Effects of dietary flaxseed on atherosclerotic plaque regression

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Francis AA, Deniset JF, Austria JA, LaValleé RK, Maddaford GG, Hedley TE, Dibrov E, Pierce GN. Effects of dietary flaxseed on atherosclerotic plaque regression. Am J Physiol Heart Circ Physiol 304: H1743–H1751, 2013. First published April 12, 2013; doi:10.1152/ajpheart.00606.2012.—Dietary flaxseed can retard the progression of atherosclerotic plaques. However, it remains unclear whether these antiatherogenic effects extend to plaque regression. In the present study, the therapeutic potential of dietary flaxseed on atherosclerotic plaque regression and vascular contractile function was evaluated using a novel rabbit model. Rabbits were randomly assigned to receive either a regular diet for 12 wk (group I) or a 1% cholesterol-supplemented diet for 4 wk followed by a regular diet for 8 wk (group II). The remaining experimental animals were treated as in group II but were fed for an additional 14 wk with either a regular diet (group III) or a 10% flaxseed-supplemented diet (group IV). Animals in group II showed clear evidence of plaque growth stabilization. Their vessels also exhibited significantly lower norepinephrine-induced contraction and an impaired relaxation response to acetylcholine compared with animals in group I. Dietary flaxseed supplementation resulted in a significant ~40% reduction in plaque formation (P = 0.033). Animals in both groups II and III displayed improved contraction and endothelium-dependent vessel relaxation. Dietary flaxseed is a valuable strategy to accelerate the regression of atherosclerotic plaques; however, flaxseed intervention did not demonstrate a clear beneficial effect on the vessel contractile response and endothelium-dependent vasorelaxation.

atherosclerosis; flaxseed; heart disease; regression; vascular contractile function

CARDIOVASCULAR DISEASE (CVD) is currently the leading cause of mortality worldwide. Its prevalence continues to increase despite improved treatments for atherosclerosis and will reach epidemic proportions within a decade due to the escalating predominance of sedentary, unhealthy lifestyles (32, 51, 52). Atherosclerosis is orchestrated by a complex array of molecular and cellular events commencing after endothelial injury. The resultant plaques may continue to progress, ultimately causing acute events such as myocardial infarction and stroke (23, 32). Recently, it has been shown that atherosclerotic plaques are highly dynamic and that their progression can be slowed, stopped, and even reversed by drug intervention (6, 18, 43). For example, aggressive risk modification with statin drug therapy can remove lipids and necrotic material, restore endothelial function and viability, and prevent vascular smooth muscle cell proliferation and phenotype reversal, three processes involved in atherosclerotic plaque formation (18, 43).

Akin to pharmaceuticals, functional foods can also be used to prevent and treat CVD. A functional food has a similar appearance to or may be a conventional food and may be consumed as part of a normal diet to have physiological benefits and/or reduce the risk of chronic disease beyond its basic nutritional effects. Functional foods, such as flaxseed, have increased in popularity and are now considered to be a viable therapeutic strategy for CVD (50). Flaxseed contains one of the highest contents of α-linolenic acid (ALA), an n-3 polyunsaturated fatty acid (PUFA) linked with CVD prevention (20, 28a, 31). Unlike drug therapy, n-3 PUFAs have fewer and milder side effects and are generally considered safe (37). Moreover, milled flaxseed has proven to be cardioprotective and antiatherogenic in animal models (7, 22, 23, 44) as well as in clinical settings (50). However, although flaxseed can diminish the progression of atherosclerotic plaques, it is unclear if dietary supplementation with flaxseed can regress established atherosclerotic plaques.

Although many studies have used nutritional interventions to induce plaque regression (5, 8, 10, 45), these studies have all prematurely initiated their interventions before there was clear evidence of the cessation of plaque growth. If a rabbit is supplemented with dietary cholesterol followed by the withdrawal of cholesterol from the diet, atherosclerotic lesions actively continue to progress. These lesions only begin to stabilize weeks after the removal of cholesterol from the diet and as the hypercholesterolemia resolves (1, 11, 26, 29, 48). Thus, if an intervention is imposed immediately after the withdrawal of cholesterol from the diet, as has been done in previous studies of regression (5, 8, 10, 44, 45), any effects observed will reflect an inhibition of plaque progression and not an effect on plaque regression.

The objective of the present study was to 1) identify a suitable dietary regimen in rabbits that showed clear evidence of plaque growth stabilization, 2) determine the ability of dietary flaxseed to induce or accelerate atherosclerotic plaque regression, and 2) discern whether flaxseed supplementation can reverse any cholesterol-induced vascular contractile abnormalities. It was hypothesized that flaxseed would accelerate the regression of atherosclerotic plaques and improve vascular abnormalities caused by cholesterol supplementation.

MATERIALS AND METHODS

Animals and Dietary Interventions

Male New Zealand White (NZW) rabbits weighing between 2.7 and 3.0 kg (Spilak Farms) were used in two related studies. The experimental protocols were reviewed and approved by the University of Manitoba Protocol Management Review committee. Upon arrival, rabbits were housed in individual cages in rooms with controlled temperatures, humidity, and a 12:12-h light-dark cycle. Animals were fed a normal cholesterol-free diet or a diet containing 1% cholesterol for 4 wk, as previously described (22, 23). After this time, the rabbits were assigned to receive either a regular diet for 12 wk (group I) or a 1% cholesterol-supplemented diet for 4 wk followed by a regular diet for 8 wk (group II). The remaining experimental animals were treated as in group II but were fed for an additional 14 wk with either a regular diet (group III) or a 10% flaxseed-supplemented diet (group IV). Animals in group II showed clear evidence of plaque growth stabilization. Their vessels also exhibited significantly lower norepinephrine-induced contraction and an impaired relaxation response to acetylcholine compared with animals in group I. Dietary flaxseed supplementation resulted in a significant ~40% reduction in plaque formation (P = 0.033). Animals in both groups II and III displayed improved contraction and endothelium-dependent vessel relaxation. Dietary flaxseed is a valuable strategy to accelerate the regression of atherosclerotic plaques; however, flaxseed intervention did not demonstrate a clear beneficial effect on the vessel contractile response and endothelium-dependent vasorelaxation.
randomly allocated to experimental groups that were administered a regular rabbit chow (Nutrena, Nature Wise Performance Rabbit Formula), 1% cholesterol-supplemented diet (Ren’s Pet Depot), or 10% flaxseed-supplemented diet, as previously described (3, 7, 22, 23). Flaxseed was provided by Glanbia Nutritionalis (Angusville, MB, Canada) and contained ~53% ALA. Flaxseed was ground, and the appropriate amount was added to moistened regular rabbit chow. The dietary components were mixed, repelleted, and fan dried. Rabbits were fed 125 g of their respective dietary regimens per day. Water was given ad libitum.

The objective of the first part of our study was to identify when the development of the atherosclerotic plaque had stabilized. NZW rabbits were randomly divided into one of eight experimental treatment groups to assess plaque dynamics after withdrawal from cholesterol feeding (Fig. 1). All animals were maintained on a 1% cholesterol-supplemented diet for 4 wk. A subset of animals was immediately euthanized to identify the extent of aortic atherosclerosis. The remaining animals were provided regular rabbit chow and euthanized at intervals of up to 28 wk after the cessation of cholesterol feeding (Fig. 1). Serum cholesterol levels, triglyceride levels, and aortic lesion areas were then measured at these time points. These data were used to identify the appropriate time to initiate an intervention to induce plaque regression in a subsequent study.

In the second study, NZW rabbits were randomly assigned to one of four experimental groups (Fig. 1). Control rabbits (group I) were maintained on regular rabbit chow for 12 wk. All other animals received 4 wk of a 1% cholesterol-supplemented diet for the initiation of plaque formation followed by 8 wk of regular rabbit chow to stabilize plaque progression (Fig. 1). A subset of animals was immediately used at this time point of plaque stabilization (group II) to establish a baseline starting point for plaque regression. The remaining animals were given an additional 14 wk of regular rabbit chow (group III) or 14 wk of a 10% flaxseed-supplemented diet (group IV; Fig. 1). Serum lipid levels, aortic fatty acid composition, vascular contractile function, and lesion areas were then evaluated in these animals.

**Serum Lipid Collection and Analyses**

Before death, blood samples were taken from the jugular vein while the animal was anesthetized with isoflurane gas (5% with O2 per minute). Samples were collected into EDTA vacutainer tubes (Becton Dickinson, Mississauga, ON, Canada) and centrifuged at 1,800 g for 15 min (4°C). Blood serum was removed and stored at −80°C. Serum cholesterol and triglyceride levels were measured enzymatically using commercial kits (Genzyme Diagnostics, Prince Edward Island, Canada). Fatty acids were extracted and derivatized as previously described (4, 39). The resulting fatty acid methyl esters were quantified by gas chromatography with flame ionization detection and identified by comparison with authentic standards (NuChek Prep, Elysian, MN) as previously described (4, 39).

**Tissue Removal**

After blood sample collection, aortas were immediately removed from the proximal aspect of the aortic arch to the base of the diaphragm. Aortas were thereafter immediately placed in Krebs-Henseleit solution containing (in mM) 115 NaCl, 25 NaHCO3, 1.38 KH2PO4, 2.5 KCl, 2.46 MgSO4, 1.9 CaCl2, and 11.2 dextrose (pH

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**Experimental design for Study #1**

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<tr>
<td>III</td>
<td>Chol 4 wks; control chow 8 wks; control chow 14 wks</td>
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<tr>
<td>IV</td>
<td>Chol 4 wks; control chow 8 wks; 10% flaxseed 14 wks</td>
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Fig. 1. Schematic of the timeline involved in the design of the dietary interventions used in this study. Chol, cholesterol-supplemented diet.
Experimental Assessment of Vascular Function

Two 3-mm rings from immediately below the aortic arch were taken for vascular function analysis. Aortic rings were subsequently fastened to an organ bath and force transducer, equilibrated with Krebs-Henseleit solution (37°C), and aerated with 95% O2-5% CO2. The extent of vasoconstriction and vasodilation was ascertained with a force transducer and recorded as mechanograms of tension [tension (in g)/tissue wet wt] as previously described (23). After equilibration, aortic rings were brought to a basal tension of 6.0 g and contracted (in g)/tissue wet wt] as previously described (23)). After an additional washout period, when rings were perfused with fresh Krebs-Henseleit solution (37°C), for the return of ring tension to basal levels. Increasing concentrations of norepinephrine (NE; 10^-8–10^-4 M) were used to construct a dose-response curve. After an additional washout period, aortic rings were contracted with 10^-6 M NE. The ability of the tissue to relax was then tested by the additional administration of 10^-8–10^-5 M ACh, an endothelium-dependent mode of relaxation. After a final washout and precontraction with 10^-4 M NE, the endothelium-independent responsiveness of vessels to relaxation was tested with sodium nitroprusside (SNP).

Evaluation of the Atherosclerotic Lesion Area

The extent of plaque accumulation on the vessel surface was determined by en face analysis (4, 22, 23). The aorta was longitudinally opened, crudely reconstructed with the rings removed to assess the vascular response, and digitally photographed. Luminal images were analyzed using Nikon imaging software (Elements). The percent lesion area was tabulated from the fraction of area covered with lesions relative to the total area of the aorta. Aortas were subsequently stored at -80°C. A subset of aortas was thawed and prepared for lipid extractions as previously described (4, 24). Lipids were resolved and quantified as described above.

Western Blot Analysis

To assess protein expression levels, aortic tissues stored at -80°C after en face analysis were used. The aortic arch (50 mg) was ground and subsequently resuspended in RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM benzamidine, and 1 mM protease inhibitor cocktail]. Samples were further processed by alternating freeze-thaw cycles followed by sonication. After a spin at 14,000 rpm, the sample supernatant was collected, and protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL). Proteins (25 μg/lane) were separated using 9% denaturing polyacrylamide gel and transferred electrophoretically onto nitrocellulose membranes. Membranes were incubated with anti-NF-kB p65 (Cell Signaling, Danvers, MA), anti-proliferating cell nuclear antigen (PCNA; Zymed Laboratories, Carlsbad, CA), and anti-actin (Sigma, St. Louis, MO) antibodies. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgGs (Jackson Immunoresearch Laboratories, West Grove, PA) were used as secondary antibodies. Bands were visualized with Supersignal West Pico Chemiluminescent Substrate (Pierce) or Luminafix Forte Western HRP Substrate (Millipore, Billerica, MA) and subsequently quantified by densitometry (Quantity One software, Bio-Rad). The level of actin was used as a loading control.

Statistical Analyses

Data are expressed as means ± SE. Statistical comparisons were made using one-way ANOVA followed by the Student-Newman-Kuels test or Fisher’s least-significant-difference post hoc test. P values of <0.05 were considered statistically significant.

RESULTS

Determination of Plaque Dynamics After Cholesterol Withdrawal

Weight gain. All animals consumed 125 g of their diet daily. Weight gain between all animals remained consistent during cholesterol feeding and after cholesterol withdrawal. There were no significant differences observed among any of the groups (data not shown; P > 0.05).

Serum lipid profiles. After 4 wk of cholesterol feeding, serum triglyceride levels increased from baseline levels of 150 mg/dl (Fig. 2A). However, after 2 wk of cholesterol withdrawal, triglyceride levels normalized (Fig. 2A). Longer durations of cholesterol withdrawal resulted in further decreases in

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Fig. 2. Decrease in triglyceride (A) and cholesterol (B) concentrations after prolonged withdrawal from cholesterol feeding. The dashed line represents baseline levels of rabbits fed exclusively regular rabbit chow for a 4-wk duration (cholesterol: 25 mg/dl; triglycerides: 150 mg/dl). Values are means ± SE; n = 6–10. *P < 0.05 vs. 0 wk of diet withdrawal; †P < 0.05 vs. 0 and 2 wk of diet withdrawal; ‡P < 0.05 vs. 0, 2, 4, and 6 wk of diet withdrawal; §P < 0.05 vs. 0, 2, 4, 6, and 8 wk of diet withdrawal.
triglyceride levels until these stabilized below baseline (Fig. 2A). Similarly, cholesterol feeding markedly increased serum cholesterol levels (Fig. 2B). Maintenance on regular rabbit chow after cholesterol feeding significantly decreased cholesterol levels in a stepwise fashion until baseline levels were achieved after 8 wk of withdrawal.

**Effects of cholesterol withdrawal on lesion development.** Atherosclerotic plaque formation initiated within 4 wk of cholesterol feeding and, thereafter, continued to progress even after the removal of dietary cholesterol (Fig. 3). Eight weeks after cholesterol withdrawal, plaque growth reached a plateau. Significant lesions were clearly visible on the aortic luminal surface. There was a gradual decrease in aortic lesion area after >8 wk of maintenance on regular rabbit chow.

**Effects of Dietary Intervention on Plaque Regression**

**Animal weight gain.** In the second part of this study, the duration of the dietary intervention was extended, and a flaxseed-supplemented nutritional arm was added after cholesterol feeding. *Group I* animals were given regular rabbit chow for 12 wk, and the remaining groups were given 4 wk of a 1% cholesterol-supplemented diet followed by 8 wk of regular rabbit chow (*group II*). *Group III* and *IV* animals were given 4 wk of a 1% cholesterol diet followed by an additional 14 wk of either regular rabbit chow or 10% flaxseed-supplemented rabbit chow, respectively (Fig. 1). Weight gain did not differ significantly (*P > 0.05*) among all experimental groups (data not shown). This was likely a reflection of the similar energy content of the different diets.

**Effects of dietary regimens on atherosclerosis.** Plaque formation was not visible on the aortic surface of control animals. However, after 8 wk of withdrawal from the cholesterol-supplemented diet, extensive atherosclerotic plaque development was evident on the luminal surface of these animals (Fig. 4). Plaque progression stabilized over the ensuing 14 wk on a regular diet (*group III*; Fig. 4). The extent of lesion area significantly decreased by 10.220.33.1 on October 30, 2017 http://ajpheart.physiology.org/ Downloaded from
The expression of biomarkers of inflammation and cell proliferation was measured by Western blot analyses in vascular tissue samples from rabbits after the completion of dietary regimens. Group I animals were given regular rabbit chow for 12 wk. The remaining groups were given 4 wk of a 1% cholesterol diet followed by 8 wk of regular rabbit chow (group II). Group III and IV animals were given 4 wk of a 1% cholesterol diet followed by an additional 14 wk of either regular rabbit chow or 10% flaxseed-supplemented rabbit chow, respectively. PCNA, proliferating cell nuclear antigen. Values are means ± SE; n = 4. *P < 0.05 vs. all other groups.

Diet-induced changes in lipid profiles. Serum triglyceride levels did not significantly differ among the four groups (Fig. 5A). The circulating cholesterol levels remained elevated (nearly 10-fold higher than controls) 8 wk after withdrawal from the high-cholesterol diet (Fig. 5B) and then slowly normalized when rabbits were placed on a regular rabbit chow for an additional 14 wk (Fig. 5B). The 10% flaxseed-supplemented diet (group IV) did not significantly affect circulating cholesterol levels compared with animals placed for a similar duration on a control diet chow (group III; Fig. 5B).

The expression of biomarkers of inflammation and cell proliferation was measured by Western blot analyses in vascular tissue samples from the four different groups. NF-κB expression was significantly elevated in group II samples compared with all other groups (Fig. 6A). PCNA is used as a marker of cell proliferation, and its expression is frequently increased under atherosclerotic conditions. PCNA expression was increased significantly in aortic tissue from group II animals compared with the control group (group I), and this returned to normal levels when animals were placed on a regular diet (group III) or a flaxseed-supplemented diet (group IV; Fig. 6B).

Total serum fatty acids (TFA) were quantified and resolved (Table 1). After 8 wk of withdrawal from dietary cholesterol, group II animals exhibited serum TFA levels and arachidonic acid (C20:4 n-6; Table 1). Fourteen weeks of regular rabbit chow and flaxseed supplementation significantly decreased levels of C20:4 n-6 and oleic acid (C18:1 O; Table 1). However, only flaxseed supplementation (group IV) was able to decreased (~40%) after an additional 14 wk of flaxseed supplementation to the diet (Fig. 4).

Table 1. Serum and aortic fatty acid concentrations of rabbits fed four different experimental diets

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<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Aorta</th>
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<tr>
<td></td>
<td>Group I</td>
<td>Group II</td>
</tr>
<tr>
<td>14:0</td>
<td>17.0±4.3</td>
<td>33.2±9.1</td>
</tr>
<tr>
<td>16:0</td>
<td>244.6±18.6</td>
<td>414±86.9</td>
</tr>
<tr>
<td>18:0</td>
<td>72.3±6.3</td>
<td>121±26.0</td>
</tr>
<tr>
<td>20:0</td>
<td>0.5†</td>
<td>3.4†</td>
</tr>
<tr>
<td>22:0</td>
<td>1.0±1.0</td>
<td>1.0±1.0</td>
</tr>
<tr>
<td>24:0</td>
<td>0.5†</td>
<td>0.5†</td>
</tr>
<tr>
<td>16:1</td>
<td>42.4±5.7</td>
<td>105.6±25.6</td>
</tr>
<tr>
<td>18:1</td>
<td>306.2±24.3</td>
<td>575.6±115.4</td>
</tr>
<tr>
<td>18:2</td>
<td>16.3±2.12*</td>
<td>17.3±3.4*</td>
</tr>
<tr>
<td>18:3</td>
<td>36.7±3.5†</td>
<td>49.7±12.5†</td>
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<tr>
<td>18:4</td>
<td>18.4±1.2*</td>
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</tr>
<tr>
<td>18:5</td>
<td>0.3†</td>
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<td>18:6</td>
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<td>19:2</td>
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Values are means ± SE [in μg/mL (serum) or μg/g (aorta)]. Samples were obtained from animals after after their dietary regimens (n = 4–7). Group I animals were given regular rabbit chow for 12 wk. The remaining groups were given 4 wk of a 1% cholesterol diet followed by 8 wk of regular rabbit chow (group II). Group III and IV animals were given an additional 14 wk of either regular rabbit chow or 10% flaxseed-supplemented rabbit chow, trace amounts present (<1 μg/100 mg or μl/100 mg fatty acids); TFA, total fatty acids (mg fatty acid in 1 ml of plasma or 1 g of aorta). *P < 0.05 vs. group II; †P < 0.05 vs. group IV.
significantly decrease levels of palmitic acid (C16:0), palmitoleic acid (C16:1), and vaccenic acid (C18:1; Table 1). The same dietary regimen significantly increased ALA levels relative to all groups. Despite the increases in ALA, its derivatives, eicosapentaenoic acid (EPA; C20:5 \( \text{n-3} \)), docosapentaenoic acid (DPA; C22:4 \( \text{n-3} \)), and docosahexaenoic acid (DHA; C22:5 \( \text{n-3} \)), were only detected in trace amounts. Flaxseed supplementation also significantly decreased the \( \text{n-6-to-n-3} \) PUFA ratio compared with all other experimental groups (Table 1).

More robust diet-induced alterations in the levels and distributions of fatty acids were observed in aortic tissue (Table 1). TFA content was significantly elevated in group II relative to all but the flaxseed-fed group. Moreover, these animals had significant increases in various long-chain fatty acids, including C16:0, C18:1 O, C18:1, linoleic acid (18:2 \( \text{n-6} \)), euric acid (C20:1), eicosadienoic acid (C20:2 \( \text{n-6} \)), and dihomo-\( \gamma \)-linolenic acid (C20:3 \( \text{n-6} \); Table 1). Fourteen weeks of regular rabbit chow or flaxseed supplementation resulted in significant reductions in the levels of various long-chain fatty acids (C16:0, C18:1, C18:1 O, C18:2 \( \text{n-6} \), C20:1, and C20:2 \( \text{n-6} \)). However, only regular rabbit chow significantly reduced the levels of C20:3 \( \text{n-6} \) fatty acid, whereas flaxseed supplementation significantly decreased serum levels of C16:1 fatty acid (Table 1). The addition of flaxseed to regular rabbit chow enhanced aortic ALA, EPA, DPA, and DHA levels. Flaxseed supplementation also significantly decreased the \( \text{n-6-to-n-3} \) PUFA ratio in aortic tissues, which was significantly increased in group II animals (Table 1).

Effects of dietary interventions on vascular function. Vascular function was first evaluated with agonist-mediated vasoconstriction. There were no significant differences in KCl-induced vasoconstriction among the various groups (Fig. 7A). A depression in the response to NE persisted during the withdrawal period after cholesterol feeding (Fig. 7B). This became significant at \( 10^{-6} \) M NE (Fig. 7B). This association...
was lost at high concentrations of NE (10⁻⁵–10⁻⁴ M). Only the regular diet was able to significantly improve responsiveness to 10⁻⁶ M NE. Likewise, this association was also lost at high concentrations of NE.

The responsiveness of aortic rings to vasoconstrictors was studied after precontraction with 10⁻⁵ M NE. Aortic rings taken from animals fed a cholesterol-supplemented diet (group II) had a significant impairment in their response to 10⁻⁵ and 10⁻⁶ M doses of ACh (Fig. 8A). Rings isolated from group III and IV animals recovered their ability to respond to ACh at concentrations of 10⁻⁵ and 10⁻⁶ M. The two groups did not differ significantly in the recovery of their vasoconstrictor responses at ≤10⁻⁶ M concentrations of ACh. However, this improvement reached statistical significance for flaxseed-supplemented animals at an ACh concentration of 10⁻⁵ M. There were no significant differences among groups in the extent of aortic relaxation caused by the endothelium-independent vasorelaxant SNP (Fig. 8B).

**DISCUSSION**

Rabbits represent an ideal model in which to study atherosclerotic plaque regression because they 1) do not require genetic manipulation to create plaques, 2) develop atherosclerotic plaques after dietary cholesterol supplementation, and 3) have similar lipoprotein transport mechanisms to humans (12, 30, 33, 34). In the present study, we observed a clear dissociation of circulating cholesterol levels from atherosclerotic plaque formation. As hypercholesterolemia was gradually resolving during the withdrawal period from cholesterol, aortic plaques continued to progress (Figs. 2 and 3). Concurrently, triglyceride levels decreased below baseline levels, a common age-dependent phenomenon observed in rabbits (23, 45, 48). The continued progression of atherosclerotic lesions despite the marked decrease in circulating cholesterol may seem paradoxical. However, cholesterol levels still remained 10-fold higher than normal when plaque progression ceased at 8 wk after cholesterol supplementation to the diet (Fig. 5B). Reidmüller et al. (48) also reported that serum cholesterol levels were 10-fold higher than baseline when the highest lesion cholesterol and macrophage/foam cell content was observed during cholesterol withdrawal. Thus, even during cholesterol withdrawal, circulating cholesterol levels remain high enough to facilitate cholesterol deposition and foam cell accumulation in growing atherosclerotic plaques. This clearly invalidates any conclusions about plaque regression in studies that initiated their intervention before the stabilization of the plaque (5, 8, 10, 45). Premature initiation of a nutritional intervention after cholesterol supplementation to the diet will only determine the ability of a food to slow plaque progression rather than its ability to induce plaque regression. Our data also demonstrate the importance of using an appropriate dietary control for nutritional interventions. Even if the nutritional interventions were initiated after the stabilization of plaque development, without an adequate experimental control it would remain unclear whether a decrease in lesion area was due to the intervention or the mild regression that normally occurs when rabbits are maintained on a regular diet after the plateau in plaque development is achieved. It is unclear if this spontaneous regression of the plaque after an extended period of time when the animal is returned to a normal diet occurs in humans as well.

The present study demonstrates that the deleterious effects of cholesterol feeding on contractile function still persisted even after 8 wk of cholesterol withdrawal (Fig. 7). Interestingly, these defects were detected with NE-induced contraction but not KCl-induced contraction. KCl has traditionally been used to stimulate smooth muscle cell contraction without activation of G protein-coupled receptors. A previous study (46) has shown that G protein-coupled receptor agonists develop greater contractile force per increase of cytosolic Ca²⁺. This suggests that the observed NE-induced defects in contractile force associated with dietary cholesterol may involve a cellular Ca²⁺ sensitization mechanism involving RhoA kinase and PKC. The data do not discount the possibility that the impairment of contractility may also involve an impairment of sarcoplasmic reticulum Ca²⁺ release, probably at the level of both vascular smooth muscle cells and vascular endothelial cells.

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**Fig. 9. Potential mechanisms of action of flaxseed to induce regression.** α-linolenic acid (ALA) is proposed to play a central role by reducing the expression of a variety of inflammatory, oxidation, and cell proliferation biomarkers directly or through alterations in the content of other fatty acid moieties, such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and docosapentaenoic acid (DPA). This reduction in inflammation, oxidation, and cell proliferation within the vascular tissue may inhibit the progression of atherosclerosis or promote its regression. SCD-1, stearoyl CoA desaturase; COX, cyclooxygenase; PPAR, peroxisome proliferator-activated receptor.
Prolonged hypercholesterolemia has been shown to impair ACh-induced endothelium-dependent vasodilation in rabbits (12, 27, 33, 34). The present findings now show, for the first time, that the deleterious effects of cholesterol feeding on contractile function persist even 8 wk after the withdrawal of cholesterol from the diet. Proinflammatory and proatherogenic environments, caused by hypercholesterolemia, promote endothelial dysfunction (9, 16, 21, 47). Because we could not detect any changes in the relaxation capacity of any of the vessels in response to nitric oxide delivered from SNAP, the changes in relaxation observed are due to an endothelium-dependent decrease in the bioavailability of nitric oxide (14, 38). Both regular rabbit chow and flaxseed feeding restored endothelium-mediated vasodilation to the same extent. Riezebos et al. (49) also showed that restoration of endothelial function occurred irrespective of pharmaceutical treatments with ramipril and isradipine. The observed improvements are likely the result of normal wound healing mechanisms that are also involved in atherosclerotic plaque regression, including endothelial cell proliferation and replacement of dysfunctional cells with endothelial progenitor cells (25).

Flaxseed supplementation significantly (P = 0.0333) accelerated the regression of atherosclerotic plaques. The high levels of n-3 fatty acids detected within the aorta may account for the observed increases in plaque regression. Inhibition of inflammation has been identified as an essential process in atherosclerotic plaque regression (25). Intake of ALA has been associated with significant decreases in inflammatory markers such as IL-6, mac-3, VCAM-1, and PCNA in aortic tissue (22). Although we did observe a decrease in NF-κB and PCNA expression in vascular tissue during flaxseed supplementation, this did not achieve a statistically significant difference from the regular diet group (group III; Fig. 6). Considerable amounts of ALA were metabolized to longer-chain n-3 fatty acids within the aorta, and a large proportion of ALA was converted to DPA. DPA has been associated with the inhibition of cyclooxygenase 1 activity, a decrease in cellular TNF-α, and the induction of gene modulatory and anti-inflammatory effects (28, 35, 36). Additionally, n-3 fatty acids such as EPA and DHA are activators of peroxisome proliferator-activated receptors (PPARs). Both PPAR-α and PPAR-γ activation have been implicated in a number of anti-inflammatory and antiatherosclerotic responses (16, 1940). Moreover, incorporation of n-3 fatty acids into cell membranes quenches ROS production, subsequently decreasing redox sensitive transcription factors, such as NF-κB, that regulate proinflammatory and proatherogenic genes (19, 41, 42). Alternatively, the decreased C16:1/18:1 content that we found (Table 1) may be interpreted to suggest that stearoyl CoA desaturase (SCD)-1 activity was inhibited by flaxseed. SCD-1 has been implicated in the formation of atherosclerosis (13, 53). Furthermore, ALA promoted cholesterol efflux from macrophages through inhibition of SCD-1 (53), a mechanism entirely consistent with its effects on plaque regression in the present study. Flaxseed also contains other functional components that have been implicated in the mediation of atherosclerosis. Flaxseed is a rich source of plant lignans such as cinnamic acid glucoside, hydroxymethylglutaric acid, and secoisolariciresinol diglucoside, which collectively form the flax lignan complex. Constituents of the flax lignan complex are antioxidants (44). Oxidized lipoproteins may play a role in atherosclerosis (15). However, in this study, the antioxidant effect of the flax diet has not been studied.

In summary, our data demonstrate that dietary flaxseed can accelerate the regression of atherosclerotic plaques in rabbits. Figure 9 shows a schematic proposed to explain the antiatherosclerotic effects of flaxseed during the advancing and regressing stages of atherosclerosis. However, further work is still required to definitively identify the mechanism(s) by which dietary flaxseed increases plaque regression.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


