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Acetylcholinesterase Inhibitors Attenuate Atherogenesis in Apolipoprotein E-Knockout Mice

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Abstract

Objective Donepezil, a reversible acetylcholinesterase inhibitor, improves cognitive function of Alzheimer's disease. Stimulation of cholinergic system was reported to improve long-term survival of rats with chronic heart failure and to attenuate inflammatory response in mice with lipopolysaccharide-induced sepsis. We sought to determine whether the pharmacological stimulation of cholinergic system by donepezil reduces atherogenesis in apolipoprotein (Apo) E-knockout (KO) mice.

Methods and Results Male ApoE-KO mice (10-week-old) were fed a high fat diet and received infusion of angiotensin (Ang) II (490 ng/kg/day). Donepezil or physostigmine was administered for 4 weeks. Oral administration of donepezil (5 mg/kg/day) or infusion of physostigmine (2 mg/kg/day) significantly attenuated atherogenesis (Oil Red O positive area) without significant changes in heart rate, blood pressure and total cholesterol levels. Administration of donepezil suppressed expression of monocyte chemoattractant protein-1 and tumor necrosis factor-a, NADPH oxidase activity and production of reactive oxygen species in the aorta.

Conclusion The present study revealed novel antioxidative and anti-atherosclerotic effects of pharmacological stimulation of cholinergic system by donepezil. Donepezil may be used as a novel therapeutics for the atherosclerotic cardiovascular diseases.

Key Words: cholinesterase inhibitor, donepezil, apolipoprotein E knockout mice, oxidative stress, cytokine

1. Introduction

Activation of vagus nerve shows various effects on hemodynamics. It slows heart rate, dilates blood vessel and reduces blood pressure. Results of the Autonomic Tone and Reflexes After Myocardial Infarction Study and the Cardiac Insufficiency Bisoprolol Study II indicate that diminished cardiac vagus activity predicts the higher mortality rate in patients with chronic heart failure [1, 2]. In addition, vagus nerve stimulation (VNS) improves long term survival of rats with chronic heart failure after experimental myocardial infarction [3]. VNS modulates the cardiac redox status and adrenergic drive, and thereby suppresses free radical generation in the failing heart [4]. However, the effect of VNS on vascular lesion formation has not been reported.

Stimulation of cholinergic system was reported to attenuate tumor necrosis factor (TNF)-a production from macrophages and hypotensive shock in lipopolysaccharide (LPS)-induced septic model [5, 6]. Stimulation of cholinergic system inhibits activation of nuclear factor-kappa B (NF-kB) [7] and induces suppressor of cytokine signal 3 expression [8], resulting in the attenuation of inflammatory responses. However, nicotine, a nicotinic acetylcholine receptor (AchR) agonist, was reported to induce endothelial dysfunction that is an initial step of atherosclerosis and to accelerate atherosclerosis in Apolipoprotein E-knockout (ApoE-KO) mice [9]. Therefore, it is not clear whether the activation of cholinergic system is atherogenic or atheroprotective.

Donepezil [diethyl(3,5-di-ter-butyl-4-hydroxybenzyl)phosphonate] is a long acting, reversible cholinesterase inhibitor and is known to improve memory and cognitive function in patients with Alzheimer's disease [10]. A recent study showed that treatment of patients with Alzheimer's disease with donepezil for 1 month reduces production of oncostatin-M, interleukin (IL)-6 and IL-1 in the peripheral blood

mononuclear cells [11], suggesting a possible anti-inflammatory effect of donepezil. However, the mechanism remains to be determined.

Angiotensin (Ang) II plays critical roles in the progression of atherosclerosis, ventricular remodeling after myocardial infarction and heart failure [12]. One of the mechanisms by which Ang II accelerates atherogenesis is the induction of oxidative stress and inflammation [13]. Ang II activates NADPH oxidase in the blood vessel resulting in the activation of redox-sensitive transcription factors such as nuclear factor (NF)-kB and activating protein (AP)-1 [14], resulting in the production of inflammatory cytokines or chemokines such as TNF-α, IL-6, and monocyte chemoattractant protein (MCP)-1.

These previous studies prompted us to examine the effect of pharmacological stimulation of cholinergic system by donepezil on the progression of atherosclerosis in ApoE-KO mice. In the present study we showed that donepezil attenuated atherogenesis in ApoE-KO mice fed a high fat diet (HFD) with or without Ang II stimulation, possibly through antioxidative and anti-inflammatory effects.

2. Materials and methods

2.1. Materials

Ang II was purchased from PEPTIDE Institute Inc. Physostigmine, Ach, lucigenin, b-Nicotinamide adenine dinucleotide 2'-phosphate reduced hydrate (NADPH) were purchased from Sigma Chemical Co. Donepezil was purchased from Toronto Research Chemicals Inc. Antibodies against p47phox and NOX1 were purchased from Santa Cruz Biotechnology, Inc. Other chemical reagents were purchased from Wako Pure Chemicals, unless mentioned specifically.

2.2. Animal model of atherosclerosis

All procedures were approved by the committee on Ethics of Animal Experiment, Kyushu University Graduate School of Medical Sciences and conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

C57BL/6J ApoE-KO mice were purchased from the Jackson Laboratory. Male ApoE-KO (10-week-old) mice were fed a HFD (35% calorie from fat, 1% cholesterol) and received infusion of Ang II (490 ng/kg/day) through an osmotic minipump (Alzet) implanted in the peritoneal cavity for 4 weeks. Mice had an ad libitum access to both food and water. Four groups were compared: control, AngII+HFD, AngII+HFD and donepezil (estimated dose of ingestion: 5 mg/kg/day via drinking water), and AngII+HFD and physostigmine (2mg/kg/day via second osmotic minipump). Blood pressure and heart rate were monitored using a computed tail-cuff system (UR-5000, UEDA, Ueda Co. Ltd.). The doses of cholinesterase inhibitors were chosen on the basis of previous studies that showed that donepezil [15] or physostigmine [16] at the doses

mentioned above did not affect heart rate or blood pressure level in mice.
In another experiment, ApoE-KO mice (12-week-old) were fed a HFD only for 8 weeks without Ang II. And the effect of donepezil was examined.

2.3. Histological and Immunohistochemical analyses

At the end of experiments, mice were anesthetized with an intraperitoneal injection of pentobarbital. The circulatory system was perfused with PBS via the left ventricle. Then, the aortic arch and the thoracic aorta was opened longitudinally, stained with Oil Red O, and pinned out on a black wax surface. The percentage of the plaque area stained by Oil Red O to the total luminal surface area was determined. Serial sections of the aortic root were prepared and were stained with the antibodies against macrophage (F4/80; Serotec Inc.) and MCP-1 (Santa Cruz Biotechnology Inc.). All images were captured with a Nikon microscope equipped with a video camera and analyzed using Adobe Photoshop and Scion Image Software.

2.4. Tissue preparation

The thoracic and abdominal aortae were immediately frozen in liquid nitrogen for RNA isolation, Lucigenin assay, and Western blot analysis. For RNA isolation, thoracic aorta was additionally perfused with RNA Later (Ambion) to prevent RNA degradation. Frozen samples of thoracic aorta were crashed on dry ice and homogenized in ISOGEN (Nippon Gene) and total RNA was prepared in accordance with the manufacturer's instruction.

2.5. Real-Time Reverse Transcription Polymerase Chain Reaction Analysis

Reverse transcription of RNA was performed with ReverTra Ace (TOYOBO). Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed using SYBR Green and the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The sequences of PCR primers used in this study are summarized in the Supplemental Table 1. Primers for GAPDH were purchased from ABI, of which sequences are not disclosed.

2.6.Lucigenin-enhanced chemiluminescence assay

NADPH-dependent superoxide production was measured by lucigenin luminescence [17]. The aorta was perfused with ice cold PBS, immediately frozen in liquid nitrogen and the assay was performed on the same day. The frozen samples of abdominal aorta were crashed on dry ice and homogenized in modified Krebs buffer (99 mmol/L NaCl, 4.7 mmol/L KCL, 1.9 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 1.0 mmol/L K_2HPO_4, 25 mmol/L $NaHCO_3$, 20 mmol/L Na-HEPES, 11 mmol/L D-glucose). A luminescence assay was performed in a balanced salt solution (137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na_2HpO_4, 1.5 mmol/L KH_2PO_4) buffer containing 5 mmol/L of lucigenin using a luminescence reader (Berthold Technology). The reaction was started by adding 100 mmol/L of β-NADPH as a substrate.

2.7. Oxidative fluorescent microphotography

Superoxide was detected in the layers of the vessel wall using fluorescent probe dihydroethidium (DHE; Molecular Probes) as described previously [18]. After perfusion with ice cold PBS, the ascending thoracic aorta were immediately frozen in OCT compound (Sakura Finetek) and stored at -80°C until preparation for the cryosection.

Cryosections (10 mm) were prepared in the next day and incubated for 30 minutes at 37°C with 2 mmol/l DHE. Images were obtained on a confocal microscope (excitation filter at 488 nm; emission filter at 550 nm).

2.8. Western blot analysis

The aorta was homogenized in modified Krebs buffer. Protein concentrations were determined with the bicinchoninic acid protein assay kit (Pierce Chemical Co). The homogenates were heated in a sample buffer at 95°C for 3 minutes, electrophoresed on 12% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore). Western blot analysis of p47phox, NOX1 and α-tubulin were performed by a conventional method and detected by ECL chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Membranes were scanned using LAS-4000mini bioimage analyzer (Fujifilm).

2.9. Statistical analysis

Statistical analysis was performed with 1-way ANOVA and Fisher's test, if appropriate. Data are shown as mean±SEM. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Cholinesterase inhibitors attenuated progression of atherosclerosis

To examine whether donepezil has anti-inflammatory and anti-atherosclerotic effects, we treated HFD-fed ApoE-KO mice with AngII that accelerates vascular inflammation and thereby atherogenesis [13]. A combination treatment with Ang II and HFD significantly increased blood pressure compared with control group. However, no significant differences in the heart rate, blood pressure and serum total cholesterol level were observed among AngII and HFD groups (Table1). Body weight was slightly

decreased in mice that received AngII and HFD compared with the control group. Oil Red O-positive area of en face aorta was reduced in mice treated with donepezil. (Figure 1A and B) Physostigmine, another cholinesterase inhibitor structurally unrelated to donepezil, also reduced Oil Red O-positive area, suggesting that inhibition of cholinesterase and a resultant increase in Ach availability are responsible for the attenuation of atherogenesis. (Figure 1A and B) Infiltration of macrophage (F4/80 antibody-positive cells) into the aortic root was also reduced in mice treated with donepezil or physostigmine. (Figure 1C) These cells expressed MCP-1 (Figure 1D) and MCP-1 positive area was diminished by treatment with either donepezil or physostigmine, suggesting that cholinesterase inhibitors may attenuate atherogenesis through suppression of MCP-1 expression and macrophage recruitment.

We also examined the effect of donepezil on ApoE-KO mice fed a HFD for 8 weeks (from 12-week-old to 20-week-old) without AngII infusion. The Oil Red O-positive area was significantly suppressed by treatment with donepezil (Figure 1E and 1F) without effects on hemodynamics and cholesterol level (Table 2).

3.2. Donepezil attenuated vascular reactive oxygen species (ROS) production and NADPH oxidase activity.

ROS play an important role in atherogenesis. We, therefore, examined the effect of donepezil on ROS production. DHE staining showed that vascular ROS production was increased in in ApoE-KO mice treated with AngII and HFD and that donepezil reduced the ROS production in the media (Figure 2A). Lucigenin assay also showed that donepezil significantly reduced NADPH oxidase activity that is increased in AngII- and HFD-treated ApoE-KO mice. (Figure 2B) However, we did not see any effect of donepzil

on the serum level of thiobarbituric acid reactive substance (data not shown), suggesting that donepezil might locally suppress oxidative stress in the aorta.

3.3. Donepezil attenuated MCP-1 and TNF-a mRNA expression

To gain insights into the molecular mechanism of anti-atherogenic effect of donpezil, mRNA expression of the aorta was examined by RT-PCR (Figure 3). The combination treatment with AngII and HFD significantly increased MCP-1 and TNF-α mRNA expression in the aorta of ApoE-KO mice. Donepezil significantly attenuated both MCP-1 and TNF-α mRNA expression.

NOX 1 is a major NADPH oxidase subunit expressed in VSMC [19]. Expression of NOX1 was slightly increased by AngII and a HFD, which was downregulated by donepezil. However, expression level of NOX1 is very low and the difference was not statistically significant.

Expression of other NADPH oxidase subunits (Nox 2, p22phox, p47phox) was significantly increased in AngII and HFD group and donepezil attenuated the mRNA expression of these molecules. However, the reduction was not statistically significant. mRNA expression of Nox 4, one of the NADPH oxidase subunit, superoxide dismutase, and catalase was not affected by the combination treatment with AngII and a HFD.

3.4. Donepezil inhibited p47phox and NOX1 protein level in the aorta.

We examined protein expression of p47phox and NOX1 in the aorta. Western blot analysis revealed that expression level of p47phox was increased in ApoE-KO mice treated with HFD and Ang II and the induction was significantly suppressed by donepezil. (Figure 4) The protein level of NOX1 was not affected among the three

groups.

4. Discussion

We showed in the present study that donepezil and physostigmine attenuated progression of AngII accelerated atherosclerosis in ApoE-KO mice fed a HFD. The anti-atherogenic effect of donepezil was also observed in ApoEKO mice fed a HFD without AngII infusion. Donepezil attenuated NADPH oxidase activity and ROS production as well as cytokine expression in the aorta. These results suggest that cholinesterase inhibitor may be a novel strategy for the treatment of atherosclerotic cardiovascular diseases.

We chose ApoE-KO mice treated with HFD and Ang II as an atherosclerotic model because AngII is known to induce inflammation and our hypothesis is that donepzil has an anti-inflammatory effect. It is of note that donepezil was more effective in HFD and AngII infusion group than HFD group. Therefore, donepezil may be more effective against AngII-induced atherogenesis.

Custodis et al showed that heart rate reduction by ivabradine, an ihibitor of I_f current in the sinoatrial node, attenuated atherogenesis in ApoE-KO mice [20]. In the present study, neither donepezil nor physostigmine significantly decreased heart rate, excluding the possible suppressive effect of bradycardia on atherogenesis. However, the reason why heart rate was not decreased by these cholinesterase inhibitors in our mice is not clear.

A recent meta-analysis by Singh et al revealed that inhalation of anticholinergics is associated with a significantly increased risk of myocardial infarction and

cardiovascular death but not with a risk of stroke in patients with chronic obstructive pulmonary disease (COPD) [21]. The results of the meta-analysis may support the idea that cholinergic system is atheroprotective. However, a very recent double-blind trial that examined the effect of tiotropium, one of the anticholinergics, in patients with COPD showed opposite results [22]. Treatment with tiotropium showed an insignificant decrease in the number of death in patients with COPD and significantly decreased the incidence of myocardial infarction compared with placebo Therefore, the issue regarding the effect of anticholinergics treatment on cardiovascular events, in particular myocardial infarction, is still controversial.

Vascular oxidative stress accelerates atherosclerosis [23]. AngII induces ROS production via activation of NADPH oxidase. Subunits of NADPH oxidase such as p47phox, p22phox, and NOX play critical role in AngII induced ROS production because knockdown of these subunit inhibited ROS production by AngII and less vascular lesion was induced in mice lacking these subunit [19]. The reduction of mRNA expression of each NADPH oxidase subunits (p47phox, p22phox and NOX1) in donepezil-treated mice was not statistically significant. However, the expression of p47phox at the protein level was significantly reduced by donepezil. Although the mechanism for the difference between mRNA and protein level of p47phox is not clear, suppression of p47phox may explain the anti-oxidative effect of donepezil.

Donepezil suppressed aortic MCP-1 expression in ApoE-KO mice received HFD and AngII-infusion. MCP-1 is well known to enhance atherogenesis. However, MCP-1 deficiency did not affect atheroslcrerotic lesion size in LDL receptor knockout mice fed a normal chow but decreased lesion size those fed a western diet [24]. The lack of the effect of MCP-1 deficiency is explained by upregulation of MCP-5, which is highly

homologous to MCP-1. This study suggests that single inhibition of MCP-1 is not sufficient to suppress atherogenesis due to activation of alternative pathways. In this regard, simultaneous suppression of TNF-α by donepezil might synergistically attenuate atherogenesis in ApoE-KO mice. A recent study showed that expression of hepatic MCP-1 mRNA was correlated with the degree of liver steatosis in LDL receptor knockout mice fed a high-fat diet [25]. Therefore, it is interesting to address in the future whether donepzil ameliorates liver steatosis in ApoE-KO mice treated with a HFD and AngII-infusion.

Acetylcholine, a major neurotransmitter of vagus nerve, is known to activate endothelial nitric oxide (NO) synthase and dilate blood vessel [26]. However, acetylcholine is rapidly degraded by cholinesterase in a few seconds. Therefore, it may be possible that donepezil and physostigmine inhibition of cholinesterase increases the availability of acetylcholine and increases NO production. However, we could not see upregulation or phosphorylation of eNOS in the aorta of ApoE-KO mice treated with donepezil (data not shown). These data suggest that an increase in NO level may not be the major mechanism for the anti-atherosclerotic effect of donepezil.

At this point, the source of acetylchloline is not clear. Amenta et al showed that cholinergic innervation of the aorta [27]. A nerve plexus in the adventitial layer has been identified in the mouse, suggesting that acetylcholine is derived from the nerve ending of the vagus. In contrast, recent studies suggest that macrophages and endothelial cells express choline acetyltransferase that produces acetylcholine from choline and acetyl-CoA [28]. Therefore, further study is needed to determine whether acetylcholine is derived from vagus nerve ending or locally produced from macrophages or endothelial cells.

The limitation of the present study is that we do not have a direct evidence that donepezil inhibited atherogenesis through an inhibition of cholinesterase. Because the dose of donepezil used in this study is very high, we could not exclude the possibility of a direct or non-specific anti-atherogenic effect of donepezil. However, physostigmine, another cholinesterase inhibitor structurally different from donepezil also suppressed atherogenesis in the same model, indicating that the anti-atherogenic effect is mediated by inhibition of cholinesterase but not by a direct or non-specific effect of the drug. Further study is needed to confirm this point.

Another limitation of the current study is that we used very high dose of donepezil compared with the dose clinically used for the treatment of Alzheimer's disease. Therefore, we must be cautious about extrapolating our results to human atherosclerosis. However, 5 mg/kg/day of donepezil is widely used to examine the effect on dementia in a rodent model [29] despite the clinical dose is 5~10 mg/day for Alzheimer's disease. Thus, differential susceptibility to the drug between human and mice, and a very short period for the development of the lesions in animal models may be the reason for the requirement for high doses to be effective.

In summary, we showed in the present study that treatment with donepezil attenuated atherogenesis in ApoE-KO mice possible through anti-oxdative and anti-inflammatory effects. Although we should be cautious in extrapolating current results to other atherosclerotic model or human atherosclerosis [30], cholinesterase inhibitors may be a novel strategy for the treatment of atherosclerotic cardiovascular diseases.

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Figure legend

Figure 1. Cholinesterase inhibitors attenuated atherogenesis in ApoE-KO mice.

The effects of donepezil and physostigmine were examined in ApoE-Ko mice fed a HFD and received AngII infusion for 4 weeks (A~D). n=6~8 (A) Oil red O staining of the en face aorta is shown. (B) Bar graph indicates the percentage of Oil red O positive area in the aorta (C) Immunohistochemical staining for macrophage by F4/80 antibody in the aortic cusp. Bar graph indicates F4/80-positive area (D) Immunohistochemical staining for MCP-1 in the aortic cusp. Bar graph indicates MCP-1-positive area. Data are expressed as mean ± S.E.M. *P<0.05 , **P<0.01 vs control, #P<0.05, ##P<0.05 vs vehicle.

The effect of donepezil was examined in ApoE-Ko mice fed a HFD for 8 weeks (E and F). (E) Oil red O staining of the en face aorta is shown. (F) Bar graph indicates the percentage of Oil red O positive area in the aorta. Data are expressed as mean ± S.E.M. **P<0.01 vs control, #P<0.05 vs vehicle. Control n=5, HFD or HFD+donepezil n=8.

Figure 2. The effect of donepezil on the oxidative stress in the aorta of ApoE-Ko mice.
(A) DHE staining revealed an increase in superoxide production in the aorta of AngII- and HFD-treated ApoE-KO mice. Donepezil reduced the extent of DHE staining. The same results were obtained in other 5 independent experiments.
(B) Lucigenin assay showed that NADPH oxidase activity was increased by treatment with AngII and HFD in the aorta of ApoE-KO mice. Donepezil reduced the NADPH oxidase activity. Data are expressed as mean ± S.E.M. *P<0.05 vs control, #P<0.05 vs vehicle. n=6~8.

Figure 3. Quantitative RT-PCR analyses for the mRNA expression in the aorta.
mRNA expression of the aorta from control, AngII- and HFD- and AngII-, HFD- and donepezil-treated ApoE-KO mice was quantified with real time RT-PCR. The primer sequences used were indicated in the supplemental table 1. Data are expressed as mean ± S.E.M. *P<0.05, **P<0.01 vs control, #P<0.05, ##P<0.01 vs vehicle. n=7~8.

Figure 4. Expression of p47phox and NOX1protein in the aorta.
Expression of p47phox and Nox1 protein was examined by Western blot analysis in the aorta of control, AngII- and HFD-, and AngII-, HFD- and donepezil-treated ApoE-KO mice. The blot was scanned and the band density was quantified. Data are expressed as mean ± S.E.M. *P<0.05 vs control, #P<0.05 vs HFD and Ang II group. n=4.

Table 1. HR, BP BW and total cholesterol levels in AngII+HFD groups

	HR (bpm)	BP (mmHg)	BW (g)	TC (mg/dl)
Control	610±20	104±2	28.8±0.6	407±21
AII+HFD	648±17	124±4**	27.2±0.4	1981±165**
AII+HFD+Donepezil	618±22	115±4*	27.7±1.0	1896±63**
AII+HFD+Physostigmine	657±8	124±3**	27.9±0.7	2250±70**

HR: heart rate, BP: blood pressure, BW: body weight, TC: total cholesterol

*P<0.05, **P<0.01 vs control

Table 2. HR, BP BW and total cholesterol levels in HFD groups

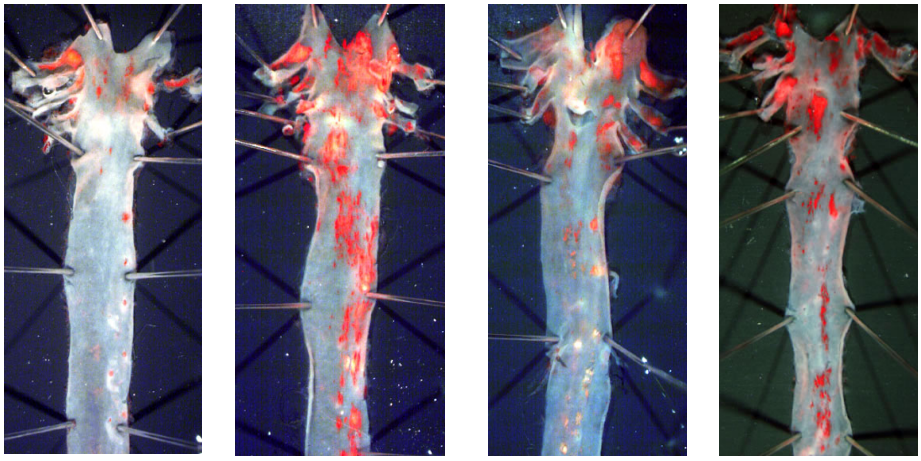
	HR (bpm)	BP (mmHg)	BW (g)	TC (mg/dl)
Control	537±13	99±1	30.8±0.6	555±28
HFD	579±15	97±5	27.5±0.7*	2173±200**
HFD+donepezil	539±19	100±4	28.6±1.2	2001±96**

HR: heart rate, BP: blood pressure, BW: body weight, TC: total cholesterol

*P<0.05, **P<0.01 vs control

Figure1-1

(A)



Control Vehicle Donepezil Physostigmine

AngII+HFD

(B)

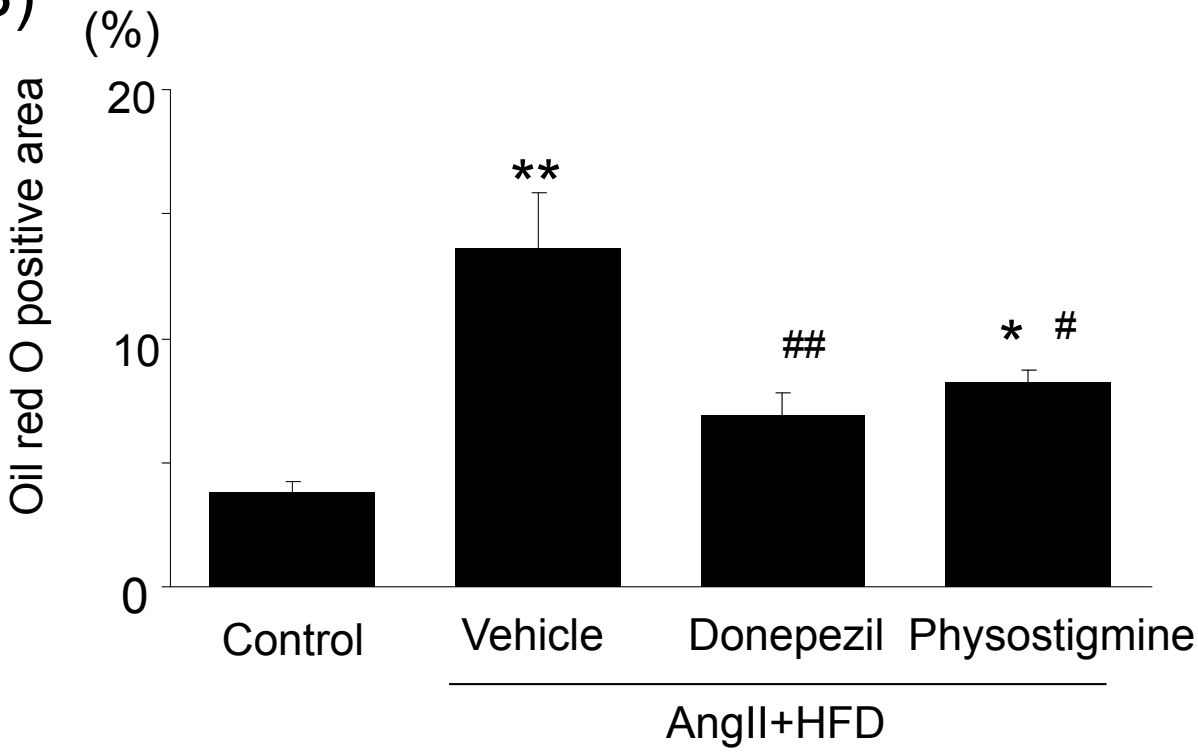
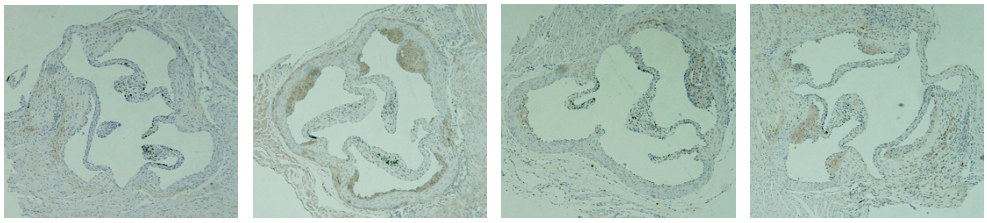
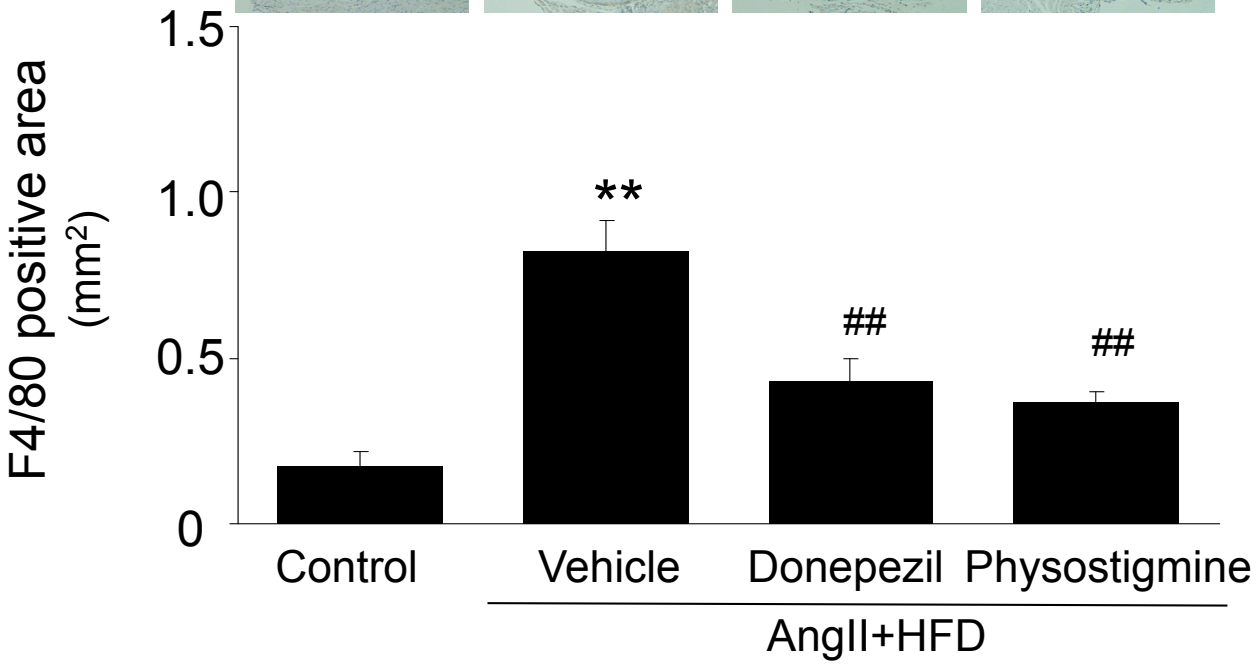


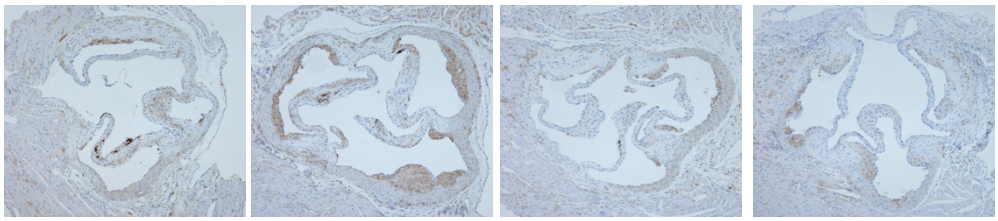
Figure1-2

(C)




F4/80 positive area
(mm²)
1.5
1.0
0.5
0
**
##
##
Control
Vehicle
Donepezil
Physostigmine
AngII+HFD

(D)



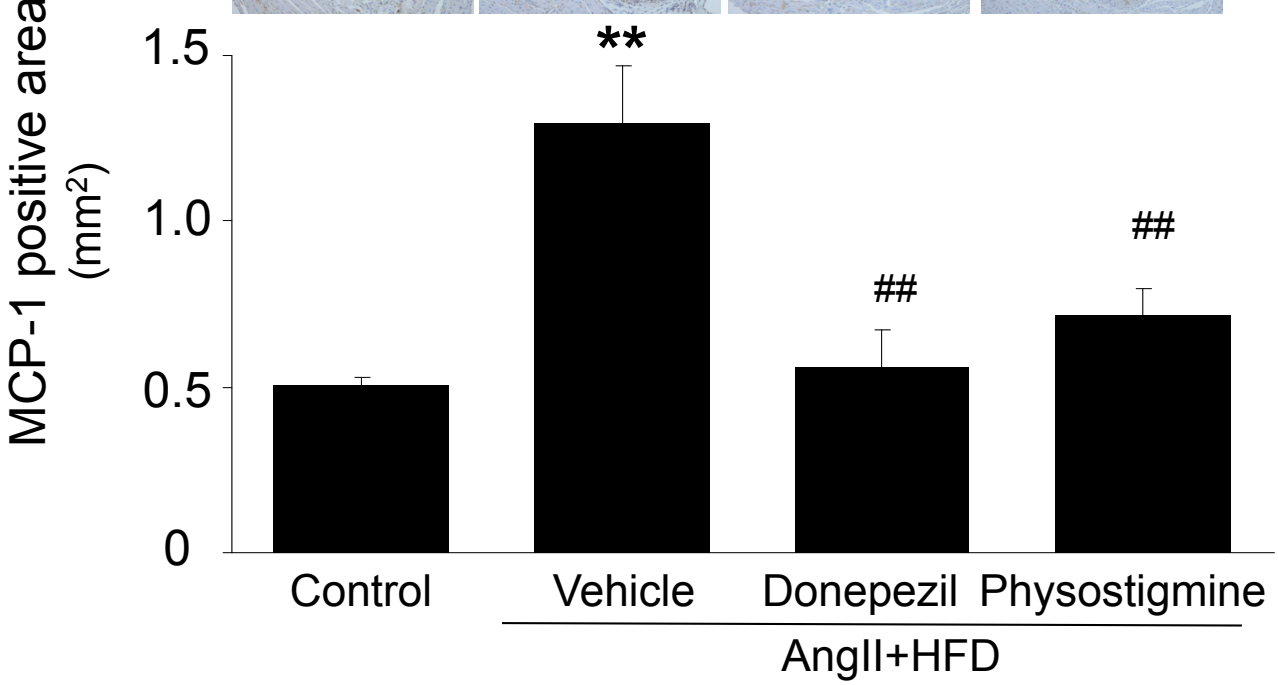
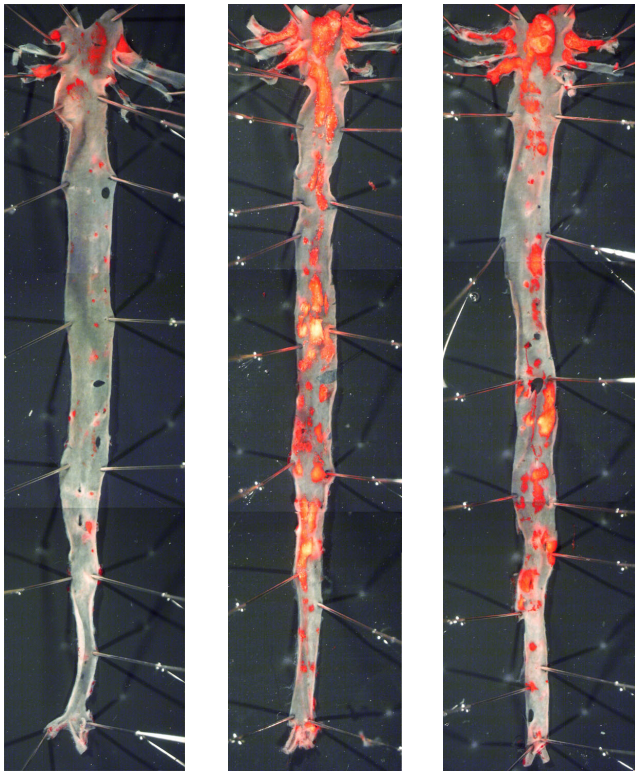

MCP-1 positive area
(mm²)
1.5
1.0
0.5
0
**
##
##
Control
Vehicle
Donepezil
Physostigmine
AngII+HFD

Figure1-3

(E)



(F)

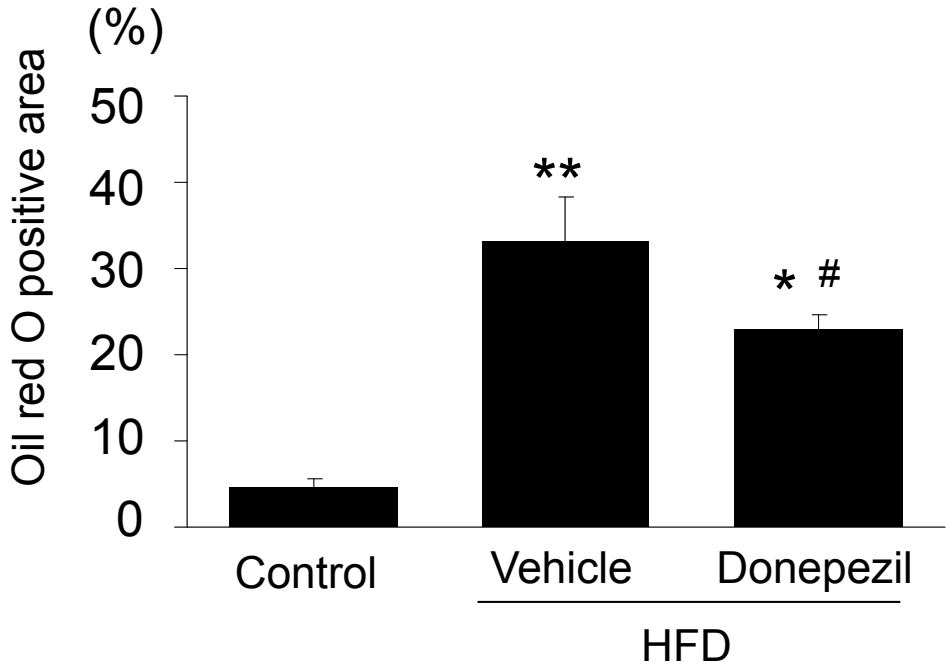
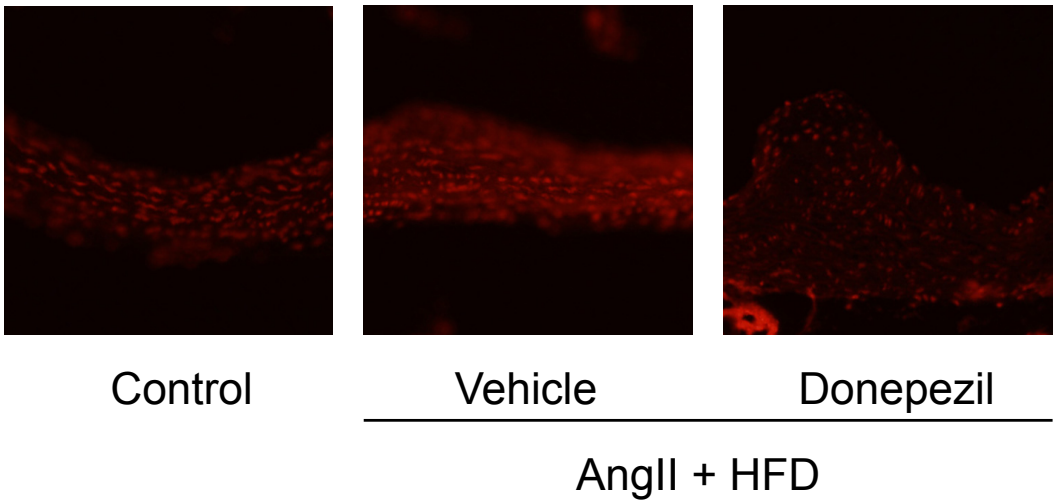

(%)
Oil red O positive area
50
40
30
20
10
0
**
* #
Control
Vehicle
Donepezil
HFD

Figure2

(A)


Control
Vehicle
Donepezil
AngII + HFD

(B)

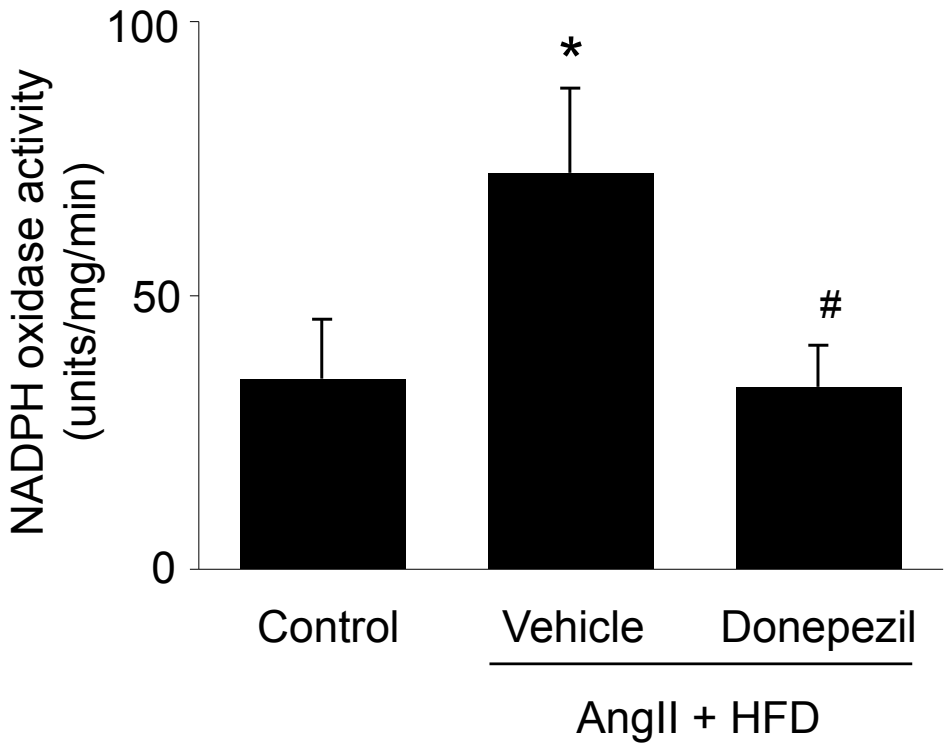

NADPH oxidase activity
(units/mg/min)
100
50
0
*
#
Control
Vehicle
Donepezil
AngII + HFD

Figure3-1

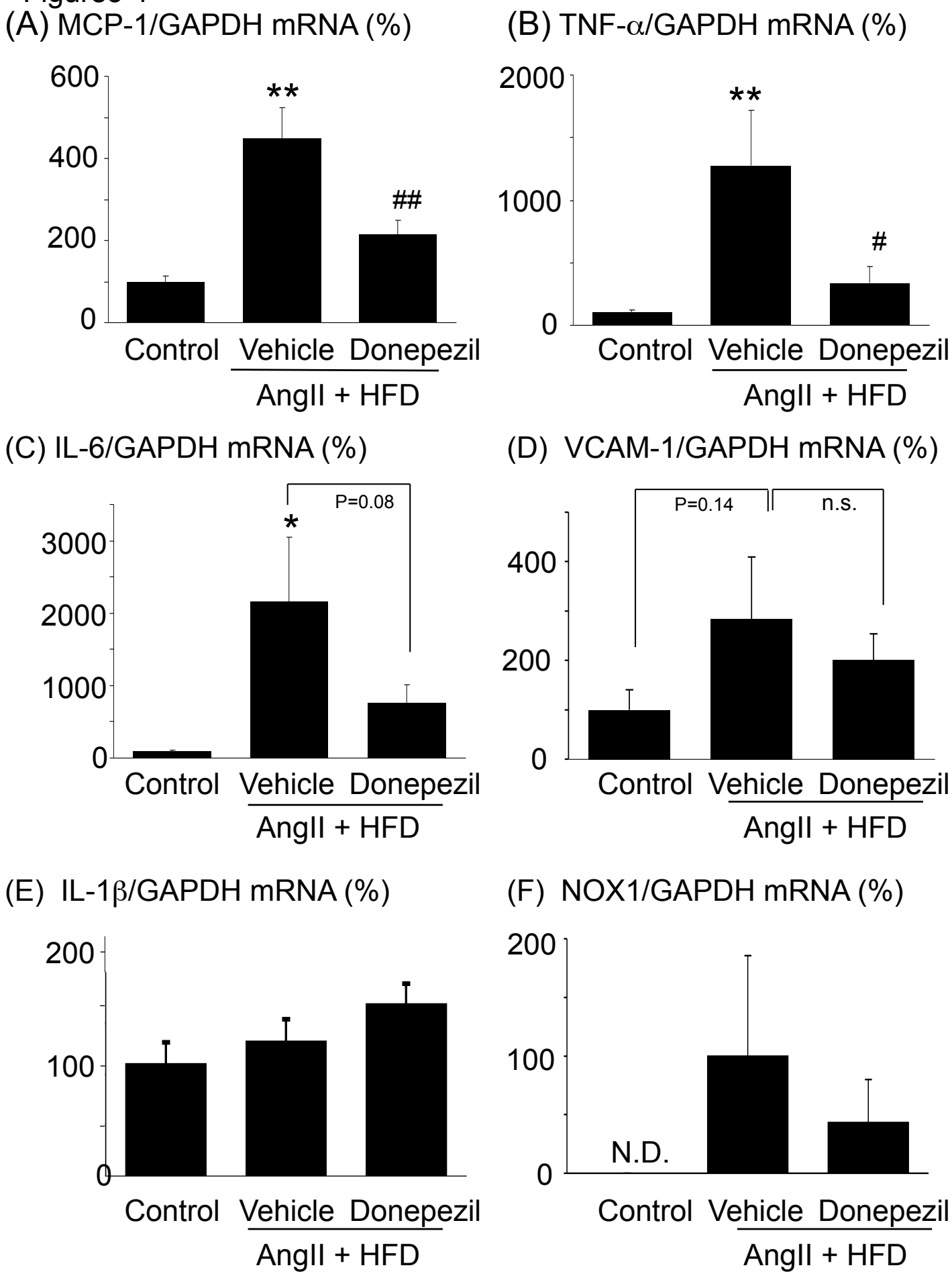

(A) MCP-1/GAPDH mRNA (%)
600
400
200
0
**
##
Control
Vehicle
Donepezil
AngII + HFD
(B) TNF-α/GAPDH mRNA (%)
2000
1000
0
**
#
Control
Vehicle
Donepezil
AngII + HFD
(C) IL-6/GAPDH mRNA (%)
3000
2000
1000
0
*
P=0.08
Control
Vehicle
Donepezil
AngII + HFD
(D) VCAM-1/GAPDH mRNA (%)
400
200
0
P=0.14
n.s.
Control
Vehicle
Donepezil
AngII + HFD
(E) IL-1β/GAPDH mRNA (%)
200
100
0
Control
Vehicle
Donepezil
AngII + HFD
(F) NOX1/GAPDH mRNA (%)
200
100
0
N.D.
Control
Vehicle
Donepezil
AngII + HFD

Figure 3-2

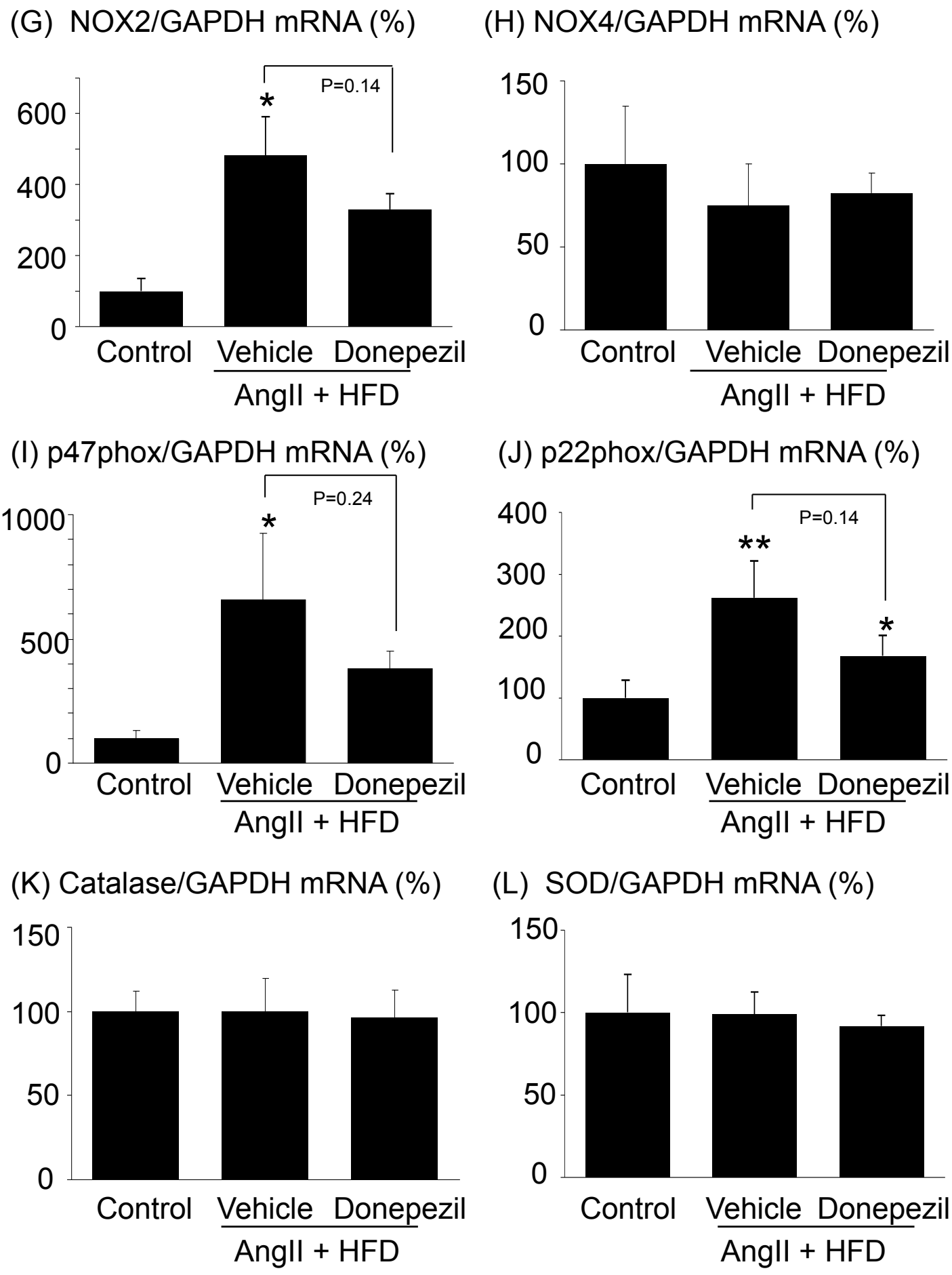
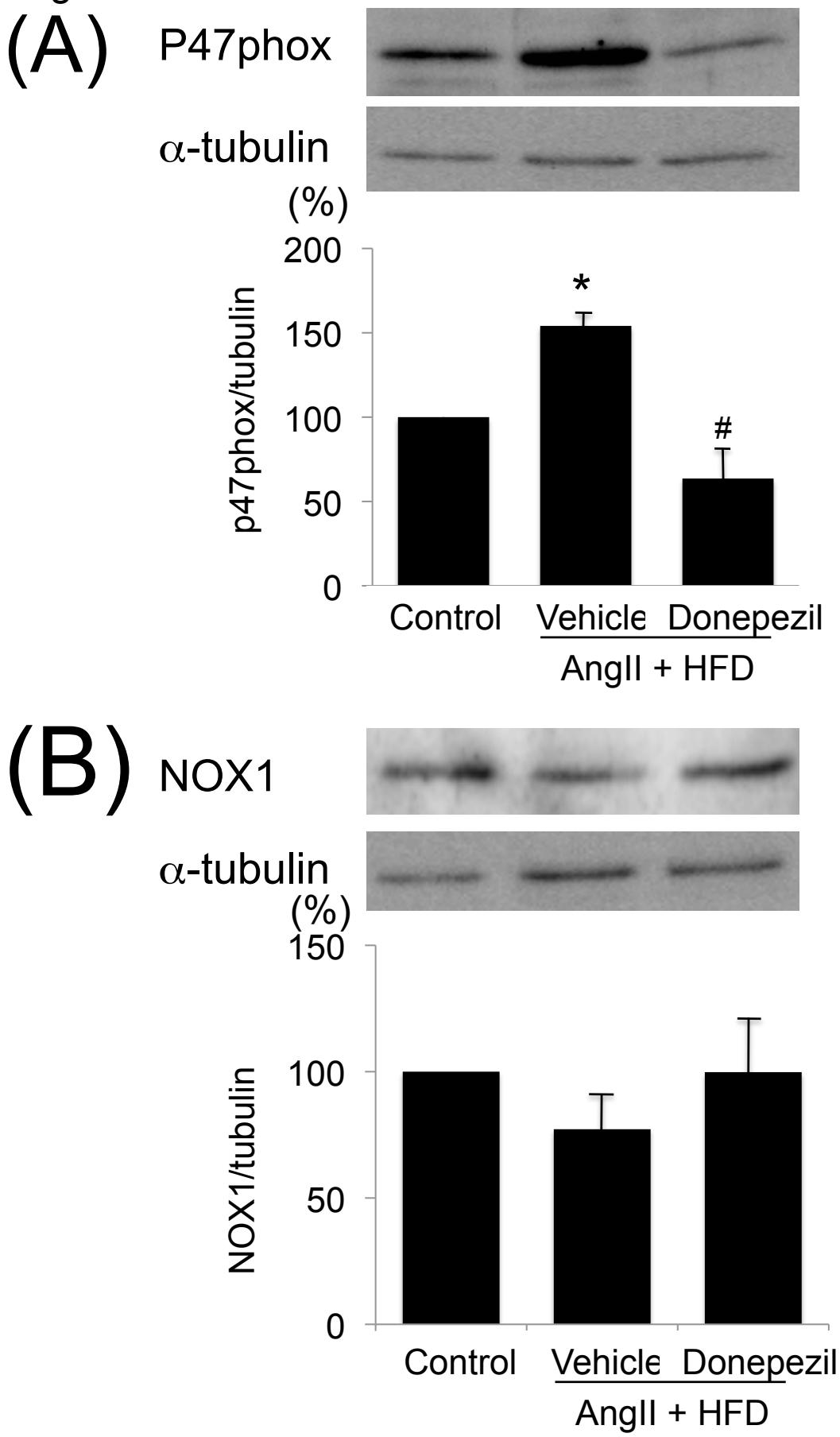

(G) NOX2/GAPDH mRNA (%)
P=0.14
*
600
400
200
0
Control
Vehicle
Donepezil
AngII + HFD
(H) NOX4/GAPDH mRNA (%)
150
100
50
0
Control
Vehicle
Donepezil
AngII + HFD
(I) p47phox/GAPDH mRNA (%)
P=0.24
*
1000
500
0
Control
Vehicle
Donepezil
AngII + HFD
(J) p22phox/GAPDH mRNA (%)
P=0.14
**
*
400
300
200
100
0
Control
Vehicle
Donepezil
AngII + HFD
(K) Catalase/GAPDH mRNA (%)
150
100
50
0
Control
Vehicle
Donepezil
AngII + HFD
(L) SOD/GAPDH mRNA (%)
150
100
50
0
Control
Vehicle
Donepezil
AngII + HFD

Figure 4


(A)
P47phox
α-tubulin
(%)
200
150
100
50
0
p47phox/tubulin
*
#
Control
Vehicle
Donepezil
AngII + HFD
(B)
NOX1
α-tubulin
(%)
150
100
50
0
NOX1/tubulin
Control
Vehicle
Donepezil
AngII + HFD