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Combinatorial effect of probucol and cilostazol in focal ischemic mice with hypercholesterolemia

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Protective effect of probucol and cilostazol on stroke

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ABSTRACT

Hypercholesterolemia may increase stroke risk by accelerating atherosclerosis, narrowing the luminal diameter in cerebral vessels and disrupting both vascular endothelial and smooth muscle function. In the present study, we investigated the beneficial effects of combinatorial therapy of probucol and cilostazol on focal cerebral ischemia with hypercholesterolemia. ApoE knockout (KO) mice were fed a high-fat diet with or without 0.5% probucol and/or 0.2% cilostazol for 10 weeks. Probucol alone and probucol + cilostazol significantly decreased total-, low density lipoprotein- and high density lipoprotein-cholesterol, while cilostazol did not affect the plasma cholesterol levels in ApoE KO mice. Administration of probucol alone and cilostazol alone significantly decreased atherosclerotic lesion area in the aorta, with a significant decrease evident using the combinatorial administration. Middle cerebral artery occlusion resulted in significantly larger infarct volumes in ApoE KO with 10 weeks of high-fat diet compared to ApoE KO fed a regular diet. The infarct volume was significantly reduced using probucol alone or cilostazol alone and was even significantly reduced by their combinatorial administration. Consistent with a larger infarct size, the combinatorial therapy prominently improved neurological function. The combinatorial administration increased cerebral blood flow during ischemia. Expression of endothelial nitric oxide synthase and adiponectin in the cortex were decreased by the high-fat diet, which were elevated by the combinatorial treatment. Adiponectin expression colocalized within the cerebral vascular endothelium. The data suggests that the combination of probucol and cilostazol prevent cerebrovascular damage in focal cerebral ischemic mice with hypercholesterolemia by up-regulation of endothelial nitric oxide synthase and adiponectin.

INTRODUCTION

Hypercholesterolemia is a major underlying cause for ischemic stroke and therapeutics targeting hypercholesterolemia decrease the risk of stroke in high-risk individuals or in patients with stroke or transient ischemic attack (Amarenco and Labreuche, 2009). Although cerebral ischemia commonly occurs in patients with hypercholesterolemia, studies have been hampered in part because the vessels are smaller and more difficult to access. In contrast, larger arteries such as the aorta and peripheral arteries have been extensively studied (Ross, 1999). In systemic vessels in humans and in animal models, hypercholesterolemia impairs endothelial and smooth muscle function (d'Uscio et al., 2001; Casino et al., 1993). Therefore, the effects of lipid lowing drugs on cerebrovascular function and pathogenic mechanisms of ischemic stroke with hypercholesterolemia should be fully elucidated *in vivo*.

Probucol is a potent lipid-soluble antioxidant that possesses anti-atherogenic properties (Kuzuya and Kuzuya, 1993) and prevents secondary cardiovascular events in patients with hypercholesterolemia (Yamashita et al., 2008). Cilostazol inhibits platelet aggregation by inhibiting the activity of phosphodiesterase III and has a demonstrated neuroprotective effect against cerebral ischemic injury in experimental studies (Choi et al., 2002; Lee et al., 2007) and clinical studies (Shinohara et al., 2010). In seeking to maximize the effects of these beneficial and useful drugs, it has been suggested that their combinatorial application might reduce atherosclerosis and prevent ischemic damage. The combinatorial strategy is supported by results of a clinical study regarding restenosis (Sekiya et al., 1998) and *in vitro* and *in vivo* experimental studies regarding atherosclerosis (Park et al., 2008; Yoshikawa et al., 2008). Moreover, concurrent treatment with probucol and cilostazol produced beneficial synergistic effects against focal cerebral ischemic injury in a rat model by reducing the

generation of superoxide (Park et al., 2007). With the aim of developing a more effective therapeutic window in the focal ischemic brain injury with hypercholesterolemia, the present study was undertaken to examine the efficacy of probucol and cilostazol combinatorial therapy.

The exact mechanisms of cerebrovascular dysfunction to ischemia during hypercholesterolemia are not completely defined but may include reduction in endothelial nitric oxide production. It has been reported that ApoE knock-out (KO) mice fed a high-fat Western diet (HFD) display endothelial dysfunction via impairment of endothelial nitric oxide synthase (eNOS)-dependent vasorelaxation (d'Uscio et al., 2001). eNOS contributes to vascular protection via increasing cerebral blood flow (CBF) and plays an important role in the regulation of brain damage after ischemia (Endres et al., 1998) and adiponectin prevents cerebral ischemic injury through eNOS-dependent mechanism (Nishimura et al., 2008). Thus, agents that regulate adiponectin-mediated eNOS signaling and increase CBF could represent a potential therapeutic target for the prevention of ischemic stroke with hypercholesterolemia.

In the present study, we explored the effects of the combinatorial use of probucol and cilostazol on focal cerebral ischemia with hypercholesterolemia using HFD-fed ApoE KO mice as an animal model. The tissue and neurologic outcomes were determined following transient middle cerebral artery (MCA) occlusion in ApoE KO mice fed the HFD for 10 weeks using treatment with probucol and cilostazol. CBF and eNOS expression levels in accordance with adiponectin expression levels were determined as the action mechanisms. The study presents evidence-based cerebrovascular protective effects of combinatorial therapy with probucol and cilostazol on the management of stroke patients who undergo hypercholesterolemia.

METHODS

General surgical preparation

Male ApoE KO mice (Japan SLC, Shizuoka, Japan) having a C57BL/6J genetic background were housed under diurnal lighting conditions and allowed food and tap water *ad libitum*. All animal procedures were in accordance with the institutional guidelines for animal research, and had been approved by the university Animal Care and Use Committee. Four-week-old ApoE KO mice were fed a Western-type HFD (42% of total calories from fat; 0.15% cholesterol; Research Diet, New Brunswick, NJ) containing 0.5% (wt/wt) probucol, 0.2% (wt/wt) cilostazol or 0.5% (wt/wt) probucol + 0.2% (wt/wt) cilostazol for 10 weeks. Anesthesia was achieved by isoflurane (2% induction and 1.5% maintenance, in 80% N_2O and 20% O_2) by face mask. The femoral artery was catheterized for the measurement of mean arterial blood pressure using a model MLT844 physiological pressure transducer (AD Instruments, Medford, MA). The data were continuously recorded using a PowerLab data acquisition and analysis system (AD Instruments) and stored in a computer. The depth of anesthesia was checked by the absence of cardiovascular changes in response to tail pinch. Rectal temperature was kept at 36.5°C-37.5°C using a Panlab^{TM} thermostatically controlled heating mat (Harvard apparatus, Holliston, MA). Arterial blood gases and pH were measured before ischemia using i-Stat System (Abbott, Abott Park, IL).

Measurement of plasma cholesterol

Blood was collected from the left ventricle under light anesthesia and stored on ice for 30 min prior to centrifugation at 13,000 rpm, 4ºC for 10 min, the plasma separated and kept at -80ºC until assayed. Lipoprotein cholesterol distribution of plasma samples was

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determined enzymatically with reagents from Kyowa MEDEX (Tokyo, Japan) using a TBA-200FR NEO apparatus (Toshiba Medical Systems, Tokyo, Japan).

Atherosclerotic lesion analysis

After 10 weeks of the HFD, mice were anesthetized and euthanized. Hearts were perfused using 10 ml of phosphate buffered saline (PBS) followed by 10 ml of 4% paraformaldehyde. After incubation in 4% paraformaldehyde overnight, the adventitia was thoroughly cleaned under a dissecting microscope, and the aorta was cut open longitudinally and pinned onto a silicone plate. To calculate the lesion area, aortas were stained with Oil red O (Sigma-Aldrich, St. Louis, MO). Fifty milliliters of an Oil red O stock solution (0.5% w/v in isopropylalcohol) was mixed with 30 ml of distilled water and filtered prior to use. Aortas were briefly rinsed with PBS containing 0.5% Tween-20 (PBS-T), incubated in the Oil red O solution for 10 min, and then destained in PBS-T for 48 min. Atherosclerotic lesion areas were quantified using iSolution full image analysis software (Image & Microscope Technology, Vancouver, Canada).

Focal cerebral ischemia

A fiber-optic probe was affixed to the skull over the middle cerebral artery (MCA) for measurement of regional CBF (rCBF) by a PeriFlux Laser Doppler System 5000 (Perimed, Stockholm, Sweden). Baseline values were measured before internal carotid artery ligation (considered to be 100% flow). Focal cerebral ischemia was induced by occluding the MCA by a previously described intraluminal filament technique (Huang et al., 1994). MCA occlusion was induced by a silicon-coated 7-0 monofilament in the internal carotid artery and the monofilament was advanced to occlude the MCA. In all animals, rCBF was measured to confirm the achievement of consistent and similar levels of ischemic induction. The filament was withdrawn 60 min after occlusion and

reperfusion was confirmed using laser Doppler. The surgical wound was sutured and mice were allowed to recover from anesthesia. Brains were removed at 24 h after MCA occlusion. Cerebral infarct size was determined on 2,3,5-triphenyltetrazolium chloride (TTC)-stained, 2-mm-thick brain sections. Infarction areas were quantified with iSolution full image analysis software (Image & Microscope Technology).

Neurological score

Neurological deficit was scored in each mouse at 24 h after the ischemic insult in a blinded fashion according to the following graded scoring system: 0 = no deficit; 1 = forelimb weakness and torso turning to the ipsilateral side when held by tail; 2 = circling to affected side; 3 = unable to bear weight on affected side; and 4 = no spontaneous locomotor activity or barrel rolling (Li et al., 2004).

Immunofluorescence

Twenty four hours after MCA occlusion, mice were deeply anesthetized with thiopental sodium and subsequently perfused transcardially with cold PBS followed by 4% paraformaldehyde for fixation. The brain of each mouse was then removed and further fixed for 48 h in 4% paraformaldehyde at 4ºC followed by cryoprotection in 20% sucrose for 24 h at 4ºC. The isolated brains were frozen and stored in the freezer at -80ºC until examined. The frozen brains were cut at 10 μm thickness with a Leica CM 3050 cryostat (Leica Microsystes, Wetzlar, Germany), immunostained with antibody against eNOS (BD Biosciences, San Jose, CA), and additionally incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody to detect eNOS. In double-fluorescence staining, sections were stained with anti-CD31 antibody (BD Biosciences), followed by treatment with FITC-conjugated secondary antibody to detect CD31, and subsequently with anti-adiponectin antibody (R&D Systems, Minneapolis,

MN), followed by treatment with Texas red-conjugated secondary antibody to detect adiponectin. Fluorescent-stained sections were analyzed by Axio Imager fluorescence microscopy (Carl Zeiss).

Drugs

Probucol [4,4'-(isopropylidenedithio)bis(2,6-di-*t*-butylophenol)] and cilostazol [OPC-13013, 6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl) butoxy]-3,4-dihydro-2-(1*H*)-quinolinone] were donated by Otsuka Pharmaceutical (Tokushima, Japan) and were added to the HFD.

Data analysis

The data are expressed as mean ± standard error of mean (SEM). Control vs. vehicle group was compared by unpaired t-test. Vehicle vs. drug alone treated groups or combinatorial group were compared using Dunnett's test. Pearson's correlation coefficient was calculated between infarct volume and total cholesterol level. The differences were considered statistically significant, when the two-tailed p values were less than 0.05. Statistical analysis was performed using SAS software (SAS Institute Japan, R9.1).

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RESULTS

Physiological parameters and plasma lipid profiles

The body weights of ApoE KO mice fed a HFD for 10 weeks were slightly higher than those in normal diet-fed mice (control) (25.90±0.97 g vs 32.39±0.71 g, respectively, $P<0.01$). Probucol and/or cilostazol did not affect body weight of HFD-fed mice (Table 1). The blood pressure did not differ among mice treated with probucol and/or cilostazol. After 10 weeks of the HFD, large increases in plasma total cholesterol and low density lipoprotein (LDL) cholesterol were observed in ApoE KO mice (Table 2, $P<0.01$ vs control). Probucol alone or in combination with cilostazol significantly decreased both total- and LDL-cholesterol levels in ApoE KO mice fed the HFD ($P<0.01$ vs vehicle). In contrast, cilostazol alone did not affect the plasma total- and LDL-cholesterol levels at all.

Effect of combinatorial therapy on atherosclerotic lesions

Measurements of lesional histomorphometry from the aorta revealed that the atherosclerotic lesion area (control 4.11±0.83 mm^2) was markedly increased to 19.41±2.81 mm^2 ($P<0.01$) in the HFD-treated group, which was significantly reduced by probucol alone (7.30±2.50 mm^2, $P<0.01$) or cilostazol alone (10.58±2.37 mm^2, $P<0.05$) (Figure 1). Moreover, when the two agents were administered in combination, the atherosclerotic lesions were more potently inhibited (4.04±1.58 mm^2, $P<0.01$).

Effect of combinatorial therapy on infarct size and neurological deficit

In order to determine whether combinatorial therapy improved the tissue outcome during cerebral ischemia in hypercholesterolemic mice, the infarct size was measured 23 h after a 1-h transient MCA occlusion. MCA occlusion resulted in 76% larger infarct

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volumes in ApoE KO fed the HFD for 10 weeks compared to ApoE KO fed the regular diet (101.75±12.06 mm^3 vs. 57.73±15.82 mm^3, $P<0.05$: Figures 2A and 2B), which was significantly reduced by probucol alone (30.32±11.19 mm^3, $P<0.01$) or cilostazol alone (52.38±19.06 mm^3, $P<0.05$), and significantly reduced by probucol + cilostazol (23.65±7.67 mm^3, $P<0.01$). Consistent with a larger infarct size, the combinatorial therapy showed prominent improvement of neurological function (Figure 2C). There was a positive correlation between plasma total cholesterol levels and infarct size after probucol treatment, and this positive correlation was noted when probucol was administered with cilostazol (data not shown). Since an increase in CBF protects against stroke, the changes in CBF during ischemia were assessed. When the CBF time course in MCA was measured, MCA occlusion was revealed to cause an abrupt reduction in CBF and CBF was higher in combination treated-mice than in age- and diet-matched ApoE KO mice, suggesting that combinatorial treatment of probucol and cilostazol lead to increased CBF during ischemia.

Effect of combinatorial therapy on eNOS and adiponectin expression

In order to explore the action mechanisms of combinatorial treatment of probucol and cilostazol on focal cerebral ischemia with hypercholesterolemia, we studied eNOS expression level in accordance with adiponectin expression levels. Few eNOS- and adiponectin-positive cells were observed in the vehicle group (Figures 4A and 4B). eNOS- and adiponectin-positive cells were increased in mice that received probucol and cilostazol in combination, whereas probucol alone or cilostazol alone showed only marginal effects on eNOS and adiponectin expression, indicating the beneficial effects of the combinatorial therapy. In addition, cilostazol alone or combinatorial treatment increased CD31, an endothelial cell marker (Figure 4B). Dual-immunofluorescence

staining was performed on adiponectin (red) and CD31 (green) and merged images demonstrated that these proteins co-localized (yellow) (Figure 4C). Collectively, these data support the suggestion that adiponectin accumulates at the endothelium in the cortex.

DISCUSSION

Presently, we evaluated the neuroprotective potential of combinatorial therapy with probucol and cilostazol to suppress the cerebral ischemic injury with hypercholesterolemia. Probucol alone and cilostazol alone significantly reduced the infarct volume in ApoE KO fed a HFD, and the combinatorial administration of probucol and cilostazol significantly reduced infarct size with neurological deficits. In addition, co-treatment with probucol and cilostazol increased CBF due to enhancement of eNOS and adiponectin expression during ischemia. These data support the view that the combination of probucol and cilostazol prevents cerebrovascular damage in focal cerebral ischemic mice with hypercholesterolemia, at least partly because of an increase of CBF via an increase of eNOS and adiponectin. This effect likely has a role in mediating the beneficial effects of such strategies in cerebrovascular disease, specifically ischemic stroke.

Hypercholesterolemia may increase the risk of stroke by accelerating atherosclerosis and segmental vessel narrowing or occlusion involving several vascular beds, as well as by disrupting vascular endothelial and smooth muscle function. The stroke-cholesterol relationship is complex and contains several paradoxes (Amarenco, 2001; Goldstein et al., 2006). Some studies showed a direct relationship between

dyslipidemia (high total cholesterol and LDL cholesterol levels, and low high density lipoprotein (HDL) cholesterol level) and stroke (Tanne et al., 2001; Jurgens et al., 1995), while other studies have not identified such an association (Sacco et al., 2001; Tanizaki et al., 2000). Few studies have evaluated ischemic stroke subtypes and patient subgroups (Tirschwell et al., 2004). Cholesterol-lowering trials have shown a decrease in the risk of cerebral infarction among patients assigned to statin treatment (White et al., 2000). In the present study, 1 h MCA occlusion and 23 h reperfusion resulted in significantly larger infarct volumes by 76% in ApoE KO fed a HFD for 10 weeks compared to ApoE KO on regular diet, consistent with a previous report (Mogi et al., 2006). This larger infarct volume was significantly reduced in accordance with significantly decreased cholesterol levels and atherosclerotic lesion areas in the aorta by probucol alone or by a combinatorial treatment of probucol and cilostazol. Taking into consideration the association between the infarct volume after MCA occlusion and serum lipid profile (total cholesterol), there was a positive linear relationship ($r=0.77$, $P<0.01$) in control, vehicle, probucol alone and the combinatorial groups. When we observed Oil red O staining in cerebral arteries of ApoE KO fed a HFD for 10 weeks, no atherosclerotic lesions were observed in cerebral arteries (data not shown). Therefore, we believed that the increase in ischemic brain damage in ApoE KO fed a HFD may not be due directly to the structure of cerebral arteries by atherosclerotic plaque formation.

Lipid lowering therapy significantly reduces the risk of stroke (Collins et al., 2003). In part, this improved outcome has been attributed to a slowed progression of intracranial (carotid) atherosclerosis consequent to a decrease in hypercholesterolemia (Amarenco and Labreuche, 2009). Some investigators have suggested that the

beneficial effects of statin in stroke may be due only in part to lipid-lowering properties, with the primary benefit derived from improved endothelial function as well as the anti-inflammatory and anti-thrombotic actions of the drugs (Vaughan et al., 2001; Ishikawa et al., 2004). Probucol (0.5%) and cilostazol (0.2%) treatment significantly decreased total cholesterol and LDL cholesterol levels, and ameliorated the progression of atheroma formation in the entire aorta in ApoE KO mice fed with the HFD. A clinical study (Sekiya et al., 1998) and experimental studies (Yoshikawa et al., 2008) have suggested the potential beneficial effects of probucol and cilostazol on restenosis and atherosclerosis. In LDL receptor-deficient mice, the combination of probucol and cilostazol more significantly decreased the atherosclerotic lesion area than either probucol or cilostazol alone (Yoshikawa et al., 2008). The clinical study resulted that treatment with a combination of probucol and cilostazol was safe and effective in preventing acute post-stenting complications and suppressing chronic restenosis (Sekiya et al., 1998). Presently, the combinatorial treatment with probucol and cilostazol modulated plasma cholesterol levels. It is possible that these effects may have inhibited atherosclerotic lesions and stroke development in ApoE KO mice fed the HFD.

Both probucol and cilostazol have been approved for use. Both are safe and efficient in their respective therapeutic categories with some different and similar action mechanisms. Probucol, which is a potent lipid-soluble antioxidant, possesses anti-atherogenic properties (Kuzuya and Kuzuya, 1993). Cilostazol increases intracellular cyclic AMP levels by inhibiting type III phosphodiesterase, and has a demonstrated *in vivo* neuroprotective effect against cerebral ischemic injury via anti-apoptotic and anti-inflammatory effects (Choi et al., 2002; Lee et al., 2007). Moreover, cilostazol has been

shown to increase plasma HDL cholesterol levels and inhibit atherosclerosis formation in ApoE KO mice fed with a Western diet and LDL receptor-deficient mice fed with a HFD (da Rosa et al., 2006; Lee et al., 2005). It is likely that two drugs that possess different mechanisms of action may result in an effective therapy. In the previous *in vitro* study, we assessed the synergistic efficacy of the combinatorial therapy with probucol and cilostazol on the antioxidant and anti-inflammatory actions in cultured human coronary artery endothelial cells (Park et al., 2008). Concurrent treatment with probucol and cilostazol had beneficial synergistic effects against focal cerebral ischemic injury in rats via reduced superoxide generation (Park et al., 2007). Consistent with these reports, we presently observed that a combinatorial therapy of probucol and cilostazol reduced infarct size with neurological deficits, providing a potential strategy for preventing ischemic stroke with hypercholesterolemia. In addition, cilostazol alone or combinatorial treatment accelerated angiogenesis which is evidenced by increased microvessels (CD31 staining, Figure 4B). Our results are consistent with the reported findings that long-term treatment of cilostazol increased angiogenesis which can increase CBF and improve brain tissue recovery (Ye et al., 2007). Therefore, additional use of cilostazol could improve brain tissue recovery and functional recovery in addition to antioxidant action of probucol.

Hypercholesterolemia is associated with decreased nitric oxide (NO) bioavailability and endothelial dysfunction, which may be very important in the altered CBF evident in stroke. Endothelial dysfunction via impairment of eNOS-dependent vasorelaxation in the aorta, carotid artery, and cerebral arterioles of ApoE KO mice fed a HFD has been reported (d'Uscio et al., 2001), and also in the forearm and coronary arteries of hypercholesterolemic patients (Casino et al., 1993). Endothelial NOS and vascular NO

maintain CBF and protect against brain injury after ischemia (Endres et al., 1998). Probucol improves the endothelium-dependent relaxation in aortic balloon injury in rabbits and a swine model of left ventricular hypertrophy by increasing NO-mediated vasodilation (Lau et al., 2003). Additionally, cilostazol causes vasodilation through an endothelial-NO-dependent pathway in rat aorta (Nakamura et al., 2001) and increases the phosphorylation of eNOS at Ser1177 in human aortic endothelial cells (Hashimoto et al., 2006). In this study, we observed an increase in CBF by combinatorial treatment with probucol and cilostazol during cerebral ischemia in ApoE KO mice fed a HFD. Moreover, hypercholesterolemia down-regulated eNOS and adiponectin in the cortex and these effects were increased by the combinatorial long-term application of probucol and cilostazol. However, we could not observe the changes of phosphorylated eNOS by combinatorial treatment with probucol and cilostazol (data not shown), suggesting that probucol and cilostazol treatment do not activate eNOS acutely by post-translational modification including phosphorylation which is approach for acute stroke treatment. We suggested that long-term probucol and cilostazol treatment leads to upregulation of eNOS, which has a combined effect of stroke prevention and prophylactic treatment for ischemic stroke with hypercholesterolemia.

Adiponectin exerts beneficial actions on cerebrovascular disease. Adiponectin is cerebroprotective by an eNOS-dependent mechanism (Nishimura et al., 2008). Adiponectin KO mice develop impaired ischemia-induced angiogenesis in a mouse model of vascular insufficiency and an excessive vascular remodeling response to injury (Matsuda et al., 2002). Recent results of a clinical study suggested an association between hypoadiponectinemia and increased mortality after ischemic stroke, and a negative correlation between adiponectin levels and initial infarct volume

(Chen et al., 2005). As well, hyperadiponectinemia is neuroprotective against ischemic stroke (Ouchi et al., 2003). Furthermore, adiponectin accumulates in the vascular endothelium during cerebral ischemia (Nishimura et al., 2008). Thus, agents that increase circulating adiponectin levels could represent a potential therapeutic target for the prevention of ischemic stroke. Probucol can significantly elevate serum adiponectin concentrations in diabetic rats (Zhang et al., 2009) and cilostazol increases adiponectin levels in type 2 diabetic patients and in a diabetic animal model (Hsieh and Wang, 2009). In the present study, the accumulation of adiponectin with eNOS in the vasculature in the combinatorial therapy group may have served to protect the vasculature. Collectively, these observations suggest that the combinatorial treatment with probucol and cilostazol regulates the adiponectin-eNOS signaling axis functions to modulate vascular function, protecting against cerebral injury after stroke.

In summary, the combinatorial therapy with probucol and cilostazol prevents ischemic stroke with hypercholesterolemia through an increase of CBF via an increase of eNOS and adiponectin. This finding may provide convincing evidence to support the cerebrovascular protective effects of the combinatorial therapy on the focal cerebral ischemic injury with hypercholesterolemia.

Authorship Contributions

Participated in research design: Shin and J.H. Kim

Conducted experiments: J.H. Kim and S.H. Park

Contributed new reagents or analytic tools: Hong, Y.D. Kim and K.P. Park

Performed data analysis: Shin, J.H. Kim and Choi

Wrote or contributed to the writing of the manuscript: Shin, J.H. Kim and Bae

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Footnotes

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Legends for figures

Figure 1. Effect of the combinatorial treatment of probucol and cilostazol on atherosclerotic lesions. (A) Representative photographs of Oil red O staining in the whole aorta showing atherosclerotic lesions of aorta from each group. ApoE KO mice were fed the HFD with or without 0.5% probucol (Pro), 0.2% cilostazol (Cilo), or both (Pro+Cilo) for 10 weeks. (B) Quantification of Oil red O-stained atherosclerotic lesion areas from each group (N=6). The lesion sizes in each group are expressed as mean ± SEM. ******, *P*<0.01 vs. age-matched ApoE KO without HFD (Con, control); [#], *P*<0.05 and [##], *P*<0.01 vs. age- and diet-matched ApoE KO (Veh, vehicle)

Figure 2. Effect of the combinatorial treatment of probucol and cilostazol on infarct volume and neurological deficit in mice with hypercholesterolemia. (A) Representative topical TTC-stained brains from ApoE KO mice fed the HFD with or without 0.5% probucol (Pro), 0.2% cilostazol (Cilo), or both (Pro+Cilo) for 10 weeks. Mice were subjected to 1 h MCA occlusion followed by 23 h reperfusion. White indicates the infarct area. (B) Quantification of infarct volume at 24 h after ischemia (N=7). Infarct volume was calculated by integrating the infarct area in 2 mm-thick coronal slices. (C) Neurological deficit was scored in each mouse at 24 h after the ischemic insult in a blinded fashion (N=11-13). Values are mean ± SEM. *****, *P*<0.05 vs. age-matched ApoE KO without HFD (Con, control); [#], *P*<0.05 and [##], *P*<0.01 vs. age- and diet-matched ApoE KO (Veh, vehicle)

Figure 3. Effect of the combinatorial treatment of probucol and cilostazol on CBF

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in response to ischemia. The time course of CBF changes in the MCA during ischemia expressed as % of baseline (N=7-9). MCA occlusion caused an abrupt reduction in CBF and CBF was higher in drug treated-mice than in age- and diet-matched ApoE KO mice. Time 0 is the onset of MCA occlusion. Values are mean ± SEM.

Figure 4. Effect of the combinatorial treatment of probucol and cilostazol on eNOS and adiponectin expression. ApoE KO mice were fed the HFD with or without 0.5% probucol (Pro), 0.2% cilostazol (Cilo), or both (Pro+Cilo) for 10 weeks. Expression of (A) eNOS (green) and (B) adiponectin protein (red) and CD31 (green), an endothelial cell marker, were determined in the cortex. In the vehicle group, few eNOS-positive cells were evident, which were increased by treatment with probucol and cilostazol in combination. Adiponectin and CD31-positive cells were increased in the mice that received probucol and cilostazol in combination as compared to the monotherapy. (C) Immunofluorescence staining of adiponectin (red) and CD31 (green) were merged (yellow), showing the colocalization of these proteins. Scale bar denotes 100 μm.

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Table 1. Physiological parameters

	Control (N=9)	Vehicle (N=10)	Probucol (N=10)	Cilostazol (N=9)	Probucol + cilostazol (N=9)
Body weight	25.90±0.97	32.39±0.71**	32.53±1.10	33.00±1.14	32.13±0.61
MABP	119.55±4.04	125.46±4.15	120.68±3.12	125.91±3.52	118.03±3.57
pH	7.42±0.02	7.43±0.02	7.39±0.02	7.38±0.01	7.36±0.01$^{\#}$
pO_2	106.56±4.49	109.10±2.53	110.40±2.73	117.44±3.34	116.56±4.36
pCO_2	29.81±0.80	24.87±1.12**	27.63±0.98	26.73±0.94	29.70±1.29$^{\#\#}$

Values are mean ± SEM. Body weight is expressed in grams. MABP (mean arterial blood pressure), pO_2, and pCO_2 are expressed in mmHg. ******, $P<0.01$ vs. age-matched ApoE KO without HFD (Control); $^{\#}$, $P<0.05$ and $^{\#\#}$, $P<0.01$ vs. age- and diet-matched ApoE KO (Vehicle)

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Table 2. Effect of combinatorial treatment of probucol and cilostazol on plasma cholesterol level

	Control (N=11)	Vehicle (N=10)	Probucol (N=10)	Cilostazol (N=10)	Probucol + cilostazol (N=11)
Total cholesterol	474.73±27.75	759.60±32.04**	230.70±24.94$^{\#\#}$	730.40±69.43	171.64±6.88$^{\#\#}$
LDL-cholesterol	321.64±24.70	663.20±73.93**	170.40±16.88$^{\#\#}$	627.50±67.35	130.18±4.94$^{\#\#}$
HDL-cholesterol	132.27±5.93	177.90±11.96**	53.50±3.52$^{\#\#}$	177.30±16.77	43.36±1.23$^{\#\#}$
Triglyceride	82.46±12.29	58.60±7.00	58.70±9.96	38.20±4.16	35.09±4.98$^{\#}$

Values are mean ± SEM. ******, *P*<0.01 vs. age-matched ApoE KO without HFD (Control); $^{\#}$, *P*<0.05 and $^{\#\#}$, *P*<0.01 vs. age- and diet-matched ApoE KO (Vehicle)

Figure 1

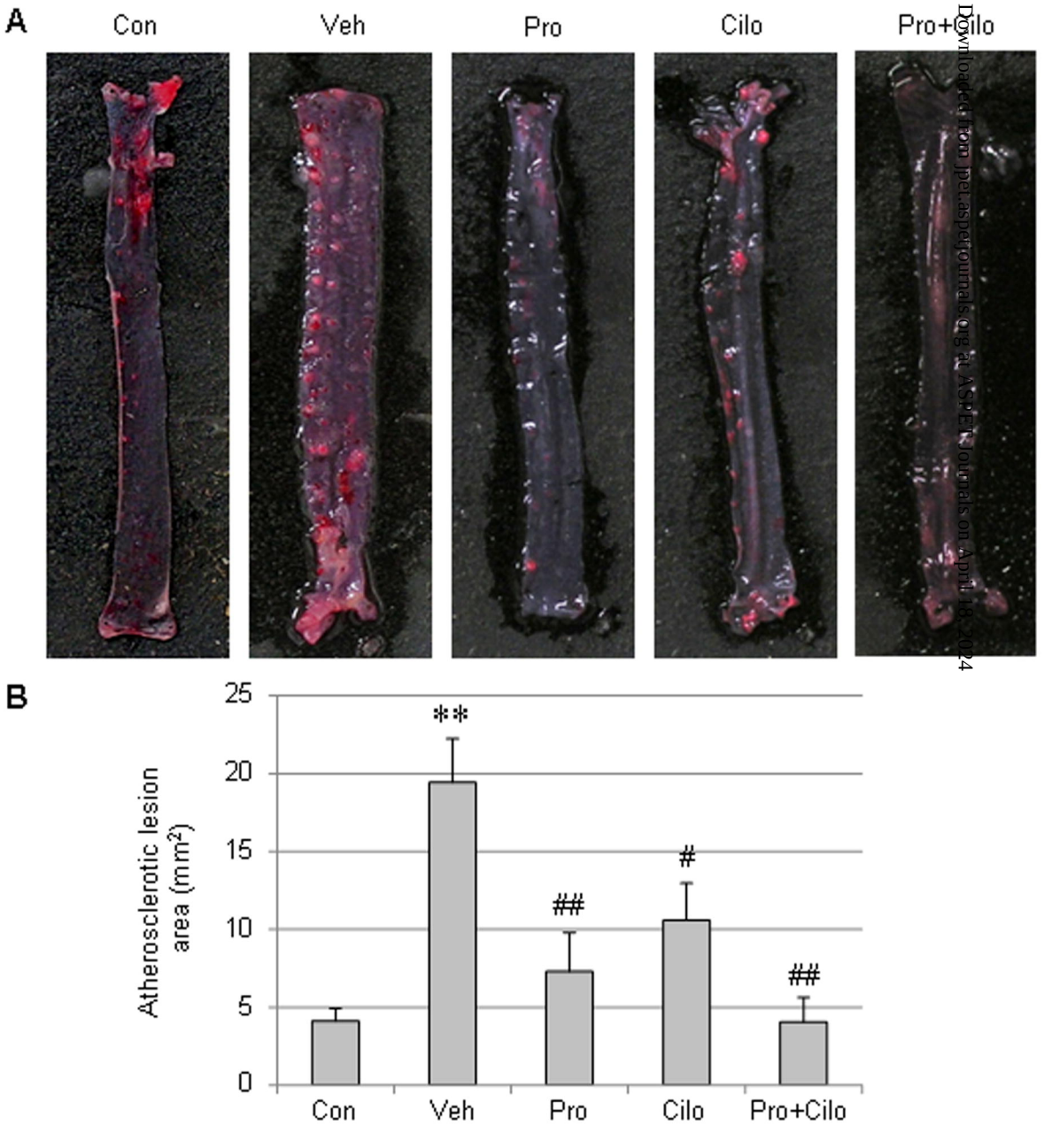


Figure 2

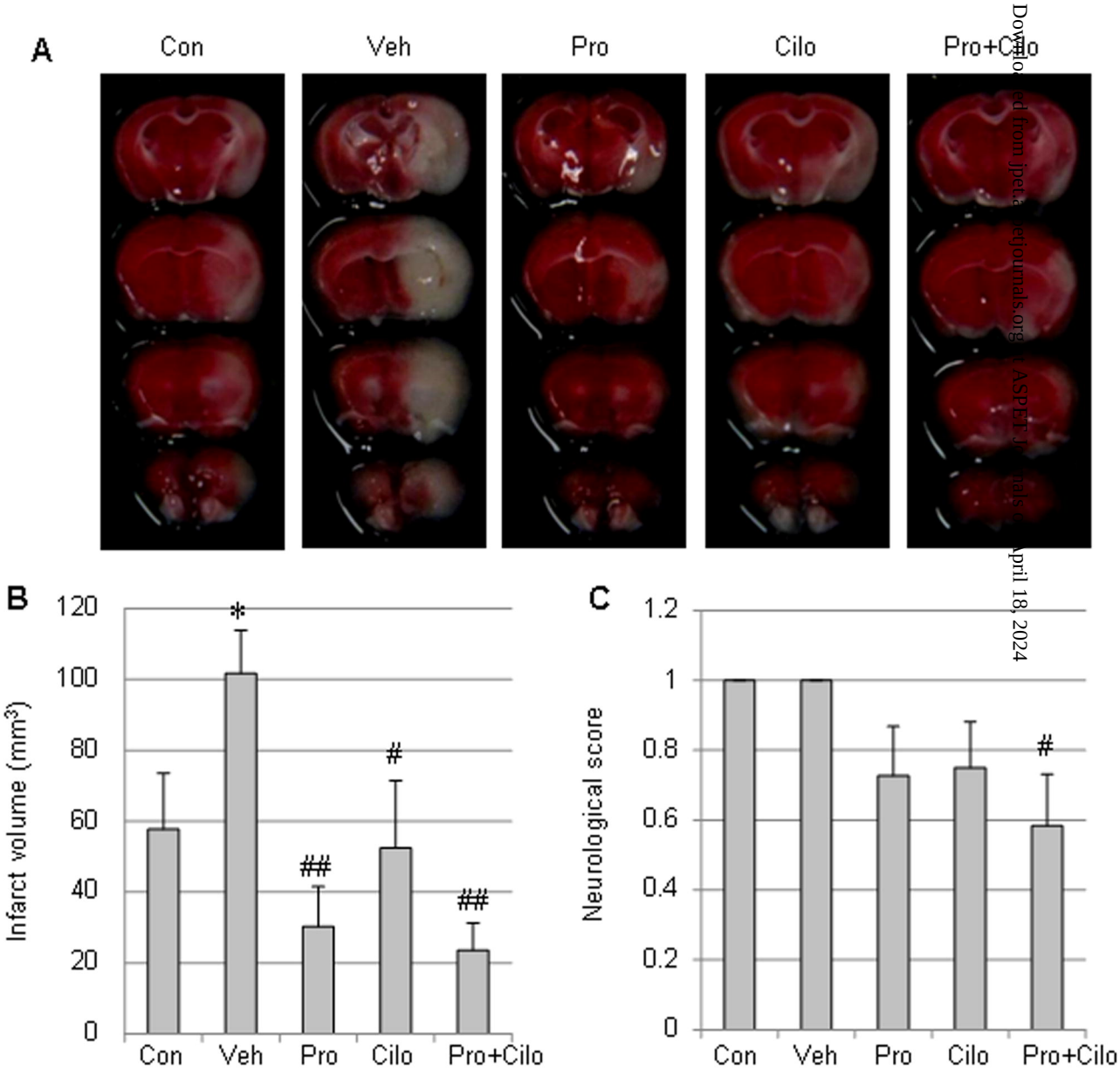


Figure 3

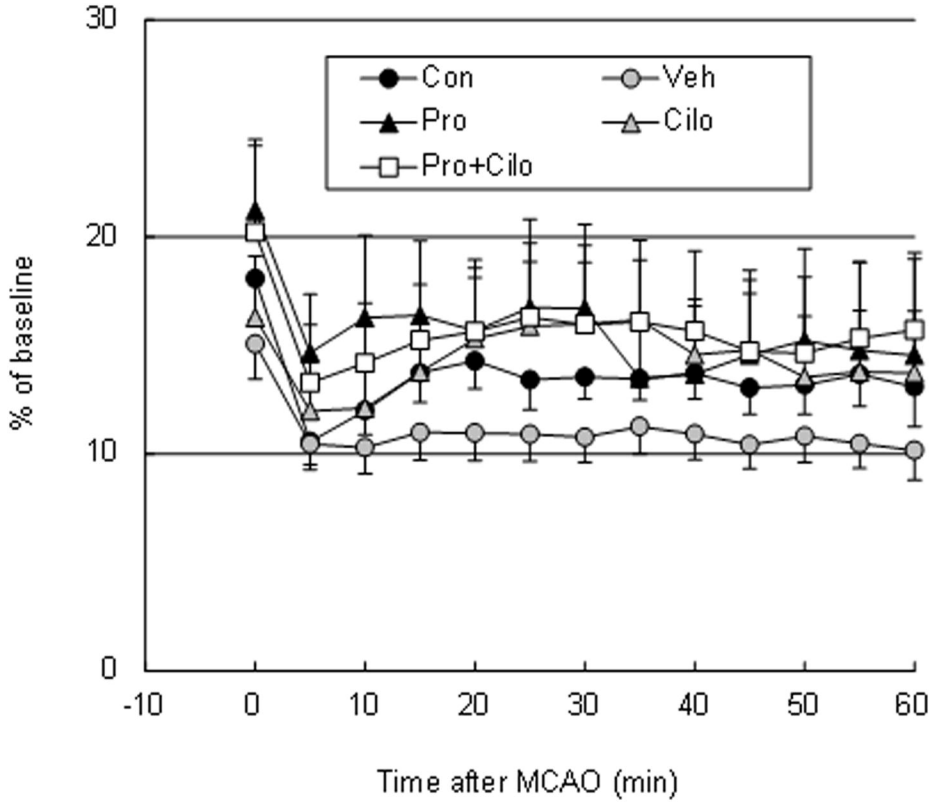
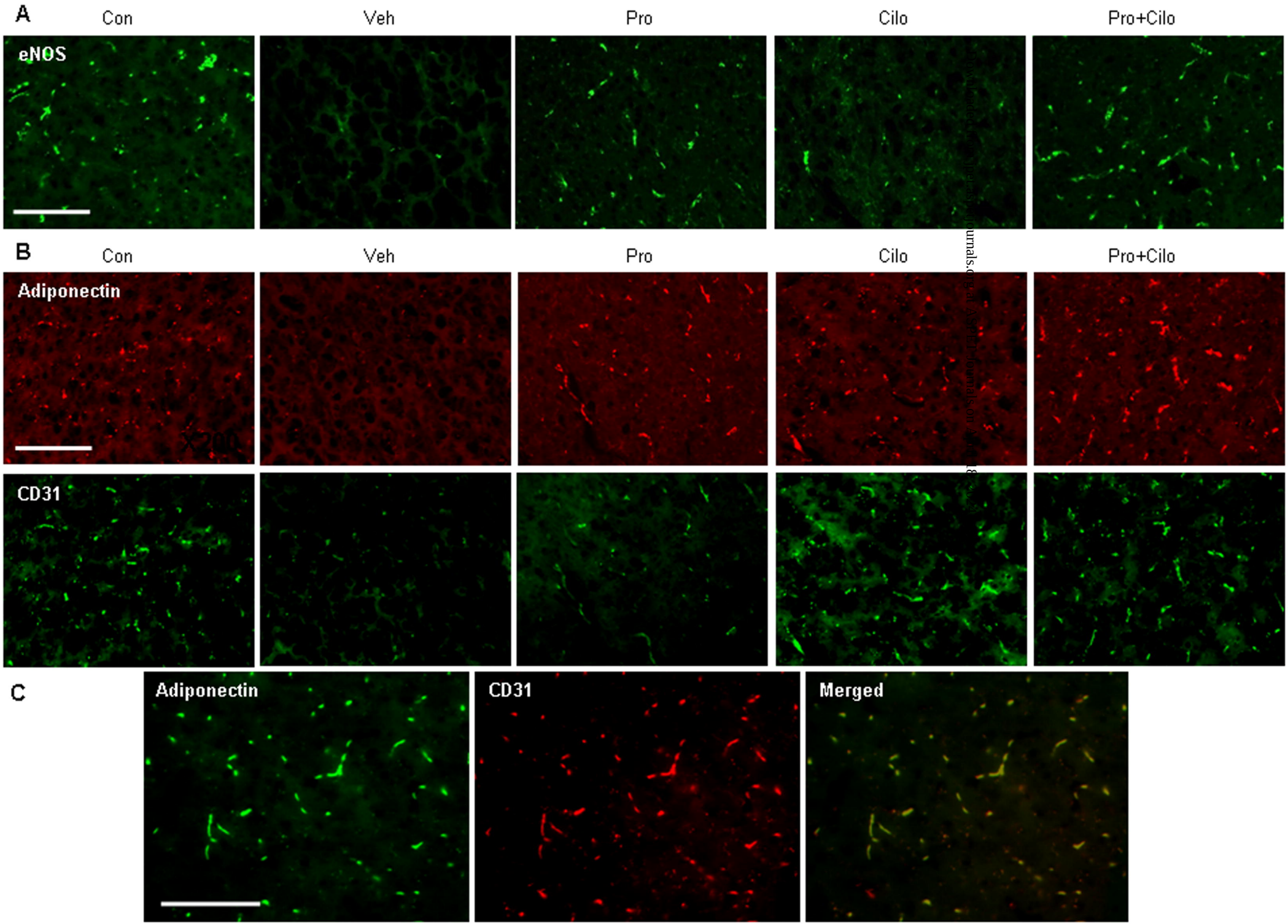


Figure 4


A
Con
Veh
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Cilo
Pro+Cilo
eNOS
B
Con
Veh
Pro
Cilo
Pro+Cilo
Adiponectin
CD31
C
Adiponectin
CD31
Merged