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Effects of Caspase Inhibitor on Angiotensin II-Induced Abdominal Aortic Aneurysm in Apolipoprotein E-Deficient Mice

Dai Yamanouchi; Stephanie Morgan; Kaori Kato; Justin Lengfeld; Fan Zhang; Bo Liu

Objective—The presence of apoptotic markers is a prominent histological feature of abdominal aortic aneurysm. To understand the role of apoptosis in the pathogenesis of this common vascular disease, we tested the effect of the pan-caspase inhibitor quinoline-Val-Asp-difluorophenoxymethylketone (Q-V_D-OPh) on aneurysm formation using a mouse angiotensin II (Ang II) model.

Methods and Results—Ang II in apolipoprotein E-deficient mice significantly induced medial cell apoptosis 3 days after infusion at the aortic region, eventually becoming aneurysmal. A daily administration of 20 mg/kg per day Q-V_D-OPh starting 6 hours before Ang II infusion reduced aneurysm incidence from 83.3% to 16.7% and maximal aortic diameter from 2.43±0.29 mm to 1.58±0.18 mm. The caspase inhibitor treated mice showed profoundly diminished levels of medial apoptosis and inflammation. In contrast, administration of Q-V_D-OPh starting 7 days after Ang II infusion had no significant impact on aneurysm development. In vitro, media conditioned by Ang II-treated smooth muscle cells (SMCs) stimulated macrophage chemotaxis in a caspase-dependent manner. Inhibition of monocyte chemoattractant protein-1 (MCP-1) in the conditioned media via a neutralizing antibody completely blocked the ability of conditioned media to attract macrophages.

Conclusions—These results indicate that medial SMC apoptosis may contribute to vascular inflammation and thus aneurysm formation, in part through production of MCP-1. (*Arterioscler Thromb Vasc Biol.* 2010;30:00-00.)

Key Words: apoptosis ■ aneurysms ■ angiotensin II ■ MCP-1 ■ migration

Abdominal aortic aneurysm (AAA) is a common age-related degenerative disease associated with high mortality. Histologically, AAA is characterized by destruction of the extracellular matrix accompanied by a depletion in medial vascular smooth muscle cells (SMCs), infiltration of lymphocytes and macrophages, and neovascularization.^{1,2} Selective depletion of neutrophils,³ lymphocytes,⁴ matrix metalloproteinases,^{5,6} or proinflammatory cytokines⁷ has been found to impair aneurysm formation in experimental models of AAAs, demonstrating the multifactorial nature of this disease.

Histological examinations of both animal and human experimental AAAs have revealed a paucity of medial SMCs in these specimens.⁸⁻¹⁰ In addition, many medial SMCs in human AAA specimens bear markers of apoptosis and demonstrate upregulation of proapoptotic initiators, such as FAS/FASL.⁹ Because medial SMCs are major sources of extracellular matrix proteins, it was postulated that depletion of medial SMCs may also make an important contribution to aneurysm by eliminating a cell population capable of directing connective tissue repair.

Apoptosis, the ordered dismantling of cells, is a multistep process that is centered by the activation of caspases, a group of structurally related cysteine proteases. Caspases can be divided into 3 groups based on their biological functions: apoptosis initiation, apoptosis execution, or cytokine activation. All caspases are synthesized and maintained as zymo-

gens, which give rise to the active forms through limited proteolysis.¹¹ In mammalian cells, apoptosis can be initiated through 2 main pathways. The extrinsic pathway involves the activation of transmembrane death receptors, such as Fas or tumor necrosis factor- α receptor, by their respective ligands, leading to activation of caspase 8. The intrinsic pathway, secondary to DNA damage or oxidative stress, involves mitochondrial depolarization that leads to the release of cytochrome *c* and activation of caspase 9. Both pathways ultimately result in activation of caspase 3, which then leads to the execution of apoptosis, including the cleavage of cell proteins, subsequent DNA fragmentation, and cell death.¹²

Multiple factors identified in human aneurysmal tissues can potentially activate the extrinsic pathway (FasL and tumor necrosis factor- α)⁹ or the intrinsic pathway (oxidative stress).¹³ In addition, activated caspase-9, the key initiator of the intrinsic pathway, was documented in experimental aneurysm,¹⁴ further supporting the presence of mitochondrial-dependent apoptosis during aneurysm development. The integral relationship between SMC apoptosis and aneurysm is further demonstrated by studies in which apoptosis was attenuated by experimental approaches such as the removal of mast cells,¹⁴ blockage of the angiotensin II (Ang II) signaling,¹⁵ and inhibition of Rho GTPase.¹⁶ However, whether and how SMC apoptosis contributes to aneurysm formation has not been directly addressed.

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In the present study, we inhibit apoptosis with a broad-spectrum caspase inhibitor, quinoline-Val-Asp-difluorophenoxymethylketone (Q-VD-OPh), in the Ang II infusion model of aneurysm. Using a combination of in vitro and in vivo approaches, we examined the potential interplay between SMC apoptosis and aneurysm formation.

Materials and Methods

Animal Model

Male, 24-week-old, apolipoprotein E-deficient (apoE KO) mice with a C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, Maine). All mice had free access to a normal diet and water. Ang II (1000 ng/kg per minute) or saline was administered subcutaneously by Alzet osmotic minipump (model 2004; Alzet, Cupertino, Calif) for up to 4 weeks.¹⁷ In the study using the caspase inhibitor Q-VD-OPh (BioVision, CA), mice were randomly selected to receive either the inhibitor or dimethyl sulfoxide (DMSO). Q-VD-OPh at 20 mg/kg per day or DMSO was administered daily via intraperitoneal injection to the Ang II-treated mice 6 hours before the beginning of the Ang II infusion or 7 days after it. The external aortic diameter was measured at the region showing maximum dilation with a digital caliper (VWR Scientific, West Chester, Pa). Aneurysm incidence is defined as an increase of 50% or greater in the external width of the suprarenal aorta as compared with that of the infrarenal region. At selected time points, mice were killed by an overdose of pentobarbital and were perfusion-fixed with a mixture of 4% formaldehyde in PBS at physiological perfusion pressure. The observers were blinded to treatment allocation. All experiments were conducted in accordance with experimental protocols that were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin Madison (Protocol M02284).

Statistical Analysis

Values were expressed as means \pm standard error. Experiments were repeated at least 3 times unless stated otherwise. Differences between 2 groups were analyzed by the Student *t* test. One-way analysis of variance (ANOVA) was used for analysis of the effects of Ang II on SMC apoptosis, and 2-way ANOVA was used for other multiple comparisons. Scheffe test was used as a post hoc test. Values of $P < 0.05$ were considered significant.

All other methods are detailed in the Supplemental Materials and Methods, available online at <http://atvb.ahajournals.org>.

Results

Apoptosis in Ang II-Induced Aneurysm

To characterize apoptotic events during the development of aneurysm, we conducted a time course study using an Ang II-induced AAA model. apoE KO mice were implanted with osmotic pumps containing either Ang II (1000 ng/kg per minute) or saline, and mice were euthanized at selected time points. In contrast to the saline control, infusion of Ang II led to rapid changes in the suprarenal region of abdominal aorta that subsequently became aneurismal (Figure 1A). At day 3, our earliest time point, most Ang II-treated mice showed grossly visible vascular hematomas, presumably resulting from medial dissection.¹⁸ Seven days after Ang II infusion, the maximal external diameter of suprarenal aorta became significantly larger than that of saline-treated controls (saline: 1.1 ± 0.1 at 28 days; Ang II: 1.6 ± 0.44 , $2.2 \pm 0.28^*$, and $2.5 \pm 0.24^*$ mm at 3, 7, and 28 days, respectively; $*P < 0.05$). By 28 days, 80% of Ang II-treated mice showed aneurysm formation, which was defined as a $\geq 50\%$ increase in external aortic diameter compared with that of the infrarenal region (Figure 1B). Immunohistological analysis showed a profound

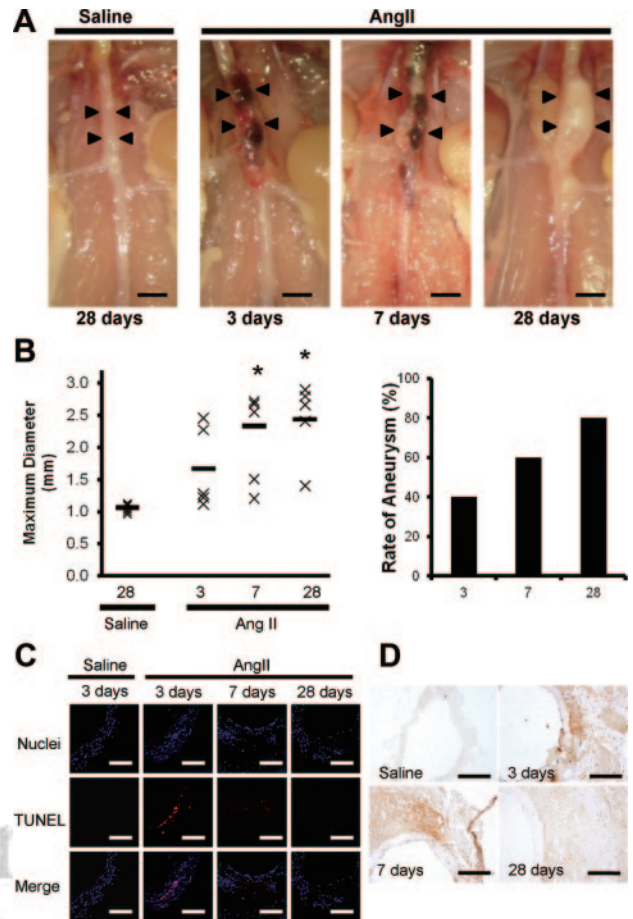


Figure 1. Medial apoptosis occurs early during the development of experimental aneurysm. A, Representative pictures of aortas removed from Ang II- or saline-infused mice 3, 7, and 28 days after pump implantation in apoE KO mice. Scale bars=2 mm. B, Maximum diameter of the suprarenal aorta of Ang II-infused or saline infused mice after 3, 7, and 28 days. The rate of AAA formation, defined as a $\geq 100\%$ increase in external aortic diameter compared with that of the infrarenal region, is also shown. $n=5$, $*P < 0.05$ compared with saline. C, Representative micrographs of TUNEL staining (red) and nuclei (blue) of aortic sections. Merged images are shown in lower panels. Scale bars=500 μm . D, Representative pictures of immunostaining using an anti-Mac3 antibody of aortic sections from saline-infused (28 days) or Ang II-infused (3, 7, and 28 days) apoE KO mice. Scale bars=250 μm .

presence of TUNEL positivity in aortic media at day 3 in Ang II-infused mice (Figure 1C). Although a significant amount of TUNEL signal persisted to day 7, most of the apoptotic cells were localized in adventitia at this point. The detection of TUNEL positivity was not restricted to the region of aortic dissection. To confirm that the TUNEL positivity detected in tunica media resulted from apoptosis of vascular SMCs, we costained the tissue sections for smooth muscle-specific α -actin. As shown in Supplemental Figure I, TUNEL staining colocalized with α -actin positivity. Furthermore, immunostaining for a macrophage marker (Mac3) showed a remarkable increase in the number of infiltrated macrophages in the aortic wall 3 and 7 days after pump implantation (Figure 1D).

In Vivo Inhibition of Apoptosis

Because robust apoptosis occurs during the early phase of aneurysm development, we hypothesized that blockage of apo-

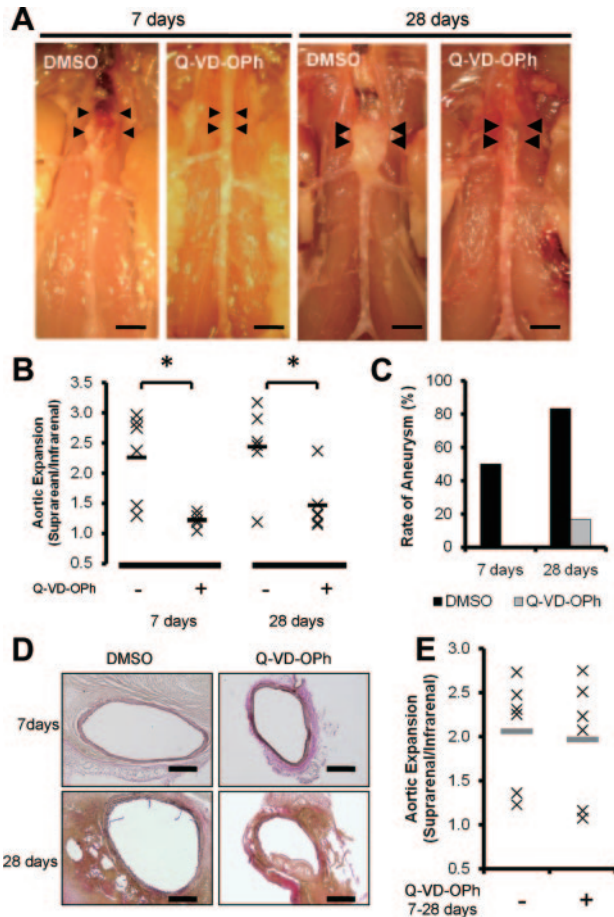


Figure 2. Inhibitor of caspase attenuates aneurysm formation induced by Ang II. A, Representative pictures of the aortas removed from Ang II-infused mice treated with Q-Vd-Oph or DMSO. Scale bars=2 mm. B and C, Morphological analyses. Aortic expansion, expressed as a ratio of the maximum diameter of the suprarenal aorta to the infra renal aorta (B) and rate of aneurysm (C) of each group are shown. n=6, **P*<0.05. D, Representative pictures of elastin staining of Q-Vd-Oph- or DMSO-injected Ang II-infused apoE mice at 7 or 28 days. Scale bars=500 μ m.

ptosis may attenuate pathogenesis of this disease process. To test this hypothesis, we used Q-Vd-Oph, a broad-spectrum inhibitor of caspases known to block apoptosis mediated by both extrinsic and intrinsic pathways.¹⁹ Indeed, an *in vitro* apoptosis assay using cultured mouse aortic SMCs stimulated with hydrogen peroxide (H₂O₂), a well-known apoptotic stimulus, confirmed the potency of this inhibitor in vascular SMCs (Supplemental Figure II, available online at <http://atvb.ahajournals.org>). Next, we administered Q-Vd-Oph (20 mg/kg per day) or DMSO via daily intraperitoneal injection to apoE KO mice starting 6 hours before Ang II infusion. Mice were euthanized 3, 7, or 28 days later. Compared with DMSO-injected mice, Q-Vd-Oph-treated mice showed remarkably impaired aneurysm formation (Figure 2A), reflected by a reduced AAA incidence (from 50% to 0% at day 7 and from 83.3% to 16.7% at day 28, respectively) (Figure 2B and Supplemental Figure III). Consistently, the maximal diameter of the suprarenal aorta was also significantly decreased by Q-Vd-Oph (Figure 2B and 2C). At day 7, vascular hematoma was noticed in only 1 of the 6 Q-Vd-Oph-treated mice, whereas 4 of 6 mice in the DMSO group showed substantial aortic

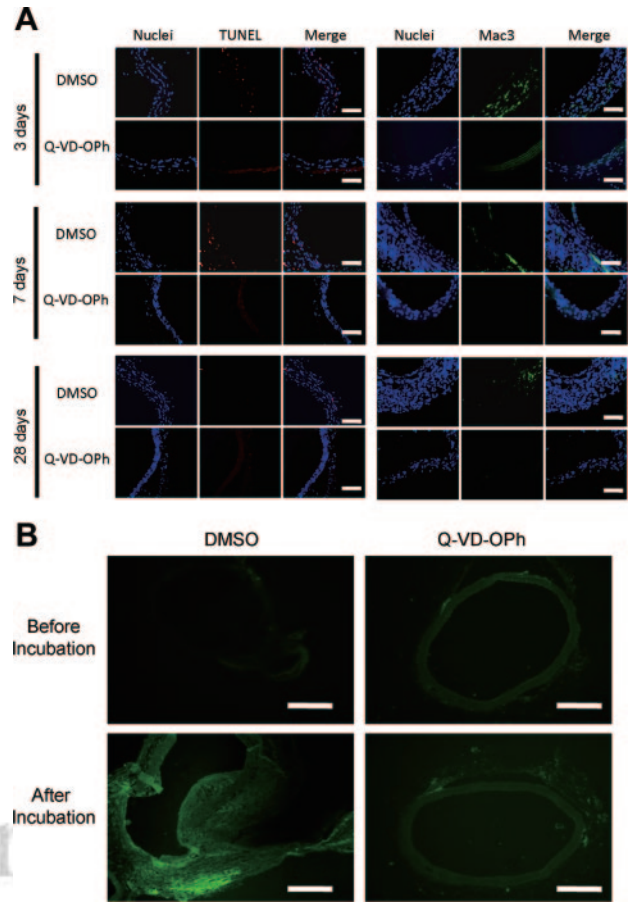


Figure 3. Q-Vd-Oph blocks aortic SMC apoptosis, macrophage invasion and elastase activation. A, Representative pictures of TUNEL staining and immunostaining for Mac3. Aortic sections of Q-Vd-Oph- or DMSO-treated Ang II-infused apoE KO mice at 3, 7, and 28 days were stained for TUNEL or anti-Mac3. Scale bars=50 μ m. n=6. B, Representative pictures of in situ zymography. Aortic sections of Q-Vd-Oph- or DMSO-treated Ang II-infused apoE KO mice at 7 days were freshly frozen and incubated with DQ elastin for 48 hours. The fluorescent intensity was obtained before and after incubation. Scale bars=200 μ m. n=6.

hemorrhage (Supplemental Figure III). In tissue cross-sections, the Ang II-induced expansion of both luminal area and external diameter of the aorta was completely eliminated by Q-Vd-Oph at day 7 and greatly reduced at day 28 (Figure 2D).

To test whether the pan-caspase inhibitor affects aneurysm growth, we began the Q-Vd-Oph treatment 7 days after Ang II infusion, a time point at which TUNEL positivity had begun to subside. In contrast to what we observed with the early drug application, the delayed administration of Q-Vd-Oph had no significant impact on aneurysmal expansion measured at day 28 (Figure 2E).

Caspase Inhibitor Diminishes Both Aortic Apoptosis and Macrophage Filtration

Further immunohistological analysis of the aortic tissues harvested after 3, 7, or 28 days of Ang II infusion confirmed that Q-Vd-Oph significantly inhibited apoptosis (Figure 3A). At day 3, the percentage of TUNEL-positive cells detected in the aortic wall was reduced from 14.8 \pm 5.4% in the DMSO control group to 0.8 \pm 0.1% in the inhibitor group (*P*<0.01;

n=6). Furthermore, Q-Vd-OPh profoundly reduced the level of macrophage infiltration, as was made evident by the nearly undetectable positivity of Mac3 in mice treated with this inhibitor (Figure 3A). Immunostaining with an anti-CD3 antibody suggests that infiltration of T cells was also decreased by the Q-Vd-OPh treatment (Supplemental Figure IV). In agreement with the diminished inflammation, arteries derived from the Q-Vd-OPh-treated mice showed profound reduction in immunostaining to interleukin-6 compared with arteries from the DMSO-treated mice (Supplemental Figure V).

We also examined the effect of the caspase inhibitor on neutrophil infiltration. At the time points of our study (days 3, 7, and 28 post-Ang II infiltration), we did not detect the significant presence of neutrophils (data not shown). It is likely that the influx of neutrophils occurs at earlier time points.

Because infiltrated macrophages are thought to be the major source of elastase activity in aneurismal tissues, we next performed an *in situ* zymography assay using aortic samples harvested from mice treated with Ang II for 7 days. Freshly frozen aortic sections were incubated with DQ elastin. Elastase activity, reflected by the fluorescence signal generated during substrate degradation, was recorded before incubation (0 hours) or after incubation (48 hours, 37°C). Arteries derived from DMSO-injected, Ang II-infused mice showed a noticeable induction of elastase activity (Figure 3B). In contrast, elastase activity was barely detectable in Q-Vd-OPh-injected, Ang II-infused mice (Figure 3B).

The Effect of Pan-Caspase Inhibitor on Macrophage Migration

The diminished macrophage infiltration resulting from the Q-Vd-OPh treatment suggests that aortic SMC apoptosis may actively contribute to vascular inflammation by attracting macrophage or monocytes to the site where active apoptosis takes place. To test this hypothesis, we turned to cultured mouse aortic SMCs. We found that Ang II dose-dependently induced SMC apoptosis (Figure 4A), an effect that was completely eliminated by Q-Vd-OPh (Figure 4B). Next, we tested the effect of Ang II on migration of RAW264.7 cells, a monocyte/macrophage cell line. As shown in Figure 4C, Ang II alone had little chemotactic effect on RAW264.7 cell migration. In contrast, media conditioned by Ang II-treated SMCs prompted a significant number of RAW264.7 to migrate through a porous membrane (Figure 4C). Inhibition of caspase activity in SMCs by Q-Vd-OPh blocked this chemotactic effect of the conditioned media, suggesting an involvement of apoptosis (Figure 4D).

To identify the potential chemoattractant factors released by apoptotic SMCs, we examined the expression of proinflammatory mediators in Ang II-treated SMCs by real-time PCR. Among the 5 inflammatory cytokines that we examined, only the expression of monocyte chemoattractant protein-1 (MCP-1) was significantly induced by Ang II in a caspase-dependent manner (Supplemental Figure VI). Subsequently, we confirmed an enhanced level of MCP-1 protein in media conditioned by Ang II-treated SMCs (Figure 4E). Similar to the mRNA induction, the accumulation of MCP-1 protein from the conditioned media was significantly reduced by Q-Vd-OPh (Figure 4E). To further show that MCP-1

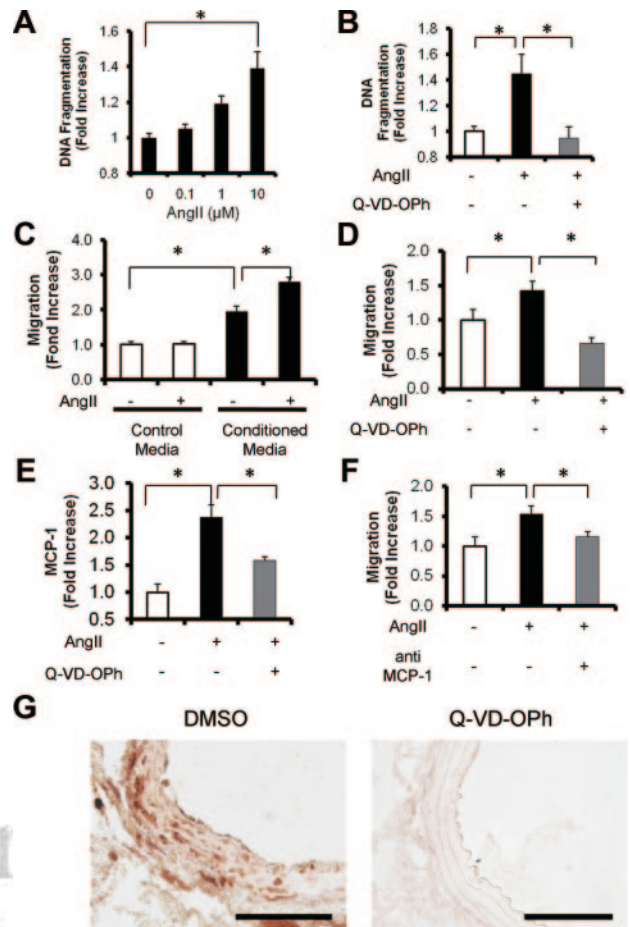


Figure 4. Apoptotic SMCs attract monocytes through release of MCP-1. A, Mouse aortic SMCs were stimulated with 0 to 10 $\mu\text{mol/L}$ Ang II for 48 hours. Apoptosis was evaluated through ELISA-measured DNA fragmentation. $*P < 0.05$. B, Mouse aortic SMCs were stimulated with 10 $\mu\text{mol/L}$ Ang II in the presence of 20 $\mu\text{mol/L}$ Q-Vd-OPh or DMSO. Apoptosis was evaluated through ELISA-measured DNA fragmentation. $*P < 0.05$. C and D, Migration of macrophages was evaluated by chemotaxis assay. Fresh media containing Ang II or media conditioned by aortic SMCs that had undergone treatment as indicated were used as chemoattractants. n=6. $*P < 0.05$. E, Aortic SMCs were stimulated with 10 $\mu\text{mol/L}$ Ang II in the presence of 20 $\mu\text{mol/L}$ Q-Vd-OPh for 48 hours. The amount of MCP-1 in media was evaluated by ELISA for MCP-1. n=6. $*P < 0.05$. F, Conditioned media were treated with a neutralizing antibody to MCP-1 (1 $\mu\text{g/mL}$) before the chemotaxis assay of monocytes. n=6. $*P < 0.05$. G, Representative pictures of immunostaining for MCP-1. Aortic sections of Q-Vd-OPh- or DMSO-treated Ang II-infused apoE KO mice at 3 days were stained for anti-MCP-1. Scale bars=250 μm . n=3.

released by Ang II-treated SMCs is responsible for attracting monocyte/macrophages, we incubated the conditioned media with a neutralizing antibody to MCP-1 before the chemotaxis assay. The migration of macrophages was significantly blocked by neutralization of MCP-1 (Figure 4F). In contrast, Q-Vd-OPh did not change levels of matrix metalloproteinase 2/9 produced by SMCs (Supplemental Figure VII).

On the basis of the above *in vitro* study, we speculate that apoptotic SMCs in the aortic wall recruit inflammatory cells by producing MCP-1. To test this hypothesis, we performed additional immunohistological analyses of the aortic tissues harvested after 3 days of Ang II infusion. As previously

reported by Ishibashi et al, Ang II infusion led to a significant elevation of MCP-1 expression in the aortic wall (Figure 4G).²⁰ Such induction was profoundly impaired in mice treated with Q-V_D-OPh (Figure 4G).

Discussion

The presence of apoptotic markers is a major pathological feature associated with AAA.^{1,8} Many experimental manipulations that have been shown to impair aneurysm formation have also caused a significant reduction in the number of apoptotic cells detected in the aortic wall.^{3,14–16} However, it is yet to be determined whether apoptotic cell death is merely a result of vascular inflammation or/and matrix destruction or whether apoptosis is a contributing cellular event necessary for the development of aneurysm. In order to determine whether there is a causal relationship between apoptotic death and the pathogenesis of aneurysm, we used a new caspase inhibitor Q-V_D-OPh, which inhibits apoptosis by blocking activation of caspases. In the Ang II-induced aneurysm model, administration of Q-V_D-OPh at the time of aneurysm induction profoundly reduced both the incidence and severity of aneurysm in apoE KO mice. To the best of our knowledge, this is the first report that directly links apoptosis to the pathogenesis of AAA.

Histological analyses of aortic tissues removed from Q-V_D-OPh-treated mice showed a remarkable reduction in the number of infiltrated macrophages and CD3+ T cells. Associated with diminished inflammation, the aortic wall of Q-V_D-OPh-treated mice displayed significantly decreased accumulation interleukin-6, as well as elastase activity. These findings suggest that inhibition of apoptosis may attenuate aneurysm formation not only by preventing SMC depletion but also by affecting vascular inflammation and matrix degradation. The potential interplay between the apoptotic event and vascular inflammation is also supported by our time course study. We showed that apoptotic SMCs were prominent throughout the tunica media 3 days after Ang II infusion, a time point at which infiltrated macrophages were most prominent in the media. We think that this early phase of apoptosis is critical to aneurysm formation, at least in the Ang II model, because application of the pan-caspase inhibitor after this window of apoptotic event had little effect on Ang II-induced aortic expansion.

Unlike necrosis, apoptosis is classically considered to be self-contained and noninflammatory. However, this conventional view has recently been challenged. Using a rat carotid angioplasty model, Schaub et al showed that activation of apoptosis in SMCs resulted in a massive inflammatory response consisting almost entirely of macrophages.²¹ Using a genetic approach, Clarke et al showed that acute induction of vascular SMC apoptosis in atherosclerotic plaques leads to intense intimal inflammation associated with thinning of fibrous caps and a loss of matrix proteins.²² More recently, this same investigative group showed that chronic low-level SMC apoptosis either during atherogenesis or within established plaques of apoE KO mice accelerates plaque growth by 2-fold and is associated with enhanced macrophage infiltration.²³ By demonstrating that the inhibition of apoptosis prevented the extensive vascular inflammation normally associated with aneurysm development, our current data further support the proinflammatory potential of apoptosis. The

inhibitory effect of Q-V_D-OPh on inflammation was substantial. The immunostaining of Mac3, CD3, or interleukin-6 was nearly undetectable in Q-V_D-OPh mice, a great contrast to the extensive inflammation observed in DMSO-treated controls.

The accumulation of macrophages in aortic media during the period of SMC apoptosis (\approx 3 days after Ang II infusion) could be caused by active recruitment via release of monocyte chemoattractants, including MCP-1. Data generated from our *in vitro* chemotaxis studies and from the immunohistochemical analysis support this notion. Media conditioned by Ang II-treated SMCs attracted monocytes/macrophages in a caspase-dependent manner. This chemoattractant property of apoptotic SMCs is mediated by MCP-1, as the release of MCP-1 is also sensitive to caspase inhibition. Neutralizing MCP-1 activity with an anti-MCP-1 antibody in the conditioned media diminished the chemotactic ability of apoptotic SMCs. Finally, *in vivo* administration of Q-V_D-OPh profoundly decreased the level of MCP-1 in Ang II-infused apoE KO mice. MCP-1-mediated infiltration of monocytes and other inflammatory cells has been previously shown to underlie Ang II-induced vascular inflammation.²⁰ Of note, MCP-1 was also identified by Schaub et al as an important chemoattractant released by Fas-induced apoptosis of SMC.²¹ We have recently reported that protein kinase C- δ mediates the expression of MCP-1 in vascular SMCs in response to another proinflammatory or apoptotic factor tumor necrosis factor- α .²⁴ Protein kinase C- δ , a well-established mediator of apoptosis,^{25,26} has also been found to be upregulated in human aneurysm tissues.²⁴ Whether protein kinase C- δ is the molecular link between SMC apoptosis and the production of monocyte chemoattractants remains to be tested.

In addition to a reduction in the number of apoptotic SMCs and infiltrated macrophages, Q-V_D-OPh resulted in dramatically diminished elastase activity; this reduction is thought to be responsible for arterial matrix destruction and loss of tissue integrity. Our observed link between SMC apoptosis and elastin degradation is supported by the finding of Clarke et al in atherosclerotic plaque. Also using apoE KO mice, Clarke et al showed that chronic induction of SMC apoptosis leads to medial expansion accompanied by increased elastic lamina breaks.²³ Because macrophages are the major source of proteases, we attribute the reduced elastase activity to the diminished macrophage invasion. This notion is supported by our finding that matrix metalloproteinase expression in cultured SMCs was not affected by Q-V_D-OPh treatment. However, we cannot exclude the possibility that caspase inhibition may directly influence the synthesis, secretion, or activation of proteases *in vivo*.

A potential limitation of our study is the use of Ang II to induce aneurysm development. This is a unique model characterized by the initial formation of dissection followed by dilatation of the suprarenal aorta. The exact cause of medial dissection in this model is not well elucidated. Our observation of reduced vascular hematoma in Q-V_D-OPh-treated mice suggests that cell apoptosis may contribute to medial dissection. Although Q-V_D-OPh is highly specific to caspases and no toxic effect associated with this inhibitor has been reported *in vivo*,^{27–29} our data do not exclude the possibility that Q-V_D-OPh might affect other classes of proteases involved in arterial wall remodeling. We do not know

whether Q-VD-OPh affected the lipid profile of apoE mice that were maintained on normal diet in our experiments. It is also possible that Ang II-induced aneurysm is more dependent on apoptosis and thus more sensitive to the caspase inhibitor as compared with other animal models of AAA, although this is unlikely because upregulated apoptosis has been detected in all experimental models of aneurysms.^{14,16,30}

In summary, our results demonstrate that the incidence and severity of Ang II-induced aneurysm in apoE KO mice was remarkably reduced by the blockage of apoptosis with a pan-caspase inhibitor, Q-VD-OPh. The recruitment of macrophage measured by immunohistochemistry, chemotaxis assay, and in situ zymography was also blocked by this inhibitor, at least in part through the reduction of MCP-1 production. These results suggest that apoptosis may be an early cellular event during aneurysm development that contributes to tissue destruction by stimulating macrophage infiltration and depletion of residential SMCs.

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Disclosure

None.

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Supplement Material

Supplemental Materials and Methods.

General Materials

Dulbecco's Modified Eagles Medium (DMEM) and cell culture reagents were from Gibco BRL Life Technologies. Chemicals, if not specified, were purchased from Sigma Chemical Co.

Cell Culture

Mouse aortic SMCs from the thoracic aorta of C57BL/6 mouse were isolated based on a protocol described by Clowes et al.¹ The isolated cells and RAW 264.7 cells (ATCC, VA) were maintained in DMEM containing 10% FBS at 37°C with 5% CO₂ and antibiotics.

Histology and Immunohistochemistry

Morphological and Immunohistochemical analysis was performed as described previously.² In brief, paraffin embedded aortas were cut into 6 µm sections for morphological and immunohistochemical analysis. The aortas of Q-VD-OPh or DMSO administered mice were embedded and frozen in OCT compound, and cut into 6µm sections. Morphometric analysis was carried out on elastic-stained arteries by elastic-Van Gieson staining. Immunostaining for IL-6, CD3 antibody (17A2) (Santa Cruz Biotechnology, CA), alpha-smooth muscle actin (DAKO, CA), MCP-1 using rabbit polyclonal anti-MCP-1 antibody (Santa Cruz Biotechnology, CA) and macrophage using monoclonal anti-Mac3 antibody (Santa Cruz Biotechnology, CA) was performed as described previously.³ TUNEL staining was performed according to manufacturer's protocol (Roche Applied Science, IN). Immunofluorescent staining was performed with donkey anti-rat Alexa 488 (Invitrogen, CA). 4',6-diamidino-2-phenylindole

dihydrochloride (DAPI) was used as a nuclear counterstain. Slides were then visualized with a Nikon Eclipse E800 upright microscope with equipped with appropriate filters. Digital images were acquired using a RetigaEXi CCD digital camera and processed and analyzed using IPLab software.⁴ TUNEL index was calculated as (number of TUNEL positive cells/number of total nuclei) per section by identifying TUNEL positive cells out of DAPI positive cells from at least 5 independent high power fields in each sample on NIH image software (ImageJ 1.36b).

In situ Zymography

For in situ zymography, freshly cut frozen aortic sections (8 μ m) were incubated at 37°C with a fluorogenic elastin substrate (DQ elastin, Invitrogen, CA) according to the manufacturer's protocol. Proteolytic activity represented by green fluorescence was measured after 48 h of incubation under a Nikon Eclipse E800 upright microscope. Fluorescent images were also acquired immediately after the addition of substrate (0 hour) and served as controls for tissue autofluorescence. Digital images were acquired using a RetigaEXi CCD digital camera.⁵

Gelatin Zymography

Mouse aortic SMCs were plated at a density of 60,000/well of 6-well plates. Cells were treated with Q-VD-OPh (20 μ M) or DMSO for 30 minutes followed by addition of TNF- α (20ng/mL or 50ng/mL). Forty-eight hours later, supernatant was collected. MMP activities in the supernatants were determined by Gelatin Zymography as described in previous publication.⁶

DNA Fragmentation ELISA (Apoptosis Assay)

DNA fragmentation ELISA was performed according to manufacturer's instruction using the Cell Death Detection ELISA system (Roche Applied Science, IN)

Monocyte chemoattractant Protein-1 (MCP-1) ELISA

ELISA for MCP-1-ELISA to detect MCP-1 secreted by SMC was performed using mouse MCP-1 ELISA kit (BD Biosciences, CA). Conditioned media of SMCs were collected by culturing SMCs at a density of 1×10^5 /ml in 1 ml of complete medium in the presence or absence of AngII in 6-well plates. After incubation for specified periods of time at 37 °C, cell-free culture supernatants were obtained. The concentrations of MCP-1 were then measured according to the manufacturer's instructions.⁷

Chemotaxis Assay

Chemotaxis assay was performed as described previously.⁷ 2×10^5 macrophages (RAW 264.7) were placed in the upper chamber of Costar 24-well transwell plates with 5- μ m pore filters (Corning, Inc., Corning, NY). Cultured conditioned medium or control media was placed into the lower chambers or wells. Anti-MCP-1 antibody (Biolegend, CA) was used for neutralization of MCP-1. After incubating plates for 6 h at 37 °C, migrated cells were collected from the lower chambers and on the bottom of the filters were counted.⁷

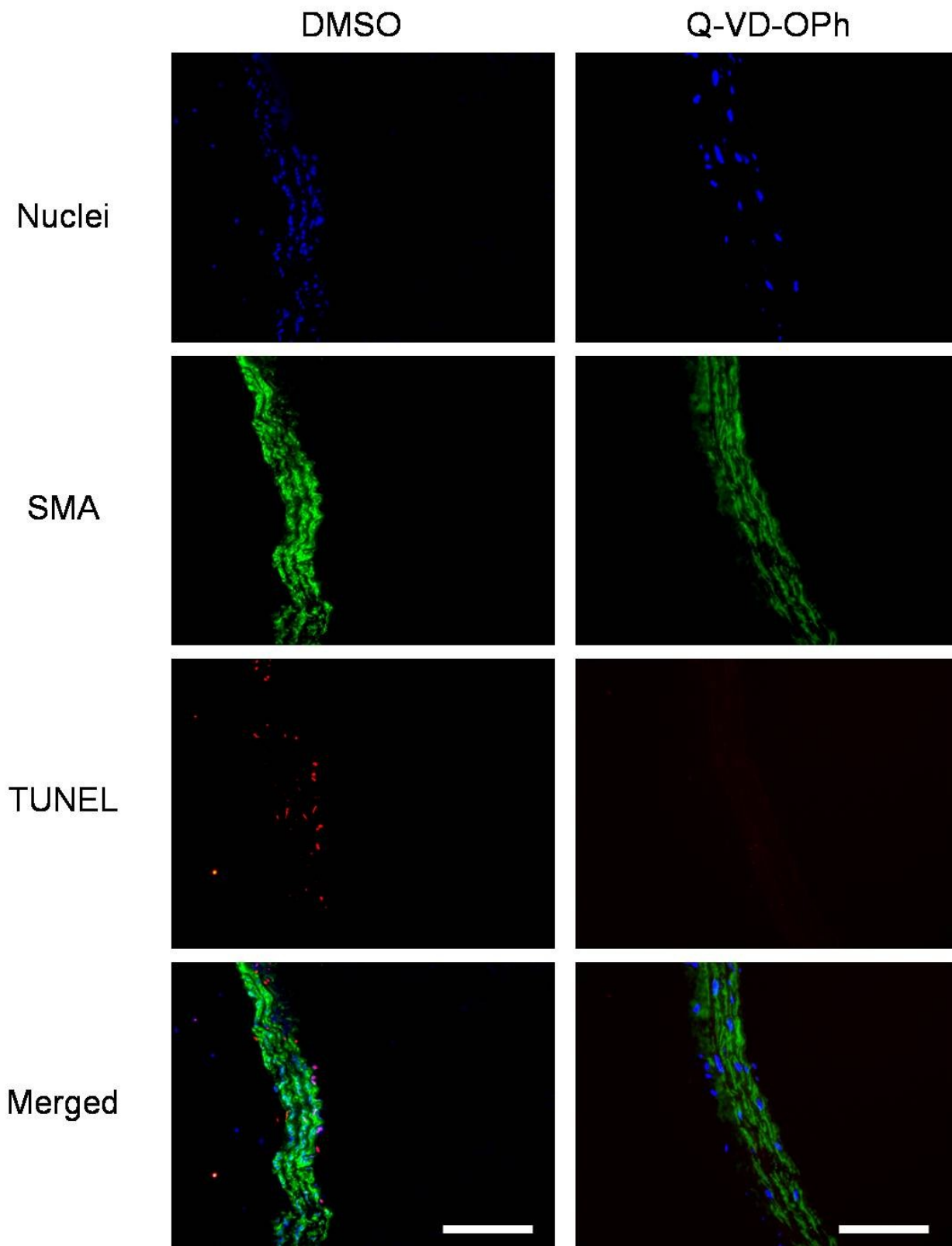
RNA Isolation and Quantification

RNA was isolated from mouse aortic smooth muscle cells (SMCs) stimulated with 10 μ M of angiotensin II (AngII) for 24 hours using an adaptation of the Trizol method (Invitrogen, CA) and the RNeasy mini column method (Qiagen) as previously described.^{8,9} RNA quality was assessed using Nano Drop 1000 (Thermo Scientific, DE).

Expression Analysis by Real-Time PCR

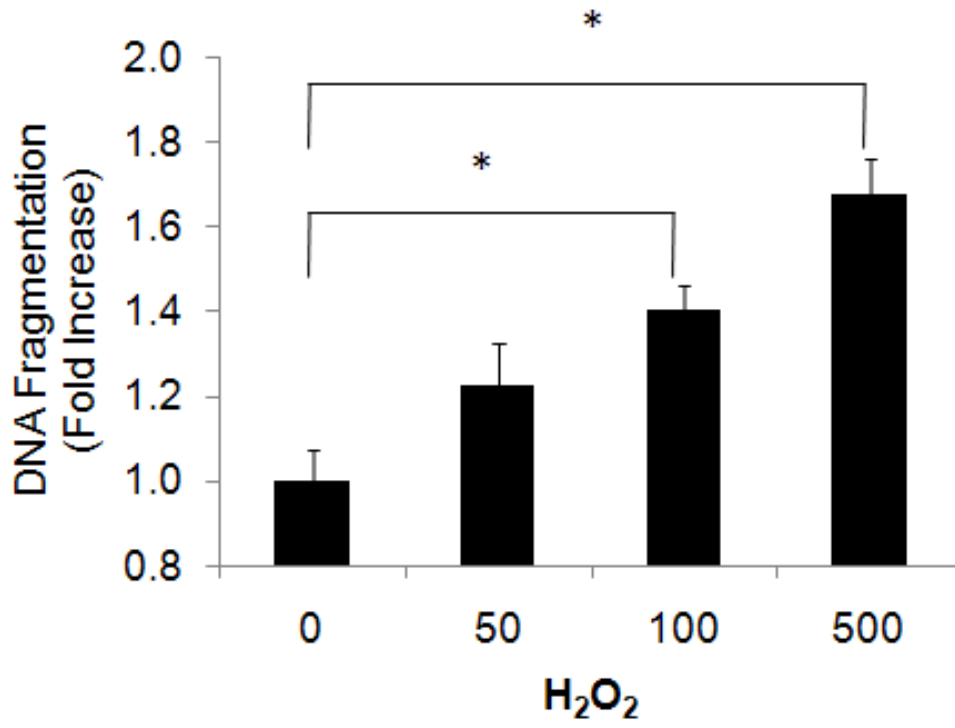
The expression of selected genes was assessed independently by quantitative real-time PCR. Total RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) and real-time PCR amplification was performed using the SYBR® Green PCR Master Mix (Applied Biosystems, CA) on 7500 Real-time PCR System (Applied Biosystems, CA). Gene expression was normalized against GAPDH. Relative gene expression (RQ) was calculated using the $\Delta\Delta C_t$. Data was expressed as fold increase of RQ. n = 3, * $P < 0.05$.

Supplemental Figure I

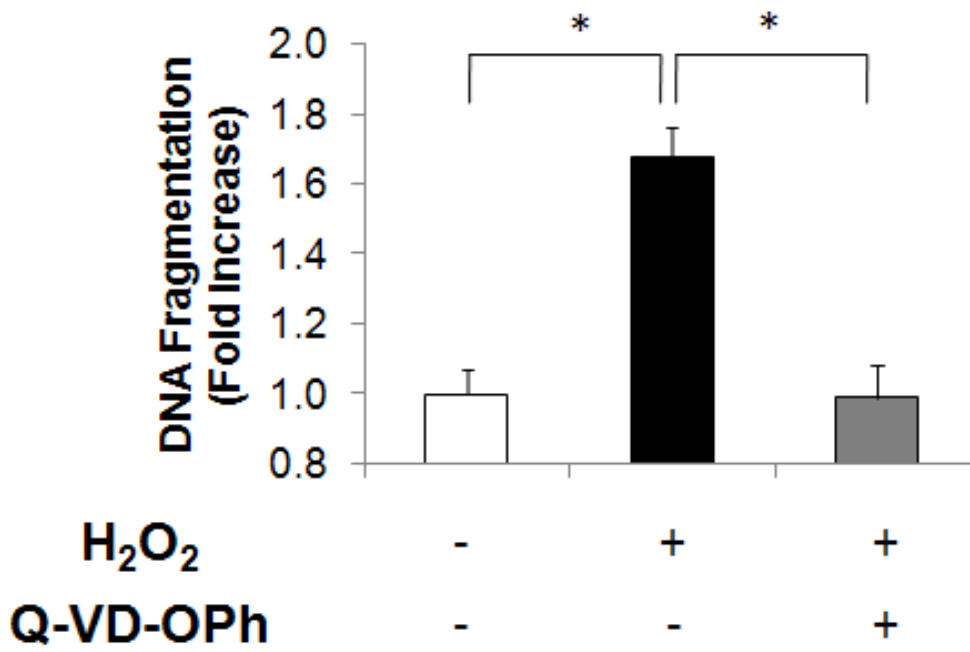


Supplemental Figure II

A.



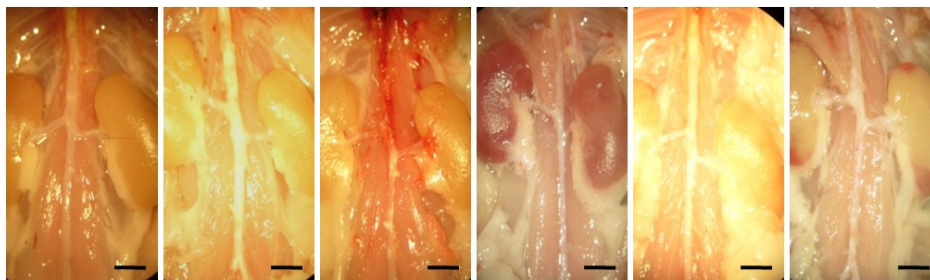
B.



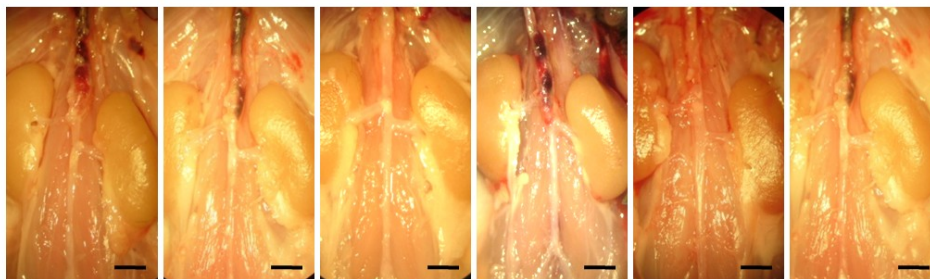
Supplemental Figure III

7 days

Q-VD-OPh

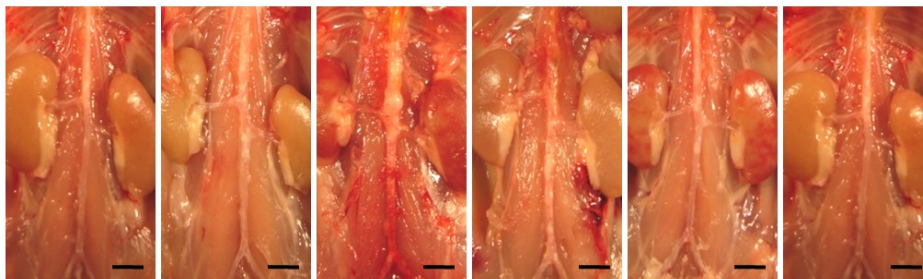


DMSO

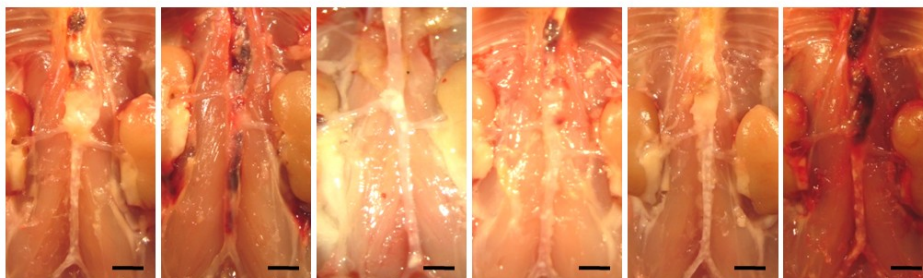


28 days

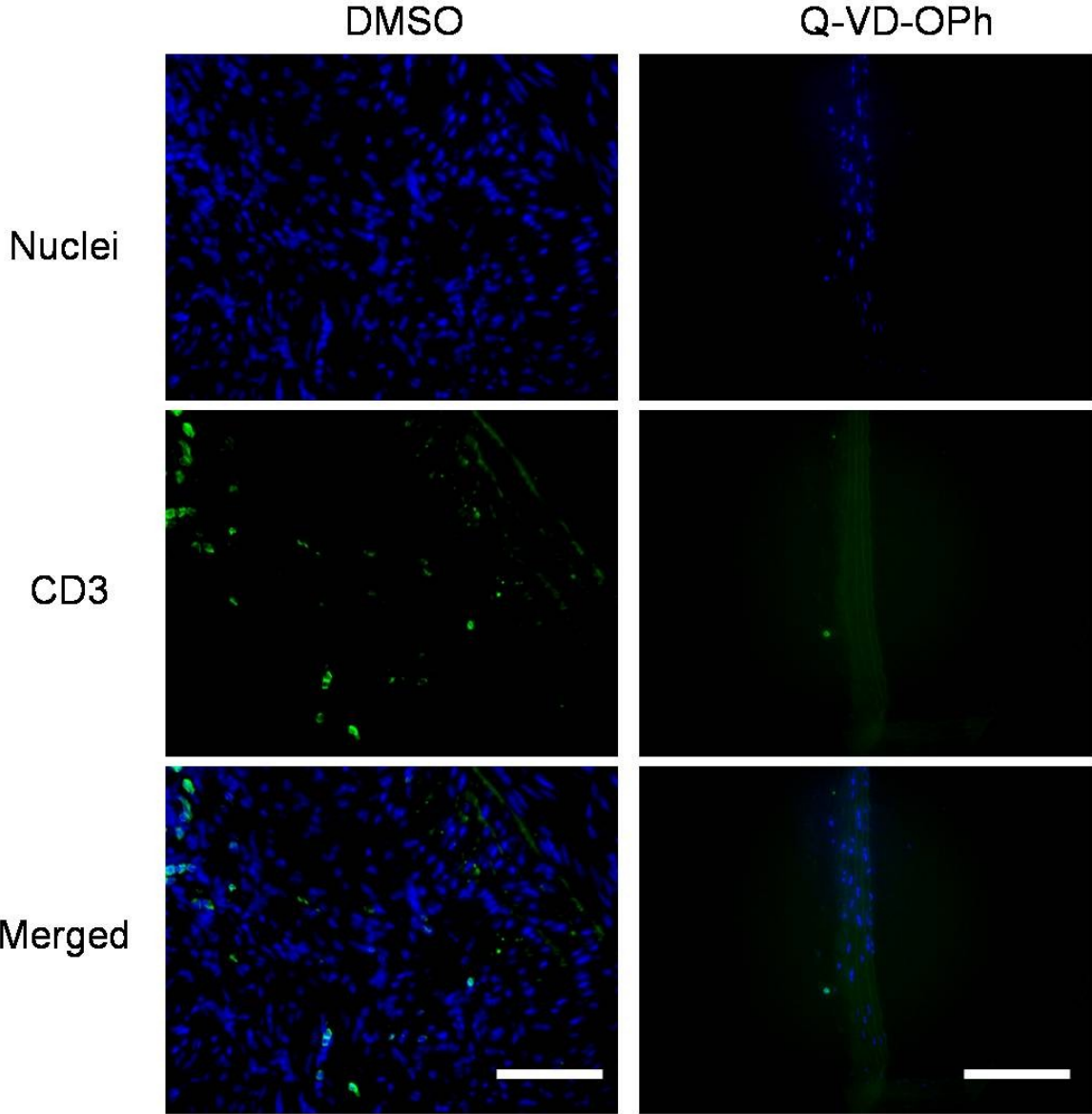
Q-VD-OPh



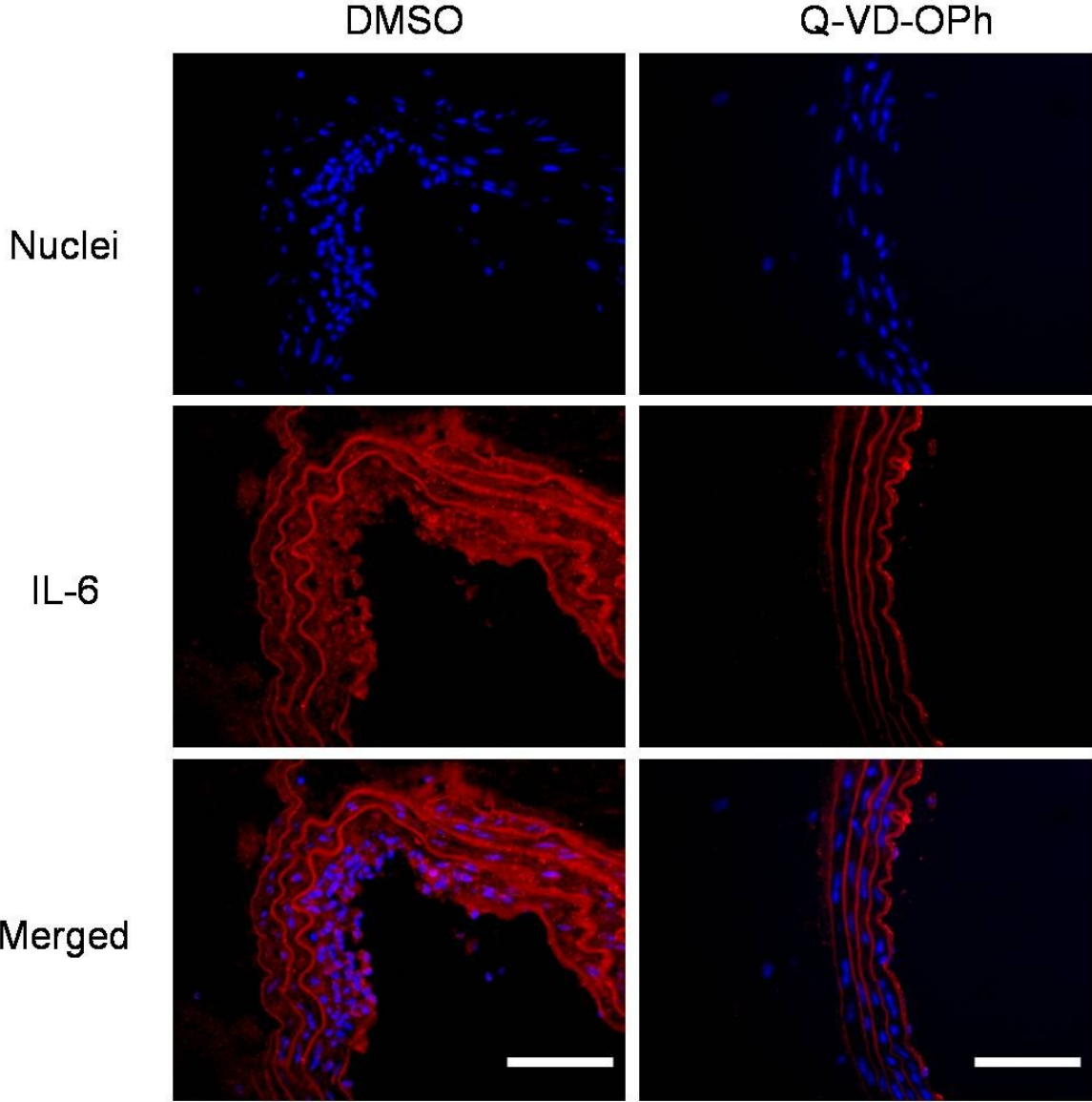
DMSO



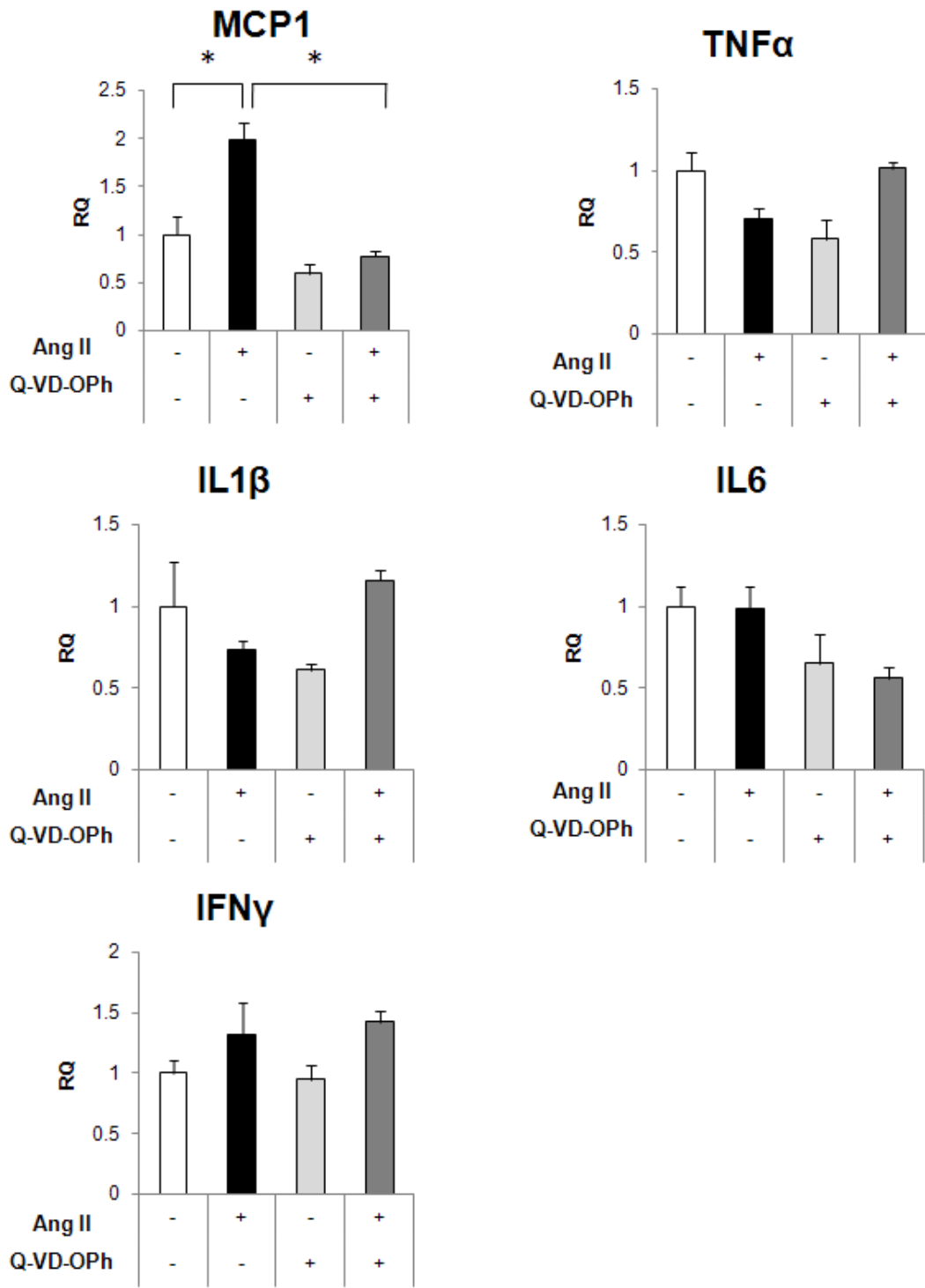
Supplemental Figure IV



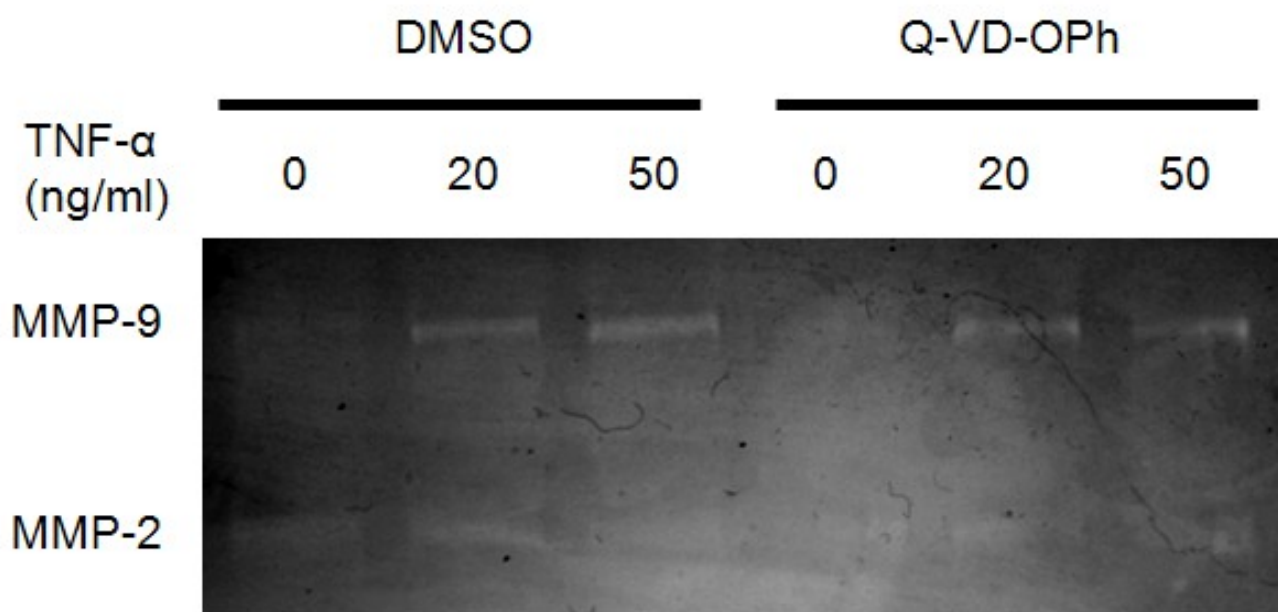
Supplemental Figure V



Supplemental Figure VI



Supplemental Figure VII



Supplemental Figure Legends.

Supplemental Figure I

Representative pictures of immunostaining for alpha-smooth muscle actin (SMA) and TUNEL. Aortic sections of Q-VD-OPh or DMSO treated AngII infused ApoE KO mice at 3 days were co-stained for SMA and TUNEL. Scale bar = 50 μm , n = 3.

Supplemental Figure II

A: Effect of Q-VD-OPh on hydrogen peroxide (H_2O_2) induced apoptosis of SMCs.

SMCs were stimulated with 0 to 500 μM H_2O_2 for 24 hours. Apoptosis was evaluated through ELISA measured DNA fragmentation. n = 3. * $P < 0.05$.

B: Effect of pan caspase inhibitor, Q-VD-OPh, on H_2O_2 induced apoptosis of SMCs. SMCs were stimulated with 500 μM of H_2O_2 in the presence of 20 μM of Q-VD-OPh or DMSO. Apoptosis was evaluated through ELISA-measured DNA fragmentation. n = 3. * $P < 0.05$.

Supplemental Figure III

Pictures of the aortas of removed from ApoE deficient mice treated with Q-VD-OPh or DMSO 7 and 28 days after AngII infusion. Scale bar = 2 mm.

Supplemental Figure IV

Representative pictures of immunostaining for CD3. Aortic sections of Q-VD-OPh or DMSO treated AngII infused ApoE KO mice at 3 days were stained for CD3. Scale bar = 50 μm , n = 3.

Supplemental Figure V

Representative pictures of immunostaining for IL-6. Aortic sections of Q-VD-OPh or DMSO treated AngII infused ApoE KO mice at 3 days were stained for IL-6. Scale bar = 50 μ m, n = 3.

Supplemental Figure VI

Effect of AngII cytokine/chemokine expression in SMCs. SMCs were stimulated with 10 μ M of AngII for 24 hours with or without 20 μ M of Q-VD-OPh. The relative gene expression (RQ) of tumor necrosis factor- α (TNF α), interleukin-1 β (IL1 β), interleukin-6 (IL6), interferon γ (IFN γ) or MCP-1 was determined using the $\Delta\Delta$ Ct. GAPDH was used as an internal control. n = 3.

P < 0.05.

Supplemental Figure VII

Effect of Q-VD-OPh on MMP expression in SMCs. A representative picture of gelatin zymography was shown. Supernatants were collected from SMCs stimulated with 0, 20, and 50 ng/ml of TNF- α for 48 hours with or without 20 μ M of Q-VD-OPh. (n = 3).

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