Membrane Potential-Dependent Inhibition of Platelet Adhesion to Endothelial Cells by Epoxyeicosatrienoic Acids

Florian Krötz, Tobias Rixinger, Martin A. Buerkle, Kasem Nithipatikom, Torsten Gloe, Hae-Young Sohn, William B. Campbell, Ulrich Pohl

Objective—Epoxyeicosatrienoic acids (EETs) are potent vasodilators produced by endothelial cells. In many vessels, they are an endothelium-derived hyperpolarizing factor (EDHF). However, it is unknown whether they act as an EDHF on platelets and whether this has functional consequences.

Methods and Results—Flow cytometric measurement of platelet membrane potential using the fluorescent dye DiBAC$_4$. Different EET regioisomers hyperpolarized platelets down to $-69\pm2$ mV, which was prevented by the non-specific potassium channel inhibitor charybdotoxin and by use of a blocker of calcium-activated potassium channels of large conductance (BK$_{Ca}$ channels), iberiotoxin. EETs inhibited platelet adhesion to endothelial cells under static and flow conditions. Exposure to EETs inhibited platelet P-selectin expression in response to ADP. Stable overexpression of cytochrome P450 2C9 in EA.hy926 cells (EA.hy2C9 cells) resulted in release of EETs and a factor that hyperpolarized platelets and inhibited their adhesion to endothelial cells. These effects were again inhibited by charybdotoxin and iberiotoxin.

Conclusions—EETs hyperpolarize platelets and inactivate them by inhibiting adhesion molecule expression and platelet adhesion to cultured endothelial cells in a membrane-potential-dependent manner. They act as an EDHF on platelets and might be important mediators of the anti-adhesive properties of vascular endothelium. (Arterioscler Thromb Vasc Biol. 2004;24:595-600.)

Key Words: epoxyeicosatrienoic acids ■ platelet adhesion ■ membrane potential ■ potassium channels ■ EDHF

Intact endothelial cells continuously release autacoids such as nitric oxide (NO), prostacyclin (PGI$_2$), or adenosine and an endothelial-derived hyperpolarizing factor (EDHF), thereby controlling vascular tone and platelet activity.$^{1,2}$ Endothelial dysfunction and the associated activation of platelets are synergistic factors in the development of cardiovascular disorders. Both may precede atherosclerosis$^{3,4}$ and are associated with an enhanced risk of adverse cardiovascular events.$^5$ Little is known about the role of EDHF in the control of platelet function, although this factor may be less susceptible to mediators that deteriorate endothelial function such as reactive oxygen species.

In several vascular beds, EDHF seems to be identical with epoxyeicosatrienoic acids (EETs),$^6$ which are products of cytochrome P450 enzymatic metabolism of arachidonic acid.$^7$ There are data indicating that EETs are released into the lumen of isolated vessels$^{8,9}$ or from endothelial cells in culture,$^{10-12}$ so they could influence not only the adjacent smooth muscle cells but also circulating blood constituents like platelets. Although in 1986, years before these compounds have been postulated to represent an EDHF in the vasculature, Fitzpatrick et al observed inhibition of platelet aggregation by EETs,$^{13}$ it was not investigated whether the platelet–endothelium interaction was affected or whether platelet membrane potential has a role in this.

In general, EETs could influence platelets by activation of calcium-activated potassium channels or by effects that are independent of the membrane potential, similar as described for endothelial cells.$^{14}$ Platelets not only contain voltage-operated potassium channels (K$_v$ channels)$^{15}$ but also calcium-activated potassium channels (K$_{Ca}$ channels), so they are potential targets of EETs.$^{16}$ In this study, we investigated whether EETs hyperpolarize platelets via K$_{Ca}$ channels and whether this has an effect on platelet activation parameters and platelet adhesion to the endothelium.

Methods

For a detailed Methods section, please see http://atvb.ahajournals.org.

Endothelial Cell Culture and Platelet Isolation

Human umbilical vein endothelial cells (HUVEC) were cultivated as described.$^{17}$ EA.hy926 cells were cultured in high-glucose DMEM supplemented with 20% fetal calf serum and 1% antibiotics. Washed platelets (WP) were prepared as described.$^{18}$

Fluorescence Measurement of Platelet Membrane Potential

Platelet membrane potential was assessed using the potential-sensitive fluorescent dye DiBAC$_4$. For calibration, other platelets...
from the same lot were re-suspended in buffer containing 0.1, 10, 20, 30, 60, or 90 mmol/L of KCl in the presence of the potassium ionophore valinomycin (2 μM, "null-point method").

Adhesion Molecule Expression
Platelet-rich plasma (PRP) (300 μL) was incubated with EETs or potassium channel blockers or both (EETs/EDHF and potassium channel blockers) for 10 minutes each, followed by addition of ADP (20 μM). FACS-staining was performed using anti-CD62P-RPE (Sero-tect, Oxford, UK) as previously described.

Platelet Aggregation
Platelet aggregation was assessed in WP or in PRP as described.

Platelet Adhesion to HUVEC Under Static Conditions
Adhesion of calcein-AM labeled platelets to confluent HUVEC was assessed by a method described by Verheul et al, with minor modifications.

Platelet Adhesion to HUVEC Under Flow
HUVEC grown on collagen-coated glass plates were placed in an airtight perfusion chamber and fluorescence-labeled platelets (200,000/μL) were continuously perfused over the cells at a shear stress of 16 dyn/cm² for 6 minutes at 37°C. Immediately before the platelet-containing superfusate was coming into contact with HUVEC, CaCl₂ (final concentration 2 mmol/L) and ADP (final concentration 100 μM) were added.

Stable Transfection of CYP2C9
EA.hy926 cells were used for stable overexpression of cytochrome P450 2C9 (CYP2C9). To analyze the release of EETs from these cells, they were washed and incubated in buffer A for 30 minutes. Thereafter, the supernatant was collected and immediately frozen to −20°C.

Immunoblotting
Immunoblotting was performed using standard techniques as previously described.

Determination of Arachidonic Acid Metabolites
The arachidonic acid metabolites 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET, and the respective DHETs were analyzed using a liquid chromatographic–mass spectrometric method (LC-MS) that has been described recently. As internal standards, octadeuterated analogues of the fatty acids were used.

Materials
The CYP2C9 plasmid and polyclonal rabbit antiserum against CYP2C9 were kind gifts from Dr B. Fisslthaler (Frankfurt, Germany). Iloprost was from Schering (Berlin, Germany). EETs were CYP2C9 were kind gifts from Dr B. Fisslthaler (Frankfurt, Germany). To analyze the release of EETs from these cells, they were washed and incubated in buffer A for 30 minutes. Thereafter, the supernatant was collected and immediately frozen to −20°C.

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Materials
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Statistical Analysis
All data are expressed as means±SEM. Data were analyzed using one-way ANOVA and/or Student t test. Differences were considered significant when the error level was P<0.05.

Results
Platelet Membrane Potential
Incubation of platelets with DIBAC₄(3) (500 nM) for 30 minutes at RT lead to a stable fluorescence signal. In platelet buffer containing 2.7 mmol/L K⁺, valinomycin (2 μM), a potassium (K⁺) ionophore, decreased fluorescence, which reached a constant level at 5 to 7 minutes, indicating that the resting platelet membrane potential was different from the K⁺ equilibrium potential. Assuming a Kᵢ of 140 mmol/L, we calculated the potassium equilibrium potential to approximately −58 mV (n=30) by use of the Nernst equation. Among all EET regioisomers tested at a concentration of 1 μM, the most pronounced hyperpolarization was caused by 11,12-EETs (to −69.4±2 mV, n=12, P<0.01) and the 8,9-regioisomer (to −66.5±2 mV, n=13, P<0.01), followed by the 14,15-regioisomer (to −63.2±4 mV, n=13, P<0.01; Figure 1A). In all subsequent experiments, the 11,12-regioisomer was used, which hyperpolarized platelets already at a concentration of 100 nM (to −62.2±3 mV, n=8, P<0.05), but significantly more at a concentration of 1 μM (P<0.01 versus 1 μM 11,12-EET). Hyperpolarization induced by 11,12-EETs was completely reversible by unspecifically blocking IKᵣ, BKᵣ, and Kᵣ channels using charybdotoxin (50 nM, n=4, P<0.01) or by blocking platelet BKᵣ, channels using iberiotoxin (500 nM, n=4, P<0.05), whereas apamin, a blocker of SKᵣ channels, had no effect (500 nM, n=4, all data, Figure 1B). Similar results were obtained for 8,9-EET and 14,15-EET (not shown). Charybdotoxin alone strongly depolarized platelets to −18.2±2 mV (50 nM, n=7). This was partly prevented by 11,12-EETs (−29.4±5 mV, n=4, P<0.05, not on graph).

EETs inhibit Platelet Adhesion
To investigate, whether EETs could affect the physiological function of platelets, we tested their influence on platelet
aggregation and on the adhesion of human platelets to cultured endothelial cells in two different assays in which adhesion was either induced by centrifugation of platelets onto HUVEC or induced by ADP-stimulation under physiological shear stress.

At the concentrations tested in our experiments (up to 10 μmol/L) 11,12-EETs did not influence platelet aggregation on stimulation with collagen (1 to 10 μg/mL), ADP (1 to 50 μmol/L), or a thrombin-receptor activating peptide (TRAP, 1 to 20 μmol/L). At least three aggregation experiments for each dose were performed in WP or PRP.

However, in adhesion experiments under non-flow conditions, pretreatment of platelets with 11,12-EETs for 10 minutes inhibited platelet adhesion to HUVEC by 22%±4% (1 μM, n=13, P<0.01). This was a dose-dependent effect that reached significant levels at a dose of 100 nM (n=6, P<0.05, Figure 1A, available online at http://atvb.ahajournals.org). It was abolished by preincubation of platelets with charybdotoxin (50 nM, n=13, P<0.01 versus 11,12-EET) or iberiotoxin (500 nM, n=13, P<0.01 versus 11,12-EET), whereas addition of apamin had no effect (n=10, all data; Figure 2A and 2B). Because charybdotoxin, besides Kᵦ₅ channels, blocks Kᵦ₆ channels, we next tested the effect of a specific Kᵦ₅ channel inhibitor, 4-aminopyridine (4-AP) (1 mmol/L), on adhesion. Platelet treatment with 4-AP also resulted in an increased basal adhesion of platelets to HUVEC (Figure IB, available online at http://atvb.ahajournals.org; n=12 to 16, P<0.05 for charybdotoxin, P<0.01 for 4-aminopyridine). Addition of an antibody against β₃-integrins, abciximab (0.2 μg/mL), but not of sham solution resulted in a decreased platelet adhesion to HUVEC by 35%±4% (n=17, P<0.01).

To corroborate these findings, we investigated the influence of EETs on ADP-induced platelet adhesion to endothelial cells under a shear stress rate of 16 dyn/cm² (Figure II, available online at http://atvb.ahajournals.org, shows representative images of these experiments.). Although high concentrations of ADP were used, basal adhesion under these conditions was much weaker than that induced by centrifugation force. However, pretreatment of platelets with 11,12-

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**Figure 2.** Membrane potential-dependent inhibition of platelet adhesion to endothelial cells. A, 11,12-EET inhibited adhesion of calcein-AM-labeled platelets to HUVEC in a static adhesion assay (1 μM, n=13, P<0.01). Abciximab (Abc) (0.2 μg/mL) was used as a positive control. B, Platelet treatment with the Kᵦ₅ channel blockers Cbtx (also blocker of Kᵦ₆ channels; 50 nM) and 1btx (500 nM, n=13, P<0.01 each) but not apamin (Apa) (500 nM, n=10) before exposure to 11,12-EETs prevented this effect. **Significantly different versus control at P<0.01; # and ## indicate P<0.05 and P<0.01 versus 11,12-EET.

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**Figure 3.** Influence of EETs on platelet adhesion under flow. Under a laminar shear stress of 16 dyn/cm² 11,12-EET (1 μM) equally inhibited basal adhesion. Mean adherence was 1.4±0.2×10³ platelets/mm² in controls (n=24, P<0.01), whereas EET-treated platelets showed only 0.7±0.1×10³ platelets/mm² (n=20, P<0.01). Platelet pretreatment with charybdotoxin (50 nM) increased adherence to 3.6±0.6×10³ platelets/mm² (n=24, P<0.01). **Significantly different versus control at P<0.01.

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**Figure 4.** Influence of EETs on platelet P-selectin expression. A, 11,12-EETs dose-dependently inhibited basal P-selectin expression in platelets (n=20, P<0.01). B, ADP-induced P-selectin expression was abrogated by 11,12-EETs (n=13, P<0.01). * and ** indicate significantly different versus control at P<0.05 and 0.01, respectively; # and ## indicate P<0.01 versus ADP.
increase in platelet CD62P expression. Pre-exposure to 11,12-EET (1 μM, n=20, P<0.01 versus ADP) blocked ADP-dependent P-selectin expression (100.4%±3%, n=19, P<0.05 versus ADP; Figure 4B). Similar to inhibition of P-selectin expression, 11,12-EETs also inhibited platelet CD41 expression as induced by ADP (data not shown).

**EA.hy926 Cells Overexpressing CYP2C9 Release EETs and a Factor That Inhibits Platelet-Endothelial Cell Adhesion**

To scrutinize the question whether EETs can be released to the extracellular space from endothelial cells and thus affect platelets, we next tested the effect of releasates from a cell line that stably overexpressed cytochrome P4502C9 (CYP2C9) on platelets. The endothelial hybridoma cell line EA.hy926 was used to overexpress a CYP2C9 plasmid. In contrast to control EA.hy926 cells, these cells (EA.hy2C9-cells) showed high expression of CYP2C9 protein (Figure 5A). EA.hy2C9-cells, but not control EA.hy926 cells in the continuous presence of L-nitro-l-arginine (L-NA 100 μM, to block NO-synthase) and indomethacin (20 μM, to block cyclooxygenase), released a factor that hyperpolarized platelets (Figure 5B; n=15, P<0.01). This hyperpolarizing effect was enhanced after stimulation with bradykinin (100 nM, n=15, P<0.01) and was inhibited by pretreatment of platelets with charybdotoxin (500 nM, n=6, P<0.01, not shown) or iberiotoxin (500 nM, n=4, NS). Analysis of regioisomers of EETs in releasates from EA.hy2C9 cells revealed high concentrations of the 8,9- (28.7±7.1 pg/μL) and the 14,15-regioisomer (18.6±4.5 pg/μL), and lower concentrations of the 11,12-regioisomer (0.2±0.05 pg/μL), whereas the 5,6-regioisomer was not detected. Stimulation with bradykinin (100 nM) increased this to 49.8±9.9 pg/μL for 8,9-EETs (n=7, P<0.01 versus control), 38.2±7.5 pg/μL for 14,15-EETs (n=7, P<0.01 versus control), or 0.3±0.05 pg/μL for 11,12-EETs (n=7, P<0.01 versus control). In all cases, the concentrations of the EET hydrolysis products, the respective DHET regioisomers (not shown), were markedly lower than that of the corresponding EET (all EET data: Figure 6).

Supernatants released from control EA.hy926 cells did not inhibit platelet adhesion to HUVEC, whereas those from EA.hy2C9 cells that were bradykinin-stimulated did so (100 nM, 27%±5% inhibition, n=10, P<0.01, Figure III, available online at http://atvb.ahajournals.org). The inhibition of platelet adhesion by an EDHF derived from bradykinin-stimulated EA.hy2C9 cells was fully prevented when platelets were pretreated with iberiotoxin (50 nM, n=8, P<0.01), and, to a lesser extent, after exposition to iberiotoxin (500 nM, n=5, P<0.05; Figure III), but not apamin (500 nM, n=9, NS).

**Discussion**

We show for the first time to our knowledge that EETs, products of endothelial CYP2C8/9 metabolism and likely mediators of EDHF effects in many vascular beds, inhibit adhesion of human platelets to the endothelium. We further show that this is at least partly a membrane potential-dependent process, which involves activation of platelet KCa channels.

EETs influencing platelet activity could be of importance as a third endothelium-derived antplatelet substance besides NO and prostacyclin.23–25 Evidence for EETs, products of cytochrome P450 metabolism, being responsible for EDHF-effects has first been found in bovine coronary arteries6 and thereafter in porcine,26 canine,27 or human coronary arteries,1 and in the hamster28 or rat microcirculation.29 Clinical data,9 bioassay experiments,10,26,30 and direct cell culture measurements show that EETs are released from the endothelium to

![Figure 5](http://atvb.ahajournals.org/) **Figure 5.** EA.hy926 cells that overexpress CYP2C9 release a factor that hyperpolarizes platelets. A, Stable overexpression of cytochrome P4502C9 in the hybridoma cell line EA.hy926 (EA.hyCYP2C9) resulted in high protein expression compared with control cells (blot representative of three experiments). B, In a NO-synthase (L-nitro-L-arginine, 100 μM) and cyclooxygenase (indomethacin, 20 μM)-independent manner, these cells released a substance that hyperpolarized platelets, which was enhanced by stimulation with bradykinin (BK) (100 nM, n=7, **P**<0.01). **Significantly different versus control at **P**<0.01.

![Figure 6](http://atvb.ahajournals.org/) **Figure 6.** Production of EETs by EA.hy926 cells transfected with CYP2C9. High amounts of 8,9-EET and 14,15-EET were released by EA.hyCYP2C9 cells. To a lesser extent, there was release of 11,12-EETs. This release (in the presence of L-NA, 100 μM and indomethacin, 20 μM) could be enhanced by bradykinin treatment (100 nM, n=7; * and ** indicate significantly different versus control at *P*<0.05 and 0.01).
the extracellular space. Although the in vivo concentrations of EETs are unknown and should be influenced by flowing blood, ex vivo measurements report that concentrations up to approximately 858 nM may be released upon acetylcholine exposure (11,12-EETs, \( \approx 275 \) pg/\( \mu L \)). Because endothelial cells have been observed to rapidly lose CYP2C mRNA and protein in cell culture, we chose to observe CYP2C9 as a source for EETs in an endothelial cell line. These overexpressing cells show high release of EETs into the supernatants (\( \mu M \) concentrations), which block platelet adhesion to the endothelium in a \( K_{Ca} \)-dependent manner. During experiments, cyclooxygenase and NO-synthase were blocked by concentrations that we have previously observed to prevent endothelial prostaglandin or NO synthesis, so confounding effects of NO or arachidonic acid metabolites of cyclooxygenase seem unlikely. To exclude that lipoxygenase metabolites of arachidonic acid were involved, we measured several HETE regioisomers, which could not be detected despite the use of sensitive assays.

In addition to endothelium-derived EETs acting as a platelet-hyperpolarizing factor, EETs released from platelet membranes on stimulation could exert similar action on platelets as endothelium-derived EETs would. Interestingly, it has been described that thrombin or platelet activating factor may hyperpolarize platelet membranes, if a depolarising sodium-influx is prevented. However, there seems to be a hyperpolarizing substance, potentially released from platelet membranes to the intracellular space, when platelets are activated by these stimuli.

In our experiments, hyperpolarization of platelets was associated with an inhibition of their adhesion to cultured endothelial cells. Both effects were membrane potential-dependent, because blockade of platelet \( K \) channels prevented them. Several potassium channels have been identified in platelets. They express a high number of \( K \) channels, and a small number of calcium-activated potassium channels \( K_{Ca} \) channels of intermediate (\( I_{KCa} \) channels) or large conductance (\( BK_{Ca} \) channels), whereas \( K_{Ca} \) channels of low conductance (\( SK_{Ca} \) channels) could not be identified. It is important to note that the effects of EETs were caused by an action on platelet \( K \) channels and not on endothelial cell ones. Hyperpolarization and inhibition of adhesion were prevented by specific blockade of platelet \( BK_{Ca} \) channel and by the less specific inhibitor of \( K_{Ca} \) and \( K \) channels, charybdoxin. Because platelets probably lack \( SK_{Ca} \) channels, apamin, which is often necessary to fully block EDHF effects in the microvasculature, had no effect in our experiments. Our data give evidence for the existence of \( BK_{Ca} \) channels in platelets, because iberiotoxin, which prevents EET effects in our study, is a highly specific inhibitor of \( BK_{Ca} \) channels. Blockade of \( BK_{Ca} \) channels, however, can only partly explain the action of charybdoxin, which is assumed to be an unspecific inhibitor of \( I_{KCa} \) channels, but also exerts its action on \( BK_{Ca} \) channels and on \( K \) channels. The strong effect of charybdoxin on platelet membrane potential exceeded the mere reversal of hyperpolarization and was most likely caused by blockade of \( K \) channels, which are abundant in platelet membranes. Interestingly, blockade of \( K \) channels by charybdoxin or 4-aminopyridine significantly depolarized platelets and, more importantly, increased their adhesion to endothelial cells in a static and in a shear-stress-dependent model of platelet adhesion to endothelial cells. Therefore, our study not only discloses a protective effect of EETs on platelets but also reveals a link between blockade of platelet \( K_{Ca} \)-channels, their membrane potential, and their adhesion to endothelial cells. The importance of EET could thus lie in regulation of the threshold potential for opening of platelet \( K \), channels and subsequent depolarisation.

Inhibition of platelet adhesion molecule expression, as induced by ADP, could present an explanatory mechanism for decreased platelet adhesion to endothelial cells, which was caused by EETs. Notably, only platelet adhesion was influenced by EETs. At the doses tested, we could not find an influence of EETs on platelet aggregation, which directs attention to the different platelet receptors involved in adhesion and aggregation. According to the current understanding of these complex processes, an initial transient adhesion is mediated via GPIb, whereas firm tethering of platelets to the endothelium is integrin-dependent and involves the GPIb/IIIa integrin. Collagen, which is used in aggregation and in adhesion assays in our study, activates platelets on binding to platelet GPVI, a molecule that seems to primarily have a signaling role, rather than serving as an adhesion receptor. Although our data suggest that the inhibition of adhesion is to some extent membrane potential-dependent, evidence for membrane potential-independent actions of EETs remains: charybdoxin treatment alone could not increase platelet P-selectin expression in our experiments (data not shown) and, although the hyperpolarization caused by EETs is moderate, there are strong effects on adhesion molecule expression and adhesion. As it has been previously observed that EETs also influence adhesion molecule expression in endothelial cells independent of membrane potential, thereby decreasing leukocyte adhesion to endothelial cells, there might be an additional membrane potential-independent effect on platelets in vivo, which will be the subject of future investigation.

Our findings draw attention toward potential physiological functions of platelet potassium channels in general. They suggest fine-tuned regulation between an influence of EETs on platelet membrane potential and strong depolarization caused by \( K_{v} \)-channel blockade. In human vessels, \( K_{v} \)-dependent inhibition of platelet-endothelium adhesion could represent an important mechanism of protection from atherosclerotic disease and its thrombotic complications. Especially in pathologically altered vessels, EDHF has pivotal importance for vasodilation. In these situations, the effects of EETs on platelet adhesion might even outbalance the importance of other endogenous antiplatelet factors. This concept, however, remains to be challenged in future studies. They will also have to clarify whether the endothelium in vivo releases EETs in amounts high enough to control platelet activation.

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