5-Aminolevulinic Acid Attenuates Atherosclerotic Plaque Progression in Low-Density Lipoprotein Receptor-Deficient Mice by Heme Oxygenase-1 Induction

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Background: Recently, 5-aminolevulinic acid (ALA) has been reported to modulate inflammatory development via an antioxidant effect. Hence, the aim of this study was to determine the anti-atherosclerotic effect of ALA.

Methods and Results: Low-density lipoprotein (LDL) receptor knockout mice were fed the following diets for 24 weeks: normal diet (n=6); 1.25% cholesterol diet (high-cholesterol diet, HCD; n=7); HCD+ALA (46 mg/kg/day; n=10); and HCD+ezetimibe (5 mg/kg/day; n=10). At 40 weeks, HCD+ALA had reduced LDL cholesterol (320±68 vs. 379±49 mg/dL), triglyceride (141±44 vs. 195±49 mg/dL) and oxidized LDL (380±40 vs. 422±64 pg/mL) compared with HCD only. En face lesion area for the entire aortic surface was significantly smaller in mice that received HCD+ALA than in mice that received only HCD (32±5% vs. 39±4%, P<0.05). ALA intake exogenously increased tissue heme oxygenase-1 (HO-1) level in plaque composite tissue of the carotid arterial wall compared with HCD only (18±8 vs. 12±3 pg/μL, P<0.05), and HO-1-positive plaque showed modest NADPH oxidase 4 expression.

Conclusions: ALA intake induces exogenous production of HO-1 at plaque sites, and improves lipid profiles and attenuation of atherosclerotic plaque progression in vivo.

Key Words: 5-Aminolevulinic acid; Atherosclerosis; Heme oxygenase-1; NADPH oxidase 4; Plaque
Methods

Animals

Inbred female low-density lipoprotein (LDL) receptor knockout mice were housed under specific pathogen-free conditions in an environmentally controlled clean room under a 12-h light/dark schedule with lights on at 8:30 p.m. in a temperature- and humidity-controlled room (22±1°C and 55±5%, respectively). The experiments were performed from 9 a.m. to 4 p.m. The animal experimental protocol was in accord with the ARRIVE guidelines. This study was conducted according to the guidelines of the Institutional Review Board for the Care of Animal Subjects of the National Defense Medical College. The Institutional Review Board for the Care of Animal Subjects of the National Defense Medical College approved this study (ethics approval no. 15049).

Experiment Protocol

Four groups of LDL receptor knockout mice (n=33 in total) were fed the following diets until 40 weeks of age (Figure 1): a normal diet until 16 weeks of age, after which 3 groups were then switched to the following diets for 24 weeks, while 1 group continued on the normal diet (CLEA Rodent Diet-2 [CE-2], n=6): a 1.25% cholesterol diet (high-cholesterol diet [HCD]; 82.7% CE-2, 15% beef tallow, 1.25% cholesterol, 1.046% α-cornstarch, n=7); an HCD + ALA diet (82.7% CE-2, 15% beef tallow, 1.25% cholesterol, 1.0% α-cornstarch, 0.046% ALA [purity >98%, SBI Pharma, Tokyo, Japan]; and an HCD+ezetimibe diet (82.7% CE-2, 15% beef tallow, 1.25% cholesterol, 1.041% α-cornstarch, 0.005% ezetimibe; CLEA Japan, Tokyo, Japan, n=10). All of the diets were purchased from CLEA Japan (Tokyo, Japan). The mice were able to access each diet any time until 40 weeks of age. Body weight was measured every week. The dose of ALA was set to 50 mg/kg/day, a dose that has been reported to have a sufficient metabolic benefit without any adverse effect, and that of ezetimibe was set to 5 mg/kg/day.

Whole blood samples obtained on orbital sinus blood sampling were analyzed for serum total cholesterol, LDL-C, TG, total bilirubin, AST and ALT as well as oxidized LDL. Oxidized LDL was measured using a Mouse Oxidized Low Density Lipoprotein (OxLDL) enzyme-linked immunosorbent assay (ELISA) kit (KT-32067, Kamiya Biomedical Company, Seattle, WA, USA).

Atherosclerotic Plaque Area

Aortas were excised at 40 weeks (24 weeks after the start of each treatment), and plaque volume was determined on oil red O staining. In brief, the thoracic ascending and abdominal aorta was removed from the heart. After cutting off minor branching arteries, the aorta was fixed in formal sucrose (4% paraformaldehyde, 5% sucrose, 20 mM EDTA, pH 7.4). After the adventitial and adipose tissue had been removed, each of the aortas was cut open longitudinally. For neutral lipid visualization, sections were rinsed in 60% isopropanol and incubated in a saturated, filtered solution of oil red O in 60% isopropanol for 1 h. The stained aorta was pinned open, and the ratio of the intimal surface stained with oil red O in the arch and thoracic regions was measured morphometrically (Win ROOF version 5.5; Mitsuya-Shoji, Tokyo, Japan).

Tissue HO-1

At 40 weeks, left carotid arteries (along with the common carotid to bifurcation of internal and external carotid arteries; approximately 2 cm in length) were excised and immediately stored at −80°C. To measure HO-1, the tissue samples were homogenized in 0.3 mL radio-immunoprecipitation assay (RIPA) lysis buffer with addition of 3 μL Halt Protease inhibitor cocktail (100×; Thermo Fisher Scientific, Tokyo, Japan) for 30 min and then homogenized (NS-310E3, 30,000 rpm for 20 s, Microtec, Tokyo, Japan) followed by disruption in an ultrasonic cell disruptor (Bioruptor, 3 times for 30 s each time with intervals of 30 s, Cosmo Bio, Tokyo, Japan) at 4°C. Thirty min later, the supernatant was ultracentrifuged (100,000×g, 10 min, twice, Tomy MX-301, Tomy Seiko, Tokyo, Japan) at 4°C. The supernatant was used to determine HO-1.

Tissue HO-1 concentration was measured in the carotid artery atherosclerotic plaque composition and in the liver using an HO-1 Mouse SimpleStep ELISA kit (Abcam 204524, Cambridge, MA, USA).
Histopathology
For histopathology, right carotid artery sections were fixed in 10% formalin and embedded in paraffin. One of 2 series of 5-μm-thick slices of paraffin-embedded tissue was placed on a slide for immunohistochemistry. Antigen retrieval was performed by heating in 10 mmol/L citrate buffer (pH 6.0) followed by treatment with 0.6% H₂O₂ in methanol. The section was incubated with a primary antibody at 4°C overnight. After washing with phosphate-buffered salts, the section was incubated with a secondary antibody (goat anti-rabbit IgG HRP; Abcam 6721) at room temperature for 1 h. Finally, the section was washed and then immunostained with DAB solution (Abcam 64238). The primary antibodies were a rabbit polyclonal antibody to HO-1 (Abcam 13243) and a rabbit polyclonal antibody to NOX4/NADPH oxidase 4 (Bioss bs-3684R, Woburn, MA, USA). The other series of slices was deparaffinized and stained with hematoxylin and eosin (HE). Using the plaque immunohistochemical samples in which an atherosclerotic plaque had formed, the ratio of the HO-1-positive area and the ratio of the NOX4-positive area in the vessel wall area were measured morphometrically for quantitative analysis (Win ROOF version 5.5, Mitsuya-Shoji, Tokyo, Japan).

Statistical Analysis
Statistical analysis was done using 1-way ANOVA–Bonferroni test. Data are presented as mean±SD, with P<0.05 considered to be statistically significant.

Results
Mouse Body Weight and Serum Lipid Profile
The HCD diet augmented mouse body weight (33±5 g at 40 weeks) compared with the normal diet (25±3 g at 40 weeks) with time (Figure 2A). From the measurement of volume of consumed diet, ALA intake was determined to

![Figure 2](image_url)
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be 46 mg/kg/day. ALA supplementation (46 mg/kg/day) as well as ezetimibe supplementation (5 mg/kg/day) had no influence on the body weight increase caused by HCD intake (31 ± 4 g and 34 ± 7 g, respectively). ALA supplementation also had no adverse effect, as indicated by the serum total bilirubin, AST and ALT being maintained in the normal range during the experiment (Figure 2B–D).

The HCD-only diet increased serum total cholesterol and LDL-C after 8 weeks of feeding (at 24 weeks of age) as shown in Figure 3. Ezetimibe supplementation completely inhibited the elevation of serum total cholesterol and LDL-C (Figure 3A,C). ALA supplementation for 24 weeks resulted in a significant decrease in serum LDL-C (320 ± 68 mg/dL) at 40 weeks of age (Figure 3C). Until 32 weeks of age, TG remained in the normal range in all 4 groups, but HCD and HCD+ALA increased serum TG at 40 weeks of age. The HCD+ALA group, however, had a small increase in TG (141 ± 44 mg/dL) compared with that in the HCD-only group (195 ± 49 mg/dL; Figure 3B).

HCD and HCD+ezetimibe resulted in an increase in serum oxidized LDL at 40 weeks of age (422 ± 64 pg/mL and 402 ± 59 pg/mL, respectively). ALA supplementation inhibited the elevation of serum oxidized LDL (380 ± 40 pg/mL), and the serum oxidized LDL level was not significantly different from that on the normal diet (345 ± 60 pg/mL; Figure 3D).

Aortic Atherosclerotic Plaque Area
Intake of the HCD-only diet for 24 weeks resulted in the development of lipid-rich atherosclerotic plaque in the aorta (Figure 4A). Plaque was dominantly seen at the aortic arch and bifurcating sites of several major branches. The ratio of en face lesion area for the entire aortic surface reached 0.39 ± 0.04 in the HCD-only group, but the HCD+ALA group had a decreased ratio of 0.32 ± 0.05. Ezetimibe supplementation prevented atherosclerotic plaque accumulation and the ratio was 0.12 ± 0.05, as low as that in mice fed a normal diet (Figure 4B).

Antioxidant Effect
HCD intake for 24 weeks resulted in compensatory induction of endogenous HO-1 production in the carotid artery, and HO-1 was recruited to plaque, which was often seen at the bifurcation site of the common carotid artery. HO-1
level in carotid tissue of HCD mice was 2-fold higher than in the normal diet mice at 40 weeks (12±3 pg/μL vs. 6.5±2 pg/μL, P<0.05, Figure 5A). ALA supplementation exogenously increased HO-1 in the carotid plaque-rich bifurcation site (18±8 pg/μL, Figure 5A) compared with the HCD-only group (Figure 5A). The HCD+ezetimibe group had less HO-1 production, probably due to smaller plaque formation.

Figure 5C shows HO-1 level in the liver. Liver HO-1 in the normal diet mice was regarded as basal HO-1 production (32±4 pg/μL). Liver HO-1 level was not increased by HCD only (36±8 pg/μL) or by HCD+ALA (38±8 pg/μL). Taken together, these results indicate that exogenous HO-1 induction seemed to be specifically augmented in atherosclerotic plaque tissue.

**Histopathology**

On HE staining of the carotid artery in HCD-only mice, typical atherosclerotic plaque consisting of thickened intima with foam cell formation was seen. These pathology findings were in accordance with human atherosclerotic grade 3 histology showing pathologic intimal thickening with extra-cellular lipids underneath a layer of foam cell macrophages (Figure 6A).21

In line with the quantitative HO-1 results, expression of immunoreactive HO-1 was markedly enhanced at carotid atherosclerotic lesions in the HCD+ALA mice, particularly in lesions in medial and intimal smooth muscle cells.
Figure 5. Heme oxygenase-1 (HO-1) in (A) the bifurcation site of the common carotid artery; (B) a plaque-poor site of the common carotid artery; and (C) in the liver, in mice according to diet. *P<0.05. ALA, 5-aminolevulinic acid; HCD, high-cholesterol diet.

Figure 6. (A) Hematoxylin and eosin (HE) staining and (B) heme oxygenase-1 (HO-1) immunostaining of the carotid arteries in mice according to diet. (A) A thickened intima with foam cells is seen in both the high-cholesterol diet (HCD) mice and the HCD+5-aminolevulinic acid (HCD+ALA) mice. (B) Expression of immunoreactive HO-1 was markedly increased in medial and intimal smooth muscle cells, especially at the carotid atherosclerotic lesions of HCD+ALA mice (arrow). Scale bar, 0.3 mm.
ALA is naturally synthesized from glycine and succinyl-CoA and is finally metabolized to heme in mitochondria in the human body. Initially, ALA was developed industrially for plant fertilizer producing chlorophyll. An et al reported that ALA inhibits abscisic acid-induced stomatal closure by reducing H\(_2\)O\(_2\) and calcium levels in guard cells, and that it simultaneously improves plant drought tolerance. This antioxidant mechanism as an inhibitor of H\(_2\)O\(_2\)-generating NADPH oxidase improved mitochondrial function in animals.

Cheng et al reported that HO-1 induction by cobalt protoporphyrin (CoPP) impeded lesion progression into vulnerable plaques, indicated by a reduction in necrotic core size and intraplaque lipid accumulation, whereas cap thickness and VSMC were increased. In their study, HO-1 protein level was 20-fold higher than in normal (Figure 6B). HCD+ezetimibe prevented plaque deposition in vessels and resulted in almost no HO-1-positive areas (Figure 6B).

Given that the core of the plaque consisted of foam cells, cells with stronger HO-1 expression had weaker expression of immunoreactive NOX4 (Figure 7). Quantitatively, there was an inverse relationship between the ratio of the HO-1-positive area and the ratio of the NOX4-positive area (Figure 7, Bottom).

**Discussion**

In this study, ALA supplementation improved serum lipid profiles with augmentation of HO-1 induction and reduction of the atherosclerotic plaque area in the whole aorta in mice fed an HCD.

![Figure 7. Heme oxygenase-1 (HO-1) and NADPH oxidase (NOX)-4 immunostaining of carotid arteries from mice treated with (A,B) a high-cholesterol diet (HCD) and (C-E) an HCD+5-aminolevulinic acid (HCD+ALA) diet. Scale bar, 0.3 mm. (Bottom) Analytic result from the immunohistochemistry samples.](image-url)
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mice. Several researchers have reported, however, that high overdose expression of HO-1 exacerbates inflammation: when HO-1 expression is low, low cellular heme and iron levels may allow for decreased oxidative injury and upregulation of important enzymes, whereas excessive accumulation of reactive iron when HO-1 expression is high would result in increased oxidative stress, cytotoxicity, and abnormal cellular proliferation. Excessive HO-1 not only leads to enhanced cell injury but also prolongs the repair process of injured endothelial tissue. From these viewpoints, long-term ALA may be practical for treating a chronic disease in a clinical situation given that, in the present study, the level of HO-1 at plaque-rich sites in mice on the HCD+ALA diet was 3-fold higher than in mice fed a normal diet.

In the present study, HO-1 was overexpressed in the atherosclerotic plaque composite of the carotid arterial wall in mice given exogenous ALA: the HCD+ALA group had a 1.5-fold higher HO-1 level than the HCD-only group. In line with this, chronic CoPP treatment induced a 5-fold higher HO-1 expression compared with that in the normal diet rats and attenuated the coronary constrictor response to ischemia-reperfusion. Modest and chronic expression of HO-1 resulted in a significant increase in serum adiponectin. The HO-1-mediated increase in adiponectin provides the heart and vascular system with tolerance and resistance not only to the oxidative stress generated in diabetes, but also to other types of vascular stress.

As a positive control group, ezetimibe supplementation completely inhibited atherosclerotic plaque formation and kept the serum lipid profile normal. It did not cause body weight gain or oxidative stress, indicated by elevation of serum oxidized LDL. These results are compatible with results obtained by Umemoto et al and Tie et al showing that ezetimibe inhibited cholesterol absorption in the intestine and kept the lipid profile normal without an anti-inflammatory effect.

In contrast, in the present study, ALA intake decreased oxidized LDL to within the normal range via induction of HO-1 production. Oxidized LDL has been shown to play a major role in atherosogenesis, and, as an antioxidant, reduces production of mitochondrial ROS and accumulation of oxidative damage to mitochondrial DNA, proteins, and lipids.

Recently, NADPH oxidase has been shown to be a key component for modifying metabolic diseases. In the present study, HO-1 expression in the core of the plaque composed of foam cells attenuated NOX4 expression. Taken together, this indicates that ALA has an antioxidant action as an inhibitor of H₂O₂-generating NOX4.

In the present study, the antioxidant agent ALA induced HO-1 production along with improvements in systemic inflammatory and lipid profiles such as plasma oxidized LDL and TG.

Study Limitations

In this study the anti-atherosclerotic effect of ALA was modest compared with the anti-atherosclerotic effect of ezetimibe, which completely inhibited plaque progression. Further study is necessary to elucidate the adjunctive efficacy of ALA added to statin therapy and also its effectiveness for regression of plaque and maintenance of vascular function.

In the present study, ezetimibe-treated mice were used as positive controls without the use of statins because the condition of LDL receptor knockout would crucially affect the pharmacological mechanism of a statin, which inhibits Hydroxymethylglutaryl-CoA and upregulates the LDL receptor. We also considered that statins have pleiotropic effects including several antioxidant effects on atherosclerosis, but the aim of this study was to elucidate the effect of HO-1 induced by ALA on atherosclerosis formation.

We evaluated local NOX4 expression in the plaque area as an essential antioxidant pathway of ALA, but NADPH oxidase subunits such as NOX2 and p22phox are also important for atherosclerotic plaque formation. Hence further studies are expected.

Conclusions

ALA exogenously induced the production of HO-1 at plaque sites, improving lipid profiles and attenuating atherosclerotic plaque progression in vivo.

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Disclosures

The authors declare no conflicts of interest.

Author Contributions

K.H. and Y.M. contributed to the conception and design of the study; K.H. and Y.M. carried out the experiments and acquired data; Y.M. performed data analysis; K.H. wrote the first draft of the manuscript; M.N., M.A. and K.I. contributed to manuscript revision and supervised the project. All authors read and approved the submitted version.

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