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Research Article

Inhibition of monoamine oxidase B reduces atherosclerosis and fatty liver in mice

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Oxidative stress is vital for pathophysiology of atherosclerosis and non-alcoholic fatty liver disease (NAFLD). Monoamine oxidase (MAO) is an important source of oxidative stress in the vascular system and liver. However, the effect of MAO inhibition on atherosclerosis and NAFLD has not been explored. In the present study, MAO A and B expressions were increased in atherosclerotic plaques in human and apolipoprotein E (ApoE)-deficient mice. Inhibition of MAO B (by deprenyl), but not MAO A (by clorgyline), reduced the atheroma area in the thoracic aorta and aortic sinus in ApoE-deficient mice fed the cholesterol-enriched diet for 15 weeks. MAO B inhibition attenuated oxidative stress, expression of adhesion molecules, production of inflammatory cytokines, and macrophage infiltration in atherosclerotic plaques and decreased plasma triglyceride and low-density lipoprotein (LDL) cholesterol concentrations. MAO B inhibition had no therapeutic effect on restenosis in the femoral artery wire-induced injury model in C57BL/6 mice. In the NAFLD mouse model, MAO B inhibition reduced lipid droplet deposition in the liver and hepatic total cholesterol and triglyceride levels in C57BL/6 mice fed high-fat diets for 10 weeks. Key enzymes for triglyceride and cholesterol biosynthesis (fatty acid synthase and 3-hydroxy-3-methylglutaryl-CoA reductase, HMGCR) and inflammatory markers were inhibited, and cholesterol clearance was up-regulated (increased LDL receptor expression and reduced proprotein convertase subtilisin/kexin type 9, PCSK9, expression) by MAO B inhibition in the liver. These results were also demonstrated in the HepG2 liver cell model. Our data suggest that MAO B inhibition is a potential and novel treatment for atherosclerosis and NAFLD.

Introduction

Cardiovascular disease is a major cause of death globally and contributes significantly to disability. The number of patients with cardiovascular diseases increased from 271 million to 523 million between 1990 and 2019, and the mortality increased from 12.1 million to 18.6 million in the same period, according to the estimation of the Global Burden of Disease Study 2019 [1]. Atherosclerosis plays a significant role in the pathophysiology of cardiovascular diseases. Traditionally, hypertension, hypercholesterolemia, diabetes, and smoking are important atherosclerosis risk factors. With advances in medications and public awareness, attention has shifted to non-traditional etiologies of atherosclerosis [2]. Among these, non-alcoholic fatty liver disease (NAFLD) is an important but less recognized risk factor for atherosclerosis [3].

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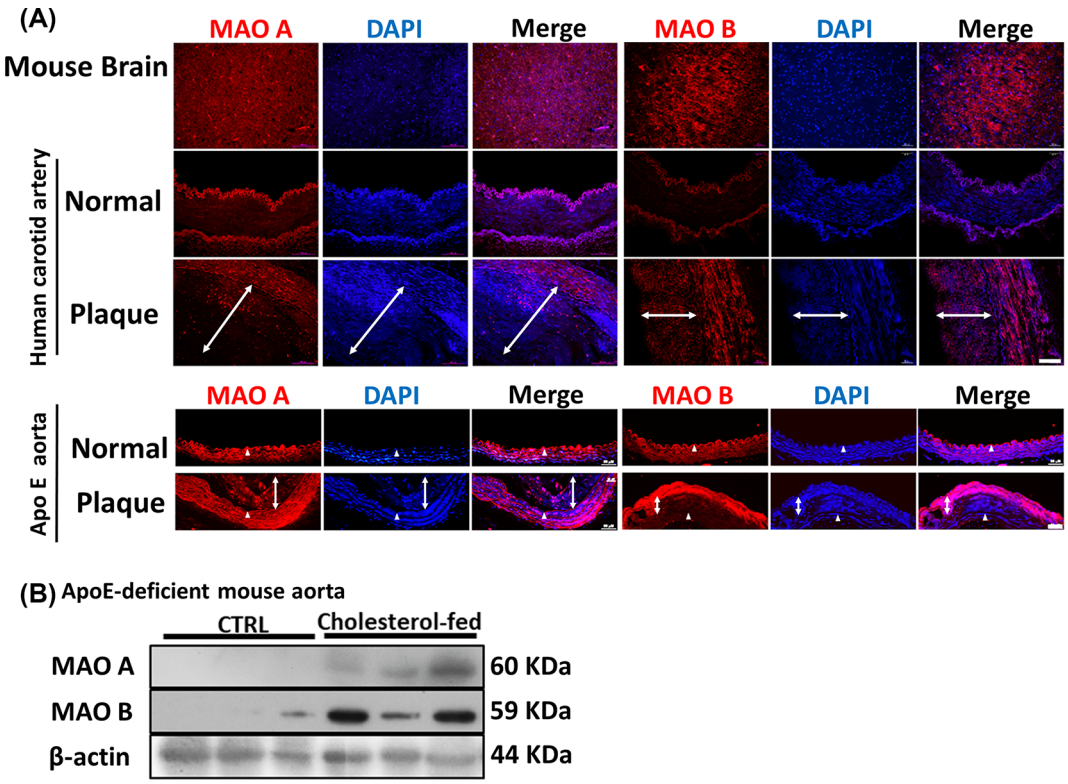

Figure 1. High expression of MAO A and B in atherosclerotic plagues

(**A**) Immunohistochemical staining of MAO A and B in mice brain, in carotid artery sections in humans, and thoracic aorta sections in ApoE-deficient mice. The scale bar = 50 μm. Tissue sections were stained with DAPI to visualize nuclei. The white arrowheads indicate the internal elastic lamina. The double-headed arrows indicate the area of atherosclerotic plaque. (**B**) Immunoblotting analysis showed that the expression level of MAO A and B was increased in the thoracic aorta in ApoE-deficient mice.

The prevalence of NAFLD is estimated to be 25% worldwide [4]. Patients with NAFLD are associated with subclinical and clinically significant atherosclerosis [5]. Additionally, these patients have a higher risk of having increased carotid intima-media thickness, calcification of coronary artery, higher arterial stiffness, and high-risk plaque features. After an acute coronary syndrome event, patients with NAFLD are more likely to have worse outcomes. As a result, cardiovascular diseases are a major cause of mortality in patients with NAFLD [6].

NAFLD increases the risk of atherosclerosis through several mechanisms, including oxidative stress, endothelial dysfunction, abnormal lipid metabolism, systemic inflammation, and insulin resistance [5]. Among these mechanisms, oxidative stress is frequently implicated in the pathophysiology of NAFLD and cardiovascular diseases [6]. Oxidative stress inhibits the hepatic secretion of very low-density lipoprotein (LDL), which results in fat accumulation in the liver. Oxidative stress also induces lipid peroxidation, inflammation, and fibrogenesis, leading to hepatic injury and fibrosis. Recently, monoamine oxidase (MAO), an enzyme first discovered in rabbit liver [7], has been identified as an important source of reactive oxygen species (ROS) in vessels [8–10] in addition to NADPH oxidases, mitochondrial respiratory chain, and other well-known sources.

MAOs, including MAO A and B, are flavoenzymes that catalyze amine substrates and produce hydrogen peroxide, ammonia, and aldehydes [11]. MAOs are expressed in the central nervous system, the heart, vasculature, and liver in humans. The physiological functions of MAOs are to protect neurons from the actions of endogenous neurotransmitters and exogenous amines and prevent dietary amines from entering circulation [11,12]. Clinically, MAO inhibitors are designed to treat Parkinson's disease, along with L-dopa [11], and several selective MAO B inhibitors are developed, including selegiline (L-deprenyl), to minimize the adverse effects. Recently, MAO inhibition has been a potential and novel therapy for heart failure [13,14]. Since MAO is an important source of ROS in the vascular system and liver, it may have a role in the development of atherosclerosis and NAFLD. However, previous studies have not focused on this correlation. Therefore, we investigated the role of MAO inhibition in atherosclerosis and NAFLD in the present study.

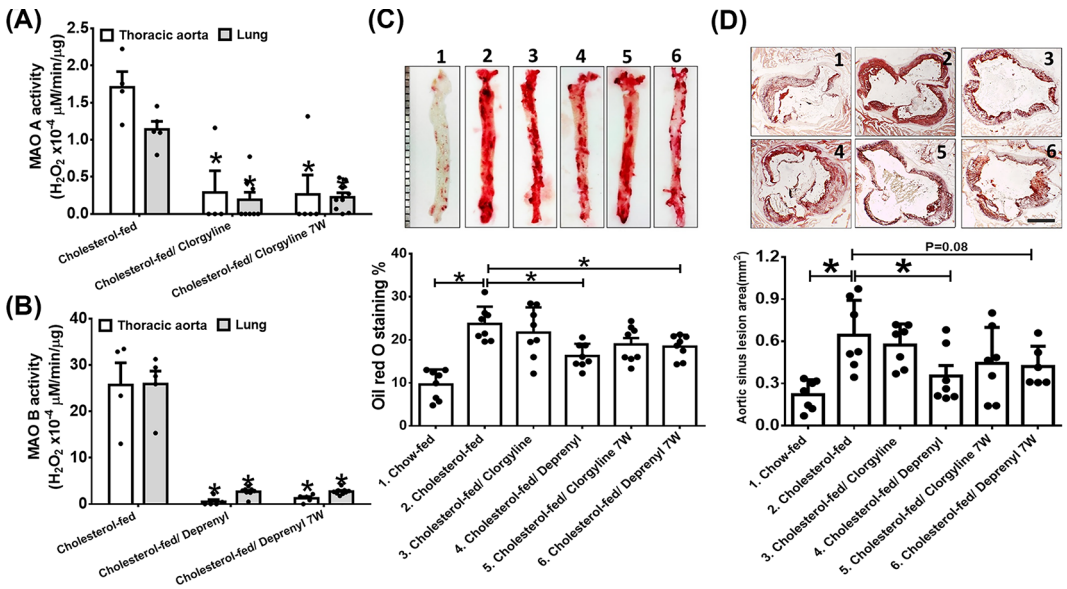

Figure 2. MAO B inhibition by deprenyl reduces MAO B activity and cholesterol-enriched diet-induced atherosclerosis in ApoE-deficient mice

(**A,B**) Effects of clorgyline and deprenyl on MAO A and B activity in the thoracic aorta and lung. *$P<0.05$ compared with the cholesterol-fed group. (**C,D**) Representative micrographs and quantification of ORO staining of the thoracic aorta and aortic sinus from ApoE-deficient mice after different treatments. All values are represented as means ± SD; *$P<0.05$.

Methods

Reagents

Polyclonal rabbit immunoglobulin G (IgG) antibodies against human glyceraldehyde 3-phosphate dehydrogenase, β-actin, α-actin, smooth muscle α-actin (α-SMA), and tumor necrosis factor-α (TNF-α), as well as horseradish peroxidase, conjugated goat anti-rabbit, and anti-mouse IgG antibodies, were purchased from GeneTex (Irvine, CA, U.S.A.). Mouse monoclonal IgG antibodies against vascular cell adhesion protein 1 (VCAM-1), intercellular adhesion protein 1 (ICAM-1), E-selectin, and proliferating cell nuclear antigen (PCNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Polyclonal rabbit IgG antibody against ionized calcium-binding adapter molecule 1 (Iba-1) was purchased from Wako (Chuo-ku, Osaka, Japan). Rabbit monoclonal IgG antibodies against MAO A and MAO B were purchased from Abcam (Cambridge, U.K.). Mouse monoclonal IgG antibody against interleukin 6 (IL-6) was purchased from PeproTech (Rocky Hill, NJ, U.S.A.). Mouse monoclonal IgG antibody against nitrotyrosine, a marker of ROS, was purchased from Millipore (Billerica, MA, U.S.A.). Palmitic acid (PA), deprenyl, 4′,6-diamidino-2-phenylindole (DAPI), and resorcin-fuchsin solution were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). 2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester was purchased from Molecular Probes (Invitrogen, Carlsbad, CA, U.S.A.). Goat anti-mouse and rabbit IgG fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate conjugated antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, U.S.A.).

Cell culture

HepG2 cells were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and cultured in Dulbecco's modified eagle medium (Life Technologies; Carlsbad, CA, U.S.A.) containing 10% fetal bovine serum and 1% antibiotic/antimycotic solution at 37°C in an incubator containing 5% CO_2. Cells were treated with deprenyl for 24 h (Figure 7) or then coincubated with 0.2 mM PA for another 48 h (Figure 8). The treated cells were used in the following experiments.

Animal study

Male ApoE-deficient or C57BL/6 mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). All protocols approved by the National Taiwan University College of Medicine and College of Public Health

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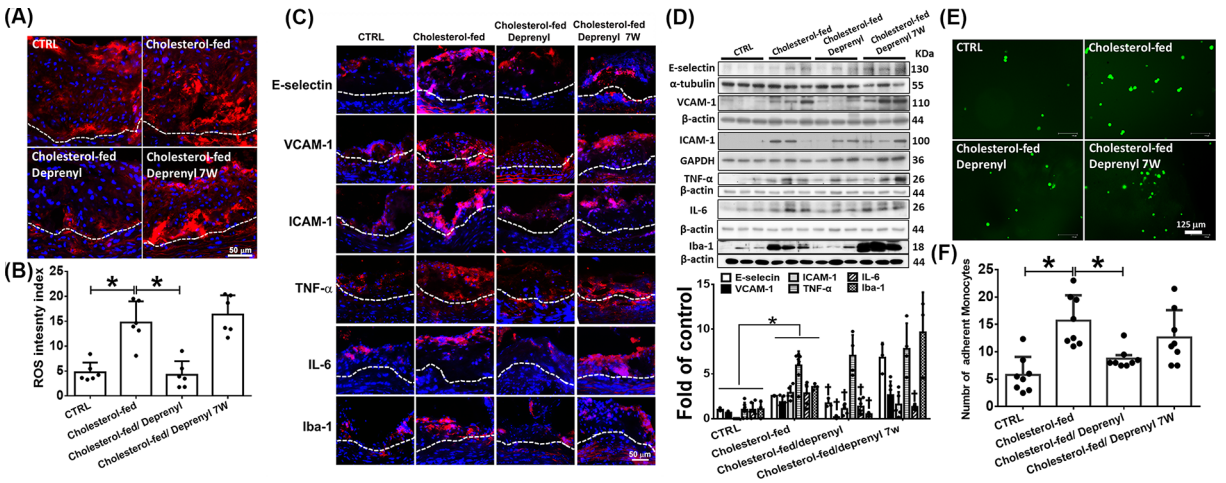

Figure 3. MAO B inhibition by deprenyl reduces the expression of ROS, adhesion molecules, inflammatory cytokines, and macrophage infiltration in atherosclerotic plaques

(**A,B**) Representative images and quantification of the intensity of nitrotyrosine staining within the thoracic aorta. (**C,D**) Immunohistochemical staining and immunoblotting analysis showed that deprenyl treatment (prevention group) decreased the expression level of E-selectin, VCAM-1, ICAM-1, IL-6, and Iba-1 in the atherosclerotic plaques, compared with the cholesterol-fed group. (**E,F**) Representative images of en face examination of the aorta and quantification of the number of adherent U937 cells to the aortae in the different groups. Tissue sections were stained with DAPI to visualize nuclei. The dashed white line represents internal elastic lamina. The scale bar represents 50 μm in (A,C) and 125 μm in panel (E). The values are the means ± SD, **P*<0.05, †*P*<0.05 vs. the cholesterol-fed group.

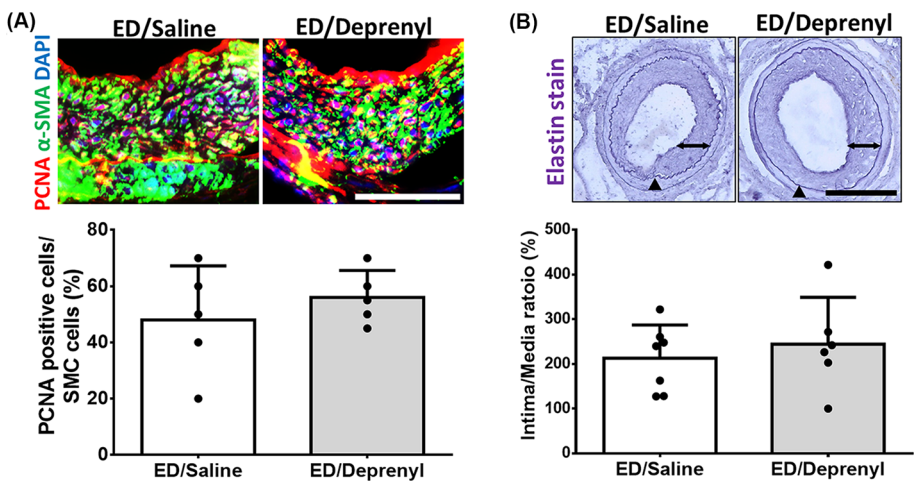

Figure 4. MAO B inhibition by deprenyl exerts no therapeutic effects on restenosis formation in the femoral artery wire-induced injury mice model

(**A**) Immunohistochemical staining for PCNA (red), α-SMA (green), and DAPI (blue) and (**B**) Elastin staining showed that deprenyl treatment had no effect on neointimal vascular smooth muscle cells proliferation and restenosis progression in denuded femoral arteries 28 days after endothelial denudation (ED). The scale bar represents 100 μm. The black arrowheads indicate the internal elastic lamina. The double-headed arrows indicate the area of neointimal hyperplasia. The values are the means ± SD.

Institutional Animal Care and Use Committee were performed in accordance with the local institutional guidelines for animal care and conducted in the Medicine Laboratory Animal Center of National Taiwan University College.

Cholesterol-diet induced atherosclerosis model

Male Apolipoprotein E (apoE)-deficient mice were randomly distributed into the following six groups: Group I (control), fed a standard chow diet; Group II (cholesterol diet), fed a cholesterol diet (0.15% cholesterol) (Purina Mills,

Inc., U.S.A.) for 15 weeks; Group III (cholesterol diet/clorgyline, the prevention group), fed a cholesterol diet and received clorgyline for 15 weeks; Group IV (cholesterol diet/deprenyl, the prevention group), fed a cholesterol diet and received deprenyl for 15 weeks; and Group V (cholesterol diet/clorgyline 7W, the treatment group), fed a cholesterol diet for 15 weeks and received clorgyline from weeks 9 to 15; and Group VI (cholesterol diet/deprenyl 7W, the treatment group), fed a cholesterol diet for 15 weeks and received deprenyl from weeks 9 to 15. Clorgyline (5 mg/kg/day in saline) or deprenyl (2.5 mg/kg/day in saline) was administered orally every day. After 15 weeks, the mice were anesthetized with pentobarbital (150 mg/kg intraperitoneally) followed by cervical dislocation, the whole thoracic aorta and aortic sinus were fixed, embedded, cryosectioned, and stained with Oil red O (ORO) for quantification of atherosclerosis. Serial sections of the thoracic aorta were stained using anti-E-selectin, VCAM-1, ICAM-1, TNF-α, IL-6, and Iba-1 antibodies for the assessment of adhesion molecules expression, inflammation, and macrophage infiltration, respectively, as previously described [15].

Femoral artery injury model

Femoral artery denuding was conducted according to the method developed by Sata et al. [16]. Acetaminophen (2.5 mg/kg) was administered in drinking water to provide analgesia. The C57BL/6 mice were divided into the endothelial denudation (ED)/saline group and the ED/deprenyl group. The deprenyl (2.5 mg/kg/day in saline) was received orally every day for 4 weeks. Twenty-eight days after the femoral artery was denuded, all mice were anesthetized with pentobarbital (150 mg/kg intraperitoneally) followed by cervical dislocation, and the injured femoral arteries were gently dissected, fixed, and cryosectioned for morphometric analysis and immunohistochemistry. Every tenth femoral artery section was stained with resorcin-fuchsin solution for neointima formation analysis. The intima/media layer ratio (I/M ratio) was calculated and used to indicate neointima formation or restenosis. Serial sections of femoral arteries were stained using anti-PCNA and α-actin antibody to assess the proliferative ratio of smooth muscle cells.

HFD-induced fatty liver model

C57BL/6 mice were randomly divided into three groups: control group (CTRL), fed with normal chow diet and vehicle orally for 10 weeks; 60% high-fat diet (HFD) group, fed with HFD and vehicle; HFD/deprenyl group (the prevention group), fed with HFD and deprenyl (2.5 mg/kg/day) orally for 10 weeks. After 10 weeks, all mice were anesthetized with pentobarbital (150 mg/kg intraperitoneally) followed by cervical dislocation, and the fasting blood and liver samples were collected for biochemical parameters analysis and liver steatosis quantification.

Histological analysis of the aorta and liver

We collected serial cryosections from the aortic sinus and liver for atherosclerotic lesions and lipid droplets deposition examination. Thoracic aortas, aortic sinus lesion, and liver sections were stained for 15 min and washed with tap water. The images of the ORO staining were recorded with a microscope. In addition, paraffin sections (5 μm) of the liver were stained with hematoxylin and eosin (H&E).

Immunohistochemical staining

The 4% paraformaldehyde-fixed tissues were blocked with 10% normal horse serum for 30 min and then incubated with the various primary antibodies (all 1:100) at 4°C overnight. Then, the tissues were washed with phosphate-buffered saline (PBS) and then incubated and reacted with appropriate secondary antibodies (all 1:200) and DAPI (1 μg/ml).

Monocytes adhesion assay

2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester labeled U937 cells were added to the thoracic aorta for 60 min. The non-adherent U937 cells were removed, washed with PBS, and observed by an inverted fluorescent microscope.

MAO activity examination

The MAO activity of tissue and cell lysates was determined by the Amplex Red Monoamine Oxidase Assay Kit (Molecular Probes).

Immunoblotting assay

Immunoblotting was conducted as previously described [17]. Briefly, the (20 μg) proteins were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were incubated with the various primary

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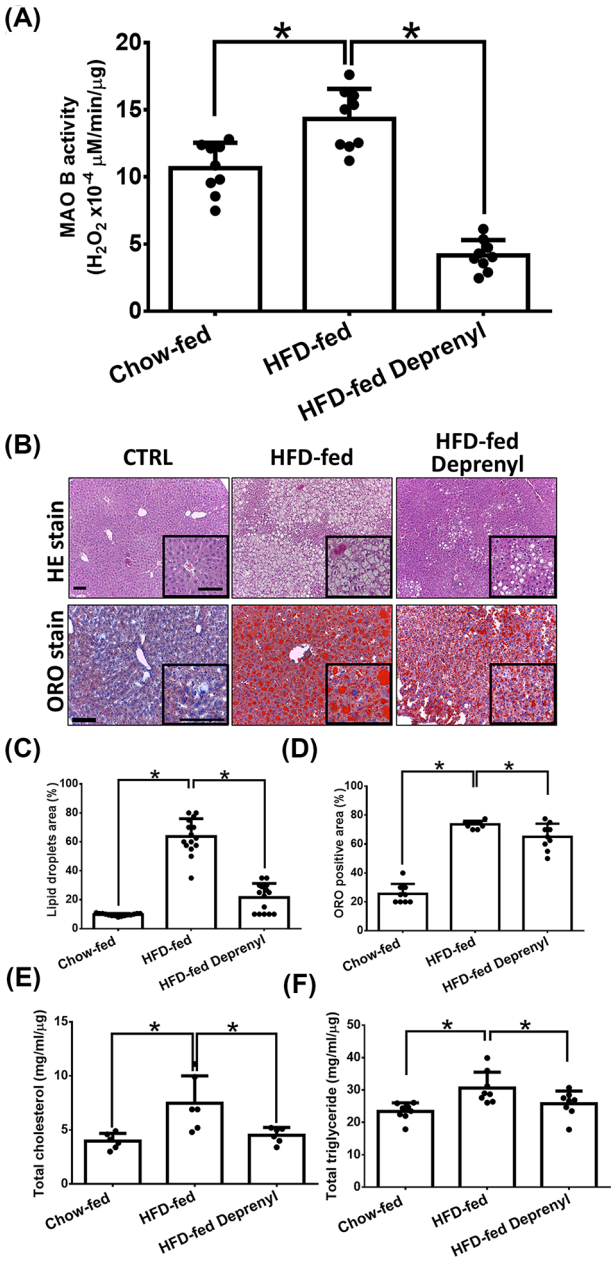

Figure 5. MAO B inhibition by deprenyl reduces lipid and cholesterol content in the liver

(**A**) Effects of deprenyl on MAO B activity in the liver. (**B**) H&E staining (upper panel) and ORO staining (lower panel) in liver sections of mice from different groups. (**C**) Quantification of lipid droplet area in different groups, measured by Image-Pro Plus software. (**D**) The expression levels of ORO were quantified by the percentage of ORO-positive areas. (**E,F**) The total cholesterol and triglyceride content in the liver in different groups. The scale bar represents 100 μm. All values are represented as means ± SD; *$P<0.05$. Chow-fed, chow diet for 10 weeks; HFD-fed, 60% fat-enriched diet for 10 weeks; HFD-fed/deprenyl, deprenyl was given along with 60% fat-enriched diet for 10 weeks.

antibodies (1:1000) overnight at 4°C. The membranes were washed with Tris-Buffered Saline-Tween (TBST) and incubated with the horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:6000) for 1 h at room temperature. Immunoreactivity was detected with enhanced chemiluminescence and quantified using Gel-Pro software. The expression level of glyceraldehyde 3-phosphate dehydrogenase, β-actin, or α-tubulin was used as the internal control.

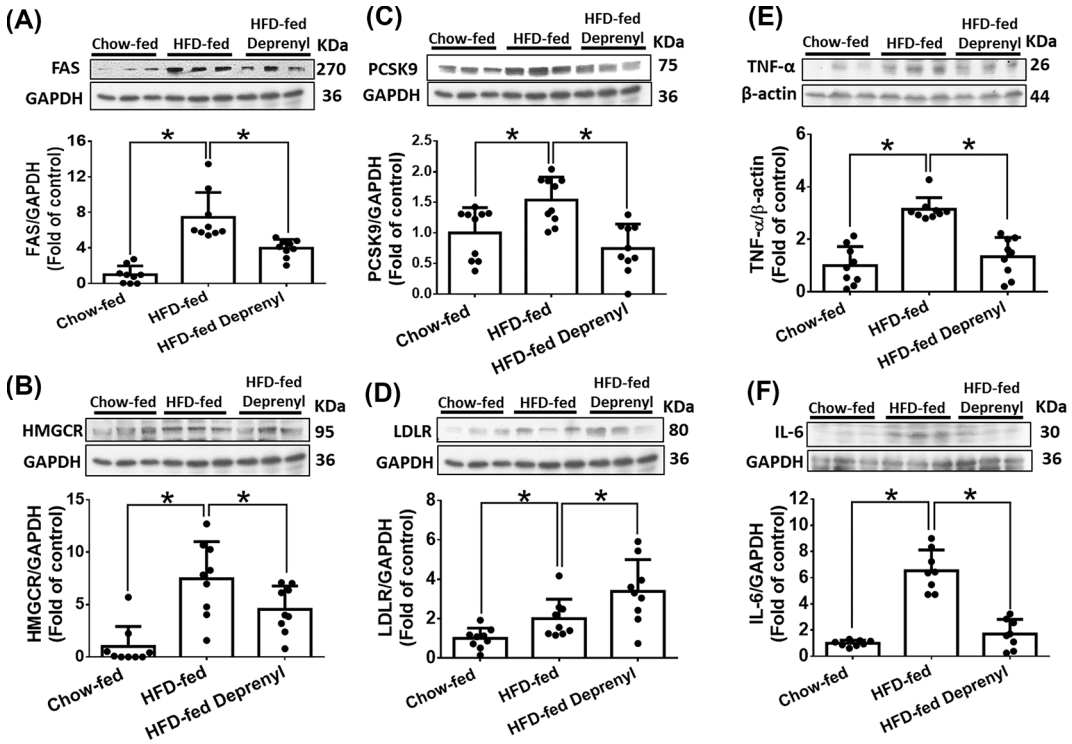

Figure 6. MAO B inhibition by deprenyl reduces lipid and cholesterol biosynthesis and inflammation, and increased cholesterol clearance in the liver

(**A–F**) Immunoblotting analysis showing the protein levels of FAS, HMGCR, PCSK9, LDLR, TNF-α, and IL-6 in the liver. All values are represented as means $\pm$ SD; *$P<0.05$.

Quantification of biochemical parameters

Fasting blood samples were collected to measure plasma triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), glucose (Randox Laboratories. Ltd., U.K.), creatinine, alanine transaminase (ALT), and aspartate aminotransferase (AST) (Fortress, Antrim, U.K.) levels. In addition, the treated cells and liver tissue lysates were collected to measure TG and TC.

Statistical analysis

All results are presented as the means $\pm$ SD. Statistical analysis was performed with one-way ANOVA followed by Tukey's multiple comparisons test and the unpaired Student's *t*-test. A P value<0.05 was defined as statistically significant.

Results

Expression of MAO in atherosclerotic lesions in human and ApoE-deficient mice

To examine MAO expression in arteries with atherosclerosis in humans and mice, immunohistochemical staining and immunoblotting were performed with antibodies against MAO A and B. Compared with the normal arteries, the expression levels of MAO A and B were significantly higher in the atherosclerotic plaques (Figure 1A,B). The data, as mentioned earlier, showed that MAO A and B might play a role in the development of atherosclerosis.

MAO B inhibition by deprenyl reduces atherosclerosis in cholesterol-fed ApoE-deficient mice

To detect the effects of clorgyline (MAO A inhibitor) and deprenyl (MAO B inhibitor) treatment on MAO A and B activity, MAO A and B-specific activity was detected in cholesterol-fed ApoE-deficient mice. As shown in Figure 2A,B, Clorgyline and deprenyl treatment significantly inhibited MAO A and B activity in thoracic aortic tissues and lungs,

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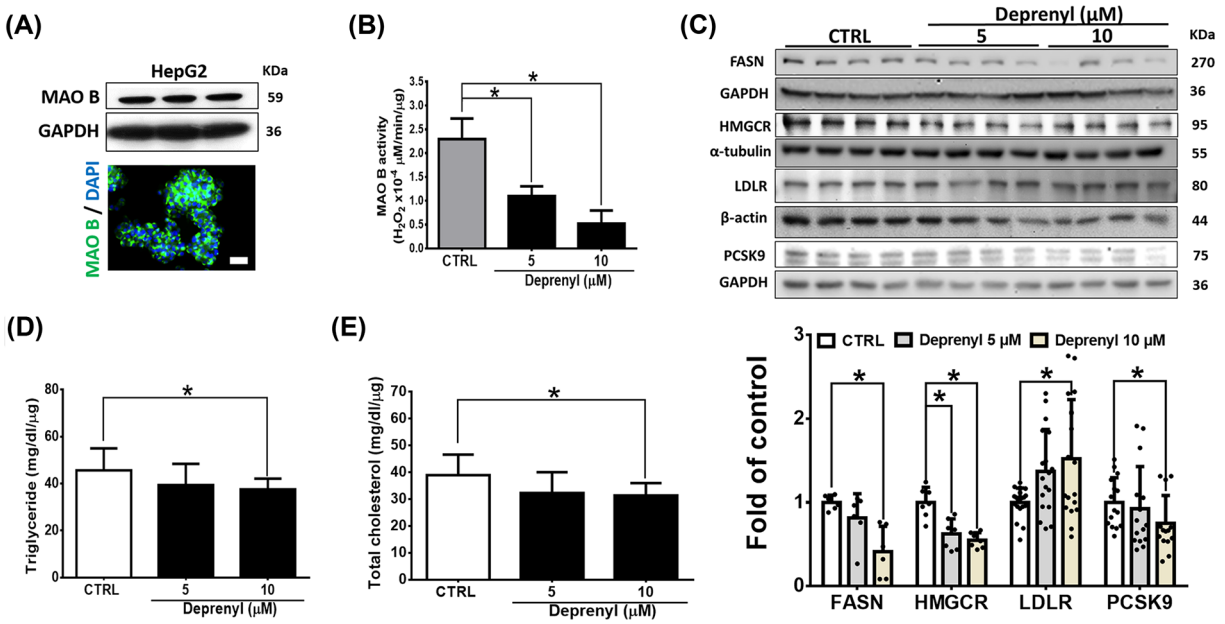

Figure 7. MAO B inhibition by deprenyl reduces lipid and cholesterol biosynthesis and increased cholesterol clearance in HepG2 liver cells

(**A,B**) Immunoblotting analysis, immunofluorescence staining, and MAO B activity assay showed that HepG2 liver cells expressed MAO B and exerted MAO B activity. (**C,D**) Immunoblotting analysis showing the expression levels of FAS, HMGCR, LDLR, and PCSK9 in HepG2 liver cells. (**E,F**) Triglyceride and total cholesterol content were measured in HepG2 liver cells. The scale bar represents 50 μm in (**A**). All values are represented as means ± SD; *$P<0.05$.

compared with the cholesterol-fed group (Figure 2A,B). Histomorphometric analysis of the thoracic aorta and aortic sinus sections was used to quantify the therapeutic effects of clorgyline and deprenyl on the atherosclerotic plaque area in ApoE-deficient mice 15 weeks after a cholesterol-enriched diet. The atherosclerotic plaque area in the thoracic aorta and aortic sinus was evaluated by ORO staining analysis (Figure 2C,D). Compared with the cholesterol-fed group, only deprenyl (prevention and treatment groups) markedly reduced atherosclerotic plaque area. The clorgyline treatment group did not reduce the atherosclerotic plaque area compared with the cholesterol-fed group. The above data showed that MAO B inhibition by deprenyl can reduce atherosclerotic plaque formation in cholesterol-fed ApoE-deficient mice.

MAO B inhibition by deprenyl reduces ROS production, expression of adhesion molecules, production of inflammatory cytokines, and macrophage infiltration in atherosclerotic plaques

Previous studies showed that ROS and inflammation play an important role in the development of atherosclerosis. The results of immunohistochemical staining and immunoblotting analysis showed that compared with the control group, the expression of nitrotyrosine (a marker of ROS), adhesion molecules, including E-selectin, VCAM-1, and ICAM-1, proinflammatory molecules IL-6, and the number of infiltrated macrophages (measured using the marker Iba-1) were increased in thoracic aorta of ApoE-deficient mice in the cholesterol-fed group. (Figure 3A–D). MAO B inhibition by deprenyl (only the prevention group) significantly decreased the ROS production, expression of these adhesion molecules and proinflammatory cytokines, and the number of infiltrated macrophages compared with the cholesterol-fed group (Figure 3A–D). Since the recruitment of leukocytes is important for the initiation and progression of atherosclerosis, the monocytes adhesion assay results showed a higher number of labeled-U937 cells in the arteries of the cholesterol-fed group than in that of the control group. In addition, a significant reduction was observed in the number of adherent monocytes subsequent to deprenyl treatment (only in the prevention group) compared with the number in the cholesterol-fed group (Figure 3E,F). The results suggest that MAO B inhibition by deprenyl reduced ROS production, the expression level of adhesion molecules, inflammatory cytokines, and leukocyte recruitment to atherosclerotic lesions in cholesterol-fed ApoE-deficient mice.

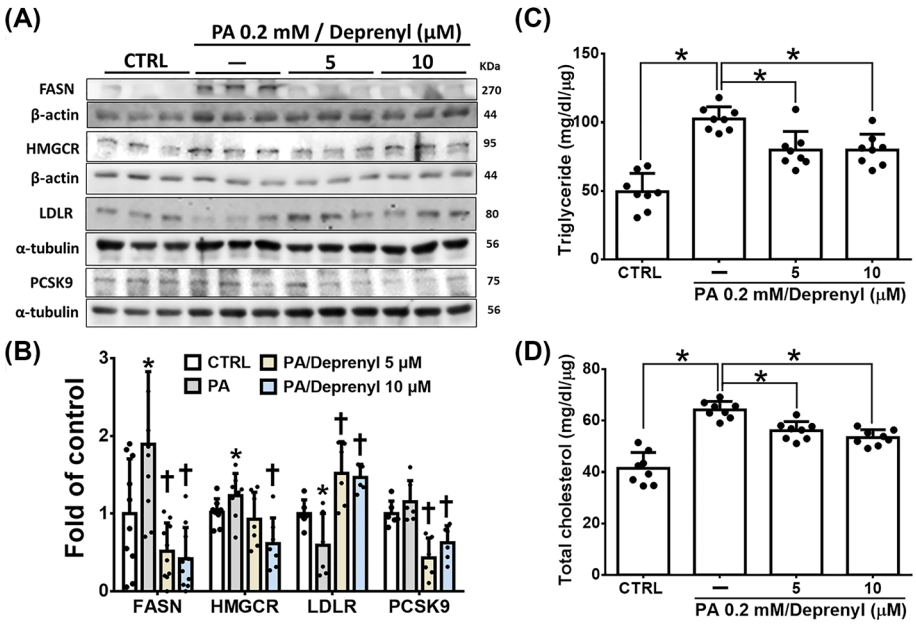

Figure 8. MAO B inhibition by deprenyl reduces lipid and cholesterol biosynthesis and increased cholesterol clearance in PA-treated HepG2 liver cells

(**A,B**) Immunoblotting analysis showing the expression levels of FAS, HMGCR, LDLR, and PCSK9 in PA-treated HepG2 liver cells, with or without deprenyl pretreatment. (**C,D**) Triglyceride and total cholesterol content were measured in HepG2 liver cells. All values are represented as means ± SD. **P*<0.05 vs. the control group (CTRL). †*P*<0.05 vs. the PA-treated group.

Table 1 Serum biochemical parameters in apolipoprotein E knockout mice fed with chow diet or cholesterol-enriched diet, with or without MAO A inhibitor clorgyline or MAO B inhibitor deprenyl

	Chow-fed	Cholesterol-fed	Cholesterol-fed + Clorgyline	Cholesterol-fed + Deprenyl	Cholesterol-fed + Clorgyline 7W	Cholesterol-fed + Deprenyl 7W
Triglyceride (mg/dl)	66.2 ± 15.6	148.0 ± 24.8*	89.5 ± 32.7†	102.7 ± 24.3†	124.3 ± 40.7	96.8 ± 22.8†
Glucose (mg/dl)	107.2 ± 14.3	145.2 ± 20.5*	144.8 ± 26.4*	148.9 ± 24.8*	144.8 ± 26.4*	136.3 ± 30.8*
Total cholesterol (mg/dl)	147.8 ± 26.6	281.1 ± 43.9*	312.5 ± 69.6*	208.0 ± 66.4†	275.7 ± 69.7*	290.3 ± 73.7*
HDL-C (mg/dl)	65.7 ± 19.2	66.6 ± 17.3	95.1 ± 39.3	66.9 ± 20.0	72.1 ± 31.7	86.2 ± 32.2
LDL-C (mg/dl)	82.1 ± 16.6	205.8 ± 34.8*	217.5 ± 72.0*	141.1 ± 57.3†	203.7 ± 89.1*	204.1 ± 62.6*
AST (U/L)	34.6 ± 2.6	47.2 ± 3.7*	39.6 ± 5.8	39.0 ± 4.6†	41.2 ± 6.3	41.2 ± 6.3
ALT (U/L)	43.8 ± 8.9	92.7 ± 9.6*	60.4 ± 9.2†	63.5 ± 11.8†	68.1 ± 15.6†	63.1 ± 11.1†

The values are expressed as the means ± SD; *N* = 5–8.
Chow-fed, chow diet for 15 weeks; cholesterol-fed, cholesterol-enriched diet for 15 weeks; cholesterol-fed/clorgyline, clorgyline (5 mg/kg/day) was given along with cholesterol-enriched diet for 15 weeks; cholesterol-fed/clorgyline 7W, the cholesterol-enriched diet was given for 8 weeks followed by cholesterol-enriched diet plus clorgyline for another 7 weeks; cholesterol-fed/deprenyl, deprenyl was given along with cholesterol-enriched diet for 15 weeks; cholesterol-fed/ deprenyl 7W, cholesterol-enriched diet was given for 8 weeks followed by cholesterol-enriched diet plus deprenyl for another 7 weeks.
Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.
**P*<0.05 compared with the chow-fed group
†*P*<0.05 compared with the cholesterol-fed group.

Effects of MAO B inhibition by deprenyl on serum biochemical parameters of cholesterol-fed ApoE-deficient mice

Plasma TC, LDL-C, TG, glucose, AST, and ALT concentrations were markedly higher in cholesterol-fed ApoE-deficient mice than in the control group (Table 1). The elevation of plasma TC, LDL-C, TG, AST, and ALT was markedly decreased by deprenyl (the prevention group). Compared with the cholesterol-fed group, plasma TC

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Table 2 Serum biochemical parameters in C57BL mice fed with chow diet, HFD, or HFD plus MAO B inhibitor deprenyl

	Chow diet	HFD	HFD + Deprenyl
Triglyceride (mg/dl)	72.6 ± 11.3	108.3 ± 18.2*	93.2 ± 10.8†
Glucose (mg/dl)	179.6 ± 30.0	258.4 ± 53.4*	177.5 ± 29.9†
Total cholesterol (mg/dl)	315.9 ± 97.4	413.2 ± 102.2*	306.7 ± 58.2†
HDL-C (mg/dl)	201.4 ± 87.3	281.5 ± 93.1	196.0 ± 53.3
LDL-C (mg/dl)	114.5 ± 11.9	131.7 ± 18.5*	110.7 ± 10.9†

The values are expressed as the means ± SD; N=10.
Abbreviations: HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.
*P<0.05 compared with the chow-fed group.
†P<0.05 compared with the HFD-fed group.

and ALT concentrations significantly decreased in the treatment group (the deprenyl 7W group). These results suggest that 15 weeks of deprenyl treatment may reduce atherosclerosis by reducing plasma LDL-C.

MAO B inhibition by deprenyl exerts no therapeutic effects on restenosis in femoral artery wire-induced injury mice model

Abnormal vascular smooth muscle cell proliferation and migration play key roles in atherosclerosis and restenosis. A femoral artery wire-induced injury model in mice was used to evaluate the therapeutic effect of deprenyl reducing atherosclerotic plaques through inhibition of proliferation and migration of vascular smooth muscle cells. In Figure 4A,B, the results showed that MAO B inhibition by deprenyl did not exert a regulatory effect on vascular smooth muscle cell proliferation and restenosis formation. These data indicate that deprenyl may act through pathways other than abnormal proliferation and migration of vascular smooth muscle cells to reduce atherosclerotic plaques.

MAO B inhibition by deprenyl reduces fatty liver, triglyceride, and cholesterol biosynthesis and inflammation and enhances cholesterol clearance in mice

We have shown that deprenyl could reduce plasma LDL cholesterol and triglyceride levels in cholesterol-fed ApoE deficient mice (Table 1). Since the liver is the major organ involved in TG and cholesterol metabolism, and NAFLD is an important risk factor for atherosclerosis, we used the NAFLD mouse model to elucidate the therapeutic role of deprenyl on cholesterol, TG metabolism, and NAFLD. Since the effect of MAO B inhibition in atherosclerosis was less significant in the treatment group, only the prevention group was used in the NAFLD mouse model. As shown in Figure 5A, deprenyl treatment significantly inhibited MAO B activity in the liver compared with the control group (Figure 5A). HFD-fed mice, administered deprenyl, exhibited marked reductions in lipid droplet deposition (Figure 5B–D) and hepatic, TC, and TG levels (Figure 5E,F) compared with HFD-fed mice. As shown in Table 2, plasma TG, TC, and LDL-C were significantly increased in HFD-fed mice compared with the control group. Deprenyl significantly decreased plasma TG, TC, and LDL-C in HFD-fed mice.

To explore the regulatory mechanism of deprenyl in lowering lipid accumulation and cholesterol concentration in HFD-fed mice, a Western blotting assay was performed for key enzymes of TG biosynthesis (fatty acid synthase [FAS]), cholesterol biosynthesis (3-hydroxy-3-methylglutaryl-CoA reductase [HMGCR]), and LDL uptake (LDL receptor, low-density lipoprotein receptor [LDLR] and proprotein convertase subtilisin/kexin type 9 [PCSK9]). LDLR in hepatocytes can bind to LDL particles in circulation and lower circulating LDL-C levels, whereas PCSK9 can bind to LDLR to prevent the recycling of LDLR [18]. Clinically, PCSK9 inhibitors are used to lower plasma LDL-C. The expressions of FAS, HMGCR, and PCSK9 decreased in mice that received deprenyl treatment, and the expression level of LDLR was markedly increased in HFD-fed mice treated with deprenyl (Figure 6A–D) compared with the HFD-treated mice. These findings suggest that deprenyl can inhibit the biosynthesis of TG and cholesterol and enhance the clearance of circulating cholesterol by LDLR.

Inflammation plays a crucial role in NAFLD and atherosclerosis progression. The expression level of inflammatory cytokine was determined to elucidate the anti-inflammatory effect of deprenyl treatment on HFD-fed mice. Compared with the HFD-treated mice, Western blotting results showed that deprenyl treatment significantly reduced TNF-a and IL-6 expression (Figure 6E,F). Furthermore, the anti-inflammatory effect of deprenyl is in concordance with the findings in the ApoE-deficient mice model.

MAO B inhibition by deprenyl decreased triglyceride and cholesterol biosynthesis and increased cholesterol clearance in HepG2 liver cells

To further elucidate the regulatory mechanism of MAO B on TG and cholesterol synthesis, deprenyl was administered to HepG2 liver cells. According to the Western blot, immunofluorescence staining, and MAO B activity assay, HepG2 cells showed a high expression level and activity of MAO B, which was significantly inhibited by treatment with deprenyl (Figure 7A,B). Immunoblotting analysis showed that deprenyl treatment significantly reduced the expression level of FAS, HMGCR, and PCSK9 and increased LDLR expression compared with the vehicle-treated group (Figure 7C,D). Furthermore, TG and cholesterol synthesis levels were markedly lower in deprenyl-treated HepG2 cells than in the vehicle-treated group (Figure 7E,F). Figure 8 shows the results of HepG2 cells treated with PA. In this model, deprenyl pretreatment significantly decreased the levels of TG and cholesterol, and the expression level of FAS, HMGCR, and PCSK9, and increased LDLR expression, compared with the group without deprenyl pretreatment (Figure 8A–D).

Discussion

In the present study, we found that MAO expression was increased in atherosclerotic plaques, especially MAO B. MAO B inhibition by deprenyl reduced atherosclerosis and fatty liver. In addition, MAO B inhibition also decreased plasma triglyceride and LDL-C concentrations, ROS production, and inflammation. In the liver, MAO B inhibition lowered the expression of FAS, which may be the mechanism of its TG-lowering activity. Furthermore, MAO B inhibition decreased the expression of HMGCR and PCSK9 and increased the expression of LDLR in the liver, which may explain its LDL-C lowering activity. These findings suggest that deprenyl is a novel treatment for atherosclerosis and NAFLD. The findings also suggest that deprenyl may act through its activity on TG and cholesterol metabolism, ROS production, and inflammation. Therefore, MAO B inhibition may be a potential and novel treatment strategy for atherosclerosis and NAFLD, which should be explored in the future.

This is the first study demonstrating MAO B inhibition's role in atherosclerosis. Atherosclerosis is initiated when endothelial cells are activated by proinflammatory cytokines or pathophysiologic processes related to cardiovascular risk factors [2]. Activated endothelial cells express adhesion molecules for leukocytes, such as monocytes, to adhere and transmigrate to the intima, where they differentiate into macrophages. Macrophages can become foam cells after engulfing modified LDL, such as oxidized LDL derived from LDL particles modified by ROS.

This present study has demonstrated that MAO B inhibition reduced ROS production, plasma LDL-C levels, and the expression of adhesion molecules and proinflammatory cytokines. In addition, in a previous study, deprenyl inhibited human LDL oxidation in *in vitro* and *in vivo* models [19]. These findings suggest that MAO B inhibition reduced atherosclerosis by inhibiting several key steps in its pathogenesis. The family of amine oxidases includes three other amine oxidases: semicarbazide-sensitive amine oxidase (SSAO), lysyl oxidase, and diamine oxidase [20]. All amine oxidases catalyze oxidative cleavage of alkylamines to aldehydes, ammonia, and hydrogen peroxide. However, the amine oxidases differ in substrate specificity, subcellular localization, and whether their cofactors contain flavin or copper. A previous study showed that inhibition of SSAO reduced atherosclerosis by inhibiting inflammation and oxidative stress in mice and rabbit models [15,21]. Lysyl oxidase participates in several pathophysiologic mechanisms of atherosclerosis and is involved in other vascular pathologies through remodeling the extracellular matrix [22]. Although there is no report regarding the role of diamine oxidase in atherosclerosis, current evidence, including findings from the present study, suggest that amine oxidases may participate in atherosclerosis through their common end-products. The various mechanisms linking amine oxidases and atherosclerosis may result from the differences in substrate specificity, subcellular localization, and aldehyde generation.

High plasma cholesterol concentration is an important risk factor for atherosclerosis, especially high LDL-C and non-HDL cholesterol levels [2]. Plasma cholesterol primarily originates from its biosynthesis in the liver [18]. β-Hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase is the key enzyme for cholesterol biosynthesis and is the drug target of statins. Plasma LDL is cleared by binding to hepatic LDLR, which is regulated by the expression of PCSK9 [18]. PCSK9 can bind to LDLR, resulting in the internalization and degradation of LDLR. Clinically, several PCSK9 inhibitors have been developed and are effective in lowering plasma LDL-C and reducing the risk of cardiovascular diseases [23]. In the present study, we demonstrated that MAO B inhibition reduced plasma total cholesterol and LDL-C levels, decreased hepatic expression of HMG-CoA reductase and PCSK9, and increased the expression of LDLR in the liver. Similarly, in HepG2 hepatocytes, MAO B inhibition decreased the expression of HMG-CoA reductase and PCSK9 and increased the expression of LDLR. The production of cholesterol was also reduced by MAO B inhibition. These findings suggest that MAO B inhibition reduced plasma LDL-C levels through the inhibition of hepatic biosynthesis of cholesterol and the enhanced clearance of LDL cholesterol in the liver.

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In the literature, treatment with deprenyl for 28 days reduced hepatic steatosis in Sprague-Dawley rats fed with a lipid-rich diet [24]. Results from the present study provide evidence from C57B6 mice and support previous findings. Several pathways have been proposed to be the pathogenesis of NAFLD [6,25], and increased hepatic *de novo* lipogenesis is important. In this pathway, FAS and acetyl CoA carboxylase are key enzymes for the biosynthesis of fatty acids. Increased levels of fatty acids could result in elevated plasma TG and the development of NAFLD. In addition, as described previously, oxidative stress is another important mechanism for NAFLD [6]. Oxidative stress inhibits the secretion of very low-density lipoprotein resulting in fat accumulation in the liver. It can also induce lipid peroxidation, inflammation, and fibrogenesis, leading to hepatic injury and fibrosis. In the present study, we found that MAO B inhibition decreased FAS expression and ROS production in the liver, reduced plasma TG concentrations, and decreased fatty liver disease severity. Additionally, MAO B inhibition reduced FAS expression and triglyceride production in HepG2 hepatocytes. These findings suggest that MAO B inhibition reduced fatty liver disease through multiple key mechanisms in the pathogenesis of fatty liver disease and NAFLD.

Deprenyl, also known as selegiline, has been used to treat Parkinson's disease [26]. It is an irreversible and selective inhibitor of MAO B, and it can increase dopamine concentrations in the synaptic gap, primarily when used in combination with levodopa. Clinically, deprenyl can improve symptoms of Parkinson's disease, as it has a levodopa-saving effect and reduces late motor complications and freezing phenomena [27–31]. Findings from the present study suggest new and potential indications for deprenyl in the treatment of atherosclerosis and NAFLD, which indications should be evaluated in future clinical studies. Currently, other MAO inhibitors are used clinically, such as rasagiline and safinamide. Since deprenyl is a relatively selective inhibitor of MAO B, the beneficial effects found in the present study may also be applied to other MAO B inhibitors. However, this correlation should be investigated in further studies.

In this study, the anti-atherosclerosis effect of MAO B inhibition was less significant in the treatment group than in the prevention group. Only IL-6 expression was reduced in the treatment group. Other key pathophysiologic steps, including ROS production, expression of adhesion molecules, and recruitment of monocytes, were not significantly suppressed in the treatment group. Similar findings were observed in our previous report, which showed that the anti-atherosclerosis effect by inhibition of SSAO was less significant in the treatment group compared with the effect in the prevention group [15]. There are two possible explanations for these findings. First, the treatment duration in the treatment group was shorter (8 weeks) than the duration in the prevention group (15 weeks). Second, in the treatment group, the pathologic process of atherosclerosis had been induced by a cholesterol-enriched diet for 7 weeks, which may also contribute to the less prominent anti-atherosclerosis effect observed in this group.

In the present study, MAO A inhibition by clorgyline did not reduce atherosclerosis. We propose several explanations for the differential effects of MAO A and MAO B inhibition in atherosclerosis. First, the expression of MAO A was significantly lower than the expression of MAO B in the aorta of ApoE-deficient mice fed with a high-cholesterol diet, as shown in Figure 1. Therefore, the production of toxic end-products by MAO A may be minimal, which may explain the lack of the anti-atherosclerosis effect of MAO A inhibition. The second lies in the difference in substrate preference. MAO A has a higher affinity for serotonin and norepinephrine, whereas MAO B prefers 2-phenylethlamine and benzylamine as their substrates [32]. Since these substrates may result in different aldehydes production and substrate availability, these could lead to different pathophysiologic responses. However, the substrate preference is not absolute. In the absence of MAO A or B, the other MAO can deaminate the non-preferred substrates [33]. Therefore, although differences in substrate preferences may be one of the mechanisms, it only partially accounts for the different effects of MAO A and B inhibition in atherosclerosis.

In conclusion, the present study has demonstrated that MAO B inhibition by deprenyl can reduce atherosclerosis and fatty liver disease through its effect on lowering plasma triglyceride and LDL-C concentrations and inhibiting ROS production and inflammation. These findings suggest that MAO B inhibition is a potential and novel treatment strategy for atherosclerosis and NAFLD.

Clinical perspectives

- Oxidative stress is a common pathophysiology for atherosclerosis and NAFLD. MAO is an important source of oxidative stress in the vasculature and liver. However, the effect of MAO inhibition on atherosclerosis and NAFLD has not been explored in the literature.

- The present study has demonstrated for the first time that MAO B inhibition can reduce atherosclerosis and NAFLD and may act through its effect on decreasing the biosynthesis of TG and cholesterol, enhancing the clearance of LDL cholesterol, lowering plasma triglyceride and LDL cholesterol concentrations, and inhibiting oxidative stress and inflammation.
- These findings suggest that MAO B inhibition is a potential and novel treatment for atherosclerosis and NAFLD.

Data Availability

The authors declare that all the data supporting the findings of this study are available within the article.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Shu-Huei Wang: Conceptualization, Resources, Data curation, Validation, Investigation, Methodology, Writing—original draft, Writing—review & editing. **Feng-Chiao Tsai:** Conceptualization, Resources, Validation, Investigation, Methodology. **Heng-Huei Lin:** Resources, Validation, Investigation, Methodology. **Tse-Ya Yu:** Conceptualization, Validation, Investigation. **Chun-Heng Kuo:** Investigation, Methodology, Writing—review & editing. **Hung-Yuan Li:** Conceptualization, Resources, Data curation, Supervision, Validation, Methodology, Writing—original draft, Writing—review & editing. **Mao-Shin Lin:** Conceptualization, Supervision, Validation, Investigation, Methodology, Writing—original draft, Writing—review & editing.

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Abbreviations

α-SMA, smooth muscle α-actin; ApoE, apolipoprotein E; HFD, high-fat diet; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; ICAM-1, intercellular adhesion protein 1; IgG, immunoglobulin G; LDL, low-density lipoprotein; MAO, monoamine oxidase; NAFLD, non-alcoholic fatty liver disease; PCNA, proliferating cell nuclear antigen; SSAO, semicarbazide-sensitive amine oxidase; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion protein 1.

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