Dietary α-Linolenic Acid Inhibits Arterial Thrombus Formation, Tissue Factor Expression, and Platelet Activation


Objective—Plant-derived α-linolenic acid (ALA) may constitute an attractive cardioprotective alternative to fish-derived n-3 fatty acids. However, the effect of dietary ALA on arterial thrombus formation remains unknown.

Methods and Results—Male C57Bl/6 mice were fed a high-ALA or low-ALA diet for 2 weeks. Arterial thrombus formation was delayed in mice fed a high-ALA diet compared with those on a low-ALA diet (n=7; P<0.005). Dietary ALA impaired platelet aggregation to collagen and thrombin (n=5; P<0.005) and decreased p38 mitogen-activated protein kinase activation in platelets. Dietary ALA impaired arterial tissue factor (TF) expression, TF activity, and nuclear factor-κB activity (n=7; P<0.05); plasma clotting times and plasma thrombin generation did not differ (n=5; P=not significant). In cultured human vascular smooth muscle and endothelial cells, ALA inhibited TF expression and activity (n=4; P<0.01). Inhibition of TF expression occurred at the transcriptional level via the mitogen-activated protein kinase p38 in smooth muscle cells and p38, extracellular signal-regulated kinases 1 and 2, and c-Jun N-terminal kinases 1 and 2 in endothelial cells.

Conclusion—ALA impairs arterial thrombus formation, TF expression, and platelet activation and thereby represents an attractive nutritional intervention with direct dual antithrombotic effects. (Arterioscler Thromb Vasc Biol. 2011;31:1772-1780.)

Key Words: fatty acids ■ platelets ■ thrombosis ■ α-linolenic acid ■ tissue factor

A rterial thrombosis is the critical step in the development of acute vascular syndromes.1–3 Circulating platelets are activated by interaction with subendothelial collagen at the site of an injury.4 In parallel, membrane-bound tissue factor (TF) expressed on vascular cells acts as a receptor for activated factor VII (VIIa).5,6 The TF/VIIa complex triggers the coagulation cascade and the formation of activated factor X (Xa), ultimately resulting in thrombin formation, which in turn cleaves protease-activated receptors on the platelet surface, boosting platelet activation and clot formation.8 The critical role of platelets and TF in the pathogenesis of acute coronary syndromes has indeed been well documented.7–9

Experimental and epidemiological studies have extensively characterized the cardioprotective and antithrombotic effects of fish-derived dietary long-chain n-3 fatty acids (FAs) eicosapentaenoic acid (EPA) (C20:5 n-3) and docosahexaenoic acid (DHA) (C22:6 n-3).10–13 Consistent with these observations, an increased risk for developing cardiovascular disease was identified in populations with low intake of EPA and DHA, prompting the American Heart Association to modify its nutrition guidelines.14

α-Linolenic acid (ALA) is an essential n-3 FA found at high concentrations in vegetable oils, in particular flaxseed oil, where it accounts for 50% of the total FA content. Following oral intake, ALA is partially converted into EPA and DHA. The rate of conversion of ALA into long-chain n-3 FAs ranges from 0.05% to 10%.15 In recent epidemiological studies, ALA consumption has been inversely associated with the incidence of myocardial infarction, sudden cardiac death, and coronary artery calcification.16–20 Despite these promising data, the precise biological mechanisms mediating the cardioprotective effects of ALA remain barely understood. In particular, it remains controversial whether the rather poor conversion of ALA to EPA and DHA can account for the beneficial effects of ALA or whether ALA exerts direct biological effects.21,22

Because increasing evidence suggests that ALA can serve as a cardioprotective nutritional supplement, this study addresses the question whether dietary ALA inhibits thrombus formation in vivo and analyzes the mechanisms involved.

Materials and Methods
For details, please see the Supplemental Materials and Methods, available online at http://atvb.ahajournals.org.
ALA Diet and Carotid Artery Thrombosis Model
C57BL/6 mice were fed a 0.21% (w/w) cholesterol diet containing either a high (7.3%) or low (0.03%) ALA concentration (Supplemental Table I).

Immunohistochemistry
Thrombus composition was analyzed in paraffin-fixed sections from occluded carotid arteries. Sections were stained for TF, fibrin, and the platelet marker CD41.

Prothrombin Time and Activated Partial Thromboplastin Time
Plasma was isolated from citrated blood (3.2%; 1/10) by centrifugation (2500g; 4°C; 15 minutes). Prothrombin time and activated partial thromboplastin time were assessed using the START 4 analyzer (Diagnostica Stago).

Thrombin Generation
Plasma thrombin generation was monitored by automated calibrated thrombography and the results analyzed using Thrombinscope software (Thrombinscope BV, Maastricht, the Netherlands).

Platelet Aggregation
Platelet aggregation was studied by whole blood impedance aggregometry (Chrono-Log, Havertown, PA).

FA Profile in Aortic Tissue
FA profiles of the pooled tissues were analyzed by gas chromatography as described. Measurements were performed in duplicate, and the mean value is displayed.

Cell Culture
Human aortic vascular smooth muscle cells (VSMC) (Clonetics, Allschwil, Switzerland) and human aortic endothelial cells (HAEC) (Clonetics) were cultured as described. Protein expression was determined by Western blot analysis and RNA by real-time polymerase chain reaction.

TF Activity
Factor Xa generation on the surface of VSMC or HAEC or in tissues was analyzed using a colorimetric assay (Actichrome, American Diagnostica Inc).

TF Promoter Activity
TF promoter activity was measured as described. A minimal TF promoter (~227 bp to +121 bp) was cloned upstream of the firefly luciferase reporter gene, and a recombinant adenoviral vector was constructed. HAEC were transduced with the vector Ad5/hTF/Luc (100 pfu/cell) for 1 hour. An adenoviral vector without reporter gene (VQAd/Empty) was used as a negative control. After transduction, HAEC were grown in medium (10% fetal bovine serum) for 24 hours and then serum-starved for 24 hours before tumor necrosis factor-α (TNF-α) stimulation with or without ALA (30 μmol/L) pretreatment. Firefly luciferase activity was determined by luminometer (Berthold Technologies, Bad Wildbad, Germany) and normalized to the protein concentration in the lysates.

Nuclear Extracts and Nuclear Factor-κB Activity
Nuclear extracts were obtained from HAEC or mouse aorta using a nuclear extraction kit (Active Motif), and nuclear factor-κB (NF-κB) activity was measured using a TransAM NF-κB p65 kit (Active Motif).

Statistics
Data are presented as mean±SEM. Statistics were performed using GraphPad Prism 4.0 software (GraphPad Software Inc, La Jolla, CA). Statistical analysis was performed using a 2-tailed unpaired Student t test or 1-way ANOVA as appropriate. A value of P<0.05 was considered significant.

Results
Dietary ALA Inhibits Arterial Thrombosis
C57BL/6 mice were fed for 2 weeks a 0.21% cholesterol diet containing either 7.3% (w/w) ALA (high ALA, treated group) or 0.03% (w/w) ALA (low ALA, control group). No difference in body weight was observed after 2 weeks of diet (n=7; P=not significant [NS]; data not shown). Aorta from animals fed a high-ALA diet showed markedly increased ALA levels (14.9% of total FA content; Supplemental Table II) compared with tissue from control animals (0.14% of total FA content; Supplemental Table II). In contrast, EPA and DHA levels did not differ between the 2 groups. Total cholesterol levels were not altered (control: 1.95±0.21 mmol/L; high ALA: 2.29±0.30 mmol/L; n=8; P=NS).

Mice on the control diet developed carotid artery thrombosis within a mean occlusion time of 40±4.2 minutes, whereas mice on a high-ALA diet occluded after 68±6 minutes (n=7; P<0.005; Figure 1A). Initial blood flow did not differ between the 2 groups (n=7; P=NS). Intravenous administration of an inhibitory anti-TF antibody before the onset of photochemical injury reduced the difference in occlusion times between control and high-ALA diets (n=7; P=NS; Figure 1B). Plasma clotting times (prothrombin time and activated partial thromboplastin time) were similar in the 2 groups (prothrombin time: 11.1±0.2 seconds [low ALA] versus 11.5±0.4 seconds [high ALA]; n=7; P=NS; activated partial thromboplastin time: 20.3±0.8 seconds [low ALA] versus 21.1±0.7 seconds [high ALA]; n=7; P=NS). Thrombin generation (endogenous thrombin potential and time to peak) did not differ between the groups (endogenous thrombin potential: 747±94 nmol/L per minute [low ALA] versus 818±80 nmol/L per minute [high ALA]; n=5; P=not significant; time to peak: 6.2±0.7 minutes [low ALA] versus 4.8±0.5 minutes [high ALA]; n=5; P=NS). Furthermore, no difference in peak thrombin values (104.4±28.7 nmol/L [low ALA] versus 118.8±22.9 nmol/L [high ALA]; n=5; P=NS) or in lag times (2.9±1.36 minutes [low ALA] versus 2.1±1.3 minutes [high ALA]; n=5; P=NS) was observed between the groups.

To analyze differences in thrombus composition, sections from occluded carotid arteries were stained for TF, fibrin, and platelets. Although thrombi were occlusive in both groups, TF staining of in the vascular media was lower in the high ALA group compared with the control group (Figure 1C; n=4). In contrast, fibrin staining was similar in thrombi from both groups. Analysis of platelet content by immunohistochemistry for CD41 demonstrated a reduced platelet content in thrombi in the ALA-treated group compared with control (Figure 1D; n=4).

Dietary ALA Inhibits Platelet Aggregation
Thrombin-induced platelet aggregation (Figure 2A to 2D) was inhibited in mice fed a high-ALA diet compared with control platelets (maximal aggregation: n=5, P<0.005, Figure 2B; area under the curve: n=5; P<0.005, Figure 2C; lag...
time: n=5, P<0.05, Figure 2D). When aggregation was induced by collagen, a similar inhibition was observed (maximal aggregation: n=5, P<0.05, Figure 2F; area under the curve: n=5, P=0.05, Figure 2G; lag time: n=5, P=0.14, Figure 2H). Platelet number did not differ significantly between the groups (1062±46 vs 911±103 L [low ALA] versus 1120±51 vs 1039±103 L [high ALA]; n=5; P=NS).

Treatment of human platelets with ALA (30 μmol/L) for 30 minutes ex vivo exerted a similar inhibition of thrombin- and collagen-induced platelet aggregation (n=5; Supplemental Table IIIA and IIIB). ALA (30 μmol/L) impaired collagen- and thrombin-induced p38 phosphorylation (n=3; P<0.01 versus collagen or thrombin alone; Supplemental Figure IA and IB).

Dietary ALA Inhibits TF Expression and NF-κB Activation
TF activity in carotid arteries was markedly decreased in mice fed a high-ALA diet compared with controls (n=7; P<0.005; Figure 3A). Inhibition of TF with a specific antibody confirmed that factor Xa generation was TF dependent (Figure 3B). This decrease in TF activity was paralleled by an impaired TF mRNA expression in aortic tissue (n=7; P<0.01; Figure 3C). In contrast, expression of TF pathway inhibitor mRNA did not differ (ΔΔCt: 0.03±0.04 low ALA group versus 0.03±0.04 high ALA; n=5; P=NS). NF-κB p65 DNA binding affinity was measured in nuclear extracts from aortic tissue. NF-κB activity was impaired in mice fed a high-ALA diet compared with controls (n=7; OD 490 nm: 0.97±0.02 [low ALA] versus 0.70±0.09 [high ALA]; n=7; P<0.05; Figure 3D).

ALA Inhibits TF Protein Expression in VSMC
Treatment with ALA (30 μmol/L) for 24 hours reduced TF expression in VSMC by 46% (n=4; P=0.05) compared with control (Figure 4A). Real-time polymerase chain reaction analysis confirmed that ALA inhibited TF expression in VSMC at the transcriptional level (n=4; P<0.005; Figure 4B). In line with this observation, ALA decreased TF activity in VSMC (n=4; P<0.001; Figure 4C). Experiments performed with an inhibitory anti-TF antibody confirmed that factor Xa generation in VSMC was TF dependent (Figure 4D). TF expression was affected by neither the n-6 FA α-linoleic acid nor the saturated FA stearic acid (n=4; P=NS; data not shown). No cytotoxic effects of ALA, α-linoleic acid, or stearic acid in VSMC were observed for any of the concentrations (data not shown).

ALA Inhibits p38 Phosphorylation and NF-κB Activity in VSMC
Analysis of the mitogen-activated protein (MAP) kinase phosphorylation demonstrated that treatment of VSMC with ALA for
24 hours resulted in a significant decrease in p38 activation (49±14% inhibition; n=4; P<0.05; Figure 5A). In contrast, activation of c-Jun N terminal kinase and extracellular signal-regulated kinase remained unaffected. Blockade of the MAP kinase p38 with SB203580 (10 μmol/L) inhibited TF expression in VSMC (n=4; P<0.01; Figure 5B). Treatment with ALA (30 μmol/L) reduced NF-κB p65 DNA binding affinity in nuclear extracts from VSMC (n=4; P<0.05; Figure 5C).

ALA Inhibits TNF-α-Induced TF mRNA Expression via MAP Kinase and Apoptosis Signal-Regulating Kinase 1 in HAEC

HAEC were treated with ALA or vehicle for 1 hour and then stimulated with TNF-α (5 ng/mL) for 4 hours. ALA inhibited TNF-α-induced endothelial TF expression in a concentration-dependent manner with a maximal effect at 30 μmol/L (86% inhibition; n=5; P<0.001 versus TNF-α alone; Figure 6A). In line with this observation, ALA decreased TF activity in HAEC (51% inhibition versus TNF-α alone; n=4; P<0.001; Figure 6B).

Real-time polymerase chain reaction analysis demonstrated that ALA (30 μmol/L) inhibited TNF-α-induced endothelial TF mRNA expression (n=4; P<0.001; Figure 6C). This decrease in TF mRNA was paralleled by an impaired activation of the MAP kinases c-Jun N-terminal kinase, p38, and extracellular signal-regulated kinase (28%, 62%, and 55% inhibition versus TNF-α alone, respectively;
n=4; P<0.05; Figure 6D). Consistent with this pattern of MAP kinase inhibition, ALA impaired activity of the MAP kinase kinase kinase apoptosis signal-regulating kinase 1 (ASK1) (n=3; P<0.005 versus TNF-α alone; Figure 6E). ALA (30 μmol/L) inhibited TNF-α-induced TF promoter activation in HAEC by 47% (n=4; P<0.05; Figure 6F). ALA (30 μmol/L) blunted TNF-α-induced IκB-α degradation (n=3; P<0.05). This effect was paralleled by reduced DNA binding affinity of NF-κB p65 protein in mouse carotid arteries.

Figure 3. Dietary ALA inhibits TF expression and NF-κB activity in vivo. Dietary ALA inhibited TF activity as assessed by factor Xa generation in carotid arteries (A, *P<0.005). B, Treatment of tissue lysates with an inhibitory anti-TF antibody (10 mg/mL) inhibited factor Xa generation (*P<0.01 vs control diet group in the presence of an IgG control antibody). Dietary ALA impaired TF mRNA expression (C, *P<0.05) and NF-κB p65 DNA binding affinity (D, *P<0.05) in mouse carotid arteries.

Figure 4. ALA inhibits TF expression in VSMC. A, Treatment with ALA (30 μmol/L) for 24 hours inhibited TF protein expression in VSMC (*P<0.05 vs control). B, ALA (30 μmol/L) inhibited TF mRNA expression (*P<0.005 vs control) after 24 hours. C, ALA (30 μmol/L) also impaired TF activity in VSMC (*P<0.001). D, Treatment of VSMC with an inhibitory anti-TF antibody inhibited factor Xa generation (*P<0.05 vs control treated with an IgG control antibody).
levels of the long-chain n-3 FA EPA and DHA. Consistent with these findings, ALA by itself impaired platelet aggregation and TF induction in different human primary cell cultures. This effect occurred at the transcriptional level, involving inhibition of MAP kinase phosphorylation, NF-κB activation, and promoter activation.

The ALA concentrations used for experiments in this study are in a clinically relevant range. Indeed, baseline plasma levels of ALA have been reported to range from 17 to 19 μmol/L, whereas a daily oral ingestion of 3 g of ALA leads to ALA plasma levels of 32±17 μmol/L and is well tolerated. Dietary supplementation of ALA for 2 weeks was sufficient to markedly increase the ALA levels in the aortic wall, whereas EPA and DHA levels did not differ from the control group. The lack of a measurable conversion to long-chain n-3 FA is in line with previous epidemiological studies demonstrating that dietary ALA poorly correlates with EPA and DHA levels in adipose tissue, erythrocytes, or plasma. Even more important, the in vivo effects of dietary ALA observed in the present study were mimicked by exogenous ALA in both human platelets and vascular cells ex vivo, where hepatic conversion of ALA to EPA and DHA does not occur. Taken together, these experiments indicate that the antithrombotic effects of ALA in vivo did not depend on the conversion of ALA to long-chain n-3 FA and provide strong evidence for a direct biological effect of ALA.

To exclude possible effects of other FA, control experiments were performed with the n-6 FA linoleic acid, as well as the saturated FA stearic acid. Neither of these FA altered TF expression, supporting the interpretation that the biological effects of the high-ALA diet on TF and thrombosis occur because of the increased ALA levels.

Dietary ALA supplementation significantly delayed arterial thrombus formation was triggered by photochemical injury in vivo. Because this effect was paralleled by a reduced platelet activation and a diminished arterial TF activity, ALA impairs thrombus formation by a dual action on both critical events involved in arterial thrombosis following vascular injury. To determine the relative contribution of platelets and TF to thrombus formation, an inhibitory anti-TF antibody was applied. Treatment with this antibody reduced the difference in occlusion times between the 2 groups, suggesting that the inhibitory effect of ALA on TF exerts a major effect on thrombus formation, whereas its effect on platelets contributes to inhibition of thrombosis. These results support previous observations demonstrating that arterial thrombosis is primarily driven by TF derived from the vessel wall in this model. TF is barely expressed under basal conditions in endothelial cells, whereas it is constitutively expressed in VSMC. Immunohistochemical analysis confirmed that TF expression in the arterial tunica media was decreased in carotid arteries derived from the ALA-treated group. Inhibition of TF was confirmed in vivo, because the ALA-rich diet decreased TF expression and NF-κB activity in arterial lysates. Consistent with these in vivo observation, treatment of VSMC with ALA for 24 hours decreased basal TF expression. This effect was mediated at the transcriptional level via the MAP kinase p38. Pharmacological inhibition of p38 confirmed the crucial role of p38 activation in constitu-
tive TF expression in VSMC. In line with p38 inhibition, as well as with previous data obtained in monocytes and macrophages, ALA also reduced NF-κB activity in VSMC.33,34 Hence, direct inhibition of TF in VSMC via p38 and NF-κB seems to play a major role in ALA’s effect on arterial thrombus formation.

To study whether ALA also affects inducible TF expression, additional experiments were performed in cytokine-activated endothelial cells. TNF-α-induced endothelial TF expression is mediated through activation of MAP kinases leading to activation of transcription factors such as activator protein-1 and early growth response factor-1, whereas the IκB kinase pathway promotes NF-κB activation.35,36 ASK1 is a redox-regulated kinase playing an essential role in stress-induced activation of MAP kinases, thus triggering various cellular processes, including inflammatory responses and TF expression.32,37 In endothelial cells, TNF-α has been shown to activate ASK1, as well as MAP kinases.6,37 Treatment with ALA significantly reduced phosphorylation of the MAP kinases p38, c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK). E, ALA (30 μmol/L) inhibited TNF-α-induced apoptosis signal-regulating kinase 1 (ASK1) phosphorylation. (*P<0.005 vs TNF-α alone). F, ALA (30 μmol/L) impaired endothelial TF promoter activation. (*P<0.05 vs TNF-α alone). Pho indicates phosphorylated.
control group. Platelet aggregation was analyzed using collagen and thrombin as agonists because they play a dominant role in platelet activation following vascular injury.3 ALA impaired both collagen- and thrombin-induced platelet aggregation, indicating that ALA inhibits glycoprotein VI and Ib-V-IX, as well as protease-activated receptor-mediated platelet activation; hence, ALA does not interfere with activation of a single pathway. Because the MAP kinase p38 plays a crucial role in collagen-induced, as well as thrombin-induced, platelet activation and adhesion, the effect of ALA on p38 phosphorylation in isolated platelets was investigated.38,39 ALA decreased collagen- and thrombin-induced p38 activation, an effect that may well account for the reduced aggregation observed in ALA-treated platelets. Taken together, these observations may provide an explanation for the remaining difference in occlusion times observed between the 2 groups after treatment with an inhibitory anti-TF antibody.

In summary, this study provides solid evidence for a potent dual antithrombotic effect of an ALA-rich diet by reducing platelet activation and impairing vascular TF expression. These effects were reproduced in a mouse model, as well as in human primary cell cultures, and were not related to an increase in EPA and DHA levels, as confirmed by our data. Hence, this study generates pathophysiological evidence for direct antithrombotic effects of dietary ALA supplementation. Because limited availability or unfavorable geographic conditions restrict access to n-3 FA from marine origin in many countries, plant-derived ALA might therefore represent an attractive cardioprotective alternative. However, keeping in mind previous clinical setbacks with experimentally promising nutritional compounds such as vitamin E, there is a great need for placebo-controlled randomized large-scale clinical trials to confirm the long-term antithrombotic potential of dietary supplemented ALA.

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Disclosures
None.

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Material and methods

ALA diet and carotid artery thrombosis model

8-week-old male C57BL/6 mice weighing on average 24 g were fed a 0.21% w/w cholesterol diet containing either a high ALA (7.3% w/w, D06080702, Research Diets, New Brunswick, NJ, USA) or low ALA concentration (0.03% w/w, D06080701, Research Diets) for 2 weeks (supplemental table 1). ALA was supplemented as flaxseed oil in the high ALA group and replaced by cocoa butter in the control group. Thrombus formation was induced by photochemical injury on the 14th day of diet. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (87 mg/kg; Butler, Columbus, OH). Rose bengal (Fisher Scientific, Fair Lawn, NJ, USA) was diluted in phosphate-buffered saline (PBS) and then injected into the tail vein at a final concentration of 50 mg/kg. The right common carotid artery was exposed following a midline cervical incision and the blood flow monitored using a Doppler flow probe (Model 0.5 VB, Transonic Systems, Ithaca, NY) connected to a flowmeter (Model T106, Transonic Systems). Photochemical injury was induced by a 1.5 mW green light laser (540 nm; Melles Griot, Carlsbad, CA) 6 minutes after intravenous rose bengal injection. From the onset of injury, blood flow was monitored until occlusion occurred, which was defined as a flow below 0.1 ml/min for at least 1 minute. Where mentioned, an inhibitory mouse anti-TF antibody obtained by immunization of rabbits against the extracellular murine TF amino acid domain 29-250 (American Diagnostica Inc., Stamford, CA, No. 4515,) was injected intravenously at a concentration of 3 mg/kg body weight 20 minutes prior to the rose bengal injection.
Immunohistochemistry

Occluded carotid arteries were harvested, fixed in 3% formalin and embedded in paraffin. Sections were stained for platelets (anti–mouse CD41; Abcam, Cambridge, UK; 1:50 dilution), fibrin (Anti-human, fibrin Fragment E, Cedarlane, Burlington, ON, Canada; 1:100 dilution) and TF (American Diagnostica; 1:50 dilution). Slides were blocked with 10% goat serum, and the primary antibody applied overnight at 4°C. Immunodetection was performed using biotinylated rabbit anti–mouse (for fibrin and TF, 1:200 dilution), or Texas red conjugated goat anti-rat secondary antibodies (1:200 dilution). Slides were counterstained with hematoxylin and eosin (TF and fibrin) or 4',6-diamidino-2-phenylindole (CD41).

Platelet preparation

For analysis of p38 expression, citrated whole blood was centrifuged at 170xg for 7 minutes to obtain platelet-rich plasma (PRP). PRP was centrifuged a second time at 170xg for 7 minutes to remove residual erythrocytes. To isolate platelets PRP was centrifuged at 350xg. Pelleted platelets were resuspended in tyrode buffer (10 mM Hepes, 12 mM NaHCO_3, 137 mM NaCl, 2.7 mM KCl, 5 mM glucose; pH 7.4) and incubated with thrombin or collagen in the presence of ALA (30 μM) or the vehicle for various time points. Reactions were stopped by addition of ice-cold lysis buffer and p38 phosphorylation assessed by western blot analysis as described below.

Platelet count and aggregation

Platelets were counted by flow cytometry using whole blood collected in EDTA tubes (B&D Diagnostics, Franklin Lakes, NJ, USA). Platelet aggregation was studied using a Chrono-Log whole blood impedance aggregometer (Chrono-Log, Havertown, PA, USA). For studies performed in murine platelets, citrated blood was drawn by
puncture from the right ventricle. For studies in human platelets, blood was obtained from healthy human volunteers and treated with ALA (30 μM; Cayman Chemical, Ann Arbor, MI, USA) or vehicle (ethanol 0.1%, Sigma Aldrich, St. Louis, USA) for 30 minutes. Aggregation studies were performed with citrated blood within 1 hour. Platelets were equilibrated under constant stirring for 1 minute prior addition of human thrombin (0.5 U/mL; Sigma Aldrich) or equine collagen type 1 (5 μg/mL; Chrono-Log). Aggregation was displayed as a function of time (AGGRO/LINK® Software; Chrono-Log). Results were monitored for 6 minutes and expressed as maximal aggregation (ohm[Ω]), area under the curve (Ωxmin), and lag time (seconds).

**Fatty acid profile in aortic tissue**

Aortic tissue was pooled (n=7 per group) and FA profiles of the tissues were analyzed by gas chromatography. Fat was extracted via hexane:isopropanol (3:2) and triglycerides were saponified using methanolic sodium hydroxide. FA were converted to fatty acid methyl ester (FAME) with methanolic boron trifluoride. FAME were separated using a gas chromatograph (Hewlett Packard HP 6890 Series, GC Systems, Waldbronn, Germany) equipped with a 200 mm x 0.25 mm CP7421-column (Varian, Middleburg, NL). For FAME identification, a FAME mixture was used as external standard (Supelco 37 component FAME mix). The proportion of different FAME was calculated using the ratio of the peak area of the respective FAME to the sum of total FAME peak areas. FA were evaluated using the HP ChemStation software (Hewlett Packard, CA, USA). Measurements were performed in duplicate and the mean value displayed.

**Prothrombin time (PT) and activated partial thromboplastin time (aPTT)**
Plasma was isolated from citrated blood (3.2%; 1/10) by centrifugation (2500xg; 4°C; 15 minutes). PT and aPTT were assessed using the START 4 analyzer (Diagnostica Stago, France).

**Thrombin generation**

Plasma thrombin generation was monitored by automated calibrated thrombography. Platelet poor plasma was mixed with human recombinant TF (5 pM) and a fluorogenic substrate for thrombin (Z-Gly-Gly-Arg-AMC Bachem Basle, Switzerland) and then recalcified. Thrombin generation was measured in a Fluoroskan® Ascent reader (Thermo Labsystems, Helsinki, Finland) and calculated using the Thrombinscope software (Thrombinscope BV, Maastricht, The Netherlands). Endogenous thrombin potential, representing the area under the thrombin generation curve and time to peak thrombin formation (TTP) were displayed using the Prism 4 software package (GraphPad Software Inc., La Jolla, USA).

**Cell culture**

Human aortic vascular smooth muscle cells (VSMC; Clonetics, Allschwil, Switzerland) and human aortic endothelial cells (HAEC; Clonetics) were cultured as described. For experiments, VSMC were serum-starved for 24 hours before pretreatment with ALA (Cayman Chemical, Ann Arbor, MI, USA), stearic acid (SA, Sigma Aldrich), linoleic acid (LA, Sigma Aldrich) or vehicle (ethanol 0.1%, Sigma Aldrich) for 6 or 24 hours. To block the mitogen-activated protein (MAP) kinase p38 MAP kinase (p38), VSMC were treated with SB203580 (Sigma Aldrich; 10) for 24 hours. For experiments with HAEC, cells were incubated with ALA for 1 hour prior stimulation with 5 ng/mL TNF-α (R&D Systems, Minneapolis, MN) for 4 hours for protein expression analysis and for 2h for analysis of TF mRNA expression. NFκB
activation was inhibited by ammonium pyrrolidinedithiocarbamate (PDTC) or BAY 11-7082 (both from Sigma Aldrich) pretreatment for 24 hours. To determine cytotoxicity, a colorimetric assay for detection of lactate dehydrogenase release was applied (Roche, Basel, Switzerland).

**Western blot**

Protein expressionin was determined by Western blot analysis. Antibodies against human TF (No. 4503) and tissue factor pathway inhibitor (TFPI, No. 4901) (both from American Diagnostica, Stamford, CT) were used at 1:2500 dilution. Antibodies against phosphorylated p38, ERK, JNK, and ASK1 (all from Cell Signaling) were used at 1:1000, 1:5000, 1:2000, and 1:2000 dilution, respectively. Antibodies against total p38, ERK, JNK, and ASK1 (all from Cell Signaling, Danvers, MA) were used at 1:2000, 1:5000, 1:2000, and 1:2000 dilution, respectively. Blots were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (1:10000 dilution, Chemicon International, Temecula, CA).

**Factor Xa generation**

To assess TF activity factor Xa generation on the surface of VSMC and HAEC was analysed using a colorimetric assay (American Diagnostica Inc, ACTICHROME). TF/FVIIa complex converted human factor X to factor Xa, which was measured by its ability to metabolize a chromogenic substrate. The absorbance of the reaction mixture was measured at 405 nm and values displayed after substraction of the background value. To confirm TF dependency of factor Xa generation in VSMC, cells were incubated with an inhibitory anti-TF antibody (American Diagnostica, No. 4509; 1:200) 30 minutes prior addition of exogenous factor VIIa.

For analysis of factor Xa generation in mouse carotid arteries, occluded right
carotid arteries from treated and control mice were homogenized in 50 µL lysis buffer (0.1% Triton-X, 100 mmol Nacl, 50 mmol Tris-Hcl, pH 7.4). 25 µg of tissue lysates were used for measurements. TF dependency of factor Xa generation in tissue lysates was confirmed by incubation with an inhibitory mouse anti-TF antibody (American Diagnostica; No. 4515; 10 mg/ml) 30 minutes prior addition of exogenous factor VIIa.

Real Time PCR

RNA was extracted from VSMC, HAEC and mouse aorta using TRIzol Reagent (Invitrogen, Carlsbad, CA) and reverse transcribed using Ready-To-Go You-Prime First-Strand Beads (Amersham, Buckinghamshire, UK). For real-time PCR analysis the following primers were used: for full length human TF (F3): sense 5’-TCCCCAGAGTTCACACCTACC–3’, antisense 5’-CCTTTCTCCTGGCCCATACAC–3’; for human ribosomal L28: sense 5’-GCATCTGCAATGGATGGT-3’, antisense 5’-TGTCTTTCGGATCATGTGT-3’; for murine full length tissue factor: sense: 5’-CAATGAATTCTCGATTGATGTG-3’, antisense: 5’-GGAGGATGATAAGATGCTGGG-3’; for murine tissue factor pathway inhibitor: sense: 5’-ACTGTGTGTCTGTTGCTTAGCC-3’, antisense: 5’-GTTCTCAGTCCCTCAGCC-3’, and for murine ribosomal S12: sense: 5’-GAAGCTGCAAGCGCTTGA-3’, antisense: 5’-AACTGCAACC-AACCACCTTC-3’. The amplification program consisted of 1 cycle at 95°C for 10 minutes followed by 35 cycles with a denaturing phase at 95°C for 30 seconds, an annealing phase at 60°C for 1 minute, and an elongation phase at 72°C for 1 minute. Melting curve analysis confirmed the accuracy of the amplicon, and PCR products were analyzed on an ethidium bromide stained 1% agarose gel. In each real-time PCR run for human TF and L28, a standard curve generated from serial dilutions of purified amplicons was
included. For murine TF and S12 the ΔΔCt threshold cycle method was used.

**Nuclear Extracts and NFκB activity**

For measurement of NFκB activity, VSMC were treated with ALA (30 μM) or the vehicle for 24 hours. Nuclear extracts were obtained from VSMC or mouse aorta using a nuclear extraction kit (Active Motif, Carlsbad, USA). Total protein (20 μg) was loaded in each well, and NFκB activity was measured using a TransAM NFκB p65 kit (Active Motif).
References


Table I

Diet composition

<table>
<thead>
<tr>
<th>Composition (per kg)</th>
<th>Low ALA</th>
<th>kcal</th>
<th>high ALA</th>
<th>kcal</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-Methionine</td>
<td>3</td>
<td>12</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Casein</td>
<td>195</td>
<td>780</td>
<td>195</td>
<td>780</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>50</td>
<td>200</td>
<td>50</td>
<td>200</td>
</tr>
<tr>
<td>Maltodextrin 10</td>
<td>100</td>
<td>400</td>
<td>100</td>
<td>400</td>
</tr>
<tr>
<td>Sucrose</td>
<td>341</td>
<td>1364</td>
<td>341</td>
<td>1364</td>
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<tr>
<td>Cellulose, BW200</td>
<td>50</td>
<td>0</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>10</td>
<td>90</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td><strong>Cocoa Butter</strong></td>
<td><strong>137</strong></td>
<td><strong>1233</strong></td>
<td><strong>0</strong></td>
<td><strong>0</strong></td>
</tr>
<tr>
<td><strong>Flaxseed Oil</strong></td>
<td><strong>0</strong></td>
<td><strong>0</strong></td>
<td><strong>137</strong></td>
<td><strong>1233</strong></td>
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<tr>
<td>Primex Shortening</td>
<td>63</td>
<td>567</td>
<td>63</td>
<td>567</td>
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<tr>
<td>Mineral Mix S10001</td>
<td>35</td>
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</tr>
<tr>
<td>Calcium Carbonate</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin Mix V10001</td>
<td>10</td>
<td>40</td>
<td>10</td>
<td>40</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>% (w/w)</th>
<th>kcal%</th>
<th>% (w/w)</th>
<th>kcal%</th>
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<tbody>
<tr>
<td>Protein</td>
<td>20</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>50</td>
<td>43</td>
<td>50</td>
</tr>
<tr>
<td>Fat</td>
<td>21</td>
<td>40</td>
<td>21</td>
</tr>
</tbody>
</table>

Total kcal/kg | 4676 | 4676 |

ALA (C18:3 n-3; % [w/w]) | 0.03 | 7.3  |
Table II

Fatty acid profile in aortas from mice fed a low and high ALA diet

<table>
<thead>
<tr>
<th></th>
<th>low ALA (% of FA)</th>
<th>high ALA (% of FA)</th>
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</thead>
<tbody>
<tr>
<td>SFA</td>
<td>29.42</td>
<td>25.07</td>
</tr>
<tr>
<td>MUFA</td>
<td>59.21</td>
<td>40.47</td>
</tr>
<tr>
<td>PUFA</td>
<td>11.37</td>
<td>34.46</td>
</tr>
<tr>
<td>Total n-3</td>
<td>1.69</td>
<td>16.09</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>6.41</td>
<td>1.18</td>
</tr>
<tr>
<td>ALA</td>
<td>0.14</td>
<td>14.99</td>
</tr>
<tr>
<td>EPA</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>DHA</td>
<td>0.42</td>
<td>0.46</td>
</tr>
</tbody>
</table>

SFA=saturated fatty acid; MUFA=monounsaturated fatty acids, PUFA=polyunsaturated fatty acids; ALA=alpha-linolenic acid; EPA=eicosapentanoic acid; DHA=docosohexanoic acid
### Table III

#### A. ALA inhibits thrombin induced human platelet activation ex vivo (n=5)

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>30 µM ALA</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max. Aggregation (Ω)</td>
<td>28.25±1.7</td>
<td>17±1.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Area under the curve (Ωxmin)</td>
<td>104.4±9.8</td>
<td>53.3±9.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lag time (seconds)</td>
<td>33.0±11.9</td>
<td>62.0±30.2</td>
<td>0.15</td>
</tr>
</tbody>
</table>

#### B. ALA inhibits collagen induced human platelet activation ex vivo (n=5)

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>30 µM ALA</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max. Aggregation (Ω)</td>
<td>35.24±1.3</td>
<td>26±1.4</td>
<td>&lt;0.005</td>
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<tr>
<td>Area under the curve (Ωxmin)</td>
<td>114.4±9.8</td>
<td>83.3±9.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lag time (seconds)</td>
<td>18.0±4.9</td>
<td>34.0±4.2</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
Legend

Figure I. ALA inhibits thrombin and collagen induced p38 activation in platelets.

**A.** ALA (30 µM) abrogates thrombin induced p38 phosphorylation (*p<0.01 versus thrombin alone). **B.** ALA inhibits collagen induced p38 phosphorylation (*p<0.01 versus collagen alone).