Objective—Earlier in vitro studies suggested a putative role for the plasma phospholipid transfer protein (PLTP) in the modulation of blood coagulation. The effect of PLTP expression on blood coagulation under both basal and oxidative stress conditions was compared here in wild-type and PLTP-deficient (PLTP−/−) mice.

Methods and Results—Under basal conditions, PLTP deficiency was associated with an extended tail bleeding time despite a significant depletion of vascular α-tocopherol content and an impairment of endothelial function. When acute oxidative stress was generated in vivo in the brain vasculature, the steady state levels of oxidized lipid derivatives, the extent of blood vessel occlusion, and the volume of ischemic lesions were more severe in wild-type than in PLTP−/− mice.

Conclusion—In addition to its recognized hyperlipidemic, proinflammatory, and proatherogenic properties, PLTP increases blood coagulation and worsens the extent of ischemic lesions in response to acute oxidative stress. Thus, PLTP arises here as a cardiovascular risk factor for the late thrombotic events occurring in the acute phase of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2010;30:00-00.)

Key Words: blood coagulation n endothelial function n lipids n thrombosis n vascular biology
Figure 1. Tail bleeding time in WT and PLTP−/− mice. Blood coagulation was assessed through measurement of tail bleeding time in WT (n=22) and PLTP−/− mice (n=23). *P<0.05 versus WT (Student t test).

Methods
PLTP-knockout homozygous (PLTP−/−) and WT mice from a homogeneous C57BL/6 background were fed a standard chow diet, and they had free access to water and food (A03 diet, Safe). All of the experiments involving animals were approved by the Ethical Committee on the Use of Laboratory Animals of the University of Burgundy (protocol number 1305), and they were performed in accordance with institutional guidelines.

Results are expressed as mean±SEM. The statistical significance of differences between data means was determined using the nonparametric Mann–Whitney U test or the Student t test, as appropriate.

Supplemental Materials and Methods (animals, bleeding time, determination of vascular reactivity, platelet preparation, platelet function, photothermolysis and infarct measurement, immunohistochemistry, cytokine and chemokine measurements, α-tocopherol assay, cholesterol/linoleic acid [LA]/hydroxyoctadecadienoic acid [HODE] assays, and protein assay) are available online at http://atvb.ahajournals.org.

Results
PLTP Deficiency and Blood Coagulation
In a first attempt to assess the effect of PLTP expression level on blood coagulation in vivo, tail bleeding time in PLTP-knockout homozygous (PLTP−/−) mice was compared with that in WT mice. As shown in Figure 1, a significant increase in tail bleeding time was observed in PLTP−/− mice compared with WT mice. Cessation of bleeding was observed on average after 83.8±10.0 seconds in WT versus 121.3±13.9 seconds in PLTP−/− mice (P<0.05). When platelet aggregation was selectively assessed in fresh whole blood using the platelet function analyzer–100 closure time test, no significant difference was observed between WT and PLTP−/− mice (mean closure time, 80.8±4.3 versus 96.9±9.3 seconds in WT [n=9] and PLTP−/− [n=9] mice, respectively; not significant). With regard to endothelium function, ie, a known determinant of vascular occlusion, endothelium-dependent contractions induced by NO-nitro-L-arginine (10 μmol/L) (measure of basal endothelial NO production) were similar in control and PLTP−/− mice (747±75 versus 884±69 mg, respectively; not significant), indicating that production of nitric oxide in the aorta was similar in WT and PLTP−/− mice under basal conditions.

We then searched for the consequences of PLTP deficiency on the vasoreactive response of thoracic aorta segments to the potent vasoconstrictor norepinephrine (NE). The aortic segments of adult mice showed similar responses to NE whether the mice expressed PLTP or not, with no differences in both Emax and pD2 values between the 2 genotypes (Figure 2B). Finally, the relaxing response curve of NE-precontracted aorta to cumulated concentrations of acetylcholine (ACH) was shifted to the right, with a significantly lower degree of endothelium-dependent relaxation induced by the 3 nmol/L to 10 μmol/L ACh doses ([ACh] log M: −8.5 to −5, respectively) in PLTP−/− mice as compared with WT mice (P<0.01 in all cases) (Figure 2A). Mean pD2 values were significantly lower in PLTP−/− mice than in WT mice (P=0.004), reflecting a significantly weaker sensitivity to ACh of the aorta from PLTP−/− animals (Figure 2B). PLTP deficiency was also associated with a significant 17% reduction in maximal ACh-induced relaxation of precontracted aorta (E\text{max}) (Figure 2B). Taken together, these observations indicate that PLTP deficiency is associated with a weaker hemostatic function in nonstimulated mice, which can be explained neither by defective platelet aggregation nor by improved endothelial function. Subsequently, intravascular thrombosis was induced in the brain through the transillumination of systemically injected Rose Bengal by filtered
Twenty-four hours after photothrombotic stroke induction, the mice were euthanized, and living versus dead cells were localized on Cresyl violet-stained sections. Fibrin-positive areas (see the 2 representative anti-fibrin brain sections) were measured on coronal sections after staining with Cresyl Violet. The distances between representative coronal sections were used to calculate a linear integration for the lesion volume. In contrast, ischemia-induced edema, measured as the ratio of ipsilateral and contralateral hemispheres 24 hours postischemia was similar in both groups (mean±SEM, 1.060±0.015 versus 1.047±0.012 in WT and PLTP−/− mice, respectively; *P<0.05), compared with PLTP−/− lesions (not shown). Finally, interleukin-6 and monocyte chemoattractant protein 1 levels were increased in plasma and in infarcted brain 24 hours postischemia, with similar observations in WT and PLTP−/− mice (not shown).

Overall, these data indicate that the extent of occlusion of blood vessels is greater in WT than in PLTP−/− mice after intravascular oxidative stimulation of thrombosis. Hypercoagulable blood accounts for differences in infarct volumes between WT and PLTP−/− animals, with no evidence that supports contributions of the intrinsic responsiveness of the brain tissue or of endothelium function to the observed phenotype.

**PLTP Deficiency Is Accompanied by Imbalanced Vitamin E Distribution Between Circulating Elements and the Vascular Wall**

In accordance with previous findings, the level of vitamin E was decreased in aortas of PLTP−/− mice compared with WT mice (α-tocopherol to artery weight ratios, 4.8±0.5 versus 7.1±0.9 ng/mg of tissue in PLTP−/− and WT mice, respectively; *P<0.05). In addition, and in accordance with previous findings,12 we observed a significant decrease in brain α-tocopherol content in PLTP−/− compared with WT mice, with consistent observations whether expressed as the ratio of α-tocopherol to cholesterol or of α-tocopherol to LA (Figure 3A). In contrast, red blood cells from PLTP−/− mice were significantly enriched with α-tocopherol compared with those of WT mice, again with consistent observations whether expressed as α-tocopherol to cholesterol or as α-tocopherol to LA ratio (Figure 4B). Plasma α-tocopherol to cholesterol ratio was higher in PLTP−/− than in WT mice, with no difference when expressed as α-tocopherol to LA ratio (Figure 4C). The latter point might relate to the fact that α-tocopherol is a significant determinant of antioxidant protection in the plasma compartment. Thus, a better preservation of LA would be ensured when α-tocopherol is high, resulting in a constant ratio. This may be less prominent in cells and tissues, where other antioxidants probably act besides α-tocopherol. Finally, the count and the α-tocopherol content of platelets did not differ between WT and PLTP−/− mice (382.3±31.6 versus 319.4±24.5 106 cells/mL and 164±18 versus 173±27 ng/mg protein, respectively; not significant in both cases). These observations are consistent with a role of PLTP in redistributing α-tocopherol from the intravascular compartment toward tissues.

**Intravascular Production of HODE Is Restrained in PLTP−/− Mice**

To assess oxidative stress, total HODE was measured in ischemic (right) and nonischemic (left) brain hemispheres of WT and PLTP−/− mice (Figure 5A), as well as in total blood (Figure 5B) 2 hours after initiation of photothrombosis. As shown in Figure 5A, the HODE level was significantly higher...
in nonischemic brain tissue of PLTP−/− mice than in that of WT mice. In the infarcted zone of the brain, a significant increase in HODE level was observed in WT mice but not PLTP−/− mice, leading in the end to comparable HODE levels in both groups. Similar trends were observed whether HODE levels were expressed as the ratio of HODE to cholesterol or HODE to LA. This suggests that in this experimental setting, brain vitamin E (which was more abundant in WT mice; see above) did not confer protection against intravascular oxidative injury. As far as the intravascular compartment is concerned, elevated amounts of HODE were measured in both genotypes 2 hours after induction of photothrombosis and as a result may influence the late, thromboembolic phase of the disease. Photoinduction of intravascular oxidative stress, like that occurring in the acute phase of atherothrombosis, was found to be less severe in PLTP−/− than in WT mice. In PLTP−/− mice, the accumulation of vitamin E in circulating elements was associated with an extended bleeding time, a greater resistance to oxidative stress, a reduction in clot formation in the brain microvasculature, and a lesser extent of ischemic lesions. This reduction of intravascular thrombosis in PLTP−/− mice occurred in spite of a significant impairment of endothelium function.

Over the past decade, and in addition to the liver α-tocopherol transfer protein (α-TTP), PLTP has emerged as an important determinant of vitamin E metabolism. Although α-TTP plays a key role in mediating the secretion of liver vitamin E into the bloodstream, PLTP has the ability to control the balance of vitamin E between intravascular circulating elements and tissues.5,6,8,9,12 The PLTP-mediated transfer of vitamin E from circulating elements toward the vascular wall has 2 concomitant but opposite effects in terms of vascular biology. On the one hand, PLTP was shown to contribute to the preservation of a normal endothelium function in ex vivo experiments on isolated arterial rings, possibly reflecting its ability to incorporate vitamin E in the vascular wall.8 On the other hand, the PLTP-mediated leakage of vitamin E out of the intravascular compartment produces vitamin E–poor lipoproteins and circulating cells, with 2 detrimental consequences: first, a weaker resistance to blood coagulation and as a result may influence the late, thromboembolic phase of the disease.

Discussion

Beyond the ability of plasma phospholipid transfer protein (PLTP) to promote and accelerate the formation of arterial lesions in the earlier steps of atherogenesis,1,4,5 it is shown here for the first time that PLTP can contribute to the modulation of arterial function and possibly to the modulation of arterial lesions.

Figure 4. α-Tocopherol distribution in brain, red blood cells, and plasma of WT and PLTP−/− mice. α-Tocopherol (α-toc) was extracted from brain, red blood cells, and plasma of WT and PLTP−/− mice and quantitated by high-performance liquid chromatography. Results are expressed as α-tocopherol to cholesterol ratio (left panels) and as α-tocopherol to LA ratio (right panels). Data are mean±SEM of n=4 WT and n=4 PLTP−/− for brain measurements, n=9 WT and n=10 PLTP−/− for red blood cell measurements, and n=9 WT and n=10 PLTP−/− mice for plasma measurements. **P<0.01 versus WT (Mann–Whitney U test).

Figure 5. HODE levels in brain and blood of WT and PLTP−/− mice. HODE levels were measured in brain and blood samples of WT (n=7; filled bars) and PLTP−/− (n=9; open bars) mice by liquid chromatography–tandem mass spectrometry, as described in Materials and Methods. A, HODE levels in ischemic (right) and nonischemic (left) brain 2 hours after photothrombosis. a, P<0.05 versus WT; b, P<0.05 versus homologous left hemisphere (Mann–Whitney U test). B, HODE levels in total blood before (t=0) and after (t=2 hours) photothrombotic ischemia. c, P<0.02; d, P<0.002 versus homologous t=0 samples; e, P<0.01 versus WT t=2 hours (Mann–Whitney U test).
intravascular oxidative transformations, and second, a higher propensity to externalize the prothrombotic PS in the outer leaflet of the erythrocyte membranes where \( \alpha \)-tocopherol normally localizes. However, the putative procoagulant effect of the PLTP-mediated redistribution of intravascular vitamin E was challenged by recent in vitro observations, which reported that PLTP can inactivate the anionic phospholipids in erythrocytes through their transfer from artificial liposomes toward plasma lipoproteins. In the context of these contrasting observations, complementary studies in animal models were needed to work out whether PLTP activity actually exerts pro- or anticoagulant properties in vivo.

In an attempt to determine the impact of PLTP and vitamin E on endothelium function, ie, a known determinant of the thrombotic risk, vasomotor tone was investigated in the present study on aortic rings from WT and PLTP-deficient mice. We observed that reduced basal vitamin E content of the vascular wall occurs in PLTP-deficient mice and is associated with impaired ability of the endothelium to vasorelax in response to ACh under standard conditions. These findings are in line with previous observations, which showed that enrichment of the vascular tissue with \( \alpha \)-tocopherol, possibly by way of the PLTP-mediated transfer reaction, has the ability to preserve normal endothelial function through the inhibition of protein kinase C stimulation.

To determine whether PLTP activity amplifies or attenuates blood coagulation, we turned to in vivo studies. Under basal/unstimulated conditions, tail bleeding time in PLTP-deficient mice was longer than that in WT mice. This is consistent with earlier in vitro observations of extended clotting time of whole blood drawn from PLTP-deficient mice compared with whole blood drawn from WT mice. In the present study, assessment of platelet function allowed to exclude that impaired hemostatic function in PLTP-deficient mice is related to defective platelet aggregation. At baseline, this effect was previously explained in terms of a nonantioxidant property of \( \alpha \)-tocopherol, which is able to modify the organization and fluidity of cell membrane and to reduce PS externalization in PLTP-deficient erythrocytes in the absence of changes in oxidation status. The brain is a highly vascularized organ, and under stimulated oxidative stress conditions the volume of the infarcted tissue is a reliable measure of the extent of thrombosis in the vascular network. Thus, homozygous PLTP-deficient mice (with no detectable PLTP activity) were next compared in a mouse model of focal cerebral photothrombosis, ie, in situ laser-mediated generation of a high oxidative stress leading to the thrombotic occlusion of brain microvasculature. The extent of in situ infarcted tissue was found to be clearly reduced in PLTP-deficient mice as compared with WT mice. In further support of a reduced thrombotic response associated with the PLTP-deficient state, the mean volume of fibrin-positive tissue measured 2 hours after photothrombosis in PLTP-deficient mice was markedly lower than that measured in WT mice. Inhibition of thrombus formation was concomitant with decreases in the vitamin E content of the aortic wall and the brain, but with a marked accumulation of vitamin E in circulating elements (present study). The accumulation of vitamin E in circulating elements from PLTP-deficient animals probably accounts for the significant decrease in oxidation-induced blood coagulation. Indeed, earlier human and animal studies reported strong negative relationships between oxidative stress and the vitamin E content of red blood cells. Alternatively, changes in lipid composition (including unsaturation and length of acyl chains as reported earlier in PLTP-knockout mice) might also contribute to differences in lipid peroxidation. Finally, PLTP-mediated direct neutralization of the procoagulant PS, as well as PLTP-dependent upregulation of endothelium activation (present study), could be excluded in this setting because they would have led to a decrease rather than an increase in tissue injury.

\( \alpha \)-Tocopherol, the main isomer of vitamin E, is mostly recognized as a potent antioxidant with pleiotropic effects. Although epidemiological and animal studies indicated a potential beneficial impact of vitamin E against cardiovascular disease and its complications, studies of vitamin E supplementation in humans have brought conflicting results. In fact, beyond total vitamin E content of the whole body, tissue levels (ie, amounts of \( \alpha \)-tocopherol in biomembranes) would be more meaningful than plasma levels, and the relative distribution between blood and tissues may well determine the extent of the expected beneficial outcome. In fact, the conclusions of most human studies were built on measurements of vitamin E concentration in plasma only, and this limitation might actually account for some of the heterogeneous observations. Interestingly, arterial injury of the common carotid artery in humans was found to be negatively associated with the vitamin E content of red blood cells, but not of plasma. In addition, total plasma vitamin E concentration was shown to be a poor predictor of cell vitamin E status. Finally, reduced vitamin E content of circulating red blood cells and lipoproteins was thought to result from faster delivery to extravascular tissues rather than from impairment of their replenishment. The present study emphasizes the validity of assessing the compartmental distribution of vitamin E in vivo. It demonstrates that the localization of vitamin E in circulating elements versus tissues relies to a great extent on the level of plasma PLTP activity, which might play a key role in accelerating the transfer of vitamin E out of the intravascular compartment.

In conclusion, PLTP has a dual role in vascular homeostasis. On the one hand, it increases the vitamin E content of the vascular wall, thus preserving endothelium function under basal conditions. On the other hand, it reduces the vitamin E content of circulating elements, thus decreasing the ability of the intravascular compartment to cope with elevated oxidative stress and increasing blood coagulation. Interestingly, high-risk patients with obesity or type 2 diabetes are known to cumulate high plasma PLTP, low vitamin E content of red blood cells, and lipoproteins, and high lipid peroxidation in erythrocyte membranes. The present study suggests that the PLTP-mediated leakage of \( \alpha \)-tocopherol out of the blood compartment may make a major contribution to increased thrombotic risk in these patients.

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Disclosures

None.

References

Plasma Phospholipid Transfer Protein Deficiency in Mice Is Associated With a Reduced Thrombotic Response to Acute Intravascular Oxidative Stress

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SUPPLEMENTARY MATERIALS AND METHODS

Animals - PLTP-/- mice were previously generated by replacing exon 2 (containing the translation start site) with a neo gene [1]. Animals were on an homogeneous C57BL/6 background (eight backcrosses), and they were a kind gift from Dr Jiang’s laboratory. When animals were received in our animal facility, PLTP-/- homozygous mice were backcrossed for eight generations with C57BL/6 wild-type mice which were purchased from the Charles River’s laboratory. The latter mice are derived from pedigreed mice from the Jackson Laboratory and are re-infused routinely with those pedigreed mice to ensure equivalent genetic quality and integrity. Both WT and PLTP-/- mice come from the same breeding colony, and every eight generations, both WT and PLTP-/- mice are routinely backcrossed in the same homogenous C57BL/6 background to avoid genetic drift.

Reagents and antibodies - Norepinephrine (NE), acetylcholine (ACh), Nω-nitro-L-arginine, Rose Bengal, heptadecanoic acid, linoleic acid, and reagents used for sample derivatization (GC analysis) were purchased from Sigma (Sigma-Aldrich, France). Tocol was from Spiral (Couternon, France). Alpha-tocopherol (HPLC calibration curve) was from Calbiochem (La Jolla, CA, USA). Standards used for HODE calibration curves (9-(S)-HODE and 13-(S)-HODE) and 13-(S)-HODE-d4 (internal standard) were purchased from Cayman Chemicals. All solvents used for chromatography and mass spectrometry were of HPLC grade. The rabbit anti-human fibrinogen/fibrin antibody was purchased from Dako. The FITC-conjugated goat anti-rabbit IgG antibodies were from Abcam.

Bleeding time - Mouse tail bleeding times were determined as previously described [2]. Briefly, the mice were maintained in a restrainer, and a 3-mm segment
of distal tail was clipped with a razor blade. The tail was immediately immersed in isotonic saline at 37°C, and time to complete cessation of bleeding was defined as the bleeding time.

Determination of vascular reactivity

-Preparation of blood vessels
Mice received a lethal injection of intraperitoneal sodium pentobarbital (40mg/kg), and blood was drawn by intracardiac puncture. We excised the aorta from the aortic arch to the celiac artery but we dissected segment only from the upper part of the aorta (thoracic) for vascular function studies. The thoracic aorta was immersed in Krebs solution (composition in mmol/L: NaCl 119; KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.17, CaCl₂ 2.5, EDTA 0.027, glucose 11, and NaHCO₃ 25) bubbled with 95% O₂ and 5% CO₂. Loose connective tissue in the adventitia was carefully removed under binocular magnification. Two or three 2-mm–wide arterial segments of thoracic aorta were dissected, with special care to preserve the integrity of the endothelium. The aortic rings were suspended horizontally between two wire hooks in organ baths containing oxygenated, 37°C Krebs solution. Changes in isometric tension were monitored continuously on a Mac Lab 8 system (AD Instruments Ltd). The resting tension of the aortic rings was set to 1 g. After a 30-minute equilibration period, the contractile response of vascular smooth muscle was checked by incubation with 30 mM of KCl.

-Basal release of NO
First, endothelium function was checked by the addition of acetylcholine (ACh) (10 µM) on one norepinephrine (NE) precontracted aortic segment. After washout and an
equilibration period, basal release of NO was estimated by N\textsuperscript{ω}-nitro-L-arginine (10 \( \mu \)M)-induced endothelium-dependent facilitation of NE (0.1 \( \mu \)M) contraction.

-Contractile and relaxing responses of arteries
Two arterial segments were used to determine the contractile and relaxing responses to ACh and NE. After washout and equilibration, the aortic rings were contracted by cumulative additions of NE in the 0.001-to 3-\( \mu \)M concentration range. After washout and a 1-hour recovery period, the aortic segments were pre-contracted with NE at a concentration that gave approximately 75% of the maximal contraction as determined from the preceding NE concentration-response curve. After the pre-contraction to NE had reached a plateau value, the aortic rings were relaxed by cumulative additions of ACh in the 0.001- to 10-\( \mu \)M concentration range.

-Data Analysis
The contraction of aortic rings to NE was expressed in mg, and the relaxation to ACh was expressed as a percentage of the contraction to NE. Maximal relaxation (\( E_{\text{max}} \)) values for NE and ACh were determined from experimental data. \( pD_2 \) values, corresponding to the negative logarithm of the agonist concentration required to produce a half maximal relaxing effect (\( EC_{50} \)), were calculated after fitting each curve according to a sigmoid equation of the form:

\[
Y = P1 + P2/(1 + e^{P3(logX-P4)})
\]

in which \( Y \) is the agonist effect, \( X \) is the corresponding agonist concentration; \( P1 \), the lower plateau response; \( P2 \), the range between the lowest and the highest plateaus of the concentration-effect curve; \( P3 \), a negative curvature index indicating the slope independently of the range; and \( P4 \), log \( EC_{50} \) [3].
Platelet preparation and quantitation - Platelet-rich plasma was obtained by a single 15 min, 100g centrifugation of fresh citrated total blood at 20°C followed by a 10 min, 1200g run at 4°C to allow platelet separation. Platelets were then washed three times with 0.15 M sodium chloride, and immediately used for tocopherol and protein quantitation. Homegeneity of platelet preparations was checked by optical microscopy and platelet counts were obtained by using the Malassez cell method.

Platelet function – Platelet aggregation time was determined on citrated fresh whole blood using the Platelet Function Analyzer (PFA)-100 closure time test (Dade Behring, Deerfield, Illinois, USA) in collagen/ADP cartridges [4].

Photothrombosis - The mice were anesthetized by an intraperitoneal injection using a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). Permanent focal ischemia was induced by photothrombotic cortical occlusion as previously described [5, 6]. Briefly, the anesthetized mice were infused for 20s with the photosensitizer dye Rose Bengal [10 mg/kg, intravenous (i.v.)] and a laser beam was focused with an optic fibre (1 mm interior diameter, emerging power 10 mW) through the skull onto the right hemisphere (1 mm posterior and 2 mm lateral relative to the bregma). The laser system was a diode-pumped solid-state laser (Laser Quantum Entertainer 200, Opton Laser International, Orsay France) working at 532 nm. The skull was irradiated for 3 min, the irradiation beginning 30 s before the dye injection. All mice were euthanized either 2h or 24 h after the onset of ischemia.

Determination of infarct size – The anesthetized mice were transcardially perfused with saline and 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) through the left ventricle at 100 mm Hg pressure until colourless perfusion fluid was obtained from the right atrium. The brains were removed, postfixed in 4% PFA
for 30 min and transferred to a 20% sucrose solution for 48 hours. They were then frozen in isopentane at −45°C and stored at −80°C. Coronal sections (30-µm thick) were cut in a cryostat (HMSSO, Microm, Francheville, France) at −20°C at 150-µm interval, collected on slides, and stained with Cresyl Violet (0.4%). Injured cortical areas (unstained tissue) were measured using a computer image analysis system (Image J), and the distances between respective coronal sections were used to calculate a linear integration for the lesion volume.

**Immunohistochemistry**- Immunostaining was performed on 30-µm thick coronal sections of perfused and fixed brains. The sections were rinsed in PBS and incubated for 30 min in 10% goat serum (Dako) in PBS to block non-specific binding sites. The sections were then incubated with either non specific rabbit IgG (control) or rabbit anti-human fibrinogen/fibrin antibodies (Dako) (3 hours, room temperature, dilution 1:200) and subsequently incubated for 2 hours with FITC-conjugated goat anti-rabbit IgG antibodies (Abcam), washed and coverslipped with a fluorescent mounting medium. Fibrin-positive tissue was quantitated using a computer image analysis system (Image J), and the distances between coronal sections were used to calculate a linear integration for the coagulated tissue volume.

**Cytokine and Chemokine Measurements** – Interleukin 6 (IL-6) and monocyte chemoattractant protein 1 (MCP-1) levels were assayed in plasma and brain homogenates by cytometric bead array using a commercially available kit (Cytometric Bead Array mouse inflammation kit; BD Biosciences) according to the manufacturer's instructions.

**Alpha-tocopherol assay** - Blood was collected on sodium citrate (1/9, vol/vol) (Sigma, St Louis, Mo) via a heart puncture and centrifuged (10 minutes, 2000g at
Erythrocytes were washed with 0.15 mol/L sodium chloride supplemented with 0.5% pyrogallol (wt/wt; Sigma), and were counted using the Malassez cell method. The thoracic and abdominal aortas of anaesthetized mice were rapidly removed and immersed in a saline solution. After the loose connective tissue had been carefully removed, the arteries were homogenized in a micropotter with 500 µL of saline containing 50 mM ascorbic acid, and 500 µL of an ethanol solution containing 50 mg/L butylated hydroxytoluene (BHT). The brains were harvested and homogenized in saline containing 50 mg/L BHT. Alpha-tocopherol was extracted from plasma, vascular wall, erythrocytes and brain and quantified using high-performance liquid chromatography (HPLC) as previously described [7-10], using tocol as an internal standard. Tocopherol analysis in platelets was performed on a HP-5MS fused silica capillary column (30 m*0.25 mm, 0.25 µm, Agilent Technologies) that was connected to a Hewlett Packard HP6890 Gas Chromatograph with a HP-7683 Injector and a HP5973 Mass Selective Detector (MSD). Gas Chromatography/Mass Spectrometry (GC/MS) conditions were as follows: helium as a carrier gas at a flow-rate of 1.1 mL/min; injector temperature 250°C; oven temperature 150°C; temperature increment 15°C/min to 280°C, then 2°C/min to 300°C that was held for 5 min. Mass spectrometer was operated under electron impact mode with an electron energy of 70 eV. Temperature of ion source and quadrupole were 230°C and 150°C, respectively. Ions at m/z 460 and 502 were used to measure tocol (internal standard) and alpha-tocopherol, respectively. Only alpha-tocopherol was detectable in platelets, and amounts were determined by comparison with a standard curve that was obtained with known amounts of alpha-tocopherol.
Cholesterol, linoleic acid and hydroxyoctadecanoic acid (HODE) assay—

Cholesterol and linoleic acid were extracted from plasma, erythrocytes and brain homogenates as described by Yoshida et al [11].

Cholesterol was quantitated by GC-EIMS using a GC 6890/MSD 5973 apparatus and a HP-5MS column (30mx250µm, 0.25µm) (Agilent Technologies) using epicoprostanol as an internal standard. The analysis was performed in the SIM mode, and selected ions were m/z=368 for cholesterol and m/z=370 for epicoprostanol, respectively.

Linoleic acid was quantitated by negative GC-CIMS using a GC 7890/MSD 5975 apparatus and a HP-5MS column (30mx250µm, 0.25µm) (Agilent Technologies). Heptadecanoic acid was used as an internal standard. The analysis was performed in the SIM mode, and selected ions were m/z=269 and m/z=279 for heptadecanoic and linoleic acid, respectively.

Total HODE (9-HODE and 13-HODE) was quantitated in blood and brain homogenates by LC-MS/MS using an HPLC 1200 apparatus coupled to a 6460 mass spectrometer (Agilent Technologies). To minimize uncontrolled oxidation during sample extraction, 0.5% pyrogallol was added to blood samples immediately after drawing. 13-(S)-HODE-d4 (Cayman Chemicals) was used as an internal standard. Extracted samples were separated on a Zorbax Eclipse Plus C18 column (2.1x100 mm, 1.8 µm). The MS analysis was performed in the MRM mode, selected transitions were 299 ->198, 295 ->195, 295 ->171 for 13-(S)-HODE-d4, 13-HODE and 9-HODE respectively.

Protein Assay – Proteins were measured lysed platelets by using the Bicinchoninic Acid assay kit (Pierce, Rockford, IL).
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