Tryptophan-Derived 3-Hydroxyanthranilic Acid Contributes to the Angiotensin II-Induced Abdominal Aortic Aneurysm Formation in Mice in Vivo

SUPPLEMENTAL MATERIAL

Online materials and methods supplement

Materials

Antibodies to MMP2 (ab37150) and alpha-smooth muscle actin (ab5694) were from Abcam. An antibody against IFN-γ (AMC4834) was from Invitrogen. Antibodies to phospho-NF-κB p65 (Ser536, 3033), NF-κB p65 (8242), and all secondary antibodies were from Cell Signaling Technology. Antibodies to human kynureninase (sc-390360), β-actin (sc-47778) and GAPDH (SC-166545) were from Santa Cruz Biotechnology. An antibody to locate IDO (MAB5412) was from Millipore. An antibody against mouse kynureninase (MAB7389) was from R&D System. An antibody to mouse 3-Hydroxyanthranilic acid was from Eagle Biosciences (IS1008). A goat anti-mouse IgG conjugated to Alexa488 green (A-11001) was purchased from Invitrogen. All primary antibodies were used in a 1:1,000 dilution for Western blot and a 1:100 dilution for immunocytochemistry and immunohistochemistry. IFN-γ recombinant human protein (PHC4031) was obtained from GIBCO. Recombinant human pro-MMP2 protein (PF037) was purchased from Millipore. 3-Hydroxy-DL-kynurenine (H1771), anthranilic acid (A89855), and angiotensin II (Ang II, A9525) were from Sigma. Kynurenic acid (sc-202683), xanthurenic acid (sc-258335), and quinolinic acid (sc-203226) were purchased from Santa Cruz Biotechnology. Control siRNA (sc-37007) and siRNAs targeting IDO (sc-45939), kynureninase (sc-95023) and NF-κB p65 (sc-29410) were from Santa Cruz Biotechnology. The transfection reagents for siRNA (Lipofectamine RNAiMax, 13778150) were from Invitrogen. Mouse IL-6 (550950) and IFN-γ (558258) ELISA kits were from BD Biosciences. TNF-α mouse ELISA kit (KMC3011) was from Life Technologies. Mouse cyclophilin A (CyPA, M7820) ELISA kit was from BIOTANG.

Patients and Sample Processing

Information of human patients and aortic sample processing were reported as previously.[1] All protocols using human aortic samples were approved by the Ethical Committee of Chinese Academy of Medical Sciences and Peking Union Medical College.

Animals

$IDO^{-/-}$ and $Apoe^{-/-}$ mice were obtained from Jackson laboratory (Bar Harbor, Maine) in a C57BL/6 background. $IDO^{-/-}$ mice were crossed with $Aope^{-/-}$ mice to generate $Apoe^{-/-}/IDO^{-/-}$ mice. Mice were housed in temperature-controlled cages under a 12-hour light-dark cycle and given free access to water and normal chow. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center and Georgia State University.

Experimental mice ($Apoe^{-/-}$ and $Apoe^{-/-}/IDO^{-/-}$ mice) at age 8 weeks or 4 weeks after bone marrow transplantation on a chow diet were infused with AngII (1,000 ng/kg/min) or physiological saline (0.9% sodium chloride) by Alzet osmotic pumps (DURECT Corp, Model 2004) as described previously.[2]

$Apoe^{-/-}$ and $Apoe^{-/-}/IDO^{-/-}$ mice at age 8 weeks on a chow diet were intraperitoneally injected with 3-hydroxyanthranilic acid (3-HAA, Santa Cuz, sc-216460) (200 mg/kg. d)[3-6] or vehicle for 6 weeks. 3-HAA was dissolved in DMSO and further diluted in Captisol (Captisol Technology).

In Vivo siRNA Delivery

The Ambion® in vivo pre-designed siRNA delivery was carried out according to Invitrogen's in vivo RNAi protocol. 0.875 mg/ml complex of control siRNA (Invitrogen, 4457289) or kynureninase siRNA (Invitrogen, 4457308) with Invivofectamine® 2.0 Reagent (Invitrogen, 1377-505) were prepared in PBS using a Float-A-Lyzer® G2 cassette (Spectrum labs, G235031) freshly before every injection. 200 μl of siRNA (7 mg/Kg) complex was delivered into an $Apoe^{-/-}$ mouse by tail vein injection every five days which started on the first day of AngII infusion.

Bone Marrow Transplantation

Bone marrow transplantation was performed as described previously.[7, 8] Bone marrow was obtained aseptically from femora and tibiae of $Apoe^{-/-}$ or $Apoe^{-/-}/IDO^{-/-}$ mice. Cells (5×10^6/mouse) were resuspended

in sterile PBS and transplanted by intravenous infusion into lethally irradiated (10 Gy) Apoe$^{-/-}$ or Apoe$^{-/-}$/IDO$^{-/-}$ mice recipients 6 hours after irradiation at the age of 8 weeks.

Analysis and Quantitation of AAA

To quantify AAA incidence and size, the maximum width of abdominal aorta was measure with Image Pro Plus software (Media Cybernetics). We quantified aneurysm incidence based on a definition of aneurysm as an external width of the suprarenal aorta that was increased by 50% or greater compared with aortas from saline-infused mice, as described previously.[9] The average diameter of the normal suprarenal aorta in control mice is 0.8 mm. We therefore set a threshold of 1.22 mm as evidence of aneurysm formation.

Histological Analysis

After hemodynamic measurements, the mice were killed. For morphological analyses, aortas were perfused with saline and fixed with 10% formalin in PBS for 5 minutes. Whole aortas were harvested, fixed and embedded in paraffin, and cross-sections (5 μm) were prepared. Paraffin sections were stained with H&E (IHC World, IW-3100), Van Gieson elastic stain (Sigma, HT25A), Masson trichrome stain (Sigma, HT15), or were used for immunostaining (DAKO, K4065). The collagen deposition areas were calculated by Image J. In terms of the determination of elastin degradation, we used a standard for the grades of elastin degradation, as described previously.[10] The grades were defined as follows: grade 1, no degradation; grade 2, mild elastin degradation; grade 3, severe elastin degradation; and grade 4, aortic rupture. Semiquantitative analysis of tissue immunoreactivity[2] was done by 4 observers blinded to the identity of the samples using an arbitrary grading system from score 1 to 4 (score 1: 0-25% positive staining in medium; score 2: 26-50% positive staining in medium; score 3: 51-75% positive staining in medium; score 4: 76-100% positive staining in medium) to estimate the degree of positive staining for each individual marker.

Blood Pressure Measurement

Blood pressure was determined by a left carotid catheter method before sacrificing the mice as described previously.[2]

Enzyme-linked immunosorbent assay (ELISA)

Serum cytokines were detected by indicated ELISA kits according to the manufacturers' instructions.

Cell Culture

Human aortic smooth muscle cells (HASMCs) (GIBCO, C0075C) were grown in M231medium (Gibco, M231500) containing 5% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (0.5 μg/mL, Gibco, 15290018), and Smooth Muscle Growth Supplement (SMGS, Gibco, S00725). All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. Cells were used between passages 3 and 8, and in all experiments were grown to 70–80% confluence before being treated with different agents.

Determination of Tryptophan (Trp), Kynureine (Kyn), and 3-Hydroxyanthranilic Acid (3-HAA)

The culture medium was centrifuged to pellet the cells to avoid contamination from the cells, and the supernatant was filtered (0.2 μM) before use. Sample preparation for plasma was operated as described previously.[11, 12] 200 μl of plasma was mixed by vortex in a small polyethylene conical tube with 1/10 volume of ice cold 2.4 mol/L perchloric acid. The cloudy suspension was chilled on ice for 15 min and then centrifuged at 10,000 g for 2 min. The clear protein-free supernatant was analyzed directly.

Kyn and Trp were measured by HPLC as described previously.[2] Kyn content was estimated along with standards ranging from 0.5-20 μM L-kynurenine (Sigma, K8625), and Trp level was assessed along standards ranging from 1-25 μM DL-Tryptophan (Sigma, 162698), using a GraceVydac C18 column (250×4.6 mm, 5.0 μM). The mobile phase was 20 mM sodium acetate at pH=4.5. The flow rate was 1 ml/min. The column effluent was monitored at 360 nm (Kyn) and 280 nm (Trp) by a UV detector.

3-HAA was measured by HPLC as described previously.[13] 3-HAA content was estimated along with standards ranging from 1-200 nM 3-Hydroxyanthranilic acid (Santa Cuz, sc-216460), using a GraceVydac C18 column (250×4.6 mm, 5.0 μM). The mobile phase consisted of 25 mM sodium acetate (pH=5.5) and methanol (90:10 v/v) was pumped at a flow rate of 1 ml/min. Fluorescent detection at excitation 320 nm and emission 420 was used.

Reverse Transcription-polymerase Chain Reaction (RT-PCR) and Real Time-PCR

Total RNA was isolated using Trizol (Invitrogen, 15596026) and Qiagen RNAeasy Columns (QIAGEN, 74204) and reverse transcribed to complementary DNA with specific antisense primers using the Bio-Rad RT-PCR and Real-time PCR system protocol, as described previously[14]. The primers of human (H) and murine (M) MMP2 and GAPDH are listed in **Supplementary Table 8.**

Western Blot Analysis

Western blots were performed as described previously.[2]

MMP Activity

The evaluation of MMP activities in cells and aortas was performed as described previously.[10, 15] 10 μg of protein in culture medium or tissue homogenates was electrophoresed in SDS-PAGE gels containing 1 mg/ml gelatin. Gels were washed in 2.5% Triton x-100 and incubated overnight in zymography developing buffer at 37^{0}C. Subsequently, the gels were stained with Coomassie brilliant blue.

Immunocytochemistry

Immunocytochemistry was performed as described previously[2] with a polyclonal antibody against Conjugated 3-HAA (Eagle BioSciences, IS1008).

Transfection of siRNA into Cells

Transient transfection of siRNA in culture cells was carried out according to Santa Cruz's protocol.

Figure Legends

Supplementary Figure 1 3-HAA mediates AngII-induced MMP2 mRNA expression in Apoe$^{-/-}$ mice.

(a) Saline or AngII (1000 ng/min per kg) was administered to Apoe$^{-/-}$ and Apoe$^{-/-}$/IDO$^{-/-}$ mice for 4 weeks. (b) Apoe$^{-/-}$ and Apoe$^{-/-}$/IDO$^{-/-}$ mice were intra-peritoneally injected with vehicle (100 μl of 20% DMSO in Captisol) or 3-HAA (200 mg/kg/d) for 6 weeks. (c) The aortas isolated from Apoe$^{-/-}$ and Apoe$^{-/-}$/IDO$^{-/-}$ mice were treated with vehicle or 400 μM 3-HAA for 48 hours ex vivo. (d) After transfection with scrambled (Scr) siRNA or KNU siRNA, Apoe$^{-/-}$ mice were infused with AngII (1000 ng/min per kg) for 4 weeks. (a-d) MMP2 and GAPDH mRNA were detected by Real time Polymerase Chain Reaction in the suprarenal aortas of the mice with indicated treatment. All results were obtained from 6–10 mice in each group. *P

<0.01 vs Apoe$^{-/-}$ saline or vehicle or Scr siRNA saline, #P <0.01 vs Apoe$^{-/-}$ AngII or Scr siRNA AngII. P values in a-d were obtained by a two-way ANOVA with Bonferroni's multiple comparisons. The error bars in a-d are s.e.m.

Supplementary Figure 2 Trp depletion inhibits MMP2 expression in HASMCs. (a, b) Cultured HASMCs were incubated with 100 μM IFN-γ for the indicated time. (c, d) Cultured HASMCs transfected with control siRNA or IDO siRNA were treated with or without IFN-γ for 48 hours. pro-MMP2 (c), IDO and GAPDH proteins were detected by immunoblotting (a, c), and the Trp and Kyn levels in the culture medium were detected by HPLC (b, d). n=3 independent experiments for all quantitative data. *P <0.01 vs control or control siRNA, #P <0.01 vs control siRNA with IFN-γ treatment. P values in b were obtained by a two-way ANOVA plus Bonferroni's multiple comparisons. P values in d were obtained by a three-way ANOVA plus Bonferroni's multiple comparisons. The error bars in b, d are s.e.m.

Supplementary Figure 3 IFN-γ induces MMP2 expression with addition of sufficient Trp in HASMCs. (a, e) Cultured HASMCs were incubated with indicated concentrations of Trp for 48 hours. (b) Cultured HASMCs were either untreated (control) or treated with IFN-γ combined with indicated concentrations of exogenous Trp for 48 hours. (c, d) Cultured HASMCs transfected with control siRNA or IDO siRNA in the treatment of vehicle (control) or IFN-γ with or without addition of 50 μM (c) or 100 μM (d) Trp for 48 hours. pro-MMP2 and IDO (b-d) proteins were detected by immunoblotting, β-actin (c, d) and GAPDH (a, b, e) were used as loading controls, and the Trp and Kyn levels in the culture medium were detected by HPLC (b-d). n=3 independent experiments for all quantitative data. The error bars in b-d are s.e.m.

Supplementary Figure 4 3-HAA Promotes MMP2 Expression in HASMCs. (a) Cultured HASMCs were incubated with indicated concentrations of 3-HAA for 48h. (b) Cultured HASMCs were incubated with 200 μM 3-HAA for indicated time. (a, b) pro-MMP2 and GAPDH proteins were detected by immunoblotting, and MMP2 activities in the culture medium were detected by zymography. In all panels, representative data from 3 independent experiments are shown.

Supplementary Figure 5 AngII activates Kyn pathway in Apoe$^{-/-}$ mice in vivo with or without KNU short-interfering RNA (siRNA) transfection. After transfections with scrambled (Scr) siRNA or kynureninase (KNU) siRNA, Apoe$^{-/-}$ mice were infused with saline or AngII (1000 ng/min per kg) for 4 weeks. (a, b) Representative immunohistochemical staining (a) and quantification (b) for IFN-γ and IDO in the suprarenal aortas of AngII-infused mice with the indicated siRNA transfections. (c, d) Serum concentrations of Trp (c) and Kyn (d) detected by HPLC in mice with the indicated siRNA transfections after AngII infusion. All results were obtained from 6–10 mice in each group. P values in b-d were obtained by a t test. The error bars in b-d are s.e.m.

Supplementary Figure 6 Proposed scheme of AngII-induced AAA formation via 3-hydroxyanthranilic acid (3-HAA) elevation in mice. AngII binds to its receptor in vascular smooth muscle cells to promote secretion of cytokine IFN-γ, which powerfully activates kynurenine (Kyn) pathway of tryptophan (Trp) metabolism. Highly induced indoleamine-2,3-dioxygenase (IDO) initially catalyzes Trp to Kyn, which is further metabolized to 3-HAA by intensely upregulated kynureninase (KNU) expression. Trp-derived 3-HAA induces the phosphorylation of the main MMP2 transcriptional factor NF-κB, which triggers MMP2 overexpression and subsequent MMP2 secretion to outside of cells and cleavage to active MMP2 by MT1-MMP. In vascular walls, active MMP2 degrades extracellular matrix resulting in the loss of the resistance of vascular wall to blood flow and consequent formation of aortic aneurysm.

Supplementary Table 1. Blood pressure and heart rate in AngII-infused mice

Supplementary Table 2. Serum lipid and glucose level in AngII-infused mice

Supplementary Table 3. Serum cytokine in AngII-infused mice

Supplementary Table 4. Serum lipid and glucose level in 3-HAA-injected mice

Supplementary Table 5. Blood pressure and heart rate in AngII-infused Apoe$^{-/-}$ mice

Supplementary Table 6. Serum lipid and glucose level in AngII-infused Apoe$^{-/-}$ mice

Supplementary Table 7. Serum cytokines in AngII-infused Apoe$^{-/-}$ mice

Supplementary Table 8. Primers for PCR

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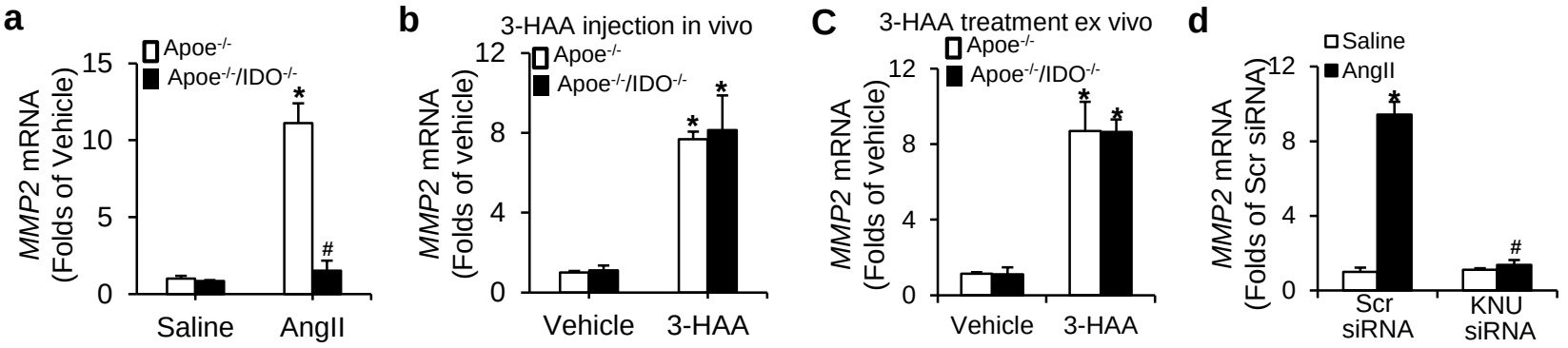

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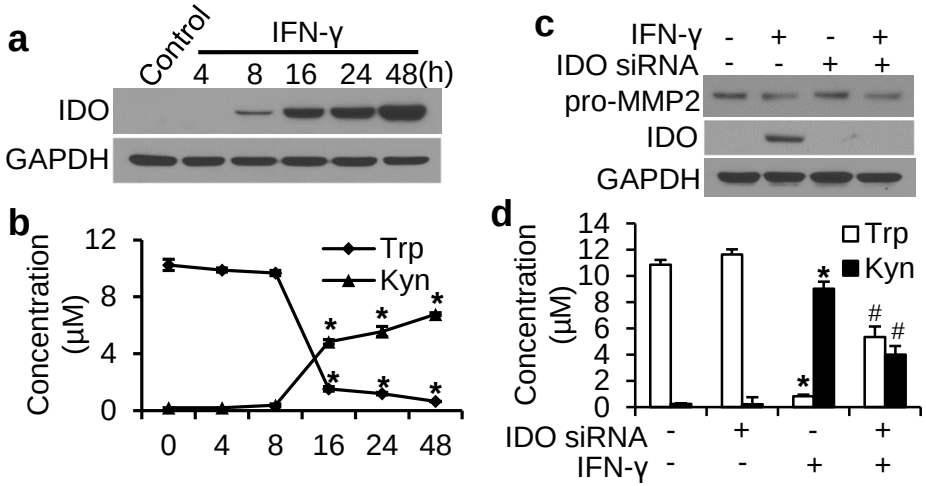

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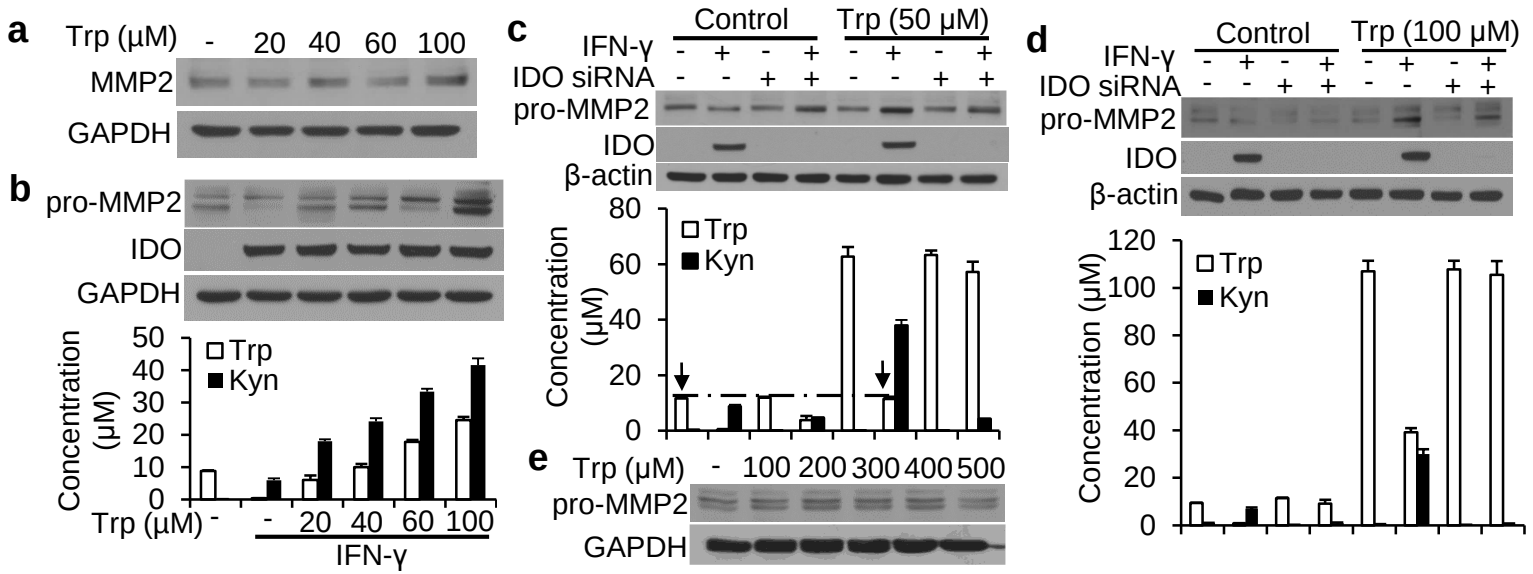

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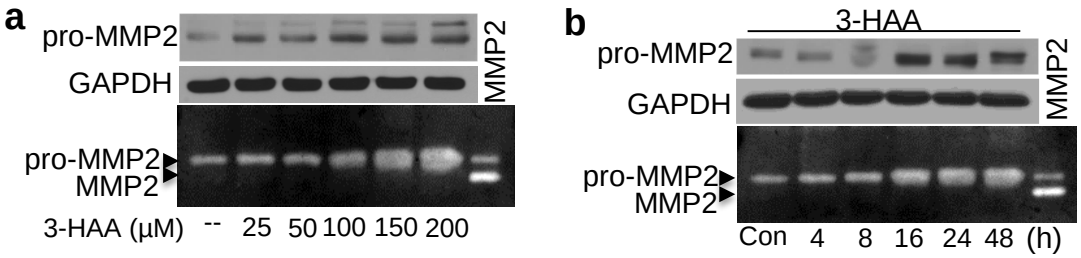

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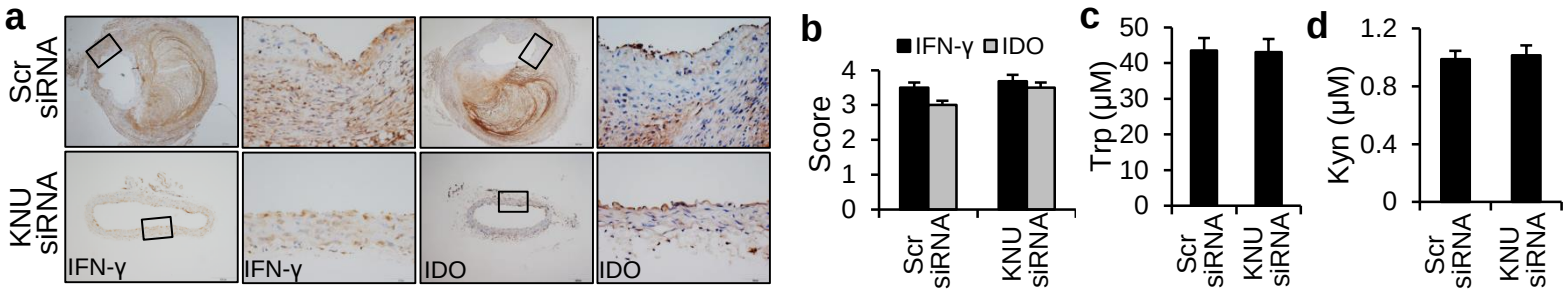

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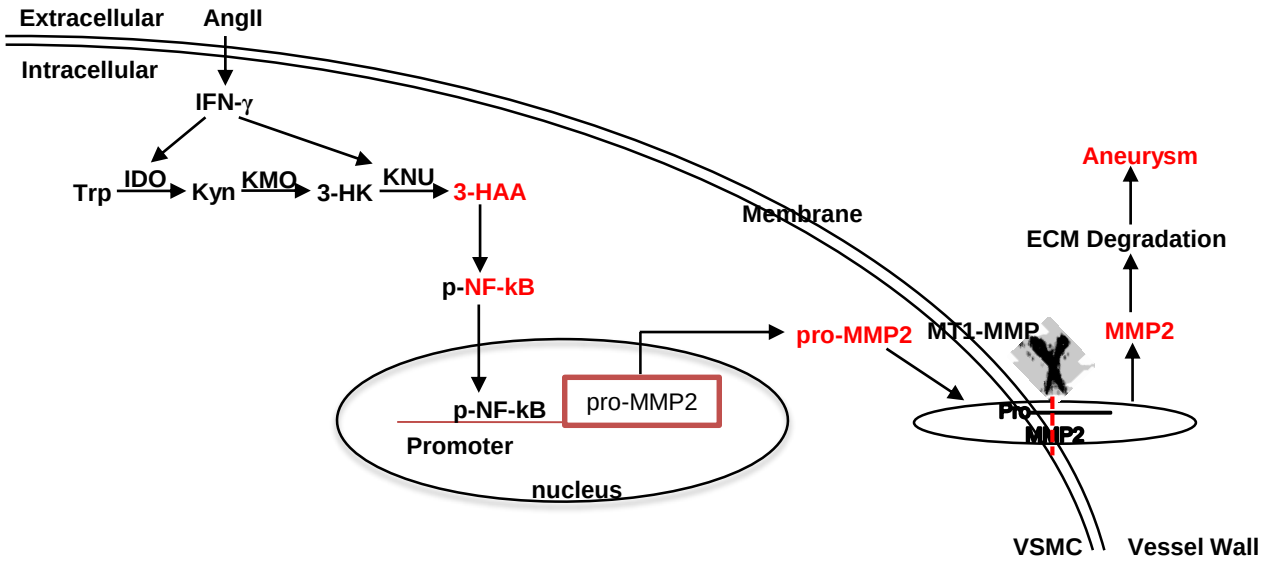

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Supplementary Table 1. Blood pressure and heart rate in AngII-infused mice

Groups	Saline		AngII	
	Apoe$^{-/-}$	Apoe$^{-/-}$/IDO$^{-/-}$	Apoe$^{-/-}$	Apoe$^{-/-}$/IDO$^{-/-}$
sBP (mmHg)	102.6±12.7	107.4±11.3	149.1±19.5*	155.6±18.4*
dBP (mmHg)	81.5±9.1	79.2±10.3	112.7±17.3*	119.1±14.9*
HR (Beats/min)	486±41	439±37	471±42	453±43

N is 6-10 in each group. Data are expressed by mean±s.e.m. The *P* values were obtained by a two-way ANOVA plus Bonferroni's multiple comparisons. * *P*<0.05 compared to saline Apoe$^{-/-}$ mice.

Supplementary Table 2. Serum lipid and glucose level in AngII-infused mice

Groups	Saline		AngII	
	Apoe$^{-/-}$	Apoe$^{-/-}$/IDO$^{-/-}$	Apoe$^{-/-}$	Apoe$^{-/-}$/IDO$^{-/-}$
Cholesterol (mg/dl)	509±37.1	498.6±31.4	496.1±22.0	497.9±24.2
Triglyceride (mg/dl)	104.9±6.9	122.5±21.3	125±12.6	108.3±4.3
BG (mg/dl)	149±31	138±23	151±27	142±19

N is 6-10 in each group. Data are expressed by mean±s.e.m. The *P* values were obtained by a two-way ANOVA.

Supplementary Table 3. Serum cytokine in AngII-infused mice

Groups	Saline		AngII	
	Apoe$^{-/-}$	Apoe$^{-/-}$/IDO$^{-/-}$	Apoe$^{-/-}$	Apoe$^{-/-}$/IDO$^{-/-}$
IL-6 (pg/ml)	6.38±1.35	6.83±1.13	20.07±3.25*	22.25±5.45*
TNF-α (pg/ml)	78.4±8.6	83.1±7.5	123.7±18.2*	118.5±16.1*
CyPA (pM)	486.7±74.0	506.6±72.0	771.8±36.8*	572.2±42.7
IFN-γ (pg/ml)	11.49±2.23	11.24±2.02	24.91±1.75*	28.32±2.73*

N is 6-10 in each group. Data are expressed by mean±s.e.m. The *P* values were obtained by a two-way ANOVA with Bonferroni's multiple comparisons. * *P*<0.05 compared to saline Apoe$^{-/-}$ mice.

Supplementary Table 4. Serum lipid and glucose level in 3-HAA-injected mice

Groups	Vehicle		3-HAA	
	Apoe$^{-/-}$	Apoe$^{-/-}$/IDO$^{-/-}$	Apoe$^{-/-}$	Apoe$^{-/-}$/IDO$^{-/-}$
Cholesterol (mg/dl)	489.7±26.6	492.5±30.0	471±26.6	470.8±30.8
Triglyceride (mg/dl)	125.4±4.8	135.8±7.0	131.5±5.5	123.4±10.1
BG (mg/dl)	127±29	142±32	137±24	132±29

N is 6-10 in each group. Data are expressed by mean±s.e.m. The *P* values were obtained by a two-way ANOVA.

Supplementary Table 5. Blood pressure and heart rate in AngII-infused Apoe$^{-/-}$ mice

Kynureninase siRNA	-	+
sBP (mmHg)	158.3±20.4	149.1±16.3
dBP (mmHg)	109.6±14.7	112.9±12.3
HR (Beats/min)	489±35	467±41

N is 6-10 in each group. Data are expressed by mean±s.e.m.

The *P* values were obtained by a *t* test.

Supplementary Table 6. Serum lipid and glucose level in AngII-infused Apoe$^{-/-}$ mice

Kynureninase siRNA	-	+
Cholesterol (mg/dl)	496.1±22.0	493.1±22.1
Triglyceride (mg/dl)	125±12.6	134.4±6.6
BG (mg/dl)	162±31	147±23

N is 6-10 in each group. Data are expressed by mean±s.e.m.

The *P* values were obtained by a *t* test.

Supplementary Table 7. Serum cytokines in AngII-infused Apoe$^{-/-}$ mice

Kynureninase siRNA	-	+
IL-6 (pg/ml)	24.1±2.8	22.7±3.0
TNF-α (pg/ml)	130.6±14.7	122.0±11.3
CyPA (pM)	739.8±50.3	605.3±62.1
IFN-γ (pg/ml)	28.3±2.1	25.9±1.8

N is 6-10 in each group. Data are expressed by mean±s.e.m.

The *P* values were obtained by a *t* test.

Supplementary Table 8. Primers for PCR

PCR production	Sequences (Forward/reverse)
H-MMP2	5′-TACAGGATCATTGGCTACACACC-3′ 5′-GGTCACATCGCTCCAGACT-3′
H-GAPDH	5′-GGAGCGAGATCCCTCCAAAAT-3′ 5′-GGCTGTTGTCATACTTCTCATGG-3′
M-MMP2	5′-CAAGTTCCCCGGCGATGTC-3′ 5′-TTCTGGTCAAGGTCACCTGTC-3′
M-GAPDH	5′-AGGTCGGTGTGAACGGATTTG-3′ 5′-TGTAGACCATGTAGTTGAGGTCA-3′

Human (H); murine (M). .