Fractalkine Activates a Signal Transduction Pathway Similar to P2Y₁₂ and Is Associated With Impaired Clopidogrel Responsiveness

Ulrike Flierl, Daniela Fraccarollo, Eva Lausenmeyer, Tim Rosenstock, Christian Schulz, Steffen Massberg, Johann Bauersachs, Andreas Schäfer

Objective—Fractalkine (FKN) activates a G protein–coupled signaling pathway similar to the one activated by ADP via P2Y₁₂, which is the drug target of clopidogrel. FKN levels are increased under several disease conditions associated with impaired clopidogrel responsiveness.

Methods and Results—Blood samples were obtained from healthy volunteers and from 40 patients under chronic clopidogrel treatment. FKN reduced prostaglandin E₁–induced vasodilator-stimulated phosphoprotein phosphorylation by ≈25% (P<0.01) at least partially mimicking the effect of ADP via P2Y₁₂. In vitro, FKN increased platelet reactivity index in clopidogrel-treated patients indicating potential activation of downstream targets of P2Y₁₂. When stratifying patients by their FKN levels, patients within the highest quartile of FKN (2042±25 pg/mL) had the weakest response to clopidogrel (platelet reactivity index, 68±4%), and patients within the lowest quartile (479±50 pg/mL) had the strongest response (platelet reactivity index, 48±7%; P=0.0106). FKN by itself induced phosphoinositide 3-kinase activation leading to Akt phosphorylation at Ser⁴⁷₃ (P<0.01 versus basal).

Conclusion—In addition to desensitizing platelets to prostaglandin E₁ via G protein-dependent Akt phosphorylation via a G protein similar to ADP signaling through P2Y₁₂, FKN increased the platelet ADP response in clopidogrel-treated patients. Once released from an atherosclerotic lesion, this mechanism could contribute locally to impaired clopidogrel responsiveness at the vulnerable plaque.

Key Words: fractalkine • G protein–coupled receptors • P2Y₁₂ • thienopyridines

A variety of chemokines including fractalkine (chemokine [C-X3-C motif] ligand 1, FKN) induce proinflammatory and prothrombotic effects in addition to their primary immunologic actions.¹⁻⁴ FKN differs from other chemokines by a polypeptide chain that carries the chemokine domain on top of an extended mucine-like stalk and allows the molecule to exist either as a membrane-anchored or as a soluble glycoprotein.⁵ FKN is expressed in endothelial cells in response to proinflammatory agents,⁶ and its expression is enhanced in vascular injury, atherosclerosis, and coronary artery disease (CAD).⁷⁻⁹ Furthermore, it induces platelet activation and adhesion via a functional FKN receptor (chemokine [C-X3-C motif] receptor 1 [CX3CR1]) expressed on the platelet surface.¹⁰ Importantly, platelet activation via the FKN/CX3CR1 axis triggers leukocyte adhesion to activated endothelium, and FKN-induced platelet P-selectin is mandatory for leukocyte recruitment under arterial flow conditions.¹¹ CX3CR1 has been described as a G protein–coupled receptor,¹² but the exact signaling pathway has not yet been explored. Bearing in mind that one of the most important platelet activating receptors, the ADP receptor P2Y₁₂, also exerts its action via a G protein–linked mechanism, a link between the receptors’ downstream signaling is suspected.

Clopidogrel, an irreversible P2Y₁₂ receptor inhibitor, has been shown to be efficient to prevent ischemic events in selected patients with atherosclerosis disease.¹⁰ Although, impaired responsiveness to clopidogrel has been observed and described,¹³ Activation of the P2Y₁₂ receptor inhibits cAMP production by adenyl cyclase.¹⁴ The platelet reactivity index (PRI) using vasodilator-stimulated phosphoprotein (VASP) phosphorylation is a standardized P2Y₁₂–specific assay that is based on the P2Y₁₂ specific inhibition of adenyl cyclase by ADP and that assesses the difference in prostaglandin E₁ (PGE₁)–induced adenyl cyclase–mediated VASP phosphorylation in the absence and presence of ADP using flow cytometry.¹⁴ There are many hypotheses about the causes of the above-mentioned impaired clopidogrel response (eg, polymorphisms in metabolizing enzymes,¹⁵ drug–drug interactions,¹⁶ certain comorbidities). The Residual Platelet Aggregation

Received on: March 15, 2011; final version accepted on: April 23, 2012.
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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org
DOI: 10.1161/ATVBAHA.112.250720
after Deployment of Intracoronary Stent score was established to estimate the likelihood of impaired clopidogrel responsiveness based on easily available patient details. Thus, among others, diabetes mellitus, renal failure, and chronic heart failure are associated with impaired response to P2Y$_{12}$ blockade. Interestingly, elevated FKN levels were observed in the above-mentioned comorbidities. Therefore, a potential link between FKN levels and clopidogrel responsiveness was assumed.

We hypothesized that FKN induces a pathway similar to that of P2Y$_{12}$, thus inhibiting cAMP production and desensitizing platelets to the endogenous platelet inhibitor PGE1. Furthermore, we evaluated a link between FKN levels and clopidogrel responsiveness in patients with CAD.

**Methods**

**Patients**

For in vitro experiments in the absence of clopidogrel pretreatment, blood samples were collected from healthy donors who gave informed consent and who had not taken any antiplatelet medication within the last 10 days.

To evaluate clopidogrel responsiveness and FKN levels, blood samples were obtained from 40 consecutive clopidogrel-treated patients with stable CAD. Clopidogrel responsiveness was determined by the P2Y$_{12}$-specific PRI with a cutoff value of 50% as previously reported. All patients had been admitted to the Cardiology Division of the University Hospital of Würzburg and gave informed consent. The study was performed in accordance with the Declaration of Helsinki.

**Blood Sample Collection**

Blood samples were collected from an antecubital vein using a 21-gauge needle. The first 5 mL of blood was discarded to avoid spontaneous platelet activation. Platelet-rich plasma (PRP) was prepared from citrated blood by centrifugation at 320g for 10 minutes, and HEPES (pH 7.4; 1-mL acid citrate dextrose/4-mL blood). PRP was kept at 37°C before use. Platelet-poor plasma was prepared by centrifuging PRP at 1000g for 5 minutes.

**Platelet Aggregation**

Platelet aggregation was performed with light transmittance aggregometry using a commercial 8-channel platelet aggregation profiler (PAP-8; Bio/Data Corp, Horsham, PA). Aggregation was induced by different concentrations of ADP in PRP. Some samples of PRP were preincubated with PGE1 and FKN (1 μg/mL and 2 μg/mL; 5 minutes) and were afterward stimulated with ADP. Light transmission was adjusted to 0% with PRP and to 100% using platelet-poor plasma for each measurement. Curves were recorded for 6 minutes. Aggregation was measured at primary, secondary, and maximal aggregation. In some experiments, the maximum aggregation of samples after preincubation with PGE1 and FKN was calculated in percentage of the maximum aggregation of PRP not preincubated with FKN, which had been stimulated in parallel with the same dose of ADP.

**Preparation of Washed Platelets**

For experiments requiring washed platelet preparations, blood was collected into acid citrate dextrose (citric acid [3.8 mmol/L] and dextrose [125 mmol/L]; 1-mL acid citrate dextrose/4-mL blood). PRP was obtained by centrifugation at 320g for 10 minutes, and HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)--modified Tyrode solution buffer 1 (NaCl, 132 mmol/L; KCl, 4 mmol/L; NaHCO$_3$, 11.9 mmol/L; NaHPO$_4$, 0.36 mmol/L; glucose, 10 mmol/L; pH 6.5; 1-mL PRP/3.5-mL buffer 1) was added. Plasma-free platelet suspensions were obtained by centrifugation (400g; 17 minutes of PRP; the resulting pellet was resuspended in HEPES-modified Tyrode solution buffer 2 (buffer 1; pH 7.4; CaCl$_2$, 1 mmol/L; MgCl$_2$, 1 mmol/L) to achieve a final platelet density of 200 000/μL. When indicated, aspirin (20 μg/mL) and the α-adrenergic receptor inhibitor BRL 44408 (10 μmol/L) were added to platelet samples.

**Western Blotting Analysis**

Washed platelets were kept in a water bath at 37°C for 15 minutes before adding 100 μL of a 3x sodium dodecyl sulfate stop buffer (consisting of Tris/HCl, 200 mmol/L; pH 6.7; 15% [vol/vol] glycerol; 6% [wt/vol] sodium dodecyl sulfate) per 100 μL of washed platelet suspension. Samples were boiled for 5 minutes at 95°C before storage at −20°C.

Platelet samples were mixed with sample loading buffer and were separated under reducing conditions on 10% sodium dodecyl sulfate polyacrylamide gel. Proteins were electrotransferred onto a polyvinylidene fluoride membrane (0.2 μmol/L; Immun-Blot, Bio-Rad, Hercules, CA). After transfer, the membranes were blocked in blotting solution (Tris-HCl, 20 mmol/L; NaCl, 150 mmol/L; 0.05% Tween 20; pH 7.6) with 5% blocking agent (Amersham, Little Chalfont, United Kingdom) overnight at 4°C followed by incubation with the primary monoclonal rabbit antiphospho-akt (Ser$^{119}$) or antiphosphoakt (Thr$^{286}$) antibody (1:1000; Cell Signaling, Danvers, MA) in blotting solution with 0.5% blocking agent for 2 hours. After washing, the blots were incubated with horseradish peroxidase–labeled goat anti-rabbit IgG antibody (1:10 000; Amersham) for 1 hour. The bands were detected using the enhanced chemiluminescence assay (ECL-Plus kit; Amersham). After autoradiography (Kodak Biomax; Kodak, Rochester, NY), densitometric analysis was performed using National Institutes of Health Image computer software. Molecular weights were determined using prestained SDS-PAGE standard and precision protein standards (Bio-Rad). All reagents for electrophoresis were purchased from Sigma.

**P2Y$_{12}$ PRI**

PRI was determined by assessing VASP status using flow cytometry (FACS Calibur; Becton Dickinson, Heidelberg, Germany). VASP phosphorylation was quantified with labeled monoclonal antibodies using a commercially available kit (PLT VASP/P2Y$_{12}$ Test kit; Biocytex, Marseille, France), for which platelets were stimulated with PGE1+ADP followed by fixation with formaldehyde. After permeabilization with Triton X-100, VASP phosphorylation at Ser$^{119}$ was determined using a monoclonal antibody (16C2) and a fluorescein isothiocyanate–labeled secondary antibody whereas platelets were counterstained with a PE-labeled anti-CD61 antibody, similar to the one described initially. The PRI was calculated after measurement of VASP phosphorylation after stimulation with PGE1 (0.5 μmol/L) by mean fluorescence intensity (MFI PGE1), as well as stimulation with PGE1 in the presence of ADP (20 μmol/L; MFI PGE1+ADP). PRI is defined as: (MFI PGE1−MFI PGE1+ADP)/MFI PGE1×100%, whereby background fluorescence is subtracted from each measurement. The lower the P2Y$_{12}$ PRI, the better the inhibition of P2Y$_{12}$ activity by clopidogrel.

**FKN ELISA**

Human serum FKN was determined using the Human CX3CL1/Fractalkine Quantikine ELISA Kit (R&D Systems, Minneapolis, MN).

**Substances**

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany) in the highest purity available. Recombinant human FKN (365-FR) was purchased from R&D Systems. Ticagrelor was provided by AstraZeneca (Mölndal, Sweden).
Statistics
Data are presented as means±SEM and analyzed using 1-way ANOVA with a Tukey post hoc test. P<0.05 was considered statistically significant.

Results
Phosphorylation of VASP in Platelets
One particular P2Y$_{12}$-specific activity of ADP signaling is G$_{ai}$-dependent inhibition of adenylyl cyclase leading to reduced sensitivity of platelets to prostanoids like PGE1.

FKN also induces platelet activation through a G$_{ai}$-coupled receptor.$^{10}$ We wondered whether FKN would also desensitize platelets for endogenous platelet inhibitors in a G$_{ai}$-dependent mechanism similar to P2Y$_{12}$. In PRP from healthy human donors, FKN significantly reduced PGE1-induced VASP phosphorylation (Figure 1A–1C; Figure I in the online-only Data Supplement).

In Vitro Effects of FKN Despite P2Y$_{12}$ Blockade
Similar to the above-mentioned PRI (see Methods), blood samples were stimulated with PGE1 and treated with ADP in the presence/absence of ticagrelor, a reversible P2Y$_{12}$ inhibitor that directly interacts with the receptor without requiring metabolism to generate an active compound.$^{24}$ The concentration of ticagrelor (1 μg/mL) was chosen to completely block P2Y$_{12}$-mediated adenylyl cyclase inhibition and to guarantee that the FKN effect is assessed in the absence of P2Y$_{12}$ signaling.

Thereafter, VASP phosphorylation was detected by flow cytometry. ADP significantly decreased PGE1-mediated VASP phosphorylation, which was blunted in the presence of ticagrelor. Interestingly, the addition of FKN significantly reduced PGE1-induced VASP phosphorylation in the presence of ticagrelor indicating a CX3CR1-mediated mechanism, which mimics the ADP effect and is functionally independent from P2Y$_{12}$ activation (Figure 2).

In Vitro Effects of FKN on Platelet Aggregation
Functionally, PGE1-induced cAMP formation suppresses the ADP-evoked, P2Y$_{12}$-mediated secondary phase of platelet aggregation. Pretreatment with FKN (1 μg/mL) abrogated the PGE1-mediated reduction of platelet aggregation.

Figure 1. Platelet vasodilator-stimulated phosphoprotein (VASP) phosphorylation at Ser$^{157}$ after stimulation with prostaglandin E1 (PGE1) and fractalkine (FKN). PGE1-induced VASP phosphorylation was significantly attenuated by preincubation with FKN (1 μg/mL) in a time-dependent pattern (A) and consistently at different concentrations of PGE1 (B and C). n=7–11. MFI indicates mean fluorescence intensity; ns, nonsignificant.
after stimulation with ADP with impact on primary, secondary, and final aggregation (Figure 3). FKN did not influence ADP-induced calcium signaling nor did it have an effect of its own (data not shown). Moreover, neither shape change nor aggregation to FKN alone was observed (data not shown).

Exploration of FKN-Mediated Platelet Signaling Pathway

Because the aforementioned results indicate an FKN-induced pathway similar to the $G_\alpha_i$-mediated signaling of P2Y$_{12}$ activation, we investigated whether there is an impact not only on $G_\alpha_i$-mediated but also on $G_{\beta\gamma}$-mediated P2Y$_{12}$-like

![Diagram](Figure 2)

**Figure 2.** Vasodilator-stimulated phosphoprotein (VASP) phosphorylation at Ser$^{157}$ (A) and Ser$^{239}$ (B) in the presence of P2Y$_{12}$ receptor inhibitor ticagrelor (Tica) was determined using flow cytometry. Samples were stimulated with prostaglandin E1 (PGE1, 1 μmol/L) and ADP (20 μmol/L) in the absence or presence of fractalkine (FKN, 2 μg/mL) and Tica (1 μg/mL). ADP significantly decreased PGE1-mediated VASP phosphorylation, which was abrogated in the presence of Tica. In the presence of full P2Y$_{12}$ blockade by Tica, FKN significantly reduced PGE1-induced VASP phosphorylation. n=5–6; MFI indicates mean fluorescence intensity.

![Diagram](Figure 3)

**Figure 3.** Modulation of prostaglandin E1 (PGE1)-induced and cAMP-mediated inhibition of the secondary phase of ADP-induced aggregation by fractalkine (FKN, 1 μg/mL) in platelet-rich plasma from healthy volunteers. ADP concentrations, which from 1.5 to 2.5 μmol/L as medium extent of aggregation, were intended for ADP induction. A. Representative aggregation response curves from a healthy donor were recorded in the presence of ADP only (trace 1), ADP+FKN (trace 2), ADP+PGE1 (trace 3), and ADP+FKN+PGE1 (trace 4). The quantitative analysis is shown for primary (B), secondary (C), and maximal (D) aggregation. *P<0.05 vs all; n=6.
signal transduction. Hence, immunoblotting experiments with washed platelets obtained from healthy volunteers were performed. Such a pathway would have to activate phosphoinositide 3-kinase (PI3K) and lead to Akt phosphorylation.²⁵ FKN by itself induced PI3K activation leading to significantly increased Akt phosphorylation at Ser⁴⁷³ compared with nonstimulated samples (Figure 4A and 4B). Moreover, FKN even potentiated PI3K activation when added to ADP compared with ADP stimulation alone (Figure 4A and 4C), although no significances after FKN incubation were observed for Akt-phosphorylation at Thr⁴⁷³ (Figure II in the online-only Data Supplement).

Furthermore, Akt phosphorylation was suppressed by preincubation of samples with TGX-221 (0.5 μmol/L; Cayman Chemical Corp, Ann Arbor, MI), a potent, selective, and cell permeable inhibitor of PI3K p110β (Figure III in the online-only Data Supplement). Four different isoforms of PI3Ks have been described; a key role for the p110β isoform was postulated in regulating thrombus formation by prevention of formation of stable integrin αⅡbβ³ adhesion contacts.²⁶ Using isoform-selective PI3K inhibitors and knockout mouse models, p110β was identified to be primarily responsible for Gαi-dependent linkage to Akt activation in ADP-stimulated platelets.²⁷ We also performed Western blotting analysis using the nonselective PI3K inhibitor Ly294002. As expected, FKN-induced signaling was completely inhibited by preincubation of samples with Ly294002 (data not shown). Taken together, these results once more indicate the impact of FKN on Gβγ activation.

Impact of FKN on Platelet Activation in P2Y₁₂ Receptor Blocker-Treated Patients
To demonstrate the assumed FKN-mediated effect in the presence of a P2Y₁₂ inhibitor, blood samples obtained from 5 patients with CAD who had been pretreated with clopidogrel were incubated with FKN in vitro. Thereafter, PRI was measured as described above. The PRI significantly increased in the presence of FKN implying reduced clopidogrel-mediated platelet inhibition in the presence of elevated concentrations of the chemokine (Figure 5A).

FKN Levels and P2Y₁₂ Inhibition in CAD
If FKN actually interacts with clopidogrel responsiveness, an association between FKN levels and the extent of clopidogrel responsiveness should be observable in clopidogrel-treated patients. Therefore, the extent of P2Y₁₂ inhibition was assessed in clopidogrel-treated CAD patients using the P2Y₁₂-specific PRI.²⁸,²⁹ In parallel, FKN serum levels were assessed by ELISA. Indeed, when stratifying the patients’ FKN serum levels, higher FKN levels were associated with impaired clopidogrel responsiveness (Figure 5B and 5C). Interestingly, the disease conditions identified by the Residual Platelet Aggregation after Deployment of Intracoronary Stent score were also associated with increased FKN levels (Figure 5D). Patient characteristics are described in the Table.

Discussion
Our data show analogies in intraplatelet P2Y₁₂- and CX3CR1-coupled pathways. Similar to P2Y₁₂ on stimulation, CX3CR1

Figure 4. Immunoblotting experiments with washed platelets obtained from healthy volunteers; (A) Western blotting demonstrating varying extent of phosphoinositide 3 (PI3)-kinase activation after stimulation with ADP (5 μmol/L and 10 μmol/L), fractalkine (FKN, 1 μg/mL and 2 μg/mL), and their combination. FKN by itself induced PI3-kinase activation leading to significantly enhanced Akt phosphorylation at Ser⁴⁷³ compared with nonstimulated samples (B). FKN even potentiated ADP-induced PI3K activation compared with ADP stimulation alone (C) demonstrating the impact of FKN not only on Gαi-mediated but also on Gβγ-mediated signal transduction. n=9; ns indicates nonsignificant.
exerts $G_{\alpha i}$-dependent inhibition of adenylyl cyclase and $G_{\beta\gamma}$-dependent activation of PI3K, whereby the extent of activation by FKN is weaker than by ADP.

ADP, one of the most important mediators of thrombosis, activates platelets through 2 G protein–coupled P2 receptors, P2Y1 and P2Y12.30 The P2Y1 receptor mediates the initiation of ADP-induced aggregation through calcium mobilization, whereas the P2Y12 receptor is involved in the completion and amplification of the aggregation response (Figure IV in the online-only Data Supplement).30 P2Y12 receptor activation $G_{\alpha i}$-dependently inhibits cAMP production by adenylyl cyclase and reduces endogenous platelet inhibition.14 Clopidogrel blocks the platelet P2Y12 receptor and prevents the secondary ADP-triggered amplification of platelet activation. Patients undergoing elective percutaneous coronary intervention display marked interindividual variability of platelet inhibition in response to clopidogrel.22,28,31 Impaired responsiveness to clopidogrel is associated with increased risk of stent thrombosis29,32 and adverse cardiovascular events after coronary stenting.33

Interestingly, CX3CR1, the FKN receptor, is coupled to the same intracellular G proteins as P2Y12, suggesting a potential overlapping downstream signaling, which could partially substitute during P2Y12 inhibition (Figure IV in the online-only Data Supplement). This is supported by the fact that FKN itself is only a weak stimulant for platelets without affecting calcium flux requiring costimulation with agonists, such as ADP.10 Similar pathways have been described previously, in which alternative signaling through $G_{\alpha i}$ in platelets could at least partially substitute for inhibition of P2Y12.34

The intact endothelium releases prostanoids (eg, prostacyclin) among other substances, which serve as endogenous platelet inhibitors by activating adenylyl cyclase. Patients with active CAD do not only have endothelial dysfunction resulting in reduced formation of these mediators, but also show decreased platelet responsiveness to the antiaggregatory effects of NO and PGE1.35,36 Such impaired responsiveness is associated with a worse outcome.37 Especially, the activity of adenylyl cyclase is inhibited by $G_{\alpha i}$-mediated signaling through P2Y12. When we assessed the effect of FKN on PGE1-mediated platelet inhibition, we found that FKN desensitizes platelets to the endogenous platelet inhibitor prostaglandin by inhibiting adenylyl cyclase. $G_{i}$ coupling of CX3CR1 has been described previously for Chinese hamster ovary cells38 and neurons.39

We could additionally show that FKN induces PI3K-dependent Akt phosphorylation via a $G_{\beta\gamma}$ protein demonstrating that not only the $G_{\alpha i}$ but also the $G_{\beta\gamma}$ subunit is involved in FKN-mediated signaling similar to ADP signaling through P2Y12. Interestingly, an FKN-dependent PI3K activation has...
In addition to acute myocardial infarction, where enhanced FKN levels could be explained by enhanced release from the ruptured atherosclerotic lesion, impaired clopidogrel responsiveness has been observed in several comorbidities to CAD (eg, diabetes mellitus, renal failure, and congestive heart failure). Interestingly, we and others have observed increased systemic FKN levels in patients with and experimental models of those diseases. Overexpression of FKN has been observed in patients with left ventricular dysfunction and in rats with heart failure. In diabetes mellitus, FKN levels are increased in animal models of type I, as well as type II, whereby induced FKN expression has been found in smooth muscle cells after high glucose conditions and in glomeruli after exposition to advanced glycation end products. FKN plays an important role in nephrogenesis and in the development of kidney diseases leading to chronic or acute renal failure. It is remarkable that clinical disease conditions related to impaired clopidogrel responsiveness do display enhanced FKN formation. Therefore, it is tempting to speculate whether higher FKN levels might locally contribute to impaired platelet inhibition under these conditions.

Of other weak agonists, epinephrine, macrophage-derived chemokine, or stromal cell–derived factor-1α have also to be taken into account when postulating alternative signaling bypassing the inhibition of P2Y12. In our patient cohort, we assessed the potential interaction between stromal cell–derived factor-1 and macrophage-derived chemokine with the PRI; however, we found no correlation with PRI (Figure VI in the online-only Data Supplement). Another limitation to be considered is the fact that the PRI, which is based on modulation of VASP phosphorylation, only provides indirect evidence with regard to cAMP and adenylyl cyclase. However, it has been established as the only clinically usable test and was used in the present context to strengthen the potential clinical relevance.

The analysis of clopidogrel-treated CAD patients revealed that patients with higher levels of FKN were more likely to have impaired clopidogrel responsiveness than patients with lower FKN levels. The bypassing of the clopidogrel-inhibited P2Y12 receptor by FKN-mediated activation of its specific receptor constitutes an important mechanism by which platelets remain hyperreactive despite using state-of-the-art antiplatelet therapy.

Hypothetically, this mechanism could be extremely relevant at the site of a ruptured plaque. First, FKN levels might be much higher at the site of release from an atherosclerotic lesion; second, platelet inhibition would be impaired at the site of a ruptured plaque. First, FKN levels might be much higher at the site of a ruptured plaque; second, platelet inhibition would be impaired at the site of a ruptured plaque. First, FKN levels might be much higher at the site of release from an atherosclerotic lesion; second, platelet inhibition would be impaired at the site of a ruptured plaque.

In conclusion, we show that increased FKN levels are associated with decreased endogenous platelet inhibition and impaired response to P2Y12 inhibition with clopidogrel. Furthermore, assumed similarities to the P2Y12 receptor...
concerning the signaling pathway could be verified. Thus, we describe a novel pathomechanism by which alternative G protein–coupled signaling induced by the proinflammatory and atherogenic mediator FKN might impede platelet inhibition by thienopyridines.

**Sources of Funding**

This work was supported by Interdisziplinäres Zentrum für Klinische Forschung (IZKF) Würzburg (projects E-39 and Z-2/25 to Dr Schäfer).

**Disclosures**

None.

**References**


Fractalkine Activates a Signal Transduction Pathway Similar to P2Y<sub>12</sub> and Is Associated With Impaired Clopidogrel Responsiveness

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Fractalkine activates a signal transduction pathway similar to P2Y\textsubscript{12} and is associated with impaired clopidogrel responsiveness

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Supplemental figure legends

Suppl. Figure I: Original flow cytometry data; A: Gating of platelets by forward and sideward scatter (FSC; SSC), then depicting extend of VASP-Ser\textsuperscript{157} phosphorylation (FITC-labelled antibody; y-axis) by gating CD61-positive cells (PE labelled antibody; x-axis). B: Histogramm overlay of the extend of VASP-Ser\textsuperscript{157} phosphorylation: grey – background, blue – stimulation with 1µM PGE\textsubscript{1}, red – pre-incubation with 1µg/mL FKN for 5min before PGE\textsubscript{1} (1µM)-stimulation.

Suppl. Figure II: Evaluation of Akt- phosphorylation at Thr\textsuperscript{308} performing immunoblotting experiments with washed platelets; in contrast to phosphorylation at Ser\textsuperscript{473} no significant changes were observed for Thr\textsuperscript{308} phosphorylation; n=8.

Suppl. Figure III: Akt- phosphorylation was suppressed by pre-incubation of samples with TGX221 (0,5µM), a potent, selective, and cell permeable inhibitor of PI3K p110β, once more indicating the impact of fractalkine on G\textsubscript{βγ} activation; n=7.

Suppl. Figure IV: Adenosine-5’-diphosphate (ADP) activates platelets through two G protein-coupled P2 receptors, P2Y\textsubscript{1} and P2Y\textsubscript{12}. The G\textsubscript{αq}-coupled P2Y\textsubscript{1} receptor initiates ADP-induced aggregation via phospholipase C (PLC\textsubscript{β}) and mobilization of calcium, while the G\textsubscript{αi}-coupled P2Y\textsubscript{12} receptor mediates amplification and completion of the aggregation response. In addition, P2Y\textsubscript{12} is G\textsubscript{αi}-dependently linked to the inhibition of cyclic adenosine monophosphate (cAMP) production by adenylyl cyclase (AC), which can be stimulated by prostaglandins (e.g. PGE\textsubscript{1} or PGI\textsubscript{2}). cAMP-dependent protein kinase (PKA) phosphorylates the vasodilator-stimulated phosphoprotein (VASP) and inhibits platelet aggregation and secretion. The extent of
ADP-induced attenuation of prostaglandin-mediated VASP phosphorylation is used in the platelet reactivity index (PRI) to determine P2Y\textsubscript{12}-specific ADP-activity. P2Y\textsubscript{12} is also G\textsubscript{\textbeta\textgamma}-dependently linked to PI3-kinase and consecutive Akt phosphorylation. Thienopyridines such as clopidogrel inhibit multiple pro-aggregatory actions of ADP, mostly by preventing the P2Y\textsubscript{12}-mediated secondary ADP-response contributing to amplification of platelet activation. Fractalkine (FKN), via its receptor CX\textsubscript{3}CR1, induces similar G-protein coupled signaling, which results in a bypassing effect to P2Y\textsubscript{12} blockade, leading to inhibition of AC and activation of PI3K.

**Suppl. Figure V:** Western blots were performed using washed platelets in the presence / absence of acetylsalicylic acid (ASA, 20\textmu g/mL) and subsequent stimulation with different concentrations of fractalkine and / or ADP (5\textmu M). ASA did not influence fractalkine mediated Akt- phosphorylation at Ser\textsuperscript{473}, furthermore, elevated FKN-concentrations did not significantly influence phosphorylation extent at Akt-Ser\textsuperscript{473}; n=4 (A&B).

(C): Immunoblotting was performed of washed human platelets generated from samples from patients taking P2Y12-receptor inhibitors and the COX-inhibitor ASA. Even in the presence of the afore mentioned medication, the effect of fractalkine on Akt- phosphorylation at Ser\textsuperscript{473} was still observable; n=3;

**Suppl. Figure VI:** PRI levels determined from blood samples from clopidogrel-treated patients with coronary artery disease after in vitro incubation with SDF1 (1\textmu g/mL, n=4) or MDC (1\textmu g/mL, n=4) showed no significant changes compared with “native” PRI (A). SDF1 (B) and MDC (C) serum levels show no correlation with PRI of patients with coronary artery disease. Scatter-plots display the individual values for SDF1 / MDC vs PRI; n=49, \( r^2=0.009295 \), \( p=0.8353 \) for SDF1 (B); n=48, \( r^2=0.007940 \), \( p=0.5495 \) for MDC (C).
Suppl. Figure I

A

B

- Background
- PGE1 (1µM)
- PGE1 (1µM) + FKN (1µg/mL)
Suppl. Figure II
Suppl. Figure III

Basal  |  FKN  | TGX221+FKN  | TGX221

Ser473/pAkt

p<0.05  p<0.05
Suppl. Figure IV

clopidogrel, ticlopidine ticagrelor, prasugrel cangrelor

ADP

P2Y₁

Gₐₚ

PLCβ

Gₜ_pool

PGE₁

FKN

CX₃CR1

Gₐ_pool

Gₜ_pool

AC

cAMP

ATP

PI3K

pAkt

Akt

secretion aggregation

pAkt

Akt

P-VASP

VASP

PKA

Akt

Suppl. Figure IV
Suppl. Figure VI

A

B

C

p = 0.8353  
$r^2 = 0.0009295$

p = 0.5495  
$r^2 = 0.007840$