Deletion of the Mas Receptor Aggravates the Development of Atherosclerosis and the Formation of Abdominal Aortic Aneurysms in Apolipoprotein E-Deficient Mice

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presented by

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Statutory declaration

I promise that I completed the dissertation “Deletion of Mas Receptor Aggravates the Development of Atherosclerosis and the Formation of Abdominal Aortic Aneurysms in Apolipoprotein E-Deficient Mice” independently. All the content is in line with the requirements of “Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine Universität Düsseldorf”. I promise that this is the first time I submit my Ph.D. works.

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Ich verspreche, dass ich die Dissertation abgeschlossen habe. "Deletion von Mas Receptor verschärft die Entwicklung der Atherosklerose und die Bildung von Bauchaortenaneurysmen in Apolipoprotein E-defizienten Mäusen" unabhängig. Der gesamte Inhalt steht im Einklang mit den Anforderungen der "Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine Universität Düsseldorf". Ich verspreche, dass dies das erste Mal meine Doktorarbeit ist.

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Abbreviations

A	
AAA	Abdominal aortic aneurysms
AA	arachidonic acid
ACE	Angiotensin converting enzyme
ACE2	Angiotensin converting enzyme 2
Ang I	Angiotensin I / Angiotensin-(1-10)
Ang II	Angiotensin II / Angiotensin-(1-8)
Ang-(1-5)	Angiotensin-(1-5)
Ang-(1-7)	Angiotensin-(1-7)
Ang-(1-9)	Angiotensin-(1-9)
Ang-(1-255)	Angiotensin-(1-255)
AT1R	Angiotensin II receptor type 1
AT2R	Angiotensin II receptor type 2
ARBs	Angiotensin receptor blockers
AKT	Protein kinase B (PKB)
B	
BCA	bicinchoninic acid assay
BSA	Bovine serum albumin
apoE-KO	apolipoprotein E knockout
apoE/Mas-KO	apolipoprotein E / Mas receptor knockout
C	
CCL2	monocyte chemoattractant protein-1
CD	Cluster of Differentiation
cGMP	cyclic guanosine monophosphate
E	
ECM	extracellular matrix proteins
ERK	extracellular signal–regulated kinases
EC	endothelial cells
eNOS	endothelial nitric oxide synthase
EGFR	epidermal growth factor receptor
F	
FMD	flow mediated vasodilatation
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
H	
HDL	high-density lipoproteins
HUVEC	human umbilical vein endothelial cells
I	
iNOS	inducible nitric oxide synthase
IMT	intima media thickness
IL	Interleukin
ICAM-1	intercellular adhesion molecule 1

IFNγ	Interferon gamma
J	
JAK2	Janus-activated kinase
L	
LDL	low-density lipoproteins
LDL-KO	Low-density lipoprotein knockout
LOX-1	lectin-like oxidized low-density lipoprotein receptor-1
LPS	lipopolysaccharide
M	
Mas-KO	Mas knockout
MRI	Magnetic resonance imaging
MR	Magnetic resonance
MMPs	Metrix metalloproteinases
MMP2	Metrix metalloproteinases-2
MMP9	Metrix metalloproteinases-9
MCP-1	monocyte chemoattractant protein-1
M1	Macrophage subtype 1
M2	Macrophage subtype 2
MAPK	Mitogen-activated protein kinase
N	
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Norepinephrine
NO	nitric oxide
NOX	NADPH oxidase
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS	neuronal nitric oxide synthase
O	
oxLDL	oxidized low-density lipoprotein
ORO	Oil red O
OD	optical density
P	
PI3K	phosphoinositide 3 kinase
PLA	phospholipase A
PAD	peripheral artery disease
PFC	perfluorocarbon nanoemulsions
PW	pulsed-wave
PCR	Polymerase chain reaction
R	
RAS	renin-angiotensin-system
RAAS	renin–angiotensin–aldosterone system
RNS	reactive nitrogen species
ROS	reactive oxygen species
Rho	Ras homolog gene family
Rho A	Ras homolog gene family member A

RT-PCR	Real-time PCR
S	
SDS	sodium dodecyl sulfate
SOCS3	suppressor of cytokine signaling 3
STAT	signal transducers and activators of transcription
SBP	systolic blood pressure
sGC	soluble guanylyl cyclase
T	
TBST	Tris-buffered saline, 0.1% Tween 20
TNF-alpha	tumor necrosis factor alpha
TGF-β	transforming growth factor beta
TAA	Thoracic aortic aneurysm
V	
VSMC	vascular smooth muscle cell
VCAM-1	vascular cellular adhesion molecule-1
W	
WD	Western type diet
WB	Western blotting
WT	Wild type, C57BL/6 strain

1. Summary

Cardiovascular disease is the leading cause of death worldwide. The renin-angiotensin-system (RAS) is one of the most important hormone systems regulating cardiovascular diseases, such as hypertension, atherosclerosis and aneurysms. Angiotensin (Ang) II, the main active metabolite in the RAS, promotes the development of hypertension, atherosclerosis and the formation of abdominal aortic aneurysms (AAA) through activation of the Ang II receptor type 1 (AT1R). Besides vasoconstriction as well as sodium and water reabsorption in the kidney, AT1R activation leads to increased oxidative stress production, decreased nitric oxide (NO) bioavailability, fibrosis, inflammation and immune cell infiltration. Recently, a novel axis of the RAS, called the alternative RAS-axis, has been described. In contrast to the Ang II / AT1R axis, there is accumulating evidence that the Ang-(1-7) / Mas axis counteracts the effects of Ang II. By activating the G-coupled 7 transmembrane receptor Mas, Ang-(1-7) induces vasodilation, inhibition of cell growth, antithrombotic, and anti-inflammatory processes. Recently, we and others have shown that Ang-(1-7) increases NO bioavailability and reduces the generation of reactive oxygen species (ROS) within the vasculature. Moreover, deletion or inhibition of the Mas receptor fails to improve the endothelial dysfunction and vascular injury caused by Ang-(1-7). Thus, it seems that Ang-(1-7) improves vascular dysfunction and injury via activation of the Mas receptor. The purpose of this study is to evaluate the role and underlying signaling pathway of Mas receptor activation in the development of atherosclerosis and the formation of AAA in apolipoprotein E knockout (apoE-KO) mice.

Here, we could show that deletion of the Mas receptor aggravated Western-type diet (WD)-induced atherosclerosis in apoE-KO mice. Evaluation of NO mediated endothelial vasodilation revealed an impairment of vascular function in apolipoprotein E / Mas receptor knockout (apoE/Mas-KO) mice, compared with apoE-KO mice. In contrast, chronic infusion of Ang-(1-7) improved endothelial dysfunction and atherosclerosis in apoE-KO mice, but not in apoE/Mas-KO mice. The improved vascular function was accompanied by a drastic increase of vascular NO-mediated cGMP generation and a significant decrease of vascular ROS production suggesting that Ang-(1-7)-induced Mas receptor activation improved vascular function and attenuated the development of atherosclerosis in part by increasing vascular NO bioavailability. Besides the differences in NO bioavailability, macrophage infiltration into the atherosclerotic plaques was significantly increased in aortas of apoE/Mas-KO mice compared to apoE-KO mice. Moreover, the increased macrophage infiltration in aortas of apoE/Mas-KO mice was associated with a proinflammatory cytokine expression profile of infiltrated macrophages suggesting that Mas receptors influence not only vascular NO bioavailability but also the phenotype of macrophages.

In addition to the data generated in the atherosclerosis model, Mas deficiency also accelerated the incidence and size as well as intima media thickness (IMT) and elastin fragmentation of Ang II-induced AAA in apoE-KO mice. Blood pressure and cardiac

hypertrophy did not differ during chronic infusions of Ang II in apoE-KO and apoE/Mas-KO mice suggesting that Mas deficiency-augmented AAA formation was blood pressure-independent. In accordance with the results seen in atherosclerotic plaques, macrophage and T cell infiltration was significantly increased in AAA of apoE/Mas-KO mice compared to apoE-KO mice suggesting that the Mas receptor of immune cells not only influences the atherosclerotic plaque formation but also plays a role in the development of abdominal aortic aneurysms. In Ang II-infused old WT mice, deletion of the Mas receptor increased AAA formation suggesting that Mas deficiency not only accelerates AAA formation in the hyperlipidemia mice but also increases the development of AAA in the normolipidemia mice.

In conclusion, our results demonstrate that the Mas receptor plays an important role in the development of vascular injury. Thus, Ang-(1-7)-induced Mas receptor activation improves endothelial dysfunction and atherosclerosis, whereas Mas receptor deficiency aggravates atherosclerosis and the development of abdominal aortic aneurysms during chronic Ang II infusion. Besides its important influence on vascular NO bioavailability and ROS production, the Mas receptor regulates inflammation and immune cell function and thereby seems to be new promising therapeutic target for treating cardiovascular diseases.

2. Zusammenfassung

Herz-Kreislauf-Erkrankungen ist weltweit die führende Todesursache. Das Renin-Angiotensin-System (RAS) ist eines der wichtigsten Hormonsysteme, die kardiovaskuläre Erkrankungen wie Bluthochdruck, Atherosklerose und Aneurysmen regulieren. Angiotensin (Ang) II, der wichtigste aktive Metabolit im RAS, fördert die Entwicklung von Hypertonie, Atherosklerose und die Bildung von Bauchaortenaneurysmen (BAA) durch Aktivierung des Ang II-Rezeptors Typ 1 (AT1R). Neben der Vasokonstriktion sowie der Natrium- und Wasserreabsorption in der Niere führt die AT1R-Aktivierung zu einer erhöhten oxidativen Stressproduktion (ROS), verringerte die Stickstoffmonoxid (NO) Bioverfügbarkeit und erhöht die Entstehung von Fibrose, Entzündungen und Immunzelleninfiltration. In letzter Zeit wurde eine neue Achse der RAS, die als alternative RAS-Achse bezeichnet wurde, beschrieben. Im Gegensatz zur Ang II / AT1R-Achse gibt es zunehmend Hinweise darauf, dass die Ang- (1-7) / Mas-Achse den Wirkungen von Ang II entgegenwirkt. Die Ang-(1-7) induzierte Aktivierung des G-gekoppelten 7 Transmembranrezeptors Mas führt zur Vasodilatation, Hemmung des Zellwachstums, antithrombotischen und entzündungshemmenden Prozesses. Kürzlich haben wir und andere zeigen können, dass Ang- (1-7) NO Bioverfügbarkeit erhöhte und die Erzeugung von reaktiven Sauerstoffspezies (ROS) im Gefäßsystem reduziert. Darüber hinaus führt die Deletion oder pharmakologische Hemmung des Mas-Rezeptors zu einer endothelialen Dysfunktion und zum vaskulären Schaden, welcher durch Ang- (1-7) nicht verbessert werden konnte. So scheint es, dass Ang- (1-7) die Gefäßdysfunktion durch Aktivierung des Mas-Rezeptors verbessert. Der Zweck dieser Studie ist es, die Funktion des Mas Rezeptors und den zugrundeliegenden Signalweg der Mas Rezeptor-Aktivierung bei der Entwicklung von Atherosklerose und der Bildung von BAAs bei Apolipoprotein E Knockout (ApoE-KO) Mäusen zu untersuchen.

In der vorliegenden Arbeit, konnten wir zeigen, dass die Deletion des Mas Rezeptor die durch Western-Diät (WD) -induzierte Atherosklerose bei apoE-KO-Mäusen verschlimmert. Die erhöhte Atherosklerosebildungs in Apolipoprotein E / Mas-Rezeptor-Knockout (apoE / Mas-KO) Mäusen geht gleichzeitig mit einer verschlechterten NO-vermittelten endothelialen Vasodilatation in diesen Tieren im Vergleich zu apoE-KO Mäusen einher. Im Gegensatz dazu verbesserte die chronische Infusion von Ang- (1-7) die endotheliale Dysfunktion und die Atherosklerose bei apoE-KO-Mäusen, aber nicht bei apoE / Mas-KO-Mäusen. Die verbesserte Gefäßfunktion wurde mit einer drastischen Zunahme der vaskulären NO-vermittelten cGMP-Bildung und einer signifikanten Abnahme der vaskulären ROS-Produktion begleitet, was darauf hindeutet, dass Ang-(1-7) induzierte Mas-Rezeptor-Aktivierung die Gefäßfunktion NO abhängig verbesserte und die Entwicklung der Atherosklerose durch Erhöhung der vaskulären NO-Bioverfügbarkeit. teilweise verbessert. Neben den Unterschieden in der NO-Bioverfügbarkeit war die Makrophagen-Infiltration in die atherosklerotischen Plaques der Aorten von apoE / Mas-KO-Mäusen im Vergleich zu apoE-KO Mäusen signifikant erhöht. Darüber hinaus war die erhöhte Makrophageninfiltration in Aorten von apoE / Mas-KO-Mäusen mit einem proinflammatorischen Zytokin-

Expressionsprofil von infiltrierten Makrophagen assoziiert, was darauf hindeutet, dass Mas-Rezeptoren nicht nur die vaskuläre NO-Bioverfügbarkeit beeinflussen, sondern auch den Phänotyp von Makrophagen.

Zusätzlich zu dem, im Atherosklerose-Modell erzeugten Daten, war nach 4-wöchiger Ang II Infusion die Inzidnenz und die Ausprägung des Bauchortenaneurysmas in apoE / Mas-KO Mäusen signifikant erhöht im Vergleich zu apoE-KO mäusen. Interessanterweise, war dabei der Blutdruck und die Herzhypertrophie nicht unterschiedlich zwischen den apoE-KO und apoE / Mas-KO Mäusen. Dies deutet darauf hin, dass der Mas Rezeptor seine Effekte bei der BAA-Bildung blutdruckunabhängig vermittelt. In Übereinstimmung mit den Ergebnissen, die bei atherosklerotischen Plaques beobachtet wurden, war die Makrophagen- und T-Zell-Infiltration in den Bauchaortenaneurysmen von apoE / Mas-KO-Mäusen im Vergleich zu apoE-KO-Mäusen signifikant erhöht. Diese Ergebnisse lassen darauf schließen, dass der Mas-Rezeptor auf Immunzellen nicht nur die atherosklerotische Plaquebildung beeinflusst, sondern auch eine maßgebliche Funktion bei der Entwicklung von Bauchaortenaneurysmata spielt. In Ang II-infundierten 8 Monate alten WT-Mäusen erhöhte die Deletion des Mas-Rezeptors die BAA-Bildung, was darauf hindeutet, dass das Fehlen des Mas-Rezeptors nicht nur die BAA-Bildung bei den hyperlipämischen Mäusen beschleunigt, sondern auch die Entwicklung von BAA bei normolipämisch, alten Mäusen erhöht

Abschließend zeigten unsere Ergebnisse, dass der Mas-Rezeptor eine wichtige Rolle bei der Entstehung von Gefäßschäden spielt. So verbessert die Ang- (1-7) induzierte Mas-Rezeptor-Aktivierung die endotheliale Dysfunktion und Atherosklerose, während die Deletion des Mas Rezeptors in unserem Knockout Modell die Atherosklerose und die Entwicklung von Bauchaortenaneurysmata verschlimmert. Neben dem bedeutenden Einfluss auf die vaskuläre NO-Bioverfügbarkeit und die ROS-Produktion reguliert der Mas-Rezeptor die Entzündungs- und Immunzellfunktion und scheint damit ein neues, vielversprechendes therapeutisches Ziel für die Behandlung von Herz-Kreislauf-Erkrankungen zu sein.

3. Introduction

Cardiovascular diseases are the leading risk factor causing cardiovascular mortality and morbidity worldwide [1]. The renin-angiotensin system (RAS) is a hormone system involved in the development and pathogenesis of cardiovascular diseases, such as hypertension, atherosclerosis, and aneurysms. Many human studies and experimental animal models have shown that activation of the angiotensin converting enzyme (ACE) / Ang II / AT1R axis promotes the development of cardiovascular diseases, accompanying by oxidative stress, decreased NO bioavailability, inflammation, fibrosis, and cell apoptosis. In contrast, to the ACE/Ang II/AT1R axis, there is also an alternative RAS pathway called ACE2/Ang-(1-7)/Mas axis which counteracts the effects of Ang II. In this regard, Ang-(1-7) which is metabolized by ACE2 and activates its own receptor Mas is considered to be an important metabolite of RAS. Thus Ang-(1-7) has been shown to ameliorate myocardial infarction, stroke, hypertension, and vascular injury by increasing NO bioavailability, decreasing oxidative stress, and reducing inflammation [2-5]. As activation of the Mas receptor seems to be an interesting therapeutic approach, we tested whether Ang-(1-7) improves the development of atherosclerosis via Mas receptor activation. And we also tested the function of the Mas receptor in an Ang II-induced abdominal aortic aneurysms model.

The first part of the thesis describes the function of the Mas receptor in the development of atherosclerosis. This part compares atherosclerosis between apoE-KO mice and apoE/Mas-KO mice, the underlying mechanism of Mas receptor function in the development of atherosclerosis, and the protective effects of Ang-(1-7)-mediated Mas receptor activation in the development of vascular injury and atherosclerosis. The second part investigates the effects of the Mas receptor in the development of aortic aneurysms. For these studies, an established aortic aneurysm model was used. This required infusion of apoE-KO and apoE/Mas-KO mice chronically with Ang II for four weeks.

3.1 The Renin-angiotensin system

The RAS is active in many tissues, including the heart, kidneys, adrenal glands, nervous system, and vasculature. Therefore, it is not surprising that it regulates very important cardiovascular functions like blood pressure control, sympathetic activity, vascular homeostasis, and metabolic syndrome. Dysregulation leads or exaggerates several diseases like hypertension, vascular injury, inflammation, fibrosis, heart diseases, and renal dysfunction [6-8].

3.1.1 The renin-angiotensin system

The RAS contains many angiotensin metabolites and receptors, such as Ang I, Ang

II, Ang III, Ang IV, Ang-(1-9), Ang-(1-7), ACE, ACE2, AT1R, Ang II type 2 receptor (AT2R) and Mas receptor. This complex system controls the balance of the RAS in healthy tissues.

Figure 1

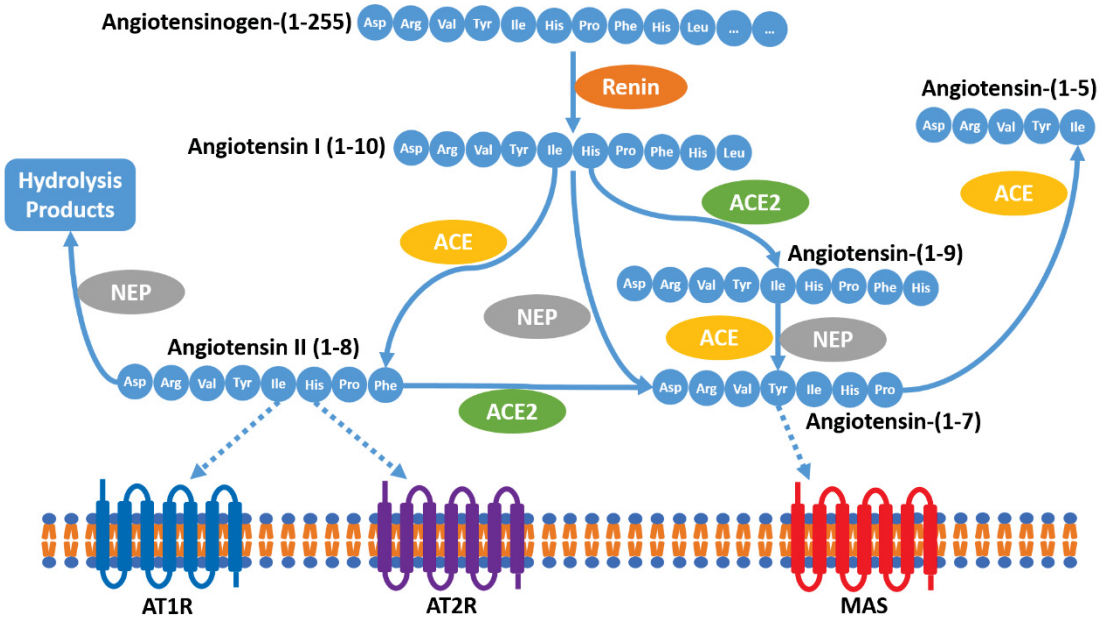

Figure 1 Detailed view of the renin-angiotensin system. Renin cleaves Angiotensinogen-(1-255) to Angiotensin I. Angiotensin converting enzyme (ACE) is the major enzyme responsible for the generation of Ang II. Ang II can activate the AT1R and AT2R. ACE, ACE2, and neprilysin (NEP) are the major peptidases involved in the generation of the heptapeptide Angiotensin-(1-7). Angiotensin-(1-7) can activate the Mas receptor. Adapted from Yang et al. [9].

Ang II generation begins with Angiotensinogen [Ang-(1-255)], which can be converted into Ang I by renin. Ang I can be directly or indirectly degraded into several metabolite peptides (Figures 1 and 2).

The octapeptide Ang II converted from Ang I by ACE, activates two different types of receptors, the AT1R and AT2R. Genetic and pharmacological studies have shown that Ang II mediates most of its effects through the activation of the AT1R. Activation of AT1R by Ang II leads to hypertension, cardiovascular diseases, and kidney failure (Figure 3) [10-15]. The function of the AT2R is not fully understood. It seems that activation of the AT2R opposes the effects of AT1R activation. The affinity of Ang II to AT1R and AT2R are similar [16], but the distribution and expression of the receptors are different [17]. Activation of AT2R antagonized AT1R-mediated effects, including hypertension and vascular diseases [18, 19]. While, deletion of AT2R exaggerated nephropathy and atherosclerosis [20, 21].

Figure 2

Figure 2 Angiotensin peptides and their receptors. Many RAS peptides share similar amino acids sequences and functions. Ang II and Ang III can induce vascular dysfunction through activating AT1R. AT2R, AT4R, Mas receptor, and MrgD activation show a protective role in vascular diseases.

In addition to Ang II, recent studies provide evidence for an alternative pathway of the RAS. In this pathway, ACE2 plays an important role, as it can convert both Ang I and Ang II into Ang-(1-9) and Ang-(1-7), respectively (Figure 1) [22-25]. Particularly, the degradation effectiveness of Ang II to Ang-(1-7) by ACE is almost 400-times greater than Ang I to Ang-(1-9) by ACE2 [25, 26]. The metabolite Ang-(1-7) activates its own receptor Mas which has been shown to counteract the effects of Ang II in cardiovascular diseases. Under baseline conditions, interruption of ACE2 did not markedly change the plasma Ang II concentration and the blood pressure levels in mice. But the deletion of ACE2 altered blood pressure responses and cardiovascular function during Ang II infusion [27, 28]. Moreover, Mas receptor deficiency caused hypertension in mice with the FVB background [29].

Recent experimental studies have shown that Ang-(1-7) mediates most of its effects through the activation of the Mas receptor, leading to anti-inflammation, anti-fibrosis, decreased oxidative stress, and improved vascular function (Figure 3). Thus, ACE2/Ang-(1-7)/Mas axis attracts more and more attention as a therapeutic intervention which may help to develop more treatment strategies for the therapy of cardiovascular diseases.

Figure 3

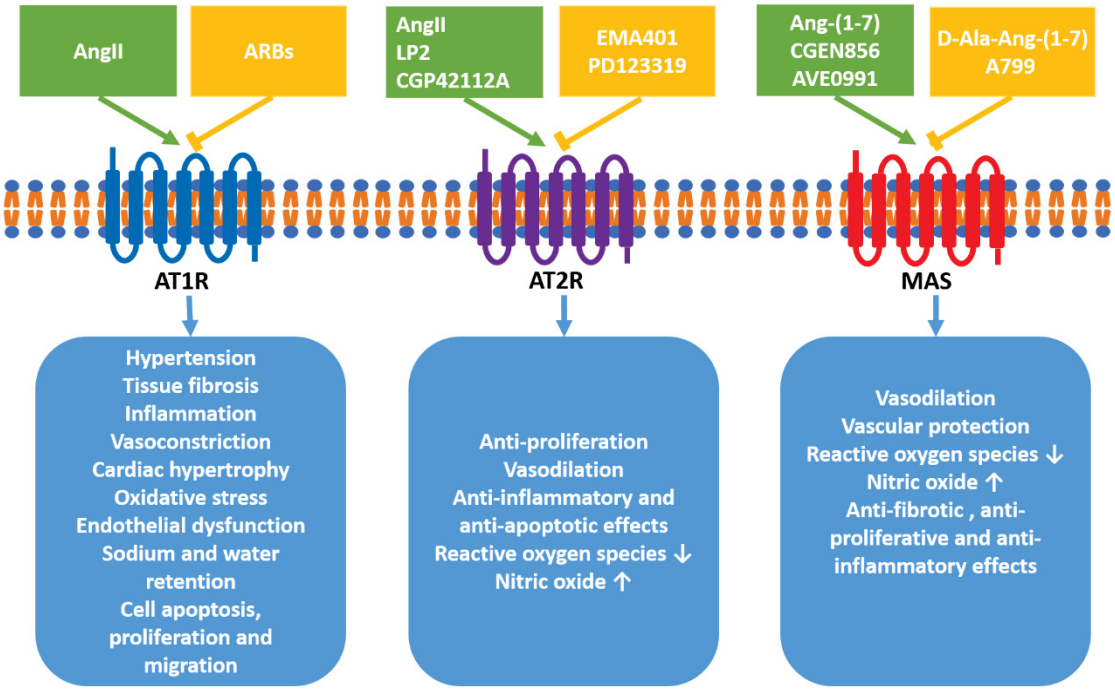

Figure 3 Effects of AT1R, AT2R and the Mas receptor activated by agonists (green) or antagonists (yellow). AT1R activation induces vascular dysfunction. Angiotensin receptor blockers (ARBs) inhibit the effects of AT1R. Activation of AT2R and the Mas receptor improve vascular function. Inhibition of AT2R and the Mas receptor may lose their protective functions in vascular diseases. Adapted from Yang et al.[9].

3.1.2 Angiotensinogen and renin

Ang-(1-255), also called angiotensinogen is primarily produced from the liver [7]. When our body is facing hypotremia or reduction of renal blood flow, the renal juxtaglomerular cells begin to secrete renin. The mechanisms controlling how renin is produced and secreted by these cells are complex and include several pathways [30]. First, the changes in renal arterial pressure near juxtaglomerular cells are sensed by intrarenal baroreceptors. Second, renin is also stimulated by sympathetic nervous stimulation through beta-adrenergic receptors. Third, the rate of NaCl ion transport and mediating information via a specific mediator to juxtaglomerular cells are sensed by a macula densa segment of the early distal tubule. Fourth, control of renin release is also stimulated by various humoral factors, including angiotensin, prostanoids, nitric oxide, and atrial natriuretic peptides [31, 32]. Then renin cleaves the 10 N-terminal amino acids of Ang-(1-255) to produce Ang I, which is the source of several active angiotensin peptides [33]. The function of the rest of the 245 amino acids derived from Ang-(255) is unknown [34].

3.1.3 ACE/Ang II/AT1R axis and its effects on vascular injury

The removal of two amino acids by the enzyme ACE converts Ang I to the octapeptide Ang II. Ang II plays an outstanding role in the pathogenesis of hypertension and vascular injury through activating AT1R [11, 35-37]. Increased Ang II levels lead to elevated blood pressure by inducing vasoconstriction, regulating sympathetic nerve activity, and increasing sodium and water reabsorption from nephron [38]. In addition to hypertension, increased Ang II-induced AT1R activation promotes vascular injury and thereby the development of atherosclerosis, aneurysms, and renal and cardiac dysfunction [10-15].

Ang II-induced vascular injury and hypertension is associated with increased oxidative stress and reduced NO bioavailability [39-41]. Oxidative stress reflects a condition of decreased antioxidant capacity and increased ROS production, which react or influence the vascular NO degradation levels, leading to vascular dysfunction.

Increased oxidative stress is a common result caused by many Ang II-induced signaling pathways in the vasculature, including phospholipase A2, phospholipase C, Ca^{2+} channels, Ras homolog gene family member A (RhoA)/ Ras homolog gene family (Rho) kinase, Janus-activated kinase-(JAK2)-signal transducers and activators of transcription (STAT) pathways, and mitogen activated protein kinase (MAPK) pathways [42-44]. Vascular ROS productions can be generated from all layers of the vessel, mainly produced by the NADPH oxidases which normally consist of five subunits (p22phox, p40phox, p47phox, p67phox, and gp91phox). Ang II-induced ROS production is not only involved in the degradation of NO, but also act as intracellular signaling molecules activating the MAPKs, which are involved in vasoconstriction, fibrosis, migration and inflammation [45-47]. In addition, some animal experiments have shown that Ang II can also cause vascular dysfunction and injury. It does this via stimulation of the cGMP-degrading phosphodiesterases 1 and 5, which leads to reduced cGMP bioavailability [48-52].

In contrast to AT1R activation, deletion or blockade of AT1R lead to attenuated blood pressure response and preserved vascular function. Thus, numerous studies have shown that AT1R antagonist Losartan attenuates endothelial dysfunction and vascular injury through reducing inflammatory responses and cell apoptosis [53-56]. Further studies have shown that Losartan suppresses Ang II-induced vascular smooth muscle cells (VSMCs) proliferation, migration and inflammation responses [57]. In this respect, absence of ACE2 leads to an increased renal NADPH oxidase activity which can be suppressed by AT1R antagonists [47]. Both inflammation and vascular remodeling are contributing to atherosclerosis and abdominal aortic aneurysms (AAA). In this regard, Ang II-induced macrophage-related inflammation is another key reason for Ang II-induced vascular injury. For example, accumulation of macrophage and releasing of pro-inflammatory factors in the vasculature induces endothelium damage, degradation of extracellular matrix proteins (ECM), as well as apoptosis, proliferation, and

migration of VSMCs [58-60]. Thus, Wenzel et al. [61] showed that deletion of monocytes reduces Ang II-dependent hypertension and vascular injury in mice. The mechanism controlling how activation of macrophages influences blood pressure and vascular injury during Ang II infusion is still not fully understood. However, it seems, that Ang II promotes the generation of pro-inflammatory M1 macrophages, leading to an increased immune cell infiltration in the vasculature.

Figure 4

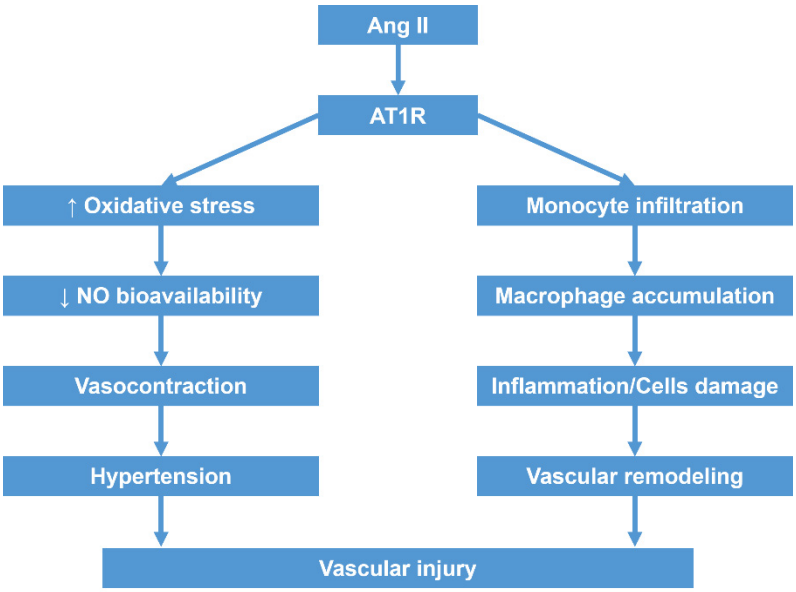

Figure 4 Ang II induces vascular injury by increasing macrophage infiltration and oxidative stress though AT1R.

3.1.4 ACE2/Ang-(1-7)/Mas axis

In addition to Ang II, another important metabolite of the RAS is Ang-(1-7). Ang-(1-7) is mainly generated from the conversion of Ang II by ACE2. It counteracts the effects of Ang II by activating Mas receptor. ACE2 mediates cardiovascular function by increasing Ang II degradation levels and Ang-(1-7) production levels. Experimentally, activation of the ACE2/Ang-(1-7)/Mas axis reduces hypertension and improves vascular injury and is highly correlated with the balancing regulation of NO bioavailability and ROS production [57, 62]. Some studies also demonstrated that the regulating the capacity of ACE2/Ang-(1-7)/Mas axis is associated with inflammatory responses [63-66].

ACE2, which shares 42% amino acid homology to the catalytic domain of ACE, is expressed in the intima and media layers of the vessel, and can also be expressed in the kidney, lung, liver, intestine, and central nervous system [67-69]. Based on its well-explained ability to convert Ang I and Ang II, it is sure that ACE2 is critically important

in the regulation of Ang II concentration levels in plasma and tissues [27]. Actually, genetic deletion of ACE2 in mice has resulted in more severe Ang II-dependent atherosclerosis, abdominal aortic aneurysms and hypertension, compared to control mice [28, 70-72]. Likewise, deletion of ACE2 increased renal superoxide generation, NADPH oxidase (NOX) 4 expression levels and some pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-alpha), interleukin (IL)-6, and IL-1beta, inducing discrete hypertension and renal dysfunction in apoE-KO mice. In addition to increased Ang II levels, reduced expression levels of the Ang-(1-7), which is responsible for vasoprotective and anti-oxidative effects through Mas receptor, have also been identified in ACE2 knockout mice [73, 74]. This vasoprotective function presents as a vascular mediating effect via increasing oxidative stress and decreasing NO bioavailability [62].

In this respect, it is not surprising that the Mas receptor is involved in the regulation of signaling pathways of NO bioavailability, such as phospholipase A (PLA) generates arachidonic acid (AA), and phosphoinositide 3 kinase (PI3K)/protein kinase B (AKT) signaling pathway activates eNOS by phosphorylation at dephosphorylation at threonine 495 and serine 1177 (Figure 5) [73]. Deletion of the Mas receptor is identified by increasing ROS production to elevate blood pressure. Ang-(1-7) infusion improves renal endothelial dysfunction by increasing NO-dependent cGMP generation and decreasing H_2O_2 production as well as gp91phox and p47phox expression [10].

The activated Mas receptor's ability to decrease vascular inflammation is a further example of its importance in vasoprotective function. Many experimental studies have shown that activation of the Ang-(1-7)/Mas axis improved endothelial dysfunction, atherosclerosis, renal disease and cardiac failure by decreasing inflammatory responses, such as decreased macrophage infiltration and production of chemokines and cytokines [75-80]. Each of these effects were shown to be reversible when treated together with Mas receptor antagonist A779. In addition, AVE0991 improved atherosclerotic plaques by inhibiting macrophage infiltration and polarization in apoE-KO mice [81]. Thus, Ang-(1-7)/Mas activation is assumed to improve vascular injury through inhibiting inflammatory responses.

Figure 5

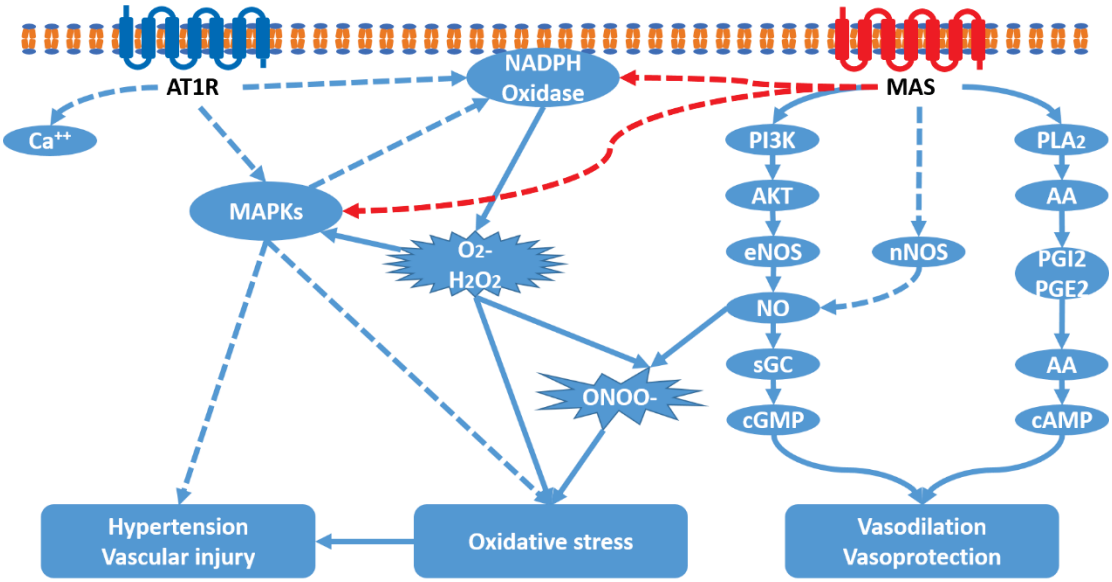

Figure 5 Schematic Mas receptor and AT1R signaling in the vessel. Mas receptor signaling protects from Ang II-induced vascular injury by increasing NO bioavailability and reducing oxidative stress. Red dotted lines indicate an inhibitory effect of Mas receptor signaling on the generation of oxidative stress. Adapted from Yang et al.[9].

3.2 Atherosclerosis and RAS

3.2.1 Atherosclerosis

Atherosclerosis is one of the leading causes of cardiovascular health-related mortality worldwide [82]. The occurrence of atherosclerosis is related to age, gender, genetics, and lifestyle. It is a slow, progressive disease that may start already in young adults [83, 84]. In some people, atherosclerosis develops quickly in their 30 years old. In others, it does not become apparent until the age of 50 years old or older. However, the arteries are common to get some hardening when people get older.

Hardening of the arteries is a condition in which plaque accumulates inside the arteries, especially in curved vessels, such as aortic arches. Plaque is made of cellular waste products, including fatty substances, cholesterol, calcium and fibrin [85]. The most critical situations of atherosclerosis are a persistent accumulation of plaque, break-off of plaque and thrombus formation, which may partially or totally block the blood's flow through an artery in an organ or limb of the host. Diseases that may develop as a result of atherosclerosis include heart attack, stroke, angina, coronary heart disease, carotid artery disease, peripheral artery disease (PAD) and chronic kidney disease. All of these diseases greatly endanger human health, especially in older people [86].

The pathogenesis of atherosclerosis is a poorly understood process. But it is evident that atherosclerosis is a form of chronic vascular inflammation and that atherosclerotic plaque begins accumulating due to endothelial dysfunction. Damage to

the intima layer of the artery, which induces an imbalance of NO bioavailability and oxidative stress, is the cause of endothelial dysfunction [85, 87, 88]. Three possible reasons for damage to the arterial wall are elevated blood pressure, high blood cholesterol/triglycerides, and smoking.

3.2.2 Atherosclerosis risk factors

The most common risk factor for atherosclerosis are smoking, diet, and genetics.

Smoking causes a seven-fold increase in the rate of atherosclerosis in the coronary arteries[86]. Inhaling cigarette smoke induces vascular injury by increasing blood presser, oxidative stress, several effects that damage the vascular system. This can increase blood pressure and increase the tendency for blood to clot.

The idea that there is a relation between dietary fat and atherosclerosis is controversial. It is known, however, that increased plasma LDL levels highly increase the risk of atherosclerosis [89], while HDL can decrease the rate of atherosclerosis development or even prevent its onset.. Some studies suggest that LDL particles are more prone to pass over the endothelial cells to the media layer of the vessel. Once LDL particles penetrate into the vessel wall, they become easier to oxidize [90]. Endothelial cells respond by attracting monocytes from blood, causing the monocytes to infiltrate the arterial walls and transform into macrophages. The continuous ingestion of oxidized LDL (oxLDL) particles by macrophage trigger a cascade of immune responses, finally forming specialized foam cells and generating an atheroma. The transformed macrophages can produce, or rupture to release, more chemokines and cytokines to attract more macrophages, resulting in a snowballing progression that continues the cycle, inflaming the blood vessel. The existing atherosclerotic plaques induce the proliferation and migration of the vascular smooth muscle cell, and the endothelial lining thickens, increasing the separation between the plaque and lumen.

Gender is another important risk factor in the development of atherosclerosis, males develop atherosclerosis more often than females. This is explained through different regulation of the immune and hormone systems [91, 92].

Figure 6

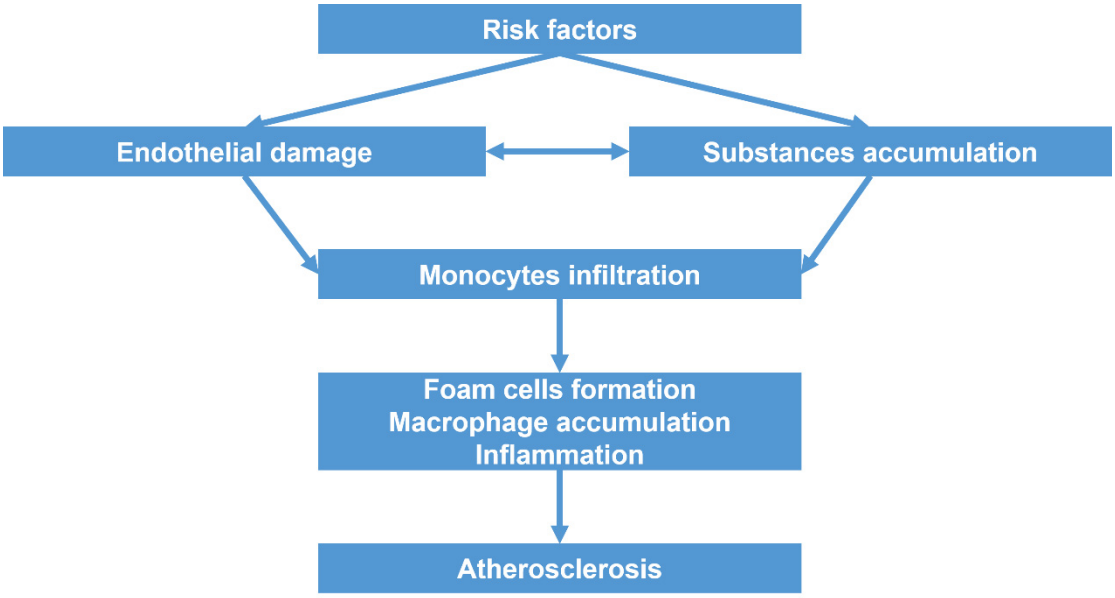

Figure 6 Brief schematic diagram of atherosclerosis.

3.2.3 Atherosclerosis and Ang II/AT1R

It is widely believed that atherosclerosis is a kind of chronic vascular inflammation, accompanied by increased ROS and reactive nitrogen species (RNS)-induced endothelial cell damage[93-95]. Activated Ang II/AT1R induces the same effects, such as increased oxidative stress, endothelial dysfunction, and macrophage infiltration. Over the past few decades, many experiments have successfully established research models of atherosclerosis induced by activation of the Ang II/AT1R axis, demonstrating that activated Ang II/AT1R contributes to the development of atherosclerosis.

The RAS plays an important role in the pathophysiology of atherosclerosis. Besides the classical regulatory effects on blood pressure and sodium homeostasis, the Ang II/AT1R is involved in the regulation of remodeling and contractility of the aortic wall. Overexpression and dysregulation of Ang II/AT1R initiate vascular injury through several signaling pathways leading to cell proliferation, apoptosis, fibrosis, oxidative stress and inflammation [96]. In contrast, deletion of AT1R markedly reduced hypercholesterolemia-induced atherosclerotic plaques, endothelial dysfunction, and oxidative stress in apoE-KO mice independent of plasma cholesterol levels and blood pressure [97].

3.2.4 Atherosclerosis and ACE2/Ang-(1-7)/Mas

As described above, Ang-(1-7) has been shown to counter-regulate the pathophysiological effects of Ang II in cardiovascular diseases by activating its own receptor Mas [98, 99]. In this regard, chronic Ang-(1-7) infusion has been shown to attenuate atherosclerosis. However, the protective mechanism of the Mas receptor in

the progression of atherosclerosis is unclear.

Vascular dysfunction as a key event in the initiation of atherosclerosis is a result of diminished release and a function of endothelium-derived nitric oxide and increased ROS production. Thus, it is not surprising that an increase in NO bioavailability and a decrease in ROS induced by an activation of the ACE2/Ang-(1-7)/Mas axis decreases blood pressure and promotes vasoprotective effects [100, 101]. In order to investigate the protective mechanism of ACE2/Ang-(1-7)/Mas in the development of atherosclerosis, researchers generated several animal models. Genetic deletion of the Mas receptor induces endothelial dysfunction by increasing activity of NADPH oxidase, as well as superoxide and peroxynitrite production, which is aggravated during Ang II-induced hypertension [29, 102, 103]. On the other hand, overexpression of vascular ACE2 in spontaneously hypertensive rats caused increased Ang-(1-7) levels, reduced blood pressure, and improved endothelial function [104]. Moreover, chronic Ang-(1-7) infusion has been shown to improve vascular function and attenuate the development of atherosclerosis by increasing NO bioavailability, reducing ROS via decreasing the gp91phox and p47phox expression level, and reducing vascular inflammation. Vascular inflammation is reduced via down-regulation of expression levels for adhesion proteins vascular cellular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) , and pro-inflammatory cytokines IL-6 and monocyte chemoattractant protein-1 (MCP-1) [64, 105].

Yang et al. [106] found that chronic infusion of Ang-(1-7) significantly decreased atherosclerosis, accompanying with decreased macrophage infiltration, oxidative stress, and improvement of endothelial dysfunction in apoE-KO mice. An *in vitro* study showed that Ang-(1-7) markedly suppressed VSMCs migration and proliferation. Beside the anti-inflammation effects, Ang-(1-7) enhanced the stability of atherosclerotic plaques [63]. Caffa et al. [63] also observed that Mas receptor gene expression levels were significantly increased in human stable plaque.

However, these studies could not confirm that Ang-(1-7) exclusively mediates its effects via the Mas receptor. Therefore, there is still no evidence that Ang-(1-7)-induced Mas receptor activation or deletion of the Mas receptor affects the development of atherosclerosis.

3.3 Abdominal aortic aneurysms and RAS

3.3.1 Abdominal aortic aneurysm

Abdominal aortic aneurysm (AAA) is defined as the expansion of aortic diameter to more than 50% of the original aortic diameter [107-111]. AAA is the most common form of aortic aneurysms. In humans, about 85% AAAs are infrarenal [112], whereas most of the AAAs in mice are suprarenal. They usually lack symptoms except when ruptured. Occasionally, abdominal, back, or leg pain may occur. If the aneurysm is large

enough, a doctor can locate it by pushing on the abdomen.

The most dangerous complication due to AAA is rupture or dissection. In patients with an aneurysm diameter of less than 5.5 centimeters, the risk of aortic rupture in the next year is less than 1%[113, 114]. Among patients with an aneurysm diameter of between 5.5 and 7 centimeters, the risk is around 9.4% to 19.1%, while for patients with an aneurysm expansion of more than 7 centimeters, the risk is around 32.5% [115]. If an AAA ruptures, the mortality rate is between 85% to 90% [111].

The pathophysiological mechanism of AAA is complex. The classical theory is that vascular remodeling, blood pressure, and blood flow play a key role in the formation of AAA [116]. The arterial wall cannot tolerate the impact of blood flow because of elevated blood pressure, resulting in the AAA formation. Increased intra-arterial blood pressure helps to enlarge the size of aneurysms. Atherosclerosis is the most common cause of AAA. Atherosclerotic plaques erode the aortic wall, causing degeneration of the elastic fibers and subsequently to thickening of the aortic wall or dissection of the aorta. Although elevated blood pressure was known to increase the growth rate of AAA, elastic lamellae disruption and inflammatory cell infiltration were also observed in the aorta [117].

There is currently no validated drug treatments that favorably affects aneurysm development and rupture; open operation and endovascular surgery are the only available treatment options for AAA [111, 118]. Therefore, it is important for research to define the mechanisms of AAA formation so that potential drug targets for this devastating disease can be identified.

3.3.2 AAA risk factors

The exact pathology of the degenerative process remains unclear. Nevertheless, some hypotheses have been proposed from well-defined risk factors, including age, smoking, hypertension, genetics, and atherosclerosis [119].

In humans, AAA occurs most commonly in those over 50 years old [120]. In 2013, aortic aneurysms resulted in 152,000 deaths, up from 100,000 in 1990 [1]. In 2009, AAA resulted in between 10,000 and 18,000 deaths in the USA. Screening with ultrasound is recommended for men between 65 to 75 years old with smoking history. While in Sweden, ultrasound screening is recommended for everyone aged and older. In the UK, screening all men over the age of 65 is recommended. Once an aneurysm is detected, further ultrasounds are typically done on a regular basis.

The influence of gender is high. AAA is 3 to 6 times more common in male patients, with a risk between 20 to 30% [121]. AAA affects between 2 and 8% of men over 65 years old. The prevalence of AAA in men is up to six times greater than in women [122]

More than 90% of people who develop AAA have smoked at some point in their lives [123]. Chronic vascular tension and inflammation-induced hypertension is also considered a long-term risk factor for the development of AAA.

The influence of genetic factors is high. Connective tissue disorders caused by Marfan syndrome and Ehlers-Danlos syndrome are strongly correlated with AAA development[124]. There are many hypotheses about the exact genetic disorder that could increase AAA incidence among male members of the families with genetic disease history. Some studies suppose that the alpha 1-antitrypsin deficiency could be a crucial factor influencing AAA formation [125]. Hereditary etiology hypotheses have also been proposed. Both relapsing polychondritis and pseudoxanthoma elasticum could be a reason for AAA formation [126, 127].

AAA was thought to be caused by atherosclerosis [128], because the suprarenal aortic wall is often affected heavily. Further studies have confirmed that AAA and atherosclerosis share many pathophysiological features, showing many potential linkages during the development of vascular diseases [128]. The most remarkable histopathological changes of AAA have been observed in all layers including intima, media, and adventitia. These include accumulation of lipids in foam cells, macrophage infiltration, calcifications, thrombosis, extracellular free cholesterol crystals, and increased intima-media thickness and ruptures of the layers. These events are also found in the development of atherosclerosis. The adventitial inflammatory infiltrate is also observed in AAA. However, the degradation of media and adventitia by means of the proteolytic process seems to be the basic pathology of aorta expansion. Some studies observed that the expression levels and activity levels of matrix metalloproteinases (MMPs) were markedly increased in patients with AAA. These MMPs, a family of endopeptidases, lead to elastin degradation in media, rendering the aortic wall more susceptible to the change of blood pressure. Some others studies have suggested AAA rupture could be induced by the serine protease granzyme B via cleavage of decorin leading to adventitial structure disruption. Compared to the thoracic aorta, researchers have also observed a decreased content of vasa vasorum in the abdominal aorta [129]. Accordingly, the media layer of the artery is also sensitive to hormone regulation and substance accumulation, which makes it more susceptible to vascular damage.

Classical theories believe that hemodynamics contributes to AAA formation. Hemodynamics has a predilection for the abdominal aorta. The histological structure and mechanical characteristics of the abdominal aorta differ from those of the thoracic aorta. This is because the diameter retains decreases from the aortic root to the bifurcation, and the aortic wall of the abdominal aorta also contains a smaller percentage of elastin. The mechanical tension in the abdominal aortic wall is, therefore, higher than in the thoracic aortic wall. The elasticity and dispensability of the artery also decrease with age, leading to gradual dilatation of the segment. Elevated blood pressure in patients with arterial hypertension certainly contributes to the development of the pathological process. Suitable hemodynamics situations may be related to

specific intra-luminal thrombus patterns along the aortic lumen, which may affect the development of AAA formation. Some recent studies observed a different pathological process that Ang II-induced AAA formation independent of blood pressure changes in mice models.

Figure 7

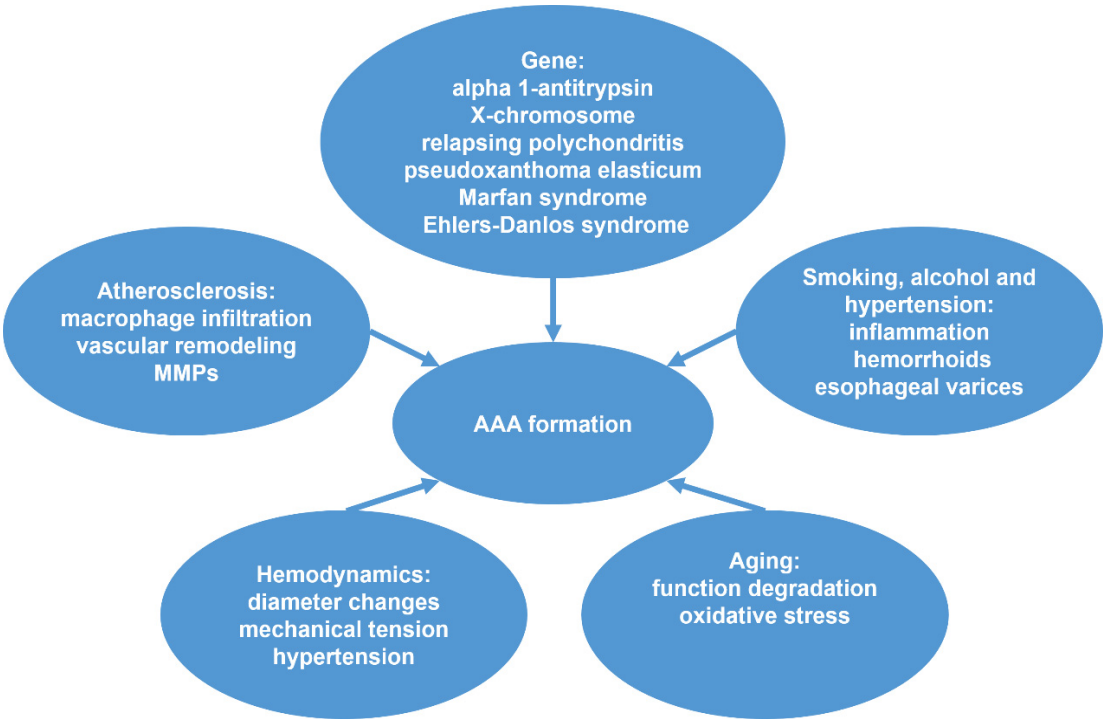

Figure 7 AAA formation and risk factors

3.3.3 AAA and Ang II/AT1R

The AAA risk factors indicate that AAA formation is highly correlated with increased oxidative stress, degenerated vascular structure, and macrophage-related inflammation. Many studies have investigated the mechanism of AAA formation under the treatment of Ang II,since Ang II-induced vascular injury and AAA have the same effects. Many animal models have been established in an attempt to understand AAA formation. Studies have demonstrated that Ang II induces AAA formation in apoE-KO and LDL-KO mice [130]. Similar to human AAA formation, increased Ang II production in mice induces immune cells to accumulate in the arterial wall. Accumulated inflammatory cells are responsible for extracellular matrix degradation and arterial expansion [131, 132]. These results in mice have contributed to insight into the mechanism of human AAA [132].

Details of the relationships between AAA risk factors, Ang II and AAA formation are still poorly understood. From humans with a high risk of AAA, the long-term

increased oxidative stress and inflammation causes vascular dysfunction and vascular injury. It eventually results in the development of atherosclerosis and the formation of aneurysms [133-136]. However, activated Ang II/AT1R has the same effects on oxidative stress and inflammation. All vascular diseases, including endothelial dysfunction, atherosclerosis and aneurysms, are correlated with elevated blood pressure, inflammation, and the imbalance of vascular NO bioavailability and oxidative stress [137-140]. Recently, some studies have suggested that inflammation plays an important role in vascular diseases by releasing chemokines, cytokines and MMPs, regulating immune cells, and stimulating macrophage polarization [141-143].

Daugherty et al. showed that chronic Ang II infusion accelerated AAA formation in ApoE-KO mice [132]. This progress of Ang II-induced AAA formation associated to a complex pathogenesis with considerable regional and temporal heterogeneity [35]. The AAA can be detected after 2 days of Ang II infusion [131]. Further studies show that macrophage infiltration is highly associated with AAA formation. Macrophage accumulation is observed in all regions of the aorta during Ang II treatment [132]. Recruitment of monocytes can cause degradation of elastin and vice versa; elastin degradation increases the availability of a chemoattractant gradient used for monocyte recruitment [131]. Monocytes/macrophages can release MMPs. MMPs are proteases, which can degrade ECM and elastic fibers. Elastin degradation may be caused by increased expression and activity levels of MMPs. Ang-(1-7) acting through Mas receptors often improves matrix metalloproteinases-2 (MMP2) and matrix metalloproteinases -9 (MMP9) expression and activity levels [144, 145].

Ang II increases oxidative stress and tissue inflammation, which are closely related to each other. Oxidative stress induces macrophages to release inflammatory factors, causing inflammation [146]. Oxidative stress has been shown to induce apoptosis of VSMCs and increase the risk of cellular inflammatory response, injury, and vascular endothelial dysfunction, including regulation of MMPs[147, 148]. ROS and RNS are also possibly involved in the formation of AAA [149, 150]. It has been shown that Ang II can cause leukocyte and monocyte infiltration, which leads to the release of a variety of chemokines, inflammatory response cytokines and proteases [46]. The infiltrated immune cells express MCP-1, which promotes vascular wall absorption of more macrophages [151]. This continuous cycle condition aggravates inflammatory response. In addition, the overexpressed MMPs and serine proteases, caused by immunoreactivity, can break down laminin, collagen, and elastic fibers, leading to thinning of the vessel wall resulting in aortic rupture or dissection [152, 153]. The persistence of inflammatory responses and increase in ROS production are detrimental to VSMCs, causing apoptosis of VSMCs, which may release more proteases from cells, and further accelerate thinning of the vessel wall [154]. Recently, Liu et al. found that ursodeoxycholic acid inhibits AngII-induced AAA rupture, primarily by reducing VSMCs apoptosis due to oxidative stress [155].

There is a view that the outer layer of vasculature is important for vascular

inflammation [156]. Adventitia, the outer layer around the blood vessels, maintains the shape of the blood vessel. Adventitia consists of loose connective tissue, mainly composed of fibroblasts, which can synthesize and secrete elastic fibers and collagen fibers, which help maintain normal vascular morphology. Vascular adventitial fibroblasts also play an important role in the vascular inflammatory response. Cell culture and animal experiments showed that AngII promoted the expression levels of P-selectin, ICAM-1, IL-6 and MCP-1 in adventitial fibroblasts, and increased the chemotactic adhesion phenomenon between outer membrane fibroblasts and macrophage [157]. MCP-1 promotes monocytes to adhere to fibroblasts, induces fibroblast proliferation, thickens the adventitia, and promotes cytokine production [158]. This cycle may regulate vascular adventitial inflammatory response. In addition, macrophages secrete a class of proteases named MMPs, which can degrade a variety of ECM components. Both MMP2 and MMP9 are abundantly expressed in the lesion locations of AAA patients. Over-expressed MMP2 and MMP9 degrade ECM, resulting in the functional loss of the vascular structure fixation due to changes in the adventitial membrane composition [159-161]. Conversely, knockout or inhibition of MMP2 and MMP9 can reduce the degradation of ECM and formation of AAA [161, 162].Therefore, macrophage-secreted MMP2 and MMP9 are considered to be one of the important causes of AAA formation.

Some current animal studies found that there is a high association between AngII-induced AAA formation and macrophage infiltration independent of blood pressure. In the region of AngII-induced AAA from apoE mice, the initial feature is the accumulation of macrophages in the media layer and the degradation of elastin [131].

Ang II also induces T and B cell infiltration in the AAA region [163, 164]. T lymphocytes and B lymphocytes promote the secretion of inflammatory response factors, stimulate macrophage infiltration, and are found in human AAA region. This may induce inflammatory response factors and cause AAA formation [165-167]. A large number of B lymphocyte infiltrations have been observed in the outer wall of the AAA location [166]. Activated B cells further activate macrophages and mast cells by releasing inflammatory response factors, immunoglobulin, and MMPs. These factors, stimulated by B cells, can cause the decomposition of collagen and matrix protein, causing vascular wall remodeling. T lymphocytes are another a key factor in the immune system, often interacting with macrophages. Macrophages activate T cells in the target tissue, while T cells also release inflammatory cytokines which further activate macrophages and T cells themselves. T lymphocytes, as important as macrophages, are preferentially involved in the inflammatory response to the immune response in human AAA assays [168, 169].

3.3.4 AAA and ACE2/Ang-(1-7)/Mas

Despite an effective suppression of this pathway by ACE inhibitors or AT1R blockers, results of end organ damage and high blood pressure induced by Ang II are

still not satisfactory [170]. This unexpected result might be caused by a potential downregulation of an alternative RAS in the development of these diseases. This alternative pathway includes the ACE2/Ang-(1-7)/Mas axis and activation of the AT2R (Figure 1), which antagonize the bad effects of activated Ang II/AT1R axis in AAA formation.

Many recent studies have demonstrated that inhibition of the ACE/Ang II/AT1R axis or activation of ACE2/Ang-(1-7)/Mas axis suppressed AAA formation in animal models [11, 171-174]. Pena et al. have shown that Ang-(1-7) activates the Mas receptor to reduce the inflammatory response induced by AngII [175]. Another human endothelial cells study found that Ang-(1-7) decreased AngII-induced ICAM-1, VCAM-1, and MCP-1 expression levels, leading to reduced vascular inflammatory response [105]. It is strongly associated with inhibition of the p38 MAPK signaling pathway and activation of NFκB pathways [105]. Ang-(1-7)/Mas axis also modulates the NFκB signaling pathway in human brain VSMCs and micro-vessels to improve AngII-induced vascular remodeling. It does so by reducing the expression levels of TNF-alpha, MCP-1 and IL-8 [176]. Many studies have shown that the beneficial effects of Ang-(1-7) in vascular injury were reversed by inhibition of A779 [75-80].

Currently, the protection mechanism of the Mas receptor in Ang II-induced AAA formation is unclear. However, many studies have shown that inhibition of ACE2 aggravates vascular diseases while infusion of ACE2 attenuates vascular diseases. Both results are highly associated with macrophage infiltration and polarization [66, 177-180]. Whole-body deletion of the Mas receptor significantly increases macrophage infiltration and pushes it to the proinflammatory M [lipopolysaccharide (LPS) + Interferon gamma (IFNγ))] subtype [181]. In a macrophage cell culture research model, Ang-(1-7) decreases the expression of LPS-induced proinflammatory cytokines [182]. It is also unclear whether macrophage also modulates the susceptibility to AngII-induced AAA.

3.4 Aim of the study

Here, in order to investigate the role of the Mas receptor in atherosclerosis and to identify possible signaling pathways regulating these effects, we generated a Mas receptor knockout mouse on an apoE-KO background (apoE/Mas-KO). And mice were fed with WD food for 12 weeks. Afterwards, in order to investigate the underlying mechanism and to clarify the role of Mas in AAA formation, apoE-KO and apoE/Mas-KO mice were infused with Ang II for 4 weeks.

4. Materials and methods

4.1 Ethics Statement and mice

All animal experimental investigations were in accordance with the federal state authority (Landesamt fuer Natur-, Umwelt- und Verbraucherschutz Nordrhein Westfalen; reference: AZ. 8.87-50.10.34.08.216 and AZ 84–02.04.2012.A250) and performed according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Mice were inspected daily for living environment and animation. The apoE-KO, apoE/Mas-KO, WT, and Mas-KO male mice were obtained from an in-house breed at the local animal care facility in Heinrich Heine Universität Düsseldorf. All mice were on a C57Bl/6 background. Littermates were used as controls. The animals were housed at 20°C to 22°C temperature and a 12-hour day and night cycle with free access to water and food.

4.2 Diet and drug administration

4.2.1 Atherosclerosis study

Induction of atherosclerosis: 6-week-old apoE-KO and apoE/Mas-KO mice were fed with WD food (Sniff, Soest, Germany) (42% fat, 0.15% cholesterol) and allowed free access to tap water. After 6 weeks of feeding, mice were treated with either saline or Ang-(1-7), delivered over 6 weeks by osmotic mini-pumps. The minipumps (Alzet Model 1004) were filled with either saline or Ang-(1-7) (82 μg/kg/h). And the osmotic mini-pumps were replaced after 3 weeks. Osmotic minipumps were inserted subcutaneously during anesthesia with ketamine (100mg/kg, i.p.) and xylazine (5 mg/kg, i.p.).

4.2.2 AAA study

Induction of AAA for Experiment with apoE-KO mice: 8-week-old apoE-KO and apoE/Mas-KO mice were infused with either saline or Ang II (1000 ng/kg/h), delivered for 4 weeks by osmotic minipumps (Alzet Model 2004).

Induction of AAA for Experiment with aged mice: 8-month-old WT and Mas-KO mice were treated with either saline or Ang II (1000 ng/kg/h), delivered 4 weeks by osmotic minipumps (Alzet Model 2004). Osmotic minipumps were inserted subcutaneously during anesthesia with ketamine (100mg/kg, i.p.) and xylazine (5 mg/kg, i.p.).

Figure 8

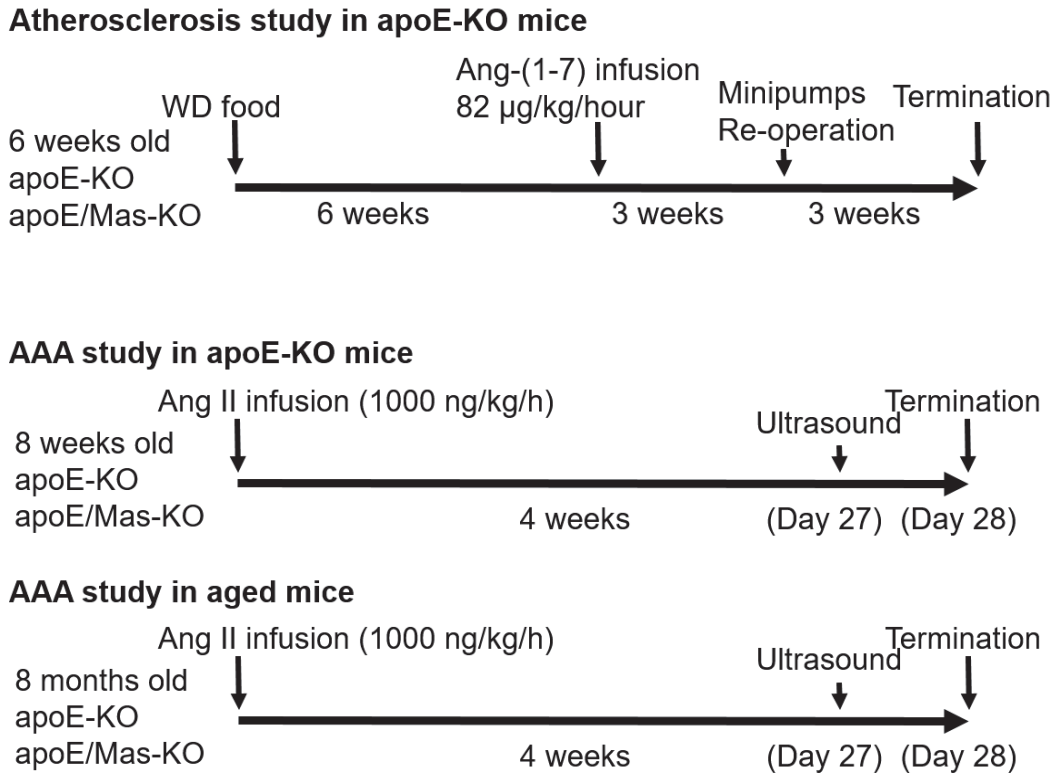

Figure 8 Experimental procedures.

4.3 Flow-mediated dilatation (Ultrasound)

Test of Ang-(1-7)/Mas axis activation on vascular function: FMD was measured on the day before termination. The details were described previously [183]. Mice were anesthetized with 2vol% isoflurane. Body temperature was kept at 37°C by using a heated examination table that was also equipped with ECG electrodes. The hair was removed from the left hind limbs. Pre-warmed ultrasound gel was daubed in access to the left hind limbs before measurement. The ultrasound probe was manually aligned with the femoral vein visible at the upper inner thigh. Images of the femoral arteries (FA) in mice were obtained using the Visual Sonics imaging platform Vevo 2100 and a 30-70 MHz linear array micro scan transducer (Visual Sonics). Using Duplex ultrasound mode, the artery and the arterial blood flow are identified by pulsed-wave Doppler (PWD). During FMD-assessment in the brachial artery in mice, a vascular occluder was placed at the left limb to induce occlusion of the distal hind limb as an ischemic trigger. Inflation occlusion (5 minutes) was carried out manually (figure 9A). Following hind limb ischemia, the cuff was deflated and femoral artery diameter measurements were continuously recorded for 3 min at 30 sec intervals. The heart rate and FA blood flow velocity were monitored by PWD was displayed as spectral Doppler curves. Acquired images analysis was performed from recorded videos using a semi-automated system (Brachial Analyzer, MIA, Iowa City) [183]. A region of interest in the longitudinal image of the FA was selected. The software automatically traces the

blood vessel lumen with accurate edge measurement algorithms. The software detects the wall boundaries of the near and the far wall (M-line) of the selected blood vessel image and quantifies the arterial lumen diameter (Figures 9B). The changes of FMD are calculated as absolute diameter difference (μm) and relative diameter difference (%) [(Diameter post-ischemic – Diameter baseline)／Diameter baseline] * 100.

Figure 9A

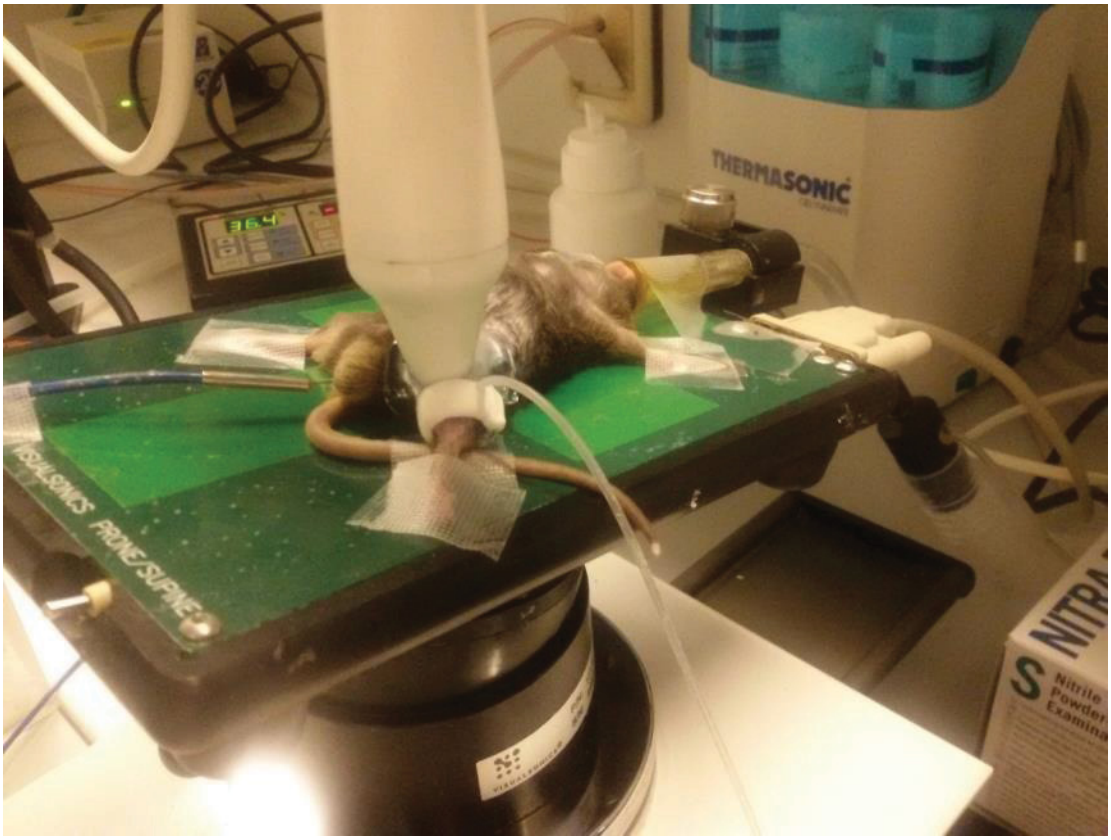

Figure 9B

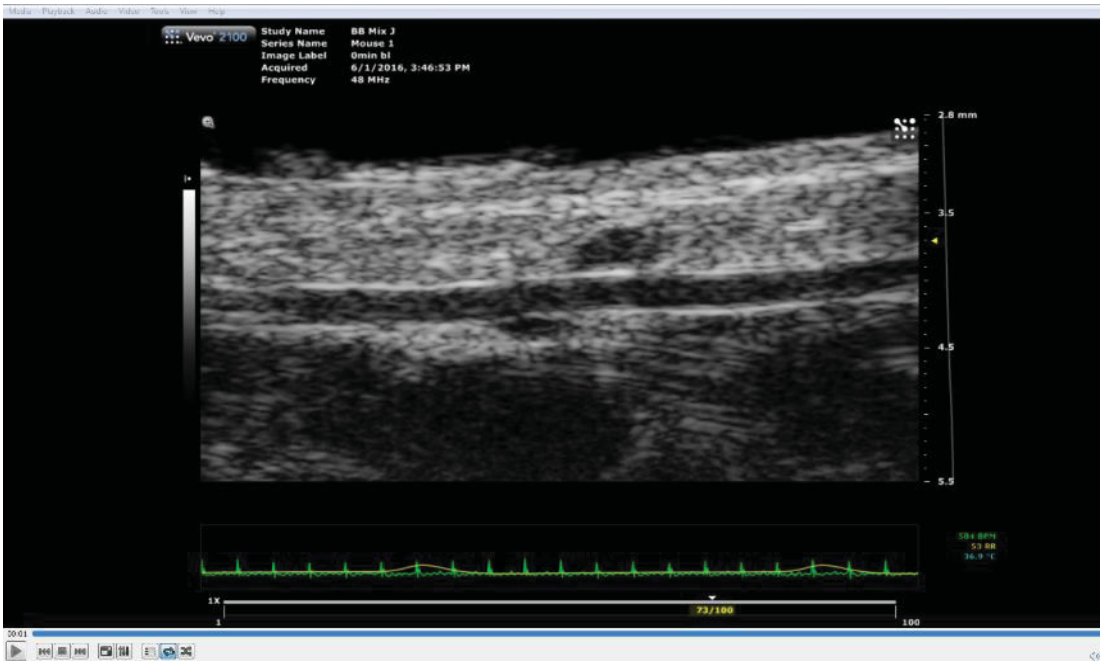

Figure 9 (A) Setup of an FMD measurement. (B) Representative video of FMD measurement.

4.4 Aortic cGMP measurement

Aortic slices were cut and equilibrated for 10 min in tempered (37°C), oxygenated (with 95% O2, 5% CO2) Krebs–solution as described before [10]. To extract cGMP, slices were frozen in liquid nitrogen, homogenized in 70% (v/v) ice-cold ethanol using a glass/glass homogenizer and then centrifuged (14 000x g, 15 min, 4°C). Supernatants were dried at 95°C and the cGMP content was measured by RIA (Brooker et al., 1979). Preparation of tracer, acetylation of samples and standards and incubation with antibody were performed as described [10]. To standardize the different samples, protein pellets were dissolved in 0.1 M NaOH/0.1% sodium dodecyl sulfate (SDS) and protein content was determined using the bicinchoninic acid method (Uptima).

4.5 Oil Red O Staining and quantification

To quantify the extent of atherosclerotic lesions, aortas (from the aortic root to 3 mm from the opening section of the aorta) were dissected from the mice. The aortas were then incubated in 4% paraformaldehyde overnight. After removal of adventitia, tissues were stained with Oil Red O solution (ORO, 5 g ORO powder in 1L methanol) histopathology staining method. Cleaned aortas were pretreated in 78% methanol for 5 minutes and then transfered to the fresh NaOH-ORO solution (1 ml NaOH: 3.5 ml ORO) for 90 minutes. After ORO staining, aortas were incubated in 78% methanol for 15 minutes and preserved in 1X PBS.

To obtain photographs, aortas were pinned and photographed under a microscope

and digital camera. The photos were captured by a camera (Coopix 4500, Nikon, Tokyo, Japan) with Carl Zeiss lens (426126 Carl Zeiss, Jena, Germany) under a Leica microscope (Leica MZ6, Wetzlar, Germany). Fiji ImageJ software (Fiji Version 2.0.0) was used to measure the atherosclerotic lesions.

4.6 Blood pressure measurement

SBP was measured in conscious mice by tail-cuff method (BP-98A; Softron Co.). For habituation, mice were trained daily for five consecutive days prior to the experiment. Thereafter, ten measurements per mouse were recorded every second day after Ang II minipump implantation.

Radiotelemetry was performed as described previously [184]. Radiotelemetry catheters [PA-C10 (Data Sciences International, Saint Paul, MN)] were implanted into the left carotid artery during anesthesia with ketamine and xylazine. The transducer unit was implanted into a subcutaneous pouch of the abdomen. After 1 week of recovery period to the normal circadian rhythms in an undisturbed environment, minipumps were implanted into the mice. Measurements during baseline and Ang II infusion were collected every 20 minutes for a 10-second interval. Data were analyzed using Dataquest A.R.T. software (Version 4.0, Data Sciences International, Saint Paul, MN).

4.7 Western Blotting (nitrotyrosine)

To measure aortic nitrotyrosine levels, Western Blotting (WB) was used as described previously [10]. After termination, mice were perfused with sterile 1X PBS for 2 minutes. The aorta was then dissected and preserved in All Protect Tissue Reagent (Qiagen, Germany) overnight. The next day, samples were transferred into -80 ℃ for preservation. Aortas were ruptured by automatic homogenizer in 1X Tissue Lysis Buffer (600 µL EMPIGENE, 10 mL phosphate buffered saline, 44 nM PMSF) containing protease and phosphatase inhibitors. Lysates were centrifuged at 4000 g for 10 min at 4°C. The supernatant sample concentrations were determined by a Bradford assay (Bioassay Systems). After protein samples mixed with Laemmli reagent and denaturation (5 min at 95°C), 20 µg of total proteins were loaded onto 8% SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane as described from the product instruction (X-Cell Blot Module, Invitrogen). The membrane was blocked in blocking buffer (5% BSA in TBST) for 1 h. Nitrotyrosine was stained by primary antibody anti-nitrotyrosine (39B6, Santa Cruz Biotechnology, Dallas, Texas, USA). The reference protein was β-actin (Sigma Aldrich, St. Luis, MO, USA). The second antibody was anti-mouse antibody (P0447, Dako, Germany). Membrane was treated with ECL solution before photo capture. WB signals were quantified using FluorChem FC2 Imager (Alpha Innotec, San Leandro, CA, USA).

4.8 Aortic shape (Ultrasound)

To observe the aortic shape for the AAA study, mice were detected by ultrasound one day before termination. Ultrasound measurements were performed using the high-resolution ultrasound imaging system Vevo2100 (Visualsonics, Toronto, Canada). The transducer MS 440 was used (18-38 MHz) for the measurements. Mice were anesthetized with isoflurane (2.5vol%). Body temperature was kept at 37°C using a heated examination table that was also equipped with ECG electrodes. The abdominal hair was removed and pre-warmed ultrasound gel was daubed on the abdominal skin. The whole aorta (from aortic root to the hind limb bifurcation) was monitored by PWD and was displayed as spectral Doppler curves. Images were analyzed by ImageJ.

4.9 Quantification of abdominal aortic aneurysms

To measure the incidence and diameter of AAA in the AAA study, aortas were flushed with sterile PBS from the left ventricle during termination. The maximal AAA diameters of aortas were measured with a digital caliper (PEARL PE-7811, Germany) *in vivo*. Then, the aortas were dissected from the aortic root to the iliac bifurcation and positioned in 4% PFA overnight. After removal of the connective tissues, aortas were pinned and photographed.

4.10 Magnetic resonance imaging (MRI)

Intravenously injected perfluorocarbon nanoemulsions (PFC) were used [185-188]. During the 28 days of Ang II infusion, mice were monitored longitudinally by $^{1}H/^{19}F$ MRI at 9.4T using a micro-imaging unit with actively shielded 40-mm gradients (1.5 T/m maximum gradient strength, 110 µs rise time at 100 % gradient switching). Mice were placed in a 25-mm $^{1}H/^{19}F$ birdcage resonator and after the acquisition of anatomical ^{1}H reference images, Magnetic resonance (MR) angiography was performed using a flow compensated 2D flash sequence (256x192 matrix size; 0.5 mm slice thickness, 0.25 mm overlap; scan time 3 min; 80° flip angle).

To monitor macrophage infiltration, PFCs were injected on day 4 and the 1H/19F MRI datasets were acquired 72 h later, to ensure optimal accumulation of PFC-loaded cells at inflammatory foci. For ^{19}F imaging, a ^{19}F RARE sequence with the following parameters was applied: 2.56´2.56 cm2 FoV, 64×64 matrix, 1 mm slice thickness, TR 4000 ms, RARE factor 32, 256 averages, 34 min acquisition time. *Ex vivo* high resolution $^{1}H/^{19}F$ MRI scans were performed using a 3D RARE sequence with the following parameters: 1.0´1.0´3.0 cm3 FoV, 128×128×192 matrix (19F = 64×64×96), TR 2500 ms, RARE factor 32, 8 averages (19F=180), 5 h (19F = 14 h) acquisition time.

4.11 Sirius red staining (collagen and media-intima thickness)

To analyze the IMT and collagen content for the AAA study, aortas were perfused and dissected during termination. Then aortas were incubated in 4% PFA overnight.

Suprarenal aortas were cut and fixed in paraffin. Paraffin samples were cut with 5 µm thickness for IMT calculation and 20 µm thickness for collagen measurement. Slides were stained with Sirius Red (No.9046 Chondrex, Redmond, WA, USA).

Slide pictures were captured under the microscope by AxioCam MRc5 (Carl Zeiss, Jena, Germany). IMT was calculated by ImageJ. The collagen content was measure using the calculation method in the manual of the Sirius Red Kit. Collagen content was detected by UV light (Nanodrop 2000c, Thermo Scientific, USA) at 750 nm (baseline), 540 nm (sample) and 605 nm (sample). The collagen content was calculated by the following formula: Collagen (µg/section) = [Optical Density (OD) 540 nm value - (OD) 605 nm value * 0.291)] / 0.0378. Non-collagen content was calculated with: Non-collagenous proteins (µg/section) = OD 605 nm / 0.00204.

$$\textbf{Collagen (µg/section)} = \frac{\textbf{OD 540 nm - OD 605 nm * 0.291}}{\textbf{0.0378}}$$

$$\textbf{Non-collagenous proteins (µg/section)} = \frac{\textbf{OD 605 nm}}{\textbf{0.00204}}$$

4.12 Immunohistochemistry (F4/80 and CD3)

For the AAA study, hearts (from the atherosclerosis study) and suprarenal aortas (from the AAA study) were isolated after termination. Samples were then incubated in 4% PFA overnight for the following paraffin fix. Paraffin samples were cut with 5 µm thickness for further staining. After the dewaxing step, slides were stained with F4/80 (macrophage) or CD3 (T cells). In addition to special notes, the remaining incubations were completed at room temperature. The stained pictures were captured by a camera under a microscope and analyzed by ImageJ.

F4/80 staining: Slides were pretreated with Proteinase K (Dako S3020, 3 Min), HRP-Block/Peroxidase-Blocking (Dako S2003, 10 Min), Biotin/Avidin Solution (Dako X0590, 10 Min), and Protein-Blocking serum free (Dako X0909, 1 hour). The slides were dried and the samples incubated with antibody “anti F4/80” [(Serotec MCA497RT) at 1:100 antibody diluent (Dako S0809)] overnight at 4°C. The following day, sections were incubated with the second antibody “anti-rat, biotiniliert” [(Dianova 112-065-003) at 1:500 antibody diluent (Dako S0809)] for 30 Min, with conjugate “Streptavidin HRP konjugiert” [(Dianova 016-030-084) at 1:500 antibody diluent (Dako S0809)] for 30 Min, DAB (Envision Kit, DM827) and substrate buffer (SM 903) for 3.5 Min. The slide backgrounds were stained with Haemalaun-Solution, and the slides were hard set with glycerol (Dako C0563).

CD3 staining: Samples were pre-treated with target retrieval solution (pH 9, 20 Min, 98°C) and left at room temperature for 30 Min. Sections were incubated with Avidin/Biotin Blocking (Dako Nr. X0590, 15 Min), AP-Block (Dako Nr. S2003, 10

Min), Serum-free block (Dako Nr. X0929, 25 Min). Sections were incubated with primary antibody (Dako Nr. N158059) for one hour, and secondary antibody anti-rabbit biotinylated [(Vectastain Elite ABC Kit #PK-6101) at 1:200 in antibody buffer] for 30 Min. Sections were incubated with conjugate solution (Vectastain Elite ABC Kit #PK-6101, 30 Min) and vector solution (Vector # SK-480, 4 Min). The slide backgrounds were stained with Hematoxylin (by Mayer), and the slides were hard set with Roti Mount (HP68.1).

4.13 MOVATs staining

To identify the elastic fibers fragmentation, MOVATs stain was performed on paraffin sections of suprarenal aortas. Sections (5 μm thickness) were fixed in Bouin's solution (No.10132 Sigma-Aldrich,;50°C for 10 min) and stained with 5% sodium thiosulfate (No.217263 Sigma-Aldrich; 5 min), 1% alcian blue (No.5268 Sigma-Aldrich; 15 min), alkaline alcohol (No.6899 Sigma-Aldrich; 10 min), Movat's Weigert's solution (Hematoxylin, No.9627 Sigma-Aldrich; Iron chloride hexahydrate, No.2877 Sigma-Aldrich; Iodine, No.3380 Sigma-Aldrich; Potassium iodide, No.901778 Chempur; 20 min), crocein scarlet-acid / fuchsin solution (Crocein Scarlett, No.0531 Chempur; Acid Fuchsin, No.8129 Sigma-Aldrich; 1min), 5 % phosphotungstic acid (No.4006 Sigma-Aldrich; 5 min) and 1% acetic acid (5 min). Between every step, the tissue samples were washed with running tap water and with distilled water. Then samples were dehydrated in 95 % (1 min) and 100 % ethanol (1 min) and stained in alcohol saffron (No.8381 Sigma-Aldrich; 8 min). Samples were washed in 100 % ethanol (1 min), moved to xylene (10 min) and covered with cytoseal mounting medium (No.8310-4 Microm). Images were analyzed using the ImageJ software. The details of method described previously [189].

4.14 Urine collection and analysis

For urine collection, 24 hours' worth of urine samples were collected by metabolic cages at the local animal care facility in Heinrich Heine Universität Düsseldorf 3 days before termination. Samples were then preserved in -80 ℃ in the presence of 0.005% BHT. 8-isoprostane was analyzed using the 8-isoprostane ELISA kit (No.516351, Cayman, USA). Creatinine was also measured by Kit (No. 500751, Cayman, USA).

4.14 Real-time PCR (RT-PCR)

Tissues were flushed by sterile 1X PBS during termination and then preserved in RNA later at -80 ℃ for future detection. To investigate gene expression levels in the aorta, mRNA was isolated from frozen tissues by mRNA mini kits (QIAGEN, Germany). The gDNA from mRNA samples were removed by wipe-out buffer (QuantiTect Kit QIAGEN, Germany). Hot Star Taq DNA polymerase was used for PCR. The cDNA samples were mixed with Syber Green (QIAGEN, Germany) for RT-PCR (15 minutes at 95 ℃; 39 times 30s 95 ℃, 30s 58 ℃, 30s 72 ℃; 2 minutes 72 ℃). The RT-PCR was performed with an ABI PRISM 7300 (Applied Biosystem, Germany).

Rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene.

All primers were obtained from Thermo Fisher Scientific.

Gene	Products reference number
NOS2	Mm00440502
MCP-1 (CCL2)	Mm00441242
IL6	Mm00446190
TNF-alpha	Mm00443258
LL1R1	Mm00434237
IL12b	Mm00434174
IL1b	Mm00434228
Chil3	Mm00657889
Fizz (Retnla)	Mm00445109
Arg1	Mm00475988
Mgl2	Mm00460844
Slamf1	Mm00443316
Mrc1	Mm00485148

4.15 Blood isolation and measurement

To measure the plasma changes under the treatment of Ang II, blood was directly isolated from the heart by syringe with heparin during termination. Blood samples were then centrifuged at 5000 RPM for 15 minutes at room temperature. Plasma samples were isolated and preserved with EDTA at -80 ℃. Cholesterol, triglyceride, HDL, and LDL were measured at the University of Duesseldorf Hospital.

4.16 Isolated perfused kidney

Experiments with isolated perfused kidneys were performed to measure the endothelial function according to the method described previously [190]. Pressure response was monitored continuously with a Statham P23 Db pressure transducer (Gould, Oxnard, CA) coupled to a Watanabe pen recorder (Graphtec Corp., Tokyo, Japan).

Mice were anesthetized with ketamine and xylazine. Whole kidneys were dissected under a microscope (Olympus CO11) and perfused with Krebs–Henseleit buffer as described previously [191]. A bolus injection of 60 mM KCl was administrated to test the contractility of the kidneys followed by a stabilization period. After this stabilization period, renal vasoconstriction was induced by a chronic infusion of 1mM norepinephrine (Sigma-Aldrich, St Louis, MO, USA). In the precontracted kidneys, renal vasorelaxation was then induced by different concentrations (0.003 mM,

0.01 mM, 0.03 mM, 0.1 mM, 0.3 mM, 1 mM, 3 mM, 10 mM, 30 mM,) of carbachol (Sigma-Aldrich, St Louis, MO, USA). Renal vascular relaxation is expressed as the ratio of pressure reduction to precontracted kidney pressure.

Figure 10

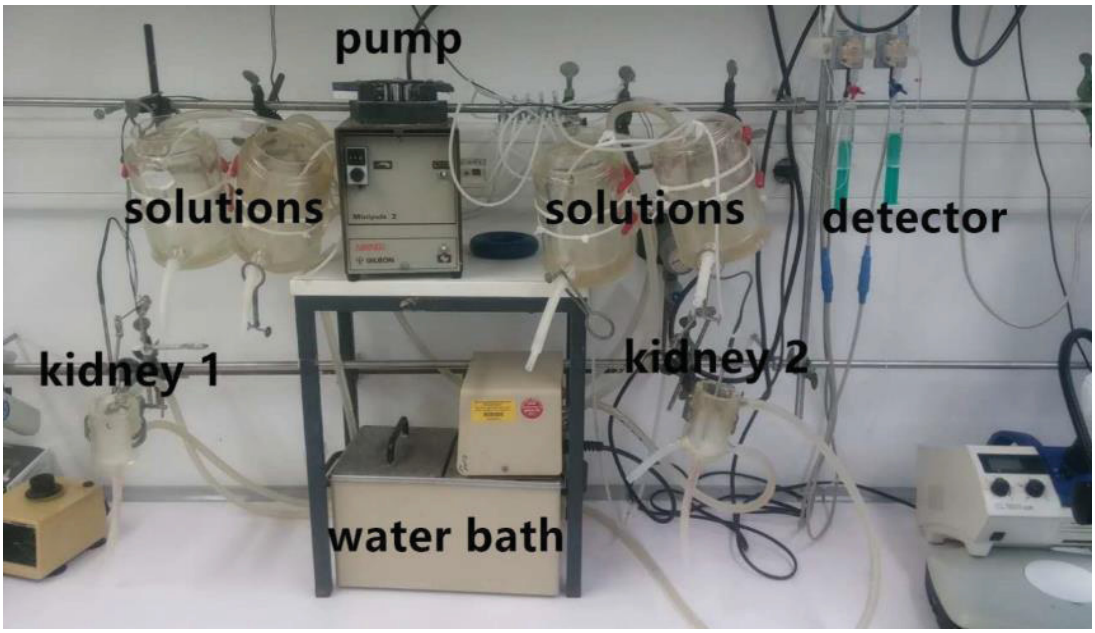

Figure 10 Isolated perfused kidney detector.

4.17 Statistical analysis

Data have been expressed as mean ± SEM (n = number of animals or samples). Version 5.0 of GraphPad Prism (San Diego, USA) was used for data analysis. Student's t-test was primarily used to compare means of two groups with a Gaussian distribution. Multiple comparisons of more than two groups with Gaussian distribution were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons posthoc test. Statistical analysis of two groups in which the data was not normally distributed (or where this could not be assumed) were analyzed by the Mann-Whitney-U-Test. Differences between dose response curves were analyzed by two-way ANOVA for repeated measurements followed by Bonferroni correction posthoc test. Results were considered statistically significant for $P \leq 0.05$ (*). Higher level of statistical significance were also labeled ($P \leq 0.01$ (**), $P \leq 0.001$ (***)).

5. Results

5.1.1 Ang-(1-7) improved atherosclerotic plaques in apoE-KO via activating Mas receptor

In order to investigate the function of the Mas receptor in the development of atherosclerosis, we stained and analyzed atherosclerotic lesions in aortic arches of mice that had been treated either with Ang-(1-7) or with saline (figure 11A). Deletion of the Mas receptor accelerated WD-induced atherosclerotic lesions in apoE-KO mice (figure 10B and C, $P < 0.005$). In addition, Ang-(1-7) reduced atherosclerotic lesion area in apoE-KO ($P < 0.005$), but not in apoE/Mas-KO mice (figure 11B and C), suggesting that Ang-(1-7) mediates its protective vascular effects solely through Mas receptor activation.

Figure 11A

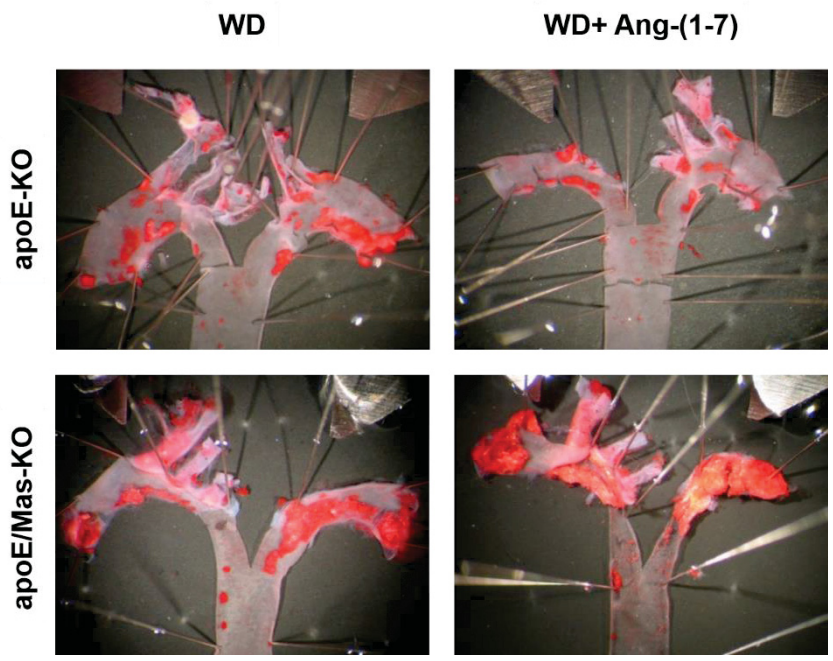

Figure 11B

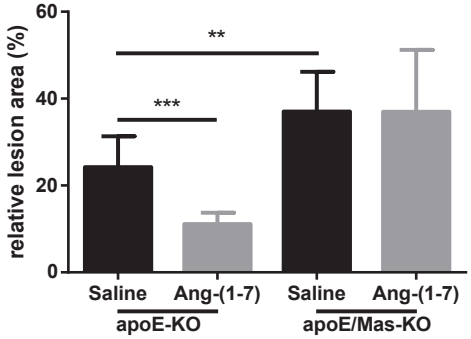

Figure 11C

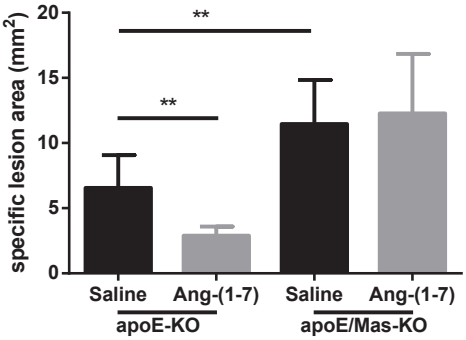

Figure 11 (A) Representative ORO photos of aortic arches. The distance between the digital calipers is 7 mm. (B) Deletion of Mas receptor increased atherosclerotic plaques in aortic arches. Ang-(1-7) improved atherosclerosis in apoE-KO, but not in apoE/Mas-KO. (n=7-11). (relative lesion area %; apoE-KO vs. apoE/Mas-KO, 24.25±2.137, n=11 vs. 37.02±2.9, n=10, P ＜0.005; apoE-KO vs. apoE-KO + Ang-(1-7), 24.25±2.137, n=11 vs. 11.15±0.9749, n=7, P ＜0.005; apoE/Mas-KO vs. apoE/Mas-KO + Ang-(1-7), 37.02±2.9, n=10 vs. 36.96±4.505, n=10) (specific lesion area mm; apoE-KO vs. apoE/Mas-KO, 6.561±0.7943, n=11 vs. 11.46±1.131, n=10, P＜0.005; apoE-KO vs. apoE-KO + Ang-(1-7), 6.561±0.7943, n=11 vs. 2.889±0.2876, n=7, P ＜ 0.005; apoE/Mas-KO vs. apoE/Mas-KO + Ang-(1-7), 11.46±1.131, n=10 vs. 12.27±1.522, n=10) *P < 0.05, **P < 0.01, ***P < 0.001.

Previous studies have shown that deletion of the Mas receptor failed to improve vascular dysfunction in Ang-(1-7) treated apoE-KO mice. Vascular dysfunction is a key event in the early stage of atherosclerosis, and this process is related to an imbalance of oxidative stress and NO bioavailability. To investigate the function of the Mas receptor in oxidative stress, urine samples were collected from apoE-KO mice and apoE/Mas-KO mice. Deletion of the Mas receptor increased urinary 8-isoprostane/creatinine levels in apoE-KO mice (figure 12A), which means that deletion of Mas receptor increased oxidative stress in apoE-KO mice. To better understand the function of the Mas receptor in modulating NO bioavailability, nitrotyrosine (a marker for oxidative stress) was measured. Deletion of Mas receptor increased nitrotyrosine levels in apoE-KO mice (figure 12B), suggesting that deletion of the Mas receptor may increase cell damage, inflammation, and NO degradation.

Figure 12A Figure 12B

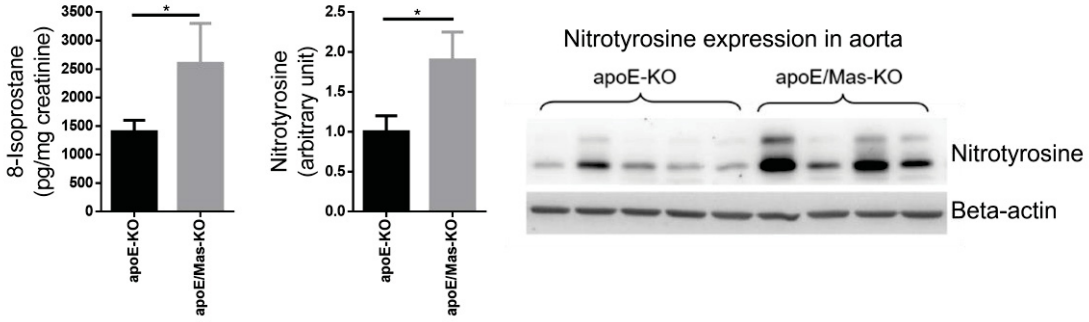

Figure 12 (A) Deletion of the Mas receptor increased urinary 8-isoprostane / creatinine levels in apoE-KO mice. n=6-8 (B) Deletion of the Mas receptor increased nitrotyrosine levels in apoE-KO mice. n=6-8, *P＜0.05.

5.1.2 Ang-(1-7) improved endothelial dysfunction in apoE-KO was associated with increased NO bioavailability

We found that deletion of the Mas receptor increased ROS production and decreased NO bioavailability in apoE-KO mice. Both are important in the breakdown of endothelial dysfunction, which is considered a key event in the development of atherosclerosis. To investigate the function of Mas in the regulation of vascular function, we measured endothelial-dependent vasorelaxation in the isolated perfused kidney. Carbachol caused renal vascular relaxation in apoE-KO mice, and this result was significantly impaired in apoE/Mas-KO mice (figure 13).

Figure 13

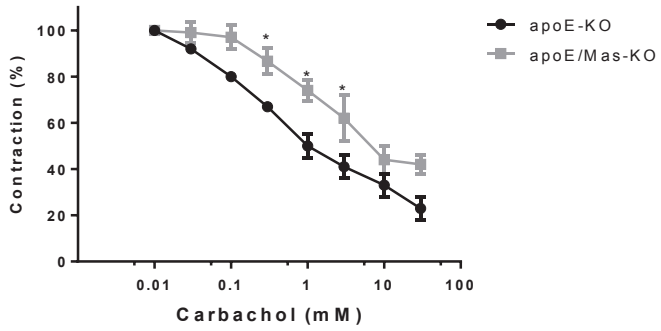

Figure 13 Deletion of Mas receptor caused vascular dysfunction in isolated perfused kidney of apoE-KO mice (n=5-10 per group). *P＜0.05.

Endothelial dysfunction can be also measured by FMD. In order to understand the correlation between endothelial-mediated vasodilation and atherosclerosis in Mas-deficienct mice, we measured FMD as an *in vivo* method for NO dependent vasodilation. Chronic Ang-(1-7) infusion increased FMD significantly in apoE-KO mice (diameter, P ＜ 0.005; percent, P ＜ 0.0001), but not in apoE/Mas-KO mice (figure 14 A and B). These results suggest that Ang-(1-7) improves vascular function through Mas receptor activation.

Figure 14A

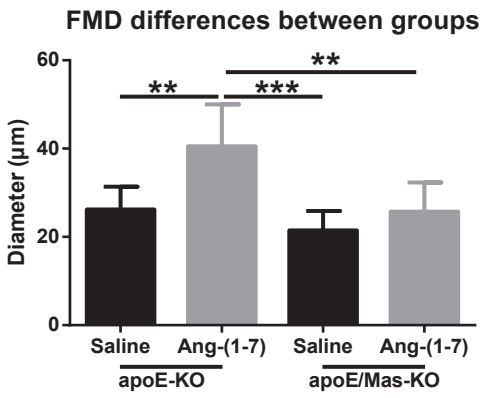

Figure 14B

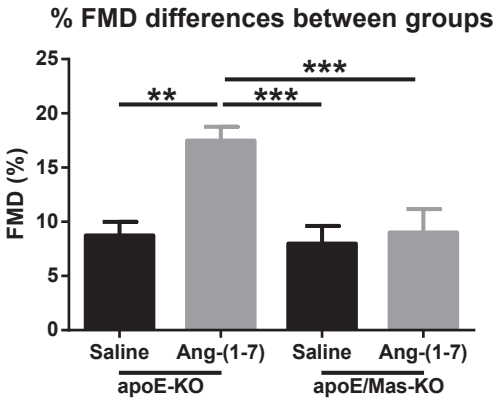

Figure 14 (A and B) Ang-(1-7) improved WD-induced FMD in apoE-KO, but not in apoE/Mas-KO. (n=3-11). (diameter μm, apoE-KO vs apoE-KO + Ang-(1-7), 26.24 ± 1.618 n=10 vs. 40.50 ± 4.253 n=5, p ＜ 0.005; apoE/Mas-KO vs apoE/Mas-KO + Ang-(1-7), 21.45 ± 1.975 n=5 vs. 25.75 ± 3.813 n=3, p ＜ 0.005) (percent %, apoE-KO vs apoE-KO + Ang-(1-7), 8.738 ± 0.3800 n=10 vs. 17.49 ± 0.5696 n=5, p=0.3069 apoE/Mas-KO vs apoE/Mas-KO + Ang-(1-7), 7.984 ± 0.7260 n=5 vs. 9.020 ± 1.247 n=3, p=0.4654) *P＜0.05, **P＜0.01, ***P＜0.001.

These results suggest that deletion of the Mas receptor decreased NO bioavailability, which may be a reason for Mas-deficiency-accelerated endothelial dysfunction and atherosclerosis. To confirm that the improvement of vascular function is caused by an increase in Ang-(1-7)-induced vascular NO bioavailability, we measured aortic cGMP levels, a marker for NO generation, in apoE-KO mice treated with Ang-(1-7). Indeed, Ang-(1-7) significantly enhanced aortic cGMP / Creatinine ratio in Ang-(1-7)-treated apoE-KO mice (Figure 15, P ＜ 0.05).

Figure 15

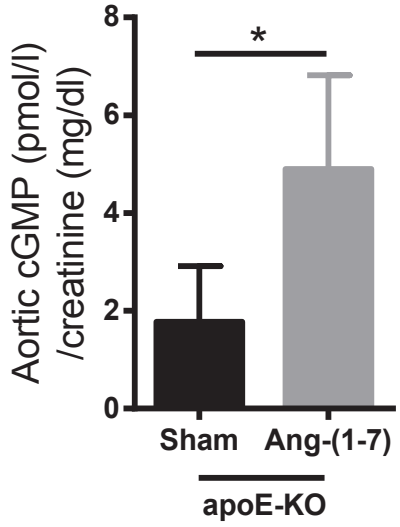

Figure 15 Chronic infusion of Ang-(1-7) increased aortic cGMP / creatinine levels in apoE-KO mice. (apoE-KO vs apoE-KO + Ang-(1-7), 1.775 ± 0.5706 (pmol/l)/(mg/dl) n=8 vs. 4.900 ± 0.9600 (pmol/l)/(mg/dl) n=8, *P < 0.05).

5.1.3 Deletion of the Mas receptor increases macrophage infiltration and polarization

Nitrotyrosine, as a product of tyrosine nitration mediated by RNS, is a known indicator of cell damage, inflammation, and NO production. The previous experiment indicated increased nitrotyrosine levels in apoE-KO mice missing their Mas receptor, and atherosclerosis is considered as a chronic inflammation process related to macrophage infiltration. In order to examine whether Mas receptor deficiency

aggravates atherosclerosis through an inflammation-related mechanism, we measured macrophage and T-cell infiltration of the aortic root from WD-fed mice (figure 16 A and B). The analysis showed that deletion of the Mas receptor increased macrophage and T-cell infiltration in the cardiac valve (F4/80 staining, $P < 0.05$; CD3 staining, $P < 0.05$).

Figure 16A

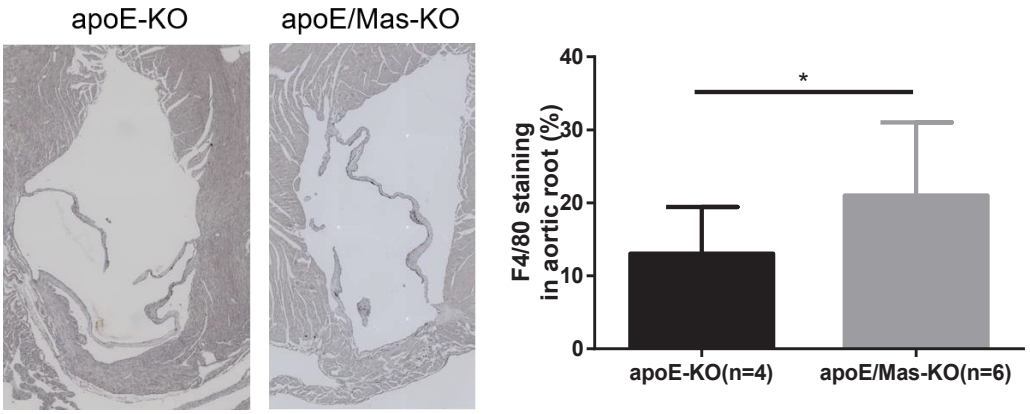

Figure 16B

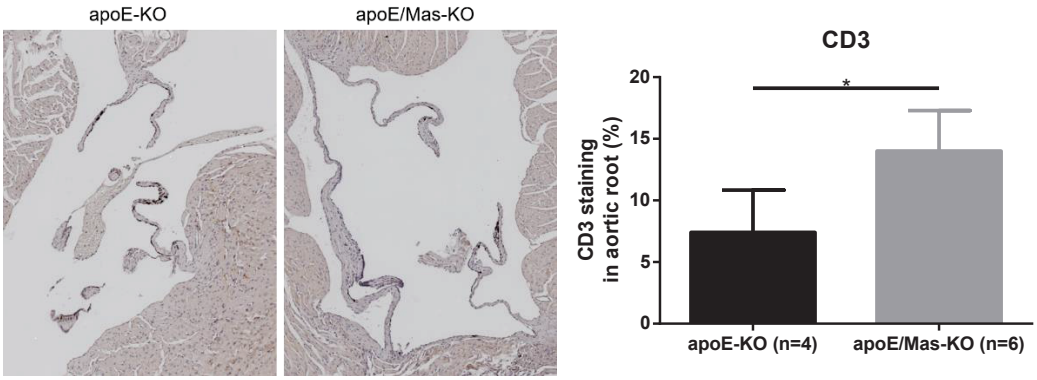

Figure 16 (A) Representative pictures of the aortic valve, which was stained with F4/80. Deletion of the Mas receptor increased macrophage infiltration in the aortic valve. (F4/80 staining, apoE-KO vs apoE/Mas-KO, 13.05 ± 3.197 % n=4 vs. 20.99 ± 4.097 % n=6, $P < 0.05$) (B) Representative pictures of the aortic valve, which was stained by CD3. Deletion of the Mas receptor increased T cell infiltration in the aortic valve. (CD3 staining, apoE-KO vs apoE/Mas-KO, 7.407 ± 1.715 n=4 vs. 14.01 ± 1.340 n=6, $P < 0.05$). *$P<0.05$.

Since Mas deficiency significantly increased atherosclerotic plaques in the aortic arch region of apoE-KO mice, and also increased Macrophage and T cell infiltration in aortic roots of apoE-KO mice, this suggests an important role for the Mas receptor in

the macrophage function of apoE-KO mice. To further determine the changes of gene expression levels from different macrophage subtypes between groups, aortas were isolated and measured by RT-PCR. Deletion of the Mas receptor significantly increased inducible nitric oxide synthase (iNOS), MCP-1, IL-6, IL12p40 and Fizz1 gene expression levels (figure 17). In more detail, Mas deficiency was associated with more pro-inflammatory macrophage cytokine expression, such as iNOS, MCP-1, IL-6 and IL12p40, and less anti-inflammatory macrophage cytokine expression, such as Fizz1, in the atherosclerotic aortas of apoE-KO mice. These results suggest that Mas deficiency accelerates atherosclerosis and is highly associated with macrophage activation and polarization (increased M1/M2 ratio).

Figure 17

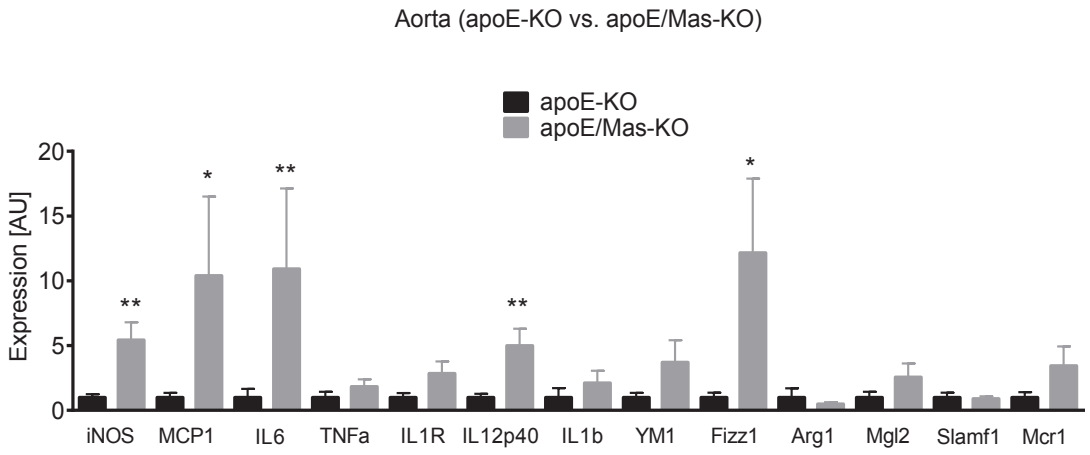

Figure 17 Normalized mRNA expression levels from the aorta. Mas deficiency activates pro-inflammatory genes (iNOS, MCP-1, IL-6, and IL12p40) expression in atherosclerotic aortas of apoE-KO mice. n=6, *P＜0.05, ** P＜0.01.

5.2.1 Deletion of the Mas receptor reduced survival proportions related to AAA formation

To evaluate the influence of the Mas receptor on Ang II-induced AAA formation in apoE-KO mice, survival rates and aorta shape were calculated. During chronic Ang II infusion, deletion of the Mas receptor significantly reduced survival rates (figure 18) in apoE/Mas-KO mice compared with apoE-KO mice.

Figure 18

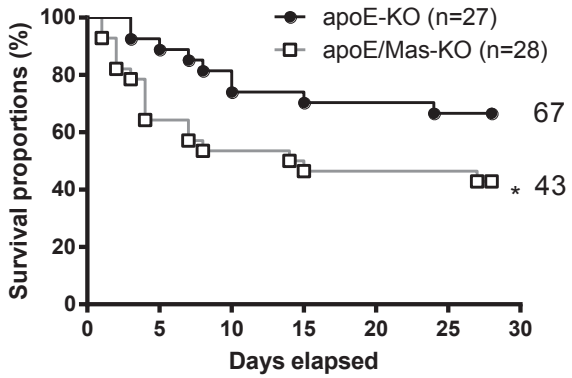

Figure 18 Ang II infusion accelerated mortality in apoE/Mas-KO mice compare with apoE-KO mice. (apoE/Mas-KO 43% survived from 28 mice vs. apoE-KO 67% survived from 27 mice; $P<0.05$) *$P<0.05$.

In order to define the reason of death, the mice that had died were dissected and examined. Autopsy surgery showed that the main causes of death were aneurysm rupture and aortic dissection (figure 19). In apoE-KO mice, Ang II-induced aortic rupture in the suprarenal aorta (50%) and the aortic arch (33.33%). Aortic dissection was the cause of death in only 8.33% of cases. In apoE/Mas-KO mice, Ang II caused 52.63% of deaths due to AAA rupture, 21.05% of deaths due to aortic arch rupture, 10.53% of deaths due to aortic dissection, and 5.26% of deaths due to thoracic aortic aneurysm (TAA) rupture.

Figure 19

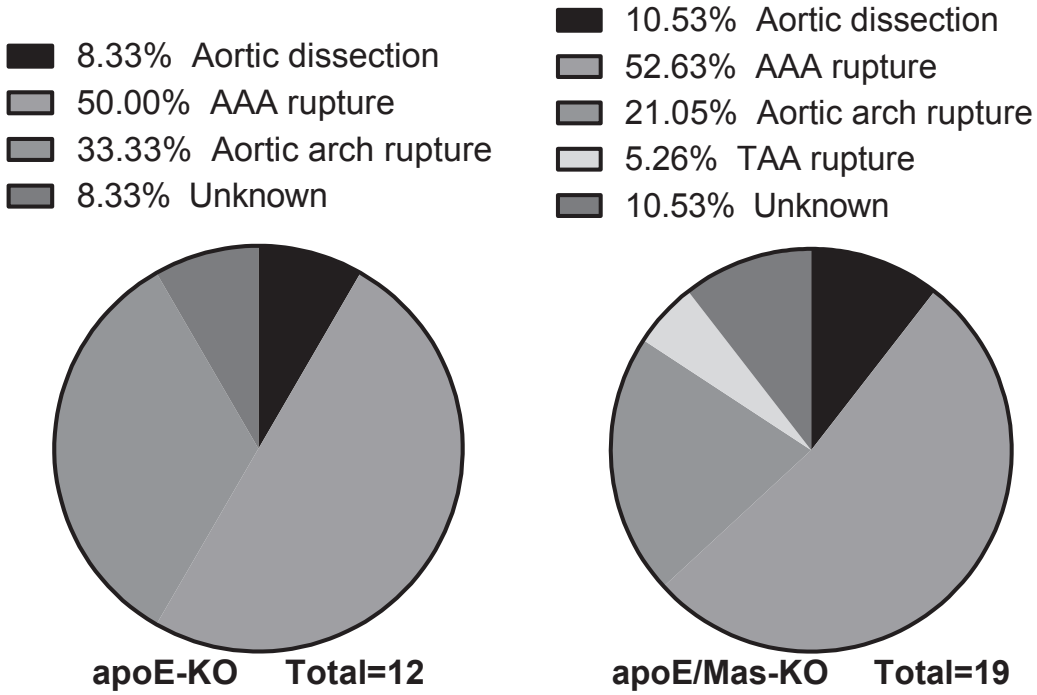

Figure 19 Cause of death in apoE-KO and apoE/Mas-KO mice.

To further analyze changes in aortic shape and the incidence of AAA *in vivo*, suprarenal aortas, and aortic arches were detected by ultrasound before termination. Ang II significantly increased maximal abdominal aortic diameters (figure 20) in Mas-deficient mice (P＜0.01)

Figure 20

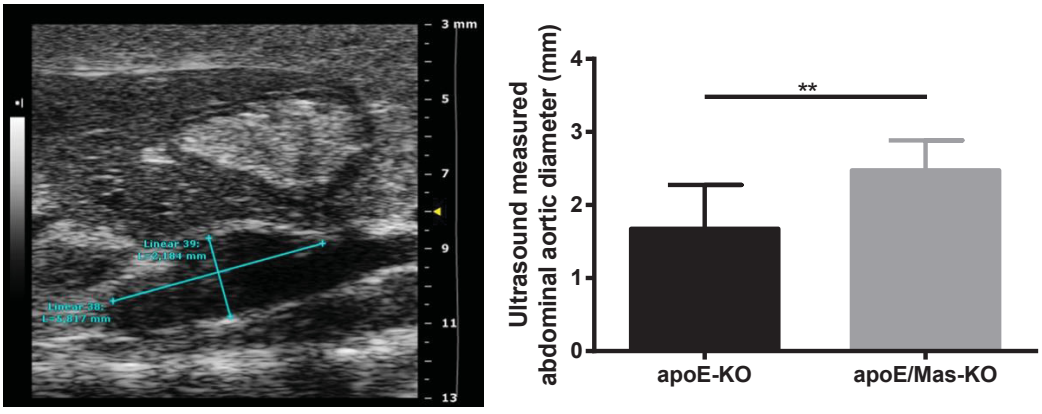

Figure 20 Representative AAA picture detected by ultrasound. Deletion of the Mas receptor increased abdominal aortic diameters in Ang II-treated apoE-KO mice measured by ultrasound. (apoE-KO vs apoE/Mas-KO; 1.674 ± 0.1504 mm, n=16 vs. 2.475 ± 0.1365 mm, n=9; p＜0.01), ** P＜0.01.

To confirm these results, maximal abdominal aortic lumen area was calculated. Ang II significantly increased maximal AAA lumen area (figure 21) in apoE/Mas-KO mice compared with apoE-KO mice (P＜0.005).

Figure 21

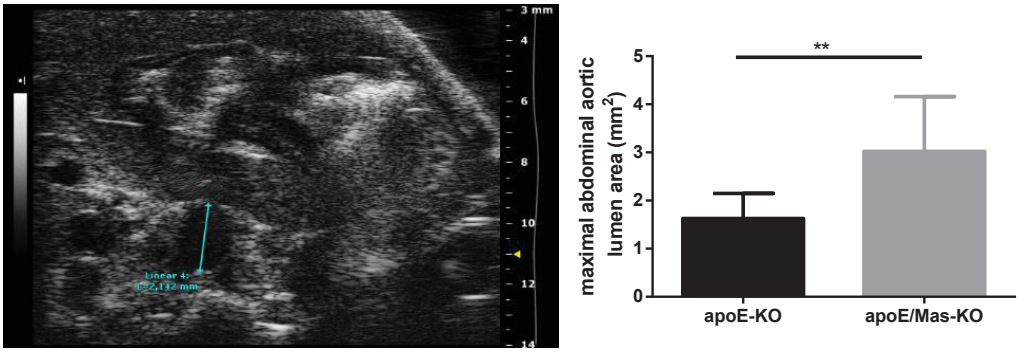

Figure 21 Representative abdominal aortic lumen area picture detected by ultrasound. Deletion of Mas receptor increased abdominal aortic lumen area in Ang II-treated apoE-KO mice measured by ultrasound. (apoE-KO vs apoE/Mas-KO; 1.627 ± 0.1566 mm^2, n=11 vs. 3.022 ± 0.4644 mm, n=6; P＜0.01), ** P＜0.01.

In contrast, Ang II infusion did not influence the maximal diameter of aortic arches (figure 22) between both groups (P=0.9036). This result suggests that deletion of the Mas receptor accelerates Ang II-induced aortic expansion only in the abdominal aorta.

Figure 22

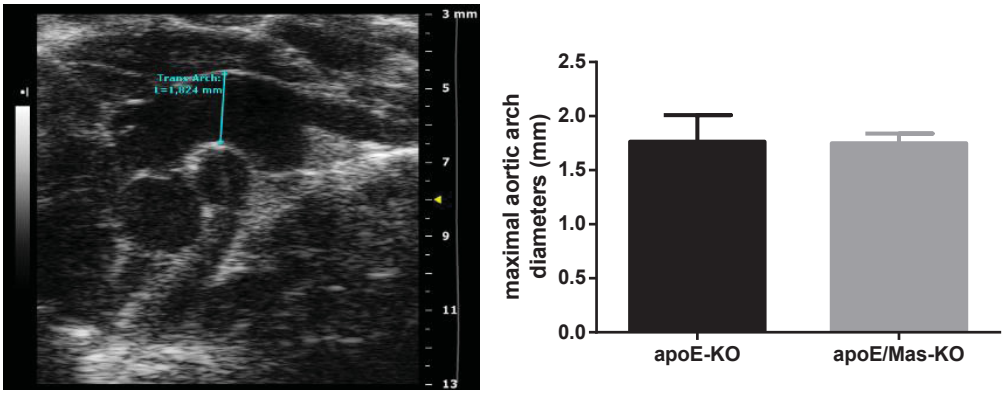

Figure 22 Representative aortic arch detected by ultrasound. Deletion of the Mas receptor did not influence maximal diameter of the aortic arch in Ang II-treated apoE-KO mice measured by ultrasound. (apoE-KO vs apoE/Mas-KO; 1.763 ± 0.09321 mm, n=7 vs. 1.75 ± 0.03625 mm, n=6; P=0.9036).

In order to further confirm our findings, maximal thoracic aortic diameters and abdominal aortic diameters of the survived mice were measured. As shown in figure 23, AAA can be observed and measured easily *in vitro*.

Figure 23

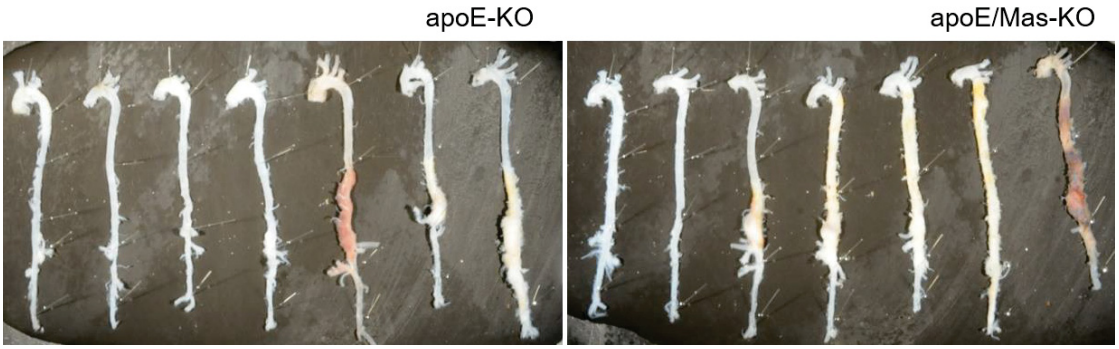

Figure 23 Representative aortas from survived mice.

Deletion of the Mas receptor increased AAA incidence (figure 24) in apoE-KO mice (50% vs. 74%).

Figure 24

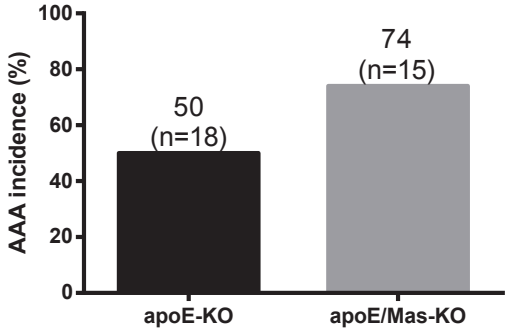

Figure 24 Mas deficiency increased AAA incidence in survived mice.

Ang II markedly increased maximal external abdominal aortic diameters (figure 25A) in apoE/Mas-KO mice, compared with apoE-KO mice ($P<0.05$). Similar to the *in vivo* data, Mas deficiency did not influence thoracic aortic diameters.

Figure 25A Figure 25B

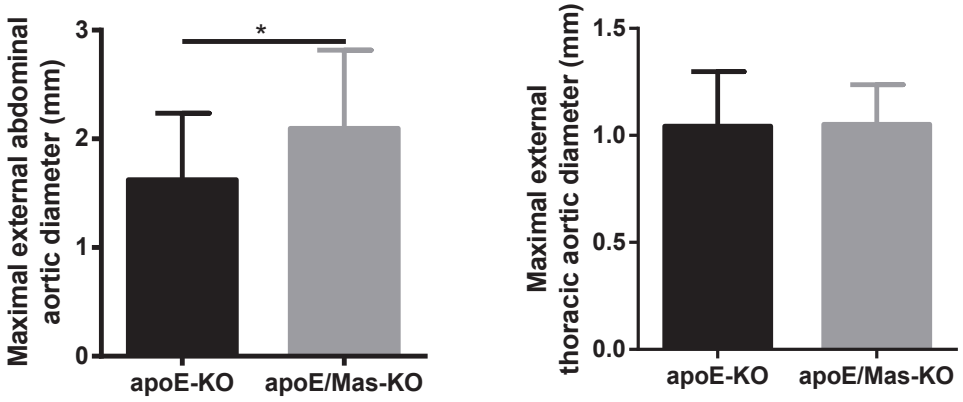

Figure 25 (A and B) Deletion of the Mas receptor increased maximal external abdominal aortic diameters (apoE-KO vs apoE/Mas-KO; 1.624 ± 0.1446 mm, n=18 vs. 2.228 ± 0.2107 mm, n=12; P＜0.05) in Ang II-treated apoE-KO mice, but did not influence thoracic aortic diameters (apoE-KO vs apoE/Mas-KO; 1.043 ± 0.06373 mm, n=16 vs. 1.051 ± 0.04959 mm, n=14). *P＜0.05.

5.2.2 Deletion of the Mas receptor accelerated IMT and the collagen content

The progression of AAA is associated with adverse functional and structural changes to the arteries. To test whether the Mas receptor affects vascular remodeling, IMT and collagen content were stained by Sirius Red (figure 26A). In Ang II-treated apoE-KO mice, deletion of the Mas receptor significantly increased IMT of the abdominal aorta (P＜0.05) (figure 26B), suggesting that deletion of the Mas receptor aggravated Ang II-induced VSMC proliferation.

Figure 26A

Figure 25B

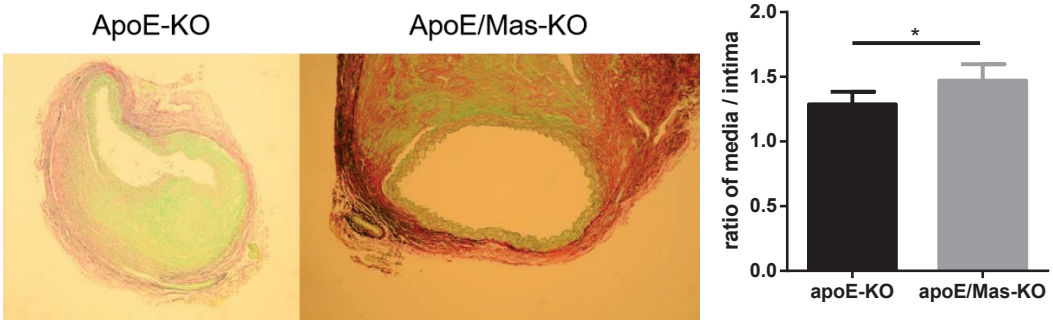

Figure 26 (A) Representative pictures of AAA section slides stained by Sirius Red. (B) Mas deficiency accelerated Ang II-induced intima-media thickness in AAA. (apoE-KO vs apoE/Mas-KO; 1.288 ± 0.03621, n=7 vs. 1.472 ± 0.05109 n=6) *P＜0.05.

To measure the component changes in the suprarenal aorta, we measured collagen content. Both collagen proportion (P＜0.05) and content (P＜0.05) from abdominal aorta were markedly increased in Ang II-treated apoE/Mas-KO mice, compared with apoE-KO mice (figure 27 A and B), suggesting that deletion of Mas receptor increased collagen content in AAA location.

Figure 27A

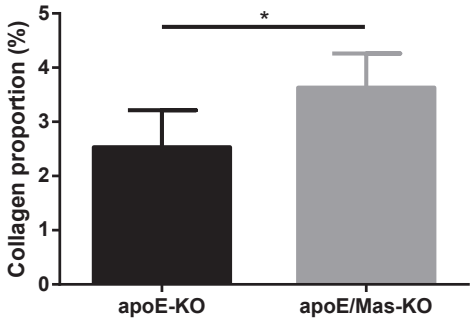

Figure 27B

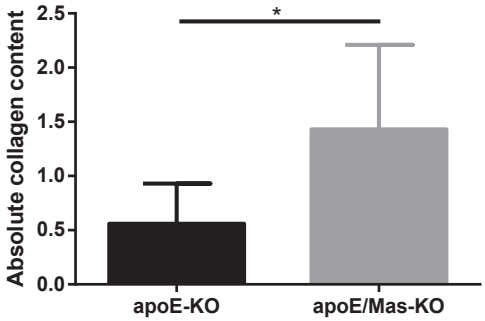

Figure 27 (A and B) Mas deficiency increases collagen proportion (apoE-KO vs apoE/Mas-KO %; 2.531 ± 0.2785, n=6 vs. 3.631 ± 0.2385, n=7; P＜0.05) and absolute content (apoE-KO vs apoE/Mas-KO; 0.5599 ± 0.1510, n=6 vs. 1.431 ± 0.2943, n=7; P ＜0.05) in suprarenal aorta. *P＜0.05.

5.2.3 Deletion of the Mas receptor accelerated elastin fragmentation

Concomitant with abdominal aortic expansion were pathological changes in the media of suprarenal aortas after 4 weeks of Ang II infusion. To test whether the Mas receptor affects elastic fibers, samples were stained by Movat' stain. Elastin fragmentation was increased greatly in apoE/Mas-KO mice compared with apoE-KO mice (figure 28, P＜0.05).

Figure 28A

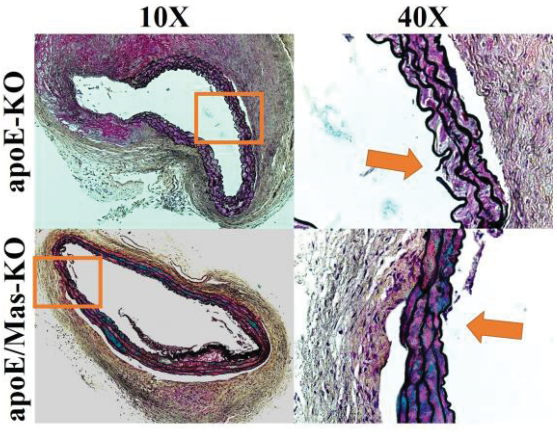

Figure 28B

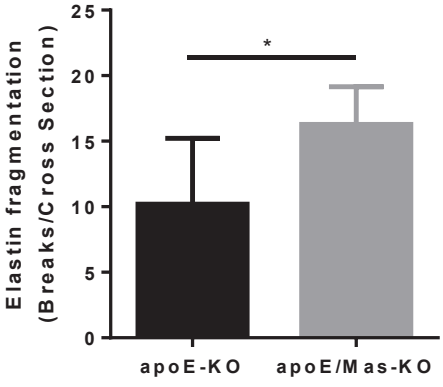

Figure 28 Suprarenal aortic sections from Ang II-infused apoE/Mas-KO mice have increased elastic fibers breaks. (A) Representative suprarenal aortic section of apoE-KO or apoE/Mas-KO mice infused with Ang II. Elastin fragmentation was examined by Movat. (B) Elastin fragmentation counted in cross sections. (apoE-KO vs apoE/Mas-KO; 10.20 ± 2.245 n=5 vs. 16.29 ± 1.085 n=7) *P＜0.05. Black, nuclei and elastic fibers; yellow, collagen and reticular fibers; blue, ground substance and mucin; intense red, fibrinoid and fibrin; red, muscle.

5.2.4 Deletion of the Mas receptor accelerates AAA formation independent of blood pressure

To examine whether differences in blood pressure are responsible for an accelerated AAA formation in apoE/Mas-KO mice, blood pressure was measured by non-invasive tail-cuff and by invasive radio telemetry. Interestingly, tail-cuff measured SBP did not differ during chronic infusions of Ang II in apoE-KO mice and apoE/Mas-KO mice (Figure 29A). To further support this observation, we measured SBP by radio telemetry detector. SBP did not show any significant difference between two groups in 4 weeks (figure 29B). Both results suggest that Mas deficiency accelerated AAA formation independent of Ang II-induced blood pressure elevation.

Figure 29A

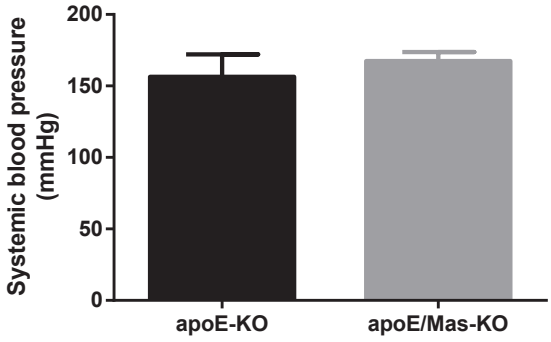

Figure 29B

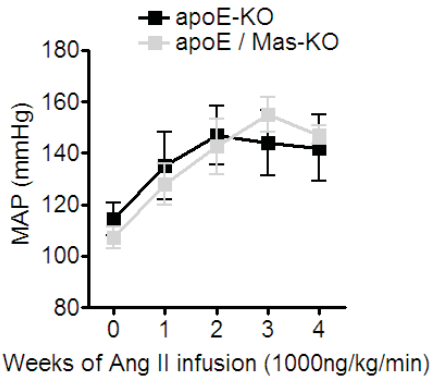

Figure 29 (A) Tail-cuff measurement of systolic blood pressure. (apoE-KO vs. apoE/Mas-KO, 156.4 ± 4.985 mmHg n=10 vs. 167.5 ± 3.126 mmHg n=4) (B) Radio telemetry measurement of mean arterial pressure during 4 weeks of Ang II treatment (n=5 per group).

In addition, Ang II infusion did not show any differences in cardiac hypertrophy of apoE and apoE/Mas-KO mice. Heart/tibia ratios and heart/body ratios were similar between apoE-KO mice and apoE/Mas-KO mice (Figure 30 A and B). Taken together, the results suggest that the accelerated AAA formation and vascular remodeling in apoE/Mas-KO mice is blood pressure independent.

Figure 30A

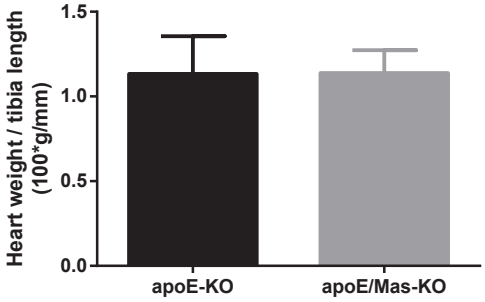

Figure 30B

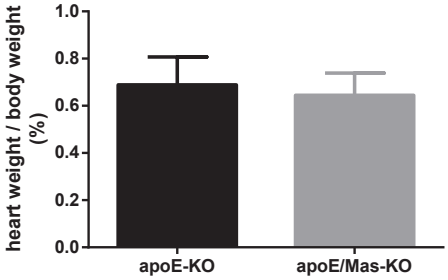

Figure 30 (A) Heart weight/tibia length. (apoE-KO vs. apoE/Mas-KO, 1.134 ± 0.05563 100*g/mm n=16 vs. 1.138 ± 0.04086 100*g/mm n=10) (B) Heart weight/body weight. (apoE-KO vs. apoE/Mas-KO, 0.6887 ± 0.03049 n=15 % vs. 0.6444 ± 0.02723 % n=12)

5.2.5 Deletion of the Mas receptor increases plasma HDL levels

To identify the influence of the Mas receptor on plasma, we isolated plasma and measured cholesterol, triglyceride, HDL, and LDL (figure 31). Deletion of the Mas receptor significantly increased HDL levels in apoE-KO mice ($P < 0.05$). Deletion of the Mas receptor does not cause significant differences in cholesterol, triglyceride or LDL levels.

Figure 31

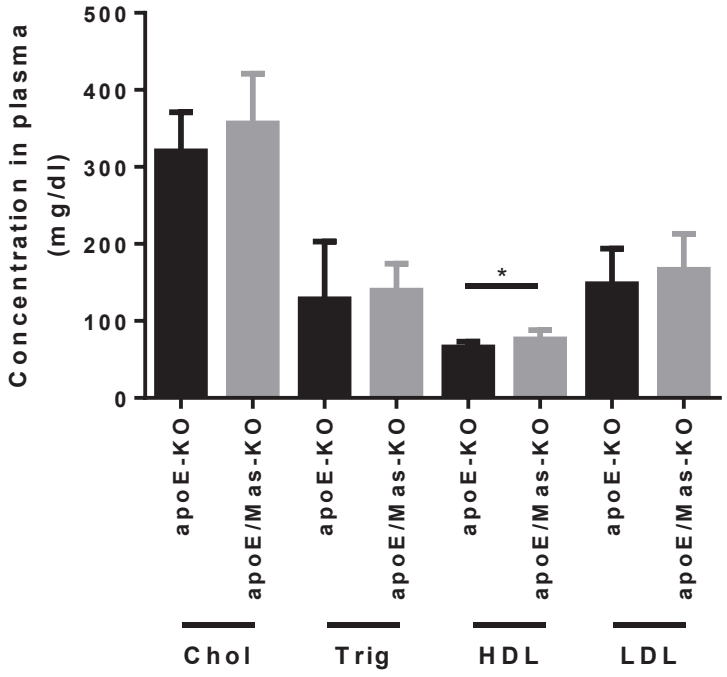

Figure 31 Deletion of the Mas receptor significantly increases plasma HDL levels in apoE-KO mice (apoE-KO, n=9 vs. apoE/Mas-KO, n=10, mg/dl; HDL 65.56 ± 2.550 mg/dl, n=9 vs. 76.10 ± 3.758; cholesterol 320.0 ± 16.96, vs. 356.3 ± 20.44; triglyceride 128.0 ± 25.11 vs. 139.2 ± 11.06; LDL 147.9 ± 15.31 vs. 166.3 ± 14.87) Chol, Cholesterol; Trig, Triglyceride; HDL, High-density lipoprotein; LDL, Low-density lipoprotein. *P＜0.05.

5.2.6 Deletion of the Mas receptor increases macrophage infiltration and inflammation

To investigate the exact course of AAA formation and the possible involvement of immune cells, aortic lumen shape and macrophage infiltration were measured by MRI at days 0, 7, 14 and 28, ^{1}H MR angiography clearly revealed that Ang II increased lumen diameter of the abdominal aorta of both apoE-KO and apoE/Mas-KO mice within 28 days (figure 32 A and B). However, this effect was more pronounced in apoE/Mas-KO mice compared to apoE-KO mice (figure 32C). The increase in aortic volume was also associated with a more frequent occurrence of AAA in apoE/Mas-KO mice (~ 72%) compared to apoE-KO mice (~ 40%) and could already be observed by ^{1}H MR angiography on day 7 of the Ang-II treatment.

Figure 32A

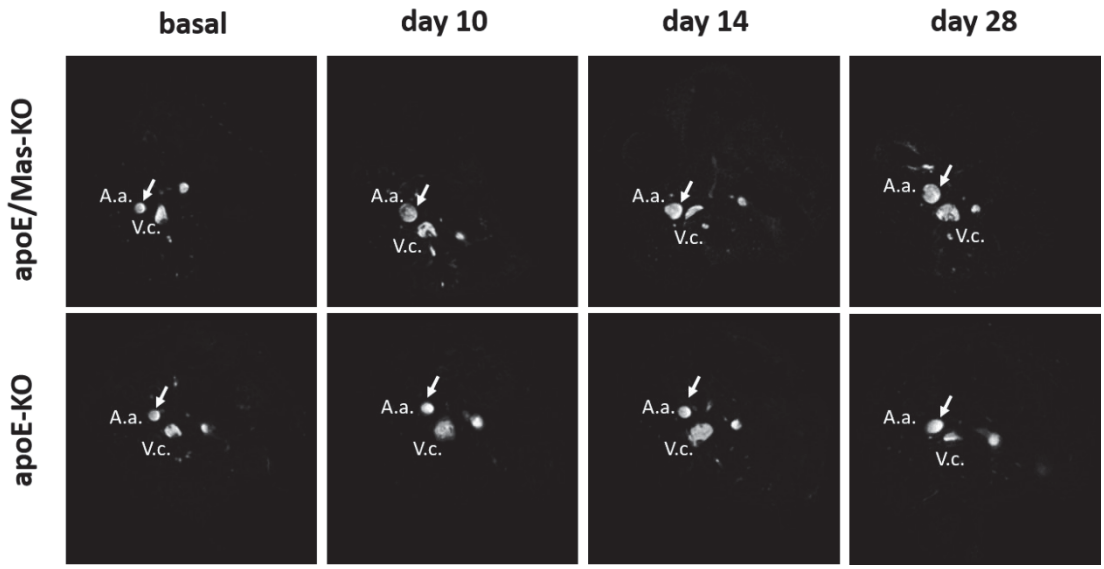

Figure 32B

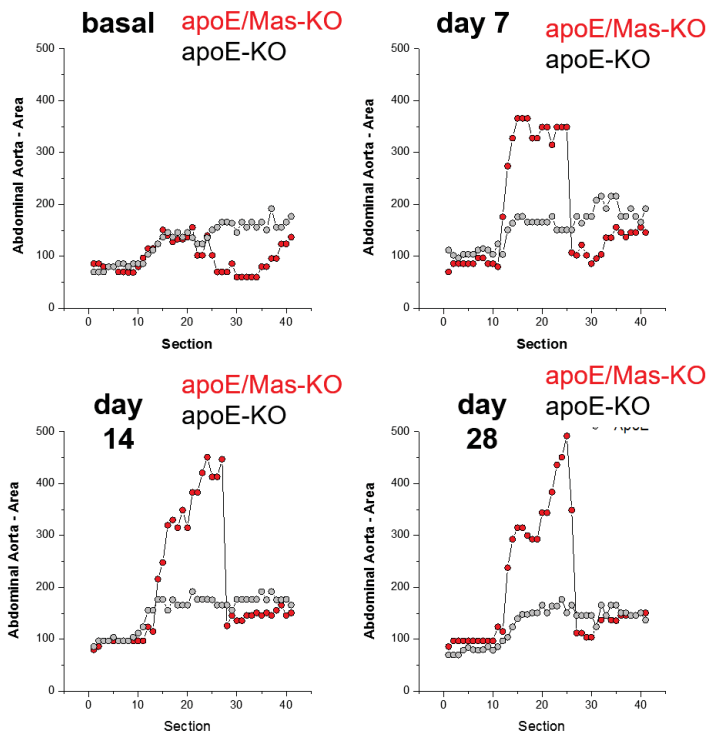
basal
apoE/Mas-KO
apoE-KO
day 7
apoE/Mas-KO
apoE-KO
day 14
apoE/Mas-KO
apoE-KO
day 28
apoE/Mas-KO
apoE-KO
Abdominal Aorta - Area
Section

Figure 32C

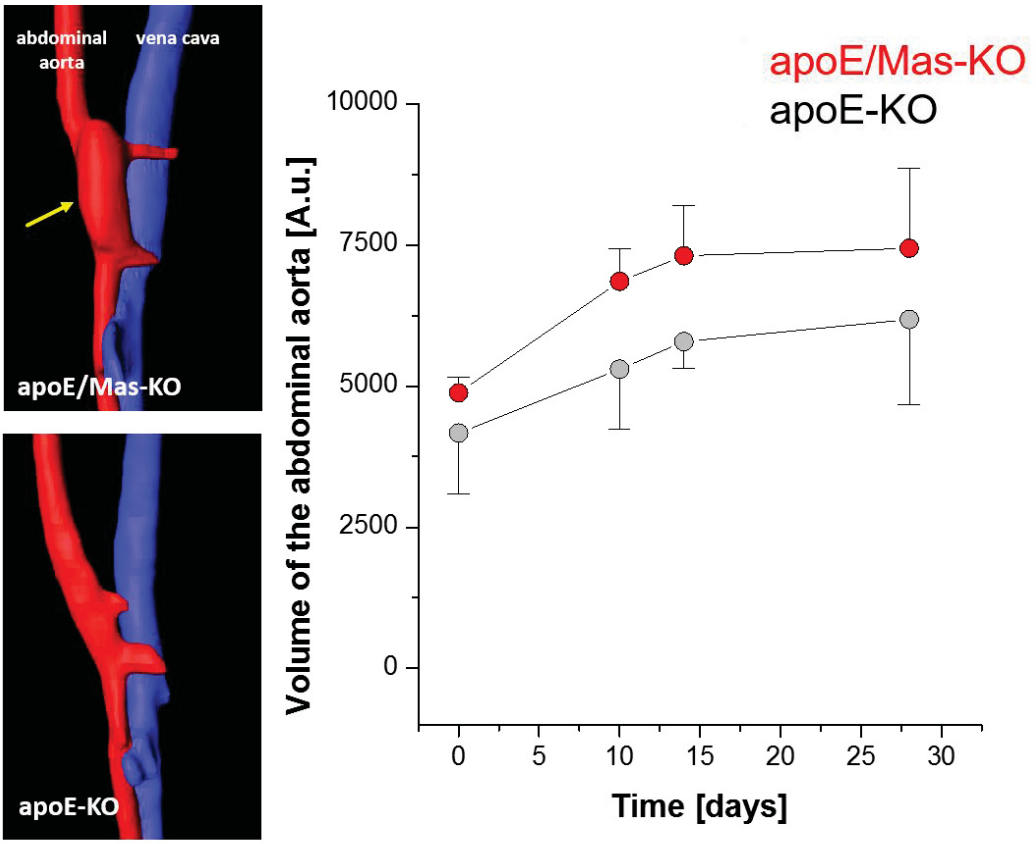
abdominal aorta
vena cava
apoE/Mas-KO
apoE-KO
apoE/Mas-KO
apoE-KO
Volume of the abdominal aorta [A.u.]
Time [days]

Figure 32 (A) ^{1}H MRI abdominal images. (B) Ang II-induced AAA occurred during the first 7 days. (C) Deletion of the Mas receptor increased AAA size in apoE-KO mice treated with Ang II.

To analyze the time point of immune cell infiltration of the abdominal aorta, we injected PFCs on day 4 after Ang-II treatment and subjected mice to ^{19}F MRI for 72 hours for efficient accumulation of PFC-loaded cells in the inflammatory foci. After only 72 hours strong ^{19}F signals surrounding the abdominal aorta in apoE/Mas-KO mice were visible, however only in those areas with abdominal aortic aneurysms (figure 33).

Figure 33

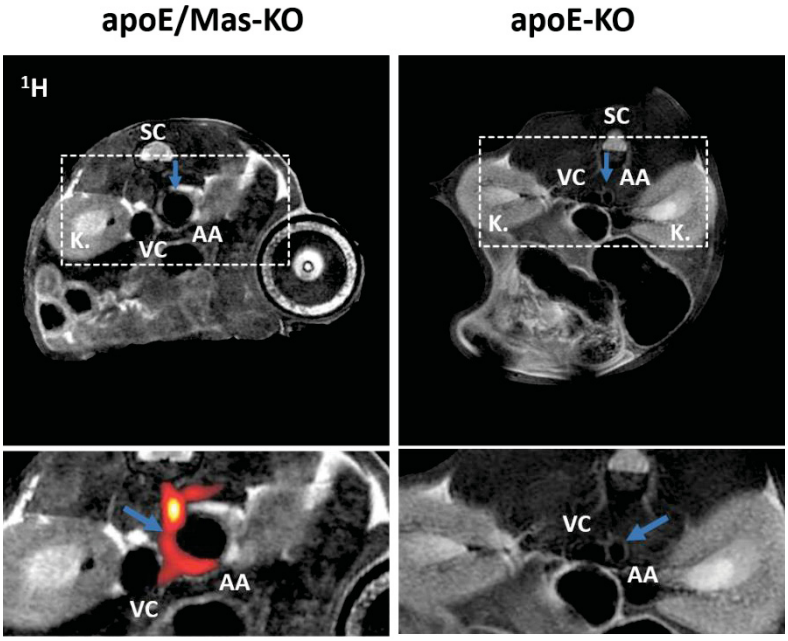

Figure 33 $^{1}H/^{19}F$ MRI measured abdominal images.

The presence of 19F signals - which indicate macrophage infiltration into the injured vessel wall - was corroborated by high-resolution *ex vivo* $^{1}H/^{19}F$ MRI of isolated and fixed abdominal aortae (figure 34), showing that aortic expansion is related to macrophage infiltration.

Figure 34

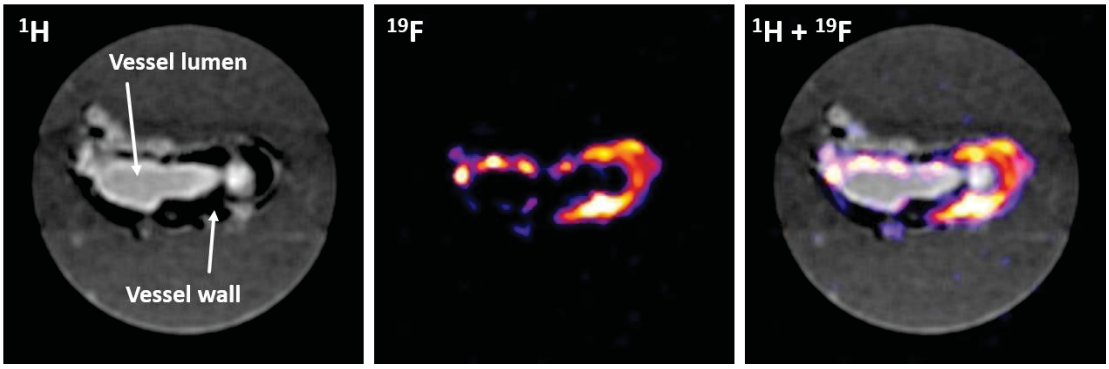

Figure 34 Representative pictures of ^{1}H/^{19}F MRI measurement from the isolated aorta.

Finally, histological analysis confirmed the presence of large amounts of macrophages within this area. To evaluate the difference of macrophage lesion area between two strains, we performed additional experiments with immunohistochemistry by F4/80 (figure 35). Deletion of the Mas receptor significantly increased Ang II-induced macrophage lesion area of AAA in the cross section ($P<0.05$).

Figure 35

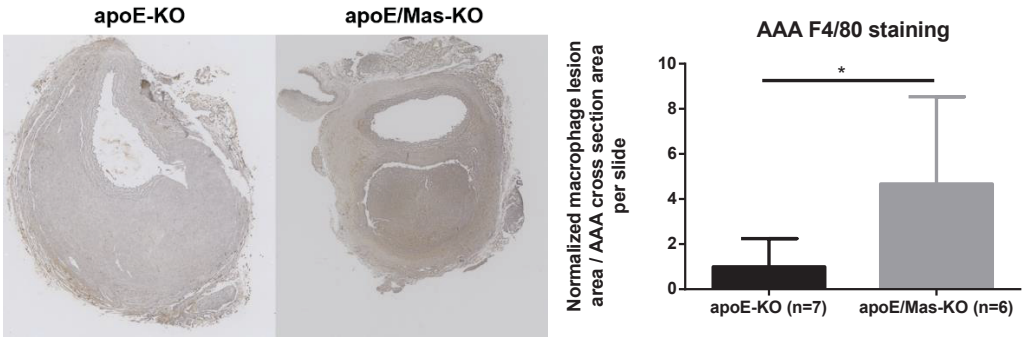

Figure 35 Representative photos of AAA stained by F4/80 antibody. Deletion of the Mas receptor increased macrophage infiltration of apoE-KO mice with Ang II-induced AAA. n=6-7 per group, *$P<0.05$.

In addition, the difference in T cell infiltration was measured between two strains, we performed experiments with immunohistochemistry by CD3 (figure 36). Deletion of the Mas receptor increased Ang II-induced T cells infiltration in AAA cross section ($P<0.05$).

Figure 36

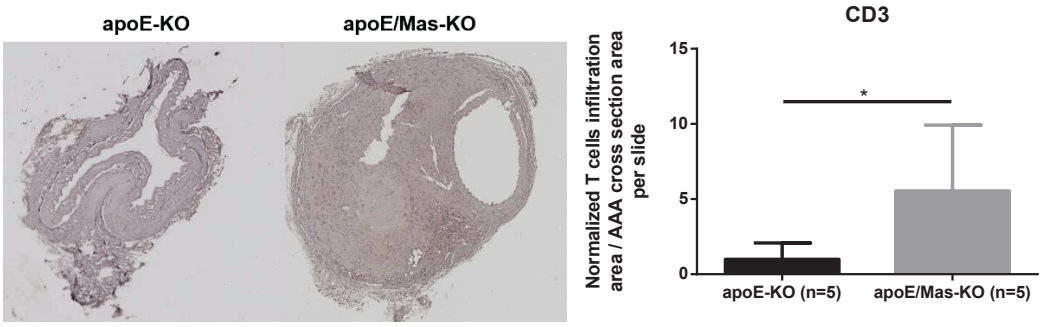

Figure 36 Representative photos of AAA stained by the CD3 antibody. Deletion of the Mas receptor increased T cell infiltration of apoE-KO mice with Ang II-induced. n=5 per group, *P＜0.05.

5.2.7 Deletion of the Mas receptor aggravates AngII-induced AAA formation in aged C57BL/6 mice

Previous results suggested that deletion of Mas receptor aggravates Ang II-induced AAA formation in apoE-KO mice. However, this process was accelerated by the apoE gene. To better understand whether a single Mas gene plays a key role in Ang II-induced AAA formation, we generated Mas-KO mice from WT mice. 8-month-old mice were infused with Ang II for 4 weeks.

To evaluate the influence of the Mas receptor on Ang II-induced AAA formation in aged WT mice, survival rates were calculated. During the chronic infusion of Ang II, deletion of the Mas receptor did not significantly reduce survival rates (figure 37) in WT mice (1 dead in WT mice, n=14 vs. 2 dead in Mas-KO mice, n=9). In order to define the cause of death during this process, the mice that had died were dissected and examined. Autopsy surgery showed that the main causes of death in this group of mice were aneurysm rupture and aortic dissection in suprarenal aorta. In WT mice, Ang II-induced AAA rupture caused the death of one mouse. In Mas-KO mice, Ang II caused the death of one mouse by AAA rupture and one further mouse by aortic dissection. However, due to the small sample size, it is difficult to be certain that Mas deficiency was the cause of the increase in mortality. The results weakly suggest that Ang II increases mortality via aortic dissection and AAA rupture.

Figure 37

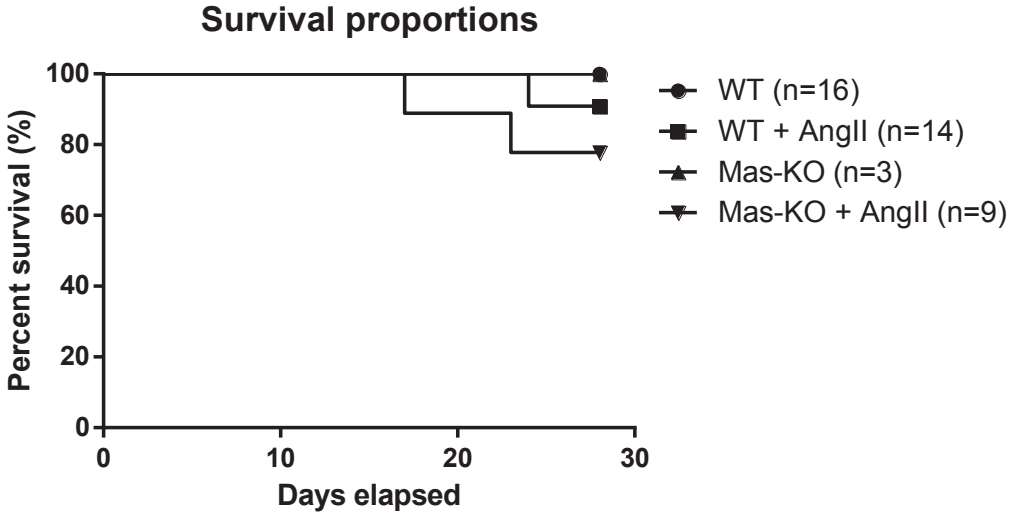

Figure 37 Survival proportions of 4 groups. Ang II-induced the deaths of 1 WT and 2 Mas-KO mice.

To further observe the change of aortic shape and formation of AAA in Ang II-treated aged mice, maximal thoracic and abdominal aortic diameters were measured in

survived mice. Ang II markedly increased maximal external abdominal aortic diameters (figure 38A) in WT mice, compared with WT mice treated with saline (P＜0.01). Ang II also markedly increased maximal external abdominal aortic diameters in Mas-KO mice, compared with Mas-KO mice treated by saline (P＜0.05). And Mas deficiency significantly accelerated Ang II-induced AAA formation (P＜0.05). While, in thoracic aorta analysis, deletion of the Mas receptor tended to increase thoracic aortic diameters in Ang II-treated C57BL/6 mice (figure 38B) (P=0.13).

Figure 38A

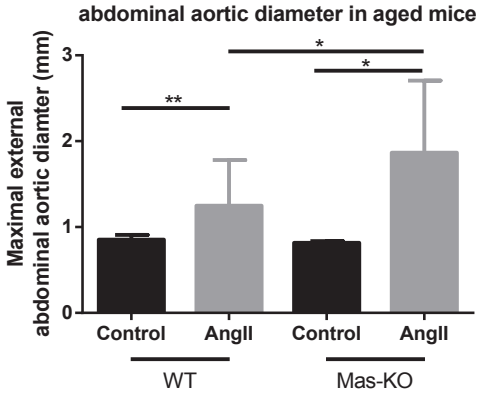

Figure 38B

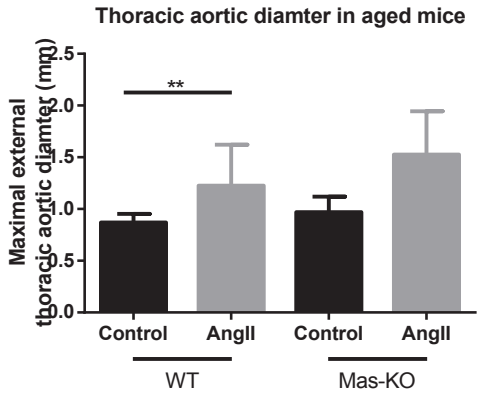

Figure 38 (A and B) Deletion of the Mas receptor accelerates Ang II-induced abdominal and thoracic aorta expansion. (abdominal aorta, WT vs. WT+Ang II, 0.8525 ± 0.01392 mm, n=16 vs. 1.249 ± 0.1473 mm, n=13 P＜0.01; Mas-KO vs. Mas-KO+Ang II, 0.8167 ± 0.01202 mm, n=3 vs. 1.867 ± 0.3167 mm, n=7 P＜0.05) (thoracic aorta, WT+Ang II vs. Mas-KO+Ang II, 1.225 ± 0.1101 mm, n=13 vs. 1.425 ± 0.1585 mm, n=7, p=0.13). *P＜0.05, **P＜0.01.

To further understand the function of the Mas receptor in AAA formation, AAA incidences were calculated. Deletion of the Mas receptor increased Ang II-induced AAA formation (figure 39) in apoE-KO mice compared to WT mice (WT vs. Mas-KO, 23% vs. 57%).

Figure 39

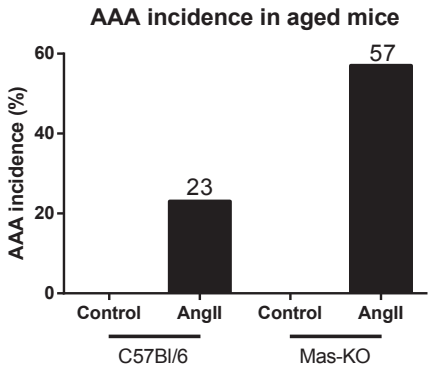

Figure 39 Deletion of the Mas receptor accelerates Ang II-induced AAA formation.

6. Discussion

6.1 Ang-(1-7) improved atherosclerosis and endothelial dysfunction through Mas receptor

Mas receptor is important in the regulation of cardiovascular diseases. Several studies have shown that treatment of Ang-(1-7) or its non-peptide mimetics improves vascular dysfunction. In addition, deletion of the Mas receptor reversed the beneficial effects of Ang-(1-7), including increased NO bioavailability and decreased oxidative stress, in apoE-KO mice[10, 190]. However, the specific function of the Mas receptor in the development of atherosclerosis and vascular function is unknown.

6.1.1 Ang-(1-7) and Mas receptor

The present study is the first of studies showing that Ang-(1-7) mediated Mas receptor activation improves vascular dysfunction and reduces the development of atherosclerosis in hypercholesterolemic apoE-KO mice. As any beneficial effects of Ang-(1-7) in apoE/Mas-KO mice could not be observed, we believe that Ang-(1-7) improves vascular function and attenuates the development of atherosclerosis solely via Mas receptor activation leading to increased NO bioavailability and reduced vascular inflammation. In addition, these results highlight the importance of the alternative axis of the RAS with its components ACE2 / Ang-(1-7) and the Mas receptor in mediating protective effects on vascular injury. However, these results are in contrast with observations showing that Ang-(1-7) mediates its beneficial effects in part by AT2 receptor activation [192, 193]. As apoE/Mas-KO mice still express AT2 receptors but do not show any improvement in vascular function during chronic Ang-(1-7) infusion, we do not believe that unselective Ang-(1-7) mediated Mas receptor activation plays a substantial role in this study.

6.1.2 Ang-(1-7)/Mas axis and vascular function

In order to investigate the underlying mechanism, vascular function was measured *in vivo* by FMD, the most widely used and validated prognostic marker in humans for NO dependent vascular dilation [194]. Impaired FMD is highly associated with atherosclerosis [195]. In the present study, Ang-(1-7) improved FMD in atherosclerotic apoE-KO but not apoE/Mas-KO mice suggesting that Ang-(1-7) improves vascular function by increasing the NO bioavailability. Increased NO-bioavailability leading to enhanced cGMP generation is a result of increased NO generation and a decreased NO degradation. And indeed, chronic Ang-(1-7) infusion increased cGMP levels in the aortas of apoE-KO mice. These findings are in line with several studies. Thus, we could clearly show that chronic Ang-(1-7) infusion increased NO dependent cGMP generation by increasing eNOS expression and reducing p47phox dependent ROS production [10, 190]. Mas receptor single knockout mice also have higher oxidative stress than WT

mice. Ang-(1-7) treatment significantly decreased oxidative stress in WT mice, and Ang-(1-7) had less inhibitory effects in Mas-KO mice [192]. Moreover, Mas receptor agonist AVE0991 also attenuated cardiac hypertrophy by reducing oxidative stress [196]. In consequence, Ang-(1-7)/Mas receptor activation improves vascular dysfunction via increasing NO bioavailability and decreasing oxidative stress.

6.1.3 Atherosclerosis and macrophages

Atherosclerosis is considered a chronic inflammatory process [197]. Besides vascular dysfunction, macrophage infiltration and polarization (increased M1/M2 ratio) have been shown to impact the development of atherosclerosis [198]. Here we could demonstrate that deletion of the Mas receptor not only impaired endothelial dysfunction in apoE-KO mice but also exaggerated the development of atherosclerotic plaques. Interestingly, the atherosclerotic plaques of apoE/Mas-KO mice were characterized by the increase of macrophage infiltration. Moreover, deletion of the Mas receptor drove macrophages into a proinflammatory M1 phenotype and increased the migration of macrophages in apoE-KO mice [181]. Here we could show that Mas receptor deficiency was associated with increased proinflammatory macrophage cytokine expression levels in apoE-KO mice suggesting again that deletion of the Mas receptor not only decreases vascular NO bioavailability but also drives macrophages into a proinflammatory phenotype leading to an aggravation of atherosclerosis.

In contrast, Ang-(1-7) or the selective Mas receptor agonist AVE 0991 reduces the expression of proinflammatory M1 macrophages and increases the expression of anti-inflammatory M2 macrophages [181]. Similar results were observed in a recent study of Skiba et al. in which the chronic administration of AVE0991 reduced atherosclerosis by affecting monocyte/macrophage differentiation and recruitment to the perivascular space during early stages of atherosclerosis in apoE-KO mice [81]. Interestingly, the pronounced effect of the Ang-(1-7)/Mas receptor axis on the macrophage function did not only affect atherosclerosis but also influenced autoimmunity. Also, deletion of the Mas receptor aggravates the course of diseases such as experimental autoimmune encephalomyelitis, a mouse model for multiple sclerosis [181], which suggests that Mas receptor function plays a pivotal role in macrophage-mediated inflammatory diseases.

Along with this direct effect on macrophage function and phenotype, Ang-(1-7) induced Mas receptor activation has been shown to reduce vascular inflammation and the development of atherosclerosis [199, 200]. For example, Ang-(1-7) attenuated Ang II increased iNOS, IL-1β, NADPH oxidase, and NF-κB levels in human VSMC [199]. Moreover, Yang et al. [200] showed that Ang-(1-7) administration attenuated atherosclerosis in apoE-KO mice on a high-fat diet. This observation was accompanied by a significant decrease of pro-inflammatory cytokines and MMPs, which has not been observed in mice treated together with A779, a selective Mas receptor antagonist. In addition, Ang-(1-7) decreased Ang II-induced vascular remodeling and inflammation [176]. This process was associated with decreased level of NFκB, TNF-alpha, MCP-1,

and IL8. Similarly, Ang-(1-7) significantly reduced diabetes-induced vascular hypertrophy and leukocyte recruitment, accompanied with decreased ICAM-1 and VCAM-1 levels suggesting that the beneficial effects of Ang-(1-7) are not dependent on the stimulus which leads to vascular injury [64].

6.1.4 Limitations

Although blood pressure in the apoE/Mas-KO mice were not measured, it seems very unlikely that the deletion of the Mas receptor affects blood pressure and therefore the development of atherosclerosis. This assumption is supported by the fact that chronic Ang-(1-7) administration does not affect blood pressure [10, 190]. Moreover, Mas receptor deficiency did not affect blood pressure on a C57BL/6 background[74].

6.1.5 Conclusions

In conclusion, Ang-(1-7) mediated Mas receptor activation improved vascular dysfunction and attenuated the development of atherosclerosis through several mechanisms. First, Ang-(1-7) increases NO bioavailability by increasing NO generation and reducing ROS production and thereby improving endothelial mediated vasodilation. Second, Ang-(1-7) mediated Mas receptor activation affects macrophage function and phenotype directly, which in turn modulates atherosclerosis. Third, these effects lead to reduced vascular inflammation resulting by decreased ROS production, macrophage infiltration and increased NO bioavailability. Taken together, these results support the activation of the Ang-(1-7)/Mas receptor axis in the prevention of atherosclerosis.

Figure 40

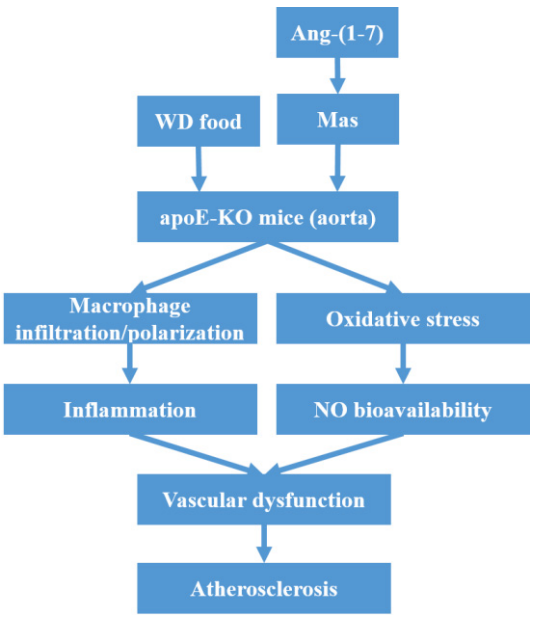

Figure 40 Diagram of the atherosclerosis study

6.2 Deletion of the Mas receptor increased Ang II-induced AAA formation

As described above, deletion of the Mas receptor significantly increased immune cell infiltration in the study of atherosclerosis. In addition, studies have shown that Ang II-induced AAA formation was accompanied by increased immune response [132]. However, it is unknown whether Mas deficiency increased proinflammatory response aggravates Ang II-induced vascular injury and thus influences AAA formation in apoE-KO mice.

In the present study, we show that deletion of the Mas receptor accelerates Ang II-induced AAA formation, including both AAA incidence and AAA size, in apoE-KO mice. The increased AAA formation leads to a higher risk of aortic rupture which leads to higher mortality in mice. In this study, the AAA formation process seems to be related to macrophage infiltration rather than blood pressure.

6.2.1 AAA and blood pressure

It is commonly agreed that blood pressure and blood flow play a key role in the formation of AAA [116]. In our study, there was no difference in blood pressure in Ang II treated apoE-KO and apoE/Mas-KO mice. Blood pressure was not only measured by the tail cuff method where small differences might be overseen. Blood pressure was also measured by radio telemetry, which is a much more sensitive method for detecting small blood pressure differences [201]. In addition to the similar blood pressure values, there were also no significant differences in heart weight, a marker for hypertensive organ damage, between both groups. These results further attest to the similar blood pressure values. In contrast to ACE2 deficient mice where the degradation of Ang II is reduced, infusion of Ang II induces an exaggeration of blood pressure in ACE2 knockout mice [27]. Mas receptor deficiency in a C57BL/6 background did not show any differences in blood pressure with or without Ang II infusion. Ang-(1-7) did not influence the blood pressure levels in apoE-KO mice [10, 106, 190]. Our findings are in accordance with these results, and show that Mas receptor deficiency accelerated AAA formation is blood pressure independent. In contrast, Mas receptor deficiency on a FVB background showed higher blood pressure values compared to control mice [29]. Moreover, different studies have observed effects of Ang-(1-7) infusion on blood pressure and renal blood flow in hypertensive rats [202-205]. These opposite results may be explained by different species, gender, background or mouse models [206-208].

6.2.2 AAA and endothelial dysfunction

The pathophysiology of AAA formation is dependent of vascular remodeling, inflammatory response, immune cell infiltration and vascular endothelial dysfunction

[209]. All these effects are seen in Mas deficient apoE-KO mice. As discussed above and previously shown, endothelial dysfunction was significantly impaired in apoE/Mas-KO compared to apoE-KO mice. And chronic Ang-(1-7) infusion improved endothelial dysfunction by increasing NO bioavailability and reducing ROS production in many different animal models [10, 29, 74, 103]. Therefore, it seems feasible that the impaired endothelial function of apoE/Mas-KO mice might be one important factor for the increased incidence of AAA compared to apoE-KO mice.

6.2.3 AAA and macrophages

In general, chronic Ang II infusion increases the accumulation of macrophages in the vasculature and induces macrophages to release more inflammatory cytokines, such as MCP-1, which increases the adhesion and invasion of more monocytes/macrophages into tissues. Therefore, it is not surprising that the deletion of ACE2 or the inhibition of the Mas receptor aggravate Ang II-induced tissue inflammation [210]. In the present study, we could show that deletion of the Mas receptor increased macrophage and T cells infiltration in the AAA region. Moreover, MRI and ultrasound measurements show that deletion of the Mas receptor increased aortic expansion and macrophage signaling. In addition, isolated AAA region shows a strong macrophage signaling. On the other hand, activation of ACE2/Ang-(1-7)/Mas axis reduced Ang II-induced tissue inflammation [5, 211, 212]. Consequently, we suggest that Mas receptor deficiency aggravates Ang II-induced inflammatory response through increasing M1/M2 ratio and the upregulation of proinflammatory cytokines leading to accelerated AAA formation. In more detail, many studies have shown evidence that the deletion or activation of Ang-(1-7)/Mas axis influences vascular function and for that reason may influence Ang II-induced AAA formation. Ang II induces vascular injury via mediating endothelial dysfunction, monocyte infiltration, inflammatory response, and vascular remodeling [131, 167, 213-215]. Ang-(1-7) inhibits Ang II-induced VSMC proliferation and migration through downregulation of ERK1/2 phosphorylation levels [216]. Ang-(1-7) reduces Ang II-induced VCAM-1 expression levels by decreasing NK-kappaB on vascular endothelium cells through the Mas receptor [213]. Ang-(1-7) reduces Ang II-induced MCP-1, VCAM-1, and ICAM-1 expression levels by downregulating NFκB and P38 MAPK in human umbilical vein endothelial cells (HUVEC) through the Mas receptor activation [105]. Ang-(1-7) also shows anti-inflammatory effects by inhibiting lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in Ang II treated HUVECs [202]. Our study serves as a proof-of-concept that the Mas receptor protects vascular structure through mediating inflammatory response during Ang II treatment in apoE-KO mice. On the other hand, the limitation of this study is that we did not investigate the macrophage subtype after Ang II infusion.

6.2.4 AAA and T cells

Generally, T cells communicate with macrophages during inflammatory response.

We have identified that deletion of the Mas receptor increased T lymphocyte infiltration in both atherosclerosis and AAA studies in apoE-KO mice, confirming that T cells play an important role in the vascular injury. It is well recognized that there is an increased infiltration of T lymphocytes into the aortic wall due to artery injury [163, 217-219]. It's not surprising that Ang-(1-7) downregulated T cells in atherosclerosis and Ang II-induced aneurysms studies [81, 207]. We believe that deletion of the Mas receptor causes an increase of T cell induced vascular inflammation, and in this way contributes to the pro-inflammatory environment, which may aggravate vascular injury through activating macrophages to polarize to the M1 phenotype. During Ang II infusion, T cells infiltrated into the aorta, kidneys, and brain, and elevated T cell-derived production of chemokines, like the chemokine C-C motif ligand 2 (CCL2) and ROS production in aorta and kidney, leading to further T cells infiltration [218]. With regard to our findings, this could be one reason why deletion of the Mas receptor increases M1/M2 ratio.

6.2.5 Mas receptor and IMT

Here, we observed that deletion of the Mas receptor increased IMT, which could explain why deletion of the Mas receptor increased endothelial dysfunction and AAA formation. Similar results were also observed by others[220]. Deletion of the Mas receptor increased intima/media ratio and intimal thickening, resulting in vascular dysfunction in C57BL/6 mice. This result is not due to cellular proliferation [220]. Here, we confirmed that deletion of the Mas receptor increases IMT during Ang II treatment. These results are in line with observations where Ang-(1-7) treatment reduces Ang II induced increase of VSMCs, which are the cellular components of the aortic media layer [57. Increased IMT often leads to aggravated vascular dysfunction and injury. The mechanisms are still not fully understood but it is suggested that Ang II induces proliferation, migration and apoptosis of VSMCs [57, 221, 222]. Ang-(1-7) reduced neo-intimal formation and VSMCs proliferation after vascular injury [193, 223, 224] whereas A779 (a selective Mas receptor inhibitor) can reverse the inhibitory effects of Ang-(1-7) on VSMCs proliferation [216]. This suggests that Mas receptor participates in the anti-proliferative action of Ang-(1-7). The next study determines whether the Mas receptor deficiency affect VSMCs and intimal thickness as well as the associated cellular signaling pathways *in vitro*.

6.2.6 Collagen, elastic fibers, and AAA

What are the mechanisms through which the Mas receptor deficiency increases AAA formation and aortic rupture during Ang II infusion? The functions of aortic elastic fibers and collagen content are likely to contribute to the aortic structure and function stability [225-227].

Collagen is a component of the aortic wall and is responsible for the stability of the aortic structure [225]. Our results show that deletion of the Mas receptor increased

collagen content and proportions in Ang II-induced AAA locations. This observation may be explained in the following manner: an increase of content of collagen is a protective response to vascular injury. This can also explain why the surviving apoE/Mas-KO mice have higher collagen contents, compared with apoE-KO mice. Other studies observed similar results with collagen content expression levels that were significantly increased under Ang II treatment [228], or decreased under Ang-(1-7) treatment [229]. There are also two more possible ways these observations could be clarified. The first would be that the increase of collagen is a protective mechanism under environmental stressors. For example, hypoxia affects adventitial fibroblasts by inducing the secretion of ACE and Ang II [230, 231]. Ang II increases collagen content to anchor the structure of blood vessel. This process can contend with elevated blood pressure and indicate the function of collagen in hypertension. However, in our experiments, there was no significant difference in SBP between groups. This suggests that elevated blood pressure might not be the key role in inducing increased collagen content in Mas receptor deficient apoE mice. Second, Ang II increased macrophage infiltration and polarization, causing high expression levels of MMP2 and MMP9 that result in the degradation of ECM. Finally, this leads to AAA formation [161, 232]. MMPs are also thought to play an essential role in cell behavior such as VSMCs migration, proliferation, differentiation, and apoptosis [233]as it seems to accelerate the degradation of ECM. Recently, we have shown that aortic MMP 9 is regulated by chronic Ang II infusion [234]. The thinning of the outer aortic wall causes it to lose its function to fix the structure of the blood vessel. Finally, elevated blood pressure may increase the risk of aortic rupture or dissection. In order to repair the unstable vascular structure, the collagen expression levels were increased in apoE/Mas-KO mice most likely as a compensatory mechanism.

In addition to the important role of ECM in the development of AAA, the alteration of connective fibers (elastin and collagen) which directly effects aortic elasticity and strength, is also responsible for the development of AAA [226, 227]. Here, we observed that deletion of the Mas receptor significantly increased elastin fragmentation in the suprarenal aortas of Ang II-infused mice. Hence, the elastic fiber breakage in apoE/Mas-KO mice could be responsible for the aggravated aortic rupture, AAA formation, and vascular dysfunction. Similar results were found in an animal model of the Loeys-Dietz syndrome, a connective tissue disorder leading to aortic aneurysms during Ang II infusion through a TGF-ß1 signaling pathway [235]. In the present study we did not measure TGF-ß1 expression but recent papers suggest that the Ang-(1-7) / Mas receptor axis is able to attenuate TGF-ß1 signaling in skeletal muscle or heart of Ang II-infused mice [236, 237]. Another explanation for the accelerated fragmentation might be that the increased inflammatory response in apoE/Mas-KO compared to apoE-KO mice by method of elastin fragmentation products, play a role in promoting macrophage infiltration [238]. Thus, the pronounced macrophage accumulation of apoE/Mas-KO mice may be associated with the presence of increased elastin fragmentation. On the other side, the accelerated fragmentation might also be the result of increased MMPs activity [239, 240].

Figure 41

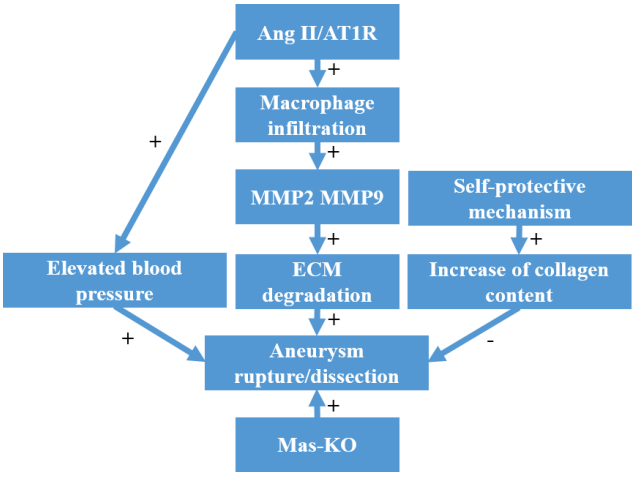

Figure 41 Diagram of collagen function

6.2.7 Vascular injury and HDL

Generally, higher levels of HDL have a protective role to the onset of cardiovascular disease, and low HDL levels are associated with a high risk of cardiovascular disease and AAA [241, 242]. Here, plasma test results have shown that deletion of the Mas receptor significantly increased plasma HDL levels in surviving apoE-KO mice, while the other three plasma components were not significantly increased. As the samples were collected from surviving mice, this could be the reason why apoE/Mas-KO mice can survive from worse vascular injury than the apoE-KO mice. We believe that this is another compensatory mechanism to modulate vascular injury in Mas receptor deficient apoE-KO mice. Clinical studies found that patients with larger AAA size have a higher monocyte/HDL ratio [243]. He et al. [244] suggested that AAA patients have lower HDL levels and impaired HDL function. Raising HDL levels and improving HDL function can decrease the AAA formation. In our study, deletion of the Mas receptor aggravated Ang II-induced AAA formation. This vascular injury may activate some self-protective actions to elevate plasma HDL levels and prevent the AAA formation, as apoE/Mas-KO mice have a higher incidence and more pronounced development of AAAs compared to apoE-KO mice. The impact of this protective mechanism in AAA formation did not seem to be significant enough in our model.

6.2.8 Age and AAA

A non-modifiable risk factor for AAA formation is age, especially for people over the age of 50 years [111]. The present study has found that deletion of the Mas receptor increased Ang II-induced AAA formation in apoE-KO mice. It was unclear whether

hyperlipidemia was a necessary factor for the more pronounced Ang II-induced AAA phenotype in Mas deficient mice. In order to test this, Mas receptor deficiency mice on a C57BL/6 background were investigated. It has been found that deletion of the Mas receptor also markedly increased Ang II-induced AAA formation in 8-month-old mice independent of hypercholesterolemia suggesting that age-related structural changes of the aorta mediated by the Mas receptor play also an important role in the development of AAA. It further demonstrates the important protective role of the Mas receptor in AAA formation in old mice. The underlying mechanisms of how the Mas receptor signaling affects structural changes in aortas of aging mice are still not clear. In general, cardiovascular aging is closely associated with increased vascular oxidative stress, reduced NO production and vascular inflammation [245, 246]. Based on these observations in aged control mice, one can speculate that Mas receptor deficiency which leads to decreased NO-bioavailability and increased vascular inflammation induces accelerated aortic vascular injury and markedly increased Ang II-induced AAA formation in the normolipidemic mice. The activation of the Mas receptor may have benefits to aging-associated vascular diseases. However, the Mas receptor-related signaling pathways and the effects of Ang-(1-7) in old mice have not be investigated. Future studies will define the role of the Mas receptor in young and old mice, and compare the gene expression levels from wild type and gene-modified mice with different age.

6.2.9 Conclusions

In summary, deletion of the Mas receptor increased mortality and AAA formation, in apoE-KO mice chronically infused with Ang II. Increased AAA formation was blood pressure independent as cardiac hypertrophy and blood pressure values did not differ between apoE-KO and apoE/Mas-KO mice. In accordance to the development of atherosclerosis, accelerated AAA formation was also associated with increased macrophages and T cells infiltration. These results suggest that immune cell activation is a common mechanism for vascular injury in Mas deficient mice. In addition, Mas receptor deficiency also increased AAA formation in 8-month-old normolipidemic mice, which provides critical evidence that AAA generation in Mas deficient mice is also not dependent on hypercholesterolemia. Taken together, these results support the hypothesis that the expression of the Mas receptor is important for the prevention of AAA formation in Ang II-infused mice.

7. Conclusions

The results demonstrate that the Mas receptor has protective functions in the regulation of vascular homeostasis and vascular injury. Its biological importance was underlined by the robust phenotype in different mouse models of vascular injury. Whole-body knockout of the Mas receptor significantly aggravated WD-induced atherosclerosis in apoE-KO mice and Ang II-induced abdominal aortic aneurysm formation in apoE-KO but also in normolipidemic mice on a C57BL6 background.

Our data reveals that deletion of the Mas receptor exacerbates the development of atherosclerosis and endothelial dysfunction, increases oxidative stress, and decreases NO bioavailability. Ang-(1-7), the physiological ligand for the Mas receptor, is able to improve vascular dysfunction and atherosclerosis in apoE-KO mice, but not in apoE/Mas-KO mice. The improved vascular function is accompanied by an increase of vascular NO-mediated cGMP generation and a decrease of vascular ROS production. This suggests that Ang-(1-7)-induced Mas receptor activation improves vascular function and attenuates the development of atherosclerosis which is related to an increase in vascular NO bioavailability. Moreover, deletion of the Mas receptor increases T lymphocyte accumulation and macrophage infiltration, and pushes macrophages to the M1 phenotype, which suggests that the Mas receptor plays an important role in the regulation of immune cell function and inflammation.

In addition, deletion of the Mas receptor increases AAA formation in apoE-KO mice. This increase is associated by an increase in intima media thickness, elastin fragmentation and accelerated inflammatory response in Ang II-infused apoE-KO mice. While there was no difference in blood pressure and cardiac hypertrophy in apoE-KO and apoE-Mas-KO mice, these results suggest that Mas deficiency-caused vascular remodeling is independent of blood pressure. In line with the results observed in the atherosclerosis study, deletion of the Mas receptor increases macrophage infiltration and T cell accumulation in the suprarenal aortas of Ang II-infused apoE-KO mice. This suggests that the modulation of immune cell function by the Mas receptor is not only important for the development of atherosclerosis but also for the formation and progression of abdominal aortic aneurysms. In addition, it is shown that Mas receptor deficiency also increased AAA formation in aged normolipidemic mice on a C57Bl/6 background suggesting that Mas receptor influences vascular structure and injury during aging.

In summary, we identified the Mas receptor as an important regulator in the development of vascular injury as Mas receptor deficiency exaggerates the development of atherosclerosis and the formation of AAA whereas Ang-(1-7) induced Mas receptor stimulation attenuates atherosclerosis. Moreover, our studies suggest that activating Ang-(1-7)/Mas axis may have therapeutic benefits in controlling atherosclerosis and aortic expansion. Future work to gain the underlying mechanism of the Mas receptor may facilitate the development of novel therapy and prevention

strategies against these vascular diseases.

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