PORH peak was significantly smaller than on AUC (–29 ± 11 vs. –45 ± 19%, respectively, P = 0.015). Lidocaine/prilocaine effect did not differ on PORH peak vs. AUC (–57 ± 11 and –52 ± 29%, respectively, not significant [NS]). The effect of the combination did not differ on PORH peak vs. AUC (–54 ± 15 and –59 ± 29%, respectively, NS).

EETs and NO Synthase Inhibition

There was no significant difference in absolute CVCmax values between treatment sites (placebo: 1.9 ± 0.5 PU/mmHg; fluniconazole: 1.9 ± 0.6; l-NMMA: 1.9 ± 0.5; fluniconazole + l-NMMA: 1.9 ± 0.6). Similarly, there was no significant difference in baseline flux values between treatment sites (placebo: 16 ± 6%CVCmax; fluniconazole: 18 ± 6%CVCmax; l-NMMA: 14 ± 4%CVCmax; and fluniconazole + l-NMMA: 14 ± 6%CVCmax).

The PORH peak at the placebo site averaged 64 ± 9%CVCmax and was significantly reduced at the fluniconazole site only (49 ± 11%CVCmax; P = 0.001) (ANOVA P < 0.001, Fig. 2). The PORH peak was significantly lower at the combination site compared with the l-NMMA site (P = 0.008). The PORH AUC was lower than placebo (4,317 ± 993%CVCmax/s) at the fluniconazole site only (2,318 ± 691%CVCmax/s; P = 0.008) (ANOVA P = 0.002, Fig. 2).

In a post hoc analysis, when data were expressed as a percentage of placebo peak and AUC, fluniconazole effect on PORH peak was significantly smaller than on AUC (–23 ± 12 vs. –43 ± 24%, respectively, P = 0.043).

DISCUSSION

Using two-dimensional LSCI coupled with skin microdialysis, we show that, in addition to sensory nerves, cytochrome epoxygenase metabolites play a major role in skin PORH.

To assess the role of EDHF in skin PORH, we used fluniconazole as an inhibitor of cytochrome epoxygenases. Lorenzo and Minson previously showed that 50 mM tetraethylammonium decreases PORH peak and AUC (24), suggesting that an EDHF is implicated in skin PORH. However, tetraethylammonium ions, especially at high concentrations, may block different types of potassium channels with varying degrees of effectiveness, targeting ATP-dependent potassium and delayed-rectifier channels (21). We tested the hypothesis that the EETs pathway, one of the EDHF pathways, is involved in skin PORH. Like tetraethylammonium, fluniconazole did not affect baseline CVC (24), suggesting that cytochrome epoxygenase metabolites do not regulate basal skin vascular tone. Similarly to Lorenzo and Minson, we were unable to demonstrate an effect of fluniconazole at the lidocaine/prilocaine-treated sites, suggesting that
while both pathways contribute to skin PORH, they do not act synergistically and that cytochrome epoxygenase metabolites are involved in the downstream actions of sensory nerves. A potential limitation is that, in addition to sodium channels, local anesthetics may block potassium channels, whether voltage sensitive or not, as well as Ca\(^{2+}\)/H\(_{11001}\) channels, yet with a low affinity (33). Therefore, while unlikely, we cannot rule out a direct effect of lidocaine/prilocaine on calcium-activated potassium channels. Recent data suggest that CYP metabolites play a role in local thermal hyperemia in skin (9). In the latter study, CYP2C9 was blocked using sulfaphenazole, which has the advantage of being highly specific but the disadvantage of being insoluble in water, and requires dimethyl sulfoxide, making experiments more complex. We chose fluconazole since it preferentially inhibits CYP2C9 and -2C19 (35), is soluble in water, is available for perfusion in humans, and has been used previously as a reference for EETs inhibition in studies on flow-mediated dilation in conductance arteries, where an inhibition of EETs production was demonstrated (2, 4). Furthermore, with the use of intra-arterial fluconazole injections in patients with essential hypertension, an impaired contribution of EETs to the flow-mediated dilation in the radial artery can be demonstrated (4). Interestingly, the inhibitory effect of fluconazole was more pronounced on the PORH AUC than on the peak, suggesting that EETs are mostly involved in the time course rather than the peak response.

Previous in vitro studies clearly indicate that an endothelium-dependent non-NO, nonprostanoid mechanism of relaxation is involved in human subcutaneous microvessels mounted on pressure myographs (10, 12). Indeed, EDHF was the major

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**Fig. 1.** A: representative laser speckle contrast image (LSCI) over the 4 microdialysis fibers during the peak of postocclusive reactive hyperemia (region of interest 1 to 4 and their respective controls 5 to 8, the latter were used only for quality control during the recording and not used for data analysis) where the effect of fluconazole, lidocaine/prilocaine, and the combination can be observed. B: mean \pm SE effect of placebo, fluconazole, lidocaine/prilocaine, and the combination on peak postocclusive reactive hyperemia (left) and area under the curve (AUC, right). PORH, postocclusive reactive hyperemia; CVC, cutaneous vascular conductance; CVCmax, maximal CVC. *P < 0.001 (peak) and <0.01 (AUC) vs. placebo. †P = 0.003 vs. fluconazole. Corresponding individual data are on bottom.
contributor to the acetylcholine-dependent endothelium vasorelaxation, and in these arteries a product of CYP metabolism of arachidonic acid was likely to be EDHF, as shown by the concentration-dependent inhibition obtained using the nonspecific CYP inhibitor ketoconazole (12). However, heterogeneity of CYP inhibitors is observed, since acetylcholine relaxation of human subcutaneous microvessels was unaffected by 17-octadecynoic acid, a CYP inhibitor, whereas econazole induced a small by significant rightward shift of the concentration-dependent curves (10). In contrast, Lenasi et al. showed that inhibition of CYP2C9 through skin microinjections did not modify acetylcholine iontophoresis-induced vasodilation of human forearm skin, whereas combined NO and COX blockade altered the response (23). However, to fully compare such data and avoid the confounding effect of current-induced vasodilation, acetylcholine and the blockers used should be infused intradermally through microdialysis fibers rather than via the less reproducible iontophoresis and microinjection techniques.

Multiple CYPs can metabolize arachidonic acids to EETs. Mammalian CYP1A, CYP2B, CYP2C, CYP2D, CYP2G, CYP2J, CYP2N, and CYP4A subfamilies have been shown to be capable of EET biosynthesis in vitro (37), but only the CYP2C and CYP2J isoforms appear to contribute to EETs formation in human endothelial cells (5). In our study it is likely that fluconazole exerted its effect through the inhibition of EETs production. Indeed, plasma EET concentrations are very low, ~10 ng/ml (4). EETs are strongly bound to proteins (95%), and the quantity available for diffusion through a microdialysis membrane is therefore very low. In addition, quantification by skin microdialysis

Fig. 2. A: representative LSCI image over the 4 microdialysis fibers during the peak of postocclusive reactive hyperemia where the effect of fluconazole, N\textsuperscript{G}-monomethyl-l-arginine (l-NMMA), and the combination can be observed (region of interest 1–4 and their respective controls 5–8, the latter were used only for quality control during the recording and not used for data analysis). B: mean +/- SE effect of placebo, fluconazole, l-NMMA, and the combination on peak postocclusive reactive hyperemia (left) and AUC (right). *P = 0.001 (peak) and 0.002 (AUC) vs. placebo. #P = 0.008 vs. l-NMMA. Corresponding individual data are on bottom.
would not be optimal given that EETs production would be transient over the 5-min PORH, while it takes ~60 min to obtain a sufficient volume of dialysate to run an assay using LC-MS MS. LSCI coupled with the use of inserted microdialysis fibers has the advantage of providing easy visual analysis throughout the protocol, with low within-subject coefficients of variation for skin PORH (13).

The human skin microcirculation is able to release NO, as suggested in the plateau phase of local thermal hyperemia (27) or following acetylcholine perfusion (38). In the present study we showed that infusion of a NO synthase inhibitor, L-NMMA, or following acetylcholine perfusion (38). In the present study LSCI coupled with the use of inserted microdialysis fibers has the advantage of providing easy visual analysis throughout the protocol, with low within-subject coefficients of variation for skin PORH (13).

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