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Effect of cytochrome P450-dependent epoxyeicosanoids on Ristocetin-induced thrombocyte aggregation

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Abstract
Epoxyeicosatrienoic acids (EETs) produced by cytochrome P450 (CYP)-dependent epoxidation of arachidonic acid (AA) inhibit thrombocyte adhesion to the vascular wall. Upon dietary omega-3 fatty acid supplementation, EETs are partially replaced by eicosapentaenoic acid (EPA)-derived epoxyeicosatetraenoic acids (EEQs) and docosahexaenoic acid (DHA)-derived epoxydocosapentaenoic acids (EDPs). We hypothesized that the omega-3 epoxy-metabolites may exhibit superior anti-thrombogenic properties compared to their AA-derived counterparts. To test this hypothesis, we analyzed the effects of 11,12-EET, 17,18-EEQ and 19,20-EDP on ristocetin-induced thrombocyte aggregation (RITA), a process that mimics thrombocyte adhesion to the vascular wall.
The eicosanoids were added for 5, 30, or 60 minutes to thrombocyte-rich plasma freshly prepared immediately after blood collection from stringently selected apparently healthy subjects. Thrombocyte aggregation was then induced by Ristocetin (0.75 mg/ml) and assessed by turbidimetric measurements. After 60 minutes of preincubation, all three epoxy-metabolites significantly decreased the rate of RITA. 17,18-EEQ and 19,20-EDP were effective already at 1 µM, whereas 5-fold higher concentrations were required with 11,12-EET. Addition of AUDA, an inhibitor of the soluble epoxide hydrolase, potentiated the effect of 17,18-EEQ resulting in a significant further decrease of the velocity as well as amplitude of the aggregation process. In contrast to their profound effects on RITA, none of the epoxy-metabolites was effective in reducing collagen- or ADP-induced thrombocyte aggregation.
These results indicate a highly specific role of CYP-eicosanoids in preventing thromboembolic events and suggest that the formation of 17,18-EEQ and 19,20-EDP may contribute to the anti-thrombotic effects of omega-3 fatty acids.

Introduction

Thromboembolic complications are still a serious problem in interventional cardiology. They were described to arise from ruptures of arteriosclerotic plaques, from blood vessel wall injuries or from the luminal surface of cardiovascular implants (e.g. of stents, grafts, mechanical heart valves, etc.). Essential components of such thrombi are thrombocytes stabilized by fibrin fibers [27, 40].
Dual anti-thrombocyte therapy using aspirin and clopidogrel is known to be essential to prevent stent thrombosis and its fatal consequences in patients undergoing percutaneous coronary intervention with stent implantation [18, 53]. This therapy decreased the rate of early (≤ 30 days) stent thrombosis after implantation of bare-metal stents (BMS) to a clinically accepted extent. While a dual thrombocyte inhibition of 4 weeks is thought to be sufficient after BMS implantation, it has to be prolonged after drug-eluting stents (DES) because the endothelialisation of their luminal stent surface will last much longer.
The efficacy of drug-eluting stent (DES) technology to avoid restenosis is based on the local release of cytotoxic drugs, which exert different biological effects, such as activation of signal transduction pathways and inhibition of cell proliferation. The drugs can successfully prevent vascular smooth muscle cell proliferation and migration, which are key factors in the development of restenosis, but also impair the reendothelialization [26, 41]. Recent studies indicate that dietary supplementation of omega-3 polyunsaturated fatty acids on top of aspirin/clopidogrel synergistically improves the anti-thrombotic therapy in patients after percutaneous coronary intervention [12, 21].
CYP-dependent AA metabolites are increasingly recognized to play an important role in the regulation of vascular, renal and cardiac function [46, 49]. Epoxyeicosatrienoic acids (EETs) are produced by CYP epoxygenases and serve as endothelium-derived hyperpolarizing factor in several vascular beds [10, 17]. In addition to regulating vascular tone, EETs have anti-inflammatory properties and reduce the activation of endothelial cells as well as thrombocytes. Recent studies revealed that EETs hyperpolarize thrombocytes and inactivate them by inhibiting adhesion molecule expression resulting in reduced thrombocyte adhesion to the vascular wall [33, 34]. Direct effects on thrombocyte aggregation have been also reported, however, the results remained partially controversial. EET-mediated inhibition of thrombocyte aggregation may be caused by inhibition of cyclooxygenase activities and stimulation of nitric oxide production in thrombocytes [15, 59]. CYP epoxygenases do not only metabolize AA but also accept the fish oil omega-3 polyunsaturated fatty acids EPA and DHA as efficient alternative substrates [32]; compare Figure 1. Dietary EPA/DHA supplementation shifts the endogenous CYP-eicosanoid profile from AA- to EPA- and DHA-derived metabolites. Under these conditions, 17,18-EEQ derived from EPA and 19,20-EDP derived from DHA become predominant epoxyeicosanoids in various tissues and in the circulation [4]. EEQs and EDPs may take over and even surpass the role of EETs as endothelium-derived hyperpolarizing factors [24, 35, 57, 59]. EEQs and EDPs also inhibited AA-induced thrombocyte aggregation at concentrations below those affecting thromboxane synthesis [54]. However, the role of EEQs and EDPs on thrombocyte aggregation has not been investigated yet. To address this question, we analyzed the influence of different CYP-dependent eicosanoids (11,12-EET, 17,18-EEQ and 19,20-EDP) on the Ristocetin-induced thrombocyte aggregation (RITA) – a process that mimics the adherence of thrombocytes to cells and structures of the vascular wall [7].

Material and Methods

Compounds

11,12-EET, 17,18-EEQ and 19,20-EDP as well as 12-(3-adamantan-1-yl-ureido) dodecanoic acid (AUDA) were purchased from Cayman Chemicals. Appropriate stock solutions were prepared in ethanol and added to thrombocyte rich plasma. The final concentration of the compounds was varied between 1 and 10 µM while keeping the final concentration of ethanol in the incubations below 0.2 vol%.

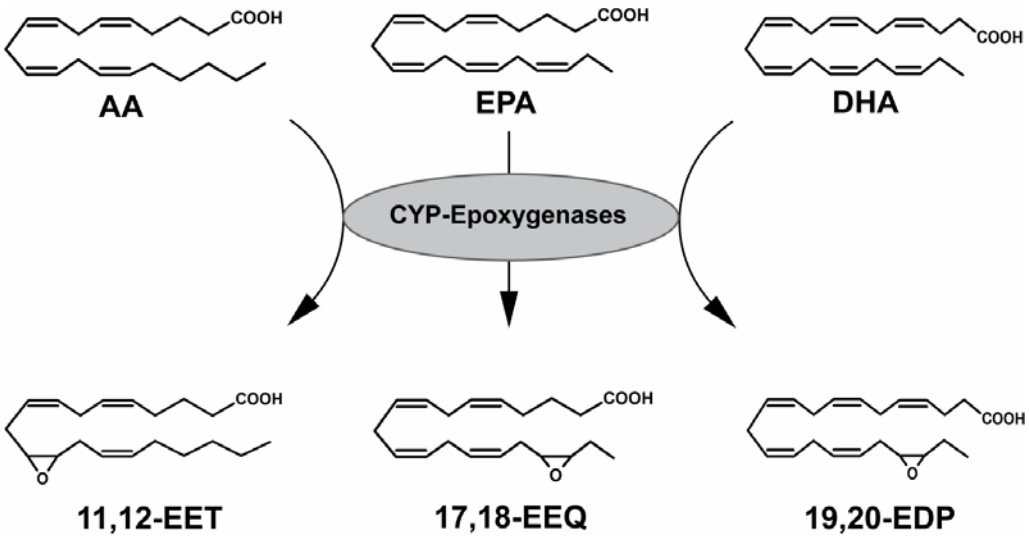

Figure 1: Cytochrome P450 epoxygenases accept arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as efficient alternative substrates. The products include isoform-specific sets of regioisomeric epoxyeicosatrienoic acids (EETs), epoxyeicosatetraenoic acids (EEQs) and epoxydocosapentaenoic acids (EDPs). Depicted are the three metabolites tested in the present study

Study participants

The stratification of apparently healthy subjects was performed according to stringent inclusion criteria [6, 28]. These were:

- no apparent illness
- no medication
- no somatic cardiovascular risk factors (e.g. diabetes, hypertension, lipid metabolism disorder)
- recorded laboratory parameters in the reference range

Blood collection, storage (e.g. due to temperature changes) and handling (e.g. during centrifugation to gain thrombocyte rich plasma) of the samples bear the risk of a pre-analytical artificial thrombocyte activation. Therefore, thrombocyte count (150.000 – 400.000 per l in the native blood), the spontaneous thrombocyte aggregation (MT < 10%), the thrombocyte reactivity index (TRI < 1.10) and the PFA-closure time (PFA < 71 sec) – using the Platelet-Function-Analyzer (Siemens PFA100™) - were checked before inclusion of the subjects. Only when all parameters were in the respective reference ranges the samples were used.

The study was carried out in accordance with the principles of the Declaration of Helsinki/Edinburgh 2000. Every subject was informed that participation in the study was voluntary. The participants were also told about the procedures and possible risks. Written informed consent was obtained from all patients. Ethical approval was received from the Ethics Committee of the Charité, Berlin, Germany and the study was performed in accordance with the ethical guidelines of Clinical Hemorheology and Microcirculation [2].

Study design

Whole blood was obtained from 10 apparently male healthy adult volunteers. To reduce artifical thrombocyte activation, blood samples were collected from an antecubital vein using a 19-gauge needle, gently inverted to ensure mixing and the first 2.5 ml were discarded. All samples were collected in Vacutainer tubes (Becton Dickinson, San Jose, CA) containing 3.8% trisodium-citrate solution as anticoagulant [5].

Immediately after venepuncture the experiments started. Thrombocyte-Poor Plasma (TPP) was prepared by centrifugation at 3345 *g* for 20 min, at room temperature. To obtain thrombocyte-rich plasma (TRP) samples were centrifuged at 120 g for 15 minutes. For experiments only the upper half of the supernatant was used to prevent leukocyte contaminations.

TRP was incubated with various concentrations of the different eicosanoids from stock solutions or the vehicle (ethanol up to 2‰ in plasma v/v) alone for 5, 30 and 60 minutes at 37 °C. Then, thrombocyte aggregation was induced by adding Ristocetin 0.75 mg/mL or 1 mg/ml. The aggregation process was monitored over 10 min starting from agonist addition to stirred TRP aliquots into glass tubes preheated to 37 °C.

Thrombocyte aggregation

The thrombocyte aggregation test was performed using the turbidimetric method described by Born and Cross [9] using the Thrombocyte Aggregometer APACT-4004 (Haemochrom Diagnostica, Essen, Germany) with four measuring channels, so that 4 aggregation measurements could be measured at the same time. Platelet aggregation was measured by light transmission, with 100% calibration as the absorbance of TPP and 0% calibration as the absorbance of unstimulated PRP on each run. The aggregation was measured by the height of aggregation (maximum inducible aggregation, MT [%]) and the rate of increase of aggregation (AT [%/time]). Figure 2 shows an example where the eicosanoid addition did not alter the maximum inducible aggregation MT, but was followed by a reduction of the rate of increase of aggregation (AT) coinciding with a delay of the aggregation process.

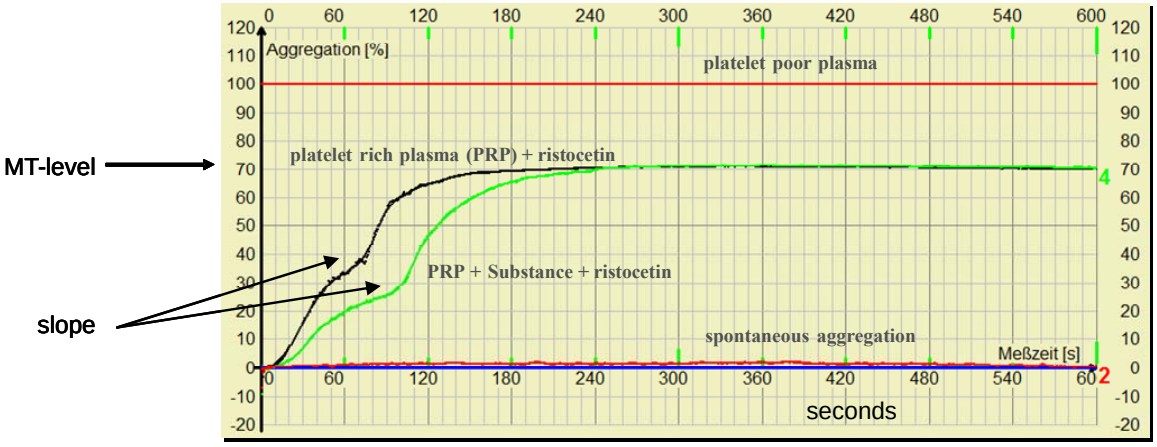

Figure 2: Individual examples of aggregation measurements (black line: control without eicosanoids; green line: platelet rich plasma preincubated for 60 min with 5 µM eicosatrienoic acid before adding 0.75 mg/ml Ristocetin)

Statistics
For all samples mean value and standard deviation are given, categorical data are described as percentage or relative frequency.
To test the efficacy of the different eicosanoids the two-sided t-test for paired samples was used. For multi-sample comparisons a variance analysis for repeated measures or a factorial variance analysis was performed. A probability value of less than 0.05 was accepted as significant. Because of the explorative character of the study no Bonferoni adjustment was performed.

Results
To include n=10 apparently healthy subjects fulfilling all inclusion criteria with normal thrombocyte function, n=16 adults between 25 and 62 years of age had to be examined.

Effect of ethanol on the Ristocetin-induced platelet aggregation
The eicosanoids used in the study were dissolved in ethanol. Therefore, it had to be examined how ethanol in this setting would influence the Ristocetin-induced thrombocyte aggregation (RITA). This analysis was performed testing TRP of six apparently healthy subjects (0 vol‰ up to 2 vol‰ final concentration of ethanol in plasma).
After addition of 1 mg/ml Ristocetin the thrombocyte aggregation increased considerably by 2,160% from 3.9±2.1% to 84.3±11.2% ($p<0.0001$). Ethanol (up to a concentration of 2 vol‰) did neither influence the maximum of the induced platelet aggregation MA, nor the rate of increase of aggregation AT after 5 minutes, 60 minutes or 120 minutes of incubation time.

Effect of 11,12-EET on the Ristocetin-induced platelet aggregation
Table 1 shows the RITA (after adding 0.75 mg/ml Risticetin) after a 5-, 30- or 60-minute incubation of the TRP at 37 °C with different concentrations of 11,12-EET.

Table 1: Ristocetin (0.75 mg/ml)-induced platelet aggregation after incubation at 37 °C of PRP with 1, 5, or 10 [µM] epoxyeicosatrienoic acid (11,12-EET) in comparison to Ristocetin-induced platelet aggregation with vehicle solution only (control)
(TRP: thrombocyte-rich plasma; MT: Maximum thrombocyte aggregation in [%]; AT: rate of increase of aggregation in [%/min]; *: $p<0.05$ compared to the Ristocetin-induced platelet aggregation at the same time points; mean value ± standard deviation)

	TRP+vehicle solution + 0.75 [mg/ml]**Ristocetin**	**TRP+11,12-EET** + 0.75 [mg/ml] **Ristocetin**		
		1 [µM]	5 [µM]	10 [µM]
	n=10	n=10	n=10	n=10
5 min incubation				
MT [%]	85.5±13.6	86.1±12.9	79.2±14.9	80.1±14.1
AT [%/min]	69.4±13.9	67.7±14.1	65.8±13.1	66.9±14.9
30 min incubation				
MT [%]	83.4±12.9	84.8±12.3	76.8±15.8	76.5±13.8
AT [%/min]	65.2±15.4	65.4±14.2	65.1±16.3	62.2±16.1
60 min incubation				
MT [%]	72.5±21.6	81.9±14.7	70.2±16.8	63.9±15.9
AT [%/min]	63.4±14.9	64.1±16.3	**58.0±17.0***	**51.1±17.2***

The incubation of TRP in the EET supplemented vehicle for 5 min or 30 minutes did not influence the platelet aggregation. A supplementation of 5 µM as well as of 10 µM 11,12-EET to the vehicle led after a 60-minute incubation to a marked decrease in the rate of increase of thrombocyte aggregation AT; compared to the TRP without addition of eicosanoids by 8.5% (p=0.0267) or 19.4% (p=0.014), respectively. The amplitude of the RITA MT was decreased, however, not significantly.

Effect of 17,18-EEQ on the Ristocetin-induced platelet aggregation

Table 2 shows the RITA (after adding 0.75 mg/ml Ristocetin) after 5-, 30- or 60-minute incubation of TRP at 37 °C with different concentrations of 17,18-EEQ.

Table 2: Ristocetin (0.75 mg/ml) induced platelet aggregation after 5, 30, and 60 minutes of incubation at 37 °C of PRP with 1 or 2 [µM] epoxyeicosatetraenoic acid (17,18-EEQ) in comparison to Ristocetin-induced thrombocyte aggregation with vehicle solution (control) only

(TRP: thrombocyte-rich plasma; MT: Maximum thrombocyte aggregation in [%]; AT: rate of increase of aggregation in [%/min]; *: p<0.05; **: p<0.005 compared to the Ristocetin-induced thrombocyte aggregation at the same time points; mean value ± standard deviation)

	TRP+vehicle solution + 0.75 [mg/ml] **Ristocetin**	**TRP+17,18-EEQ** + 0.75 [mg/ml] **Ristocetin**	
		1 [µM]	2 [µM]
	n=10	n=10	n=10
5 min incubation			
MT [%]	76.2±14.3	76.7±15.6	75.9±15.2
AT [%/min]	67.6±13.4	65.7±15.5	66.4±14.6
30 min incubation			
MT [%]	75.1±16.3	75.4±63.3	69.0±15.4
AT [%/min]	65.5±15.6	64.3±15.1	57.2±18.5
60 min incubation			
MT [%]	70.0±19.6	62.7±21.2	51.6±18.7
AT [%/min]	62.1±15.7	**45.1±18.1****	**43.5±19.1***

17,18-EEQ had a significant influence on the thrombocyte aggregation after 60 minutes of incubation. The rate of increase of aggregation **AT** was clearly decreased by 27.4% (p=0.0014) after incubation with 1 µM or by 30.0% (p=0.0018) after incubation with 2 µM compared to the slope of the aggregation curve without adding eicosanoids. The maximum of the thrombocyte aggregation **MT** was decreased merely in tendency by 10.4% (p= 0.3210) or by 26.3% (p=0.124).

Effect of 19,20-EDP on the Ristocetin-induced platelet aggregation

Table 3 shows the Ristocetin-induced (0.75 mg/ml) platelet aggregation after a 5-, 30- and 60-minute incubation of TRP with 1 µM 19,20-EDP. Compared to the thrombocyte aggregation without adding eicosanoids the slope of the aggregation curve was significantly decreased by 40.4% (p<0.001) after 60 minutes of incubation.

Table 3: Ristocetin (0.75 mg/ml) induced platelet aggregation after 5, 30, and 60 minutes of incubation at 37 °C of platelet-rich plasma with 1 [µM] 19,20-EDP in comparison to Ristocetin-induced thrombocyte aggregation with vehicle solution only (control)
(TRP: thrombocyte-rich plasma; MA: Maximum platelet aggregation in [%]; AT: rate of increase of aggregation in [%/min]; **: p<0.001 compared to the Ristocetin-induced platelet aggregation at the same time point; mean value ± standard deviation)

	PRP +vehicle solution + Ristocetin	**PRP+19,20-EDP + Ristocetin**
	0.75 [mg/ml] n=10	1 [µM] + 0.75 [mg/ml] n=10
5 min incubation		
MA [%]	75.5±19.6	75.7±21.5
AT [%/min]	85.7±4.0	86.0±4.0
30 min incubation		
MA [%]	75.4±20.3	74.0±12.0
AT [%/min]	75.9±11.2	63.0±7.0
60 min incubation		
MA [%]	70.2±21.6	63.3±7.0
AT [%/min]	71.2±10.5	**42.4±11.8****

Table 4 shows the Ristocetin-induced (0.75 mg/ml) platelet aggregation after a 5-, 30- and 60-minute incubation of the TRP with 5 µM AUDA or 10 µM AUDA (12-(3-adamantan-1-yl-ureido)dodecanoic acid) an inhibitor of the soluble epoxide hydrolase (sEH).

Table 4: Ristocetin (0.75 mg/ml) induced platelet aggregation after 5, 30, and 60 minutes of incubation at 37 °C of platelet-rich plasma with 5 [µM] or 10 [µM] AUDA in comparison to Ristocetin-induced thrombocyte aggregation with vehicle solution only (n=6)
(TRP: thrombocyte-rich plasma; MA: Maximum platelet aggregation in [%]; AT: rate of increase of aggregation in [%/min]; mean value ± standard deviation)

	TRP + vehicle solution + 0.75 mg/ml Ristocetin	**TRP + vehicle solution + 5 µM AUDA + 0.75 mg/ml Ristocetin**	**TRP + vehicle solution + 10 µM AUDA + 0.75 mg/ml Ristocetin**
5 min incubation			
MA [%]	80.3±19.2	78.4±19.7	85,7±6,0
AT [%/min]	73.9±23.3	71.8±10.9	68,3±13,0
30 min incubation			
MA [%]	80.2±13.2	80.5±15.2	84,1±10,7
AT [%/min]	73.7±24.1	74.8±10.4	66,1±11,8
60 min incubation			
MA [%]	71.9±23.9	72.9±21.8	69,6±27,0
AT [%/min]	64.7±23.4	62.3±14.6	60,8±19,7

Compared to the thrombocyte aggregation without adding eicosanoids there was no influence of AUDA on the RITA. Even at the highest concentration of 10 µM AUDA no significant influence was visible.

Effect of the Combination of 17,18-EEQ with AUDA on the Ristocetin-induced platelet aggregation

AUDA (12-(3-adamantan-1-yl-ureido)dodecanoic acid) is an inhibitor of the soluble epoxide hydrolase (sEH). It inhibits the hydrolysis of EPA-, DHA- und AA-epoxides (e.g. 11,12-EET, 17,18-EEQ, 19,20-EDP), extends the plasma half life time and thereby prolongs the effective period of these eicosanoids [31].

Table 5: Ristocetin-induced (0.75 mg/mL) thrombocyte aggregation after 5, 30, and 60 minutes of incubation of TRP with 1 [µM] 17,18-EEQ or with 10 [µM] 17,18-EEQ in combination with 10µM AUDA at 37 °C compared to Ristocetin-induced platelet aggregation with vehicle solution (control) only
(TRP: thrombocyte-rich plasma; MA: maximal platelet aggregation [%]; AT: slope of the aggregation graph in [%/min]; **: $p < 0.005$ ***; $p<0.0001$; compared to the Ristocetin-induced platelet aggregation without eicosanoids at the same time point; mean value ± standard deviation)

	TRP + Ristocetin 0.75 [mg/ml]	**TRP + 17,18-EEQ + AUDA** 1 [µM] +10 [µM]	**TRP +17,18-EEQ + AUDA** 10 [µM] + 10 [µM]
	n=10	n=10	n=10
5 min incubation			
MA [%]	89 ± 3	89 ± 3	89 ± 3
AT [%/min]	86 ± 4	88 ± 4	88 ± 4
30 min incubation			
MA [%]	79 ± 9	**59 ± 11****	59 ± 13
AT [%/min]	79 ± 10	**51 ± 31****	**59 ± 18**
60 min incubation			
MA [%]	69 ± 9	**49 ± 16****	62 ± 19
AT [%/min]	71 ± 11	**37 ± 7*****	54 ± 16

The simultaneous incubation of 1 µM 17,18-EEQ together with 10 µM AUDA led to a significant decrease of the rate of increase of aggregation **AT** as well as of the maximum amplitude of platelet aggregation **MA**.

The addition of 11,12-EET or 17,18-EEQ did not show any effects on the ADP- or on the collagen-induced platelet aggregation (see table 6).

Table 6: Ristocetin-induced (0.75 mg/mL) platelet aggregation after 60 minutes of incubation of PRP with 1 [µM] 11,12-EET or with 0.25 [µM] 17,18-EEQ at 37 °C compared to Ristocetin-induced platelet aggregation with vehicle solution (control) only
(TRP: thrombocyte-rich plasma; MA: maximal platelet aggregation [%]; AT: slope of the aggregation graph in [%/min]; mean value ± standard deviation)

	TRP	**TRP + 11,12-EET**	**TRP +17,18-EEQ**
	n=10	n=10	n=10
ADP [10µM]			
MA [%]	84.5±7,2	84.3±5.0	80.9±6.8
AT [%/min]	139.0±16.7	134.3±12.0	136.3±16.2
Kollagen [5µg/ml]			
MA [%]	84.5±5.9	79.7±9.8	83.1±6.0
AT [%/min]	136.1±16.4	114.4±37.4	128.6±19.3
spontaneous aggregation			
MA [%]	5.3±3.5	3.5±1.4	3.2±2.2
AT [%/min]	5.7±2.5	8.3±4.4	6.7±3.6

At a concentration of 1 mg/ml Ristocetin all effects were still recognizable in the trend but not longer significant.

Discussion

The study presented here revealed a marked concentration-dependent influence of different CYP-dependent eicosanoids - 11,12-EET, 17,18-EEQ and 19,20-EDP - on the Ristocetin-induced thrombocyte aggregation (RITA). After 60 minutes of incubation of the thrombocytes with the respective eicosanoids the increase of the aggregation was significantly delayed.
19,20-EPD and 17,18-EEQ were effective already at a concentration of 1 µM whereas 5-fold higher concentrations were required to achieve significant reduction of the thrombocyte aggregation rate with 11,12-EET. Simultaneous pre-incubation with 17,18-EEQ and AUDA [31], an inhibitor of the soluble epoxide hydrolase – sEH converts the epoxy-metabolites to their less active diols [49, 58] - potentiated the effect of 17,18-EEQ and was followed by a significant decrease of the amplitude MA of the RITA.

At the site of a vessel wall injury, the tethering of thrombocytes to the subendothelium is - under high shear flow - almost exclusively mediated by von Willebrand factor (vWF) which is found in the subendothelial extracellular matrix of the vessel wall, is circulating in blood plasma and is stored in the α-granula of thrombocytes [52]. vWF binding is dependent upon a specific thrombocyte receptor, the GPIb-IX-V complex [1]. Its binding to the GPIb-IX-V complex was shown to trigger intracellular signalling pathways, calcium flux and the calcium dependent activation of the thrombocyte membrane aggregation receptor for fibrinogen GPIIb/IIIa [11, 13, 29]. Via fibrinogen bridging a thrombocyte aggregate or thrombus then can develop.
The interaction between vWF and its thrombocyte receptor can well be studied *in vitro* using Ristocetin, a peptide antibiotic of the vancomycin group, described to mimic the interaction between the subendothelium and the vWF-factor so that thrombocytes can be bound [7, 8] with no differences in the spreading of and actin assembly in thrombocytes following their activation [37]. Therefore, in the present study Ristocetin was chosen as agonist to induce thrombocyte aggregation. If eicosanoids should interfere with the Ristocetin-induced thrombocyte aggregation, this would be a substantial reference to a possible effect on the

adherence of thrombocytes to dysfunctional blood vessel intimae or to body foreign surfaces of implants.
Clinical data [3], as well as cell culture measurements showed that after stimulation EETs, EEQs and EDPs were released from the endothelium and from thrombocyte membranes to the extracellular space [14, 35, 45, 61], relaxing vascular smooth muscle cells in a paracrine manner. Evidence that they are responsible for vasodilatory effects has been found in different vascular beds like mesenteric, renal, cerebral and coronary arteries [35, 39, 43, 56]. Substances causing vasodilation often also control thrombocyte function [25, 39]. While the vasodilatory capacity of these substances has been shown repeatedly, studies of their effects on thrombocyte function are rare.

The molecular mechanisms of the CYP450-dependent eicosanoids on thrombocyte aggregation are only partially understood. It was discussed that eicosanoids act on the thrombocyte membrane but not via receptor-coupled signal transduction [30]. Human thrombocytes can offer ~ 160.000 negatively charged phospholipids [48], such as (phosphatidylserine which is usually found on the cytoplasmic side of the outer cell membrane) on the extracellular side of the plasma membrane [36], generating a strongly negative net charge of platelets preventing – at a neutral pH – their clustering. Ristocetin seems to reduce the net charge in the vicinity of the GPI-receptors so far, that an interaction between thrombocytes and soluble vWF factor can proceed [22, 62]. Since the GPIb-IX-V-receptor complex also mediates the adherence of thrombocytes to body foreign surfaces also, it is well conceivable, that this process might be influenced by eicosanoids. Also in favour of this hypothesis is that eicosanoids do not influence the thrombocyte aggregation after stimuli which are thought not to be depending on the membrane-potential like ADP or collagen. An early study reporting a direct anti thrombocyte-aggregation effect of 11,12-EET *in-vitro* [15] could neither be confirmed in a later study by Kroetz et al. nor in the present study [33]. The group of Pohl reported that EETs are able to induce a mild hyperpolarisation of the platelet membrane by a blockade of thrombocyte K channels (mainly the large conductance BK_{Ca} channels) [30, 33] thereby preventing the influx of Ca^{++} (not the intra-cellular release via the PLC signal pathway [30, 38]). The hyperpolarisation of the thrombocyte resting potential was accompanied by an inhibition of the P-selectin expression [19, 33, 34, 42], a cell adhesion molecule, which is exposed on the outside of the thrombocyte membrane during activation, then promoting platelet aggregation [20].
Since the hyperpolarisation of thrombocytes by eicosanoids is a process, which needs considerably more than 10 minutes [44], this might explain the time necessary to produce the anti-aggregatory effects of the tested compounds. In the case of a blockade of Ca^{2+}-influx [34], platelet activation is thought to depend solely on the release of Ca^{2+} from the dense tubular system. Therefore, eicosanoid treated thrombocytes probably face a relative Ca^{2+} shortage compared to untreated platelets, possibly resulting in a deceleration of platelet activation and aggregation.

Additionally, eicosanoids are able to increase the activity of heme-oxygenase with a subsequent generation of carbon monoxide (CO) [47]. This gasotransmitter inhibits thrombocyte activation at low concentrations [51]. It acts through the same mechanism as NO but is less potent.
Last but not least it could be shown that EETs decreased the thrombocyte aggregation in vivo (in cerebral arterioles of mice) [23; 50]. It is discussed whether this effect was caused by a reduction of the release of thromboxane B2 - which could also explain the effect of EETs on thrombocyte aggregation.

The effects of monoepoxides - metabolized CYP-epoxygenases – derived from EPA, like 17,18-EEQ, or from DHA, like 19,20-EDP, are widely unknown. VanRollins reported that EEQs were able to influence the thrombocyte aggregation in concentrations where no inhibition of the thromboxane B2 release occurred [54]. The delayed increase in thrombocyte aggregation was markedly greater after incubation with 17,18-EEQ with 30.1% ($p=0.0018$) compared to 10,9% with 11,12-EET. This is in good agreement with former studies showing that 17,18-EEQ compared to 11,12-EET initiated an activation of calcium-dependent potassium channels double as high [35].
19,20-EDP has also been reported to be more potent than EET in activating BK_{Ca} channels [32, 55] and in mediating vasorelaxation [60] which is thought to be in line with our results. This epoxy-eicosanoid seems to be the most effective one regarding the anti-aggregatory efficacy since a significant delay of the thrombocyte aggregation could be obtained already at a concentration of 1 µM.
A significant decrease of the maximum thrombocyte aggregation was not observed until the metabolization of EEQ was suppressed by AUDA, an inhibitor of the soluble epoxide hydrolase (AUDA: 12-(3-adamantan-1-yl-ureido) dodecanoic acid).

Limitations of the Study
The sample size is with n=10 per group, too small, to be able to suggest the application of the eicosanoids used in this study for the prevention of thrombocyte aggregation. It cannot be excluded that by increasing the sample size significant differences also for other concentrations and eicosanoids could have been found.
A comparison with other studies is limited since in those studies other platelet aggregation agonists were used.

Conclusion
The main finding of our study is that CYP-dependent epoxyeicosanoids interfere with ristocetin-induced thrombocyte aggregation indicating that these metabolites may counteract the adhesion of thrombocytes to dysfunctional blood vessel intimae or to body foreign surfaces of implants. Our data are in line with studies from Krötz et al. who showed that EETs inhibited the adhesion of human thrombocytes to endothelial cells [33, 16] and from Sudhahar et al demonstrating that EETs led to a decrease of the in vivo thrombocyte aggregation [50].
In addition, we could show in this study that the epoxyeicosanoids derived from omega-3 polyunsaturated fatty acids (17,18-EEQ and 19,20-EDP) are clearly more potent inhibitors of Ristocetin-induced thrombocyte aggregation compared to the AA-derived 11,12-EET. The finding that AUDA potentiated the effect of 17,18-EEQ suggests that the anti-aggregatory activity of the epoxyeicosanoids is abolished by sEH-mediated hydrolysis.

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References

[1] R.K. Andrews, E.E. Gardiner, Y. Shen, J.C. Whisstock, and M.C. Berndt, Molecules in focus. Glyoprotein Ib-IX-V. *International Journal of Biochemistry & Cell Biology* **35** (2003), 1170 – 1174.

[2] Anonymous. Ethical Guidelines for Publication in Clinical Hemorheology and Microcirculation. *Clinical Hemorheology and Microcirculation* **44** (2010), 1-2.

[3] S.L. Archer, F.S. Gragasin, X. Wu, S. Wang, S. McMurtry, D.H. Kim, M. Platonov, A. Koshal, K. Hashimoto, W.B. Campbell, J.R. Falck, Michelakis ED. Endothelium-derived hyperpolarizing factor in human internal mammary artery is 11,12-epoxyeicosatrienoic acid and causes relaxation by activating smooth muscle BK(Ca) channels, *Circulation* **107** (2003), 769–776.

[4] C. Arnold, M. Markovic, K. Blossey, G. Wallukat, R. Fischer, R. Dechend, A. Konkel, C. von Schacky, F.C. Luft, D.N. Muller, M. Rothe, and W.H. Schunck, Arachidonic acid-metabolizing cytochrome p450 enzymes are targets of omega-3 fatty acids, *J Biol Chem* **285** (2010), 32720-31733.

[5] O.K. Baskurt, M. Boynard, G.C. Cokelet, P. Connes, B.M. Cooke, S. Forconi, F. Liao, R. Hardeman, F. Jung, H.J. Meiselman, G. Nash, N. Nemeth, B. Neu, B. Sandhagen, S. Shin, G. Thurston, and J.L. Wautier, International Expert Panel for Standardization of Hemorheological Methods. New guidelines for hemorheological laboratory techniques, *Clin Hemorheol Microcirc* **42** (2009), 75-97.

[6] B. Berg, H.E. Solberg, J.E. Nilsson, and N. Tryding, Practical experience in the selection and preparation of reference individuals: empirical testing of the provisional scandinavian recommendations. In: Reference values in laboratory medicine. Gräsbeck R, Alström T. (eds). John Wiley & sons, Chichester (1981), pp. 55-64.

[7] M.C. Berndt, and J.P. Caen, Platelet glycoproteins. *Prog Haemost Thromb* **7** (1984), 111 – 150.

[8] M.C. Berndt, X. Du, and W.J. Booth, Ristocetin-dependent reconstitution of von Willebrand factor to purified human platelet membrane glycoprotein IB-IX complex. *Biochemistry* **27**; 1988: 633 – 640.

[9] G.V. Born, and M.J. Cross, The Aggregation of blood platelets. *J Physiol* **168** (1963), 178-195.

[10] W.B. Campbell, and J.R. Falck, Arachidonic acid metabolites as endothelium-derived hyperpolarizing factors. *Hypertension* **49** (2007), 590-596.

[11] K.J. Clemetson, Platelet activation: signal transduction via membrane receptors. *Thromb Haemostas* **74** (1995), 111 – 116.

[12] M.G. Cohen, J.S. Rossi, J. Garbarino, R. Bowling, A.A. Motsinger-Reif, C. Schuler, A.G. Dupont, and D. Gabriel, Insights into the inhibition of platelet activation by omega-3 polyunsaturated fatty acids: Beyond aspirin and clopidogrel. *Thrombosis Research* **128** (2011), 335-340.

[13] B.S. Coller, and H.R. Gralnick, Biochemical mechanism of ristocetin-induced platelet agglutination, *Clin Res* **24** (1976), 305A.

[14] B. Fisslthaler, R. Popp, L. Kiss, M. Potente, D.R. Harder, I. Fleming, and R. Busse, Cytochrome P450 2C is an EDHF synthase in coronary arteries, *Nature* **401** (1999), 493–497.

[15] F.A. Fitzpatrick, Ennis M. D., Baze M. E., Wynalda M. A., McGee J. E., and Liggett W. F, Inhibition of Cyclooxygenase Activity and Platelet Aggregation by Epoxyeicosatrienoic Acids, *J Biol Chem* **261** (1986), 15334-15338.

[16] I. Fleming, Cytochrome p450 and vascular homeostasis, *Circ Res* **89** (2001), 753-762.

[17] I. Fleming, Epoxyeicosatrienoic acids, cell signaling and angiogenesis, *Prostaglandins Other Lipid Mediat*. **82** (2007), 60-67.

[18] Fourth American College of Chest Physicians Consensus Conference on Antithrombotic Therapy, *Chest* **108**(4 Suppl) (1995), 225S-522S.
[19] L.T. Friedhoff , E. Kim, M. Priddle, and M. Sonenberg, The effect of altered transmembrane ion gradients on membrane potential and aggregation of human platelets in blood plasma, *Biochem Biophys Res Commun* **102** (1981), 832 – 837.
[20] B. Furie, and B.C. Furie, Role of platelet P-selectin and microparticle PSGL-1 in thrombus formation, *Trends in molecular medicine* **10** (2004): 171–178.
[21] G. Gajos, P. Rostoff, A. Undas, and W. Piwowarska, Effects of polyunsaturated omega-3 fatty acids on responsiveness to dual antiplatelet therapy in patients undergoing percutaneous coronary intervention: The omega-pci (omega-3 fatty acids after pci to modify responsiveness to dual antiplatelet therapy) study, *J Am Coll Cardiol* **55** (2010), 1671-1678.
[22] D. Green, and E.V. Potter, Platelet-bound ristocetin aggregation factor in normal subjects and patients with von Willebrand's disease, *J Lab Clin Med* **87** (1976), 976-986.
[23] M.L. Heizer, J.S. McKinney, and E.F. Ellis 14,15-Epoxyeicosatrienoic acid inhibits platelet aggregation in mouse cerebral arterioles, *Stroke* **22** (1991), 1389-93.
[24] Hercule HC, Salanova B, Essin K, Honeck H, Falck JR, Sausbier M, Ruth P, Schunck WH, Luft FC, Gollasch M. The vasodilator 17,18-epoxyeicosatetraenoic acid targets the pore-forming bk alpha channel subunit in rodents. *Exp Physiol* **92** (2007), 1067-1076.
[25] L.J. Ignarro, Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein, *Circ Res* **65** (1989), *1*–21.
[26] M. Joner, A.V. Finn, A. Farb, E.K. Mont, F.D. Kolodgie, E. Ladich, R. Kutys, K. Skorija, H.K. Gold and R. Virmani, Pathology of rertug-eluting stents in humans: delayed heaöling and late thrombotic risk, *J Am Coll Cardiol* **48** (2006), 193 – 202.
[27] F. Jung, C. Mrowietz, U.T. Seyfert, R. Grewe and R.P. Franke, Influence of the direct NO-donor SIN-1 on the interaction between platelets and stainless steel stents under dynamic conditions, *Clin Hemorheol Microcirc* **28** (2003), 189-99.
[28] F. Jung, C. Wischke and A. Lendlein, Degradable, multifunctional cardiovascular implants: challenges and hurdles, *MRS Bull* **35** (2010), 607 – 613.
[29] H.E. Kattlove and M.H. Gomez, Studies on the mechanism of ristocetin-induced platelet aggregation, *Blood* **45** (1975), 91-96.
[30] C. Kenneth, C. Malcolm and F.A. Fitzpatrick, Epoxyeicosatrienoic Acids Inhibit $Ca2^{+}$ Entry into Platelets Stimulated by Thapsigargin and Thrombin, *J Biol Chem* **267** (1992), 19854-13858.
[31] I.H. Kim, K. Nishi, H.J. Tsai, T. Bradford, Y. Koda, T. Watanabe, C. Morisseau, J. Blanchfield, I. Toth, B.D. Hammock, Design of bioavailable derivatives of 12-(3-adamantan-1-yl-ureido)dodecanoic acid, a potent inhibitor of the soluble epoxide hydrolase. *Bioorg Med Chem* **15** (2007), 312-23.
[32] A. Konkel, and W.H. Schunck, Role of cytochrome P450 enzymes in the bioactivation of polyunsaturated fatty acids, *Biochim biophys acta* **1814** *(*2011), 210-222.
[33] F. Krötz, T. Riexinger, M.A. Buerkle, K. Nithipatikom, T. Gloe, H.Y. Sohn, W.B. Campbell and U. Pohl, Membranepotential-dependent inhibition of platelet adhesion to endothelial cells by epoxyeicosatrienoic acids, *Arterioscler Thromb Vasc Biol* **24** (2004), 595-600.
[34] F. Krötz, N. Hellwig, M.A. Bürkle, S. Lehrer, T. Riexinger, H. Mannell, H.Y. Sohn, V. Klauss, and U. Pohl, A sulfaphenazole-sensitive EDHF opposes platelet-endothelium interactions in vitro and in the hamster microcirculation in vivo, *Cardiovasc Res* **85** (2010), 542-50.
[35] B. Lauterbach, E. Barbosa-Sicard, M.H. Wang, H. Honeck, E. Kärgel, J. Theuer, M.L. Schwartzman, H. Haller, F.C. Luft, M. Gollasch, M.H. Schunck, Cytochrome P450-

dependent eicosapentaenoic acid metabolites are novel BK channel activators, *Hypertension* **39** (2002), 609-613.
[36] T. Lhermusier, H. Chap, and B. Payrastre, Platelet membrane phospholipid asymmetry: from the characterization of a scramblase activity to the identification of an essential protein mutated in Scott syndrome, *J Thromb Haemost* **9** (2011), 1883-91.
[37] O.J.T. McMarty, S.D.J. Calaminus, M.C. Berndt, L.M. Machesky, and S.P. Watson, Von Willebrand factor mediates platelet spreading through glycoprotein IB and GPIIbIIIa in the presence of botrocetin and ristocetin, respectively, *Thromb Haemostas* **4** (2006), 1367 – 1378.
[38] E.P. Milner, Q. Zheng, and J.C. Kermode, Ristocetin-mediated interaction of human von Willerand factor with platelet glycoprotein Ib evokes a transient calcium signal: observations with FURA-PE3, *J Lab Clin Med* **131** (1998), 49 – 62.
[39] H. Miura, and D.D. Gutterman, Human coronary arteriolar dilation to arachidonic acid depends on cytochrome P-450 monooxygenase and Ca2+-activated K+ channels, *Circ Res* **83** (1998), *501*–507.
[40] E. Morgenstern, A. Ruf, and H. Patscheke, Ultrastructure of the interaction between human platelets and polymerizing fibrin within the first minutes of clot formation, *Blood Coagul Fibrinolysis* **1** (1990), 543-6.
[41] G. Nakazawa, A.V. Finn, M.C. John, F.D. Kolodgie, and R. Virmani, The significance of preclinical evaluation of sirolimus-, paclitaxel- and zotarolimus-slitung stents, *Am J Cardiol* **100** (2007), 36 – 44.
[42] E. Pipili, Platelet membrane potential: simultaneous measurement of diSC3(5) fluorescence and optical density, *Thromb Haemostas* **54** (1985), 645 – 649.
[43] J. Quilley, and J.C. McGiff, Is EDHF an epoxyeicosatrienoic acid? *Trends Pharmacol Sci* **21** (2000), 121–124.
[44] T. Riexinger, Ein Cytochrom P450 2C8/9-abhängig gebildeter EDHF hyperpolarisiert Thrombozyten und hemmt deren Adhäsion an Endothelzellen. Dissertationsschrift, Ludwig-Maximilians-Universität, München, 2008.
[45] G.E. Revtyak, M.J. Hughes, A.R. Johnson, and W.B. Campbell, Histamine stimulation of prostaglandin and HETE synthesis in human endothelial cells, *Am J Physiol* **255** (1988), C214–*C225.*
[46] R.J. Roman, P-450 metabolites of arachidonic acid in the control of cardiovascular function, *Physiol Rev* **82** (2002), 131-185.
[47] D. Sacerdoti, C. Colombrita, M. Di Pascoli, M.L. Schwartzman, M. Bolognesi, J.R. Falck, A. Gatta, and N.G. Abraham, 11,12-epoxyeicosatrienoic acid stimulates heme-oxygenase-1 in endothelial cells, Prostaglandins *Other Lipid Mediat* **82** (2007), 155-61.
[48] G.V.F. Seaman, and D.E. Brooks, Electrochemical aspects of platelet adhesion and aggregation. *Thromb Diath Haemorrh* **42** (1970), 93-107.
[49] A.A. Spector, and A.W. Norris, Action of epoxyeicosatrienoic acids on cellular function. *Am J Physiol Cell Physiol* **292** (2007), C996-1012
[50] V. Sudhahar, S. Shaw, and J.D. Imig, Epoxyeicosatrienoic acid analogs and vascular function, *Curr Med Chem* **17** (2010), 1181-90.
[51] N.J. Truss, and T.D. Warner, Gasotransmitters and platelets, *Pharmacol Ther* **132** (2011), 196-203.
[52] V.T. Turitto, H.J. Weiss, T.S. Zimmerman, I.I. Sussman, FactorVIII/vonWillebrand factor in subendothelium mediates platelet adhesion. *Blood* **65** (1985), 823 – 831.
[53] B. van der Loo, S. Spring, R. Koppensteiner, High-dose atorvastatin tratment in patients with peripheral arterial disease: effects on platelet aggregation, blood rheology and plasma homocysteine, *Clin Hemorheol Microcir* **47** (2011), 241 – 251.

[54] M. VanRollins, Epoxygenase metabolites of docosahexaenoic and eicosapentaenoic acids inhibit platelet aggregation at concentrations below those affecting thromboxane synthesis, *J Pharmacol Exp Ther* **274** (1995), 798-804.
[55] R.X. Wang, Q. Chai, T. Lu, and H.C. Lee, Activation of vascular BK channels by docosahexaenoic acid is dependent on cytochrome P450 epoxygenase activity, *Cardiovascular research* **90** (2011), 344-352.
[56] M.D. Widmann, N.L. Weintraub, J.L. Fudge, L.A. Brooks, and K.C. Dellsperger, Cytochrome P-450 pathway in acetylcholine-induced canine coronary microvascular vasodilation in vivo, *Am J Physiol* **274** (1998*), H283*–H289.
[57] D. Ye, D. Zhang, C. Oltman, K. Dellsperger, H.C. Lee, and M. VanRollins, Cytochrome p-450 epoxygenase metabolites of docosahexaenoate potently dilate coronary arterioles by activating large-conductance calcium-activated potassium channels, *J Pharmacol Exp Ther* **303** (2002), 768-776.
[58] D.C. Zeldin, J. Kobayashi, J.R. Falck, B.S. Winder, B.D. Hammock, J.R. Snapper, and J.H. Capdevila, Regio-, and enantiofacial selectivity of epoxyeicosatrienoic acid hydration by cytosolic epoxide hydrolase, *J Biol Chem* **268** (1993), 6402–6407.
[59] L. Zhang, Y. Cui, B. Geng, X. Zeng, and C. Tang, 11,12-Epoxyeicosatrienoic acid activates the L-arginine/nitric oxide pathway in human platelets, *Mol Cell Biochem* **308** (2008), 51-56.
[60] Y. Zhang, C.L. Oltman, T. Lu, H.C. Lee, K.C. Dellsperger, and M. VanRollins, EET homologs potently dilate coronary microvessels and activate bk(ca) channels, *Am J Physiol Heart Circ Physiol* **280** (2001), H2430-2440.
[61] Y. Zhu, E.B. Schieber, J.C. McGiff, and M. Balazy, Identification of arachidonate P-450 metabolites in human platelet phospholipids, *Hypertension* **25** (1995), 854–859.
[62] M. Zucker, K. Sook-Ja, J. McPherson, and R. Grant, A. Binding of factor VIII to platelets in the presence of ristocetin, *Br J Haematol* **35** (1977), 535-549.