Original Article

Cilostazol Attenuates AngII-induced Abdominal Aortic Aneurysms But Not Atherosclerosis in ApoE Deficient Mice

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Running Title:
Cilostazol attenuates AngII-induced AAA (39 characters)

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Abstract

Objective: Abdominal aortic aneurysm (AAA) is a permanent dilation of the abdominal aorta associated with rupture which frequently results in fatal consequences. AAA tissue is commonly characterized by localized structural deterioration accompanied with inflammation, profound accumulation of leukocytes; although the specific function of these cells is unknown. Cilostazol, a phosphodiesterase III (PDEIII) inhibitor, is commonly used for patients with peripheral vascular disease or stroke due to its anti-inflammatory effect which is vasoprotective effects through its anti-inflammatory effect. In this study, we evaluated the effects of cilostazol on angiotensin II (AngII) -induced AAA formation.

Approach and Results: Male apolipoprotein E deficient mice were fed either normal diet or a diet containing cilostazol (0.1% wt/wt). After 1 week of diet consumption, mice were infused with AngII (1,000 ng/kg/min) for 4 weeks. AngII infusion increased maximal diameters of abdominal aortas; whereas cilostazol administration significantly attenuated dilatation of abdominal aortas thereby reducing AAA incidence. Cilostazol also reduced macrophage accumulation, matrix metalloproteinases activation, and inflammatory gene expression in the aortic media. In cultured vascular endothelial cells, cilostazol reduced expression of inflammatory cytokines and adhesive molecules through activation of the cyclic AMP – protein kinase A pathway.

Conclusions: Cilostazol attenuated AngII-induced AAA formation by its anti-inflammatory effect through PDEIII inhibition in the aortic wall. Cilostazol may be a promising new therapeutic option for AAAs.

Abbreviations;
AAA Abdominal aortic aneurysm
AngII Angiotensin II
MCP-1 monocyte chemoattractant protein-1
PDEIII phosphodiesterase III
PKA protein kinase A

Introduction

Abdominal aortic aneurysm (AAA) is a permanent dilation of the abdominal aorta with an onset of symptoms that are often insidious until the overt manifestation of rupture. Associated with advanced age, male sex, cigarette smoking, and genetic predisposition, AAA is a common disease with a prevalence of 3-5 % in males over 60 years of age[1]. AAA and aortic dissection is the 9th leading cause of death in the Population Survey Report from Ministry of Health, Labour and Welfare in Japan, as well as countries of the Western world. Open and endovascular surgical repairs are the only available treatments for large AAAs (> 5 cm). There is no proven medical therapy available, particularly for small AAAs[2]. The effects of anti-hypertensive and anti-dyslipidemic therapies to prevent aneurysm growth and rupture in patients with AAA are still controversial [3-5].

The pathogenesis of AAA is multifactorial with complex interactions between MMP driven enzymatic degradation of aortic elastin and damage to medial smooth muscle cells. Loss of smooth muscle cells, elastin degradation medial thinning, adventitial hypertrophy, accumulation of inflammatory cells, atherosclerosis and thrombi are common pathological characteristics in human AAAs[6, 7]. AAA is regarded as a chronic inflammatory disease as previous studies have demonstrated accumulation of macrophages and lymphocytes in the aortic wall[8], and elevated plasma concentrations of IL-1β, IL-6, TNF-α, and IFN-γ in AAA patients[9, 10].

Angiotensin II (AngII)-induced AAA is a commonly used model of aneurysm formation in mice. Chronic AngII infusion induces AAA in normo- and hyperlipidemic mice, with some succumbing to aneurysm rupture[11 12]. Some pathological features of this model resemble human AAAs, including medial elastin degradation and accumulation of inflammatory cells, including macrophages, and T and B lymphocyte cells in the aortic wall. During the early phase of AngII-induced AAAs, macrophage accumulation occurs in the medial and adventitia layers of the suprarenal aorta. Macrophage accumulation into the medial layer is often accompanied by disruption of elastin fibers[7, 13]. Previous studies have demonstrated the roles of inflammation in aneurysmal formation in this model as deletion of cyclooxygenase-2, osteopontin, and microsomal prostaglandin E synthase-1 attenuated AngII-induced AAA formation[14-16]. Bone marrow-derived monocyte chemoattractant protein-1 (MCP-1) receptor, caspase-1-dependent IL-1β production, IL-6 and TNF-α are also involved in formation of AngII-induced AAA[17-20].

Cilostazol, a selective inhibitor of phosphodiesterase III (PDEIII), is commonly used in clinical practice as an anti-platelet drug for peripheral artery disease and stroke. PDEIII is expressed in platelets, heart, adipose tissue, liver, kidney and arterial tissue[21-23]. PDEs degrade cAMP synthesized by adenylate cyclase. By

inhibiting PDEIII enzymatic activity, cilostazol increases intracellular cAMP and activates protein kinases, including protein kinase A (PKA) and exchange protein directly activated by cAMP. This leads to myriad effects, including vasodilation and inhibition of proliferation in the vasculature, inotropic and chronotropic effects in heart, anti-restenosis effects in coronary arteries, reduction of triglycerides and increases of high-density cholesterol concentration in plasma, in addition to an anti-platelet effect[24, 25]. Previous studies have shown that cilostazol has anti-inflammatory, anti-oxidative stress, and anti-proliferative effects on vasculature[26-29]. Despite these vasoprotective effects, the effect of cilostazol on AAA development remains unknown. In this study, we determined the effects of cilostazol on AngII-induced AAAs in apoE deficient (apoE -/-) mice. Our study demonstrated that cilostazol reduced inflammatory changes in the aortic wall and attenuated AAA development. These effects are associated with PDEIII inhibition reducing inflammatory properties of endothelial cells.

Materials and Methods

Mice and Study Protocol:

Male, 8-12 week-old, apoE -/- mice were purchased from The Jackson Laboratory (Bar Harbor, Cat.#2052). All mice were maintained in a barrier facility, and ambient temperature ranged from 20 °C to 24 °C. Mice were fed diet and water ad libitum. Only male mice were studied because female mice have very low incidence of AngII-induced AAA as detailed in an ATVB Council statement[30]. Cilostazol was a generous gift from Otsuka Pharmaceutical Company. Either vehicle or cilostazol-containing (0.1 %wt/wt) diet were started 1 week prior to AngII infusion. For aneurysm quantification and histological analysis, saline or AngII (1,000 ng/kg/min, Bachem, Cat. #H-1705) were infused via Alzet mini-osmotic pumps (Model 2004, Durect Corp) for 28 days. Mini-osmic pumps were implanted subcutaneously on the right flank, as described previously[12, 31]. For mRNA and protein (Western blotting and gelatin zymography) analyses, AngII or saline was infused into mice for 7 days. The experimental protocol was approved by the Ethics Review Committees for Animal Experimentation of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical sciences.

Blood Pressure Measurement:

Systolic blood pressure and pulse rate were measured by sphygmomanometry using a tail cuff system (BP-98A, Softron) following a published protocol[32]. Conscious mice were introduced into a small holder mounted on a thermostatically controlled warming plate and maintained at 37°C during measurement.

Lipids Measurements:

After an overnight fast, blood was obtained by cardiac puncture under anesthesia. Total cholesterol, triglycerides, and high-density lipoprotein-cholesterol concentrations were determined in individual plasma samples using commercially available enzymatic-based kits (Wako Chemicals Cat #439-17501, #465-56701, #432-40201).

Cilostazol Concentrations:

Mice were fed either control or ciostazol-containing diet for 5 weeks. For systemic cilostazol concentration measurements, blood was obtained during the daytime without fasting and plasma was separated. Cilostazol concentrations were measured using high performance liquid chromatography (Otsuka Pharmaceutical Company).

Quantification of Aneurysms and Atherosclerosis:

After 28 days of either saline or AngII infusion, mouse aortas were harvested for aneurysm and atherosclerosis quantification following the AHA statement on atherosclerosis[33]. Aortas were perfused with saline by left ventricular puncture and were fixed in 10 % formalin overnight. Adventitial fat was removed and the maximum external width of the suprarenal aorta was measured using computerized morphometry (Lumina Vision software, Mitani Corp) as described previously[34,35]. Aneurysm was defined as a 50 % increase compared to saline-infused aorta. In saline-infused mice, the mean suprarenal width was 0.87 mm, consequently we defined AAA as > 1.30 mm. Atherosclerosis was quantified on intima on aortic arches as % lesion area by *en face* method as described previously[36-38].

Histological Analysis:

Mouse abdominal aortas were fixed in formalin, embedded in OCT, and were sectioned serially at 10 μm thickness. Verhoeff's staining was used to examine elastin fiber integrity. Immunoperoxidase staining was performed to examine macrophage infiltration and localization of phosphodiesterase III using a CD68 antibody (SEROTEC, Cat.#MCA1957) and PDE3A antibody (Abcam, Cat.#ab99236) respectively. Reactivity of the antibodies with tissue antigens was detected using AEC and ImmPACT AEC HRP Substrate (Vector Laboratories) as described previously[39,40].

Gelatin Zymography:

Either control or cilostazol-containing (0.1 %wt/wt) diet were started 1 week prior to AngII infusion, and aortas were harvested 1 week after infusions. Proteins were extracted from aortas without any distinguishable aneurysms. Samples were resolved under nonreducing condition on a 7.5 % polyacrylamide gel containing 0.1% gelatin as a substrate for MMP activity.

Isolation and Culture of Endothelial Cells:

Aortic endothelial cells were isolated from apoE -/- mice by an explant technique as described previously[41, 42]. Briefly, under sterile conditions, aortas were removed from apoE -/- mice and adventitia removed. Aortas were cut into 3 mm-long rings, placed on Matrigel (BD, Cat. #356234), and incubated in EGM-2 medium (Lonza) supplemented with 10 % FBS. Once cell outgrowth was observed, vessel rings were removed. The cells were passaged with dispase (BD, Cat. #354235) and then plated onto collagen-coated culture dishes. Subsequent passages were performed with 0.25 % trypsin-EDTA, and cells were split in a 1:4 ratio. The identification of cells was confirmed by immnostaining for von Willebrand factor.

Cell Culture Conditions:

Endothelial cells isolated from aorta of apoE -/- mice were used for between passages 4-6. After overnight serum starvation, endothelial cells were incubated with selected concentrations of cilostazol (0, 1, 10 μM) / H89(Cayman chemical, Cat. #130964-39-5) / forskolin (Cell Signalling, Cat. #9803) for 30 min and incubated in the presence of TNF-α (1 ng/ml, R&D, Cat. #410-MT) for 24 hours, then media and cells were collected. Medium was used for the MCP-1 ELISA assay. Cell lysates were used for Western blotting and quantitative PCR.

Realtime Polymerase Chain Reaction:

mRNAs were extracted from aortas or cell lysates using RNeasy Fibrous Tissue Mini kits (Qiagen) or RNeasy Mini kits (Qiagen), respectively. Reverse transcription was performed using iScript cDNA synthesis kit (Bio Rad). PCR reactions were performed with an ABI Step One Real-Time PCR System (Applied Biosystems) using Fast SYBR Green Real time PCR Mixture (Applied Biosystems). Primers for *Ccl2/Mcp-1*, *Il-1β*, *Cox2*, *iNos*, *Spp1*, *Icam-1*, *Mmp-2*, *Mmp-9* and *18s* were available commercially (Takara Bio Inc.). Each sample was normalized to values for *18s* mRNA expression (ΔΔCT method).

Western Blotting:

Cultured endothelial cells were placed in lysis buffer (Cell Signaling, Cat #9803). Samples were loaded onto 10 % SDS-PAGE and transferred to PVDF membrane, immunoblotted with primary antibodies (ICAM-1, R&D, Cat.#AF796 and β-actin, Sigma, Cat.#A1978), followed by secondary antibodies conjugated with HRP. Bands were detected by use of an enzyme-linked chemiluminesence detection kit (Merck Millipore). The density was quantified with luminescent image analyzer (Fujifilm) and normalized to β-actin.

MCP-1 ELISA assay:

MCP-1 in culture media was quantified with a sandwich ELISA technique using the Quantikine Mouse/Rat CCL2/JE/MCP-1 immunoassay kit (R&D, Cat.#EMJ 00) according to the manufacturer's instructions.

Statistics:

All plot and bar graphs were created with SigmaPlot v11.0 (Systat Software Inc.). All statistical analyses were performed using SigmaStat v3.5, incorporated into SigmaPlot v11.0. Data are presented as mean ± standard errors (SE). Statistical significance between multiple groups was assessed by Two Way analysis of variance

(ANOVA) with Holm-Sidak post hoc or One Way ANOVA with Student–Newman-Keuls post hoc, One Way ANOVA with Tukey's post hoc, One Way ANOVA on Ranks with a Dunn's post hoc, where appropriate. Percentage incidence and mortality of AAA were analyzed by Fisher's Exact test.-A p value less than 0.05 was considered statistically significant.

Results

Cilostazol had no effect on body weight, pulse rate, blood pressure and lipid concentrations

Male, 8-12 week-old, apoE -/- mice were fed either control or cilostazol-containing (0.1 %wt/wt) diet. After 1 week of cilostazol administration, either saline or AngII (1,000 ng/kg/min) were infused for 28 days. Plasma cilostazol concentrations in mice fed cilostazol-containing diet were 198 ± 10 ng/ml; while being undetectable in mice fed a control diet. AngII infusion had no effect on plasma cilostazol concentrations. The plasma cilostazol concentrations in mice were equivalent to plasma concentrations 12 to 24 hours after oral administration of 100 mg in humans. There were no significant differences in body weight and pulse rate in saline or AngII infusions among the 4 groups. As expected, AngII infusion increased systolic blood pressure significantly, but there was no difference between the two groups (AngII + control diet and AngII + cilostazol containing diet; Supplemental Table I). In addition, cilostazol had no effect on plasma concentrations of total cholesterol, triglyceride, and high density lipoprotein-cholesterol in apoE -/- mice during saline or AngII infusion (Supplemental Table II).

Cilostazol attenuated formation of AngII-induced AAA but did not influence AngII-accelerated atherosclerosis

To determine the effects of cilostazol on development of AngII-induced AAAs, male apoE -/- mice fed control or cilostazol-containing diet were infused with either saline or (saline: n = 5, cilostazol: n = 6) AngII for 28 days (AngII: n = 20, AngII + cilostazol: n = 17). No mice died of aneurysm rupture in saline-infused mice. In the AngII + control diet group, 1 mouse died of thoracic aortic aneurysm (TAA) rupture and 3 died of AAA rupture. In the AngII + cilostazol group, 1 mouse died of TAA rupture and 2 mice died of AAA rupture. At the end of study, *ex vivo* suprarenal aortic width was measured (Fig. 1A). In saline-infused mice, cilostazol had no effect on *ex vivo* suprarenal aortic width (mean width of abdominal aorta: 0.87 ± 0.04 mm in saline mice versus 0.88 ± 0.03 mm in cilostazol mice). AngII infusion significantly increased aortic width (mean width of abdominal aorta: 1.94 ± 0.16 mm, Fig. 1A, representative photo in Fig. 1B left). Cilostazol administration attenuated dilatation of aortic width and decreased AAA formation (mean width of abdominal aorta: 1.53 ± 0.17 mm, p = 0.049, Figure1A, representative photo in Fig.1B right). Based on AAA being defined as a > 50 % increase in suprarenal aorta width, the incidence of AAAs in AngII-infused mice not administered cilostazol was 79%. Cilostazol significantly decreased the incidence to 43% (p = 0.029, Fig. 1C). However, there was no significant difference in the aortic rupture rate between the AngII infused group

(mortality rate: 15% and 11%, respectively, n.s. Fig. 1D). There were no differences in atherosclerotic lesion area as evaluated by *en face* method among the 4 groups (Supplemental figure I).

Cilostazol reduced medial disruption and macrophage infiltration

To evaluate the histological characteristics of abdominal aortas, we performed Verhoeff's staining on tissue sections from suprarenal aortas. Pronounced disruptions of medial layers were observed in AngII-infused mice;, however, elastin fragmentation induced by AngII infusion were markedly reduced in mice consuming cilostazol. (Fig. 2D, E, F). Since it is known that macrophage-mediated inflammation plays an important role in AngII-induced AAA formation, we performed immunohistochemistry to determine the localization of macrophages in aortas. Immunohistochemistry of CD68 revealed increased macrophage accumulation in the region of medial disruption in aorta from AngII-infused mice, whereas less macrophage infiltration in medial layer was observed in mice consuming cilostazol (Fig. 2G, H, I).

Cilostazol reduced matrix metalloprotease activity in the aorta

Gelatin zymography was performed to detect MMP-2 and MMP-9. After 7 days of AngII infusion, aortas without any discernable aneurysms were used for gelatin zymography. Pro-form MMP-2 (65 and 70 kDa), active-form MMP-2 (58 kDa) and MMP-9 (92 kDa) were increased by AngII infusion and reduced by cilostazol administration (Fig. 3A, B, C).

Cilostazol administration reduced mRNA abundance of inflammatory cytokines in aortas

Gene expression of inflammatory cytokines such as *Ccl2/Mcp-1*, *Il-1β*, *Cox2*, and *Spp1* were increased significantly in aortas of AngII mice. Cilostazol administration reduced mRNA abundance of these molecules (Fig. 4). These results suggested that cilostazol suppressed inflammation in aortic walls including macrophage accumulation in the media, leading to the reduction of dilatation of aortic width and incidence of AAAs.

Cilostazol reduced MCP-1 and ICAM-1 expression in aortic endothelial cells

Since cilostazol decreased AAA formation in our model and is known to inhibit PDEIII function, we sought to investigate the site of PDE III expression in developing AAAs. We performed immunohistochemistry for PDEIII in the aorta. PDE III localized mainly to the intima and partly in media (Supplemental figure II). Therefore, we investigated the effects of cilostazol on aortic endothelial cells.

Endothelial cells isolated from aortas of apoE -/- mice were incubated with selected concentrations of cilostazol (0, 1, 10 μM) for 30 min, and then incubated with TNF-α (1 ng/ml) for 24 hours. mRNA was extracted for real-time PCR. TNF-α elevated *Ccl2/Mcp-1* and *Icam-1* mRNA expression; while cilostazol suppressed mRNA expression of them in a concentration-dependent manner (Fig. 5A). Furthermore, cilostazol reduced TNF-α-stimulated *Vcam-1* mRNA expression (Fig. 5B). In agreement with this endothelial cell mRNA expression, concentrations of CCL2/MCP-1 in cultured media were reduced by cilostazol in a concentration-dependent manner (Fig. 5C). By Western blotting, the abundance of ICAM-1 was increased during incubation with TNF-α but reduced by cilostazol, as expected (Fig. 5D).

Cilostazol exerted protective effects on aortic endothelial cells through PDEIII inhibition

PDEIII inhibition increases intracellular cAMP, and cAMP exerts anti-inflammatory effects through the PKA pathway in vascular endothelial cells [43]. Therefore, we hypothesized that activation of the cAMP-PKA pathway might be the target cilostazol during AAA development. To further elucidate the mechanism, we next performed an in vitro study using forskolin, an activator of adenylate cyclase, and H89, an inhibitor of protein kinase A. Endothelial cells were incubated with cilostazol (10 μM) or forskolin (10 μM), cilostazol and H89 (10 μM) for 30 min, incubated with TNF-α (1 ng/ml) for 24 hours, then mRNA was harvested from cell lysates and subjected to real-time PCR. TNF-α increased mRNA expression of *Ccl2/Mcp-1* and *Icam-1*, but they were reduced by cilostazol and forskolin. Co-incubation of H89 blunted the effects of cilostazol (Fig.6). These results demonstrated that cilostazol reduced the expression of inflammatory cytokines and adhesive molecules, such as CCL2/MCP-1 and ICAM-1, through the cAMP-PKA pathway.

Discussion

Chronic infusion of AngII into hyperlipidemic mice consistently induces AAA formation. The characteristics of this AAA model include activation of an inflammatory response and stimulation of a proteolytic cascade[11]. In the present study, we demonstrated that cilostazol attenuated AAA development and reduced AAA incidence induced by chronic AngII infusion. Furthermore, this inhibitory effect of cilostazol on AngII-induced AAA formation was, at least in part, due its anti-inflammatory effect to reduce activation of the cAMP-PKA pathway in endothelial cells.

Cilostazol is used widely as an anti-platelet drug. In addition, cilostazol has been reported to have many pleiotropic effects including anti-inflammatory, anti-proliferative and vasodilatory. Previous studies have shown that cilostazol inhibited vascular inflammation by downregulation of VCAM-1 expression in a diabetic rat model[27], and attenuated MCP-1 and MMP-9 expression in a balloon-injury model[28] in vivo. Cilostazol exerted anti-inflammatory effects by reducing MCP-1 production *in vitro*[44], TNF-α production induced by lipopolysaccharide stimulation[45], NF-kB activation and VCAM-1 expression[46]. Genetic depletion of cyclooxygenase-2 and osteopontin (*Spp1*) attenuated AngII-induced AAA formation, indicating these molecules play pivotal role in development of AAA[14, 16]. Consistent with these previous studies, in our study, gene expression of *Ccl2/Mcp-1*, *Il-1β*, *Cox2*, *Spp1* were reduced in the aortic wall during cilostazol administration, resulting in AAA attenuation.

The AngII-induced AAA model provides similar features found in human AAA including medial degeneration, inflammation, intraluminal thrombus, rupture and atherosclerosis[11]. However, AngII-induced AAAs are formed in the suprarenal aorta, which differs in location from human AAAs. In other AAA models, the elastase infusion model and $CaCl_2$ model induces AAA in infra-renal aorta. Zhang et al. reported that cilostazol retarded AAA development by inhibiting MMP activities, expression of NF-κB and oxidative stress using rat elastase AAA model[47]. Taken together, cilostazol may provide a new therapeutic option for AAA.

During development of AngII-induced AAA, an initial event is medial accumulation of macrophages, which may lead to subsequent medial rupture[7]. We investigated how cilostazol prevented vascular inflammation induced by AngII infusion. First, we investigated PDEIII localization in the murine aortic wall. PDEIII was reported previously to be localized in vascular smooth muscle cells, endothelium, and activated macrophages[21, 23, 48]. In our study, PDEIII was localized mainly in endothelial cells. Accordingly, we investigated the effect of cilostazol on endothelial cells using murine primary cultured aortic endothelial cells. In vascular endothelium,

cilostazol has been reported previously to increase NO synthesis[49] to exert anti-inflammatory effects including reduction of monocyte chemoattractant protein-1 secretion[44] and VCAM-1 expression[46]. Furthermore, it is well established that increases in intracellular cAMP promote endothelial barrier integrity[43]. We confirmed that cilostazol reduced expression of adhesion molecules and the production of inflammatory cytokines, thereby reducing the endothelial response to inflammation. Given these findings, we conclude that cilostazol, in part, reduced macrophage accumulation into the media through activation of the cAMP-PKA pathway in endothelial cells.

Matrix metalloproteinases are increased in aortic tissue from AAA patients[50-52]. Thus, these proteinases were considered to exert a pivotal role in aneurysm formation. In AngII-induced AAA, both MMP-2 and MMP-9 are also activated, and are thought to be important for AAA development[53]. Genetic disruption of MMP-9 has been reported to suppress elastase induced AAA[54]. Inhibition of MMP-2 also has been reported to attenuate AAA formation in $CaCl_2$ induced AAAs[55]. A previous experimental study using elastase induced AAA model revealed cilostazol reduced MMP-2 and MMP-9 activities in the aortic wall[47]. In our study, cilostazol administration suppressed AngII-induced MMP activity and thereby prevented matrix protein degradation and protected the structural integrity of the aorta.

Although AngII promotes atherosclerosis as well as aneurysms in hypercholesterolemic mice [7], the occurrence and degree of severity varies in previous reports [36,38,56,57]. Indeed, atherosclerosis and AAA can respond differently to specific interventions[7, 20, 36,38,56,57]. These findings imply that the mechanisms underlying atherosclerosis and aneurysms are different. In previous studies, cilostazol reduced atherosclerosis in low-density lipoprotein receptor -/- mice[58] and apoE -/- mice[59] fed a high-fat diet. However, in our study, cilostazol had no effect on AngII-accelerated atherosclerosis in the aortic arch.

AngII infusion also induces AAAs in normolipidemic mice, but the incidence is 3-4 fold lower than in hyperlipidemic mice[60]. However, there is only weak evidence correlating total cholesterol concentrations and aneurysmal growth in humans[61, 62]. In the AngII model, concentrations of plasma apolipoprotein B-containing lipoproteins are more important to AAA formation than HDL-cholesterol[63]. Treatments with statin or Omega 3 poly unsaturated fatty acids have a certain effect on aneurysm development and growth[64, 65]. Both animal and clinical studies have shown that cilostazol decreases concentrations of serum triglyceride and increases high-density lipoprotein[24]. However, in our study, cilostazol had no effect on serum lipid concentrations, implying that cilostazol attenuated AAA through other pharmacological effects. Additionally, this finding was consistent with no improvement of atherosclerosis by cilostazol administration.

Several clinical studies reported that blood pressure is considered as an important player in AAA formation and growth[66]. Several animal studies have indicated that propranolol, a β-blocker, might attenuate aneurysmal growth on the bases of its hemodynamic properties and its biochemical effect on matrix cross-linkage[67, 68]. However, clinical studies failed to show the efficacy of propranolol on aneurysmal expansion. On the other hand, cilostazol sometimes causes tachycardia in clinical situations. In our animal study, cilostazol had no effect on blood pressure and pulse rate. It is likely that pulse rate may not influence AAA formation and growth.

There are several reports regarding usefulness of other anti-platelet therapy on AAA development and progression[69-71]. Owens et al. reported platelet inhibitors, aspirin and clopidogrel reduced death from AAA rupture but had no effect on dilatation of abdominal aorta in established AngII-induced AAAs. Furthermore, they also showed that administration of aspirin and P2Y12 inhibitors were associated with reduced death in AAA patients in a human retrospective study[72]. Since cilostazol exerts its anti-platelet effect in a mechanism different from that of aspirin and clopidogrel, it may offer a beneficial effect on the reduction of aortic dilation in an established AAA model. However, further studies are warranted to test and clarify the effect of cilostazol regarding the aspect of anti-platelet effect on established AAA growth and rupture.

Conclusion

Cilostazol attenuated AngII-induced AAA formation by reducing inflammatory changes in the aortic wall. Cilostazol exerted this anti-inflammatory effect through PDEIII inhibition in aortic endothelial cells. Cilostazol could be a therapeutic agent for abdominal aortic aneurysms.

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Figures and Supplemental Files

Cilostazol Attenuates AngII-induced Abdominal Aortic Aneurysms But Not Atherosclerosis in ApoE Deficient Mice

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Running Title:
Cilostazol attenuates AngII-induced AAA (39 characters)

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Supplemental Table I. Characteristics of Mice

Study Groups		N	Body Weight (g)	Systolic Blood Pressure (mmHg)	Pulse Rate (bpm)
	Treatment				
Saline	Vehicle	6	26.9 ± 1.0	98 ± 3	621 ± 34
	Cilostazol	5	26.1 ± 1.0	97 ± 3	651 ± 21
AngII	Vehicle	16	27.3 ± 0.7	126 ± 24*	600 ± 14
	Cilostazol	14	27.7 ± 0.6	132 ± 19†	608 ± 15

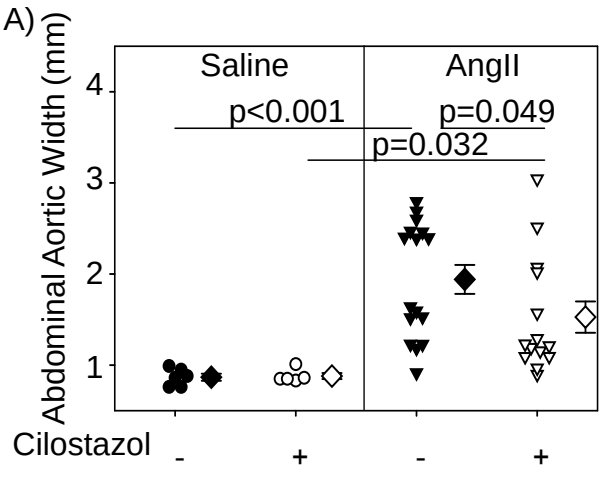
Body weight, systolic blood pressure and pulse rate were measured at the end of the study. AngII elevated systolic blood pressure in AngII and AngII + cilostazol group (*$p = 0.004$, $^{\dagger}p = 0.001$ respectively versus saline infused groups) Values are represented as mean ± SE. Statistical analyses were performed by Two Way ANOVA.

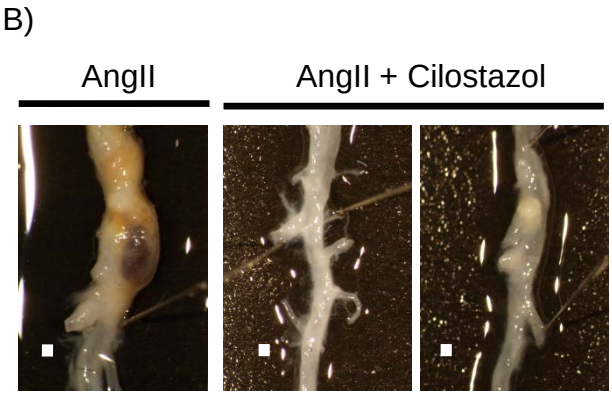
Supplemental Table II. Effects of Cilostazol on Plasma Lipid Concentration

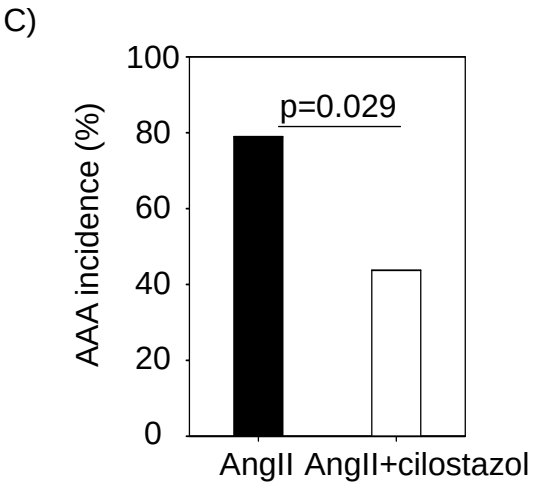
Study Groups		N	Total Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL-Cholesterol (mg/dl)
	Treatment				
Saline	Vehicle	5	484.2 ± 39.8	340.4 ± 45.9	13.0 ± 2.4
	Cilostazol	5	584.0 ± 23.1	391.0 ± 42.1	17.1 ± 2.9
AngII	Vehicle	16	536.3 ± 29.5	282.0 ± 27.5	17.1 ± 2.1
	Cilostazol	14	512.7 ± 30.7	304.6 ± 35.7	17.6 ± 1.5

Total cholesterol, triglyceride, HDL-cholesterol concentrations in plasma were measured after 16 hour food deprivation at the end of study. Values are represented as mean ± SE. No statistical differences were seen in values using Two Way ANOVA.

Figure 1


A)
Abdominal Aortic Width (mm)
Saline
AngII
p<0.001
p=0.049
p=0.032
4
3
2
1
Cilostazol
-
+
-
+


B)
AngII
AngII + Cilostazol


C)
AAA incidence (%)
100
80
60
40
20
0
p=0.029
AngII
AngII+cilostazol

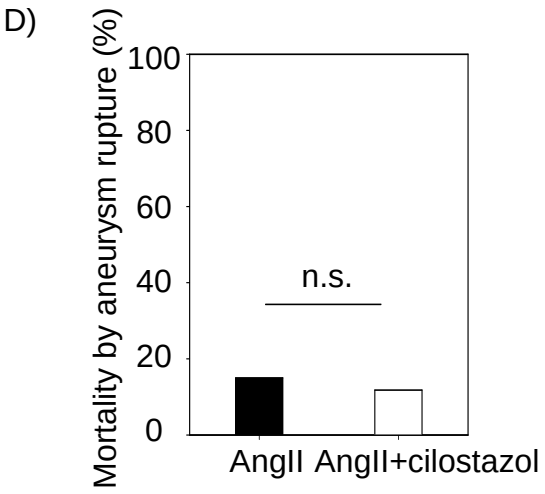

D)
Mortality by aneurysm rupture (%)
100
80
60
40
20
0
n.s.
AngII
AngII+cilostazol

Figure 2

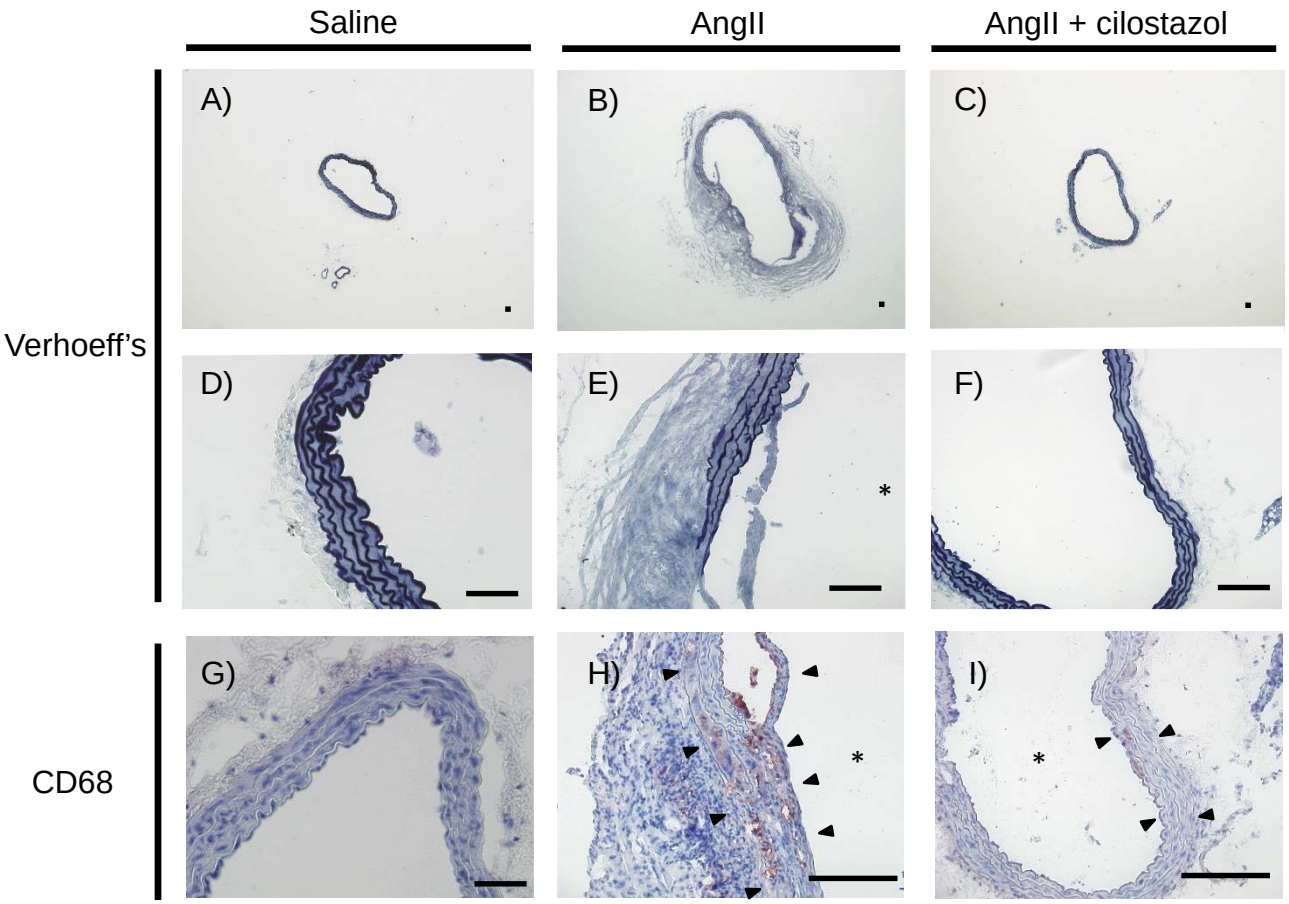
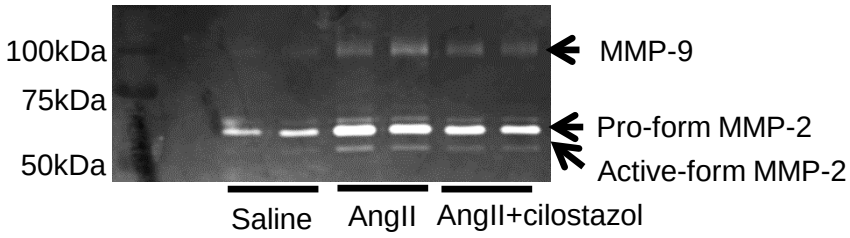


Figure 3

A)


100kDa
75kDa
50kDa
MMP-9
Pro-form MMP-2
Active-form MMP-2
Saline
AngII
AngII+cilostazol

B)

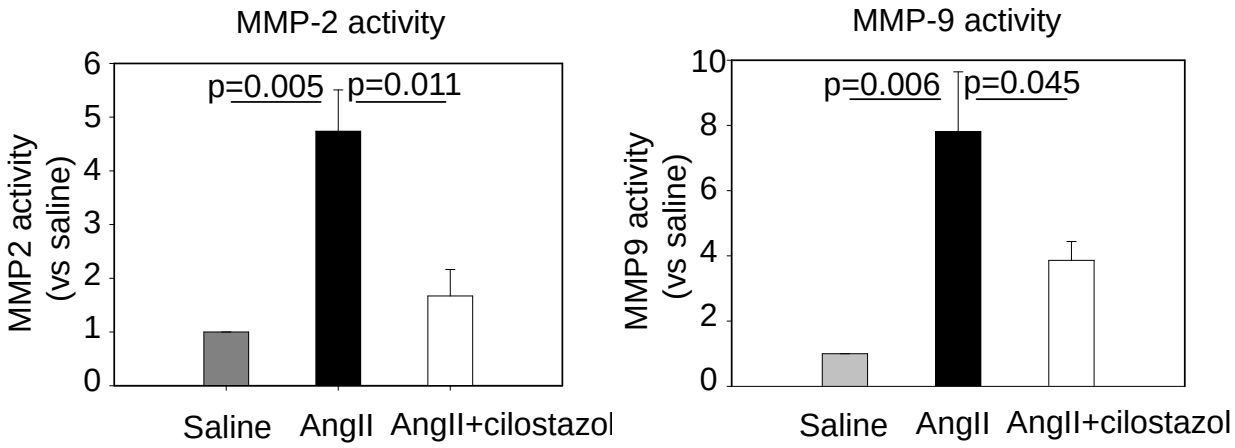

MMP-2 activity
MMP2 activity (vs saline)
p=0.005
p=0.011
0
1
2
3
4
5
6
Saline
AngII
AngII+cilostazol
MMP-9 activity
MMP9 activity (vs saline)
p=0.006
p=0.045
0
2
4
6
8
10
Saline
AngII
AngII+cilostazol

Figure 4.

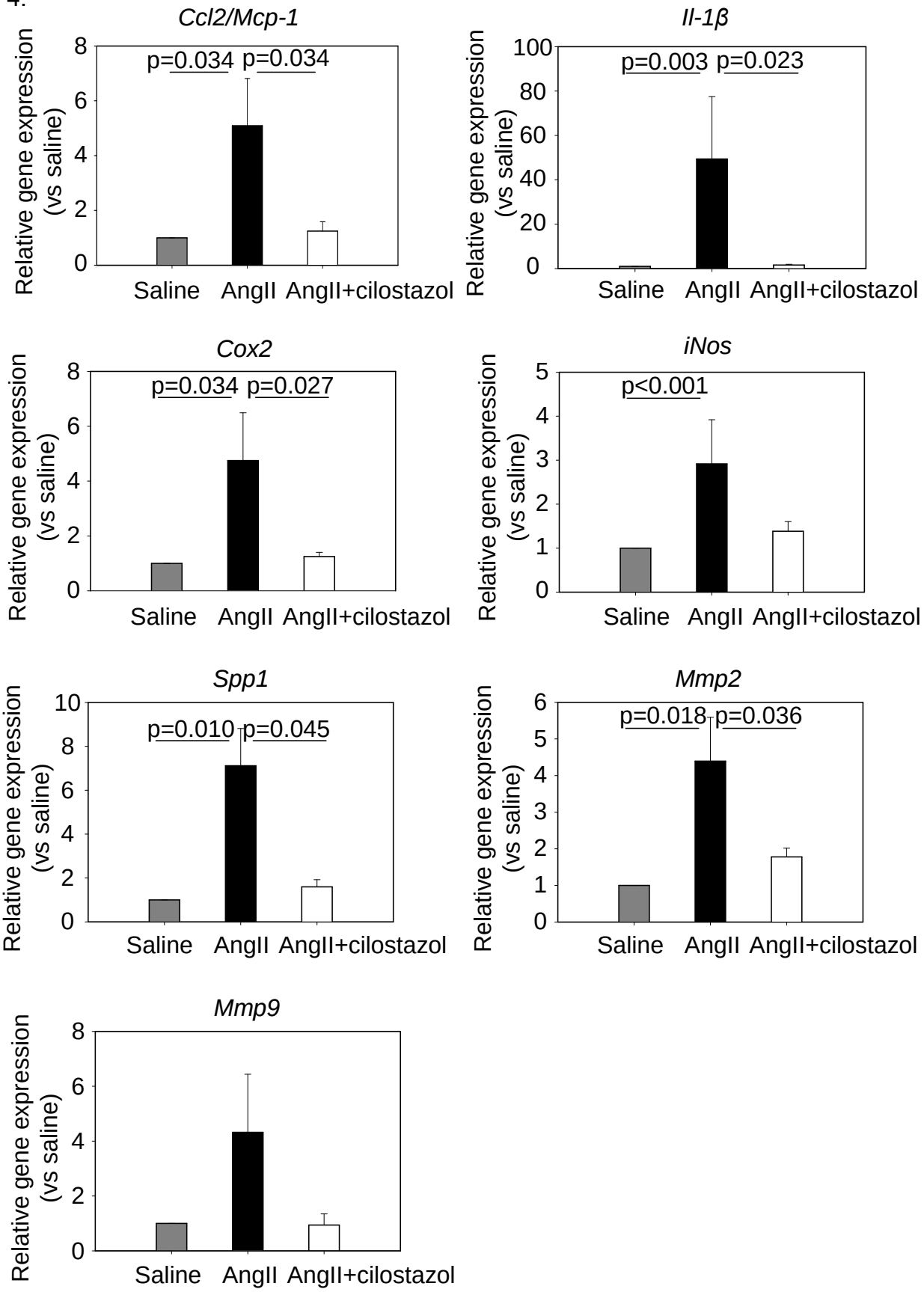

Ccl2/Mcp-1
Relative gene expression (vs saline)
p=0.034 p=0.034
Saline AngII AngII+cilostazol
Il-1β
p=0.003 p=0.023
Cox2
p=0.034 p=0.027
iNos
p<0.001
Spp1
p=0.010 p=0.045
Mmp2
p=0.018 p=0.036
Mmp9

Figure 5.

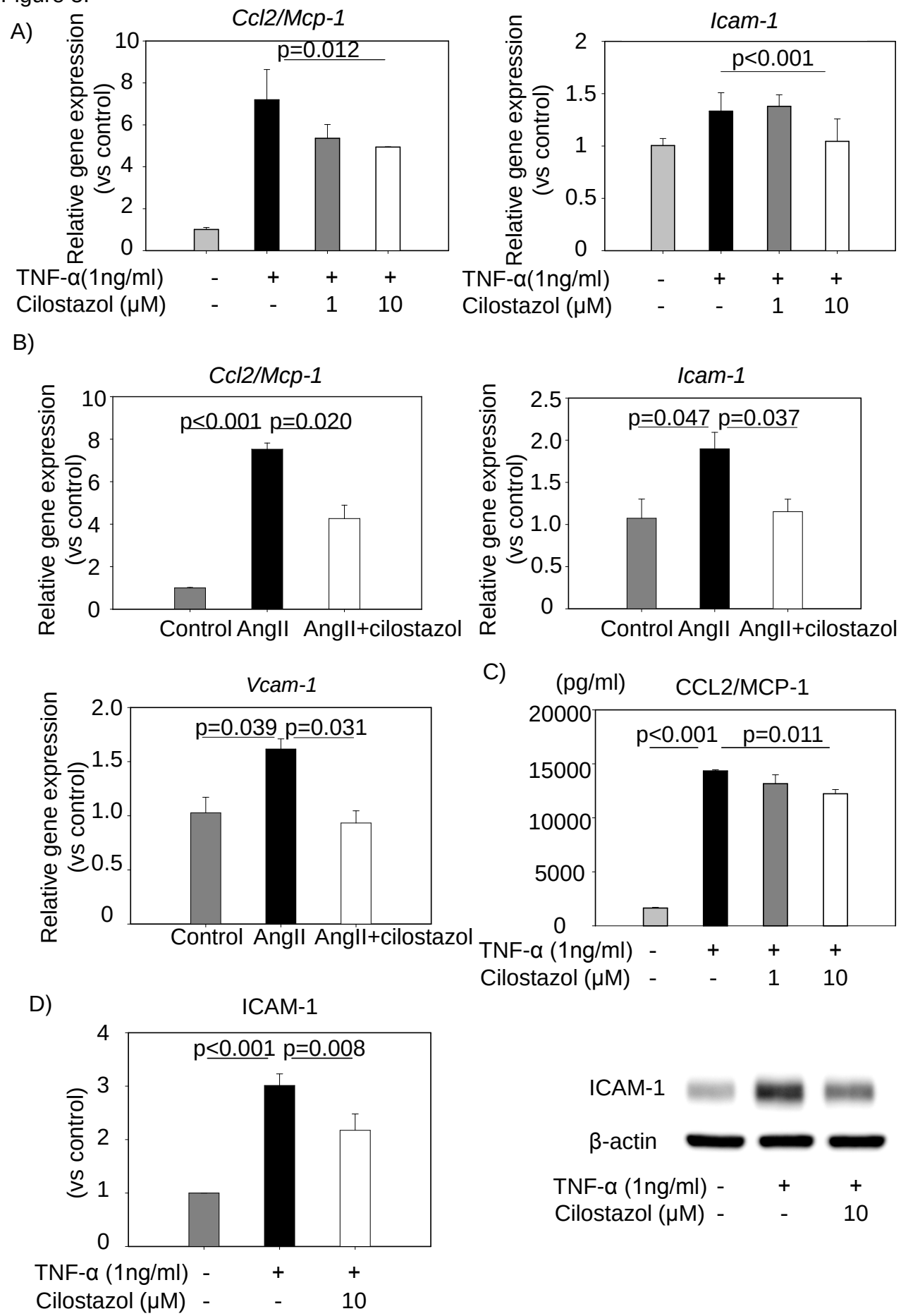

A)
Ccl2/Mcp-1
Relative gene expression (vs control)
p=0.012
TNF-α(1ng/ml) - + + +
Cilostazol (μM) - - 1 10
Icam-1
Relative gene expression (vs control)
p<0.001
TNF-α(1ng/ml) - + + +
Cilostazol (μM) - - 1 10
B)
Ccl2/Mcp-1
Relative gene expression (vs control)
p<0.001 p=0.020
Control AngII AngII+cilostazol
Icam-1
Relative gene expression (vs control)
p=0.047 p=0.037
Control AngII AngII+cilostazol
Vcam-1
Relative gene expression (vs control)
p=0.039 p=0.031
Control AngII AngII+cilostazol
C)
(pg/ml)
CCL2/MCP-1
p<0.001 p=0.011
TNF-α (1ng/ml) - + + +
Cilostazol (μM) - - 1 10
D)
ICAM-1
(vs control)
p≤0.001 p=0.008
TNF-α (1ng/ml) - + +
Cilostazol (μM) - - 10
ICAM-1
β-actin
TNF-α (1ng/ml) - + +
Cilostazol (μM) - - 10

Figure 6.

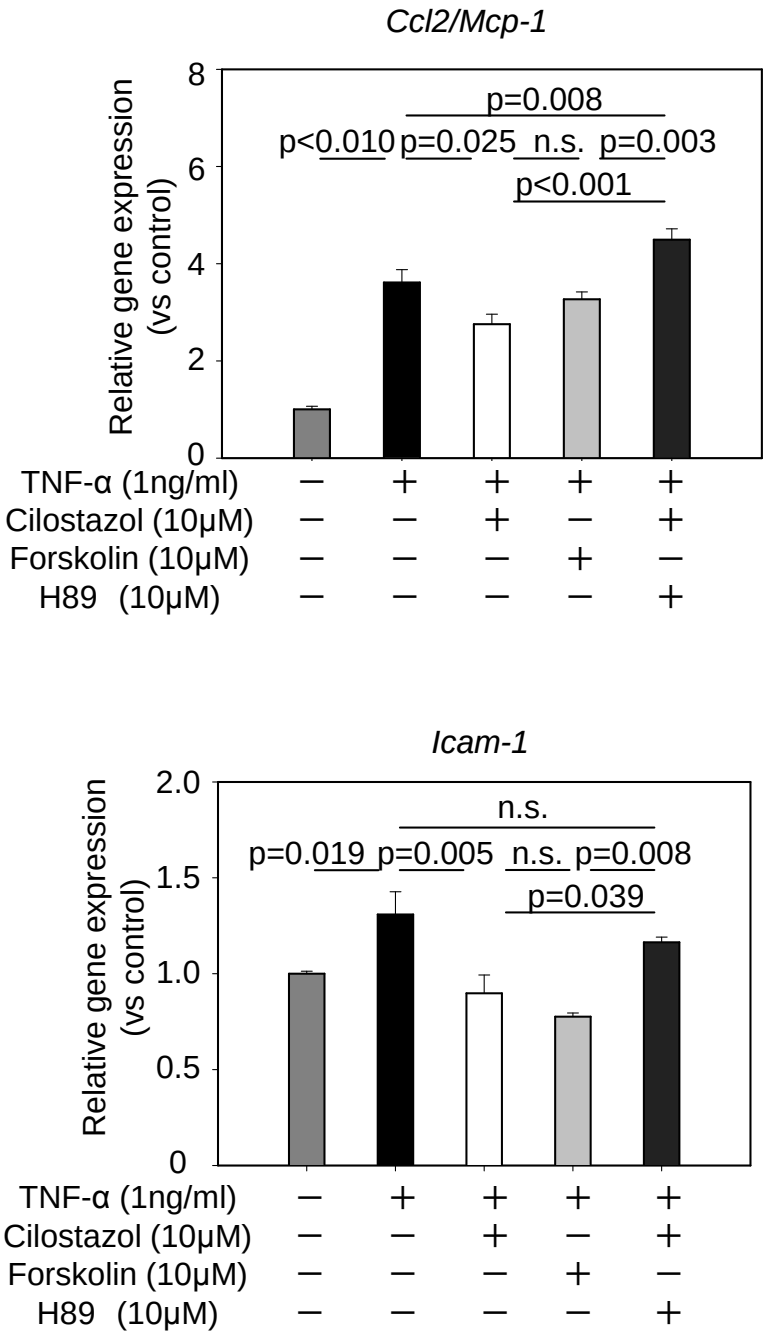

Ccl2/Mcp-1
Relative gene expression (vs control)
8
6
4
2
0
p=0.008
p<0.010
p=0.025
n.s.
p=0.003
p<0.001
TNF-α (1ng/ml) − + + + +
Cilostazol (10μM) − − + − +
Forskolin (10μM) − − − + −
H89 (10μM) − − − − +
Icam-1
Relative gene expression (vs control)
2.0
1.5
1.0
0.5
0
n.s.
p=0.019
p=0.005
n.s.
p=0.008
p=0.039
TNF-α (1ng/ml) − + + + +
Cilostazol (10μM) − − + − +
Forskolin (10μM) − − − + −
H89 (10μM) − − − − +

Figure Legends

Figure 1. Cilostazol attenuated AngII-induced AAA formation.

A) AngII infusion significantly increased ex vivo maximal diameters of abdominal aortas from AngII and AngII + cilostazol mice ($p < 0.001$ and $p = 0.032$, respectively when comparing saline infused group); while cilostazol attenuated enlargement of aortic diameter ($p = 0.049$ when comparing the AngII-infused group). Aortic diameter measurements from each mouse are represented by a circle or an inverted triangle (black circles represent saline-infused mice without cilostazol administration, white circles represent-saline-infused mice with cilostazol administration, black triangles represent AngII-infused mice without cilostazol administration, white triangles represent AngII-infused mice with cilostazol administration, diamonds represent means, and bars represent SE. Statistical analysis was performed by two-way ANOVA.

B) Representative images of abdominal aortas from experimental mice infused with AngII and fed control (left) or cilostazol-containing diets (right).

C) The incidence of AAA (> 50% increase in aortic width) was 79% in AngII mice (black bar), and was decreased significantly to 43% in AngII + cilostazol mice (white bar). Statistical analyses were performed by Fisher's Exact test ($p = 0.029$).

D) The mortality ratio from AAA rupture in AngII-infused mice is shown in black bars, and the mortality ratio in AngII-infused mice administered cilostazol are shown in white bars. There was no significant difference in mortality from aneurysm rupture between these two groups by Fisher's exact test.

Figure 2. Cilostazol reduced elastin degradation and macrophage accumulation in the aortic media.

Elastic fibers were stained with Verfoeff's staining in tissues sections of suprarenal aortas (A-F). A, D, G were samples from saline-infused mice. B, E, H were samples from AngII-infused mice. C, F, I were samples from AngII-infused + cilostazol mice.

Disruption of elastic fibers and enlargement of aortic diameters was seen in aortas from AngII-infused mice (B, E). Degradation of elastic fibers and enlargement of aortic diameters were reduced in aorta from AngII-infused + cilostazol mice (C, F).

Representative immunostaining images of macrophages with an anti-mouse CD68 antibody (G-I). Macrophages were localized predominantly in the media of suprarenal aortas from AngII-infused mice (H), but were only seen rarely in aortas from AngII-infused + cilostazol mice (I). * indicates the lumen of aorta, arrow head indicates the perimeter of the media. (bar = 200 μm)

Figure 3. Cilostazol reduced enzymatic activity of MMP-2 and -9 in the aorta.
A) Gelatin zymography detected MMP-2 and MMP-9 activity in tissue extracts from aortas (n = 3, each group). Pro-form MMP-2 was detected in the murine aorta without neither AngII nor cilostazol administration. Pro-form and active-form of MMP-2 (72 and 59 kDa), MMP-9 (92 kDa) were detected in AngII-infused mice. Activities of MMP-2 and MMP-9 were reduced in aortas from AngII + cilostazol mice. Cilostazol diminished AngII-induced MMP-2 and MMP-9 activation.
B) Quantitative data for MMP-2 and MMP-9 activity. Each bar represents the mean enzymatic activity of MMP-2 (top graph) or MMP-9 (bottom graph) in aortas from saline mice (gray bar), AngII mice (black bar), and AngII + cilostazol mice (white bar). AngII-infusion increased MMP-2 and MMP-9 activity ($p = 0.005$, $p = 0.011$ when compared to saline-infused mice, respectively); cilostazol reduced the abundance ($p = 0.006$, $p = 0.045$). Statistical analysis was performed by One Way ANOVA.

Figure 4. Cilostazol reduced relative mRNA abundance of inflammatory cytokines and MMPs in aortas.

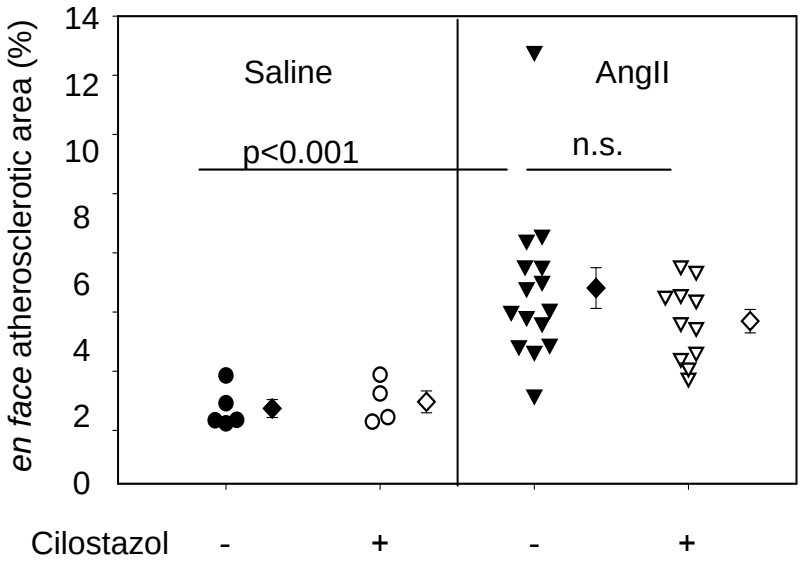
Aortas were harvested after 1 week of AngII infusion, with or without cilostazol (n = 5-7). Each bar shows relative gene expression normalized to 18S in saline mice (gray bar), AngII-infused mice (black bar), and AngII-infused + cilostazol mice (white bar). These inflammatory cytokines and MMPs were elevated in aortas from AngII-infused mice, but were reduced by cilostazol administration. Statistical analyses were performed by One Way ANOVA.

Figure 5. Cilostazol reduced mRNA abundance of inflammatory cytokines and adhesion molecules in cultured aortic endothelial cells isolated from apoE -/- mice.
A) mRNA abundance of *Ccl2/Mcp-1* and *Icam-1* were increased by TNF-α incubation (1 ng/ml, 24 hours), but suppressed by cilostazol (1 μM, 10 μM) in a concentration-dependent manner (n = 3 in each group). Statistical analyses were performed by One Way ANOVA.
B) Quantitative mRNA abundance of inflammatory cytokines and adhesion molecules in aortic endothelial cells (n = 3 in each group). Expression of *Ccl2/Mcp-1*, *Icam-1* and *Vcam-1* were upregulated by TNF-α stimulation (black bar), and were significantly reduced by cilostazol incubation (white bar). Statistical analyses were performed by One Way ANOVA.
C) CCL2/MCP-1 concentrations in culture medium were measured by using a commercial ELISA kit. Aortic endothelial cells from apoE -/- mice were incubated with the indicated conditions for 24 hours. Cilostazol (10 μM) administration reduced

CCL2/MCP-1 in culture medium. Statistical analyses were performed by One Way ANOVA.
D) ICAM-1 expression in aortic endothelium was evaluated by Western blotting (n = 3 in each group). The relative abundance of ICAM-1 protein was quantified by densitometry and normalized to the abundance under the control condition. Statistical analyses were performed by One Way ANOVA.

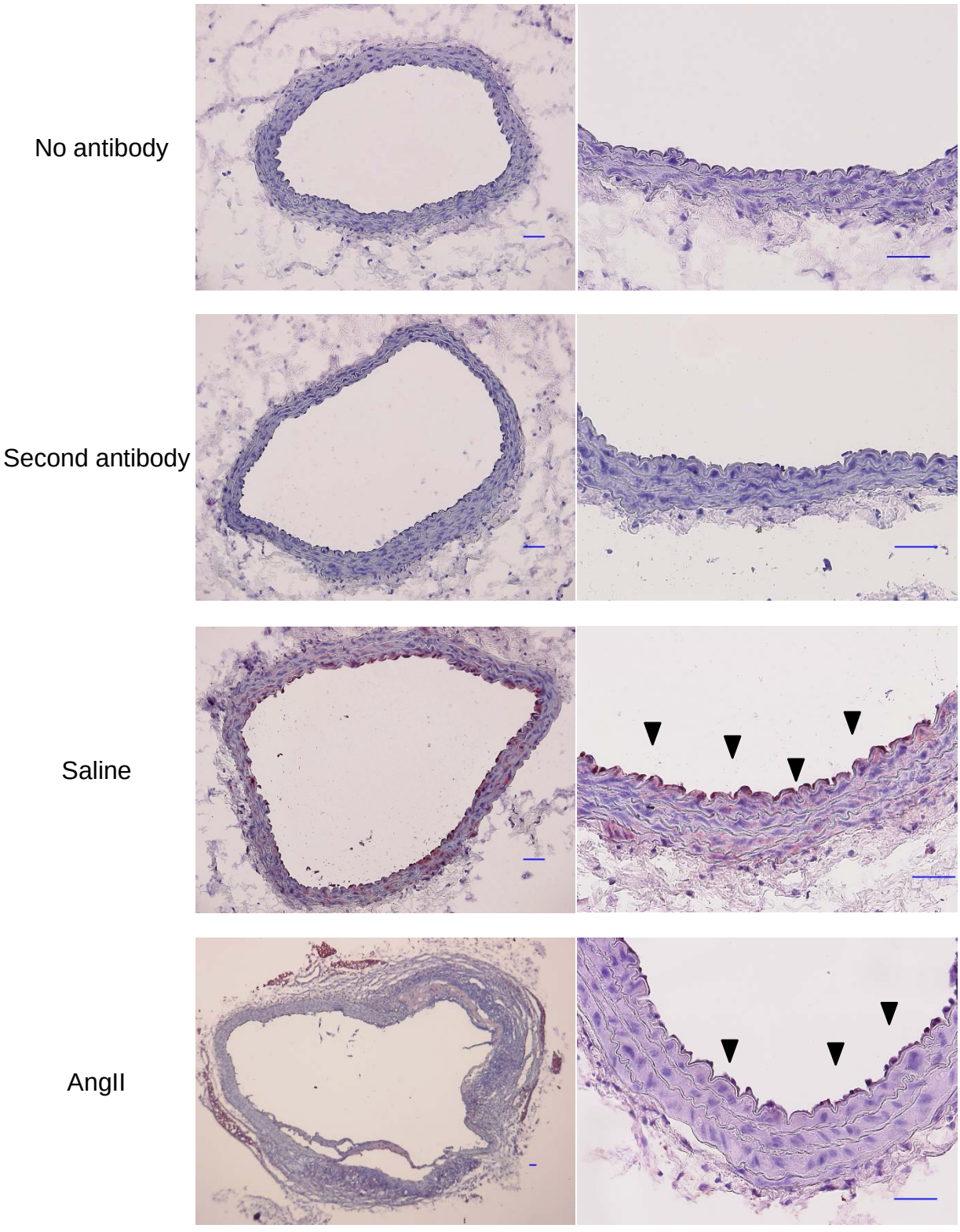
Figure 6. Cilostazol suppressed Ccl2/Mcp-1 and Icam-1
mRNA abundancethrough the cAMP-PKA pathway in aortic endothelial cells isolated from apoE -/- mice. Aortic endothelial cells were incubatedwith indicated conditions (n = 3 in each group). The effects of cilostazol were mimicked by forskolin (10 μM), an activator of adenylate cyclase, and were abrogated by H89 (10 μM), an inhibitor of PKA. Statistical analysis was performed by One Way ANOVA.

Supplemental Figure I.



Supplemental Figure I. Cilostazol did not reduce atherosclerosis induced by AngII infusion. While AngII infusion significantly increased *en face* atherosclerosis area, cilostazol had no effect on lesion areas. Values for each individual mouse is represented by a circle or an inverted triangle (closed circle represents saline-infused mice, closed circle represents cilostazol mice, closed triangle represents AngII-infused mice, opened triangle represents AngII-infused + cilostazol mice); diamonds represent means and bars represent SE. Statistical analysis was performed by Two Way ANOVA.

Supplemental Figure II.



Supplemental Figure II. Localization of PDEIII in aortic wall
A) Immunostaining of PDEIII in tissuesections of abdominal aorta from apoE -/- mice, with or without AngII-induced AAA. No positive staining was observed in negative controls (no antibody; used neither 1st antibody nor 2nd antibody, 2nd antibody; used only 2nd antibody without 1st antibody), PDEIII were expressed mainly in aortic intima (arrow head). (bar = 50 μm)