Activation of NAD(P)H Oxidase by Tryptophan-Derived 3-Hydroxykynurenine Accelerates Endothelial Apoptosis and Dysfunction In Vivo

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Rationale: The kynurenine (Kyn) pathway is the major route for tryptophan (Trp) metabolism in mammals. The Trp–Kyn pathway is reported to regulate several fundamental biological processes, including cell death.

Objective: The aim of this study was to elucidate the contributions and molecular mechanism of Trp–Kyn pathway to endothelial cell death.

Methods and Results: Endogenous reactive oxygen species, endothelial cell apoptosis, and endothelium-dependent and endothelium-independent vasorelaxation were measured in aortas of wild-type mice or mice deficient for nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase subunits (p47phox or gp91phox) or indoleamine-pyrrole 2,3-dioxygenase 1 with or without angiotensin (Ang) II infusion. As expected, AngII increased plasma levels of Kyn- and 3-hydroxykynurenine-modified proteins in endothelial cells in vivo. Consistent with this, AngII markedly increased the expression of indoleamine-pyrrole 2,3-dioxygenase in parallel with increased expression of interferon-γ. Furthermore, in wild-type mice, AngII significantly increased oxidative stress, endothelial cell apoptosis, and endothelial dysfunction. These effects of AngII infusion were significantly suppressed in mice deficient for p47phox, gp91phox, or indoleamine-pyrrole 2,3-dioxygenase 1, suggesting that AngII-induced enhancement of Kynurenines via NAD(P)H oxidase–derived oxidants causes endothelial cell apoptosis and dysfunction in vivo. Furthermore, interferon-γ neutralization eliminates AngII-enhanced superoxide products and endothelial apoptosis by inhibiting AngII-induced Kynurenines generation, suggesting that AngII-activated Kyn pathway is interferon-γ–dependent. Mechanistically, we found that AngII-enhanced 3-hydroxykynurenine promoted the generation of NAD(P)H oxidase–mediated superoxide anions by increasing the translocation and membrane assembly of NAD(P)H oxidase subunits in endothelial cells, resulting in accelerated apoptosis and consequent endothelial dysfunction.

Conclusions: Kyn pathway activation accelerates apoptosis and dysfunction of the endothelium by upregulating NAD(P)H-derived superoxide. (Circ Res. 2014;114:480-492.)

Key Words: 3-hydroxykynurenine ▪ apoptosis ▪ indoleamine-pyrrole 2,3-dioxygenase ▪ kynurenine ▪ NAD(P)H oxidase ▪ oxidative stress ▪ tryptophan

Endothelial dysfunction is characterized by increased endothelial cell apoptosis, impaired endothelium-dependent relaxation, and aberrant oxidative stress. Endothelial dysfunction accounts for a large portion of all cardiovascular diseases and is associated with significant morbidity and mortality.1-3 Recent evidence indicates that a specific endothelial dysfunction associated with cardiovascular diseases is the local formation of reactive oxygen species (ROS). Although ROS are well-known inducers of apoptosis,4-6 in cultured cells, a causal link between ROS and endothelial cells (ECs) in vivo and the inducing factors have not yet been fully established.

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Tryptophan (Trp) is an essential amino acid that functions as a biochemical precursor for neurotransmitters and other key biosynthetic intermediates, such as niacin. The kynurenine (Kyn) pathway is the major route for Trp metabolism in mammals. L-Tryptophan is first transformed into Kyn in a reaction catalyzed by 2 dioxygenases: tryptophan 2,3-dioxygenase, and indoleamine-pyrrole 2,3-dioxygenase (IDO). Expression of tryptophan 2,3-dioxygenase is normally restricted to the liver, whereas IDO, which catalyzes the rate-limiting step in the catabolism of Trp along the Kyn pathway, is found in other tissues. Next, kynurenine 3-hydroxylase converts Kyn to 3-hydroxykynurenine (3-OHKyn). This molecule is further metabolized in a series of steps to ultimately produce nicotinamide adenine dinucleotide (NAD).3 IDO is induced by
γ-unsequently increasing the formation of neurotoxic 3-OHKyn12,13 in endothelial apoptosis and dysfunction in mice in vivo. Whether the activation of the Kyn pathway contributes to no reports that AngII alters Trp or 3-OHKyn in vascular cells in vivo. Although AngII is a well-known inducer of NAD(P)H oxidase, but also induce the expressions of genes encoding inflammatory cytokines and adhesion molecules, such as interleukin-6,6,7 and interferon-γ (IFN-γ) in several cell types.5,6,8–11 For example, IFN-γ enhances the expression and activity of kynurenine 3-hydroxylase, consequently increasing the formation of neurotoxic 3-OHKyn12,13 in human macrophages and microglia cells. The pathways of Trp and the potentially pathological roles of Trp metabolites in ECs are largely unknown.

Overwhelming evidences suggest that angiotensin II (AngII) induces constitutive nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase in the vascular wall and, therefore, intracellular ROS production, resulting in excessive apoptosis and dysfunction of epithelium and endothelium.14–17 ROS not only act directly as proinflammatory cytokines in the vascular wall,4,18 but also induce the expressions of genes encoding inflammatory cytokines and adhesion molecules, such as interleukin-C,4,19 Although AngII is a well-known inducer of NAD(P)H oxidase, the molecular mechanisms by which AngII induces NAD(P)H oxidase activation are not fully elucidated. Currently, there are no reports that AngII alters Trp or 3-OH Kyn in vascular cells in vivo. Whether the activation of the Kyn pathway contributes to endothelial cell apoptosis and endothelial dysfunction by AngII remains unknown. Thus, the aim of the present study was to establish the roles of Trp-derived Kyn in AngII-induced endothelial apoptosis and dysfunction in mice in vivo.

**Methods**

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Data are reported as mean±SEM. An unpaired Student t test was used to detect significant differences between 2 groups. A 1-way ANOVA followed by Bonferroni multiple comparison tests, as applicable, was used to compare differences among ≥3 groups. A P value <0.05 was considered significant. A full description of Materials and Methods can be found in the Online Data Supplement.

**Results**

**Inhibition of NAD(P)H Oxidase–Derived ROS Ablates AngII-Induced Endothelial Apoptosis and Dysfunction In Vivo**

AngII is known to cause vascular endothelial dysfunction and endothelial injury.15,16,20,21 We thus decided to first determine if AngII increased endothelial cell apoptosis in AngII-infused mice in vivo. AngII infusion had no effect on heart rates or blood glucose (Online Table I), but did significantly increase blood pressure and cardiac hypertrophy (Online Table I; Figure 1A). As expected, AngII significantly increased superoxide anion levels (Figure 1A and 1B; Online Figure IB and IC). Importantly, a deficiency of p47phox or gp91phox remarkably suppressed AngII-induced superoxide formation.

AngII infusion significantly increased endothelial cell apoptosis (Figure 1C) and impaired endothelium-dependent relaxation in wild-type (WT) C57BL/6J mice (Figure 1D and 1E). Both these effects of AngII infusion were significantly attenuated in mice deficient for the NAD(P)H subunits (p47phox or gp91phox). Specifically, endothelial cell apoptosis was reduced from 52.6±2.7% in WT AngII-treated mice to 25.6±3.6% and 25.7±1.8% in p47phox- and gp91phox-deficient mice, respectively, whereas endothelium-dependent relaxation was 63.3±2.1% and 59.6±6.6%, respectively, versus 35.3±6.4% in WT AngII-treated mice (Figure 1C–1E; Online Figure II). These results suggest that NAD(P)H-derived ROS are likely responsible for AngII-induced increases in endothelial apoptosis and dysfunction. AngII did not affect the endothelium-independent vasorelaxation in mice induced by sodium nitroprusside at concentrations of 1×10−10 to 1×10−6 mol/L (Figure 1F and 1G). Taken together, these results suggest that AngII acts via NAD(P)H oxidase–derived ROS to trigger endothelial cell apoptosis and impair endothelium-dependent relaxation.

**IDO Deletion Eliminates AngII Infusion–Induced Kyn Generation**

Increased levels of Trp metabolites such as Kynurenines are associated with oxidative stress, endothelial dysfunction, and pathological vascular remodeling.22–26 Thus, we reasoned that AngII causes endothelial cell apoptosis and NAD(P)H oxidase activation by increasing Kynurenines in vascular cells. To test this idea, we first assayed plasma levels of Trp and Kyn. As depicted in Figure 2A, AngII significantly increased plasma Kyn in WT mice but not in IDO1-deficient mice. AngII infusion did not alter plasma Trp levels in any mouse genotype (data not shown), possibly reflecting the high content of Trp in mouse diets. Plasma 3-OHKyn was not detectable, possibly explained by the fact that 3-OHKyn could have reacted with intracellular proteins.10,27 On the basis of our plasma data, we conclude that IDO deletion eliminates the increase in plasma Kyn induced by AngII infusion in vivo (Figure 2A).

ECs are reported to be the primary site for Kynurenines generation in malaria- infected tissue in vivo.28,29 As depicted in Figure 2B, IDO protein expression was only observed in the endothelium of WT mice infused with AngII, and not in WT mice treated with saline, or IDO1−/− mice with or without AngII. Concomitant with increased staining of IDO in ECs, AngII infusion markedly increased endothelial cell staining with an antibody against 3-OH Kyn (Figure 2C).
ROS production in AngII-infused mice in vivo. As shown in Figure 2D and Online Figure IIIA, IDO deletion partially inhibited AngII-induced ROS formation. Next, we determined whether the Kyn pathway contributed to AngII-induced endothelial cell apoptosis. As expected, AngII administration greatly accelerated endothelial cell apoptosis compared with controls (76.0±6.8% and 9.6±1.5% for WT AngII and WT saline, respectively; \( P < 0.01 \)), whereas IDO deletion significantly decreased the numbers of apoptotic ECs induced by AngII. Endothelial function was assayed by acetylcholine (ACH)-induced endothelium-dependent relaxation in WT, p47phox−/−, and gp91phox−/− mouse aortas. F and G, Sodium nitroprusside (SNP)-induced endothelium-independent relaxation in WT, p47phox−/−, and gp91phox−/− mouse aortas are not impaired by AngII. \( * P < 0.05 \) vs WT-saline; \( ** P < 0.01 \) vs WT-saline; \( # P < 0.05 \) vs WT-AngII; n=4 to 10 per group.

Figure 1. p47phox or gp91phox deletion attenuates angiotensin II (AngII) infusion–mediated endothelial apoptosis and dysfunction in vivo. Wild-type (WT), p47phox−/−, and gp91phox−/− mice were infused with saline or AngII (1000 ng/min per kg) for 2 weeks. A and B, Deletion of p47phox and gp91phox ablates AngII-induced superoxide production in vivo. AngII-stimulated superoxide anions were detected by using DHE staining, as described in Materials and Methods section. C, Deletion of p47phox and gp91phox abolishes AngII-induced endothelial cell apoptosis. Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells in endothelial cells (n=4–6 per group; n=4 sections for each sample). D and E, p47phox−/− or gp91phox−/− deficiency rescues endothelial dysfunction induced by AngII. Endothelial function was assayed by acetylcholine (ACH)-induced endothelium-dependent relaxation in WT, p47phox−/−, and gp91phox−/− mouse aortas. F and G, Sodium nitroprusside (SNP)-induced endothelium-independent relaxation in WT, p47phox−/−, and gp91phox−/− mouse aortas are not impaired by AngII. \( * P < 0.05 \) vs WT-saline; \( ** P < 0.01 \) vs WT-saline; \( # P < 0.05 \) vs WT-AngII; n=4 to 10 per group.

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Next, we decided to determine if the Kyn pathway was involved in AngII-induced endothelial dysfunction. The aortas of WT AngII-infused mice exhibited severely impaired endothelium-dependent vasorelaxation compared with controls (Figure 2G). In contrast, IDO deletion markedly improved the endothelium-dependent response to acetylcholine (1×10–6 mol/L) in mice receiving AngII infusion (57.1±7.8% and 30.6±5.3% for IDO1−/− and WT, respectively; \( P < 0.05 \); Figure 2G). However, AngII did not alter the endothelium-independent vasorelaxation induced by sodium nitroprusside (1×10–10–1×10–6 mol/L) in WT or IDO1−/− mice (Figure 2H). These data suggest a role for the Kyn pathway in AngII-induced ROS generation, which results in endothelial apoptosis and dysfunction.

AngII Increases the Expression of IFN-γ in ECs In Vivo

Infusion of AngII in mice rapidly induces the cardiac expression of IFN-γ,30,31 a cytokine that has been reported to induce IDO expression in neurons, dendritic cells, macrophages, and lens epithelial cells.7,9,10,13 As shown in Figure 3A, AngII markedly induced IFN-γ expression in WT and IDO1−/− mice, suggesting that AngII increases IFN-γ in ECs in vivo. We postulated that AngII acted via increased IFN-γ expression to promote IDO-derived Kyn.

IFN-γ Neutralization Abrogates AngII-Induced ROS Production and Endothelial Cell Apoptosis by Inhibiting Kyn Pathway

To this end, we first measured plasma levels of Kyn in WT mice injected with anti-IFN-γ or rat control IgG antibody after AngII infusion. As shown in Figure 3B, IFN-γ neutralization remarkably eliminated AngII-increased plasma Kyn, but did not change plasma Trp level (data not shown). Consistently,
Figure 2. Indoleamine 2,3-dioxygenase (IDO) deletion alleviates endothelial apoptosis and dysfunction via superoxide anion elimination in vivo. Wild-type (WT) and IDO−/− mice were administrated with saline or angiotensin II (AngII; 1000 ng/min per kg) for 4 weeks. A, AngII increases kynurenine (Kyn). The serum concentrations of Kyn were detected by HPLC. B, AngII increases IDO expression in vivo. C, AngII increases the detection of 3-hydroxykynurenine (3-OHkyn)-modified proteins in WT but not in IDO−/− mice. B and C, Representative immunohistochemical staining (original magnification, ×400) and quantifications for IDO (B) and 3-OHkyn-modified proteins (C) in aortas. D, AngII increases superoxide anions in WT but not in IDO−/− mice. Superoxide anions were detected by using DHE staining. E and F, IDO deletion abrogates AngII-mediated endothelial apoptosis. Triple immunofluorescence photographs show apoptotic endothelial cells (green) in WT and IDO−/− mice aortic endothelium (CD31; red) and nuclei (Hoechst; blue). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells colocalized with CD31-positive cells, and CD31-positive cells were counted in all aortic sections (original magnification, ×25). Histogram shows the mean number of TUNEL–CD31-positive nuclei/CD31-positive nuclei. Images are representative of a study conducted in a separate set of animals (n=6 per group; n=4 sections for each sample). G and H, Endothelium-dependent relaxation and endothelium-independent relaxation in mouse aortas of WT and IDO−/− with or without AngII. G, Sodium nitroprusside (SNP)-induced endothelium-independent relaxation in mice aortas of WT and IDO−/− are not impaired by AngII. *P<0.01 vs WT-saline; #P<0.05 vs WT-AngII; n=4 to 10 per group.
AngII-induced IDO protein expression was ameliorated in endothelium of WT mice with anti-IFN-γ antibody injection (Figure 3C), as well as the expressions of AngII-induced 3-OHkyn modified proteins (Figure 3D), suggesting that AngII-activated Kyn pathway is IFN-γ-dependent.

Next, we tested if IFN-γ-derived Kynurenines participated in AngII-induced ROS generation. As expected, IFN-γ neutralization significantly decreased superoxide anions in endothelium of WT mice with AngII infusion (Figure 3E; Online Figure IIIIB). Consequently, cleaved caspase-3, a maker for apoptosis, was significantly increased in AngII-induced ECs (Figure 3F), whereas AngII-induced cleaved caspase-3 was markedly inhibited by anti-IFN-γ treatment (Figure 3F). Taken together, IFN-γ mediates AngII-induced Kynurenines generation, resulting in oxidative stress and endothelial apoptosis in vivo.

**IFN-γ Accelerates Apoptosis of ECs Via Induction of the Kyn Pathway**

Accordingly, we determined if endogenous Kynurenines contributed to IFN-induced cell death in human aortic endothelial cells (HAECs) in vitro. We first detected the cleaved forms of poly (ADP-ribose) polymerase (PARP), caspase-7, and caspase-3 (key factors in apoptosis) in HAECs. As shown in Figure 4A and 4B, IFN-γ increased the amount of cleaved PARP, caspase-7, and caspase-3 in a concentration- and time-dependent manner. In addition, pretreatment with IDO inhibitor (1-methyl-β-d-tryptophan; 1-MT) or kynurenine 3-hydroxylase inhibitor (Ro61-8048) attenuated the IFN-γ–induced increases in cleaved PARP, caspase-7, and caspase-3 (Figure 4C).

We next quantified apoptosis using caspase-3 activity assay and TUNEL staining in IFN-γ–stimulated HAECs. Consistent with this, pretreatment with 1-MT suppressed IFN-γ–induced increases in caspase-3 activity (27.3%) and TUNEL-positive cell numbers (53.1%); a similar degree of suppression was produced by Ro61-8048 (21.6% and 62.7%, respectively; Figure 4D and 4E; Online Figure IV). As we expected, pretreatment with 1-MT or Ro61-8048 decreased IFN-γ–induced ROS production (Figure 4F). Taken together, these results indicate that IFN-γ acts through the Kyn pathway to trigger oxidative stress and apoptosis in HAECs, which was further confirmed in mouse lung ECs (MLECs) isolated from WT
and IDO−/− mice in vitro (Figure 4G–4J; Online Figure VA and VB). IDO deficiency significantly ablated IFN-γ–induced ROS and cell apoptosis.

**IFN-γ Induces the Kyn Pathway of Trp Metabolism in ECs**

Next, to determine the effects of IFN-γ on Trp catabolism and Kyn formation in ECs in vitro, we incubated HAECs with different concentrations of IFN-γ for 24 hours or with 100 ng/mL IFN-γ for the indicated times. As shown in Figure 5A and 5D, the protein levels of IDO and kynurenine 3-hydroxylase increased with increasing concentrations of IFN-γ and incubation time. We also determined the effects of IFN-γ on supernatant concentrations of Kyn and Trp in HAECs using HPLC. IFN-γ (0.25–25 ng/mL) induced a concentration- and time-dependent increase in endogenous Kyn formation with a concomitantly dramatic decrease in Trp consumption (Figure 5B and 5E). The reciprocal changes in Kyn and Trp content in the culture medium were first evident 8 hours after treatment with IFN-γ (100 ng/mL; Figure 5E). Notably, the decrease in Trp in the supernatant was more rapid than the increase in Kyn after 48 hours of IFN-γ stimulation (Figure 5E). The changes in supernatant Kyn/Trp concentration ratio, which reflects IDO activity, and IDO protein expression exhibited the same trend under the indicated conditions (Figure 5C and 5F). Treatment with 1-MT (200 μmol/L) significantly inhibited the IFN-γ–induced Kyn generation (37.1%) and Trp decline (33.4%) in the culture medium (Figure 5G), which was further confirmed in MLECs. IDO deletion totally ablated IFN-γ–induced Kyn generation and Trp consumption (Figure 5H and 5I).

We next determined if the difference in the Trp decrease and Kyn increase might be because of increased association of 3-OHKyn with intracellular proteins. To this end, we incubated HAECs with exogenous 3-OHKyn (60 μmol/L) for 24 hours and immunostained cells for 3-OHKyn–modified proteins. Staining for 3-OHKyn–modified proteins was detected using a terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay. Data were reported as %TUNEL-positive in a microscopic field±SE. F and J, Superoxide production was assessed by DHE/HPLC. *P<0.05 vs control; **P<0.01 vs control; #P<0.05 vs IFN-γ (A–D) or WT IFN-γ (G–J); ##P<0.01 vs IFN-γ (A–F) or WT IFN-γ (G–J).
much more striking in cells treated with extracellular 3-OHKyn than in control cells (Figure 6A and 6B). Because 3-OHKyn content was virtually undetectable in supernatants by HPLC, we attempted to determine whether IFN-γ treatment resulted in 3-OHKyn modification of proteins in HAECs. As expected, IFN-γ treatment induced a marked increase of 3-OHKyn–modified proteins (Figure 6C and 6D), which was inhibited by incubation with 1-MT (200 μmol/L) or Ro61-8048 (50 μmol/L; Figure 6C and 6D).

Furthermore, immunoblot analyses of cell lysates with an anti-3-OHKyn-KLH antibody revealed a significant increase in 3-OHKyn–modified proteins (molecular weights, ≈ 50 and 65 kDa) in IFN-γ–stimulated cells compared with control cells (Figure 6E). This was further confirmed in MLECs. IDO knockout remarkably eliminated the formation of 3-OHKyn–modified proteins induced by IFN-γ (Figure 6F–6H).

**3-OHKyn Induces Endothelial Cell Apoptosis**

To determine the effects of Kynurenines on endothelial cell apoptosis, we cultured confluent HAECs in serum-free medium overnight and exposed them to exogenous Kyn or 3-OHKyn at 60 μmol/L for 24 hours. Exposure to Kyn had no effect on HAEC apoptosis (Figure 7A). In contrast, treatment with 3-OHKyn markedly increased protein cleavage of PARP, caspase-7, and caspase-3; elevated caspase-3 activity; and enhanced TUNEL staining (Figure 7A–7C; Online Figure VI), indicating that 3-OHKyn potently promoted apoptosis in HAECs.

These effects of 3-OHKyn appeared to be concentration-dependent (Figure 7D–7F; Online Figure VIIA). Cleaved PARP, caspase-7, and caspase-3 were detectable at 3-OHKyn concentrations as low as 5 μmol/L and increased with increasing concentration (Figure 7D). Consistent with this, caspase-3 activity was significantly increased at concentrations of ≥ 20 μmol/L (P<0.01), with greater increases observed at 60 μmol/L (≈2.1-fold) than at 20 and 40 μmol/L (≈1.5-fold; Figure 7E). Similarly, the percentage of TUNEL-positive cells increased progressively with increasing concentrations of 3-OHKyn (P<0.01); the percentage of TUNEL-positive cells was ≈ 3-fold greater than that in controls at 20 μmol/L 3-OHKyn and increased to ≈ 4.5-fold at 60 μmol/L 3-OHKyn (Figure 7F; Online Figure VIIA).
3-OHkyn–induced HAEC apoptosis was also time-dependent. The 3-OHkyn–induced increases in cleaved PARP, caspase-7, and caspase-3 were detectable <4 hours, reached a peak after 8 hours of treatment, and declined slightly after 24 hours of treatment (Figure 7G). A similar time course was also observed for changes in caspase-3 activity and TUNEL.
staining (Figure 7H and 7I; Online Figure VIB). Finally, chelation of transition metals with diethylenetriaminepentaacetic acid did not affect cell apoptosis induced by 3-OHKyn (data not shown), suggesting that transition metals are not required for 3-OHKyn–induced apoptosis.

3-OHKyn Acts Via ROS to Increase Mitochondrial Cytochrome c Release

The induction of endothelial cell apoptosis by 3-OHKyn treatment was further confirmed by measuring changes in the number of viable ECs by MTT assay after treatment with different concentrations of 3-OHKyn for 24 hours. As shown in Figure 8A, 3-OHKyn reduced endothelial cell viability in a concentration-dependent manner, with significant effects observed at the lowest concentration tested (5 μmol/L).

Next, we investigated how 3-OHKyn triggered apoptosis in ECs. Previous studies had reported that glutathione and NADH play a major role in preventing Kynurenines-induced modifications in human lens. Thus, we hypothesized that 3-OHKyn may induce intracellular ROS generation. As expected, 3-OHKyn increased ROS formation in a concentration-dependent manner, with a minimal effective concentration of 20 μmol/L (Figure 8B).

ROS are reported to increase mitochondrial cytochrome c release, which activates caspase-9 and accelerates apoptosis. Accordingly, we tested whether 3-OHKyn altered cytochrome c release in HAECs. As shown in Figure 8C, cytochrome c was detectable in the cytoplasm of 3-OHKyn–treated cells, but not in control cells. Mitochondrial cytochrome c release increased with increasing concentrations of 3-OHKyn (Figure 8C).

Overexpression of Superoxide Dismutase Attenuates 3-OHKyn–Induced Increase in Apoptosis

The damaging effects of ROS are normally kept under control by endogenous antioxidant systems, including glutathione, ascorbic acid, and enzymes, such as superoxide dismutase (SOD), glutathione peroxidase, and catalase. In the presence of mitochondrial SOD, superoxide anion ($O_2^-$) can be converted to $H_2O_2$, which can diffuse out of mitochondria into the cytoplasm. Overexpression of MnSOD, an SOD isoform located in the mitochondrial matrix, attenuated 3-OHKyn–induced mitochondrial cytochrome c release in HAECs in association with a concomitant decrease in 3-OHKyn–induced cleavage of PARP, caspase-7, and caspase-3 (Figure 8D). Taken together, these results suggest that 3-OHKyn increases ROS generation, which subsequently causes cytochrome c release from mitochondria and endothelial cell apoptosis in HAECs.

NAD(P)H Oxidase Is a Major Source for 3-OHKyn–Induced ROS Generation

We next determined the sources of 3-OHKyn–derived ROS in ECs. HAECs were pretreated with tempol (10 μmol/L), a SOD

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**Figure 7.** 3-Hydroxykynurenine (3-OHKyn) is apoptosis-inducing kynurenine. A to C, 3-OHKyn induces cell apoptosis. Human aortic endothelial cells (HAECs) were treated with complete serum medium or serum-free medium alone or containing kynurenine (60 μmol/L) or 3-OHKyn for 24 hours. D to F, 3-OHKyn induces cell apoptosis in a dose-dependent manner. HAECs were incubated for 24 hours with various concentrations of 3-OHKyn. G to I, 3-OHKyn induces cell apoptosis in a time-dependent manner. HAECs were treated with 60 μmol/L 3-OHKyn for different times. A, D, and G, Cell lysates were subjected to immunoblot analysis to detect the protein cleavage of poly (ADP-ribose) polymerase (Parp), caspase-7, and caspase-3. B, E, and H, Results of caspase-3 activity were the mean±SE of 3 independent measurements. C, F, and I, Data were reported as %TUNEL-positive in a microscopic field±SE. TUNEL represents terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling. *P<0.05 vs SFM or control; **P<0.01 vs control.
mimetic, and apocynin (100 μmol/L), a selective NAD(P)H oxidase inhibitor, for 1 hour before exposure to 3-OHkyn for 24 hours. As shown in Figure 8E, both tempol and apocynin markedly inhibited ROS generation induced by 3-OHkyn in HAECs (Figure 8E). In contrast, l-NAME, a nonselective NO synthase inhibitor, had no inhibitory effect on 3-OH–induced ROS (data not shown). Consequently, 3-OHkyn–induced cleavage of PARP, caspase-7, and caspase-3 were significantly attenuated by tempol or apocynin pretreatment (Figure 8F), which was further confirmed by the overexpression of Cu/Zn SOD (SOD1), a SOD isoform located in the cytosol (Figure 8G).

Because apocynin blocked 3-OHkyn–induced ROS, we next determined whether 3-OHkyn altered the expression or subcellular localization of NAD(P)H oxidase subunits p47phox and p67phox in HAECs. 3-OHkyn significantly increased the expression of both subunits in the membrane fraction (Figure 8H) and increased NAD(P)H oxidase activity (Figure 8I). Taken together, these results indicate that 3-OHkyn promotes the translocation of p47phox and p67phox subunits from the cytosol to the plasma membrane, where they become assembled into an O₂⁻-producing complex.

Finally, we determined how 3-OHkyn promoted the translocation of p47phox and p67phox NAD(P)H oxidase subunits. Western blot analyses of p47phox and p67phox immunoprecipitates using an anti-3-OHkyn-KLH antibody revealed that p47phox and p67phox coimmunoprecipitated with 3-OHkyn in stimulated HAECs but not in control cells (Figure 8J), suggesting that 3-OHkyn can bind to p47phox and p67phox, resulting in their membrane translocation and consequent NAD(P)H oxidase activation.
Discussion

In this study, we show for the first time that Kynurenines accelerate apoptosis in ECs in vitro and in vivo through the induction of NAD(P)H oxidase–derived ROS generation. Our results elucidate the IFN-γ–induced signaling pathway of Kynurenines formation and the molecular mechanism responsible for the proapoptotic role of 3-OHKyn in ECs in vitro (Online Figure VIII). 3-OHKyn, which reacts with and chemically modifies cytosolic NAD(P)H oxidase subunits p47phox and p67phox, increases intracellular ROS by promoting the translocation of NAD(P)H oxidase subunits. ROS subsequently induces mitochondrial cytochrome c release and endothelial cell apoptosis. In line with these observations, we found that infusion of AngII-activated Kyn metabolic pathway in the mouse aortic endothelium is IFN-γ–dependent. Activation of the Kyn pathway is shown to have an important effect on AngII-induced NAD(P)H oxidase–dependent oxidative stress, which contributes to the endothelial apoptosis and dysfunction in vivo triggered by AngII infusion (Online Figure VIII).

One of the major findings of this study is that 3-OHKyn is an apoptotic product of Trp metabolism through the Kyn pathway. Exogenous 3-OHKyn, but not Kyn, caused apoptosis of HAECS in a concentration- and time-dependent manner. Consistent with this, inhibition of 3-OHKyn formation caused by IFN-γ also significantly abolished IFN-γ–induced apoptosis. Furthermore, IFN-γ–induced apoptosis was found in MLECs from WT, but significantly less in MLECs from IDO−/− mice. Taken together, these results indicate that endogenous 3-OHKyn formed in response to IFN-γ is responsible for increased apoptosis in ECs.

Consistent with previous reports showing that 3-OHKyn plays a proapoptotic role in other cell lines through the induction of ROS, we also found that 3-OHKyn induced ROS formation and cytochrome c release into the cytosol. ROS scavengers and inhibitors of ROS formation markedly attenuated 3-OHKyn–induced endothelial cell apoptosis. Mechanistically, we found that 3-OHKyn chemically modified p47phox and p67phox, resulting in increased translocation and assembly of p47phox and p67phox within the plasma membrane. As a result, 3-OHKyn increased the activity of NAD(P)H oxidase, leading to increased production of ROS in ECs.

We also demonstrated for the first time that infusion of AngII induces endogenous formation of Kynurenines in the circulation and endothelium by increasing IFN-γ expression. A causative link of AngII and IFN-γ is well supported by the results obtained from the IFN-γ neutralization antibody. The administration of the antibody against IFN-γ rather than control IgG1 antibody markedly reduced the plasma levels of Kyn and the expressions of IDO and 3-OHKyn modified proteins, superoxide production, and apoptosis in the endothelium. Consistently, IDO deletion attenuated the formation of superoxide products induced by AngII infusion, thereby ameliorating endothelial death and impaired endothelium-dependent vasorelaxation in vivo, whereas impaired NAD(P)H oxidase–derived ROS rescued AngII-induced endothelial apoptosis and dysfunction. In addition, IFN-γ neutralization eliminates AngII-increased superoxide products and endothelial apoptosis by inhibiting AngII-induced Kynurenines generation. Our results are in line with other studies showing that the Kyn pathway has an important role in several fundamental biological processes, including central nervous system disorders, peripheral disorders, infections, immunoregulation, and ultraviolet protection and cataract formation in the lens. Kynurenines were recently reported to mediate apoptosis in lens and renal tubular epithelial cells and fiber cells. Recently, an increase in Kynurenines has been implicated in oxidative stress, immune/inflammatory responses, endothelial dysfunction, progression of atherosclerosis, and vascular remodeling in cardiovascular diseases.

Because NAD(P)H oxidase–derived ROS are considered common mediators of endothelial dysfunction and vascular inflammation, the modification of NAD(P) H oxidase by Trp-derived 3-OHKyn might be a common mechanism for these diseases.

In summary, we have demonstrated that 3-OHKyn, a product of Trp metabolism, increases NAD(P)H oxidase–derived ROS, endothelial cell apoptosis, and endothelial dysfunction in vivo. Thus, inhibiting 3-OHKyn formation might be a potential therapeutic strategy for preventing endothelial dysfunction in cardiovascular diseases.

Sources of Funding

This study was supported by funding from the National Institutes of Health RO1 (HL110488, HL105157, HL096032, HL080499, HL089920, HL079584, and HL074399), the American Diabetes Association, the Warren Chair in Diabetes Research of the University of Oklahoma Health Science Center (all to M.-H.Z.), and a Scientist Development Grant (11SDG5560036) from American Heart Association (to P.S.). M.-H.Z. is a recipient of the National Established Investigator Award of the American Heart Association.

Disclosures

None.

References


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**Novelty and Significance**

**What Is Known?**

- An increase in Kynurenines has been implicated in oxidative stress, immune and inflammatory responses, and endothelial dysfunction.
- Angiotensin II (AngII) induces apoptosis and epithelial and endothelial dysfunction.
- Interferon-γ (IFN-γ) powerfully induces indoleamine 2,3-dioxygenase (IDO) expression in several cell types.

**What New Information Does This Article Contribute?**

- AngII induces endogenous formation of Kynurenines in the circulation and the endothelium by increasing IFN-γ expression.
- Kynurenines accelerate apoptosis in endothelial cells (ECs) in vitro and in vivo through induction of nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase–derived reactive oxygen species (ROS) generation.

- 3-Hydroxykynurenine (3-OHKyn) is a product of tryptophan metabolism through the kynurenine (Kyn) pathway that promotes apoptosis.

This study shows that the activation of the Kyn pathway by AngII is IFN-γ–dependent. The activation of the Kyn pathway promotes AngII-induced NAD(P)H oxidase–dependent oxidative stress, leading to endothelial apoptosis and dysfunction in vivo. In addition, our results elucidate a proapoptotic role for 3-OHKyn in ECs in vitro. 3-OHKyn reacts with and chemically modifies the cytosolic NAD(P)H oxidase subunits p47phox and p67phox, subsequently increasing intracellular ROS by promoting their translocation and resulting in endothelial cell apoptosis. Taken together, these data show that Kynurenines accelerate apoptosis in ECs in vitro and in vivo through the induction of NAD(P)H oxidase–derived ROS generation.
Activation of NAD(P)H Oxidase by Tryptophan-Derived 3-Hydroxykynurenine Accelerates Endothelial Apoptosis and Dysfunction In Vivo
Qiongxin Wang, Miao Zhang, Ye Ding, Qilong Wang, Wencheng Zhang, Ping Song and Ming-Hui Zou

Circ Res. 2014;114:480-492; originally published online November 26, 2013;
doi: 10.1161/CIRCRESAHA.114.302113
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/114/3/480

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/11/26/CIRCRESAHA.114.302113.DC1
Supplemental Material

Online Materials and methods supplement

Reagents.

Human aortic endothelial cells (HAECs) and Dynabeads® Sheep Anti-Rat IgG were obtained from Invitrogen™ (Life Technologies, Grand Island, NY). Antibodies to IDO, Cytochrome C, CD31, active Caspase-3, and Zn SOD (SOD1) were from Abcam (Cambridge, MA). Antibodies to p47phox, Na+ K+ ATPaseα, β-actin and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against KMO was from Proteintech Group (Chicago, IL). An antibody to locate 3-Hydroxykynurenine (P3UI) was from LifeSpan BioSciences (Seattle, WA). An antibody to Mn SOD (SOD2) was from Enzo Life Sciences (Farmingdale, NY). An antibody against IFN-γ was from Life Technologies. Antibodies against Parp, Cleaved Caspase-7, Cleaved Caspase-3 and p67phox, and all secondary antibodies were from Cell Signaling (Boston, MA). Ultra-LEAF™ Purified anti-mouse IFN-γ (XMG1.2) and Ultra-LEAF™ Purified Rat IgG1 antibodies were from Biolegend (San Diego, CA). Rat anti-Mouse CD102 (ICAM-2) and Rat anti-Mouse CD31 (PECAM-1) antibodies were from BD Pharmingen™ (BD Biosciences, San Jose, CA). A goat anti-mouse IgG conjugated to Alexa594 red, a goat anti-Rabbit IgG conjugated to Alexa555 red, Dihydroethidium (DHE), and Hoechst 33342 were purchased from Molecular Probes (Life Technologies). IFN-γ Recombinant Human, IFN-γ Recombinant Mouse and Fibronectin Human Protein were obtained from Gibco (Life Technologies). L-kynurenine, DL-Tryptophan, 3-Hydroxy-DL-kynurenine, 1-Methyl- DL-tryptophan (1-MT) to inhibit IDO activity and Angiotensin II (Ang II) human were from Sigma-Aldrich (St. Louis, MO). 3, 4-Dimethoxy-N- [4-(3-nitrophenyl)-2-thiazolyl] benzenesulfonamide (Ro61-8048) to block the activity of kynurenine 3-hydroxylase was from TOCRIS Bioscience (Ellisville, MO). MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide) cell proliferation assay kit was obtained from ATCC (Manassas, VA); Caspase-3 activity assay kit was obtained from Molecular Probes (Invitrogen); In Situ Cell Death Detection kit (TMR red) was obtained from Roche Applied Science (Indianapolis, IN). Qproteome Cell Compartment Kit was purchased from Qiagen (Valencia, CA). Mini-Osmotic pumps (Model 2004) were obtained from Alzet Durect Corp (Cupertino, CA). EnVision®+ Dual Link System-HRP (DAB+) was from DakoCytomation (Carpinteria, CA). DeadEnd™ Fluorometric TUNEL System was purchased from Promega (Madison, WI). Other chemicals, if not indicated, were from Sigma-Aldrich.

Animals.
IDO1−/− mice, p47phox−/− mice, gp91phox−/− mice, and C57BL/6J wild-type (WT) mice (genetic controls) were obtained from Jackson laboratory (Bar Harbor, ME). 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.

AngII (1,000 ng/kg/min) or physiological saline (0.9% NaCl) was infused continuously by subcutaneously implanted Alzet osmotic pumps (Model 2004) from weeks 8-10 or 12, as described previously1. WT mice were intraperitoneally injected with 0.5 ml of anti-IFN-γ (XMG1.2) or control rat IgG1 antibody at a concentration of 0.5 mg/ml every 7 days for 4 weeks beginning on the day of AngII infusion. After 2 or 4 weeks of infusion, mice were anesthetized with inhaled isoflurane and weighed. Hearts and aortas were removed and immediately separated carefully. Hearts were dried and weighed. At the time of harvest, a portion of aortic samples was incubated in 10% buffered formalin for 24 h, and in reagent alcohol for subsequent permanent paraffin embedding and sectioning; another portion was freshly embedded in O.C.T. compound for subsequent cryosectioning.

**Cell Culture.**

Human aortic endothelial cells (HAECs) were grown in endothelial cell basal medium (Lonza, Walkersville, MD) containing 5% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), and growth factor. All cells were incubated at 37°C in a humidified atmosphere of 5% CO2. Cells were used between passages 3 and 10, and in all experiments were grown to 70–80% confluence before being treated with different agents.

**Isolation of mouse Lung Endothelial Cells (MLEC).**

Isolation of primary MLEC was performed as described previously2. Purified rat anti-mouse CD31 and ICAM-2 antibodies were covalently coupled to pre-washed Dynabead® sheep anti-rat IgG during an overnight incubation. Lung tissue was excised, minced, digested with collagenase A, triturated with a 30-cc syringe, and passed through a 70 μm cell strainer. Anti-CD31 coated beads were added to the cell suspension, mixed, and incubated for 10 min at room temperature. The bead-bound cells were isolated with a magnet, resuspended in DMEM, and plated on Fibronectin-coated T75 flasks. Cells were detached, suspended and incubated with anti-ICAM-2
coated Dynabeads, when the cells approached confluence. The bead-bound cells were isolated with a magnet, resuspended in EBM and plated in Fibronectin-coated T75 flasks.

**Blood pressure measurement.**

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

**Immunofluorescence (IF).**

CD31/TUNEL fluorescent double Staining was preceded as described previously. The procedures began with CD31 Staining. The thoracic aorta cryosections were fixed in cold acetone, followed by acetone: chloroform (1:1) and acetone alone again for 5 min each. Samples were blocked with protein block solution (Protein Block), and then incubated with primary antibody (anti-CD31) overnight at 4°C. For negative control, added the protein block solution without anti-CD31 antibody. Slides were rinsed with washing buffer and incubated with secondary antibody Alex555 Red (goat anti-rabbit) for 1h. Rinsed the slides and skipped to TUNEL Staining procedures. Samples were fixed with 4% paraformaldehyde (diluted in PBS) for 10 min, then incubated with 0.2% Triton-X-100 for 15 min after washing. Slides were rinsed and incubated with equilibration buffer (Promega kit), and then with incubation buffer containing the nucleotide mix (100 ul/slide containing equilibration buffer 90 ul, nucleatide mix 10 ul, TdT enzyme 2 μl) at 37°C for 1 hour. For negative control, prepare the incubation buffer without TdT enzyme. The slides were immersed in 2X SSC for 15 min and rinsed with washing buffer to remove unincorporated fluorescein-dUTP. Counterstain with Hoechst 33342 (1 μg/ml in PBS) for 5 min to locate nuclei. Mounted the slide with prolong anti-fade mounting medium (Invetrogen) and a glass cover slip. All the images are captured by Zeiss Confocal Laser Scanning Microscope (CLSM). The results are quantified using LSM510 image analysis software.

**Assays of endothelium-dependent and endothelium-independent vasorelaxation.**

Vessel bioactivity was assayed by organ chamber, as described previously. Contractile responses of aortic rings were evoked using 30 nM U46619 to elicit reproducible responses. At the plateau of contraction, Ach (1×10⁻⁸ to 1×10⁻⁴ mol/L) or SNP (1×10⁻¹⁰ to 1×10⁻⁶ mol/L) was progressively added to the organ bath to induce endothelium-dependent relaxation, respectively.

**Immunohistochemistry.**

Thoracic aortas were fixed in 4% paraformaldehyde overnight, and then processed, embedded in paraffin, and sectioned at 5 μM. Aortic sections were stained as described previously with modifications.
Semiquantitative analysis of tissue immunoreactivity 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 intima; score 2: 26-50% positive staining in intima; score 3: 51-75% positive staining in intima; score 4: 76-100% positive staining in intima) to estimate the degree of positive staining for each individual marker.

**Detection of ROS.**

Cell $O_2^-$ levels were measured according to the DHE fluorescence/ High-performance liquid chromatography (HPLC) assay$^6$ with minor modifications.

To measure ROS production in isolated aortas in situ, frozen descending thoracic aorta sections were stained with DHE as described previously with slight modification$^6$. Results were quantified using Image J Image software.

**Determination of tryptophan (Trp) and kynureine (Kyn).**

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. Samples preparation for plasma was operated as described previously$^7$. 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 10,000g for 2 min. The clear protein-free supernatant was analyzed directly.

Kyn and Trp were measured by HPLC as described previously with slight modification$^8$. Kyn content was estimated along with standards ranging from 0.5-20 μM L-kynurenine (Sigma-Aldrich), and Trp level was assessed along standards ranging from 1-25 μM DL-Tryptophan (Sigma-Aldrich), using a GraceVydac C18 column (250×4.6 mm, 5.0 μM). The mobile phase consisted of 20 mM sodium acetate (PH=4.5) and 20mM sodium acetate containing 20% methanol was pumped at a flow rate of 1 ml/min. The column effluent was monitored at 360 nm (Kyn) and 280 nm (Trp) by a UV detector (JASCO MD-2015 Plus Multiwavelength).

**Adenoviral infection.**

Cultured HAECs at 60% confluence were inflected with adenovirus encoding green fluorescence protein (GFP) and super oxide dismutase 1/2 (SOD1/2), and then washed and incubated in fresh medium for an additional 12 h before experimentation. Under these conditions, the infection efficiency typically exceeded 80% as determined by GFP expression.

**Cell Fractionation.**
Subcellular fractions were performed using the Qproteome Cell Compartment Kit, according to manufacturer’s protocol.

**Cell Viability Assay.**

Cell viability was measured by MTT assay according to the manufacturer’s protocol.

**Assay of Caspase-3 Activity.**

Caspase-3 Activity was quantified according to the manufacturer’s instructions by using highly sensitive assay kits obtained from Molecular Probes.

**NAD(P)H Oxidase Activity Assay.**

NADPH oxidase activity of the cell lysate was measured using a modified assay. Briefly, photon emission from the chromogenic substrate lucigenin as a function of acceptance of electron/O₂⁻ generated by the NADPH oxidase complex was measured every 15 s for 20 min in a microplate reader with a Berthold luminometer. 100μM NADH and 5μM lucigenin were used in each sample.

**Western Blot and Immunoprecipitation.** Immunoprecipitation and Western blotting were performed as described previously.

**TUNEL Staining.**

Terminal deoxynucleotidyltransferase-mediated nick-end labeling assay for HAECs was carried out according to a commercially available kit from Roche Applied Science.

**Immunocytochemistry.**

Cells were cultured in 12-well plates and fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton® x-100. After blocking with Protein Block (BioGenex), cells were incubated with a monoclonal antibody against 3OHKyn-modified KLH or STAT1 for 1 h at RT. A goat anti-mouse IgG conjugated to Alexa594 red or goat anti-rabbit IgG conjugated to Alexa488 was used as the secondary antibody, and cells were permanently mounted with DAPI (Invitrogen). As a negative control, cells were incubated with only the secondary antibody.

**Statistics.**

Data are reported as means ± standard errors of the means. An unpaired Student’s t-test was used to detect significant differences between two groups. A one-way analysis of variance followed by Bonferroni’s
multiple comparison tests, as applicable, was used to compare differences among three or more groups. P-values less than 0.05 were considered significant.

References


Online Figure I. Deficiency of p47phox and gp91phox abrogates AngII-stimulated superoxide anions in vivo. A, AngII increases cardiac hypertrophy. Histogram showed ratios of heart weights/bodyweights in WT and IDO1−/− mice infused with or without AngII for 4 weeks. B & C, Deletion of p47phox and gp91phox eliminates AngII-stimulated superoxide anions in vivo. Representative images of DHE staining (original magnification, X400) in aortas from WT, p47phox−/− and gp91phox−/− mice infused with or without AngII for 2 weeks.

Online Figure II. Deletion of p47phox and gp91phox ablates AngII-induced endothelial apoptosis in vivo. Triple immunofluorescence photographs show apoptotic endothelial cells (green) in aortic endothelium (CD 31, red); nuclei (Hoechst, blue) of WT, p47phox−/− and gp91phox−/− mice infused with or without AngII for 2 weeks. Images are representatives of a study conducted in a separate set of animals (n=4-6 per group, n=4 sections for each sample). TUNEL positive cells colocalized with CD31 positive cells and CD31 positive cells were counted in all aortic sections (original magnification, 25×).
Online Figure III. IDO inhibition ameliorates AngII-induced superoxide anions in vivo. A, IDO deletion inhibits AngII-induced superoxide anions. Representative images of DHE staining (original magnification, X400) in aortas from WT and IDO−/− mice infused with or without AngII for 4 weeks. B, IFN-γ neutralization ameliorates AngII-induced superoxide anions in vivo. Representative images of DHE staining (original magnification, X400) in aortas from WT mice intraperitoneally injected with anti-IFN-γ or control rat IgG1 antibody for 4 weeks beginning on the day of AngII infusion.

Online Figure IV. Inhibition of IDO or KMO eliminates IFN-γ-induced cell apoptosis. HAECs were pretreated for 1 hour with medium alone or medium containing MT (200µM) or Ro61-8048 (50 µM) before incubation with or without IFN-γ (100ng/ml) for 48 hours. Nuclear DNA fragment as a sign of apoptosis was detected using a TUNEL assay and indicated by red fluorescence. Nuclei were stained with DAPI (Blue) (From up: Death, DAPI and merge).
Online Figure V. IDO deficiency ablates IFN-γ-induced cell apoptosis. MLECs isolated from WT and IDO−/− mice were challenged with or without 100ng/ml IFN-γ for 48 hours. A, Endothelial cell specific staining (CD31) with primary MLECs isolated from WT and IDO−/− mice. Immunocytochemistry was performed to localize ECs by rabbit anti-CD31 monoclonal primary antibody and Alexa555 red-conjugated goat anti- rabbit IgG secondary antibody. Cells were counterstained with a nuclear stain, DAPI (blue) (From left: CD31, DAPI and merge). B, IDO
deficiency inhibits IFN-γ-induced cell apoptosis. Nuclear DNA fragment as a sign of apoptosis was detected using a TUNEL assay and indicated by green fluorescence. Nuclei were stained with DAPI (Blue) (From up: Death, DAPI and merge).

**Online Figure VI. 3-OHkyn induces cell apoptosis.** HAEC cells were treated with complete serum medium or serum free medium alone or containing Kyn (60µM) or 3-OHkyn for 24 hours. Nuclear DNA fragment as a sign of apoptosis was detected using a TUNEL assay and indicated by red fluorescence. Nuclei were stained with DAPI (Blue) (From up: Death, DAPI and merge).
**Online Figure VII.** 3-OHkyn induces cell apoptosis in a dose- and time-dependent manner. A, HAEC cells were incubated for 24 hours with various concentration of 3-OHkyn. HAEC cells were treated with 60µM 3-OHkyn for different time. Nuclear DNA fragment as a sign of apoptosis was detected using a TUNEL assay and indicated by red fluorescence. Nuclei were stained with DAPI (Blue) (A and B, from up: Death, DAPI and merge).
Online Figure VIII. Proposed mechanism for kynurenine pathway activation in endothelial apoptosis and dysfunction. AngII up regulates IFN-γ expression in endothelial cells. IFN-γ induces IDO and KMO expressions which catalyze tryptophan metabolism in Kyn pathway. 3-OHkyn, the important product of this pathway, which reacts with and chemically modifies cytosolic proteins, increases intracellular ROS by promoting the translocation and membrane assembly of NAD(P)H oxidase subunits (p47phox and p67phox) and induces mitochondrial cytochrome c release, resulting in cell apoptosis. The excessive superoxide, leading to endothelial apoptosis and NO bioactivity deficiency, contributes to endothelial dysfunction, and finally impairs vascular control.
Online Table I. AngII increases blood pressure in WT and IDO⁻/⁻ mice. Table showed blood glucose, systolic blood pressure, diastolic blood pressure and heart rate in WT and IDO⁻/⁻ mice with AngII infusion for 4 weeks. N is 6-10 in each group. Data were expressed by mean±s.e.m. *P<0.05 compared to WT Saline mice.

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