ORIGINAL RESEARCH ARTICLE

Nuclear Receptor NR1D1 Regulates Abdominal Aortic Aneurysm Development by Targeting the Mitochondrial Tricarboxylic Acid Cycle Enzyme Aconitase-2

Ling-Yue Sun, MS*; Yu-Yan Lyu, MD, PhD*; Heng-Yuan Zhang, MS; Zhi Shen, BS; Guan-Qiao Lin, BS; Na Geng, BS; Yu-Li Wang, BS; Lin Huang, PhD; Ze-Hao Feng, MS; Xiao Guo, PhD; Nan Lin, MS; Song Ding©, MD, PhD; An-Cai Yuan©, MD, PhD; Lan Zhang, MD, PhD; Kun Qian, PhD; Jun Pu©, MD, PhD

BACKGROUND: Metabolic disorder increases the risk of abdominal aortic aneurysm (AAA). NRs (nuclear receptors) have been increasingly recognized as important regulators of cell metabolism. However, the role of NRs in AAA development remains largely unknown.

METHODS: We analyzed the expression profile of the NR superfamily in AAA tissues and identified NR1D1 (NR subfamily 1 group D member 1) as the most highly upregulated NR in AAA tissues. To examine the role of NR1D1 in AAA formation, we used vascular smooth muscle cell (VSMC)-specific, endothelial cell–specific, and myeloid cell–specific conditional Nr1d1 knockout mice in both AngII (angiotensin II)– and CaPO4-induced AAA models.

RESULTS: Nr1d1 gene expression exhibited the highest fold change among all 49 NRs in AAA tissues, and NR1D1 protein was upregulated in both human and murine VSMCs from AAA tissues. The knockout of Nr1d1 in VSMCs but not endothelial cells and myeloid cells inhibited AAA formation in both AngII- and CaPO4-induced AAA models. Mechanistic studies identified ACO2 (aconitase-2), a key enzyme of the mitochondrial tricarboxylic acid cycle, as a direct target trans-repressed by NR1D1 that mediated the regulatory effects of NR1D1 on mitochondrial metabolism. NR1D1 deficiency restored the ACO2 dysregulation and mitochondrial dysfunction at the early stage of AngII infusion before AAA formation. Supplementation with αKG (α-ketoglutarate, a downstream metabolite of ACO2) was beneficial in preventing and treating AAA in mice in a manner that required NR1D1 in VSMCs.

CONCLUSIONS: Our data define a previously unrecognized role of nuclear receptor NR1D1 in AAA pathogenesis and an undescribed NR1D1-ACO2 axis involved in regulating mitochondrial metabolism in VSMCs. It is important that our findings suggest αKG supplementation as an effective therapeutic approach for AAA treatment.

Key Words: alpha-Ketoglutarate • abdominal aortic aneurysm • mitochondria • nuclear receptor • vascular smooth muscle

Abdominal aortic aneurysm (AAA) is a life-threatening disease characterized by permanent regional dilation of the abdominal aorta, resulting in catastrophic events of rupture and sudden death. The majority of patients with AAA are asymptomatic until lethal rupture, which impedes AAA diagnosis at an early stage. At...
Clinical Perspective

What Is New?

- NR1D1 (Nuclear receptor subfamily 1 group D member 1) ranks the highest in fold change among all 49 nuclear receptors in abdominal aortic aneurysm (AAA) tissues, and global or vascular smooth muscle cell–specific knockout of Nr1d1 represses AAA formation.
- Mitochondrial tricarboxylic acid cycle enzyme ACO2 (aconitase-2) is identified as a direct target repressed by NR1D1 that mediates the regulatory effects of NR1D1 on mitochondrial metabolism.
- Our study unveils a previously undescribed NR1D1-ACO2-αKG (α-ketoglutarate) axis in regulating mitochondrial metabolism and AAA pathogenesis, and provides proof-of-concept evidence indicating the therapeutic value of αKG (a metabolite of the tricarboxylic acid cycle) in AAA prevention and treatment.

What Are the Clinical Implications?

- αKG is a biological compound found naturally in the human body and available in dietary supplement form, allowing for rapid clinical translation.
- On the basis of these findings, a prospective and randomized study (Alpha-Ketoglutarate in Abdominal Aortic Aneurysm, 4A Study; Registration: https://www.clinicaltrials.gov; Unique identifier: NCT04723888) to assess the efficacy and safety of αKG in patients with AAA has been initiated.

Nonstandard Abbreviations and Acronyms

AAA abdominal aortic aneurysm
ACO2 aconitase-2
αKG α-ketoglutarate
AngII angiotensin II
DM-αKG dimethyl α-ketoglutarate
ETC electron transport chain
HDAC3 histone deacetylase 3
IL-6 interleukin-6
MASMC mouse aortic smooth muscle cell
MCP-1 monocyte chemoattractant protein-1
MMP matrix metalloproteinase
mtDNA mitochondrial DNA
NCoR1 nuclear receptor corepressor 1
NR nuclear receptor
NR1D1 nuclear receptor subfamily 1 group D member 1
RT-qPCR real-time quantitative polymerase chain reaction
TCA tricarboxylic acid
TNF-α tumor necrosis factor-α
VSMC vascular smooth muscle cell

Present, open surgical repair and endovascular placement of a stent graft are the mainstays of treatment for AAA, and there are no US Food and Drug Administration–approved medical therapies to limit the progression or reduce the risk of rupture. To identify novel therapeutic targets, a profound understanding of the underlying molecular mechanism regulating AAA formation and progression is essential.

Metabolic pathways, including glucose metabolism, lipid metabolism, and amino acid metabolism, have indispensable roles in normal and dysfunctional vasculature. As the powerhouse of cells, mitochondria play a fundamental role in regulating these metabolic pathways through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, and tight control of mitochondrial functions is critical to maintaining metabolic homeostasis. A recent single-cell RNA sequencing study demonstrated that extensive mitochondrial dysfunction occurs in different aortic cell types and is a feature of aortic aneurysms; however, the regulatory mechanisms of mitochondrial metabolic pathways in aortic aneurysm development remain poorly understood.

Metabolic nuclear receptors (NRs) are a class of ligand-inducible transcription factors that regulate gene expression during various physiological/pathological processes, especially basal metabolic function and energy metabolism. Several members of this superfamily have been demonstrated to be present in the vasculature, with important roles in vascular physiology/pathology. NR1D1 (NR subfamily 1 group D member 1), also known as Rev-erbα, belongs to the NR superfamily, which was first cloned in pituitary tissue and recognized as a vital regulator of circadian rhythms. Recently, several new functions of NR1D1 beyond its roles in circadian rhythms were identified (ie, regulating cell metabolism and inflammatory responses) and implicated in the pathogenesis of several metabolic diseases and cardiovascular diseases. Although NR1D1 has recently been demonstrated to be expressed in vascular cells, the functional role of NR1D1 in the pathology of AAA has never been investigated.

In this study, we aimed to investigate whether NR1D1 is involved in the pathogenesis of AAA and explore the underlying mechanisms. We report our findings that vascular smooth muscle cell (VSMC)-derived NR1D1 contributes to AAA formation by targeting the mitochondrial TCA cycle enzyme ACO2 (aconitase-2). Moreover, αKG (α-ketoglutarate), a downstream metabolite of ACO2, rescued mitochondrial dysfunction and restricted AAA formation.

METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.
NR1D1 Is Upregulated in Human and Murine Aortic SMCs From AAA Tissues

To date, 49 members of the NR superfamily have been documented; however, the role of the majority of NRs in AAA is unknown. To examine which NRs may be involved in the pathogenesis of AAA and identify NR...
Figure 1. NR1D1 is upregulated in human and murine aortic SMCs from AAA tissues.

A, Relative mRNA expression of 49 NRs measured by Nanostring profiling in saline- or AngII-induced AAA mice (n=5 per group). The raw data were normalized to the mean expression levels of the housekeeping genes, and the normalized data were log2-transformed. The log2 (fold change) and P values were calculated, and multiple testing using the Benjamini-Hochberg method was applied to adjust the P values. With a Benjamini-Hochberg adjusted P<0.05 and |log2(fold change)|≥1 as the criteria, 3 genes (Nr1d1 [adjusted P=0.03], Nr4a1 [adjusted P=0.04], and Nr1f3 [adjusted P=0.04]) were identified as differentially expressed NRs in mouse AAA tissues. *Benjamini–Hochberg adjusted P<0.05.

B, Top, Quantification of NR1D1 mRNA and NR1D1 protein expression measured by RT-qPCR and Western blot in human AAA and non-AAA segments (n=6 per group). Data were analyzed by unequal variance t test. (Continued)
genes altered during AAA, we first used high-throughput NanoString profiling to interrogate the expression profiles of all NR genes in the AngII-induced AAA mouse aorta. NR1D1 was identified as the member with the highest fold change in expression among 49 NR superfamily members in murine AAA tissues (Figure 1A). The upregulation of both Nr1d1 mRNA and NR1D1 protein was confirmed in human AAA tissues (Figure 1B and 1C) and murine AAA tissues (Figure 1D and 1E), as shown by RT-qPCR and Western blot. Time-course studies suggested that NR1D1 expression was significantly elevated as early as 3 days in AngII-induced AAA tissues (Figure S1). Moreover, both Nr1d1 mRNA and NR1D1 protein levels were significantly increased in CaPO4-induced AAA tissues (Figure S2A through S2C). It is important that in situ immunofluorescence staining showed that elevated NR1D1 protein was predominantly located in aortic smooth muscle cells from both human (Figure 1C) and murine (Figure 1E) AAA tissues. To further confirm the upregulation of NR1D1 in VSMCs, MASMCs were isolated and treated with AngII. As shown by RT-qPCR, Western blot, and immunofluorescence staining, both Nr1d1 mRNA and NR1D1 protein levels were significantly increased in AngII-treated MASMCs (Figure 1F and 1G). Together, our data indicated that NR1D1 was upregulated in aortic smooth muscle cells in AAA. Recent studies indicated that the activities of some NRs could be regulated by a redox mechanism. We further demonstrated that AngII induced NR1D1 upregulation in VSMCs in a reactive oxygen species–dependent manner (detailed in the Expanded Results and Figure S3).

**VSMC-Specific NR1D1 Deficiency Represses AAA Formation In Vivo**

To determine whether the upregulation of NR1D1 mediates AAA injury or acts as a self-defense anti-AAA signal, we tested the effects of global Nr1d1 knockout (Nr1d1−/−), VSMC-specific Nr1d1 knockout (Nr1d1flox/flox), endothelial cell–specific Nr1d1 knockout (Nr1d1fEC), and myeloid cell–specific Nr1d1 knockout (Nr1d1fMO) on AAA formation (Figure S4). We found that global Nr1d1 knockout and VSMC-specific Nr1d1 knockout significantly ameliorated AAA formation (Figure S4), suggesting that NR1D1 acted as a positive regulator of AAA pathogenesis. However, we did not observe any significant effects of endothelial cell–specific Nr1d1 knockout or myeloid cell–specific Nr1d1 knockout on AAA formation (Figure S4). Thus, we focused on the role of VSMC-derived NR1D1 in AAA formation and development in the following experiments.

In an AngII-induced AAA model, AngII infusion was administered to homozygous ApoE−/−/Nr1d1fASCmice and their littermate controls (ApoE−/−/Nr1d1fASCmice) for 28 days (Figure 2A), and blood pressure, heart rate, and plasma lipids were monitored. AngII increased systolic and diastolic blood pressure similarly in ApoE−/−/Nr1d1fASCmice and ApoE−/−/Nr1d1fASCmice, and no significant difference was observed in heart rate and plasma lipids between ApoE−/−/Nr1d1fASCmice and ApoE−/−/Nr1d1fASCmice (Table S3). However, both male (Figure 2B through 2F) and female (Figure S5A through S5C) ApoE−/−/Nr1d1fASCmice exhibited significantly ameliorated AAA formation in the AngII-induced AAA model. Compared with those in ApoE−/−/Nr1d1fASCmice, decreased maximal diameter of suprarenal abdominal aortas and total aortic weight/body weight were observed in ApoE−/−/Nr1d1fASCmice (Figure 2G and 2H). Ameliorated AAA formation was further validated by in vivo multimodal imaging analysis, including micro-ultrasound imaging and magnetic resonance imaging (Figure 2I through 2K).

Morphologically, histological analysis results revealed that VSMC-specific Nr1d1 knockout mitigated arterial wall thickening and reduced elastic fiber degradation and collagen deposition in AngII-administered mouse aortas (Figure S6). To evaluate the degradation of the extracellular matrix in the aortic wall, we determined the expression and activity of MMP-2 (matrix metalloproteinase-2). MMP-2 expression and MMP activity were substantially increased in AngII-induced ApoE−/−/Nr1d1fASCmice, whereas enhanced MMP-2 expression and MMP activity were dramatically abolished in the aorta of AngII-infused ApoE−/−/Nr1d1fASCmice (Figure S7). Moreover, NR1D1 deficiency suppressed the expression of inflammatory factors (ie, IL-6 [interleukin-6], TNF-α [tumor necrosis factor-α], and MCP-1 [monocyte chemotractant protein-1]) in the abdominal aortas (Figure S8A through S8F).
Figure 2. VSMC-specific NR1D1 deficiency represses AngII-induced AAA formation.

A, Schematic protocol: ApoE−/− Nr1d1fl/fl and ApoE−/− Nr1d1ΔSMC mice were subcutaneously injected with saline or AngII by a mini osmotic pump for 28 days (n=30 per group). B, Representative images of the macroscopic features of AAA formation in indicated groups (n=30 per group). Survival data were analyzed by the Kaplan-Meier method and compared using log-rank tests. *P<0.05. E, The incidence of AngII-induced AAA in indicated groups (n=30 per group). Data were analyzed by a Fisher exact test. **P<0.01; ***P<0.001. F, The incidence of AngII-induced aneurysm rupture in indicated groups (n=30 per group). Data were analyzed by a Fisher exact test: *P<0.05. G and H, Quantification of the maximal diameter of suprarenal abdominal aortas measured by a Digital Vernier Caliper and total aortic weight/BW in indicated groups (n=22–30 per group). Data were analyzed by 2-way ANOVA followed by the Bonferroni post hoc test. ***P<0.001. I, Representative images of abdominal aortas visualized by MRI using the B mode in indicated groups. J, Quantification of the maximal diameter of suprarenal abdominal aortas measured by MRI using the B mode in indicated groups. K, Representative images of abdominal aortas visualized by MRI in indicated groups. Top, Abdominal aortas visualized using 3-dimensional time of flight fast low angle shot sequence (TOF-3D-Flash). Bottom, Abdominal aortas visualized by T2-weighted, PD-weighted imaging with multiple-echo multishot sequence (MEMS-PD-T2). Data are expressed as mean ± SEM. AAA indicates abdominal aortic aneurysm; AngII, angiotensin II; BW, body weight; EVG staining, elastin van Gieson staining; HE staining, hematoxylin and eosin staining; MUI, micro-ultrasound imaging; MRI, magnetic resonance imaging; NR1D1, nuclear receptor subfamily 1 group D member 1; and SMC, smooth muscle cell.
Figure 3. NR1D1 perturbs mitochondrial metabolism by the TCA cycle in AAA.

A. Heatmap of differentially expressed genes in ApoE−/−/Nr1d1floxed/AngII and ApoE−/−/Nr1d1ΔSMC-AngII groups. Each column represents an individual replicate, and each row represents an individual gene. Upregulated genes are shown in red, and downregulated genes are displayed in blue. n=3 for both groups. Differentially expressed genes were defined as genes with a Benjamini-Hochberg adjusted P-value <0.05 and |log2(fold change)|≥1.

B. Volcano plot reveals the magnitude and significance of altered genes in ApoE−/−/Nr1d1floxed/AngII and ApoE−/−/Nr1d1ΔSMC-AngII groups. Differentially expressed genes were defined as genes with a Benjamini-Hochberg adjusted P-value <0.05 and |log2(fold change)|≥1.

C. The top 10 Kyoto Encyclopedia Genes and Genomes (KEGG) pathways of differentially expressed genes. (Continued)
To further confirm the pathogenic role of VSMC-derived NR1D1 in AAA formation, we performed experiments in another AAA model induced by CaPO₄. Compared with littermate Nr1d⁻¹/⁻ mice, Nr1d⁻¹/⁻/ΔSMC mice showed significantly ameliorated AAA formation and decreased maximal diameter of infrarenal abdominal aortas in the CaPO₄-induced AAA models in both male (Figure S2D through S2H) and female (Figure S5D through S5F) mice. Nr1d⁻¹/⁻/ΔSMC mice exhibited significantly decreased arterial wall thickness and reduced elastic fiber degradation and collagen deposition (Figure S9A through S9C) compared with littermate Nr1d⁻¹/⁻/ΔSMC controls. Furthermore, Nr1d⁻¹/⁻/ΔSMC mice displayed decreased MMP-2 expression and activity, and suppressed expression of inflammatory factors in the abdominal aorta compared with littermate Nr1d⁻¹/⁻/ΔSMC mice (Figure S9A, S9D, and S9F through S9H). Taken together, our data provided convincing evidence that VSMC-derived NR1D1 was a positive regulator that contributed to the pathogenesis of AAA.

NR1D1 Regulates Mitochondrial Metabolism by the TCA Cycle in AAA

To obtain deeper insight into the underlying mechanisms linking NR1D1 to the pathogenesis of AAA, high-throughput RNA-sequencing of mouse aortas was performed. Bioinformatics analyses of differentially expressed genes suggested that the upregulated genes were enriched in metabolism pathways (including propanoate metabolism, TCA cycle, and oxidative phosphorylation) (Figure 3A through 3C). Furthermore, metabolic profiling was performed using plasma from AngII-infused ApoE⁻⁻/⁻/Nr1d⁻¹/⁻/ΔSMC mice and littermate ApoE⁻⁻/⁻/Nr1d⁻¹/⁻/ΔSMC mice by plasmonic nanoshells enhanced laser desorption/ionization mass spectrometry, and the top 75 differentially expressed metabolites were shown (Figure 3D). To screen the potential NR1D1 downstream candidates, we performed a combined analysis of transcriptomes (differentially expressed genes, Benjamini-Hochberg adjusted \( P < 0.05 \) and \( \log_2(\text{fold change}) \geq 1 \)) and metabolomes (metabolites, variable important in projection \( > 1.2 \) using orthogonal partial least squares–discriminant analysis) with the MetaboAnalyst online tool (https://www.metaboanalyst.ca). As shown in Figure 3E, the TCA cycle ranked as the top “core pathway” according to the joint pathway analysis. Collectively, the bioinformatics analyses implied that dysregulation of the mitochondrial TCA cycle caused by NR1D1 played a pivotal role in the pathogenesis of AAA. In addition, the joint pathway analysis suggested mitochondrial ACO2 as the downstream target for NR1D1 (Figure 3F). Because mitochondrial ACO2 dysfunction may result in decreased abundances of (iso)citrate, cis-aconitate, and αKG and reduced ratios of αKG to (iso)citrate/cis-aconitate, an indicator of TCA cycle breakdown, we further examined the downstream signaling of ACO2. As expected, NR1D1 deficiency increased the abundances of isocitrate, cis-aconitate, and αKG, and the ratios of αKG to isocitrate/cis-aconitate (Figure S10). To consolidate these findings in humans, metabolomic profiling was performed in patients with AAA and matched controls. In agreement with the animal findings, differentially expressed metabolites and enrichment analysis demonstrated that the TCA cycle was the top-ranked core pathway (Figure 3G and 3H). The abundances of (iso)citrate, cis-aconitate, and αKG, the downstream metabolites of ACO2, were downregulated in human AAA plasma samples (Figure 3I through 3K). Moreover, patients with AAA had significantly lower αKG to (iso)citrate/cis-aconitate ratios (Figure 3L and 3M).

Aco2 Is the Direct Trans-Repression Target of NR1D1 in Regulating Mitochondrial Metabolism

We further examined the expression and activity of the key TCA cycle enzyme ACO2 in both human and mouse AAA samples. Compared with those in non-AAA sections, ACO2 expression and activity were dramatically decreased in human AAA sections (Figure 4A through 4D). Consistent with these results in human AAA tissues, the expression and activity of ACO2 were reduced in AngII-treated AAA mouse aortas (Figure 4E through 4H; Figure S11). To further validate that mitochondrial ACO2 was the nodal point involved in the pathogenesis of AAA regulated by NR1D1, we examined the effect of Nr1d⁻¹/⁻/ΔSMC...
Figure 4. Aco2 is the direct trans-repression target of NR1D1 in regulating mitochondrial function. A and B, Aco2 mRNA expression and ACO2 activity measured by RT-qPCR and ELISAs in human AAA and non-AAA segments (n=6 per group). Data were analyzed by Student t test. **P<0.01; ***P<0.001. C, Representative images of ACO2 expression by immunofluorescence staining of human AAA and non-AAA segments and costaining with the key smooth muscle cell–associated marker αSMA and DAPI. D, Left, ACO2 protein expression assessed by Western blot in human AAA and non-AAA segments. Right, Quantification of ACO2 protein expression measured by Western blot in indicated groups (n=6 per group). Data were analyzed by Student t test. **P<0.01. E and F, Aco2 mRNA expression and ACO2 activity measured by RT-qPCR and ELISAs in abdominal aortic segments from saline- and AngII-infused ApoE−/− mice (n=6 per group). (Continued)
on mitochondrial ACO2 expression and activity in mouse aortas after AngII treatment. The mRNA and protein expression levels and enzymatic activity of mitochondrial ACO2 were significantly elevated in ApoE−/− AAA mice compared with those in ApoE−/−/Nr1d1Δ/Δ mice (Figure 4I through 4L). Furthermore, mitochondrial ACO2 expression was up-regulated in AngII-treated MASMCs isolated from Nr1d1Δ/Δ mice after stimulation with AngII for 48 h (n=6 per group). Data were analyzed by Student t test. **P<0.01; ***P<0.001.

NR1D1 functions as a transcriptional repressor by recruiting NCoR1 (nuclear receptor co-repressor 1) and HDAC3 (histone deacetylase 3). As expected, co-immunoprecipitation and immunoblotting experiments indicated that AdNr1d1 transfection-mediated overexpression of NR1D1 enhanced the interaction between NCoR1−1−HDAC3 and NR1D1 (Figure S12A and S12B). Consistently, enhanced interactions between NR1D1 and NCoR1−1−HDAC3 complexes were observed in MASMCs treated with AngII compared with those in MASMCs treated with saline (Figure S12C). The putative binding site of mouse NR1D1 (through its recruitment of NCoR1) in the promoter region of the mouse Aco2 locus (GCCCCAC, −65 bp to −58 bp) was predicted by the Catalog of Inferred Sequence Binding Preferences (CIS-BP) database (http://cisbp.ccbr.utoronto.ca), which was confirmed by chromatin immunoprecipitation–polymerase chain reaction (Figure 4P enrolled patients with both thoracic and 4Q; Figure S12D). Moreover, dual luciferase reporter assays showed that NR1D1 overexpression repressed Aco2 promoter activity (Aco2-wt, −2000 to +98 bp) (Figure 4R), and mutant promoter activity (Aco2-mut) was higher than Aco2-wt promoter activity in MASMCs (Figure 4S). Taken together, these data identified ACO2 as the direct trans-repression target of NR1D1 in regulating mitochondrial metabolism.

Protective Role of NR1D1 Deficiency in Mitochondrial Function Is Mediated by ACO2

TCA cycle metabolites contribute to the maintenance of mitochondrial homeostasis, and alterations in mitochondrial metabolism can lead to impaired mitochondrial respiration, mtDNA damage, and electron transport chain (ETC) defects. Thus, we further investigated the effect of Nr1d1 deficiency on mitochondrial function in AAA development. Oxygen consumption analysis revealed significantly increased basal, maximal, and ATP-coupled mitochondrial oxygen consumption rates in AngII-treated MASMCs isolated from Nr1d1Δ/Δ mice compared with those in MASMCs from Nr1d1Δ/Δ littermates (Figure 5A through 5D). Moreover, Nr1d1 deficiency decreased AngII-induced mtDNA damages in cultured MASMCs isolated from Nr1d1Δ/Δ mice compared with those in MASMCs from Nr1d1Δ/Δ littermates after AngII treatment (Figure 5F and 5G). In addition, RNA-sequencing data validated that Nr1d1 deficiency significantly up-regulated the RNA levels of nuclear-encoded mitochondrial genes and mtDNA-encoded genes of ETC complexes in Nr1d1Δ/Δ/MASMCs compared with those in Nr1d1Δ/Δ-MASMCs after AngII treatment (Figure 5F and 5G). As a result, the results were further confirmed by RT-qPCR and Western blot (Figure 5J through 5L). Moreover, in situ dihydroethidium staining and terminal deoxynucleotidyl transferase dUTP nick-end labeling assays indicated significantly reduced mitochondrial apoptosis and reactive oxygen species production in Aco2−/−/Nr1d1Δ/Δ mouse aortas (Figure 5M and 5N; Figure S8G through S8I). Consistent results were observed in Nr1d1Δ/Δ mice after CaPO4 treatment (Figure S9A, S9D, and S9E). Collectively, these data indicated that NR1D1 regulated mitochondrial homeostasis in AAA.

Figure 4 Continued. Data were analyzed by Student t test. **P<0.01; ***P<0.001. G, Representative images of ACO2 expression by immunofluorescence staining in abdominal aortas from saline- and AngII-infused ApoE−/− mice. H, Left, ACO2 protein expression measured by Western blot in abdominal aortas from saline- and AngII-infused ApoE−/− mice. Right, Quantification of ACO2 protein expression measured by Western blot in indicated groups (n=6 per group). Data were analyzed by Student t test. **P<0.01. I and J, Aco2 mRNA expression and ACO2 activity measured by RT-qPCR and ELISAs in abdominal aortas from AngII-infused ApoE−/−/Nr1d1Δ/Δ and ApoE−/−/Nr1d1Δ/Δ mice. J, Left, ACO2 protein expression measured by Western blot in abdominal aortas from AngII-infused ApoE−/−/Nr1d1Δ/Δ and ApoE−/−/Nr1d1Δ/Δ mice (n=6 per group). Data were analyzed by Student t test. **P<0.01. K, Representative images of ACO2 expression by immunofluorescence staining in abdominal aortas from AngII-infused ApoE−/−/Nr1d1Δ/Δ and ApoE−/−/Nr1d1Δ/Δ mice. L, Left, ACO2 protein expression measured by Western blot in abdominal aortas from AngII-infused ApoE−/−/Nr1d1Δ/Δ and ApoE−/−/Nr1d1Δ/Δ mice after stimulation with AngII for 48 h (n=6 independent experiments). Data were analyzed by Student t test. **P<0.01. M, Aco2 mRNA expression measured by RT-qPCR in MASMCs isolated from Nr1d1Δ/Δ and Nr1d1Δ/Δ mice after stimulation with AngII for 48 hours. Bottom, Quantification of ACO2 protein expression measured by Western blot in MASMCs isolated in indicated groups (n=6 independent experiments). Data were analyzed by Student t test. **P<0.01. O, Representative images of ACO2 expression by immunofluorescence staining in MASMCs isolated from Nr1d1Δ/Δ and Nr1d1Δ/Δ mice after stimulation with AngII for 48 hours.
Figure 5. VSMC-specific NR1D1 deficiency regulates the expression of mitochondria-related genes and inhibits mitochondrial ROS production.

A, Summarized OCR tracings in MASMCs isolated from Nr1d1^flox/flox and Nr1d1^ΔSMC mice after stimulation with AngII for 48 hours (n=4 independent experiments). B through D, Basal, maximal, and ATP-coupled OCRs in indicated groups (n=4 independent experiments). Data were analyzed by Student t test. **P<0.01; ***P<0.001. E, mtDNA damage detection in MASMCs isolated from Nr1d1^flox/flox and Nr1d1^ΔSMC mice after stimulation with AngII for 48 hours (n=6 independent experiments). Data were analyzed by Student t test. *P<0.05. F and G, Relative mRNA expression of nuclear- and mitochondrial-encoded mitochondrial genes in MASMCs isolated from Nr1d1^flox/flox and Nr1d1^ΔSMC mice with AngII treatment for 48 hours, normalized to β-actin (n=6 independent experiments). Data were analyzed by Student t test. (Continued)
To determine whether these findings were direct effects of NR1D1 deletion or the consequence of AAA inhibition, abdominal aortas from ApoE−/−/Nr1d1flox/flox and ApoE−/−/Nr1d1ASM C mice were harvested on the morning of day 3 after AngII infusion before a change in AAs. NR1D1 deficiency restored the ACO2 dysregulation and mitochondrial dysfunction, as well as inhibited reactive oxygen species levels and inflammatory markers before a change in AAs on day 3 after AngII infusion (Figure 6).

To further study whether the protective effects of Nr1d1 deficiency on mitochondrial function were mediated by ACO2, a small-interfering RNA targeting Aco2 mRNA (siAco2) was transfected into cultured MASMCs (Figure 7A). ACO2 silencing reduced basal, maximal, and ATP-coupled oxygen consumption rates (Figure 7B; Figure S13), exacerbated AngII-induced mtDNA damage (Figure 7C), and reversed the protective effects of Nr1d1 knockout in MASMCs (Figure 7B and 7C; Figure S13).

Furthermore, Nr1d1 deficiency reversed the decrease in mitochondrial membrane potential (ΔΨm), mitochondrial apoptosis, and mitochondrial ETC complexes, and inhibited extracellular matrix degradation (MMP-2 expression and activity) in MASMCs after AngII treatment (Figure 7D through 7I). It is important that these protective effects could be nullified by Aco2 knockdown (Figure 7B through 7I; Figure S13). Taken together, these data suggested that the protective role of Nr1d1 deficiency against mitochondrial dysfunction was mediated by ACO2.

**Supplementation With the ACO2 Downstream Metabolite αKG Protects Against AAA Formation**

In the TCA cycle, ACO2 catalyzes the reversible transformation of citrate to isocitrate, and isocitrate dehydrogenase triggers the conversion of isocitrate to αKG (a rate-limiting step of the TCA cycle) (Figure 3F).31 Thus, we wondered whether the impaired TCA cycle in AAs would be reactivated by supplementing the key metabolic downstream product αKG. To verify this hypothesis, saline- or AngII-infused ApoE−/−/Nr1d1flox/flox and ApoE−/−/Nr1d1ASM C mice were intraperitoneally administered vehicle or DM-αKG (dimethyl α-ketoglutarate) daily for 4 weeks32 (Figure 8A). DM-αKG supplementation reduced AngII-induced AAs in ApoE−/−/Nr1d1flox/flox mice, as evidenced by the significant difference in AAA formation in DM-αKG− or saline-treated ApoE−/−/Nr1d1flox/flox mice (Figure 8B through 8G). Moreover, DM-αKG supplementation significantly reduced pathological collagen deposition, elastin degradation, and extracellular matrix degradation (MMP-2 expression and activity) and increased mitochondrial ETC complex expression in AngII-induced AAA tissues (Figure S14). Moreover, αKG supplementation attenuated aneurysm progression in a preestablished AAA mouse model33 (detailed in the Expanded Results and Figure 15). The vasoprotective effects of DM-αKG supplementation against AAA formation were further validated in the CaPO4-induced AAA model (Figure S16). Consistently, in vitro experiments showed that the addition of DM-αKG32 significantly reversed AngII-induced injuries in MASMCs (Figure S17).

These data provided convincing evidence that supplementation with the ACO2 downstream metabolite αKG protected against AAA injuries.

In the next series of experiments, MASMCs were transfected with AdCon or AdNr1d1 for 48 hours and treated with AngII. NR1D1 overexpression in MASMCs decreased mitochondrial respiratory capacity, mitochondrial membrane potential, and mitochondrial ETC complexes, and promoted mitochondrial apoptosis and extracellular matrix degradation after AngII treatment (Figure S18). However, DM-αKG treatment reversed the detrimental effects of NR1D1 overexpression on mitochondrial respiratory capacity, mitochondrial membrane potential, mitochondrial ETC complexes, mitochondrial apoptosis, and extracellular matrix degradation (Figure S18).

These results suggested that αKG supplementation nullified the detrimental effect of NR1D1 on mitochondrial function.

**DISCUSSION**

In the present work, we identified a novel role of the nuclear receptor NR1D1 in the pathogenesis of AAA. The novel contributions included the following. First, we identified that Nr1d1 gene expression exhibited the highest fold change among all 49 NRs in AAA tissues, and NR1D1 protein was upregulated in both human and
Figure 6. NR1D1 deficiency results in early changes in mitochondrial function on day 3 after AngII infusion before a change in AAAs.

Abdominal aortas from ApoE<sup>−/−</sup>/Nr1d<sup>Δ<sub>SMC</sub></sup> and ApoE<sup>−/−</sup>/Nr1d<sup>Δ<sub>SMC</sub></sup> mice were harvested on the morning of day 3 after AngII infusion before a change in AAAs. A, Representative images of the macroscopic features and HE staining of suprarenal abdominal aortas in ApoE<sup>−/−</sup>/Nr1d<sup>Δ<sub>SMC</sub></sup> and ApoE<sup>−/−</sup>/Nr1d<sup>Δ<sub>SMC</sub></sup> mice on day 3 after AngII infusion. B, Quantification of the maximal diameter of suprarenal abdominal aortas in indicated groups (n=10 per group). Data were analyzed by Student t test. C, Representative images of MMP activity and in situ DHE staining in indicated groups. D, Left, MMP-2 protein expression measured by Western blot and MMP activity measured by zymography in indicated groups. Right, Quantification of MMP-2 protein expression and MMP activity in indicated groups (n=6 per group). (Continued)
MASMCs from AAs tissues. Moreover, global and VSMC-specific knockout of Nr1d1 protected mice against AAA formation in both AngII- and CaPO4-induced AAA models. Furthermore, mechanistic studies identified ACO2, a key enzyme of the mitochondrial TCA cycle, as a direct transcriptional downstream target trans-repressed by NR1D1 that mediated the negative effects of NR1D1 on mitochondrial function and AAA formation. Last, supplementation with αKG, a downstream metabolic product of ACO2, was sufficient to protect against AAA formation and abolish the detrimental effect of NR1D1 overexpression on mitochondrial function (Figure 8H). Collectively, the results of the present study provided the first evidence for a novel regulatory NR1D1-ACO2-αKG signaling axis in AAA formation and progression.

As transcription factors, NRs regulate gene expression not only during physiological processes (metabolic and immunology response programs) but also during several pathological conditions, including cancer, metabolic disorders, and cardiovascular diseases. NR1D1 is a special member of the metabolic NR superfamily known as a core regulator of circadian rhythms. In addition to its classical functions in controlling circadian rhythms, recent studies have uncovered several new functions of NR1D1 (ie, regulating cell metabolism and inflammatory responses) in the pathogenesis of metabolic disease, cancer, and muscular dysfunction. It is important that emerging evidence has shown that NR1D1 regulates cardiovascular physiology/pathology. Although NR1D1 has been reported to inhibit atherosclerotic development, we unexpectedly found a profound detrimental effect of NR1D1 on AAA development. NR1D1 was upregulated in human and MASMCs from AAA tissues, with its gene expression ranked the highest in fold change among all 49 NRs. VSMC-specific Nr1d1 knockout significantly ameliorated AAA formation in 2 different animal models. The detrimental effect of NR1D1 was further confirmed by in vitro MASMC experiments. These data provided convincing evidence of a previously unrecognized role of VSMC-derived NR1D1 as a positive regulator contributing to the pathogenesis of AAA. In the present study, global Nr1d1 knockout and VSMC-specific Nr1d1 knockout (but not endothelial cell–specific Nr1d1 knockout or macrophage-specific Nr1d1 knockout) significantly inhibited AAA formation, suggesting a cell-specific role of NR1D1 in different vascular diseases.

NR1D1 has been shown to control glucose and lipid metabolism tightly in several organs and cells. In the present study, through integrated transcriptomic, metabolomic, and epigenomic profiling analyses, the key mitochondrial TCA cycle enzyme ACO2 was identified as a direct transcriptional downstream target trans-repressed by NR1D1 and found to mediate the detrimental effects of NR1D1 on mitochondrial metabolism. The TCA cycle, also known as the citric acid cycle or the Krebs cycle, is a series of reactions in a closed loop that forms a metabolic engine to control cell function and cell fate by regulating the generation of ATP and metabolites. Aberrant TCA cycle function is implicated in a wide variety of pathological conditions, such as cancer and diabetes. The enzyme ACO2 is indispensable for the TCA cycle, and it reversibly converts citrate to isocitrate, maintains mitochondrial function, and regulates metabolism. Dysregulated ACO2 is associated with oxidant-/lipopolysaccharide-induced mtDNA damage and apoptosis. Our results consistently revealed that ACO2 downregulation mediated by NR1D1 decreased mitochondrial respiratory capacity, increased mtDNA damage, reduced mitochondrial membrane potential, and aggravated mitochondria-mediated apoptosis in VSMCs. These data provided novel evidence supporting a role of the mitochondrial TCA cycle in the pathogenesis of AAs, and identified the NR1D1-ACO2 axis as a potential novel target for regulating mitochondrial metabolism and function in AAA treatment.

TCA cycle metabolites are important for the biosynthesis of macromolecules, such as nucleotides, lipids, and proteins, and are involved in physiology and disease. Recent evidence revealed several new and important roles for TCA cycle intermediates as signaling molecules, gene expression effectors, and stress response modulators in controlling cellular function and fate. As the downstream factor of mitochondrial function, ACO2 maintains mitochondrial function and participates in the pathogenesis of AAA.
Figure 7. ACO2 silencing abolishes the protective effects of NR1D1 deficiency on mitochondrial function.

A. Top, Quantification of Aco2 mRNA and ACO2 protein expression assessed by RT-qPCR and Western blot in MASMCs after transfection with SiCon and SiAco2-1, normalized to β-actin (n=6 independent experiments). Data were analyzed by Student t test. ***P<0.001. Bottom, ACO2 protein expression assessed by Western blot in indicated groups.

B, Summarized OCR tracings in AngII-treated MASMCs isolated from Nr1d1 flox/flox and Nr1d1ΔSMC mice pretreated with SiCon and SiAco2 (n=6 independent experiments). Data were analyzed by 2-way ANOVA followed by the Bonferroni post hoc test. *P<0.05; ***P<0.001.

C, mtDNA damage assay in indicated groups (n=10 independent experiments).

D, MitoTracker Red CMXRos staining was used to measure mitochondrial membrane potential for the assessment of early-stage apoptosis. (Continued)
The identification of NR1D1 as a novel functional receptor in mitochondrial metabolism regulation in vascular cells might broaden our understanding of the multiple biological functions of NR1D1. Our observations, along with recent findings about the role of NR1D1 in cardiovascular tissues from several research groups, provide support for NR1D1 as an essential regulator of cardiovascular biology. It is important that our study unveils a previously undescribed NR1D1-αKG axis in regulating mitochondrial metabolism, and provides proof-of-concept evidence indicating the therapeutic value of αKG supplementation in AAA prevention and treatment. αKG is a biological compound found naturally in the human body and available in dietary supplement form. On this basis of these findings, we initiated a prospective, randomized, parallel-group, controlled study to assess the efficacy and safety of αKG in patients with AAA (Alpha-Ketoglutarate in Abdominal Aortic Aneurysm, 4A Study; Registration: https://www.clinicaltrials.gov; Unique identifier: NCT04723888).

Limitations of the Study

Several study limitations should be considered. First, although a detrimental role of NR1D1 in AAA has been confirmed in our study in 2 AAA mouse models (subcutaneous AngII infusion and adventitial CaPO₄ incubation) using both global and conditional knockout mice, each animal model is limited in its ability to mimic the extremely complex process of AAA formation in patients. In addition, although Tagln-Cre drivers have been commonly used to study VSMC-specific expression, findings with this strain need to be interpreted with caution because its VSMC specificity is controversial. Second, the human AAA tissues obtained from advanced AAA lesions during open surgical repair provide limited insight into the earlier stages. Third, the mechanism underlying the discrepancy between our findings (detrimental effect of NR1D1 on AAA) and previous reports (protective effects of NR1D1 on atherosclerosis) remains unclear. Whether this discrepancy is a result of the distinct roles in different vascular cell types induced by various pathological stimuli must be further defined. Last, our findings highlight αKG supplementation as a potential treatment for...
Figure 8. Supplementation with the ACO2 downstream metabolite αKG protects against AAA formation.

A. Schematic protocol: saline- or AngII-infused ApoE−/−/Nr1d1+/+ and ApoE−/−/Nr1d1ΔSMC mice were intraperitoneally injected with vehicle or DM-αKG (100 mg/kg/d) for 28 days. B. Representative images of the macroscopic features of AAA formation in indicated groups. C. The incidence of AAA formation in indicated groups (n=30 per group). Data were analyzed by a Fisher exact test. *P<0.05; **P<0.01; ***P<0.001. D. Quantification of the maximal diameter of suprarenal abdominal aortas measured using a Digital Vernier Caliper (n=22–30 per group). Data were analyzed by 2-way ANOVA followed by the Bonferroni post hoc test. **P<0.01; ***P<0.001. E. Quantification of total aortic weight/BW (n=22–30 per group). Data were analyzed by 2-way ANOVA followed by the Bonferroni post hoc test. ***P<0.001. F. Representative images of suprarenal abdominal aortas visualized by MUI using the B mode in indicated groups. G. Quantification of the maximal diameter of suprarenal abdominal aortas measured by MUI using the B mode in indicated groups (n=22–30 per group). Data were analyzed by 2-way ANOVA followed by the Bonferroni post hoc test. **P<0.01; ***P<0.001. Data are expressed as mean ± SEM. H. Schematic representation of the molecular mechanisms. Under a pathological state during AAA development, upregulation of NR1D1 represses the nuclear transcription of Aco2 in VSMCs with recruitment of the NCoR1-HDAC3 corepressor complex. Consequently, reduced ACO2 expression and activity suppress the TCA cycle, disrupt mitochondrial homeostasis, and subsequently trigger VSMCs apoptosis, eventually resulting in the formation of AAA. Supplementation with αKG, a downstream metabolite of ACO2, alleviates mitochondrial dysfunction and restricts AAA formation. AAA indicates abdominal aortic aneurysm; ACO2, aconitase-2; αKG, α-ketoglutarate; AngII, angiotensin II; DM-αKG, dimethyl α-ketoglutarate; HDAC3, histone deacetylase 3; MUI, micro-ultrasound imaging; NCoR1, nuclear receptor corepressor 1; NR1D1, nuclear receptor subfamily 1 group D member 1; NS, nonsignificant; SMC, smooth muscle cell; TCA, tricarboxylic acid; and VSMCs, vascular smooth muscle cells.
AAA. More clinical research is warranted to confirm the benefit on AAA observed in this study.

**ARTICLE INFORMATION**

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**Affiliations**

State Key Laboratory for Oncogenes and Related Genes, Department of Cardiology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China (L.-Y.S., Y.-Y.L., Y.-L.W., L.H., L.Z., K.Q., and J.P.). Department of Vascular Surgery, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China (L.H., K.Q.). School of Biomedical Engineering and Med-X Research Institute, Shanghai Jiao Tong University, Shanghai, China (L.H., K.Q.).

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**Disclosures**

None.

**Supplemental Material**

Expanded Methods

Expanded Results

Tables S1–S4

Figures S1–S18

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