Platelet-Derived 12-Hydroxyeicosatetraenoic Acid Plays an Important Role in Mediating Canine Coronary Thrombosis by Regulating Platelet Glycoprotein IIb/IIIa Activation

Atsushi Katoh, MD; Hisao Ikeda, MD; Toyoaki Murohara, MD; Nobuya Haramaki, MD; Hideki Ito, MS; Tsutomu Imaizumi, MD

Background—In the thrombotic process of acute coronary syndromes, the pathophysiological role of thromboxane A₂ via cyclooxygenase is well established; however, the role of 12-HETE via 12-lipoxygenase is little known. Therefore, we used OPC-29030, a novel specific inhibitor of 12-HETE synthesis, to test whether platelet-derived 12-HETE is involved in mediating cyclic flow variations (CFVs) and platelet aggregation in stenosed and endothelium-injured canine coronary arteries.

Methods and Results—After developing CFVs, dogs received a vehicle or OPC-29030 intravenously. Plasma and intraplatelet 12-HETE levels increased after CFVs. OPC-29030 but not vehicle reduced CFVs, which was associated with decreases in plasma and intraplatelet 12-HETE levels. Cessation of OPC-29030 restored CFVs in association with increases in plasma and intraplatelet 12-HETE levels. ADP and U46619 induced ex vivo platelet 12-HETE production and aggregation. After OPC-29030 administration, the ADP- and U46619-induced increases in ex vivo platelet 12-HETE production and aggregation were inhibited significantly. Platelet aggregation was linearly correlated with platelet 12-HETE production. OPC-29030 suppressed activation of human platelet glycoprotein IIb/IIIa.

Conclusions—OPC-29030 reduced intraplatelet 12-HETE levels, resulting in the inhibition of coronary thrombosis in vivo in dogs. OPC-29030 inhibited human platelet glycoprotein IIb/IIIa activation in vitro. Thus, platelet-derived 12-HETE may play an important role in mediating thrombotic process. (Circulation. 1998;98:2891-2898.)

Key Words: platelets ■ hydroxyeicosatetraenoic acid ■ platelet aggregation ■ glycoproteins ■ thrombosis

Plaque disruption with subsequent thrombus formation has been implicated as a major pathogenic mechanism for the acute coronary syndromes, including unstable angina and acute myocardial infarction. In clinical studies, coronary angiographic observations have shown the presence of platelet thrombi in these syndromes. Experimental studies have also demonstrated that platelet-mediated coronary thrombosis produces pathophysiological manifestations similar to these syndromes. Thus, acute vascular injury at the site of coronary atherosclerotic plaque substantially facilitates thrombus formation through a complex cascade of cellular interactions between platelets and vascular endothelial cells.

Metabolites of arachidonic acid play an important role in mediating thrombus formation and in regulating vascular tone. Arachidonic acid, which increases dramatically via action of the phospholipase A₂-mediated phospholipid hydrolysis, is metabolized through 2 major catalytic pathways mediated by 2 different enzymes in platelets. One is cyclooxygenase, which produces thromboxane A₂ (TXA₂), and the other is 12-lipoxygenase, which produces 12-hydroperoxyeicosatetraenoic acid (12-HPETE), which is rapidly converted to 12-hydroxyeicosatetraenoic acid (12-HETE). In experimental and clinical studies, the pathophysiological role of TXA₂ in platelets is well established in thrombus formation at the culprit lesion of the coronary artery. However, it is still unknown whether 12-HETE plays an active role in mediating thrombus formation in vivo, although 12-HETE has been shown to potentiate platelet aggregation in vitro.

Accordingly, to further investigate the pathophysiology of the acute coronary syndromes, we tested our hypothesis that platelet-derived 12-HETE is involved in mediating thrombus formation in vivo by use of a well-established experimental canine model. (Circulation. 1998;98:2891-2898.)

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our hypothesis in dogs. We also examined whether OPC-29030 affects plasma and intraplatelet levels of 12-HETE and thromboxane B₂ (TXB₂) in vivo, inhibits ex vivo platelet aggregation and 12-HETE production induced by ADP and U46619, and inhibits human platelet glycoprotein (GP) IIb/IIIa activation in vitro.

Methods

Surgical Preparation and Experimental Protocol

All experimental protocols were conducted in accordance with the guidelines for animal experimentation issued from the Human and Animal Research Committee of Kurume University School of Medicine. Our canine model of cyclic flow variations (CFVs) was prepared as described previously.14,15 CFVs developed in 40 of 51 dogs. The remaining 11 dogs were excluded from this protocol.

Dogs (n=40) were divided into 4 groups. After 40 minutes of stabilization of CFVs, drugs were administered intravenously. Group 1 (n=12) received a continuous infusion of the vehicle as control (dimethylformamide; Wako Co), baicalein (a 12-lipoxygenase inhibitor; 5 mg/kg/min), or indomethacin (2.5 mg/kg/min). Group 2 (n=12) received a continuous infusion of OPC-29030 (3, 10, and 20 mg/kg/min, respectively) (Otsuka Co) for 40 minutes. All dogs were monitored for 40 minutes after cessation of the treatment. To assess the effects of each treatment, the severity of CFVs was evaluated by their coronary blood flow (CBF) velocity (% control) as previously described.14,15

Measurements of Plasma and Intraplatelet 12-HETE and TXB₂ Levels In Vivo

In groups 1 (n=10) and 3 (n=12), to examine the effects of OPC-29030 on intraplatelet cAMP levels, blood was collected into a prechilled siliconized glass tube containing EDTA and isobutylmethylxanthine 0.5 mmol/L (Sigma) and was kept at 4°C. The platelet pellet was obtained as described above. The residue pellet was resuspended in modified HEPES–Tyrode’s buffer without Ca²⁺ or Mg²⁺ and containing EDTA and isobutylmethylxanthine. The platelets were washed twice with the same buffer, and the number of platelets was adjusted to 100 000/mm³. Measurements of intraplatelet cAMP levels were performed in duplicate with a commercially available competitive enzyme immunoassay kit (Titer Zyme, PerSeptive Diagnostics).

Ex Vivo Platelet Aggregation and 12-HETE Production

In group 1 (n=9) and group 3 (n=10), to assess the effects of OPC-29030 and of the vehicle on platelet function, ex vivo platelet aggregation was studied before and after the treatments. Platelet aggregation study, ADP- and U46619-stimulated platelet-rich plasmas (PRPs) were centrifuged. Supernatants were then frozen and stored at –80°C until assay. The concentration of 12-HETE was determined as described above. Platelet 12-HETE production was expressed as the difference between the eicosanoid concentrations of agonist-stimulated PRP and those of nonstimulated PRP.

Measurements of Plasma OPC-29030 Levels

In group 3 (n=12), plasma OPC-29030 levels were measured during and after the OPC-29030 treatment. The measurements were performed with high-performance liquid chromatography as previously described.16

Measurements of Intraplatelet cAMP Levels

In group 3 (n=10), to examine the effects of OPC-29030 on intraplatelet cAMP levels, blood was collected into a prechilled siliconized glass tube containing EDTA and isobutylmethylxanthine 0.5 mmol/L (Sigma) and was kept at 4°C. The platelet pellet was obtained as described above. The residual pellet was resuspended in modified HEPES–Tyrode’s buffer without Ca²⁺ or Mg²⁺ and containing EDTA and isobutylmethylxanthine. The platelets were washed twice with the same buffer, and the number of platelets was adjusted to 100 000/mm³. Measurements of intraplatelet cAMP levels were performed in duplicate with a commercially available competitive enzyme immunoassay kit (Biotrak, Amersham Pharma- cia Biotech).

Analysis of Human Platelet GP IIb/IIIa Activation

With peripheral blood collected from 5 healthy volunteers, washed platelets were prepared according to the previously described method20 and incubated at 22°C for 10 minutes with 300 ng/mL OPC-29030, 1 µmol/L baicalein, or buffer. Then, the samples were incubated with a saturating concentration of the FITC-conjugated monoclonal antibody PAC-1 (Becton-Dickinson), followed by incubation at 22°C for 10 minutes with ADP at 0.05 to 5 µmol/L, U46619 at 0.05 to 5 µmol/L, thrombin at 5 to 500 µU/mL, or buffer. The PAC-1 antibody has a specificity for an epitope that is exposed on the fibrinogen receptor GP IIb/IIIa only after an activation-dependent conformational change.21 The samples were then fixed in paraformaldehyde 1% at 22°C for 30 minutes and were analyzed within 2 hours by flow cytometry (Becton-Dickinson). Background binding, obtained from parallel samples run with FITC-conjugated immunoglobulin M (Pharmingen) was subtracted from each test sample. PAC-1 binding in the presence of maximal thrombin (500 µU/mL) and buffer alone was assigned to be 100 U fluorescence.

Statistical Analysis

Values are presented as mean±SEM. Statistical comparisons between groups were performed with a paired Student’s t test. Multiple comparisons were analyzed by repeated-measures ANOVA. The relationship between 2 parameters was analyzed by use of a linear regression analysis. Differences were considered statistically significant at a value of P<0.05.
Hemodynamic Variables Before, During, and After Treatments

<table>
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<th>Heart Rate, bpm</th>
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Phasic flow indicates peak phasic CBF velocity; mean flow, mean CBF velocity (both phasic and mean flow are expressed as percent of unconstricted CBF velocity); peak, average high flow during CFVs; nadir, average low flow during CFVs; frequency, frequency of CFVs/40 min; baseline, time period before constrictor placement; stenosis, time period after constrictor placement and before development of CFVs; initial CFVs, 40-min observation of CFVs before treatment; during treatment, 40-min observation during treatment; and after cessation of treatment, 40-min observation after cessation of treatment. Values are expressed as mean±SEM.

*P<0.05 vs baseline; †P<0.05 vs initial CFVs; ‡P<0.05 vs during treatment.

Results

Hemodynamics

Before Development of CFVs
Endothelial injury and coronary constriction significantly decreased the averaged peak phasic CBF velocity to 35% to 38% of baseline and the mean CBF velocity to 47% to 52% of baseline. These figures are similar to those reported by Ashton et al. Heart rate, aortic pressure, and peak phasic and mean CBF velocities were comparable among the 4 groups (Table).

Initial CFVs
No significant changes were observed in heart rate or in systolic and diastolic aortic pressures after development of CFVs. The peak phasic and mean CBF velocities were decreased similarly among the groups. The phasic CBF velocity was decreased to 12% to 14% of baseline and mean nadir CBF velocity to 11% to 14% of baseline. The averaged frequency of CFVs was 4.1 to 4.3 cycles/40 min. These values were also similar among the 4 groups. Thus, the severity of CFVs before the treatment was similar among the 4 groups. These figures were similar to those reported by Ashton et al. and us. (Table).

During Treatment
The treatments did not cause significant changes in heart rate or aortic pressure in groups 1, 2, and 3, but they did in group 4. In groups 1 and 2, the treatment did not cause significant changes in the nadir CBF velocity or the frequency of CFVs. In group 3, OPC-29030 significantly increased the nadir CBF velocity and significantly decreased the frequency of CFVs in association with increases in plasma OPC-29030 levels (212±30 ng/mL at 20 minutes and 286±48 ng/mL at 40 minutes) (Figure 2, Table).

After Cessation of Treatment
In groups 1, 2, and 3, cessation of treatment did not cause significant changes in heart rate or aortic pressure. In group 4, cessation of treatment decreased heart rate and increased aortic pressure, although statistically insignificantly. In groups 1 and 2, cessation of treatment did not cause significant changes in either nadir CBF velocity or frequency of CFVs. In group 3, cessation of OPC-29030...
treatment returned the nadir CBF velocity and the frequency of CFVs to the levels before treatment. Plasma OPC-29030 levels after cessation of treatment decreased to $68 \pm 16$ and $31 \pm 8$ ng/mL at 20 and 40 minutes, respectively (Figure 2). In group 4, cessation of the OPC-29030 treatment significantly restored CFVs (Table).

**Plasma and Intraplatelet 12-HETE and TXB₂ Levels**

In group 1 (Figure 3A), plasma 12-HETE levels after CFVs were significantly increased compared with those before CFVs. Neither treatment nor cessation of treatment caused significant changes in plasma 12-HETE levels. In group 3 (Figure 3B), plasma 12-HETE levels after CFVs were significantly increased compared with those before CFVs. OPC-29030 significantly decreased plasma 12-HETE levels. After cessation of treatment, plasma 12-HETE levels were restored to those observed during initial CFVs. In group 3, intraplatelet 12-HETE levels changed to the same degree as plasma levels (Figure 4).

In group 3, plasma and intraplatelet TXB₂ levels were not changed by OPC-29030 treatment (plasma TXB₂, $0.91 \pm 0.14$ ng/mL during CFVs versus $0.99 \pm 0.21$ ng/mL during treatment, $P=NS$; intraplatelet TXB₂, $0.22 \pm 0.05$ ng/mL during CFVs versus $0.24 \pm 0.05$ ng/mL during treatment, $P=NS$).

**Figure 2.** Relationship between concentrations of OPC-29030 and its antithrombotic effects after OPC-29030 treatment. Note that OPC-29030 reduces CFVs in association with increases in plasma OPC-29030 levels and that cessation of treatment restores CFVs in association with decreases in plasma OPC-29030 levels.

**Figure 3.** Plasma 12-HETE levels before (baseline) and after (initial CFVs) development of CFVs, during treatment with vehicle (A) or OPC-29030 (B), and after cessation of each treatment. Plasma 12-HETE levels significantly increased after development of CFVs. OPC-29030 significantly reduced plasma 12-HETE levels. Cessation of treatment returned plasma 12-HETE levels to pretreatment levels. Vehicle did not have such effects.

**Figure 4.** Intraplatelet 12-HETE levels before (baseline) and after (initial CFVs) development of CFVs, during treatment with OPC-29030, and after cessation of treatment. Intraplatelet 12-HETE levels significantly increased after development of CFVs. OPC-29030 significantly reduced intraplatelet 12-HETE levels. Cessation of treatment returned intraplatelet 12-HETE levels to pretreatment level.
Effects of Treatments on Ex Vivo Platelet Aggregation and 12-HETE Production

In group 1, both ADP and U46619 caused comparable concentration-dependent platelet aggregation before and after treatment (Figure 5A). In group 3, both agonist-induced platelet aggregation (Figure 5B) and platelet 12-HETE production (Figure 6) were significantly suppressed by OPC-29030 compared with those before treatment. There were significant linear relationships between platelet aggregation and 12-HETE production after stimulation with both agonists (Figure 7).

Intraplatelet cAMP Levels

The intraplatelet cAMP levels did not change at any given time point (Figure 8).

Effects of OPC-29030 and Baicalein on Human Platelet GP IIb/IIIa Activation

As shown in Figure 9, ADP, U46619, and thrombin caused dose-dependent platelet GP IIb/IIIa activation. OPC-29030 and baicalein significantly inhibited the platelet GP IIb/IIIa activation induced by all these agonists.

Discussion

As originally described by Folts et al., focal platelet aggregation and subsequent dislodgment at the coronary stenotic site result in brief and repeated episodes of coronary occlusion that are characterized by CFVs. The present model is now established as an in vivo model of coronary arterial thrombosis. We previously reported that conscious dogs...

Figure 5. A, Ex vivo platelet aggregation in response to various concentrations of ADP and U46619 during initial CFVs (□) and treatment with vehicle (■). B, Ex vivo platelet aggregation during initial CFVs (□) and OPC-29030 treatment (○). ADP and U46619 induced platelet aggregation in a concentration-dependent manner. OPC-29030 significantly reduced platelet aggregation, whereas vehicle caused no significant changes.

Figure 6. Ex vivo platelet 12-HETE production in response to various concentrations of ADP and U46619 during initial CFVs (□) and OPC-29030 treatment (○). ADP and U46619 induced platelet 12-HETE production in a concentration-dependent manner. OPC-29030 significantly reduced platelet 12-HETE production.
with CFVs manifest a pathophysiology similar to that of the acute coronary syndromes in humans. The major findings of the present study were that (1) there were increases in both plasma and intraplatelet 12-HETE levels after development of CFVs; (2) OPC-29030 reduced CFVs in association with decreases in both plasma and intraplatelet 12-HETE levels, whereas the cessation of OPC-29030 restored CFVs in association with increases in both plasma and intraplatelet 12-HETE levels; (3) OPC-29030 inhibited ex vivo platelet aggregation and 12-HETE production induced by ADP and U46619; and (4) OPC-29030 inhibited agonist-induced activation of human platelet GP IIb/IIIa in vitro. Thus, our results suggest that platelet-derived 12-HETE is critically involved in mediating platelet aggregation and CFVs in vivo in stenosed and endothelium-injured coronary arteries.

In the present study, OPC-29030 at 3 \(\mu g \cdot kg^{-1} \cdot min^{-1}\) did not affect CFVs, but OPC-29030 at 20 \(\mu g \cdot kg^{-1} \cdot min^{-1}\) significantly reduced CFVs, although this dosage caused systemic hemodynamic changes. Thus, decreases in CFVs by OPC-29030 at 20 \(\mu g \cdot kg^{-1} \cdot min^{-1}\) might not be attributable solely to its effects on platelets. In contrast, OPC-29030 at 10 \(\mu g \cdot kg^{-1} \cdot min^{-1}\) significantly reduced CFVs without systemic hemodynamic changes. Therefore, the dosage of 10 \(\mu g \cdot kg^{-1} \cdot min^{-1}\) was used for the subsequent protocols. The cessation of OPC-29030 treatment significantly restored CFVs. Our data therefore indicate that OPC-29030 reduces CFVs by its antiplatelet effects. To further investigate the role of 12-HETE in the pathogenesis of the thrombotic process of CFVs, we measured plasma and intraplatelet 12-HETE levels. In this connection, a previous in vitro study showed that 12-HETE was produced by aggregated platelets, and a previous study using the same dog model with CFVs demonstrated that the 12-HETE synthesis was increased by 10-fold in the stenosed and endothelium-injured coronary arteries. In this study, both plasma and intraplatelet 12-HETE levels during CFVs increased significantly compared with those before CFVs. The inhibitory effects of OPC-29030 on CFVs were associated with decreases in both plasma and intraplatelet 12-HETE levels. The IC\(_{50}\) of OPC-29030 for ex vivo platelet 12-HETE inhibition was 0.73 \(\mu mol/L\) (315 ng/mL). In this study, when CFVs were inhibited, the plasma concentration of OPC-29030 after treatment was 212±30 and 286±48 ng/mL at 20 and 40 minutes, respectively. Thus, the effective plasma OPC-29030 levels in vivo were close to the value of IC\(_{50}\) in the ex vivo experiment. The cessation of OPC-29030 treatment decreased the plasma OPC-29030 levels to <70 ng/mL and restored CFVs in association with significant increases in plasma and intraplatelet 12-HETE levels. Taken together, our data clearly suggest that platelet-derived 12-HETE plays an important role in the thrombotic process of CFVs in this model.
We used an in vivo model of coronary arterial thrombosis in this study. Although OPC-29030 treatments significantly reduced CFVs in association with decreases in intraplatelet 12-HETE levels, it is not known whether OPC-29030 treatment inhibits platelet aggregation in association with inhibition of platelet 12-HETE production. To address this issue, we investigated the effects of OPC-29030 on ex vivo platelet aggregation and 12-HETE production. ADP and U46619, a thromboxane mimetic, were chosen as platelet agonists in this study because ADP and TXA₂ are important mediators of CFVs.⁹,¹⁰ In the present study, ADP and U46619 induced platelet aggregation (Figure 5) and 12-HETE production (Figure 6) in a concentration-dependent manner. Both ex vivo platelet aggregation (Figure 5B) and 12-HETE production (Figure 6) were significantly suppressed after OPC-29030 treatments, whereas the vehicle did not have this effect (Figure 5A). Importantly, there was a close linear relationship between platelet aggregation and intraplatelet 12-HETE production for ADP and U46619 (Figure 8). These findings further support the previous studies demonstrating that 12-lipoxygenase inhibitors such as baicalein²⁵ inhibit in vitro human platelet aggregation induced by the same agonists as used in this study. Thus, our data suggest that OPC-29030 inhibits platelet aggregation through the inhibition of platelet 12-HETE production via the lipoxygenase pathway. Moreover, our data provide evidence that, in the cascade of arachidonic acid metabolism in activated platelets, the lipoxygenase pathway plays an important role in mediating platelet aggregation in addition to the cyclooxygenase pathway, which is implicated in platelet aggregation in the Folts dog model.⁹,¹⁰

OPC-29030 is a novel specific inhibitor of 12-HETE synthesis in activated platelets and can be administered in vivo. Indeed, this agent does not inhibit purified cyclooxygenase activity and does not affect platelet TXA₂ production stimulated with thrombin (H. Ito, MS, and coworkers, unpublished work). In the present study, OPC-29030 did not have any effects on the plasma and intraplatelet TXB₂ levels in vivo. Thus, OPC-29030 does suppress the 12-HETE synthesis in platelets without a significant effect on the cyclooxygenase pathway.¹⁶ OPC-29030 is a derivative of cilostazol,¹⁶ which is known to inhibit platelet aggregation by elevating intraplatelet cAMP levels.²⁶ In this study, OPC-29030 at 10 μg · kg⁻¹ · min⁻¹ did not affect intraplatelet cAMP levels. Therefore, the beneficial effects of OPC-29030 on CFVs are unlikely to be mediated via the increases in intraplatelet cAMP levels but are more likely to be mediated via the specific inhibition of 12-HETE synthesis in platelets. In this study, OPC-29030 did not increase plasma and intraplatelet TXB₂. The exact mechanisms are unclear. However, previous studies have demonstrated that the inhibition of platelet 12-HETE production prevents the increase in intraplatelet [Ca²⁺],²³ which would have nonspecifically inhibited the activation of phospholipase A₂ in platelets, leading to no significant changes in plasma and intraplatelet TXB₂ levels in this study. Furthermore, it is possible that 12-HPETE, the precursor of 12-HETE, was a platelet-active lipoxygenase metabolite. However, it has been shown that 12-HPETE inhibits platelet activation²⁷ and 12-HETE potentiates platelet activation.²³ Hence, this possibility is unlikely.

There are several possible mechanisms by which 12-HETE causes platelet aggregation. First, 12-HETE may potentiate platelet GP Ib/IIa activation, which is the final common pathway of platelet aggregation²⁹ and a key factor for coronary thrombosis.²⁹ Once a platelet is activated, GP Ib/IIa takes on a new 3-dimensional conformational state and reveals binding sites for fibrinogen.²¹ In this work, we studied the effects of inhibition of platelet 12-HETE synthesis on activation of human platelet GP Ib/IIa because there is no available antibody that reacts with canine platelets. Consequently, OPC-29030 as well as baicalein significantly inhibited human platelet GP Ib/IIa activation induced by ADP, U46619, and thrombin (Figure 9). The concentration of OPC-29030 used in this in vitro study was comparable to that achieved in a canine model of coronary arterial thrombosis in vivo. Thus, the present findings suggest that platelet 12-HETE causes platelet aggregation via GP Ib/IIa activation. Second, 12-HETE may upregulate platelet P-selectin,¹⁰ which potentiates the thrombotic process.¹⁰ Recently, we¹⁵ and others²² have shown that P-selectin plays an important role in

Figure 9. Human platelet surface expression of activated GP Ib/IIa in response to various concentrations of ADP (left), U46619 (center), and thrombin (right). Washed platelets were incubated with buffer (C), 300 ng/mL OPC-29030 ( ●), or 1 μmol/L baicalein ( □). ADP, U46619, and thrombin induced platelet GP Ib/IIa activation in a concentration-dependent manner. Both OPC-29030 and baicalein significantly reduced platelet GP Ib/IIa activation.
mediating CFVs in the present model. Because P-selectin is an important adhesion molecule for the interaction of platelets and endothelial cells with leukocytes and because it has been demonstrated that OPC-29030 significantly decreases platelet adhesion onto the denuded surface of rat aorta under in vivo dynamic flow conditions (T. Igawa, PhD, and coworkers, unpublished work), the antithrombotic effect of OPC-29030 in this study may be due to the inhibition of platelet adhesiveness through the downregulation of P-selectin.

In conclusion, the present study first demonstrates that OPC-29030, a novel specific inhibitor of 12-HETE synthesis, inhibits platelet aggregation and abolishes CFVs in vivo in association with decreases in both plasma and intraplatelet concentrations of 12-HETE. Furthermore, OPC-29030 inhibited human platelet GP IIb/IIIa activation. Our results strongly suggest that platelet-derived 12-HETE contributes to the pathophysiology of thrombus formation in acute coronary syndromes. Thus, the inhibition of 12-HETE via the lipoygenase pathway in activated platelets could become an attractive therapeutic modality of these syndromes in humans.

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