Renin–Angiotensin System

Angiotensin-(1–7) Modulates Renal Vascular Resistance Through Inhibition of p38 Mitogen-Activated Protein Kinase in Apolipoprotein E–Deficient Mice

Sebastian A. Potthoff, Michael Fähling, Tilman Clasen, Susanne Mende, Bassam Ishak, Tatsiana Suvorava, Stefanie Stamer, Manuel Thieme, Sema H. Sivritas, Georg Kojda, Andreas Patzak, Lars C. Rump, Johannes Stegbauer

Abstract—Apolipoprotein E–deficient (apoE(−/−)) mice fed on Western diet are characterized by increased vascular resistance and atherosclerosis. Previously, we have shown that chronic angiotensin (Ang)-(1–7) treatment ameliorates endothelial dysfunction in apoE(−/−) mice. However, the mechanism of Ang-(1–7) on vasoconstrictor response to Ang II is unknown. To examine Ang-(1–7) function, we used apoE(−/−) and wild-type mice fed on Western diet that were treated via osmotic minipumps either with Ang-(1–7) (82 μg/kg per hour) or saline for 6 weeks. We show that Ang II–induced renal pressor response was significantly increased in apoE(−/−) compared with wild-type mice. This apoE(−/−)-specific response is attributed to reactive oxygen species–mediated p38 mitogen–activated protein kinase activation and subsequent phosphorylation of myosin light chain (MLC20), causing renal vasoconstriction. Here, we provide evidence that chronic Ang-(1–7) treatment attenuated the renal pressor response to Ang II in apoE(−/−) mice to wild-type levels. Ang-(1–7) treatment significantly decreased renal inducible nicotinamide adenine dinucleotide phosphate subunit p47phox levels and, thus, reactive oxygen species production that in turn causes decreased p38 mitogen-activated protein kinase activity. The latter has been confirmed by administration of a specific p38 mitogen-activated protein kinase inhibitor SB203580 (5 μmol/L), causing a reduced renal pressor response to Ang II in apoE(−/−) but not in apoE(−/−) mice treated with Ang-(1–7). Moreover, Ang-(1–7) treatment had no effect in Mas(−/−)/apoE(−/−) double-knockout mice confirming the specificity of Ang-(1–7) action through the Mas-receptor. In summary, Ang-(1–7) modulates vascular function via Mas-receptor activation that attenuates pressor response to Ang II in apoE(−/−) mice by reducing reactive oxygen species–mediated p38 mitogen-activated protein kinase activity. (Hypertension. 2014;63:265–272.) • Online Data Supplement

Key Words: angiotensin-(1–7) • apolipoprotein E • p38 mitogen-activated protein kinase

The increasing prevalence of obesity represents a significant health burden in modern societies. It significantly contributes to the cardiovascular morbidity and mortality.1 Hypercholesterolemia, atherosclerosis, and hypertension are common in obese subjects. Atherosclerosis and hypertension are characterized by increased reactive oxygen species (ROS) production, affecting vascular smooth muscle cell migration and proliferation, and, therefore, play a pivotal role in their pathogenesis.2,3

Apolipoprotein E–deficient mice (apoE(−/−)), an animal model of severe hypercholesterolemia, are prone to atherosclerosis and are characterized by severe vascular dysfunction.3,4 This is accompanied by increased vasoconstrictor response to angiotensin II (Ang II).6 This development can be drastically accelerated by a high-fat Western diet.7

Angiotensin II, the main effector of the renin–angiotensin system, contributes to the development of impaired vascular function in apoE(−/−) mice.8 Deficiency of the angiotensin II type 1A receptor (AT1aR) in apoE(−/−) mice ameliorates atherosclerosis and endothelial dysfunction, highlighting the importance of the renin–angiotensin system in the pathogenesis of vascular injury.9,10

Recent studies indicate that p38 mitogen-activated protein kinase (MAPK) activation is involved in increased Ang II–dependent vasoconstriction. Inhibition of p38 MAPK has been shown to improve endothelial function and decrease Ang II–dependent vasoconstriction.11–13 Ang II activates the p38 MAPK through increased ROS generation in vitro.14,15 p38 MAPK activation is thought to be a calmodulin–calcium-independent pathway of Ang II–mediated vasoconstriction. Activation of p38 MAPK through phosphorylation enhances Ang II–dependent phosphorylation of the myosin light chain (MLC20), thus increasing contractile response.16

Received August 23, 2013; first decision September 11, 2013; revision accepted October 4, 2013.
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The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.113.02289/-/DC1/.
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Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.113.02289

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Besides Ang II as the main classical effector of the renin–angiotensin system, Ang-(1–7) has been shown to play a significant role in physiological and pathological states. The heptapeptide Ang-(1–7) is derived from Ang I or Ang II by several peptidases, including the carboxypeptidases angiotensin-converting enzyme (ACE) and ACE2. However, the generation of Ang-(1–7) from Ang II by ACE2 seems to be the preferred pathway. In fact, deletion of ACE2 accentuates atherosclerosis in apoE(−/−) mice, which is contributed to decreased Ang-(1–7) formation. Ang-(1–7) acts through its only receptor called Mas. Previously it has been shown that atherogenesis in apoE(−/−) mice can be inhibited by pharmacological Mas activation. Mas-deficient mice show increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, leading to endothelial dysfunction and strain-dependent hypertension through an increase in ROS abundance.

Previously, we and others have shown that chronic Ang-(1–7) treatment ameliorates endothelial dysfunction in apoE(−/−) mice on a high-fat diet. However, the effect of Ang-(1–7) on vasoconstrictor response to Ang II is yet unknown. Therefore, the aim of the present study was to examine whether chronic Ang-(1–7) treatment affects the renal vasoconstrictor response to Ang II in apoE(−/−) mice. Furthermore, we examined the effect of Ang-(1–7) treatment on p38 MAPK activity and ROS abundance in these animals. To confirm that Ang-(1–7) mediates its effects through the Mas-receptor, we performed additional experiments in Mas(−/−)/apoE(−/−) double-knockout mice.

### Methods

#### Animal Care

Wild-type (WT), apoE(−/−), and Mas(−/−)/apoE(−/−) mice were obtained from an in-house breeding at the local animal care facility. Mice were on a C57Bl/6j background. littermates were used as controls. The animals were housed in type III Makrolon polycarbonate cages at 45% humidity, 20°C to 22°C temperature and a 12-hour day and night cycle with free access to water and food.

The investigations were conducted to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996). Detailed Materials and Methods are available in the online-only Supplement.

#### Results

Six-week-old WT (group 1), apoE(−/−) (groups 2–4), and Mas(−/−)/apoE(−/−) double-knockout (groups 5 and 6) mice were fed a high fat, cholesterol-enriched diet. After 6 weeks, apoE(−/−) mice (groups 2 and 3) were treated with either saline or Ang-(1–7) (82 µg/kg per hour) via osmotic mini-pumps subcutaneously, as described previously. Another group of apoE(−/−) mice was treated with a p38 MAPK inhibitor (BIRB796, 50 mg/kg per day) from the age of 6 weeks (group 4). In addition, Mas(−/−)/apoE(−/−) double-knockout mice (groups 5 and 6) were treated as groups 2 and 3. In all groups, osmotic minipumps were replaced after 3 weeks.

No significant differences were observed in blood pressure and lipid profile between Ang-(1–7)–treated and untreated apoE(−/−) mice after 12 weeks of high-fat diet. Interestingly, in Mas(−/−)/apoE(−/−) double-knockout mice, cholesterol levels were significantly higher when compared with the Ang-(1–7)–treated double-knockout group. Untreated Mas(−/−)/apoE(−/−) double-knockout mice also showed a higher level of triglycerides compared with the WT control group (Table).

### Chronic Ang-(1–7) Treatment Ameliorates Increased Pressor Response to Ang II in ApoE(−/−)

To evaluate the influence of Ang-(1–7) treatment on Ang II–induced pressor response in renal resistance vessels of apoE(−/−) mice, kidneys were isolated and perfused as described previously. Renal pressor response to Ang II was significantly increased in apoE(−/−) compared with WT mice. Interestingly, chronic treatment with Ang-(1–7) attenuates renal pressor response significantly (Figure 1A; Emax: WT, 114.2±6.1 mm Hg; apoE(−/−), 149.6±4.7 mm Hg; apoE+ApoE(−/−), 107.6±9.3 mm Hg). In WT mice, chronic Ang-(1–7) treatment had no effect on Ang II–dependent pressor response (Figure S1 in the online-only Data Supplement).

Consistently, Ang-(1–7) treatment significantly attenuated phospho-MLC20 levels in apoE(−/−) mice (Figure 1B and 1C). In contrast to chronic Ang-(1–7) treatment, acute administration of Ang-(1–7) (0.1 µmol/L) had no significant effect on renal pressor response to Ang II (Figure S2). To examine a potential influence of chronic Ang-(1–7) treatment on gene expression, mRNA levels of ACE2, AT1aR, AT2R, and the Mas-receptor were determined by quantitative polymerase chain reaction. As shown in Figure S3, chronic Ang-(1–7) treatment did not influence the expression levels of these candidate genes.

### Ang-(1–7) Reduces ROS Generation Through a p47phox-Dependent Mechanism

Recently, it has been reported that ROS production is increased in apoE(−/−) mice. To test whether Ang-(1–7) affects ROS production, renal oxygen radical levels and

### Table. Systolic Blood Pressure and Lipid Profile of Groups on High-Fat Diet at Age of 18 weeks (12 Weeks of High-Fat Diet)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>WT</th>
<th>apoE(−/−)</th>
<th>apoE(−/−)+Ang-(1–7)</th>
<th>Mas(−/−)/apoE(−/−)</th>
<th>apoE(−/−)+Ang-(1–7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>110±2</td>
<td>108±2</td>
<td>107±2</td>
<td>113±2</td>
<td>114±2</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>4.4±1.0</td>
<td>34.0±2.5†</td>
<td>34.9±2.6†</td>
<td>40.5±3.4†</td>
<td>27.6±3.0†</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>0.4±0.1</td>
<td>1.4±0.3</td>
<td>0.9±0.2</td>
<td>1.68±0.3*</td>
<td>1.28±0.2</td>
</tr>
</tbody>
</table>

| Data are mean±SEM (for lipid profile: WT, n=5; apoE(−/−): n=10; apoE(−/−)+Ang-(1–7): n=9; Mas(−/−)/apoE(−/−): n=8; Mas(−/−)/apoE(−/−)+Ang-(1–7): n=10 and for blood pressure, each group: n=5). Significant differences are marked: *P<0.05, †P<0.001, group comparison vs WT. ‡P<0.05: Mas(−/−)/apoE(−/−) vs Mas(−/−)/apoE(−/−)+Ang-(1–7). One-way ANOVA followed by Bonferroni multiple comparison post hoc test. Ang-(1–7) indicates Angiotensin-(1–7); apoE(−/−), apolipoprotein E–deficient mice; and WT, wild type.
urinary 8-isoprostane excretion were measured. As shown in Figure 2A and 2B, chronic Ang-(1–7) treatment significantly reduced urinary 8-isoprostane levels and lucigenin-enhanced chemiluminescence in renal cortex samples.

To examine the influence of Ang-(1–7) treatment on ROS production, we analyzed the expression levels of subunits of the NADPH enzyme family in renal cortex lysates. Expression of the NADPH oxidase subunit p47phox, an Ang II inducible NADPH subunit, was significantly increased in apoE(−/−) compared with WT mice.28 Interestingly, Ang-(1–7) treatment restored p47phox expression to WT levels, suggesting that Ang-(1–7) affects ROS abundance through modulation of NADPH oxidase expression (Figure 2C).

Chronic Ang-(1–7) Treatment Improves Vascular Function by Reducing MAPK p38 Activation

To evaluate whether Ang-(1–7) reduces vascular reactivity through a p38 MAPK-mediated mechanism, we measured p38 MAPK activity in renal cortex and preglomerular resistance vessel samples. Phospho-p38/p38 ratio was increased in apoE(−/−) compared with WT mice (Figure 3A and 3B).

Chronic Ang-(1–7) treatment restored the level of phospho-p38 MAPK to WT levels (Figure 3A and 3B). To confirm whether the increased p38 MAPK activation is responsible for the augmented vascular reactivity to Ang II, renal pressor response to Ang II was measured in the presence or in the absence of the specific p38 MAPK inhibitor SB203580 (5 µmol/L). In
kidneys of WT mice, pressor response induced by Ang II was not significantly different with or without MAPK p38 inhibition (Figure 3C). However, in apoE(−/−) mice, pressor response to Ang II was significantly attenuated in the presence of SB203580 (5 μmol/L; Figure 3D). In contrast, acute p38 MAPK inhibition did not significantly affect Ang II–induced pressor response in apoE(−/−) mice chronically treated with Ang(1–7), confirming the effect of Ang(1–7) treatment on p38 MAPK-mediated vascular reactivity (Figure 3E). To evaluate whether the increased renal pressor response to Ang II is solely related to increased p38 activity, we performed additional experiments with the specific extracellular-signal-regulated kinase (ERK) 1/2 inhibitor PD98059 (5 μmol/L). PD98059 had no effect in WT or apoE(−/−) mice (Figure S4).

**Deletion of Mas-Receptor Abolishes Ang-(1–7) Effect in ApoE(−/−)**

To test whether the observed effects of Ang-(1–7) were mediated by Mas-receptor activation, we generated Mas(−/−)/apoE(−/−) double-knockout mice. In Mas(−/−)/apoE(−/−) double-knockout mice, chronic Ang(1–7) treatment did not influence renal pressor response (Figure 4A) and urinary 8-isoprostane excretion (Figure 4B). SB203580 (5 μmol/L) significantly reduced pressor responses to Ang II in kidneys of apoE(−/−) mice treated with Ang-(1–7) (Figure 4C). Western blot analysis of renal cortex lysates evaluating the phospho-p38/p38 mitogen-activated protein kinase (MAPK) ratio. Ratio was increased in apolipoprotein E–deficient (apoE(−/−)) mice when compared with wild-type (WT) and apoE(−/−) mice treated with angiotensin (Ang)-(1–7). WT vs apoE(−/−): 1.00±0.03 vs 2.83±0.24, *P<0.05; apoE(−/−) vs apoE(−/−)+Ang-(1–7): 2.83±0.24 vs 1.29±0.10, ++P<0.01; WT vs apoE(−/−)+Ang-(1–7): 1.00±0.03 vs 1.29±0.10, #P=NS; n=4; each group. One-way ANOVA followed by Bonferroni multiple comparison post hoc test. A representative Western blot of p38 and phospho-p38 MAPK expression in renal cortex lysates is shown.

**Figure 3. A**, Western blot analysis of renal cortex lysates evaluating the phospho-p38/p38 mitogen-activated protein kinase (MAPK) ratio. Ratio was increased in apolipoprotein E–deficient (apoE(−/−)) mice when compared with wild-type (WT) and apoE(−/−) mice treated with angiotensin (Ang)-(1–7). WT vs apoE(−/−): 0.65±0.11 vs 2.00±0.18, ***P<0.001; apoE(−/−) vs apoE(−/−)+Ang(1–7): 2.00±0.18 vs 1.14±0.10, +++P<0.001; WT vs apoE(−/−)+Ang(1–7): 0.65±0.11 vs 1.14±0.10, #P=NS; n=8; each group. Data represent mean±SEM. One-way ANOVA followed by Bonferroni multiple comparison post hoc test. C, Ang II–induced pressor response is not altered in the presence of the p38 MAPK inhibitor SB203580 (5 μmol/L) in kidneys of WT mice. (WT, n=23; WT+SB203580, n=14). D, In kidneys of apoE(−/−) mice, p38 MAPK inhibition (SB203580; 5 μmol/L) significantly attenuated Ang II–induced pressor response. (apoE(−/−), n=14; apoE(−/−)+SB203580, n=11). E, Pressor response to Ang II in isolated, perfused kidneys of apoE(−/−) mice treated with Ang(1–7): p38 MAPK inhibition had no significant effect on Ang II–induced vasoconstriction (apoE(−/−)+Ang(1–7), n=9; apoE(−/−)+Ang(1–7)+SB203580, n=6). C–E, Data represents mean±SEM; (C) P=NS WT vs WT+SB203580; (D) **P<0.01, ***P<0.001: apoE(−/−) vs apoE(−/−)+SB203580; and (E) P=NS, apoE(−/−)+Ang(1–7) vs apoE(−/−)+Ang(1–7)+SB203580. Two-way ANOVA for repeated measurements followed by Bonferroni correction post hoc test.
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Mas(−/−)/apoE(−/−) double-knockout mice (Figure 4C) in a similar manner as seen in single apoE(−/−) mice (Figure 3D).

Chronic Inhibition of p38 MAPK Ameliorates Ang II–Dependent Vasoreactivity and Systemic Pressor Response

To elucidate whether decreased p38 MAPK activity observed in Ang(1–7)–treated mice is causative for the reduced renal pressor response to Ang II, we tested vascular reactivity in vivo and ex vivo after chronic p38 MAPK inhibition. Therefore, apoE(−/−) mice (group 4) were fed either on an oral p38 MAPK inhibitor (BIRB796, 50 mg/kg per day) or a placebo. In acute infusion experiments (in vivo) and in isolated perfused kidneys (ex vivo), Ang II–dependent pressor response was significantly attenuated in apoE(−/−) mice chronically treated with BIRB796 compared with placebo-treateed animals (Figure 5A and 5B).

Discussion

Hypercholesterolemia is an important risk factor for vascular dysfunction, atherosclerosis, and hypertension. ApoE(−/−) mice subject to high-fat Western diet are characterized by a pronounced vascular dysfunction and rapid progression of atherosclerosis, which is a result of impaired clearance of low-density lipoprotein, leading to downregulation of ROS scavenging mechanisms. In these mice, mechanisms of proper vasorelaxation and vasoconstriction are compromised because of hypercholesterolemia. Previously, we showed that apoE(−/−) fed on a high-fat diet exhibit impaired endothelial-dependent vasorelaxation. Chronic Ang(1–7) treatment improved NO bioavailability and, therefore, ameliorated endothelial dysfunction in these mice.

In the present study, we show that pressor response to Ang II is significantly increased in apoE(−/−) renal resistance vessels compared with WT animals and that chronic Ang(1–7) treatment abolished the elevated pressor response to Ang II. Therefore, Ang(1–7) does not only ameliorate endothelial dysfunction but also normalizes vasoconstriction in response to Ang II.

Ang II induces vasoconstriction through the activation of the AT1R. ApoE(−/−) mice are susceptible to Ang II–induced hypertension because chronic Ang II infusion causes increased endothelial dysfunction and accelerated progression of atherosclerosis. This influence is attributed to higher ROS abundance and a sustained inflammatory response in the vasculature. Because Ang(1–7) seems to counteract AT1R–mediated effects, it is coherent that inhibition of Ang(1–7) generation in ACE2-deficient apoE(−/−) mice accentuated vascular inflammation. Hence, Ang(1–7)–mediated activation of the Mas-receptor attenuated atherogenesis in apoE(−/−) mice. These studies clearly indicate a beneficial role of Ang(1–7) in atherogenesis. However, little is
known on changes in vascular function in apoE(−/−) mice and the effect of Ang-(1–7) treatment.

Here, we demonstrate that increased contractility in apoE(−/−) mice is accompanied by an increased phosphorylation of MLC20 in preglomerular resistance vessels. Higher basal MLC20 phosphorylation increases contraction at a given Ca2+ concentration.34 Because the p38 MAPK/MK2 pathway causes MLC20 phosphorylation, and p38 MAPK is activated by ROS, inflammatory cytokines, and hypertonicity, we hypothesized that ROS-mediated activation of p38 MAPK might have a direct effect on vessel contractility by increased MLC20 phosphorylation.15,16

We show that p38 MAPK activity is significantly elevated in preglomerular resistance vessels and renal cortex of apoE(−/−) mice. Because the expression of the inducible NADPH subunit p47phox was significantly increased in apoE(−/−) mice compared with WT mice, our data indicate an increase in ROS production capability, which in turn activates p38 MAPK. p47phox is a key component of NADPH oxidase enzyme complex and plays an important role in ROS production in the vasculature.35 Accordingly, specific markers of ROS are increased in apoE(−/−) compared with WT mice. It has been demonstrated that p47phox contributes significantly to vascular damage in apoE(−/−) mice because it is essential for the development of Ang II-dependent hypertension and atherosclerosis.36–38 In addition, deletion of p47phox resulted in an attenuated vasoconstrictor response to Ang II and decreased ROS production.39 In line with these observations, our data confirm that the elevated pressor response to Ang II seen in apoE(−/−) mice is caused by ROS-mediated p38 MAPK activation and subsequent MLC20 phosphorylation.

Accordingly, we demonstrate that acute inhibition of p38 MAPK with SB203580, as well as chronic inhibition of p38 MAPK by oral application of BIRB796, reduced pressor response to Ang II (in vivo and ex vivo). This effect was not observed using an ERK 1/2 inhibitor. Our results are in line with previous studies showing that increased p38 MAPK activity enhances vascular contractility in vitro and in vivo.38,41

A possible contributor counteracting the exaggerated pressor response to Ang II might be increased Ang-(1–7)–mediated NO bioavailability. However, we previously showed that Ang-(1–7) treatment cannot even closely restore NO bioavailability in apoE(−/−) as found in WT mice.24 Recently, it has been acknowledged that Ang-(1–7) plays an important role in the control of ROS level in the renal vasculature and affects p38 MAPK signaling in pathophysiological conditions.42–44 For instance, chronic Ang-(1–7) treatment improved endothelial dysfunction and attenuated the progression of atherosclerosis in apoE(−/−) mice by reducing ROS production.24,25,33 In contrast, ACE2 deficiency causes a marked increase in oxidative stress, which has been attributed to the diminished generation of Ang-(1–7) by ACE2 deficiency as a counter regulatory mechanism to Ang II.45 In addition, cardiac overexpression of Ang-(1–7) improved Ang II–dependent cardiac hypertrophy by reducing p38 MAPK activity.46 Therefore, we hypothesized that other mechanisms, independent from NO, might contribute to the attenuated pressor response to Ang II seen in Ang-(1–7)–treated apoE(−/−) mice.

Here, we demonstrate that chronic Ang-(1–7) in apoE(−/−) mice reduced the abundance of phosphorylated MLC20 in preglomerular resistance vessels of apoE(−/−) mice. Hence, these mice showed attenuation of renal pressor response to Ang II. This was accompanied by reduced renal p38 MAPK activity in renal cortex and preglomerular resistance vessels and a reduction in ROS generation. In addition, chronic treatment with Ang-(1–7) decreased the expression of the NADPH oxidase subunit p47phox in apoE(−/−) mice and subsequently ROS generation in renal cortex samples. Thus, our study clearly indicates that chronic Ang-(1–7) application attenuated the elevated pressor response to Ang II through a ROS/p38 MAPK-dependent mechanism.

The present study cannot give a clear statement whether the effects of Ang-(1–7) are the result of immediate actions or because of the chronic treatment. However, acute Ang-(1–7) infusion did not affect Ang II–dependent pressor response in apoE(−/−) significantly and, thus, our results suggest no direct effect on p38 MAPK activity. We demonstrate that chronic Ang-(1–7) treatment reduces p47phox-dependent ROS generation and thereby decreases p38 MAPK activation to a basal level seen in WT mice. This is supported by the fact that p38 MAPK inhibition had no effect in WT mice. It has been suggested that Ang-(1–7) exerts its beneficial effects through AT2R activation or AT1R inhibition.19,47,48 Nevertheless, there is growing evidence that Mas-receptor signaling seems to be the predominant pathway for Ang-(1–7)–mediated beneficial effects on atherosclerosis and vascular function.25,33 In Mas(−/−)/apoE(−/−) double-knockout mice, chronic Ang-(1–7) treatment neither affected Ang II–induced pressor response nor decreased urinary 8-isoprostane excretion. These results support the importance of Mas-receptor–mediated action of Ang-(1–7) in this model and exclude any nonspecific effect or, for example, the dependency on AT2R signaling. Interestingly, cholesterol levels in untreated double-knockout mice were significantly higher when compared with Ang-(1–7)–treated mice. Because vascular function did not differ between Ang-(1–7)–treated and untreated Mas(−/−)/apoE(−/−) mice, the relevance of this finding remains unclear.

In conclusion, our experiments offer strong evidence that increased ROS-mediated p38 MAPK activity is the underlying cause for the elevated pressor response to Ang II in hypercholesterolemic apoE(−/−) mice. Chronic Ang-(1–7) treatment restores the pressor response to Ang II through a p47phox-dependent reduction of ROS. Because increased ROS is a key mechanism of p38 MAPK activation, Ang-(1–7)–mediated decrease of ROS is one of the underlying mechanisms in restoration of normal vascular function. Deficiency of the Mas-receptor abolishes any action of Ang-(1–7). This indicates the crucial role of Mas-receptor activation in this model and supports the view that the Mas-receptor could be a future target for pharmaceutical treatment in cardiovascular disease.

Perspectives
In the present study, we demonstrated that increased vasoreactivity to Ang II in renal resistance vessels of hypercholesterolemic apoE(−/−) mice is caused by an increased p38 MAPK activity. Acute and chronic inhibition of p38 MAPK inhibition in vivo and ex vivo confirms its pivotal role. In these mice, the
p38 MAPK is activated through a ROS/p47phox mechanism. This mechanism might explain the crucial role of p47phox in Ang II–dependent hypertension seen by others. However, more studies are needed to show a direct effect of tissue-specific deletion of p38 MAPK on blood pressure regulation.

In addition, our study showed that chronic Ang-(1–7) treatment attenuates the increased pressor response to Ang II by a significant reduction in ROS generation and p47phox abundance and hence attenuation of p38 MAPK activity. In this setup, for the first time, using double-knockout mice deficient of the Mas-receptor and apoE, we showed that the beneficial effects of Ang-(1–7) treatment were dependent on Mas-receptor signaling. Hence, any effects on vasoactivity and ROS were abolished in the double-knockout mice. Therefore, the Mas-receptor seems to be a promising target for intervention in hypercholesterolemia.

Acknowledgments

The excellent technical assistance of Blanka Duvnjak, Nicola Kuhr, and Christina Schwandt is greatly acknowledged.

Sources of Funding

This work was supported by the Forschungskommission der Heinrich-Heine-University Düsseldorf (grant 9772478 to J. Stegbauer and S.H. Sivritas) and the German Society of Hypertension (grant to J. Stegbauer)

Disclosures

None.

References


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**Novelty and Significance**

**What Is New?**

- In apolipoprotein E-deficient mice (apoE−/−), vasoreactivity to angiotensin II (Ang II) is increased in renal resistance vessels by reactive oxygen species/p47phox-mediated increase of p38 mitogen-activated protein kinase activity. Chronic Ang-(1–7) treatment restores the Ang II pressor response to levels seen in wild-type animals. Ang-(1–7) exerts its effects via the Mas-receptor, causing decreased oxidative stress and subsequent reduction of p38 mitogen-activated protein kinase activity.

**What Is Relevant?**

- Ang-(1–7) counteracts increased vasoreactivity to Ang II in apoE−/− mice in a Mas-receptor-dependent manner.

**Summary**

Increased p38 mitogen-activated protein kinase activity is responsible for increased vasoreactivity to Ang II in apoE−/−. Ang-(1–7) treatment attenuates the increased vasoreactivity by reducing reactive oxygen species and consequently p38 mitogen-activated protein kinase activity.
Angiotensin-(1–7) Modulates Renal Vascular Resistance Through Inhibition of p38 Mitogen-Activated Protein Kinase in Apolipoprotein E–Deficient Mice

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Hypertension. 2014;63:265-272; originally published online November 4, 2013; doi: 10.1161/HYPERTENSIONAHA.113.02289

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/63/2/265

Data Supplement (unedited) at:
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Angiotensin-(1-7) Modulates Renal Vascular Resistance Through Inhibition Of P38 Mitogen-Activated Protein Kinase In Apolipoprotein E Deficient Mice

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Supplement – Materials and Methods

Animal treatment

In this study, apoE (-/-) mice were fed a high fat diet in order to accelerate the progression of endothelial dysfunction due to progressing atherosclerosis. WT animals fed a high fat diet served as healthy controls. Ang-(1-7) treatment was introduced 6 weeks after the start of the high fat diet as an interventional treatment. The rational of this study design was to treat after occurrence of vascular injury.

At age 6 weeks, all groups were set on a high fat “western diet” (Sniff, Soest, Germany) (42 % fat, 0.15 % cholesterol) for 12 weeks. Group 1: WT untreated for 6 weeks and treated with saline for consecutive 6 weeks (osmotic minipump, Alzet Model 1004)

Group 2: apoE (-/-) untreated for 6 weeks and treated with saline for consecutive 6 weeks (osmotic minipump, Alzet Model 1004)

Group 3: apoE (-/-) untreated for 6 weeks and treated with Ang-(1-7) (82 µg/kg/hr) for consecutive 6 weeks (osmotic minipump, Alzet Model 1004)

Group 4: apoE (-/-) treated for 12 weeks with an oral p38-inhibitor (BIRB796, 50 mg/kg/day, high fat diet preparation containing the inhibitor), after 6 weeks additional treatment with saline for consecutive 6 weeks (osmotic minipump, Alzet Model 1004). 26

Group 5: Mas (-/-) / apoE (-/-) untreated for 6 weeks and treated with saline for consecutive 6 weeks (osmotic minipump, Alzet Model 1004)

Group 6: Mas (-/-) / apoE (-/-) untreated for 6 weeks and treated with Ang-(1-7) (82 µg/kg/hr) for consecutive 6 weeks (osmotic minipump, Alzet Model 1004)

All animals were sacrificed at the age of 18 weeks.

Isolated perfused mouse kidney

Mice were anesthetized by i.p. injection with Ketamin (100 mg/kg) and Xylazin (5 mg/kg). Kidneys were isolated microscopically (Olympus CO11) and perfused with Krebs-Henseleit buffer according to an amended method described previously.¹ Changes in perfusion pressure reflected changes in vascular resistance of renal resistance vessels immediately after preparation, a bolus injection of 60 mM KCl was
delivered to test the viability of the preparation followed by a stabilization period of 30 min. After the stabilization period, renal vasoconstriction was induced by increasing concentrations of Angiotensin II (Sigma-Aldrich) in the presence or absence of the specific p38 MAPK inhibitor SB203580 (5 µM; Sigma-Aldrich), the ERK1/2 inhibitor PD98059 (5 µM; Sigma-Aldrich) or Ang-(1-7) (0.1 µM; Bachem). Increase in pressor response was measured in mmHg.

**Acute infusion experiments**

18 weeks old apoE (-/-) (group 2 and 4) mice were anesthetized by i.p. injection with Ketamin (100 mg/kg) and Xylazin (5 mg/kg). Mice were placed on a heating plate to ensure constant body temperature at approx. 36 °C. Left carotid artery was cannulated for continuous measurement of mean arterial pressure (MAP). Left jugular vein was cannulated for application of increasing doses of Ang II (bolus of 0.1, 1, 10 µM/kgBW). Change in mean arterial pressure (MAP) was measured for each concentration of Ang II.

**Systolic blood pressure measurement**

Systolic blood pressure (BP) was measured non-invasively by tail-cuff sphygmomanometer using a BP-98A device (Softron, Japan). Mice were trained for 4 days prior to evaluation of BP. BP was measured for 5 days at the end of the observational period (week 18).

**Isolation of preglomerular vessels**

Preglomerular vessels, containing mainly interlobular arteries and afferent arterioles, were isolated by a modified iron oxide-sieving technique as described previously. The kidneys were perfused via cannulation of the aorta, smaller needles (G20, G23) and pores sieves (100 µm) were used for tissue separation and separation of renal particles, respectively.

**Quantification of urinary 8-isoprostane concentration**

24-h urine samples were collected in metabolic cages at the end of the experimental period. Urinary concentrations of 8-isoprostane were measured using a colorimetric-assay kit (Cayman Chemical Company) and normalized to urinary creatinine concentration.

**Immunoblotting for p38 MAPK and phospho-p38 MAPK**

Renal cortex tissue of pre-glomerular vessels were placed into ice-cold 1 % Triton lysis buffer (containing a protease inhibitor cocktail (Sigma Aldrich)) and were immediately homogenized. Lysates were centrifuged at 15,000 x g for 10 min at 4 °C. Protein concentrations from the supernatant were measured using a Bradford assay (Bioassay Systems). After dithiothreitol treatment (100 mM) and denaturation (5 min at 95 °C), 30 µg of total protein were loaded onto 10 % SDS-PAGE gels and then transferred to nitrocellulose membranes according to manufacturer’s instructions (X-Cell Blot Module, Invitrogen). Membranes were treated with blocking buffer (5 % BSA, and 0.1 % tween 20 in PBS) for 1h at room temperature and then incubated either with primary monoclonal rabbit anti-p38 antibody (1:750) (Cell Signalling Technology) or primary monoclonal rabbit anti-phospho-p38 antibody (1:1300) (Cell Signalling Technology), and rabbit anti-β-actin (1:2000), (Santa Cruz Biotechnology) over-night. Bound primary antibody was detected with anti-rabbit HRP conjugated
secondary antibody (1:10000) (Dako, Germany) by 60 min incubation at room temperature. Antibody labelling was visualized by the addition of a chemiluminescence reagent. Chemiluminescence was visualized using a FluorChem FC2 Imager (Alpha Innotec, USA). Immunoblots from each tissue were performed in triplicates.

Immunoblotting for phospho-MLC$_{20}$

Pre-glomerular vessel tissue was lysed and processed as described above. Polyclonal rabbit anti-phospho-MLC$_{20}$ antibody was used as primary antibody (1:500) (Acris Antibodies GmbH). Bound primary antibody was detected with anti-rabbit HRP conjugated secondary antibody (1:50000) (Dako, Germany) by 60 min incubation at room temperature. Assessment of chemiluminescence was performed as described above. Reference protein was alpha-actin (1:2000).

Immunoblotting for p47phox (renal cortex)

Renal cortex tissue was lysed and processed as described above. Polyclonal rabbit anti-p47phox antibody was used as primary antibody (1:200) (Santa Cruz Biotechnology). Bound primary antibody was detected with anti-rabbit HRP conjugated secondary antibody (1:10000) (Dako, Germany) by 60 min incubation at room temperature. Assessment of chemiluminescence was performed as described above.

Quantitative real time RT-PCR (qPCR)

Kidney cortex samples were analyzed for relative expression levels (mRNA) of AT1a-receptor (AT1a), AT2-receptor (AT2), angiotensin converting enzyme 2 (ACE2) and Mas-receptor (MasR). After homogenization of tissue with a Tissue Ruptor (Qiagen, Germany), total RNA was isolated using a RNA Micro Kit (Qiagen, Germany) according to the manufacturer's instructions. Quantitative real time RT-PCR was performed with an ABI PRISM 7300 (Applied Biosystem, Germany) and the SYBR Green master mix (Qiagen, Germany). The PCR reaction was performed in a total volume of 20 µl with 1 µl cDNA corresponding to 50 ng RNA as template.

The PCR conditions were 15 min at 95 °C, followed by 40 cycles (denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s, extension at 72 °C for 34 s, detection at 79 °C for 34 s). Experiments were performed in duplicates. 18S ribosomal RNA was chosen as the endogenous control (housekeeping gene). The levels of targeted genes were normalized to 18S rRNA expression. Data was analyzed using the DeltaCT-method. Statistical analysis was performed with a one-way ANOVA for repeated measurements followed by Bonferroni correction post-hoc test.

The following primer sequences were used: 18S ribosomal RNA (QuantiTect Primer Assays, Qiagene, Cat-N° QT00324940), MasR: forward: TTGTGGCAGCAGTAAAGAGA, reverse: ATGGATACAGTGTTGCCTTG. ACE2: forward: TCTGGGCAAACTCTATGCTGA, reverse: TGATGGGCTGTCAAGAAGTTG. AT2: forward: ACCCTGATGAGTGTCTG, reverse: CTGACATCCGGAAATAATGT. AT1a: forward: GCTTGGTGTTGATCGTACC, reverse: GGCGAGATTTAGAAGACG.

Lucigenin superoxide detection
Lucigenin-enhanced chemiluminescent detection of superoxide production in kidney cortex of apoE (-/-) on western diet treated with or without Ang-(1-7) (group 2 and 3) was performed as described previously. Briefly, freshly cleaned and harvested thoracic aortic and kidney cortex segments were equilibrated in Krebs-HEPES buffer for 30 min at 37 °C. Aortic and kidney segments were transferred to vials containing albumin buffer enriched with 5 μM lucigenin, and chemiluminescence was recorded every 2 min for 20 min using Packard Luminometer Analyzer (Picolite A6112, Packard, Downers Grove, IL, USA). Background readings were subtracted from sample reading and results are expressed as counts/min/mg tissue dry weight (mean ± SEM).

Statistics

Data are expressed as mean ± SEM (n=number of animals). Student's t-test was used to compare means of two groups with Gaussian distribution. Multiple comparison of more than two groups with Gaussian distribution were analysed by one-way ANOVA followed by Bonferroni's multiple comparison post-hoc test. Statistical analyses of data of two groups in which Gaussian distribution was not normal (or could not be assumed) were analysed by the Mann-Whitney-U-Test. Statistical analyses of data of more than two groups in which Gaussian distribution was not normal (or could not be assumed) were analysed by the Kruskal-Wallis-Test followed by Dunn's multiple comparison post-hoc test. Differences between dose-response curves were analysed by two-way ANOVA for repeated measurements followed by Bonferroni correction post-hoc test.

Probability levels of p < 0.05 were considered statistically significant. If applicable, a higher level of statistical significance is stated (p < 0.01, p < 0.001). The number of experiments (n) refers to the number of mice or the number of individual samples.
Supplement – References


S1: Ang II-induced pressor response in isolated perfused kidney from WT mice was not significantly different if treated chronically with Ang-(1-7) (82 µg/kg/hr) (WT: n=5; WT + Ang-(1-7): n=7) (p=NS). Two-way ANOVA for repeated measurements followed by Bonferroni correction post-hoc test.

S2: Acute Ang-(1-7) (0.1 µM) administration did not influence Ang II-induced pressor response in isolated perfused kidney of non-treated apoE (-/-) mice significantly (n=6; each group) (p=NS). Two-way ANOVA for repeated measurements followed by Bonferroni correction post-hoc test.
S3: Comparison of relative gene expression (mRNA level) of RAS-related genes in WT vs. apoE (-/-) vs. apoE (-/-) + Ang-(1-7). (n=4; each group) There was no significant difference in gene expression. (real time PCR data for AT1aR, AT2R, ACE2, MasR. deltaCT to 18s expression normalized to WT expression). p=NS. One-way ANOVA for repeated measurements followed by Bonferroni correction post-hoc test.
S4: Ang II-induced pressor response is not influenced by MEK 1/2-inhibition neither in kidneys of WT (A; n=11 each group) nor apoE (-/-) (B; n=5 each group) mice. P=NS for apoE (-/-) versus apoE (-/+) + PD98059. P=NS for WT versus WT + PD98059. Two-way ANOVA for repeated measurements followed by Bonferroni correction post-test.