An adverse action of varenicline, a prescription medication for smoking cessation, on the atherosclerotic plaque formation

禁煙補助薬バレニクリンの心血管系有害作用に関する研究

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Contents

Abbreviations

ABC; adenosine triphosphate-binding cassette transporters

ApoE KO mice; apolipoprotein E knockout mice

CD36; cluster of differentiation 36

DHβE; dihydro-β-erythroidine hydrobromide

ERK1/2; extracellular signal-regulated kinase 1/2

HDL; high density lipoprotein

LOX-1; lectin-like oxidized LDL receptor-1

MLA; methyllycaconitine

nAChR; nicotinic acetylcholine receptor

NF-κB; nuclear factor-kappa B

oxLDL; oxidized low density lipoprotein

SR-A; scavenger receptor class A

General introduction

Smoking is an important risk factor for the induction and development of cardiovascular disease, ischemic heart disease, chronic obstructive pulmonary disease and cancer [1]. Among tobacco users, cardiovascular disease occurs with high morbidity, and is a leading cause of death. Smoking cessation is well known to produce long-term cardiovascular benefits and is strongly recommended for patients with various diseases [2].

Atherosclerosis, one of the major causes for cardiovascular disease, is characterized as chronic inflammatory disease. The subendothelial retention of oxidized low density lipoprotein (oxLDL) has been known to actively participate in the atherosclerotic process through various mechanisms including monocytes adhesion and activation on the endothelial cells, endothelial cell dysfunction, foam cell formation from macrophages and migration and proliferation of the vascular smooth muscle cells [3]. Atherosclerotic plaque rapture and destabilization occur due to incorporated oxLDL in macrophages in the atherosclerotic plaque. Macrophages located in the arterial intima layer express several scavenger receptors (lectin-like oxidized LDL receptor-1 (LOX-1), cluster of differentiation 36 (CD36) and scavenger receptor class A (SR-A)) for an uptake of the oxLDL and adenosine triphosphate-binding cassette transporters (ABC) A1 and G1 for an efflux of the excess cholesterol to the extracellular acceptors such as high density lipoprotein (HDL). Cigarette smoking-induced decreased HDL and increased LDL levels in the blood are the risk factors of atherosclerosis. In particular, atherosclerosis is the leading cause of smoking-induced cardiovascular diseases [4]; these adverse effects could be ameliorated by smoking cessation [5]. Smoking cessation is well known to produce

long-term cardiovascular benefits and also this is strongly recommended for patients with various diseases.

Varenicline has recently been introduced as an aid to smoking cessation. It is a partial agonist of $\alpha_4\beta_2$ nicotinic acetylcholine receptors (nAChR), and a full agonist of α_7 nAChR [6,7]. Varenicline is more effective than nicotine replacement therapy and frequently used clinically for smoking cessation [8,9,10]. However, varenicline shows adverse effects, most commonly headache, nausea, abnormal dreams, and insomnia [11,12]. In addition to these central adverse reactions, increased risk of cardiovascular events has been observed in patients taking varenicline [13,14], although there are also reports showing no significant increase in severe cardiovascular adverse events [15,16,17]. Thus, involvement of varenicline and increased cardiovascular risk remains controversial.

The present thesis aimed at elucidating an adverse effect of varenicline on the atherosclerotic plaque formation as a causal factor for cardiovascular events and its mechanism of action. In the chapter 1, to determine if varenicline treatment is associated with cardiovascular events, we examined the effect of long-term varenicline treatment on atherosclerotic plaque formation in apolipoprotein E knockout (ApoE KO) mice. In the chapter 2, we elucidated whether varenicline promotes oxLDL uptake in macrophages and clarify its mechanism in the present in vitro study.

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Chapter 1

Varenicline aggravates plaque formation through alpha7 nicotinic acetylcholine receptors in ApoE KO mice

1. Introduction

Smoking is an important causative and/or risk factor for the induction and development of cardiovascular disease, ischemic heart disease, chronic obstructive pulmonary disease, and cancer [1,2]. Among tobacco users, cardiovascular disease occurs with high morbidity, and is a leading cause of death. Smoking cessation is well known to produce long-term cardiovascular benefits and is strongly recommended for patients with various diseases [3].

Varenicline has recently been introduced as an aid to smoking cessation. It is a partial agonist of $\alpha_4\beta_2$ nicotinic acetylcholine receptors (nAChR), and a full agonist of α_7 nAChR [4,5]. Varenicline is more effective than nicotine replacement therapy and frequently used clinically for smoking cessation [6,7,8]. However, varenicline shows adverse effects, most commonly headache, nausea, abnormal dreams, and insomnia [9,10]. In addition to these central adverse reactions, increased risk of cardiovascular events has been observed in patients taking varenicline [10,11], although there are also reports showing no significant increase in severe cardiovascular adverse events [12,13,14]. Thus, involvement of varenicline and increased cardiovascular risk remains controversial. To determine if varenicline treatment is associated with cardiovascular events, we examined the effect of long-term varenicline treatment on atherosclerotic plaque formation in apolipoprotein E knockout (ApoE KO) mice.

2. Materials and methods

2-1. Animals

C57BL/6J ApoE KO mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and housed under standard conditions for humidity, room temperature, and dark-light cycles. Mice were given free access to food and water throughout the study. The study protocol was approved by the Laboratory Animal Care and Use Committee of Fukuoka University.

2-2. Drugs

Varenicline tartrate was purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada) and dissolved in saline (Otsuka Pharmaceutical Co., Tokyo, Japan).

2-3. Experiment 1

ApoE KO mice (8 weeks old) were fed a high-fat diet (1.25% cholesterol, 15% cacao butter, and 0.5% sodium cholate, F2HFD1; Oriental Yeast Co., Tokyo, Japan). Mice were randomized into varenicline-treated and non-treated groups, and subcutaneously injected with either saline (vehicle, n = 8) or 0.05/0.5 mg kg^{-1} day^{-1} varenicline (n = 9/8, respectively) for a 3-week period starting from 8 weeks old. At 11 weeks of age, mice were killed and perfused with ice-cold phosphate-buffered saline. The heart and aorta were excised and analyzed histologically.

2-4. Experiment 2

ApoE KO mice (8 weeks old) were fed a high-fat diet, and randomized into the

following three groups: (1) vehicle group treated with saline (n = 10), (2) varenicline group treated with 0.5 mg kg^{-1} day^{-1} varenicline (n = 10), and (3) methyllycaconitine (MLA) plus varenicline group injected with 5 mg kg^{-1} day^{-1} MLA (an α_7 nAChR antagonist) into the abdominal cavity and 0.5 mg kg^{-1} day^{-1} varenicline (n = 9) for 3 weeks. At 11 weeks of age, mice were euthanized and the *en face* plaque area examined.

2-5. *En-face* plaque area

To quantify the extent of atherosclerotic lesions, immediately after mice were killed, the whole aortic length was excised for quantification of the *en face* plaque area, as previously described [15,16,17]. Briefly, after carefully removing fat and adventitial tissue, the aortic arch and thoracic to abdominal aorta were opened longitudinally, pinned on a black wax surface, and stained with oil red O (Sigma, St. Louis, MO, USA). *En face* images were obtained using a stereomicroscope and analyzed with the public domain software, Image J (NIH Image, Bethesda, MD, USA). Percentage of the luminal surface plaque area stained by oil red O was determined.

2-6. Histological analysis and oil red O staining

Lipid accumulation in atherosclerotic plaques at the aortic root in the heart were analyzed. The aortic root was embedded in O.C.T. Compound (Sakura FineTech, Tokyo, Japan). Serial cryostat sections (6 μm thick) were prepared as described previously [15,16,17]. Briefly, atherosclerotic plaques were investigated in five separate sets of sections, with each set separated by 60 μm. Oil red O staining was performed to examine lipids. The oil red O-positive area, a marker of lipid accumulation, was analyzed using Image J. Average values for the five sections in each animal were used for analysis.

2-7. Measurement of total plasma cholesterol levels

At 11 weeks of age, blood was collected to measure plasma total cholesterol levels. Plasma total cholesterol levels were measured using WAKO Cholesterol E Assay kit (Wako Chemical Co., Osaka, Japan).

2-8. Statistical Analysis

Histological quantitative analyses were performed by a single observer blinded to the experimental protocol. Data are expressed as mean ± standard deviation. Bonferroni analysis was used for comparison between the three groups.

P values < 0.05 were considered statistically significant.

3. Results

3-1. Experiment 1

We determined if varenicline causes progression of plaque formation in ApoE KO mice. Varenicline at a dose of 0.5 (but not 0.05) mg kg^{-1} day^{-1} for 3 weeks significantly progressed plaque formation in the aortic arch and whole aorta, in ApoE KO mice compared with vehicle and low-dose (0.05 mg kg^{-1} day^{-1}) varenicline groups (Figs. 1 and 2). Plaques in the aortic arch and whole aorta formed by varenicline treatment (0.5 mg kg^{-1} day^{-1} for 3 weeks) were 1.6- and 1.5-fold increased, respectively, compared with vehicle. This higher dose of varenicline showing significant and marked aggravation in plaque formation was employed in the following experiment.

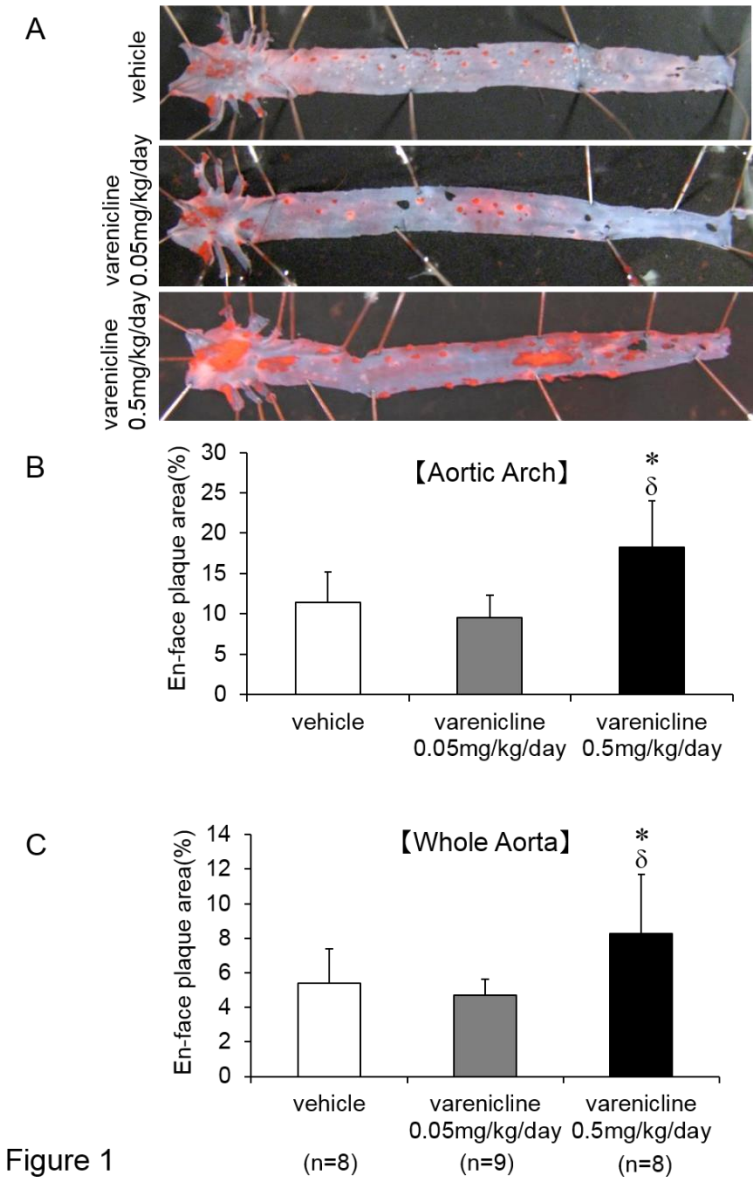

Fig. 1. Atherosclerotic plaques in the aorta of varenicline-treated ApoE KO mice.

(A) Representative *en face* photographs of the aorta showing oil red O-stained atherosclerotic plaques. Quantitative measurement of *en face* plaque area (%) in the aortic arch (B) and whole aorta (C). Each bar indicates mean ± S.D. $^{*}P < 0.05$ vs vehicle group, $^{\delta}P < 0.05$ vs 0.05 mg kg^{-1} day^{-1} varenicline treatment group.

3-2. Experiment 2

To determine the mechanism by which varenicline aggravates atherosclerotic plaque formation, we examined the effect of MLA, an α_7 nAChR antagonist, on varenicline-induced aggravation of atherosclerotic plaque formation in ApoE KO mice. With combined varenicline and MLA treatment, varenicline-induced aggravation of atherosclerotic plaque formation in the aortic arch and whole aorta of ApoE KO mice were not observed (Fig. 2). Moreover, atherosclerotic plaque formation was markedly increased by 178% in the aortic root of the varenicline group (Fig. 3). MLA also inhibited varenicline-induced aggravated plaque formation in the aortic root (Fig. 3). There were no significant differences in body weight and total plasma cholesterol levels between vehicle, varenicline, and MLA plus varenicline groups (Table 1).

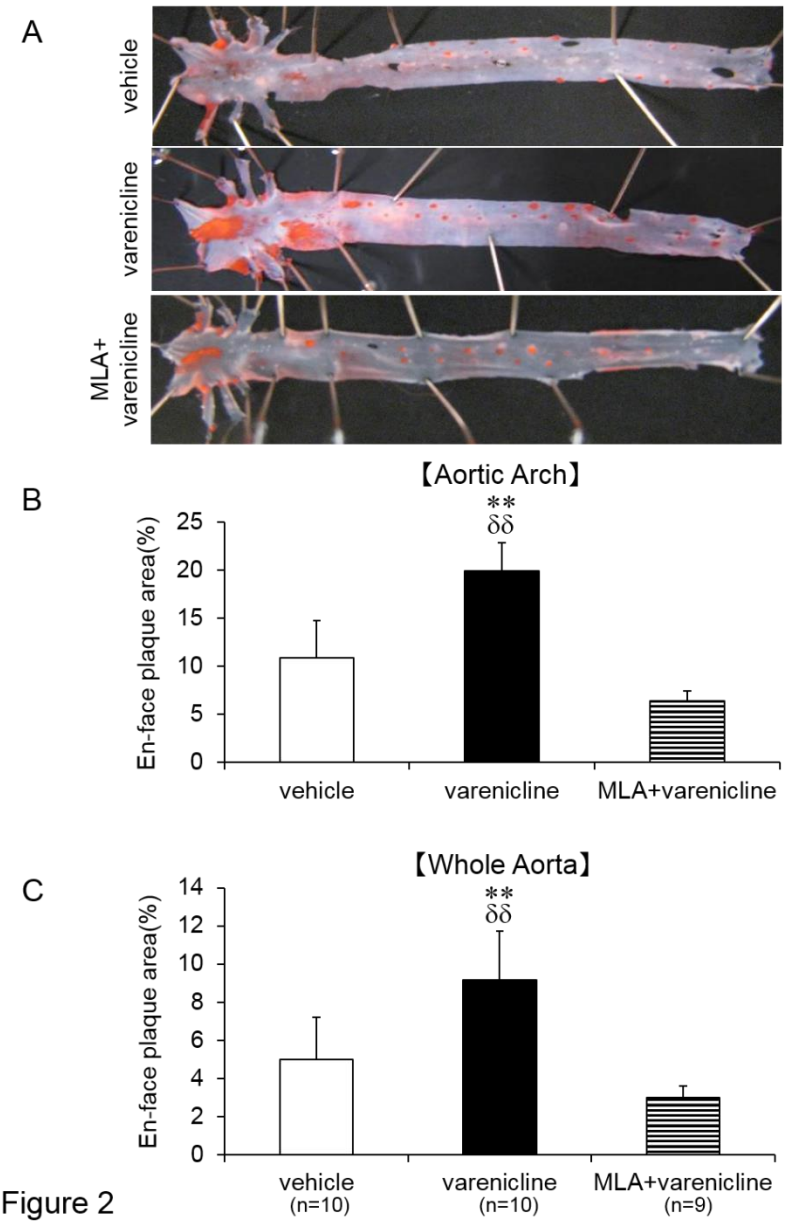

Fig. 2. Atherosclerotic plaques in the aorta of varenicline- and varenicline plus MLA-treated ApoE KO mice.

(A) Representative *en face* photographs of the aorta showing oil red O-stained atherosclerotic plaques from vehicle, varenicline, and varenicline + MLA-treated ApoE KO mice. Pooled data showing the effect of varenicline and MLA treatment on oil red O-stained positive areas in the aortic arch (B) and whole aorta (C). Each bar indicates mean ± S.D. $^{**}P < 0.01$ vs vehicle group, $^{\delta\delta}P < 0.01$ vs varenicline treatment group.

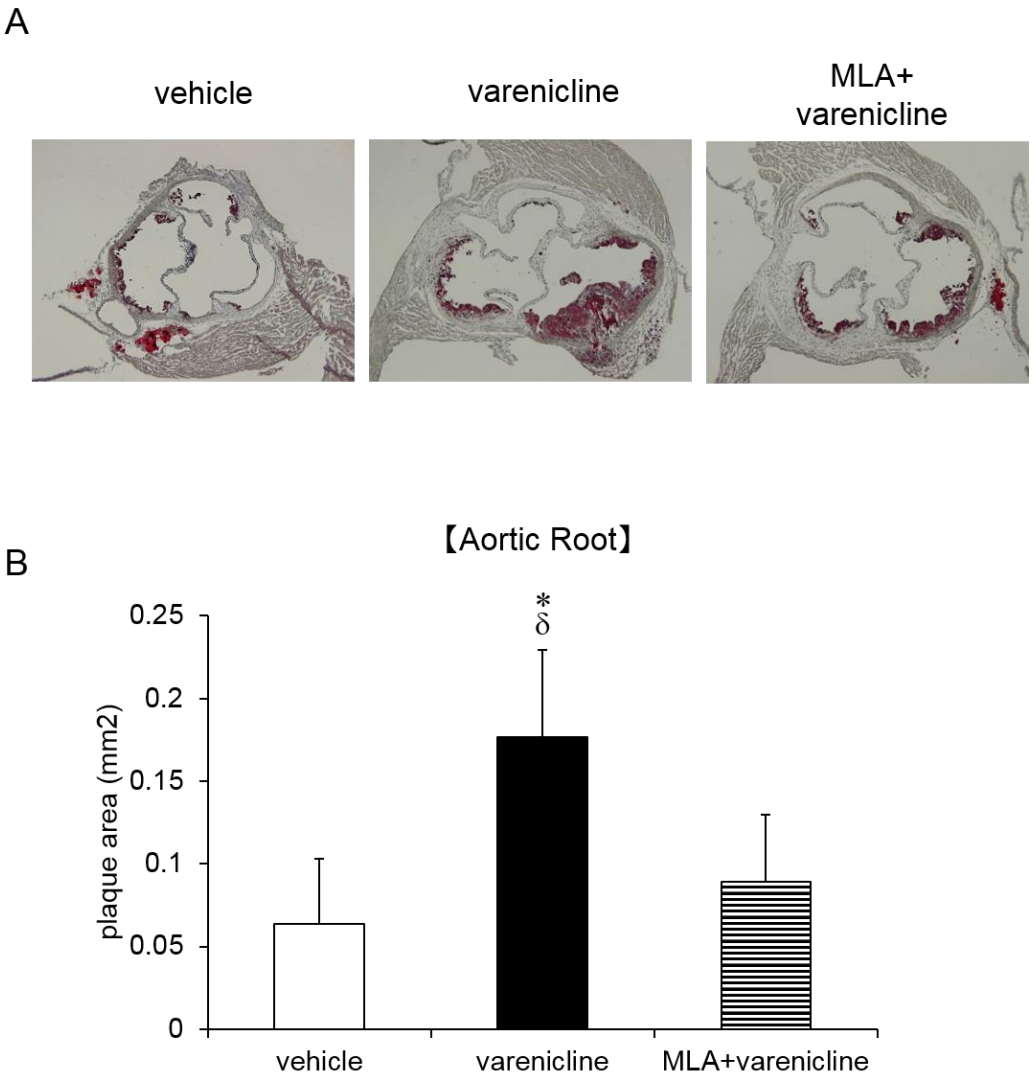

Figure 3

Fig.3. Atherosclerotic plaques in the aorta of varenicline- and varenicline plus MLA-treated ApoE KO mice.

(A) Representative cross-sections of oil red O-stained plaques in the aortic root. (B) Pooled data of plaque area in the aortic root. Each bar indicates mean ± S.D. $^{*}P < 0.05$ vs vehicle group, $^{\delta}P < 0.05$ vs varenicline treatment group.

Table 1. Background Profiles

	vehicle	varenicline	MLA + varenicline
Body Weight, g	18.4±2.6	18.4 ± 1.9	18.7 ± 2.6
Total Cholesterol, mg/dl	1952 ± 306	1920 ± 374	1997 ± 454

Table 1

There are no significant differences in body weight or total cholesterol between groups. Data are expressed as mean ± S.D. *N* = 10 (vehicle), 10 (varenicline), and 9 (MLA plus varenicline).

4. Discussion

Patients with cardiovascular disease, even though receiving drug therapy, are at high risk for the occurrence of cardiovascular events. To decrease this risk, patients are strongly recommended to stop smoking [1]. Varenicline is one of the most effective drugs for smoking cessation [6,7,8]; however, when compared with a placebo, an increased risk of cardiovascular events has been reported in patients taking varenicline [10,11]. Therefore, in this study, we determined if varenicline causes progression of plaque formation in ApoE KO mice. Long-term varenicline treatment at a dose of 0.5 mg kg^{-1} day^{-1} progressed plaque formation in the aortic arch and whole aorta of ApoE KO mice (Fig. 1), suggesting that varenicline may increase the risk for occurrence of cardiovascular events.

Varenicline is an $\alpha_4\beta_2$ nAChR partial agonist and an α_7 nAChR full agonist [5]. Varenicline efficacy in smoking cessation is due to its partial agonistic activity at neuronal $\alpha_4\beta_2$ nAChR in the brain mesolimbic dopaminergic system. Varenicline prevents nicotine from binding to and moderately stimulates these receptors, leading to inhibition of nicotine reinforcement during smoking and reduced craving during smoking abstinence. Nevertheless, varenicline efficacy in α_7 nAChR activation is 8-fold less potent than at $\alpha_4\beta_2$ nAChR [5]. Considering that in the clinic long-term varenicline treatment is ongoing for several months, it is highly likely that a significant effect of varenicline occurs because of activation of α_7 nAChR. The α_7 nAChR is expressed on cells associated with atherosclerotic plaque formation, including human vascular smooth muscle cells, aortic endothelial cells, platelets, macrophages, and T and B lymphocytes. Among these human cell types, the α_4 subunit is expressed on vascular smooth muscle cells and B lymphocytes,

while the β_2 subunit is expressed on aortic endothelial cells and T lymphocytes [18,19,20,21]. Therefore, varenicline appears to aggravate plaque formation by acting on α_7 rather than $\alpha_4\beta_2$ nAChR. This is likely associated with varenicline-induced cardiovascular events, although further experiments are required to clarify the target cell of varenicline-induced aggravation of plaque formation.

To determine if varenicline aggravates atherosclerosis formation via α_7 nAChR, varenicline was administered to ApoE KO mice in combination with MLA, an α_7 nAChR antagonist. As shown in Figs. 2 and 3, MLA inhibited varenicline-aggravated plaque formation in the aortic arch, whole aorta, and aortic root of ApoE KO mice. These results strongly support the notion that varenicline aggravates plaque formation through α_7 nAChR, and thereby causes adverse cardiovascular reactions. In addition, there are reports suggesting that the α_7 nAChR play a crucial role in the mediation of nicotine-induced dendritic cell maturation, platelet activation, and E-selectin expression in aortic endothelial cells [21,22,23]; all phenomena involved in the progression of atherosclerotic plaque formation. Furthermore, one case report found that varenicline has a prothrombotic effect via brainstem α_7 nAChR [24]. This is similar to the effects of nicotine, because acute nicotine administration is capable of inducing thrombosis. These results support our present findings that demonstrate α_7 nAChR involvement in adverse cardiovascular reactions of varenicline.

In conclusion, varenicline aggravates plaque formation by stimulating α_7 nAChR, and consequently may increase the risk for cardiovascular events. The possibility that varenicline is involved in adverse cardiovascular effects through α_7 nAChR must be considered.

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Chapter 2

Varenicline enhances oxidized LDL uptake by increasing expression of LOX-1 and CD36 scavenger receptors through α_7 nAChR in macrophages

1. Introduction

Smoking is an important risk factor for the onset and development of cardiovascular disease, ischemic heart disease, chronic obstructive pulmonary disease and cancer (Boyle 1997; Benowitz, 2008). One of the major causes of cardiovascular disease, atherosclerosis, is characterized as a chronic inflammatory disease.

Subendothelial retention of oxidized low-density lipoprotein (oxLDL) is known to participate actively in the atherosclerotic process through the following: adhesion and activation of monocytes on endothelial cells; dysfunction of endothelial cells; formation of foam cells from macrophages; and migration and proliferation of vascular smooth muscle cells (Gleissner et al., 2007). Rupture and destabilization of atherosclerotic plaques occur as a result of incorporation of oxLDL in macrophages in atherosclerotic plaques. Macrophages located in the arterial intima layer express several scavenger receptors (lectin-like oxidized LDL receptor-1 (LOX-1), cluster of differentiation (CD) 36 and scavenger receptor class A (SR-A)) for uptake of oxLDL (Endemann et al., 1993; Fuhrman et al., 2002; Lu et al., 2011) and adenosine triphosphate-binding cassette transporters (ABC) A1 and G1 for efflux of excess cholesterol to extracellular acceptors such as high-density lipoprotein (HDL). Cigarette smoking-induced decreased levels of HDL and increased levels of LDL in blood are risk factors for atherosclerosis. In particular, atherosclerosis is the leading cause of smoking-induced cardiovascular diseases (Ambrose and Barua, 2004; Faxon et al., 2004); these adverse effects could be ameliorated by smoking cessation (Johnson et al., 2010). The latter is known to produce long-term cardiovascular benefits, and this is strongly recommended for patients with various diseases (Fiore et al., 2008).

Varenicline is a selective partial agonist of the $\alpha_4\beta_2$ nicotinic acetylcholine receptor (nAChR) and full agonist of the α_7 nAChR. Varenicline is the first non-nicotine drug therapy developed specifically for smoking cessation, and is used widely and frequently for this purpose. The efficacy and safety of varenicline used for smoking cessation is remarkably higher than those for nicotine replacement therapy (Nakamura et al., 2007; Gonzales et al., 2006; Jorenby et al., 2006; Sood et al., 2009). However, varenicline shows adverse effects such as nausea, headache, abnormal dreams and insomnia (Oncken et al., 2006; Hays et al., 2008). There are the contradictory results regarding the risk of adverse cardiovascular events associated with varenicline (Singh et al., 2011; Prochaska et al., 2012; Ware et al., 2013; Mills et al., 2014).

Recently, we reported that varenicline aggravates formation of atherosclerotic plaques through α_7 nAChR in apolipoprotein E knockout mice (Koga et al., 2014), but its mechanism of action was not determined. Our findings suggest that varenicline increases the risk of cardiovascular events as a result of aggravated formation of atherosclerotic plaques. In the present study, we determined whether varenicline promotes oxLDL uptake in macrophages, and clarified its mechanism of action *in vitro*.

2. Method and materials

2.1. Drugs

Varenicline tartrate was purchased from Toronto Research Chemicals (Toronto, ON, Canada) and dissolved in dimethyl sulfoxide (Wako Pure Chemicals, Osaka, Japan). Methyllycaconitine citrate salt (MLA) and dihydro-β-erythroidine hydrobromide (DHβE) were obtained from Sigma–Aldrich (Saint Louis, MO, USA) and Tocris Bioscience (Bristol, UK), respectively.

2.2. Cell culture

The murine macrophage line RAW 264.7 was purchased from Riken (Saitama, Japan). These cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and 100 U/mL penicillin/streptomycin (Nacalai Tesque, Kyoto, Japan). Cells were seeded in 60-mm dishes at 8×10^5 and cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were serum-starved for 3 h and then treated with varenicline.

2.3. Western blotting

Proteins from RAW 264.7 cells were isolated using lysis buffer (50 mM HEPES, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM 50 mM NaF, 10 mM sodium pyrophosphate decahydrate, and 1 mM phenylmethylsulfonyl fluoride) containing 1% phosphatase inhibitor cocktail 2 (Sigma–Aldrich), 1% phosphatase inhibitor cocktail 3 (Sigma–Aldrich), and 2% protease inhibitor cocktail (Sigma–Aldrich). Protein concentrations were measured using a protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of total protein (30 μg/sample) were subjected to sodium dodecyl sulfate–

polyacrylamide gel electrophoresis (10%) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) by electrophoretic means. Non-specific antibody binding was blocked by incubation of polyvinylidene difluoride membranes with Blocking One (Nacalai Tesque) for 60 min at room temperature. Primary antibodies used were goat polyclonal anti-LOX-1 (1:100 dilution; Santa Cruz Biotechnology. Santa Cruz, CA, USA), anti-CD36 (1:200; R&D Systems, Minneapolis, MN, USA), rabbit polyclonal anti-SR-A (1:500; Gene Tex, Los Angeles, CA, USA), anti-phospho-extracellular signal-regulated kinase 1/2 (ERK1/2) (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-phospho-nuclear factor-kappa B p65 (NF-κB p65) (1:1000; Cell Signaling Technology), anti- ERK1/2 (1:1000; Cell Signaling Technology), anti-NF-κB p65(1:1000; Cell Signaling Technology), and mouse polyclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:2000 dilution; Millipore, Billerica, MA, USA). Blots were incubated overnight at 4°C with primary antibodies, and then incubated with horseradish peroxidase-conjugated secondary antibodies (1:10000 dilution) for 60 min at room temperature. Blots were developed using an ImmunoStar® LD (Wako Pure Chemicals). Signal intensities were normalized using GAPDH. Band images were captured digitally with a FluorChem® Q imaging system (Alpha Innotech, San Leandro, CA, USA) and band intensities quantified using ImageJ software.

2.4. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA from RAW264.7 cells was extracted using an RNeasy Mini RNA Extraction kit (Qiagen, Tokyo, Japan) according to manufacturer instructions. RNA was reverse-transcribed with a Superscript® VILO™ Synthesis kit (Invitrogen, Carlsbad, CA, USA)

using random primers. cDNA extraction was determined by real-time qPCR using a MX3000P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA) and normalized using the housekeeping gene GAPDH. PCR products were quantified using a Thunderbird™ SYBR® qPCR Mix (Toyobo, Osaka, Japan).

Primers used for PCR reactions (forward and reverse, respectively) were: LOX-1 (5′-TGA AGC CTG CGA ATG ACG AG-3′ and 5′-GTC ACT GAC AAC ACC CAG GCA GAG-3′); CD36 (5′-GAA CCT ATT GAA GGC TTA CAT CC-3′ and 5′-CCC AGT CAC TTG TGT TTT GAA C-3′); SR-A (5′-TGA ACG AGA GGA TGC TGA CTG-3′ and 5′-GGA GGG GCC ATT TTT AGT GC-3′); GAPDH (5′-AAA GAC CCC TTC ATT GAC-3′ and 5′-TCC ACG ACA TAC TCA GCA C-3′). cDNAs were amplified through 50 cycles (denaturation at 95 °C for 60 s; annealing at 53 °C for 60 s; extension at 72°C for 90 s).

2.5. oxLDL uptake into peritoneal macrophages

To isolate mouse peritoneal macrophages, C57BL/6 mice were injected (i.p.) with 2 mL of 4% thioglycollate (Sigma–Aldrich). Cells were collected from the peritoneal cavity 3 days after injection. Then, cells were incubated in 24-well plates in complete medium (RPMI 1640 media containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin). After 3 h, cells were washed three times with phosphate-buffered saline and cultured in media. Adherent cells, which were considered to be peritoneal macrophages, were used in experiments (Koga et al., 2013). For a single experiment, peritoneal macrophages were collected from one mouse. OxLDL was purchased from Alfa Aesar (Haverhill, MA, USA). Peritoneal macrophages were serum-starved for 3 h and then were treated with oxLDL (80 μg/mL) and 10 μM varenicline for 24 h. After washing thrice with phosphate-buffered saline, cells were fixed in 4%

paraformaldehyde and stained with oil red O and hematoxylin to evaluate oxLDL uptake into peritoneal macrophages. The number of oil red O-labeled macrophages was counted. Then, the mean number of cells in 15 random microscopic fields from three independent wells in each experiment was determined. The experiment was repeated four times and the mean percentage from four separate experiments is shown in our figures.

2.6. Statistical analyses

All quantitative analyses were undertaken by a single observer blinded to the experimental protocol. Data are the mean ± S.D. Differences among two groups were compared using unpaired Student's *t*-test. Comparison among four groups was done using ANOVA followed by Tukey's multiple comparison tests. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of varenicline on expression of scavenger receptors (LOX-1, CD36 and SR-A) in RAW264.7 cells

Treatment with varenicline (1, 5, and 10 μM) for 24 h increased the protein levels of LOX-1 by 10%, 35% and 45% of vehicle, respectively, in RAW264.7 cells in a dose-dependent manner (Fig. 1A). Protein levels of CD36 were also increased upon varenicline (1, 5, and 10 μM) treatment by 36%, 93% and 110% of vehicle, respectively, in a dose-dependent manner (Fig. 1B). However, varenicline failed to alter SR-A expression at protein or mRNA levels (data not shown). Subsequently, 10 μM varenicline was used for all experiments. The mRNA levels of LOX-1 and CD36 were increased significantly with varenicline (10 μM) by 40% and 60% of vehicle, respectively, in RAW264.7 cells ($P<0.01$ and $P<0.01$, respectively) (Fig. 1C, D). This is the first evidence that varenicline increases expression of LOX-1 and CD36 in macrophages.

Figure.1

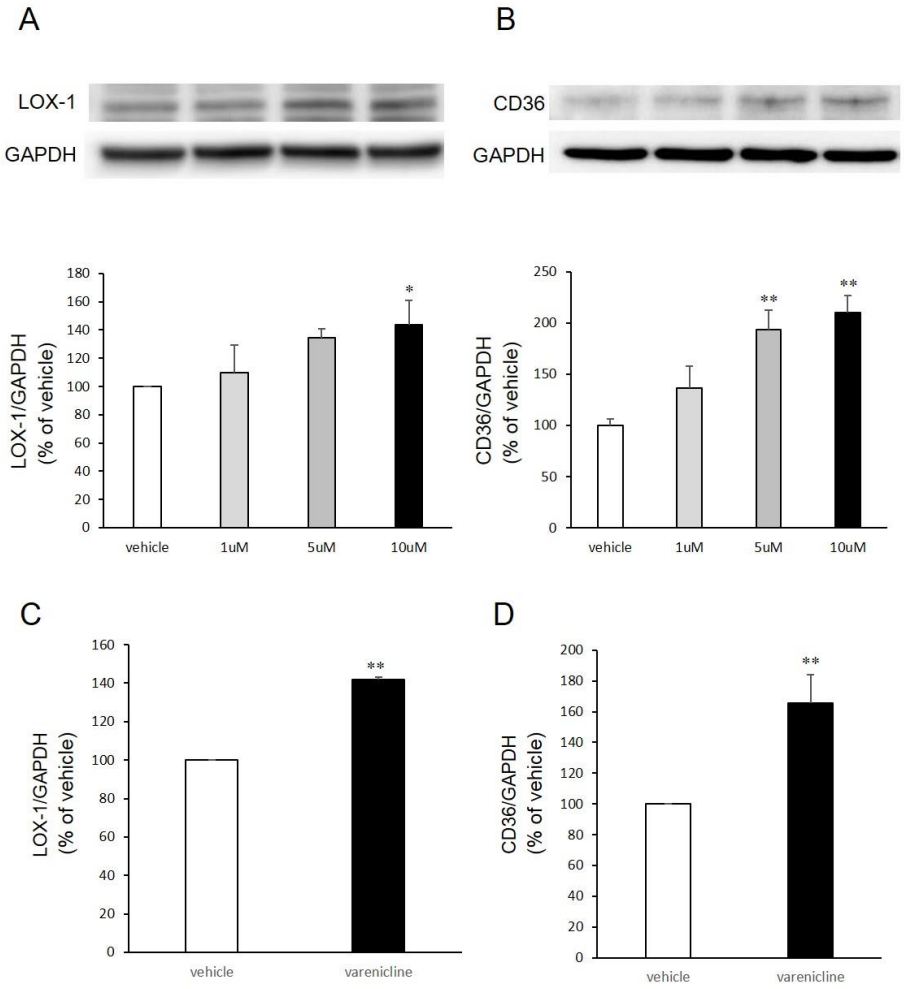


Fig. 1. Effect of varenicline on expression of LOX-1 and CD36 in RAW264.7 cells. (A and B) Representative immunoblots (upper panel) and quantitative analyses of band intensities (bottom panel) showing protein expression of LOX-1 and CD36, respectively. Cells were treated with vehicle or varenicline (1–10 μM) for 24 h. (C and D) Bar graphs showing quantitative real-time RT-PCR analyses of the mRNA expression of LOX-1 and CD36, respectively. Cells were treated with vehicle or varenicline (10 μM) for 24 h. Results are expressed as percentage of vehicle-treated cells. GAPDH was used as a loading control. Each bar indicates mean ± S.D (n=3). *P<0.05, **P<0.01: significant difference from vehicle.

3.2. Varenicline increased oxLDL uptake in the peritoneal macrophages

Few oil red O-positive macrophages were observed in the absence of oxLDL (80 μg/mL) for 24 h in vehicle- and varenicline-treated cells (Fig. 2A). Conversely, the number of oil red O-positive cells markedly was increased in the presence of oxLDL compared with that in the absence of oxLDL. Varenicline (10 μM) treatment for 24 h significantly increased oxLDL uptake in macrophages by 56% of vehicle in the presence of oxLDL ($P<0.01$) (Fig. 2B).

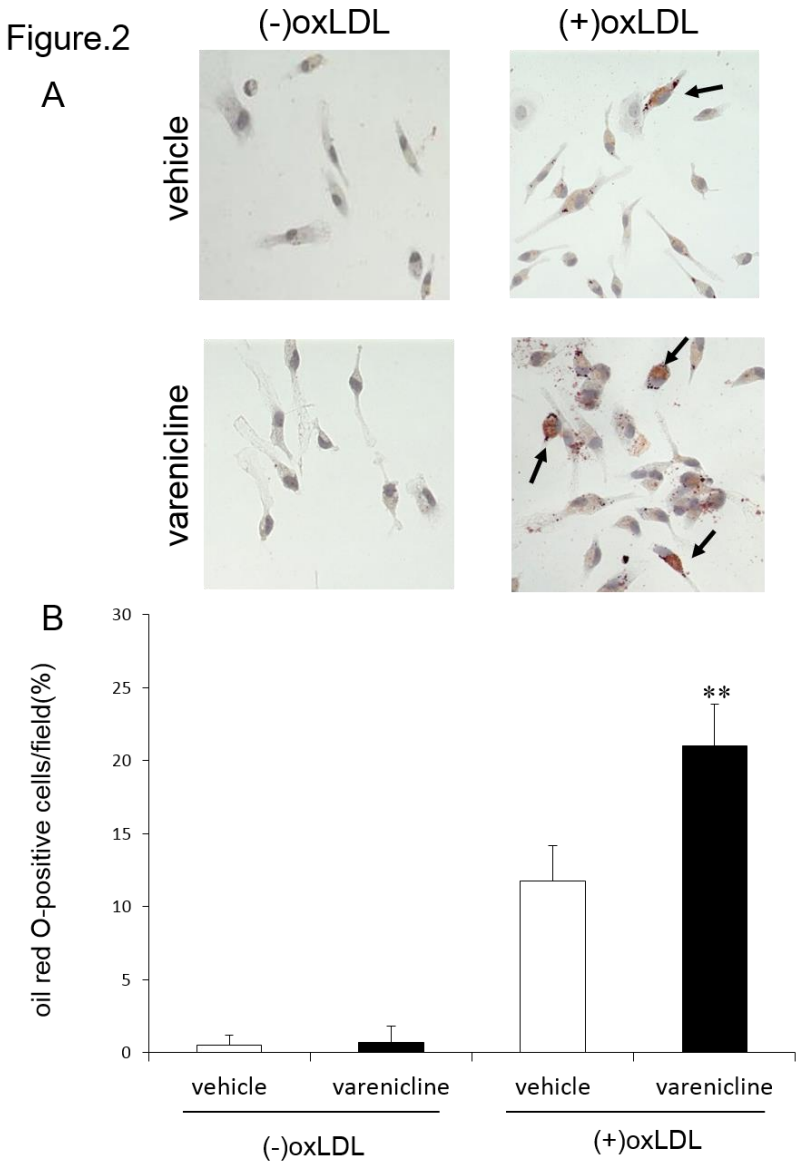


Fig. 2. (A) Representative photographs showing macrophages stained with oil red O and hematoxylin in the absence (−) and presence (+) of oxLDL (80 μg/mL) for 24 h (left and right panel, respectively). Peritoneal macrophages were treated with vehicle or varenicline (10 μM) for 24 h (upper and bottom panel, respectively). Arrowheads show oil red O-positive cells in representative images. (B) Quantitative analyses of oxLDL uptake in macrophages. Results are expressed as a percentage of oil red O-labeled cells in total cells. Each bar indicates mean ± S.D (n=4). **P<0.01: significant difference from (+) oxLDL-vehicle.

3.3. Involvement of α_7 (but not $\alpha_4\beta_2$) nAChR in varenicline actions

An antagonist of α_7 nAChR, MLA, (50 nM), significantly suppressed varenicline-induced expression of LOX-1 and CD36 by 65% and 36%, respectively, in RAW264.7 cells ($P<0.01$ and $P<0.05$, respectively) (Fig. 3A, B). However, an antagonist of $\alpha_4\beta_2$ nAChR, DHβE, (1 μM), did not change varenicline-induced expression of LOX-1 and CD36 (Fig. 3C, D). Treatment with MLA (50 nM), but not DHβE (1 μM), suppressed varenicline-increased oxLDL uptake by 45% in macrophages (Fig. 4).

Figure.3

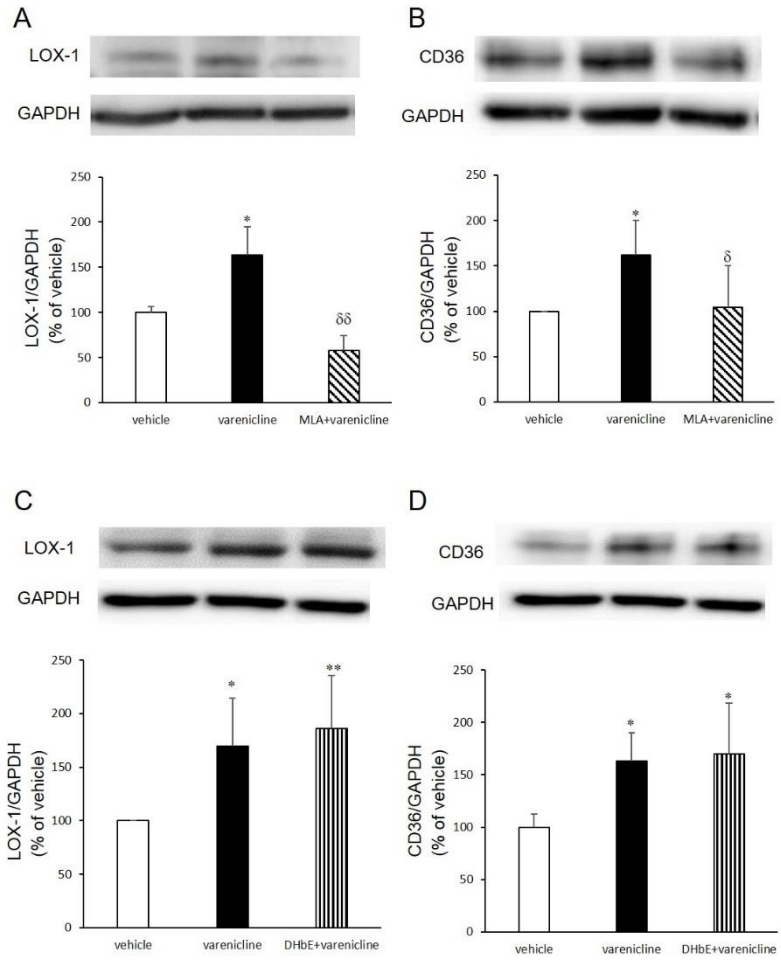
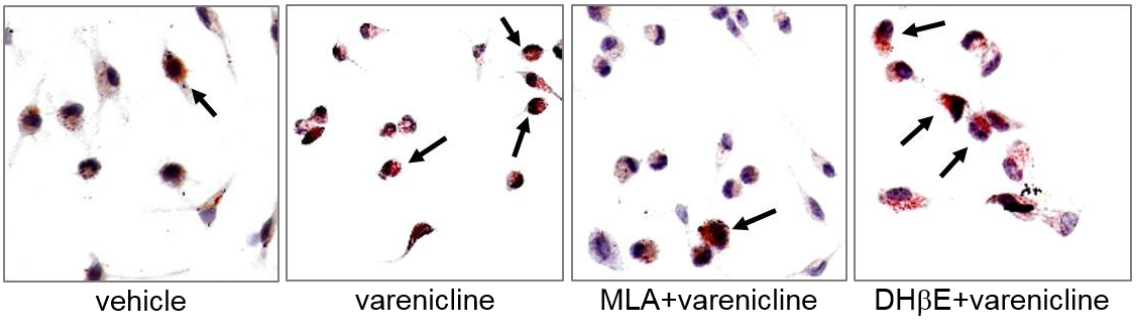


Fig. 3. Effect of α_7 (A and B) and $\alpha_4\beta_2$ nAChR (C and D) antagonists on varenicline-increased expression of LOX-1 and CD36 protein (left and right panel, respectively) in RAW 264.7 cells. Cells were treated with vehicle or varenicline (10 μM) for 24 h in the absence or presence of the α_7 nAChR antagonist MLA (50 nM) or the $\alpha_4\beta_2$ nAChR antagonist DHβE (1 μM). MLA and DHβE were added to cells 30 min before treatment with vehicle or varenicline. Representative immunoblots (upper) and quantitative analyses of band intensities (lower) showing protein expression of LOX-1 and CD36 in RAW264.7 cells. Each bar indicates mean ± S.D (n = 3–6). *P<0.05, **P<0.01: significant difference from vehicle. $^{\delta}$P<0.05, $^{\delta\delta}$P<0.01: significant difference from varenicline.

Figure.4

A



B

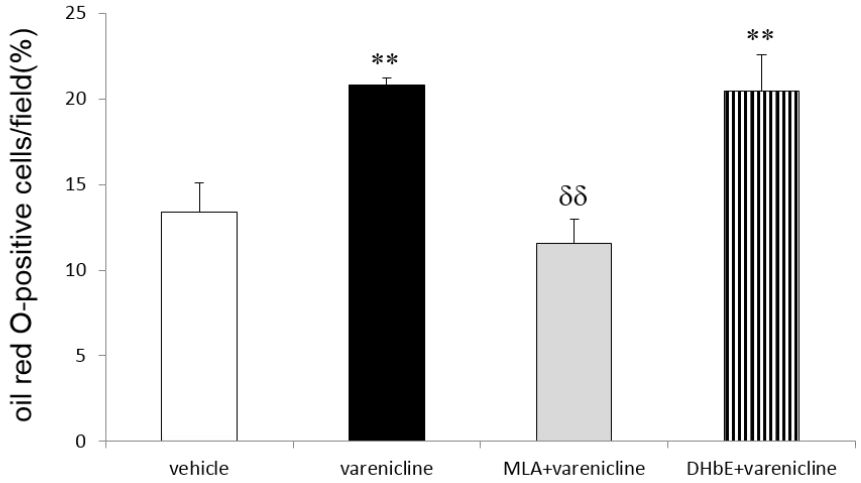


Fig. 4. Effect of α_7 and $\alpha_4\beta_2$ nAChR antagonists on varenicline-increased oxLDL uptake in macrophages. (A) Representative photographs showing macrophages stained with oil red O and hematoxylin. Arrowheads show oil red O-positive cells in representative images. (B) Quantitative analyses of oxLDL uptake in macrophages incubated with oxLDL (80 μg/mL) for 24 h. Cells were treated with vehicle or varenicline (10 μM) for 24 h in the absence or presence of the α_7 nAChR antagonist MLA (50 nM) or the $\alpha_4\beta_2$ nAChR antagonist DHβE (1 μM). MLA and DHβE were added to cells 30 min before treatment with vehicle or varenicline. Each bar indicates mean ± S.D (n = 4). **P<0.01: significant difference from vehicle. $^{\delta\delta}$P<0.01: significant difference from varenicline.

3.4. Varenicline activated the ERK1/2 and NF-κB signaling pathway through α_7 nAChR in RAW 264.7 cells

To determine the role of intracellular signaling pathway in varenicline-treated macrophages, we examined activation of ERK1/2 and NF-κB signaling. Varenicline induced phosphorylation of ERK1/2 and NF-κB in a time-dependent manner compared with 0 min (ERK1/2 and NF-κB: 2.8-fold increase at 5 min and 4.7-fold increase at 10 min after varenicline treatment, respectively) (Fig. 5). Pretreatment with MLA (50 nM) significantly suppressed varenicline-induced activation of ERK1/2 and NF-κB by 47% ($P<0.01$) and 45% ($P<0.05$), respectively, in RAW264.7 cells (Fig. 6A, B). However, DHβE (1 μM) pretreatment did not block these activations (Fig. 6C, D).

Figure.5

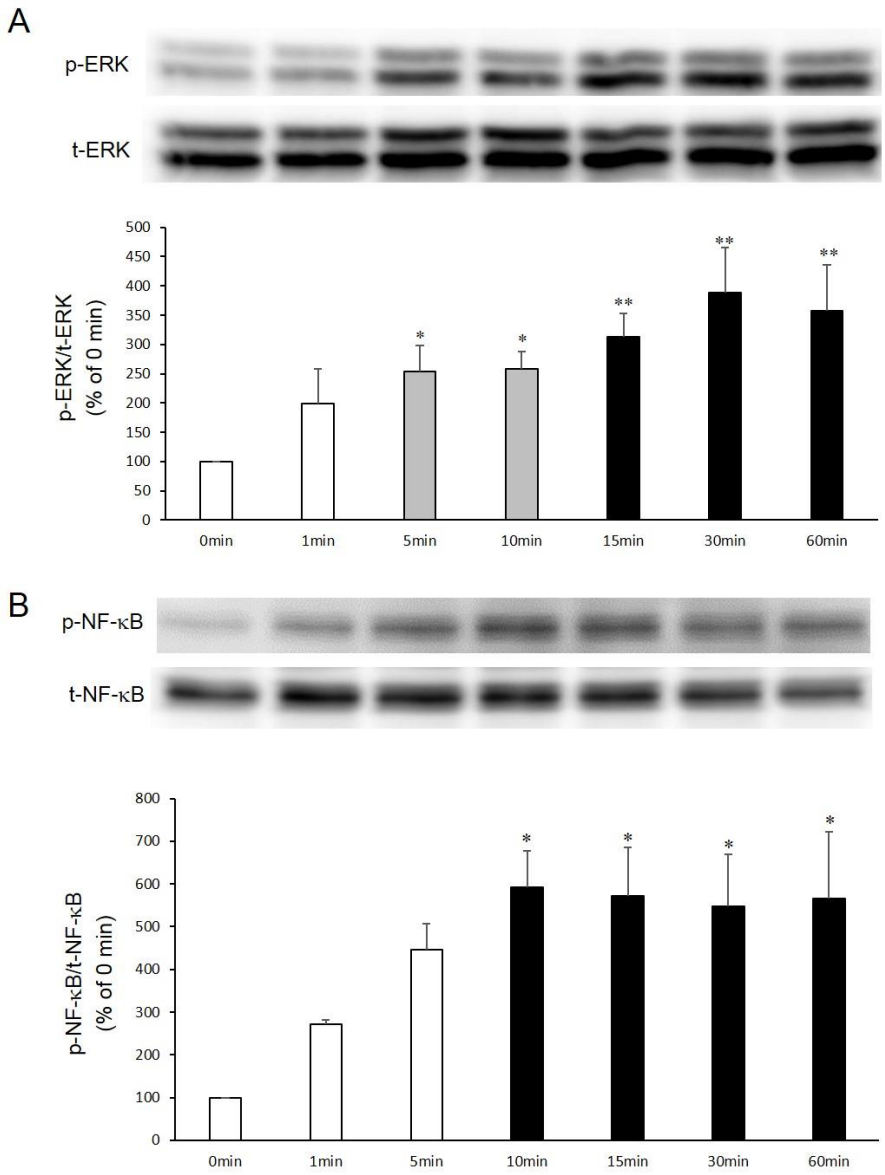


Fig. 5. Varenicline-induced phosphorylation of ERK1/2 (A) and NF-κB (B) in RAW 264.7 cells. Cells were treated with varenicline (10 μM) for the indicated times (1, 5, 10, 15, 30 and 60 min). Representative immunoblots (top in each panel) show the protein expression of total and phosphorylated ERK1/2 and NF-κB in A and B, respectively. At the bottom of each panel, values are the relative ratio of phosphorylated proteins to total proteins in untreated control cells as a percentage (% of 0 min). Each bar indicates mean ± S.D (n=3). *P<0.05, **P<0.01: significant difference from 0 min.

Figure.6

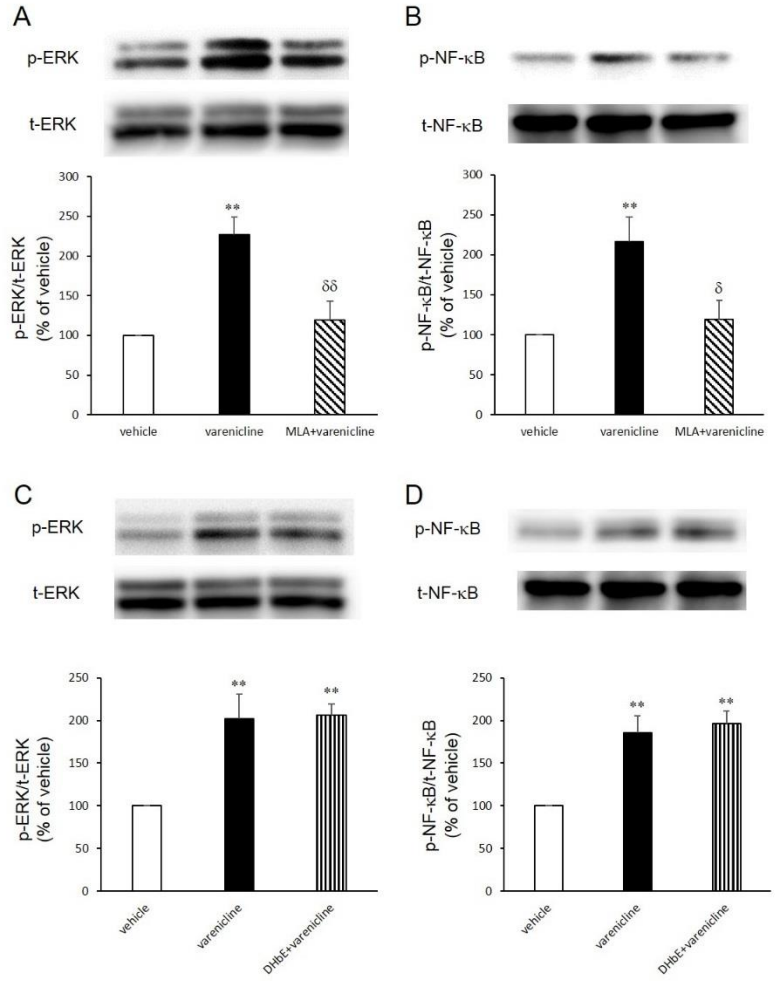


Fig. 6. Effect of MLA (α_7 nAChR antagonist) and DHβE ($\alpha_4\beta_2$ nAChR antagonist) on varenicline-induced activation of ERK1/2 and NF-κB in RAW 264.7 cells. Cells were treated with vehicle or varenicline (10 μM) for 30 min in the absence or presence of MLA (50 nM) (A and B) or DHβE (1 μM) (C and D). MLA and DHβE were added to cells at 30 min before treatment with the vehicle or varenicline. Representative immunoblots (top in each panel) show the protein expression of total and phosphorylated ERK1/2 (left) and NF-κB (right) in RAW264.7 cells. At the bottom of each panel, values are the relative ratio of phosphorylated proteins to total proteins in vehicle-treated cells as a percentage (% of vehicle). Each bar indicates mean ± S.D (n = 3–6). *P<0.05, **P<0.01: significant difference from vehicle; $^{\delta}$P<0.05, $^{\delta\delta}$P<0.01: significant difference from varenicline.

4. Discussion

Previously, we reported that varenicline aggravates formation of atherosclerotic plaques in apolipoprotein E knockout mice, suggesting that varenicline increases the risk of adverse cardiovascular events (Koga et al., 2014). However, the mechanisms underlying varenicline-aggravated plaque formation are not known.

In the present study, we provide *in vitro* evidence that varenicline increases oxLDL uptake in macrophages probably as a result of increased levels of LOX-1 and CD36 through α_7 nAChR. This mechanism could contribute to varenicline-induced aggravation of formation of atherosclerotic plaques.

Zhou *et al.* showed that nicotine stimulates nAChR to increase expression of CD36, but not SR-A, in macrophages (Zhou et al., 2013). That report supports the findings of the present study whereby varenicline increased expression of LOX-1 and CD36, but not SR-A, in RAW264.7 cells (Fig. 1). Considering that varenicline promotes oxLDL uptake in peritoneal macrophages markedly (Fig. 2), the increased expression of LOX-1 and CD36 in the protein and mRNA levels is highly likely to be closely associated with increased oxLDL uptake in macrophages. Formation of foam cells from macrophages is known to play a key part in early atherosclerosis (Stary et al., 1994; Bobryshev 2006).

The α_7, rather than $\alpha_4\beta_2$, nAChR has been shown to contribute preferentially to varenicline-aggravated formation of atherosclerotic plaques in apolipoprotein E knockout mice (Koga et al., 2014). An α_7 nAChR antagonist (MLA), but not an $\alpha_4\beta_2$ nAChR antagonist (DHβE), reversed varenicline-increased protein expression of LOX-1 and CD36 in RAW264.7 cells and oxLDL uptake in peritoneal macrophages (Fig. 3, 4). These results demonstrate that the adverse cardiovascular effects of varenicline are mediated preferentially by α_7 nAChR.

It has been reported that activation of α_7 nAChR upregulates expression of NF-κB genes by stimulation of the ERK1/2 signaling pathway in Het-1A cells (Chernyavsky et al., 2010). The ERK1/2 signaling pathway mediates increases in CD36 expression through increased NF-κB activity in RAW264.7 cells (Liang et al., 2016). Also, lipopolysaccharide has been shown to upregulate LOX-1 expression through activation of an ERK signaling pathway in RAW264.7 cells (Zhao et al., 2014; Hossain et al. 2015). We found that varenicline significantly activated ERK1/2 and NF-κB signaling in RAW264.7 cells at 5 and 10 min after varenicline treatment, respectively (Fig. 5), and that varenicline-induced these activations were significantly blocked by MLA but not DHβE (Fig. 6). These data demonstrated that ERK1/2 and NF-κB signaling are activated by varenicline through α_7 nAChR but not $\alpha_4\beta_2$ nAChR. Therefore, varenicline may upregulate expression of LOX-1 and CD36 by activating of an ERK1/2–NF-κB signaling pathway through α_7 nAChR.

LOX-1 is a member of the C-type lectin superfamily. It promotes attachment of monocytes to endothelial cells as a result of increased production of monocyte chemoattractant protein-1 (Li and Mehra, 2000). LOX-1 leads to apoptosis of endothelial cells by decreasing expression of anti-apoptotic proteins such as B-cell lymphoma 2 and baculoviral IAP repeat-containing protein 2, and also by activating an apoptotic caspase 9–caspase 3 pathway (Li and Mehra, 2009). LOX-1 is known to promote proliferation of vascular smooth muscle cells (Eto et al. 2006) and to produce inflammatory cytokines in macrophages (Moriwaki et al., 1998; Sun et al. 2016). In addition, the class-B scavenger receptor CD36 stimulates release of inflammatory cytokines such as interleukin-1β and tumor necrosis factor-α in macrophages (Yamashita et al., 2007). Therefore, the release of these inflammatory cytokines from macrophages may occur because of the

upregulation of LOX-1 and CD36. Consequently, this process may be involved in varenicline-aggravated atherosclerotic plaque formation. Further study is needed to clarify these points.

In the present study, we examined the effect of varenicline on oxLDL uptake in macrophages. Formation of foam cells is associated with oxLDL uptake and cholesterol efflux; the latter is mediated by the ABC transporters ABCA1 and ABCG1. However, the effect of varenicline on cholesterol efflux from macrophages is not known. α_7 nAChR is present in the membranes of some aortic walls, including endothelial cells, vascular smooth muscle cells and macrophages (Wada et al., 2007; Gotti et al., 2004). It has been reported that nicotine upregulates expression of vascular cell adhesion molecule-1, matrix metalloproteinase-2 and matrix metalloproteinase-9 through α_7 nAChR in RAW264.7 cells (Li et al., 2015). Therefore, varenicline may upregulate expression of these proteins through α_7 nAChR and, consequently, aggravate formation of atherosclerotic plaques. Further studies are needed to clarify these hypotheses.

5. Conclusions

We demonstrated that varenicline upregulates expression of LOX-1 and CD36 significantly through α_7 nAChR, thereby promoting oxLDL uptake in macrophages. Varenicline-aggravated formation of atherosclerotic plaques may be attributed to these sequential events. An increased risk of cardiovascular events upon varenicline treatment must be considered in patients, especially those suffering from cardiovascular diseases.

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General discussion

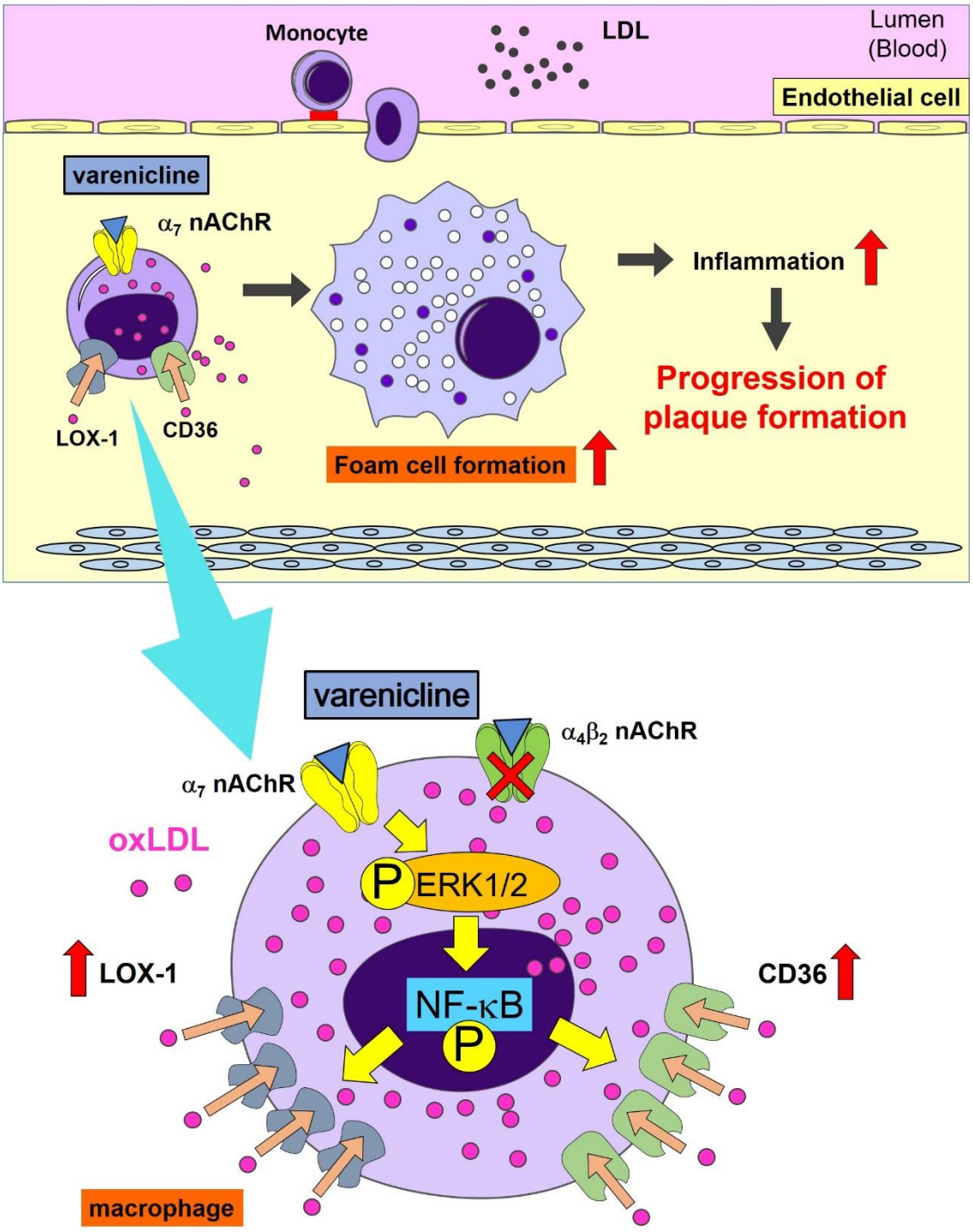
In the first chapter, we determined if varenicline increases the risk of cardiovascular events using ApoE KO mice. ApoE KO mice (8 weeks old) were injected with varenicline 0.5 mg kg^{-1} day^{-1} for 3 weeks. Varenicline aggravated atherosclerotic plaque formation in whole aorta from ApoE KO mice compared with vehicle. MLA, α_7 nicotinic nAChR antagonist, inhibited varenicline-induced aggravated plaque formation. Our findings show that varenicline progresses atherosclerotic plaque formation through α_7 nAChR.

In the second chapter, we have reported that varenicline aggravates formation of atherosclerotic plaques through α_7 nAChR in ApoE KO mice. However, little is known about its effects on macrophages in atherosclerotic plaques. Here, we ascertained whether varenicline promotes oxLDL uptake in mouse peritoneal macrophages *in vitro* and clarified its mechanism. We investigated the effects of varenicline (1–10μM) on expression of scavenger receptors (LOX-1, CD36 and SR-A) in RAW264.7 cells. Expression of protein and mRNA was determined by western blotting and qRT-PCR, respectively. Effects of varenicline (10μM) on oxLDL uptake were examined by counting the number of macrophages stained with oil red O and hematoxylin. Varenicline significantly increased expression of the protein and mRNA of LOX-1 and CD36, but not SR-A, in RAW264.7 cells, and increased oxLDL uptake in macrophages. These effects of varenicline were blocked significantly by an α_7 nAChR antagonist, MLA (50nM), but not by an $\alpha_4\beta_2$ nAChR antagonist, DHβE (1 μM). These data suggest that varenicline promotes oxLDL uptake by upregulating of expression of LOX-1 and CD36 through α_7 nAChR in macrophages. We found that varenicline significantly activated ERK1/2 and NF-κB signaling pathways in RAW264.7 cells. This activation was blocked by MLA but

not DHβE. Therefore, ERK1/2-NF-κB signaling pathway is highly likely to be responsible for varenicline-induced upregulation of LOX-1 and CD36 expression through α_7 nAChR in macrophages. These processes probably contribute to varenicline-aggravated atherosclerotic plaque formation.

To summarize the present findings, we present the hypothesis for varenicline-aggravated atherosclerotic plaque formation shown as the following diagram. Varenicline stimulates α_7 nAChR to upregulate LOX-1 and CD36 expression by activating ERK1/2-NF-κB signaling pathway in macrophages. This event induces an acceleration of oxLDL uptake in macrophages and consequently aggravates plaque formation. Varenicline is conceivable to increase the risk for cardiovascular events due to this aggravated plaque formation.

In light of the present findings, the possibility that increased risk of the cardiovascular events for varenicline treatment could occur has to be considered in patients especially suffering from cardiovascular diseases.


Monocyte
LDL
Lumen
(Blood)
Endothelial cell
varenicline
α7 nAChR
LOX-1
CD36
Foam cell formation
Inflammation
Progression of
plaque formation
varenicline
α4β2 nAChR
α7 nAChR
oxLDL
P ERK1/2
LOX-1
NF-κB
P
CD36
macrophage

Published and submitted papers

1. **Kanaoka Y**, Koga M, Sugiyama K, Ohishi K, Kataoka Y, Yamauchi A., Varenicline enhances oxidized LDL uptake by increasing expression of LOX-1 and CD36 scavenger receptors through α_7 nAChR in macrophages. Toxicology 2017;380(1), 62-71

2. Koga M, **Kanaoka Y**, Sugiyama K, Ohishi K, Ejima Y, Hisanaga M, Kataoka Y, Yamauchi A. Varenicline promotes endothelial cell migration by lowering VE-cadherin levels via activated α7 nAChR-ERK1/2 axis. (submitted to Microvascular Research)

3. Koga M, **Kanaoka Y**, Ohkido Y, Kubo N, Ohshi K, Sugiyama K, Yamauchi A, Kataoka Y. Varenicline aggravates plaque formation through α7 nicotinic acetylcholine receptors in ApoE KO mice. Biochem Biophys Res Commun. 2014;455(3-4), 194-197

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