Mechanisms of Mas1 Receptor-Mediated Signaling in the Vascular Endothelium

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Objective—Angiotensin II (AngII) has been shown to regulate angiogenesis and at high pathophysiological doses to cause vasoconstriction through the AngII receptor type 1. Angiotensin 1 to 7 (Ang-(1–7)) acting through the Mas1 receptor can act antagonistically to high pathophysiological levels of AngII by inducing vasodilation, whereas the effects of Ang-(1–7) signaling on angiogenesis are less defined. To complicate the matter, there is growing evidence that a subpressor dose of AngII produces phenotypes similar to Ang-(1–7).

Approach and Results—This study shows that low-dose Ang-(1–7), acting through the Mas1 receptor, promotes angiogenesis and vasodilation similar to a low, subpressor dose of AngII acting through AngII receptor type 1. In addition, we show through in vitro tube formation that Ang-(1–7) augments the angiogenic response in rat microvascular endothelial cells. Using proteomic and genomic analyses, downstream components of Mas1 receptor signaling were identified, including Rho family of GTPases, phosphatidylinositol 3-kinase, protein kinase D1, mitogen-activated protein kinase, and extracellular signal–related kinase signaling. Further experimental antagonism of extracellular signal–related kinases 1/2 and p38 mitogen-activated protein kinase signaling inhibited endothelial tube formation and vasodilation when stimulated with equimolar, low doses of either AngII or Ang-(1–7).

Conclusions—These results significantly expand the known Ang-(1–7)/Mas1 receptor signaling pathway and demonstrate an important distinction between the pathological effects of elevated and suppressed AngII compared with the beneficial effects of AngII normalization and Ang-(1–7) administration. The observed convergence of Ang-(1–7)/Mas1 and AngII/AngII receptor type 1 signaling at low ligand concentrations suggests a nuanced regulation in vasculature. These data also reinforce the importance of mitogen-activated protein kinase/extracellular signal–related kinase signaling in maintaining vascular function.

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Key Words: angiotensin II ■ endothelium, vascular ■ phenotype ■ renin-angiotensin system ■ vasodilation

The renin–angiotensin system is crucial for regulation of sodium homeostasis, vascular tone, angiogenesis, and overall cardiovascular function. Within this complex system, angiotensinogen is released into circulation from the liver and is cleaved into the decapeptide angiotensin I by renin.[1,2] Angiotensin I is further cleaved into the active octapeptide angiotensin II (AngII) by angiotensin-converting enzyme and the heptapeptide angiotensin 1 to 7 (Ang-(1–7)) by either angiotensin-converting enzyme 2 or various endopeptidases.[2–4] AngII is the most characterized of the renin–angiotensin system peptides and has been shown to mediate the majority of its physiological functions through the AngII type 1 receptor (AT_1R).[2,5] AT_1R activation occurs through heterotrimeric G proteins ($G_{q/11}$, $G_{i/o}$, and $G_{12/13}$), which in turn activate phospholipase C, inhibit adenylate cyclase, and activate RHO GTPases.[2] Although normal AngII signaling contributes to vascular homeostasis, elevated levels of AngII are deleterious, causing vasoconstriction and oxidative stress, whereas insufficient levels like those seen in the absence of renin activity result in endothelial dysfunction.[2,5–7]

Ang-(1–7) signaling, mediated by the Mas1 receptor,[8] has been shown to exhibit several protective cardiovascular effects that act antagonistically to elevated pathophysiological levels of AngII.[9–12] These antagonistic effects include the induction of vasodilation, preservation of endothelial function, the promotion of antifibrotic conditions, and facilitating antihypertrophic effects.[9,11,13–17] However, under conditions of renin-angiotensin system suppression, such as in low renin forms of hypertension and during periods of elevated sodium intake, low-dose AngII infusion, which normalizes levels of circulating AngII to physiological concentrations, also improves

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Nonstandard Abbreviations and Acronyms	
AngII	angiotensin II
AT_1R	angiotensin II receptor type 1
Ang-(1–7)	angiotensin 1 to 7
ERK	extracellular signal–related kinases
MAPK	mitogen-activated protein kinase
MCA	middle cerebral artery
MS/MS	tandem mass spectrometry
NFκB	nuclear factor κ-light-chain-enhancer of activated B cells
PI3K	phosphatidylinositol 3-kinase
RMVEC	rat microvascular endothelial cell
VEGFR1	vascular endothelial growth factor receptor 1
VEGFR2	vascular endothelial growth factor receptor 2

endothelial function and restores impaired vasodilator ability.[6,7] These studies suggest that there is a delicate balance of the levels of AngII and Ang-(1–7) in the renin–angiotensin system that is more complex than once thought.

Despite the growing evidence that Ang-(1–7)/Mas1 signaling is biologically important to the renin–angiotensin system, studies on the downstream signaling mechanisms have been limited, especially compared with the more studied AngII/AT_1R-axis. It has been shown that attenuation of extracellular signal–related kinases 1/2 (ERK1/2) activation through Ang-(1–7)–induced Mas1 receptor signaling in glomerular mesangial cells occurs through both cAMP and protein kinase A.[3,18] In addition, Ang-(1–7) treatment preserves endothelial function and regulates vascular oxidative stress through mechanisms involving endothelial nitric oxide synthase and nitric oxide bioavailability.[3,6,12] Studies involving human endothelial cells constitutively expressing the Mas1 receptor also suggest that Ang-(1–7) regulation of endothelial nitric oxide synthase signaling may be occurring through the regulation of PI3K (phosphatidylinositol 3-kinase) and serine/threonine protein kinase AKT pathways.[3,10] In addition, a study using quantitative phosphoproteomics provided insights into Ang-(1–7)–induced phosphorylation changes[19] and a second study using an antibody-based protein assay to monitor Ang-(1–7)–altered protein expression[20] in human endothelial cells. Both these studies indicated global changes in signal transduction, apoptosis, cell cycle, and gene expression regulation. These studies provide a strong initial basis for understanding certain aspects of Ang-(1–7)–mediated Mas1 receptor signaling; however, they do not reveal the components of the proximal signaling complex generated from early Ang-(1–7) stimulation of the Mas1 receptor.

To improve our understanding of the signaling mechanisms behind the protective effects of Ang-(1–7) in the vasculature, we characterized the effects of Ang-(1–7) and compared them with the vascular phenotypes resulting from a low, subpressor dose of AngII treatment in renin-suppressed rat models on high-salt diet. We hypothesized that (1) Ang-(1–7) would display similar effects on angiogenesis and vasodilation compared with the low, subpressor dose of AngII, (2) Ang-(1–7)/Mas1 and AngII/AT_1R signaling pathways would overlap, and (3) these pathways converge on a common mechanistic pathway regulating both endothelium-dependent vasodilation and angiogenesis. To test these hypotheses, this study used a combination of investigative techniques including an in vivo electric stimulation rat angiogenesis model, ex vivo middle cerebral artery (MCA) vasodilation in response to acetylcholine, and in vitro endothelial tube formation. These functional analyses were supplemented with proteomic and genomic pathway comparisons, followed by pharmacological targeting of implicated pathways contributing to Ang-(1–7)–mediated angiogenesis and vasodilation in the functional assays. This combined systematic approach provided a detailed insight into the intersection between Ang-(1–7)/Mas1 receptor signaling and low-dose AngII/AT_1R signaling in the vascular endothelium.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

AngII and Ang-(1–7) Enabled Angiogenesis in Response to Electric Stimulation

We have previously shown that 7 days of electric stimulation in vivo produces a robust angiogenic response in rat models that is eliminated by a high-salt diet and restored by a low, subpressor dose AngII infusion[7,21]; this result was replicated for a control in this study (Figure 1A). In vivo administration of the AT_1R antagonist losartan abolished the restorative effect of low-dose AngII infusion, whereas Mas1 receptor antagonist A779 had no effect on the ability of low-dose AngII infusion to restore angiogenesis. This experiment was repeated with Ang-(1–7) infusion and cotreatment with losartan, A779, or vehicle (Figure 1A). Vessel density was significantly increased in the Ang-(1–7)–infused rats, as observed with AngII infusion; however, losartan was unable to block the increase in vessel density, resulting from Ang-(1–7) treatment. Rats infused with Ang-(1–7) plus A779 exhibited no significant increase of vessel density, suggesting Ang-(1–7)–restored angiogenesis directly through the Mas1 receptor. Vehicle treatment (without low-dose AngII or Ang-(1–7) infusion) was unable to restore stimulated angiogenesis in Sprague Dawley rats on a high-salt diet. These data suggest that Ang-(1–7) restores stimulated angiogenesis through a Mas1 receptor-dependent signaling pathway and does not depend on AngII activation of the AT_1R axis (Figure 1A).

AngII and Ang-(1–7) Mediated Vasorelaxation Response to Acetylcholine

The effects of low-dose AngII, Ang-(1–7), or vehicle infusion on isolated maximum MCA responses to acetylcholine in Sprague Dawley rats on a high-salt diet is summarized in Figure 1B. As in previous studies,[6,7] this study shows that acetylcholine does not induce MCA vasodilation in vehicle-treated control rats on a high-salt diet (renin suppressed). A chronic infusion of a low, subpressor dose of AngII or Ang-(1–7) in these renin-suppressed rats restored endothelium-dependent vasodilation in response to acetylcholine. AngII-induced vasodilation was eliminated by cotreatment with AT_1R antagonist losartan; however, it was not affected by the Mas1 receptor antagonist A779. Similarly, Ang-(1–7)–induced vasodilation in response to acetylcholine was inhibited by cotreatment with

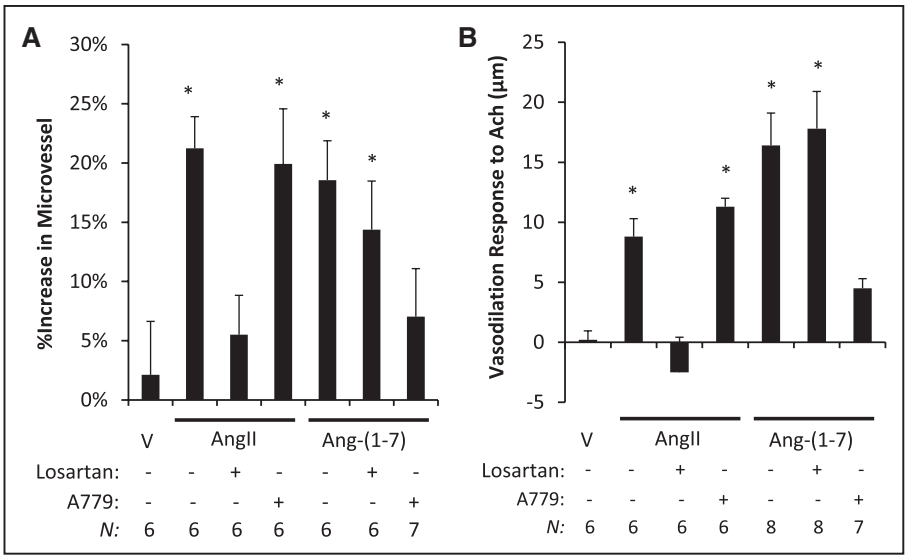

Figure 1. Angiogenesis and vasodilation were restored in Sprague Dawley (SD) rats undergoing high-salt diet (4% NaCl) suppression of the renin–angiotensin system. **A**, Rats were unilaterally stimulated whereas intravenously treated with angiotensin II (AngII), angiotensin 1 to 7 (Ang-(1–7)), or vehicle (V), and cotreated with AngII receptor type 1 antagonist (losartan) or Mas1 antagonist (A779). Both AngII through the AT1 receptor and Ang-(1–7) through the MAS1 (mas-related G-protein–coupled receptor 1) receptor significantly restored angiogenesis vs vehicle (*P<0.05). **B**, Maximum change of isolated rat middle cerebral artery diameter in response to acetylcholine (Ach; 10^{-5} mol/L) after AngII, Ang-(1–7), or vehicle cotreatment with losartan and A779 was measured. Both AngII acting through AngII receptor type 1 and Ang-(1–7) acting through the Mas1 receptor restored the vasodilation response to Ach (*P<0.05; N is indicated above for each condition).

A779, but not by losartan. As observed with angiogenesis in the Sprague Dawley rat hindlimb (Figure 1A), these data suggest that Ang-(1–7)–mediated vasodilation operates exclusively through a Mas1 receptor-dependent signaling pathway, whereas low-dose AngII-mediated vasodilation is exclusively AT_1R dependent (Figure 1B).

Analysis of Endothelial Cell Mas1 Receptor Signaling Pathways

The in vivo experiments suggested an endothelium-dependent Ang-(1–7)/Mas1 receptor proangiogenesis and provasodilator response. This endothelium-dependent result was recapitulated in preliminary tube formation assays in which rat microvascular endothelial cells (RMVECs) treated with Ang-(1–7) displayed a 16% in tube formation versus the nontreated (P<0.05; n=5). To directly analyze the endothelium-dependent response in relation to Mas1 receptor signaling, a combination of cryolysis, immunoprecipitation, and tandem mass spectrometry (MS/MS) analysis was used on signaling complexes isolated from RMVECs (Figure 2A). Fluorescence microscopy experiments confirmed that the Mas1 receptor antibody (sc-135063; Santa Cruz) epitope was intracellular (Figure I in the online-only Data Supplement). Signaling complexes were then verified for the presence of Mas1 by immunoblot (Figure 2B; Figure II in the online-only Data Supplement) according to previous protocols[22–25] using rabbit anti-rat Mas1 primary antibody (sc-135063; Santa Cruz) at 1:1000 and secondary horseradish peroxidase–conjugated goat anti-rabbit IgG antibody (BioRad) at 1:2000, as well as in the raw MS/MS data (Figure II in the online-only Data Supplement). In the Ang-(1–7)–stimulated RMVEC signaling complexes, 50 proteins were identified as significantly increased (P<0.05; n=8 plus a technical replicate; 16 total MS runs) compared with nonstimulated RMVECs after application of stringent filters (Figure 2C; Table 1; Table I in the online-only Data Supplement). Further bioinformatic pathway analysis indicated that the top represented biological signaling networks involved hematologic system development/function, hematopoiesis, nervous system development/function, and

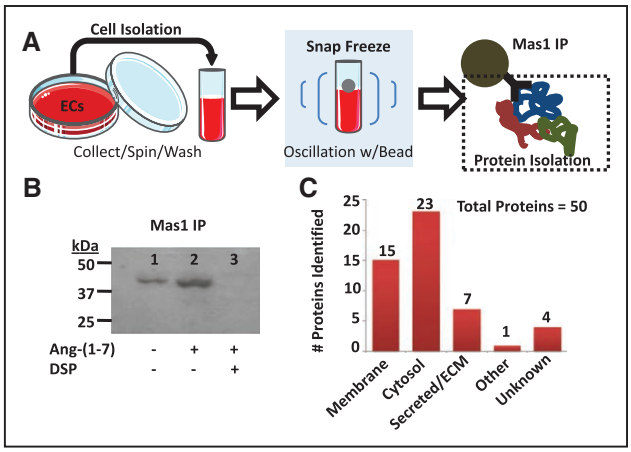

Figure 2. Mas1 protein signaling complexes were isolated by immunoprecipitation (IP). **A**, Indicated is an overview of the identification of angiotensin 1 to 7 (Ang-(1–7))–activated Mas1 receptor signaling complexes. **B**, Isolated signaling complexes from rat microvascular endothelial cells (RMVECs) were immunoblotted for Mas1 in unstimulated, stimulated with 100 nmol/L Ang-(1–7), and stimulated with 100 nmol/L Ang-(1–7) plus 1 mmol/L DSP cross-linker. Immunoblots revealed that Ang-(1–7) stimulated without cross-linker was optimal. **C**, Mass spectrometry analysis of Ang-(1–7)–stimulated Mas1 immunoprecipitation (IP) complexes revealed a total of 50 associated proteins (N=8 plus technical replicate, 16 total runs; see Methods for filtering criteria). DSP indicates dithiobis (succinimidyl proponate); EC, endothelial cells; and ECM, extracellular matrix.

Table 1. Ang-(1–7)–Stimulated MAS1 Receptor Immunoprecipitation Top Proteomic Hits

Accession Number	Annotated Protein	Ang-(1–7) IP Peptides/Scans	NormLog2Ratio* (Norm. *P* Value)	Notable Signaling Involvement
Q99MV5	Putative helicase Mov10l1 (M10L1)	9/63	2.34 (4.27E-8)	Cell cycle regulation (–)
P25095	Type 1 angiotensin II receptor (AGTR1)	2/19	0.9173 (0.034)	G-protein and renin–angiotensin system signaling
Q64438	Angiogenin-2 (ANG2)	4/102	Unique (5.41E-26)	Transcription regulation
P70478	Adenomatous polyposis coli protein (APC)	38/62	0.63 (0.046)	WNT signaling (–)
Q80YF9	Rho GTPase-activating protein 33 (RHG33)	5/57	0.72 (0.031)	G-protein signaling (CDC42, RHO/RAC)
P58660	Caspase recruitment domain-containing protein 10 (CARD10)	13/35	0.86 (0.051)	IKK/NFκB signaling
Q66K08	Cartilage intermediate layer protein 1 (CILP-1)	13/60	1.63 (2.34E-5)	TGFβ1 and IGF1 signaling (–)
P49025	Citron Rho-interacting kinase (CRIK)	28/54	0.71 (0.041)	G-protein signaling (RHO/RAC1)
P08081	Clathrin light chain A	7/57	0.91 (0.0090)	Endosomal pathway
Q5PPG7	Eukaryotic translation initiation factor 2D (EIF2D)	11/27	1.29 (0.017)	Translation initiation
Q69ZL1	FYVE, RhoGEF, and PH domain-containing protein 6 (FGD6)	14/51	1.51 (0.00023)	G-protein signaling (CDC42, +)
Q00342	Receptor-type tyrosine-protein kinase FLT3	6/60	0.63 (0.049)	SHC1(+), AKT1(+), mTOR (+), RAS(+), MAPK/ERK(+), PLCG1(+), and STAT5 signaling
P35439	Glutamate receptor ionotropic, NMDA 1 (GluN1)	11/43	1.15 (0.0058)	NMDA signaling
P97879	Glutamate receptor-interacting protein 1 (GRIP-1)	13/28	1.02 (0.044)	Signaling complex scaffold
Q61754	Glandular kallikrein K24 (mGK-24)	2/37	Unique (2.15E-10)	Kallikrein/bradykinin signaling
Q60682	Killer cell lectin-like receptor 8	6/64	0.83 (0.010)	Class I MHC signaling
D3ZBP4	Protein-methionine sulfoxide oxidase MICAL1	7/67	1.08 (0.0011)	Apoptosis signal regulation (–)
P12526	Proto-oncogene Mas (MAS1)	4/13	Unique (1.67E-4)	G-protein and renin–angiotensin system signaling
P42346	Mammalian target of rapamycin (mTOR)	24/53	1.26 (0.0010)	G-protein (RHO/RAC1) and PI3K-AKT signaling
Q99466	Neurogenic locus notch homolog protein 4 (NOTCH4)	27/33	1.58 (0.0022)	Cell survival signaling (+)
Q99MR9	Protein phosphatase 1 regulatory subunit 3A	7/93	0.55 (0.031)	Glycogen synthesis
Q9WTQ1	Serine/threonine-protein kinase D1 (PRKD1)	10/25	1.86 (0.0029)	PKC (+), DAG (+), ERK1/2 (+), IKK/NFκB (+), p38MAPK (+), and EGF (–) signaling
Q91YA2	Serine/threonine-protein kinase H1	7/52	0.91 (0.012)	Trafficking/pre-mRNA processing
Q9Z268	RasGAP-activating-like protein 1 (RASL1)	10/66	0.58 (0.057)	Ras-cAMP pathway
P27671	Ras-specific guanine nucleotide-releasing factor 1 (Ras-GRF1)	23/26	1.07 (0.044)	G-protein signaling (RHO/RAC/RAS)
P05545	Serine protease inhibitor A3K	5/32	2.21 (1.61E-4)	Kallikrein signaling (–)
P84551	SKI family transcriptional corepressor 1 (SKOR1)	12/34	1.04 (0.024)	BMP signaling (–)
P50592	TNF ligand superfamily member 10	2/68	1.92 (4.9E-7)	TNFα signaling
Q8CIR4	Transient receptor potential cation channel subfamily M member 6 (TPRM6)	17/26	1.43 (0.012)	Ion channel and kinase
P68255	14-3-3 protein theta (1433T)	6/47	Unique (8.22E-13)	G-protein signaling (RHO/RAC), PDK1, and PI3K-AKT signaling
Q80U44	Zinc finger FYVE domain-containing protein 16	14/36	2.12 (9.70E-5)	Endosomal pathway

Signaling involvement includes but is not limited to those above. All proteins indicated passed all stringent filters indicated in the Methods; a prefiltered protein list can be found in Table I in the online-only Data Supplement. AKT indicates serine/threonine protein kinase AKT; Ang-(1–7), angiotensin 1 to 7; CDC42, cell division control protein 42 homolog; ERK, extracellular signal–related kinase; FLT, fms-like tyrosine kinase; GAP, guanine nucleotide activating protein; GEF, guanine nucleotide exchange factor; MAPK, mitogen-activated protein kinase; MAS1, mas-related G-protein–coupled receptor 1; mTOR, mechanistic target of rapamycin; NFκB, nuclear factor κ-light-chain-enhancer of activated B cell; NMDA, N-methyl-D-aspartate receptor 1; p38MAPK, p38 mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PRKD1, serine/threonine protein kinase D1; RAS, Ras GTPase; and RHO, Rho GTPase.

*Hundred nanomoles per liter Ang-(1–7) stimulated vs unstimulated Mas1 IP (N=8; 16 total runs).

tissue morphology after Ang-(1–7) stimulation of RMVECs. Within the exclusive protein signaling complexes from Ang-(1–7)–stimulated RMVEC immunoprecipitations, numerous G-protein signaling components were detected, including those involved in Rho family of GTPase (RHO, RAS [Ras GTPase], RAC signaling; Table 1). Calcium signaling regulators essential for G-protein and other signaling pathways were also observed, such as diacylglycerol kinase, PI3K, and protein kinase C (Table 1). AKT1 and nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) regulators, such as mTOR (mechanistic target of rapamycin) and CARD10 (caspase recruitment domain family member 10), essential for cell regulation of inflammatory response were present as well (Table 1). CARD10, an important activator of NFκB, was detected in significantly lower amounts in the stimulated receptor complex, suggesting a decrease in activation and a potential anti-inflammatory effect. In addition, regulators of cellular reorganization and initiation of nascent protein synthesis were detected.

Analysis of Gene Expression in Ang-(1–7)–Treated RMVECs

To add depth to the Ang-(1–7)–stimulated MAS1 receptor signaling pathway data from the proteomic MS/MS analysis of the immunoprecipitation results, expression of common angiogenesis-related genes in response to 100 nmol/L Ang-(1–7) stimulation of RMVECs was examined according to previous literature.[26] The response to Ang-(1–7) treatment of RMVECs was assessed (Table 2) and related to the proteomic MS/MS data. Significant gene expression changes ($P<0.05$) were observed in 25 of the 84 genes (30%) analyzed (Table 2), including growth factor/G-protein signaling, signal transduction regulation, and transcriptional regulation. Interestingly, vascular endothelial growth factor receptor 1 (VEGFR1, *Vegfr1/Flt1*, 3.02-fold; $P=0.050$) and vascular endothelial growth factor receptor 2 (VEGFR2, *Vegfr2/Kdr*, 7.65-fold; $P=0.011$) were upregulated, suggesting a link to components of RAS signaling identified in the proteomic analyses that have been shown to mediate angiogenesis through vascular endothelial growth factor family signaling.[27] Numerous other significant changes in gene expression of important angiogenesis and vasoreactive signal molecules were observed related to G-protein signaling, including CDC42 (cell division control protein 42; *Cdc42*; 1.22-fold; $P=0.047$) and RAC2 (Ras-related C3 botulinum toxin substrate 2; *Rac2*; 6.65-fold; $P=0.009$), ERK1/2 and mitogen-activated protein kinase (MAPK) signaling, including MEK2 (mitogen-activated protein kinase kinase 2; *Map2k2*; 1.57-fold; $P=0.005$) and p38MAPK (*Mapk13*; 5.57-fold; $P=0.002$), phosphoinositol signaling, and phospholipase signaling (Table 2; $P<0.05$). As with Ang-(1–7) stimulation, the low-dose AngII stimulation upregulated CDC42 (*Cdc42*; 1.26-fold; $P=0.028$), VEGFR1 (*Vegfr1/Flt1*; 1.17-fold; $P=0.020$), p38MAPK (*Mapk13*; 4.83-fold; $P=0.026$), and RAC signaling (Rac1; 1.23-fold; $P=0.022$). Interestingly, the high-dose AngII (10× low-dose AngII) stimulation of the RMVECs exhibited a marked decrease in AKT, VEGFR, MAPK, phosphoinositol, and phospholipase signaling molecules ($P<0.05$) that were upregulated by Ang-(1–7) (Table 2) or low-dose AngII stimulation (Table 3).

Bioinformatics Signaling Pathway Analysis of Ang-(1–7)–Mediated Mas1 Signaling in the Endothelium

The results of a comprehensive bioinformatic analysis of the proteomic and real-time-polymerase chain reaction data set for pathway mapping of Ang-(1–7)–stimulated Mas1 receptor signaling in RMVECs is summarized in Figure 3. On the basis of the proteomic data, a cell surface complex consisting of extracellular matrix proteins, the Mas1 receptor, and AT_1R seem to form and signal through the Rho family of GTPases (RHO, RAC, and RAS). Further bioinformatics pathway analysis of the data indicated that G-protein signaling acted upstream of the PI3K/PRKD1 (serine/threonine protein kinase D1)/AKT and MAPK/ERK signaling pathways important for endothelial function, angiogenesis, and vasodilation. General trends observed implicated p38MAPK and ERK1/2 signaling as the important mediators of angiogenesis and vasodilation processes. This analysis suggests a possible convergence of the Ang-(1–7)/Mas1 receptor signaling in this study with previously known AngII/AT_1R signaling, especially on the p38MAPK and ERK1/2 signal cascades, leading to upregulation of VEGFR signaling as indicated by downstream gene expression analysis (Figure 3). The Ang-(1–7)/Mas1 receptor signaling complex pathway data presented here is supported by previous literature showing that early stage changes in Ang-(1–7) induced phosphorylation and protein expression related to global changes in ERK1/2, AKT1, NFκB, and vascular endothelial growth factor signaling[19,20] and further confirm the molecular basis of the cell survival and anti-inflammatory role of Mas1 receptor activation.

Ang-(1–7)–Dependent Activation of RMVEC Signaling

The bioinformatics signaling pathway analysis of Ang-(1–7)–mediated signaling in RMVECs indicated pathways crucial for angiogenesis and vasodilation. ERK1/2 and p38MAPK were 2 important protein regulators indicated by the activated Mas1 receptor immunoprecipitation proteomic MS/MS data and the Ang-(1–7)–stimulated RMVEC gene expression arrays. Therefore, the activation of ERK1/2 and p38MAPK was tested by monitoring Ang-(1–7)–induced phosphorylation in the RMVECs. Immunoblotting of ERK1/2 and p38MAPK, along with the phosphorylation of these molecules, in serum-starved (1% fetal bovine serum) RMVECs not treated and treated with Ang-(1–7) samples was used to monitor pathway activation (Figure 4). RMVECs in complete media exhibited high ERK1/2 phosphorylation but little p38MAPK phosphorylation. However, the serum-starved RMVEC samples showed little ERK1/2 phosphorylation and high p38MAPK phosphorylation, suggesting that serum starvation suppressed ERK1/2 in our system but enhanced p38MAPK. Treatment of serum-starved RMVECs with 100 nmol/L Ang-(1–7) was able to significantly recover ERK1/2 phosphorylation back to levels of complete media after 15 minutes, whereas p38MAPK remained steady in activation. These data suggest that ERK1/2 is activated in an Ang-(1–7)/Mas1 receptor-dependent manner, whereas p38 MAPK is activated during serum starvation and maintained after Ang-(1–7) treatment.

Table 2. Analysis of an Angiogenesis RT-PCR Gene Expression Array After Ang-(1–7) Stimulation of Rat Microvascular Endothelial Cells

Gene	Protein Annotation	Fold Regulation*	*P* Value	Signaling Pathway Involvement*
Cdc42	Cell division control protein 42 homolog (CDC42)	1.22	0.047	Rho GTPase, p21 signaling
Flt-1	Vascular endothelial growth factor receptor 1 (VEGFR1)	3.02	0.050	VEGF(+), PLCG(+), PKC(+), MAPK/ERK(+), AKT1(+) signaling
Flt-4	Vascular endothelial growth factor receptor 4 (VEGFR4)	–5.97	0.050	VEGF(+), PLCG(+), PKC(+), MAPK/ERK(+), AKT1(+) signaling
Hspb1	Heat shock protein β-1 (HSPB1)	2.25	0.015	Chaperone, thermotolerance, apoptosis(–), NFκB(+)
Kdr	Vascular endothelial growth factor receptor 2 (VEGFR2)	7.65	0.011	Angiogenesis(+), mitogenesis(+), cell migration(+) signaling
Map2k2	Dual specificity mitogen-activated protein kinase kinase 2 (MEK2)	1.57	0.005	MAPK/ERK(+) signaling
Mapk1	Mitogen-activated protein kinase 1 (MAPK1)	–1.16	0.031	integration of signaling
Mapk12	Mitogen-activated protein kinase 12 (MAPK12)	2.57	0.017	p38 MAPK, inflammatory(+) signaling
Mapk13	Mitogen-activated protein kinase 11 (MAPK13)	5.57	0.002	p38 MAPK, ATF2(+), ELK1(+), PRKD1(–) signaling
Pgf	Placental growth factor (PGF)	–4.38	0.004	FLT-1(+) signaling
Pik3cb	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform (PI3K beta isoform)	–1.46	0.007	PIP3(+), AKT1(+), PDPK1(+) signaling
Pik3cd	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform (PI3K delta isoform)	–2.68	0.044	PIP3(+), AKT1(+), PDPK1(+), RAS(+), MAPK/ERK(+), PI3K(+) signaling
Pik3cg	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma isoform (PI3K gamma isoform)	1.28	0.022	PIP3(+), AKT1(+), PDPK1(+), cAMP(–), β-adrenergic(–) signaling
Pla2g12a	Group XIIA secretory phospholipase A2	1.65	0.007	Phospholipid(+), PKC(+), inflammatory(+) signaling
Pla2g2e	Group IIE secretory phospholipase A2	2.73	0.002	Phospholipid(+), PKC(+), inflammatory(+) signaling
Pla2g4a	Cytosolic phospholipase A2	–1.46	0.014	Phospholipid(+), PKC(+), inflammatory(+) signaling
Ppp3ac	Calcineurin subunit B type 2 (CANB2)	2.37	0.027	Calcium signaling(+)
Prkca	Protein kinase C alpha (PKCα)	–2.19	0.003	Calcium(+), DAG(+), RAF1(+), MAPK/ERK(+), proliferation(–), apoptosis(–) signaling
Ptgs2	Prostaglandin G/H synthase 2 (cyclooxygenase-2, COX-2)	–4.59	0.043	Prostaglandin signaling (+)
Ptk2	Focal adhesion kinase 1	1.76	0.033	Migration(+), PI3K(+), AKT1(+), MAPK/ERK(+), Rho GTPase signaling
Rac2	Ras-related C3 botulinum toxin substrate 2	6.65	0.009	Rho GTPase signaling
Sh2d2a	SH2 domain-containing protein 2A (SH2D2A)	7.18	0.017	VEGFR2(+), MAPK(+), PKC(+) IKK/NFκB(+) signaling
Sphk1	Sphingosine kinase 1 (SPHK1)	1.78	0.023	Sphingosine(+), TNFα(+), NFκB(+) signaling
Sphk2	Sphingosine kinase 2 (SPHK2)	1.62	0.045	Sphingosine(+), DAG(+), PKC(+), VEGF/MAPK/RAS(+) signaling
Vegfa	Vascular endothelial growth factor A (VEGF-A)	–2.93	0.014	FLT1(+), KDR(+) signaling

All genes indicated significant fold change in gene expression ($P \leq 0.05$); biological processes include but are not limited to those above (N=3). AKT indicates serine/threonine protein kinase AKT; Ang-(1–7), angiotensin 1 to 7; ERK, extracellular signal–related kinase; FLT, fms-like tyrosine kinase; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor κ-light-chain-enhancer of activated B cell; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase; PRKD1, serine/threonine protein kinase D1; RAF, rapidly accelerated fibrosarcoma serine/threonine protein kinase; RAS, Ras GTPase; and VEGF, vascular endothelial growth factor.

*Hundred nanomoles per liter Ang-(1–7) stimulated vs unstimulated endothelial cells.

Effects of ERK1/2 and p38MAPK Inhibition on Ang-(1–7)–Stimulated Mas1 Receptor-Mediated Angiogenesis

To validate specific signaling pathways shown to be of importance in the Ang-(1–7) stimulation of the Mas1 receptor by proteomic and real-time-polymerase chain reaction analyses, we tested the functional effects of key modulators ERK1/2 and p38MAPK. RMVEC tube formation was performed for 24 hours in the presence or absence of 100 nmol/L Ang-(1–7) or AngII plus or minus ERK1/2 or p38MAPK antagonists according to the methods (Figure 5). Ang-(1–7) and AngII stimulated an upward trend in RMVEC tube formation; however, only Ang-(1–7) resulted in a significant increase ($P<0.001$) versus RMVECs alone. The AngII data, coupled with the whole animal data in Figure 1, suggest that

Table 3. Analysis of an Angiogenesis RT-PCR Gene Expression Array After AngII Low- and High-Dose Stimulation of Rat Microvascular Endothelial Cells

Gene	Protein Annotation	Fold Change*	*P* Value	Signaling Pathway Involvement*
Low	AngII			
Cdc42	Cell division control protein 42 homolog (CDC42)	1.26	0.028	Rho GTPase, p21 signaling
Hif1a	Vascular endothelial growth factor receptor 1 (VEGFR1)	1.17	0.020	Master regulator of hypoxia response
Mapk13	Mitogen-activated protein kinase 11 (MAPK13)	4.83	0.026	p38 MAPK, ATF2(+), ELK1(+), PRKD1(–) signaling
Pdgfc	Platelet-derived growth factor C	1.31	0.016	Proliferation(+), migration(+), cell survival(+) signaling
Pik3r1	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit	1.39	0.007	Insulin(+), FGF, KIT, PDGF signaling
Ppp3cb	Calcineurin subunit B type 2 (CANB2)	1.15	0.047	Calcium signaling
Prkcb	Protein kinase C beta (PKCβ)	1.47	0.019	Calcium(+), DAG(+), NFκB(+), ANDR(+), RAF/MAP/ERK(+) signaling
Rac1	Ras-related C3 botulinum toxin substrate 1	1.23	0.022	Rho GTPase signaling
High	AngII			
Akt1	V-akt murine thymoma viral oncogene homolog 1	–3.68	0.019	PDGF(+), cell survival, angiogenesis, insulin signaling
Akt2	V-akt murine thymoma viral oncogene homolog 2	–4.83	0.015	PDGF(+), cell survival, angiogenesis, insulin signaling
Arnt	Aryl hydrocarbon receptor nuclear translocator	–2.61	0.040	Transport ligand to nucleus
Bad	Bcl2 associated agonist of cell death	–4.16	0.037	Apoptosis(+) signaling
Cdc42	Cell division control protein 42 homolog (CDC42)	1.46	0.014	Rho GTPase, p21 signaling
Flt-4	Vascular endothelial growth factor receptor 4 (VEGFR1)	–5.97	0.050	VEGF(+), PLCG(+), PKC(+), MAPK/ERK(+), AKT1(+) signaling
Map2k1	Dual specificity mitogen-activated protein kinase kinase 1	–4.74	0.009	MAPK/ERK(+) signaling
Mapk11	Mitogen-activated protein kinase 11 (MAPK11)	–13.02	0.005	p38MAKP(+) signaling
Mapk14	Mitogen-activated protein kinase 14 (MAPK14)	–2.52	0.009	p38MAKP(+) signaling
Mapkapk2	Mitogen-activated protein kinase-activated protein kinase 2	–2.37	0.045	p38MAPK/MAPK14(+), TNFα(+), HSP27(+) signaling
Mapkapk3	Mitogen-activated protein kinase-activated protein kinase 3	–4.73	0.018	p38MAPK/MAPK14(+), TNFα(+), ERK(+), JNK(+) signaling
Nrp2	Neuropilin 2	–3.07	0.018	VEGF(+), PLGF-2(+) signaling
Pgf	Placental growth factor (PGF)	–8.12	0.002	FLT-1(+) signaling
Pik3cb	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform (PI3K beta isoform)	–2.03	0.024	PIP3(+), AKT1(+), PDPK1(+) signaling
Pik3cd	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform (PI3K delta isoform)	–5.74	0.013	PIP3(+), AKT1(+), PDPK1(+), RAS(+), MAPK/ERK(+), PI3K(+) signaling
Pik3r2	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit	–4.25	0.020	PIP3(+), AKT1(+), PDPK1(+), p110 signaling
Pla2g2f	Phospholipase A2, group IIF	–3.80	0.009	Hydrolyzes phosphatidylglycerol
Pla2g4b	Phospholipase A2, group IVB	–2.27	0.015	Hydrolyzes glycerophospholipids
Pla2g6	Phospholipase A2, group VI (cytosolic, calcium-independent)	–12.40	0.008	Arachidonic acid release, apoptosis
Plcg1	Phospholipase C, gamma 1	–5.11	0.013	IP3(+), DAG(+) signaling
Ppp3ca	Calcineurin subunit B type 2 (CANB2)	3.88	0.022	Calcium, DNM1L, HSPB1, SSH1 signaling
Prkca	Protein kinase C alpha (PKCα)	–6.34	0.003	RAF1, BCL2, CSPG4, TNNT2/CTNT, MAPK/ERK(+) signaling
Prkcg	Protein kinase C gamma (PKCγ)	–7.12	0.025	Calcium, DAG, p53/TP53 signaling

All genes indicated significant fold change in gene expression ($P \leq 0.05$); biological processes include but are not limited to those above (N=3). AKT indicates serine/threonine protein kinase AKT; Ang-(1–7), angiotensin 1 to 7; ERK, extracellular signal–related kinase; FLT, fms-like tyrosine kinase; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor κ-light-chain-enhancer of activated B cell; p38MAPK, p38 mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PRKD1, serine/threonine protein kinase D1; RAF, rapidly accelerated fibrosarcoma serine/threonine protein kinase; RAS, Ras GTPase; RT-PCR, real-time polymerase chain reaction; and VEGF, vascular endothelial growth factor.

*Hundred nanomoles per liter Ang-(1–7) stimulated vs unstimulated endothelial cells.

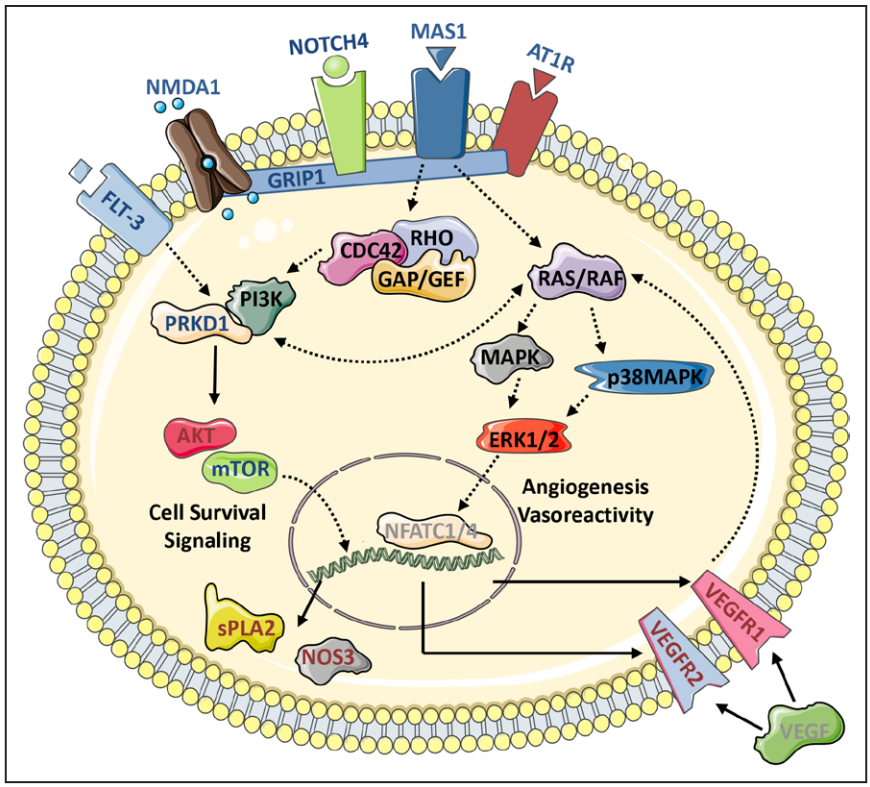

Figure 3. Using a combination of immunoprecipitation and tandem mass spectrometry (MS) protein identification, complemented by gene expression analysis, a comprehensive angiotensin 1 to 7 (Ang-(1–7))–stimulated Mas1 receptor signaling pathway was generated. Signaling proteins annotated with blue lettering indicate those identified in the tandem MS analysis of the immunoprecipitation, annotated in red lettering were significantly increased during gene expression analysis, and annotated in black lettering were identified through both methods. Gray lettering indicates inferred signaling molecules. AKT indicates serine/threonine protein kinase AKT; AT1R, angiotensin II receptor type 1; CDC42, cell division control protein 42 homolog; ERK1/2, extracellular signal-related kinases1/2; FLT-3, fms-like tyrosine kinase 3; GAP/GEF, guanine nucleotide activating protein/guanine nucleotide exchange factor; MAPK, mitogen-activated protein kinase; MAS1, mas-related G-protein–coupled receptor 1; mTOR, mechanistic target of rapamycin; NFATC1/4, nuclear factor of activated T-cells 1/4; NMDA1, N-methyl-D-aspartate receptor 1; NOS3, nitric oxide synthase 3; NOTCH4, neurogenic locus notch homology protein 4; p38MAPK, p38 mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PRKD1, serine/threonine protein kinase D1; RAF, rapidly accelerated fibrosarcoma serine/threonine protein kinase; RAS, Ras GTPase; RHO, Rho GTPase; sPLA2, secretory phospholipase A2; VEGF, vascular endothelial growth factor; VEGFR1, vascular endothelial growth factor receptor 1; and VEGFR2, vascular endothelial growth factor receptor 2.

other factors are required in vivo to aid in the AngII promotion of endothelial angiogenesis. Further experiments indicated that ERK1/2 inhibition resulted in complete loss of tube formation ability under all conditions. p38MAPK inhibition also resulted in significantly decreased tube formation versus control and stimulated conditions, but to a lesser extent than ERK1/2 inhibition. These data suggest that ERK1/2 is essential for angiogenesis processes in general and Ang-(1–7) signals directly through it, whereas p38MAPK may be one of the many MAPK upstream regulators of ERK1/2 making it a less essential molecule.

Effects of ERK1/2 and p38MAPK Inhibition on Low-Dose Ang-(1–7)–Stimulated MAS1 Receptor-Mediated Vasodilation

The influence of ERK1/2 and p38MAPK on ex vivo MCA endothelium-dependent Ach (10^{-5} mol/L)-induced vasodilation was tested. Sprague Dawley rats on a high-salt diet to suppress the renin–angiotensin system were treated in vivo with AngII (5 ng·kg^{-1}·min^{-1}), Ang-(1–7) (4 ng·kg^{-1}·min^{-1}), or DMSO vehicle (20 µL/h) and cotreated with ERK pathway inhibitor PD-98059 (PD; 10 µg/h) or p38MAPK pathway inhibitor SB-203580 (SB; 10 µg/h) for 3 to 5 days as described in the methods. MCAs were then isolated and tested for vasoreactivity ex vivo. The ex vivo studies revealed that a chronic infusion of Ang-(1–7) or AngII restored MCA vasodilation responses to acetylcholine in comparison with the vehicle treatment; this phenotype was blocked by ERK1/2 inhibition (Figure 6). Conversely, p38MAPK inhibition exhibited only slight ablation of acetylcholine-induced vasodilation in the MCA from rats receiving chronic AngII or Ang-(1–7) treatments. These data suggest that ERK1/2 is essential for the vasodilation response in conjunction with upstream MAPKs, not just those in the p38MAPK family.

Discussion

This study examined the impact of the administration of low-dose Ang-(1–7) in comparison with a low, equimolar dose of AngII on vascular dysfunction in an animal model with low renin–angiotensin system activity. The data here significantly expand the Ang-(1–7)/Mas1 receptor signaling pathway and add to the growing body of work, demonstrating an important distinction between the pathological effects of elevated or suppressed AngII and the beneficial vascular effects

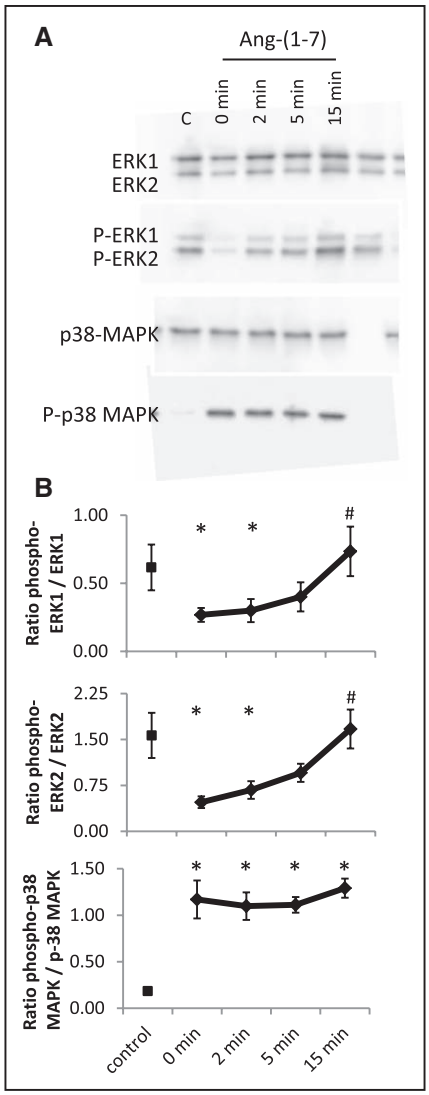

Figure 4. Phosphorylation of extracellular signal–related kinases (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK) in rat microvascular endothelial cells (RMVECs) treated with angiotensin 1 to 7 (Ang-(1–7)). **A**, Immunoblot of RMVECs serum starved in 1% fetal bovine serum and treated with 100 nmol/L Ang-(1–7) (C=positive control, cells in complete media). Membranes were blotted against ERK1/2, phospho-ERK1/2, p38 MAPK, and phospho-p38 MAPK. **B**, Quantification of immunoblots indicated that there was an Ang-(1–7)–dependent activation of ERK1/2 and that serum starvation activated p38MAPK (p38 mitogen-activated protein kinase), which remained active after Ang-(1–7) treatment. Values are expressed as ratio of phosphoprotein/protein mean area (*P<0.05 vs control cells; #P<0.05 vs zero-minute time point).

of AngII normalization. It is well established that abnormally elevated levels of AngII stimulate superoxide production and endothelial dysfunction[2,5,29–32]; however, increasing evidence indicates that pathologically suppressed AngII levels also increase oxidant stress and endothelial dysfunction relative to physiologically normal plasma AngII levels.[6,7,33,34] Numerous studies have now shown that suppression of AngII via high-salt diet disrupts vascular function and low-dose AngII infusion restores function via AT_1R.[6,7,27] This challenges the belief that reduction in AngII is universally beneficial to vascular health, but it does not contradict the finding that normalizing pathologically elevated AngII levels is an effective intervention. Our data support this concept of an ideal range of plasma angiotensin peptides to promote effective endothelial function and suggest that an equimolar, low dose of Ang-(1–7) recapitulates the effects of this subpressor dose of AngII.

In previous work, we have consistently observed altered phenotypes of microvessel angiogenesis in response to electric stimulation by directly, genetically, or environmentally manipulating AngII levels and through pharmacological interventions, including angiotensin-converting enzyme inhibition and AT_1R inhibition.[7,24,34–39] Here for the first time, we

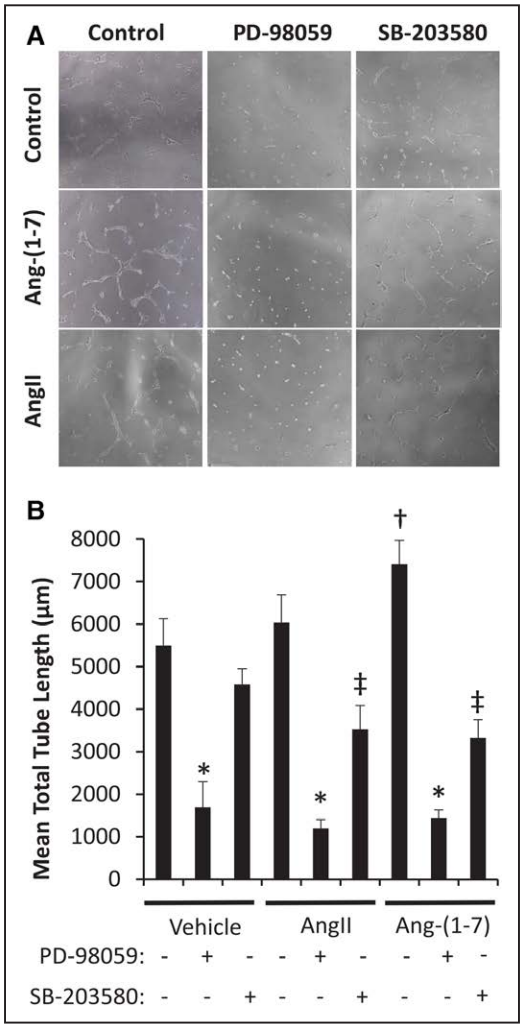

Figure 5. Endothelium-dependent tube formation. **A**, Matrigel tube formation with 20 000 rat microvascular endothelial cells (RMVECs) treated with 100 nmol/L AngII, 100 nmol/L angiotensin 1 to 7 (Ang-(1–7)), or vehicle and cotreated with extracellular signal–related kinase (ERK) pathway inhibitory PD-98059 (PD; 50 μmol/L) or p38MAPK (p38 mitogen-activated protein kinase) pathway inhibitor SB-203580 (SB; 10 μmol/L) are shown (n=12). **B**, Ang-(1–7) significantly increased (†P<0.05) tube formation compared with vehicle treatment, whereas AngII treatment produced no significant change. ERK pathway inhibition completely eliminated tube formation in all treatments (*P<0.05), whereas p38MAPK only exhibited significant inhibition in the angiotensin II (AngII) and Ang-(1–7) treatments (‡P<0.05). MAPK indicates mitogen-activated protein kinase.

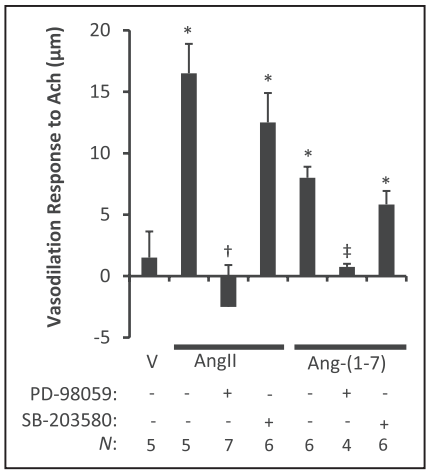

Figure 6. Mediation of ex vivo endothelium-dependent acetylcholine (Ach; 10^{-5} mol/L) induced vasodilation. Rats on a high-salt diet leading to renin–angiotensin system suppression were treated in vivo with angiotensin II (AngII; 5 ng·kg^{-1}·min^{-1}), angiotensin 1 to 7 (Ang-(1–7)) (4 ng·kg^{-1}·min^{-1}), or DMSO vehicle (20 μL/h) and cotreated with extracellular signal–related kinase (ERK) pathway inhibitory PD-98059 (PD; 10 μg/h) or p38MAPK (p38 mitogen-activated protein kinase) pathway inhibitor SB-203580 (SB; 10 μg/h) for 3 to 5 days. After treatments, the middle cerebral arteries were tested ex vivo for response to Ach (10^{-5} mol/L) induced vasodilation. ERK pathway inhibition eliminated AngII and Ang-(1–7)–mediated vasodilation, whereas p38MAPK inhibition only exhibits slight ablation on angiotensin peptide restored vasodilation (AngII data replotted from McEwen et al 2009[28] with permission of the publisher. Copyright ©2009, the American Physiological Society). MAPK indicates mitogen-activated protein kinase. *Significantly different (P<0.05) from vehicle control (V); †significantly different (P<0.05) from AngII treatment alone; ‡significantly different (P<0.05) from Ang-(1–7) treatment alone.

demonstrate that Ang-(1–7), acting through the Mas1 receptor and not AT_1R, is able to restore microvessel angiogenesis in response to electric stimulation (Figure 1A). Many studies have shown continuous infusion of Ang-(1–7) at levels ≤160× higher than used here generate Ang-(1–7)/Mas1 receptor-dependent effects that counteract the deleterious actions of high levels of AngII.[4,13,16] Our data suggest that at equimolar low doses, Ang-(1–7) and AngII have some complementary rather than antagonistic functions as seen with elevated AngII.[12–14] High levels of Ang-(1–7) inhibit vessel growth in tumor xenografts[40]; however, a recent study has shown Ang-(1–7) stimulating a significant increase in sinusoidal endothelial cell sprouting and forming tubule structures out of cavernosal strips in culture.[41] We show enhancement of in vitro endothelial cell tube formation, in vivo skeletal muscle angiogenesis, and ex vivo MCA vasodilation by treatment with low-dose physiological concentrations of both Ang-(1–7) and AngII.

Although AT_1R signaling has been well characterized,[2] Mas1 receptor signaling mechanisms are only beginning to be elucidated. To further investigate the actions of low-dose Ang-(1–7) for comparison, we examined cellular signaling processes activated by Ang-(1–7) stimulation of the Mas1 receptor signaling complex. We then compared the results against low- and high-dose AngII RMVEC gene expression data (Table 3) and with known pathways influenced by AngII stimulation of AT_1R.[2] We found substantial overlap in Ang-(1–7)–stimulated Mas1 receptor signaling and AngII-stimulated AT_1R signaling. Gene expression analysis showed that equimolar, low doses of Ang-(1–7)/Mas1 and AngII/AT1 receptor-ligand interactions lead to the activation of similar pathways, whereas high-dose AngII exhibited downregulation of the same pathways (Tables 2 and 3). Initial functional tests showed that Ang-(1–7) significantly increased the ability of RMVECs to form tube-like structures in vitro (data not shown). Importantly, this verified that Ang-(1–7) stimulation had a functional effect on the specific endothelial cell population used for subsequent proteomic and gene expression signaling pathway analysis. Proteomic MS/MS analysis of Ang-(1–7)–stimulated Mas1 receptor signaling complexes implicated proteins involved in the regulation of G-protein, ERK/MAPK, PI3K/AKT/mTOR, CARD10/NFκB, and phosphoinositol signaling (Table 1).

Gene expression analysis indicated that there were significant increases in expression of the VEGFR family, G-protein signaling, including CDC42, ERK1/2, and MAPK signaling, phosphoinositol signaling, and phospholipase signaling (Table 2). The significant increase in expression of the VEGFR1 and VEGFR2 is significant because these are important downstream effectors resulting from RAS/MAPK/ERK signaling and have been shown to mediate angiogenesis through vascular endothelial growth factor signaling in other models.[27] Together these data suggest that the Mas1 receptor signals through the RHO family of GTPases (RHO, RAS, RAC), ERK/MAPK, PI3K/PRKD1/AKT/mTOR-mediated cell survival signaling to promote normal endothelial function, cell survival, angiogenesis, and vasodilation (Figure 3). In addition, the decreased detection of CARD10 after Ang-(1–7) stimulation suggests a downregulation of NFκB signaling and an anti-inflammatory response. These Ang-(1–7)/Mas1 receptor signaling complex pathway data are supported by previous literature showing that Ang-(1–7) induced global changes in phosphorylation and protein expression related to ERK1/2, AKT1, NFκB, and vascular endothelial growth factor signaling.[19,20] The direct signaling complex and pathways implicated by this data set correlate with early stage, but not later stage, global alterations of phosphorylation detected in the previous phosphoproteomic study on Ang-(1–7) signaling.[19] Interestingly, the gene expression analysis presented here for low-dose AngII stimulation demonstrated a similar upregulation in CDC42, VEGFR1, p38MAPK, and RAC signaling to that of Ang-(1–7) suggesting signaling overlap, whereas high-dose AngII exhibited a marked decrease in these signaling pathways supporting previously observed pathophysiologic phenotypes (Table 3).

Modulation of AKT-dependent pathways by Ang-(1–7) stimulation of the Mas1 receptor, including increased mTOR and decreased NFκB activity, is supported by previous literature demonstrating that Ang-(1–7) regulates endothelial nitric oxide synthase activation through PIK3/PKB/AKT-dependent pathways.[10,19,20] In addition, it is known that AngII increases p38MAPK and ERK1/2 activities.[42] Pathway data presented here suggest p38MAPK and ERK1/2 involvement in Ang-(1–7)–mediated Mas1 signaling in the endothelium (Figure 3). Previous studies have shown that Ang-(1–7) enhances AngII

activation of ERK1/2 signaling in bone marrow–derived dendritic cells; a result that is blocked with the Mas1 antagonist A779.[43] These studies all point to a convergence between the AngII/AT_1R and the Ang-(1–7)/Mas1 receptor signaling networks through ERK1/2 and p38MAPK signaling, as well as NFκB-mediated inflammatory responses.[3,19,44] Furthermore, our data implicate ERK1/2 and p38MAPK as candidate points of convergence for AngII/AT_1R and Ang-(1–7)/Mas1 receptor signaling in the endothelium important for vasodilation and angiogenesis (Figures 5 and 6). However, our pathway activation data suggest that p38MAPK is activated in our system but not dependent only on Ang-(1–7), whereas ERK1/2 is an essential molecule for the Ang-(1–7)/Mas1 receptor vasodilation and angiogenic processes shown here (Figure 4). Further analysis of these targets using in vitro RMVEC tube formation after no stimulation, AngII stimulation, or Ang-(1–7) stimulation plus or minus ERK1/2 or p38MAPK inhibition indicated that AngII/AT_1R and Ang-(1–7)/Mas1 receptor signaling converge on these molecules for the promotion of in vitro angiogenesis (Figure 5). These data also suggest that ERK1/2 is a common point of convergence for angiogenesis pathways in general as it also inhibited in the vehicle control, whereas p38MAPK seems more specific for these renin–angiotensin system pathways. Similar conditions applied to rat MCAs indicated that AngII/AT_1R and Ang-(1–7)/Mas1 receptor signaling through ERK1/2 was essential for vasodilation, whereas p38MAPK contributed but was not essential for this process, suggesting that there may be other MAPKs involved in signaling to ERK1/2 in this instance (Figure 6). These results suggest that ERK1/2 is a point of essential convergence for acetylcholine-induced vasodilation in these pathways and angiogenesis signaling processes, including Ang-(1–7)/Mas1 and AngII/AT_1R signaling.

It is important to note that AT_1R was consistently identified as part of the Ang-(1–7)/MAS1 receptor signaling complex. It is well known that Ang-(1–7) does not signal through AT_1R. Santos et al[8] showed that Ang-(1–7) is an endogenous ligand for the G protein–coupled receptor Mas independent of AT_1R. Our data in Figure 1 also demonstrate the specificity of Ang-(1–7) for the Mas1 receptor independent of AT_1R stimulation. However, it is important to note that there have been previous reports of a functional interaction among the Mas1 receptor, AT_1R, and AT_2R that may be of importance to the receptor signaling.[45,46] It has also been shown that EGF receptor transactivation is essential for ERK1/2 signaling mediated by AT_1R.[27,28] We cannot rule out a similar paradigm here given the presence of the AT_1R in the Ang-(1–7)/MAS1 receptor signaling complex and the convergence of AT_1R and Mas1 signaling. Although our vasodilation and angiogenesis data using AT_1R and Mas1 receptor inhibitors show that Ang-(1–7) acts specifically through the Mas1 receptor (Figure 1), we cannot rule out that there could be formation of a complex between the receptors at the cell surface required for signaling. This transactivation and the potential for tissue-/cell-specific differences in the balance between AT_1R and Mas1 receptor signaling warrant examination in future studies.

The results of the current study suggest that AngII/AT_1R and Ang-(1–7)/Mas1 receptor signaling converge on an essential common pathway of importance for both angiogenesis and vasodilation in the renin–angiotensin system involving ERK1/2 and p38MAPK signaling. The study also indicated ERK1/2 signaling related specifically to phenotypes directed by AT_1R and Mas1 receptor; it seems to be essential for global angiogenesis signaling processes as a point of convergence and demonstrated an Ang-(1–7) activation dependence in our system (Figure 4). These data, along with inhibition studies in Figures 5 and 6, suggested that ERK1/2 was essential for Ang-(1–7)/Mas1 receptor-mediated vasodilation and angiogenesis. Overall, the innovative approach used for signal pathway analysis in combination with in vitro, ex vivo, and in vivo functional assays allowed for an increased understanding of Ang-(1–7)–stimulated Mas1 receptor signaling in relation to low, subpressor dose AngII (normal physiological levels) signaling through AT_1R. Our data in endothelial cells and data from other laboratories in other cell types have shown that dosage of the peptide is a significant factor in how a cell responds to AngII or Ang-(1–7).[47,48] There is also growing evidence that AngII/AT1and Ang-(1–7)/Mas1 signaling are co-occurring, dose-dependent, and more complex than once thought.[13] The complimentary action of equimolar, low-dose AngII and Ang-(1–7) suggests a delicate balance in the regulation of these 2 peptides at both the receptor and the intracellular signaling level.

Acknowledgments

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Disclosures

None.

References

1. Moon JY. Recent update of renin-angiotensin-aldosterone system in the pathogenesis of hypertension. *Electrolyte Blood Press*. 2013;11:41–45. doi: 10.5049/EBP.2013.11.2.41.
2. Balakumar P, Jagadeesh G. A century old renin-angiotensin system still grows with endless possibilities: AT1 receptor signaling cascades in cardiovascular physiopathology. *Cell Signal*. 2014;26:2147–2160. doi: 10.1016/j.cellsig.2014.06.011.
3. Xu P, Sriramula S, Lazartigues E. ACE2/ANG-(1-7)/Mas pathway in the brain: the axis of good. *Am J Physiol Regul Integr Comp Physiol*. 2011;300:R804–R817. doi: 10.1152/ajpregu.00222.2010.
4. Wang W, Bodiga S, Das SK, Lo J, Patel V, Oudit GY. Role of ACE2 in diastolic and systolic heart failure. *Heart Fail Rev*. 2012;17:683–691. doi: 10.1007/s10741-011-9259-x.
5. Kamo T, Akazawa H, Komuro I. Pleiotropic effects of angiotensin II receptor signaling in cardiovascular homeostasis and aging. *Int Heart J*. 2015;56:249–254. doi: 10.1536/ihj.14-429.
6. Durand MJ, Raffai G, Weinberg BD, Lombard JH. Angiotensin-(1-7) and low-dose angiotensin II infusion reverse salt-induced endothelial dysfunction via different mechanisms in rat middle cerebral arteries. *Am J Physiol Heart Circ Physiol*. 2010;299:H1024–H1033. doi: 10.1152/ajpheart.00328.2010.

7. Petersen MC, Munzenmaier DH, Greene AS. Angiotensin II infusion restores stimulated angiogenesis in the skeletal muscle of rats on a high-salt diet. *Am J Physiol Heart Circ Physiol*. 2006;291:H114–H120. doi: 10.1152/ajpheart.01116.2005.
8. Santos RA, Simoes e Silva AC, Maric C, et al. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A*. 2003;100:8258–8263. doi: 10.1073/pnas.1432869100.
9. Benter IF, Yousif MH, Anim JT, Cojocel C, Diz DI. Angiotensin-(1-7) prevents development of severe hypertension and end-organ damage in spontaneously hypertensive rats treated with L-NAME. *Am J Physiol Heart Circ Physiol*. 2006;290:H684–H691. doi: 10.1152/ajpheart.00632.2005.
10. Sampaio WO, Souza dos Santos RA, Faria-Silva R, da Mata Machado LT, Schiffrin EL, Touyz RM. Angiotensin-(1-7) through receptor Mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. *Hypertension*. 2007;49:185–192. doi: 10.1161/01.HYP.0000251865.35728.2f.
11. Wang Y, Qian C, Roks AJ, Westermann D, Schumacher SM, Escher F, Schoemaker RG, Reudelhuber TL, van Gilst WH, Schultheiss HP, Tschöpe C, Walther T. Circulating rather than cardiac angiotensin-(1-7) stimulates cardioprotection after myocardial infarction. *Circ Heart Fail*. 2010;3:286–293. doi: 10.1161/CIRCHEARTFAILURE.109.905968.
12. Raffai G, Durand MJ, Lombard JH. Acute and chronic angiotensin-(1-7) restores vasodilation and reduces oxidative stress in mesenteric arteries of salt-fed rats. *Am J Physiol Heart Circ Physiol*. 2011;301:H1341–H1352. doi: 10.1152/ajpheart.00202.2011.
13. Xue H, Zhou L, Yuan P, Wang Z, Ni J, Yao T, Wang J, Huang Y, Yu C, Lu L. Counteraction between angiotensin II and angiotensin-(1-7) via activating angiotensin type I and Mas receptor on rat renal mesangial cells. *Regul Pept*. 2012;177:12–20. doi: 10.1016/j.regpep.2012.04.002.
14. Souza ÁP, Sobrinho DB, Almeida JF, Alves GM, Macedo LM, Porto JE, Vêncio EF, Colugnati DB, Santos RA, Ferreira AJ, Mendes EP, Castro CH. Angiotensin II type 1 receptor blockade restores angiotensin-(1-7)-induced coronary vasodilation in hypertrophic rat hearts. *Clin Sci (Lond)*. 2013;125:449–459. doi: 10.1042/CS20120519.
15. Loot AE, Roks AJ, Henning RH, Tio RA, Suurmeijer AJ, Boomsma F, van Gilst WH. Angiotensin-(1-7) attenuates the development of heart failure after myocardial infarction in rats. *Circulation*. 2002;105:1548–1550.
16. Guimaraes PS, Santiago NM, Xavier CH, Velloso EP, Fontes MA, Santos RA, Campagnole-Santos MJ. Chronic infusion of angiotensin-(1-7) into the lateral ventricle of the brain attenuates hypertension in DOCA-salt rats. *Am J Physiol Heart Circ Physiol*. 2012;303:H393–H400. doi: 10.1152/ajpheart.00075.2012.
17. Fraga-Silva RA, Da Silva DG, Montecucco F, Mach F, Stergiopulos N, da Silva RF, Santos RA. The angiotensin-converting enzyme 2/angiotensin-(1-7)/Mas receptor axis: a potential target for treating thrombotic diseases. *Thromb Haemost*. 2012;108:1089–1096. doi: 10.1160/TH12-06-0396.
18. Liu GC, Oudit GY, Fang F, Zhou J, Scholey JW. Angiotensin-(1-7)-induced activation of ERK1/2 is cAMP/protein kinase A-dependent in glomerular mesangial cells. *Am J Physiol Renal Physiol*. 2012;302:F784–F790. doi: 10.1152/ajprenal.00455.2011.
19. Verano-Braga T, Schwämmle V, Sylvester M, Passos-Silva DG, Peluso AA, Etelvino GM, Santos RA, Roepstorff P. Time-resolved quantitative phosphoproteomics: new insights into Angiotensin-(1-7) signaling networks in human endothelial cells. *J Proteome Res*. 2012;11:3370–3381. doi: 10.1021/pr3001755.
20. Meinert C, Gembardt F, Böhme I, Tetzner A, Wieland T, Greenberg B, Walther T. Identification of intracellular proteins and signaling pathways in human endothelial cells regulated by angiotensin-(1-7). *J Proteomics*. 2016;130:129–139. doi: 10.1016/j.jprot.2015.09.020.
21. Karcher JR, Greene AS. Bone marrow mononuclear cell angiogenic competency is suppressed by a high-salt diet. *Am J Physiol Cell Physiol*. 2014;306:C123–C131. doi: 10.1152/ajpcell.00164.2013.
22. Hoffman MJ, Flister MJ, Nunez L, Xiao B, Greene AS, Jacob HJ, Moreno C. Female-specific hypertension loci on rat chromosome 13. *Hypertension*. 2013;62:557–563. doi: 10.1161/HYPERTENSIONAHA.113.01708.
23. Freed JK, Greene AS. Proteomic analysis of shear stress-mediated protection from TNF-alpha in endothelial cells. *Microcirculation*. 2010;17:259–270. doi: 10.1111/j.1549-8719.2010.00031.x.
24. Moreno C, Kaldunski ML, Wang T, Roman RJ, Greene AS, Lazar J, Jacob HJ, Cowley AW Jr. Multiple blood pressure loci on rat chromosome 13 attenuate development of hypertension in the Dahl S hypertensive rat. *Physiol Genomics*. 2007;31:228–235. doi: 10.1152/physiolgenomics.00280.2006.
25. Widlansky ME, Gokce N, Keaney JF Jr, Vita JA. The clinical implications of endothelial dysfunction. *J Am Coll Cardiol*. 2003;42:1149–1160.
26. Hoffmann BR, Wagner JR, Prisco AR, Janiak A, Greene AS. Vascular endothelial growth factor-A signaling in bone marrow-derived endothelial progenitor cells exposed to hypoxic stress. *Physiol Genomics*. 2013;45:1021–1034. doi: 10.1152/physiolgenomics.00070.2013.
27. McEwen ST, Schmidt JR, Somberg L, Cruz Lde L, Lombard JH. Time-course and mechanisms of restored vascular relaxation by reduced salt intake and angiotensin II infusion in rats fed a high-salt diet. *Microcirculation*. 2009;16:220–234. doi: 10.1080/10739680802544177.
28. McEwen ST, Balus SF, Durand MJ, Lombard JH. Angiotensin II maintains cerebral vascular relaxation via EGF receptor transactivation and ERK1/2. *Am J Physiol Heart Circ Physiol*. 2009;297:H1296–H1303. doi: 10.1152/ajpheart.01325.2008.
29. Ghiadoni L, Versari D, Magagna A, Kardasz I, Plantinga Y, Giannarelli C, Taddei S, Salvetti A. Ramipril dose-dependently increases nitric oxide availability in the radial artery of essential hypertension patients. *J Hypertens*. 2007;25:361–366. doi: 10.1097/HJH.0b013e3280115901.
30. Hitomi H, Kiyomoto H, Nishiyama A. Angiotensin II and oxidative stress. *Curr Opin Cardiol*. 2007;22:311–315. doi: 10.1097/HCO.0b013e3281532b53.
31. Ogawa S, Mori T, Nako K, Kato T, Takeuchi K, Ito S. Angiotensin II type 1 receptor blockers reduce urinary oxidative stress markers in hypertensive diabetic nephropathy. *Hypertension*. 2006;47:699–705. doi: 10.1161/01.HYP.0000203826.15076.4b.
32. Touyz RM, Schiffrin EL. Increased generation of superoxide by angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients: role of phospholipase D-dependent NAD(P)H oxidase-sensitive pathways. *J Hypertens*. 2001;19:1245–1254.
33. Zhu J, Drenjancevic-Peric I, McEwen S, Friesema J, Schulta D, Yu M, Roman RJ, Lombard JH. Role of superoxide and angiotensin II suppression in salt-induced changes in endothelial Ca2+ signaling and NO production in rat aorta. *Am J Physiol Heart Circ Physiol*. 2006;291:H929–H938. doi: 10.1152/ajpheart.00692.2005.
34. de Resende MM, Amaral SL, Moreno C, Greene AS. Congenic strains reveal the effect of the renin gene on skeletal muscle angiogenesis induced by electrical stimulation. *Physiol Genomics*. 2008;33:33–40. doi: 10.1152/physiolgenomics.00150.2007.
35. Kaczorowski CC, Stodola TJ, Hoffmann BR, Prisco AR, Liu PY, Didier DN, Karcher JR, Liang M, Jacob HJ, Greene AS. Targeting the endothelial progenitor cell surface proteome to identify novel mechanisms that mediate angiogenic efficacy in a rodent model of vascular disease. *Physiol Genomics*. 2013;45:999–1011. doi: 10.1152/physiolgenomics.00097.2013.
36. Stodola TJ, de Resende MM, Sarkis AB, Didier DN, Jacob HJ, Huebner N, Hummel O, Saar K, Moreno C, Greene AS. Characterization of the genomic structure and function of regions influencing renin and angiogenesis in the SS rat. *Physiol Genomics*. 2011;43:808–817. doi: 10.1152/physiolgenomics.00171.2010.
37. de Resende MM, Greene AS. Effect of ANG II on endothelial cell apoptosis and survival and its impact on skeletal muscle angiogenesis after electrical stimulation. *Am J Physiol Heart Circ Physiol*. 2008;294:H2814–H2821. doi: 10.1152/ajpheart.00095.2008.
38. Amaral SL, Linderman JR, Morse MM, Greene AS. Angiogenesis induced by electrical stimulation is mediated by angiotensin II and VEGF. *Microcirculation*. 2001;8:57–67.
39. Linderman JR, Kloehn MR, Greene AS. Development of an implantable muscle stimulator: measurement of stimulated angiogenesis and poststimulus vessel regression. *Microcirculation*. 2000;7:119–128.
40. Soto-Pantoja DR, Menon J, Gallagher PE, Tallant EA. Angiotensin-(1-7) inhibits tumor angiogenesis in human lung cancer xenografts with a reduction in vascular endothelial growth factor. *Mol Cancer Ther*. 2009;8:1676–1683. doi: 10.1158/1535-7163.MCT-09-0161.
41. Singh N, Vasam G, Pawar R, Jarajapu YP. Angiotensin-(1-7) reverses angiogenic dysfunction in corpus cavernosum by acting on the microvasculature and bone marrow-derived cells in diabetes. *J Sex Med*. 2014;11:2153–2163. doi: 10.1111/jsm.12620.
42. Tamarat R, Silvestre JS, Durie M, Levy BI. Angiotensin II angiogenic effect *in vivo* involves vascular endothelial growth factor- and inflammation-related pathways. *Lab Invest*. 2002;82:747–756.
43. James PA, Oparil S, Carter BL, et al. 2014 Evidence-based guideline for the management of high blood pressure in adults: report from the panel members appointed to the Eighth Joint National Committee (JNC 8). *JAMA*. 2014;311:507–520. doi: 10.1001/jama.2013.284427.
44. Nie W, Yan H, Li S, Zhang Y, Yu F, Zhu W, Fan F, Zhu J. Angiotensin-(1-7) enhances angiotensin II induced phosphorylation of ERK1/2 in mouse bone marrow-derived dendritic cells. *Mol Immunol*. 2009;46:355–361. doi: 10.1016/j.molimm.2008.10.022.

45. Von Bohlen und Halbach O, Walther T, Bader M, Albrecht D. Interaction between Mas and the angiotensin AT1 receptor in the amygdala. *J Neurophysiol*. 2000;83:2012–2021.
46. Castro CH, Santos RA, Ferreira AJ, Bader M, Alenina N, Almeida AP. Evidence for a functional interaction of the angiotensin-(1-7) receptor Mas with AT1 and AT2 receptors in the mouse heart. *Hypertension*. 2005;46:937–942. doi: 10.1161/01.HYP.0000175813.04375.8a.
47. Zimpelmann J, Burns KD. Angiotensin-(1-7) activates growth-stimulatory pathways in human mesangial cells. *Am J Physiol Renal Physiol*. 2009;296:F337–F346. doi: 10.1152/ajprenal.90437.2008.
48. Zhou LM, Shi Z, Gao J, Han Y, Yuan N, Gao XY, Zhu GQ. Angiotensin-(1-7) and angiotension II in the rostral ventrolateral medulla modulate the cardiac sympathetic afferent reflex and sympathetic activity in rats. *Pflugers Arch*. 2010;459:681–688. doi: 10.1007/s00424-010-0793-5.

Highlights

- This study demonstrates the importance of the synergistic effects of ideal plasma concentrations of angiotensin 1 to 7 (Ang-(1–7)) and angiotensin II (AngII) in normal physiological homeostasis.
- Low, subpressor doses of angiotensin 1 to 7 on the Mas1 receptor and AngII on AngII receptor type 1 exhibit similar signaling effects contributing to angiogenesis and endothelial-dependent vasodilation.
- At low doses, the effects of the angiotensin 1 to 7 on the Mas1 receptor and AngII on the AngII receptor type 1 exhibit similar pathway points of convergence that are opposed to high-dose AngII.
- Extracellular signal–related kinases 1/2 was essential for vasodilation and angiogenesis after angiotensin 1 to 7 signaling through the Mas1 receptor, whereas p38 can contribute but was not essential.