Renin-Angiotensin-Aldosterone System

Angiotensin-(1–7) Recruits Muscle Microvasculature and Enhances Insulin’s Metabolic Action via Mas Receptor

Zhuo Fu, Lina Zhao, Kevin W. Aylor, Robert M. Carey, Eugene J. Barrett, Zhenqi Liu

Abstract—Angiotensin-(1–7) [Ang-(1–7)], an endogenous ligand for the G protein–coupled receptor Mas, exerts both vasodilatory and insulin-sensitizing effects. In skeletal muscle, relaxation of precapillary arterioles recruits microvasculature and increases the endothelial surface area available for nutrient and hormone exchanges. To assess whether Ang-(1–7) recruits microvasculature and enhances insulin action in muscle, overnight-fasted adult rats received an intravenous infusion of Ang-(1–7) (0, 10, or 100 ng/kg per minute) for 150 minutes with or without a simultaneous infusion of the Mas inhibitor A-779 and a superimposition of a euglycemic insulin clamp (3 mU/kg per minute) from 30 to 150 minutes. Hind limb muscle microvascular blood volume, microvascular flow velocity, and microvascular blood flow were determined. Myographic changes in tension were measured on preconstricted distal saphenous artery. Ang-(1–7) dose-dependently relaxed the saphenous artery (P<0.05) ex vivo. This effect was potentiated by insulin (P<0.01) and abolished by either endothelium denudement or Mas inhibition. Systemic infusion of Ang-(1–7) rapidly increased muscle microvascular blood volume and microvascular blood flow (P<0.05, each) without altering microvascular flow velocity. Insulin infusion alone increased muscle microvascular blood volume by 60% to 70% (P<0.05). Adding insulin to the Ang-(1–7) infusion further increased muscle microvascular blood volume and microvascular blood flow (∼2.5 fold; P<0.01). These were associated with a significant increase in insulin-mediated glucose disposal and muscle protein kinase B and extracellular signal–regulated kinase 1/2 phosphorylation. A-779 pretreatment blunted the microvascular and insulin-sensitizing effects of Ang-(1–7). We conclude that Ang-(1–7) by activating Mas recruits muscle microvasculature and enhances the metabolic action of insulin. These effects may contribute to the cardiovascular protective responses associated with Mas activation and explain the insulin-sensitizing action of Ang-(1–7).

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Key Words: angiotensins ■ endothelial cells ■ microvasculature ■ muscles

Muscle accounts for 70% to 80% of insulin-stimulated whole-body glucose disposal during insulin infusion. A rate-limiting step in muscle insulin action is the delivery of insulin to the muscle interstitium that contributes up to 40% of insulin’s metabolic action.1 This process is critically regulated by the skeletal muscle microvasculature that not only regulates the delivery of nutrients and insulin to the muscle microcirculation but also provides the endothelial surface area for their exchanges between plasma and muscle interstitium.2–4 Insulin also actively regulates its own delivery in muscle by increasing muscle microvascular blood volume (MBV) and stimulating its own transendothelial transport.4

The renin–angiotensin system (RAS) critically regulates vascular tone, tissue perfusion, and water balance. Angiotensin II (Ang II), the main RAS component, exerts not only a potent vasoconstrictive action via its type 1 receptors (AT1R) but also a vasodilatory effect via its type 2 receptors (AT2R).5,6 In addition, the RAS actively regulates insulin action.7–9 Previous evidence mainly focused on the antagonistic action of Ang II on insulin action via AT1R-mediated nicotinamide adenine dinucleotide phosphate oxidase/oxidative stress pathway,10,11 and we have recently demonstrated that Ang II also regulates insulin action via its effects on muscle microvascular perfusion. Although basal AT1R activity restricts muscle microvascular perfusion and decreases muscle insulin delivery and action, AT1R activation potently recruits muscle microvasculature and rescues insulin action during systemic lipid infusion via increased insulin delivery in rodents.12–14 In mildly hypertensive humans, acute AT1R blockade with irbesartan increases insulin-induced skin microvascular perfusion.15 Interestingly, in healthy humans Ang II has been shown to enhance insulin-stimulated whole-body glucose disposal but impair insulin-induced skin capillary recruitment.16

In addition to AT1R, an important RAS pathway poised to oppose AT1R is the angiotensin-(1–7) [Ang-(1–7)]–angiotensin-converting enzyme-2–Mas receptor (Mas) hormonal cascade. Ang-(1–7) is a heptapeptide generated primarily from Ang II by the actions of angiotensin-converting enzyme-2. Similar to the action of Ang II on the AT1R, Ang-(1–7) exerts potent vasodilatory actions on conduit arteries and microvessels via a nitric oxide (NO)–dependent mechanism.17,18 Recent evidence suggests that, in addition to its vasodilatory action,
Ang-(1–7) also reduces Ang II– or fructose-induced insulin resistance.19–21 Transgenic rats that express an Ang-(1–7)–releasing fusion protein have elevated circulating Ang-(1–7) levels with improved glucose tolerance and insulin-stimulated glucose uptake.22 This action does not seem to be mediated via either AT1R or AT2R. Rather, Ang-(1–7) acts on a unique G-protein–coupled receptor Mas23,24 that is encoded by the Mas proto-oncogene and expressed constitutively in endothelial cells.25 Murine Mas-deficient aortae lose their Ang-(1–7)–induced relaxation response.25 Similarly, Ang-(1–7)–mediated relaxation of wild-type mesenteric arteries is equally impaired in both wild-type arteries pretreated with Mas inhibitor A-779 and arteries isolated from Mas-deficient mice.26 However, the mechanisms underlying the insulin sensitization of Ang-(1–7) remain unknown.

Given the critical role that microvasculature plays in regulating muscle insulin action and that Ang-(1–7) has been demonstrated to exert both vasodilatory and insulin-sensitizing actions, it is possible that Ang-(1–7) exerts its insulin-sensitizing action via regulating muscle microvascular endothelial surface area. This was examined in the current study, and our results indicate that Ang-(1–7) potently recruits muscle microvasculature and increases insulin-stimulated whole-body glucose disposal via an Mas-dependent mechanism.

Methods

Animal Preparations and Experimental Protocols

Overnight-fasted adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 250 to 380 g were studied under 1 of the 3 protocols (Figure 1). Before the study, rats were fed standard laboratory chow and water ad libitum and housed at 22±2°C on a 12-hour light–dark cycle. Rats were anesthetized with pentobarbital sodium (50 mg/kg IP; Abbott Laboratories, North Chicago, IL), placed in a supine position on a heating pad to ensure euthermia, and intubated to maintain a patent airway. The carotid artery and the jugular vein were cannulated with polyethylene tubing (PE-50; Fisher Scientific, Newark, DE) for arterial blood pressure monitoring, arterial blood sampling, and various infusions.

After a 30-minute baseline period, to ensure hemodynamic stability and a stable level of anesthesia, baseline microvascular blood sampling, and various infusions. This was examined in the current study, and our results indicate that Ang-(1–7) potently recruits muscle microvasculature and increases insulin-stimulated whole-body glucose disposal via an Mas-dependent mechanism.

Figure 1. Experimental protocols. Protocol 1 examines the effects of angiotensin-(1–7) [Ang-(1–7)] on muscle microvasculature. Protocol 2 studies whether Ang-(1–7) alters the microvascular and metabolic actions of insulin. Protocol 3 determines whether Mas mediates the microvascular actions of Ang-(1–7). CEU indicates contrast-enhanced ultrasound.
Assessment of the Vasorelaxant Effect of Ang-(1–7) on Preconstricted Small Arteries

The distal saphenous artery (≤250 μm) was dissected, cleaned of adhering connective tissues, and cut into segments of ≤2 mm in length. Each segment was mounted in a Multi Myograph System (Danish Myo Technology, Aarhus, Denmark). The organ chamber was filled with 6 mL of physiological salt solution buffer (NaCl 130 mmol/L, KCl 4.7 mmol/L, CaCl2 1.6 mmol/L, MgSO4 1.17 mmol/L, KH2PO4 1.18 mmol/L, NaHCO3 14.9 mmol/L, EDTA 0.026 mmol/L, glucose 5.5 mmol/L; pH 7.4), which was constantly bubbled with 95% O2–5% CO2 and maintained at 37°C. Each ring was stretched initially to 5 mN and then allowed to stabilize at baseline tone. In some arteries, the endothelium was mechanically removed by rubbing the luminal surface of the ring with human hair. Functional removal of the endothelium was verified by the lack of relaxation response to acetylcholine. After preconstriction of the arterial ring with 2 μmol/L phenylephrine, Ang-(1–7) and insulin at various concentrations were added into the chamber. For the Mas inhibitor study, preconstricted arterial rings were incubated with A-779 (20 μmol/L) for 30 minutes before Ang-(1–7) was added. Changes in arterial tone were recorded.

Statistical Analysis

All data are presented as means±SEM. Statistical analyses were performed with SigmaStat 11.0 software (Systat Software, Inc) using either the Student t test or ANOVA with post hoc analysis as appropriate. A value of P<0.05 was considered statistically significant.

Results

Ang-(1–7) Induces Arterial Relaxation via Mas in Endothelium

Previous evidence has strongly suggested a direct vasodilatory action of Ang-(1–7) on microvessels, likely via an Mas-mediated, NO-dependent pathway.34,35 Similarly, insulin also exerts potent vasodilatory action via increased NO production.34,35 As such, we first examined whether Ang-(1–7) and insulin could potentiate each other’s vasodilatory actions in vitro (Figure 2). Using phenylephrine-preconstricted distal saphenous arteries, we found that Ang-(1–7) and insulin each induced dose-dependent vasorelaxation (P<0.05). Interestingly, insulin at high physiological concentrations (1 mmol/L) did not induce significant vasorelaxation, but it markedly potentiated the vasodilatory effect of Ang-(1–7) (by ≤40%; P<0.01). Both endothelium denudement and pretreatment with Mas inhibitor A-779 completely abolished Ang-(1–7)–induced vasorelaxation.

Ang-(1–7) Recruits Skeletal Muscle Microvasculature

To examine the effect of Ang-(1–7) on the precapillary arterioles that control muscle microvascular perfusion,3,36 we next used contrast-enhanced ultrasound to measure 3 important indices of the muscle microcirculation (ie, MBV, microvascular flow velocity, and MBF before and during a continuous infusion of Ang-(1–7) at various concentrations). As shown in Figure 3, Ang-(1–7) dose-dependently increased muscle microvascular recruitment by increasing MBV within the first 30 minutes, and this effect lasted for the entire 150-minute infusion period while microvascular flow velocity was not affected. The increase in MBV led to a maximum increase in muscle MBF (by ≈80%; P<0.05). Neither mean arterial blood pressure nor blood glucose levels changed during Ang-(1–7) infusion (Tables 1 and 2).

Ang-(1–7) Enhances the Vascular and Metabolic Actions of Insulin

Knowing that Ang-(1–7) could recruit muscle microvasculature and we and others have previously demonstrated that microvascular recruitment regulates insulin delivery and action in muscle,1,4 we next examined whether Ang-(1–7) could modulate the microvascular and metabolic actions of insulin in vivo. Similar to previous reports, insulin infusion alone increased both muscle MBV and MBF by ≈60% to 70% (P<0.05) without affecting microvascular flow velocity (Figure 4). Administration of Ang-(1–7) before the initiation of insulin infusion recruited significantly more microvasculature than insulin alone. This was associated with an ≈30% increase (P<0.05) in insulin-mediated whole-body glucose disposal (Figure 5). Neither mean arterial blood pressure nor blood glucose levels changed during the entire procedure (Tables 1 and 2).

In Vivo Effects of Ang-(1–7) Are Mas Dependent

Ang-(1–7) is an endogenous ligand for the G protein–coupled receptor Mas, and our ex vivo myographic studies showed that the vasodilatory effect of Ang-(1–7) is indeed Mas dependent. We, therefore, tested whether the in vivo effects of Ang-(1–7) on the muscle microvasculature and insulin’s metabolic action are also mediated by Mas. As shown in Figure 6, inhibition of Mas with its specific antagonist A-779 abolished Ang-(1–7)–induced microvascular recruitment without affecting insulin-mediated increase in muscle MBV, evidenced by a lack of increase in MBV and MBF 30 minutes after Ang-(1–7) infusion (time, 0 minutes) but a significant increase in MBV and MBF 30 minutes after beginning the insulin infusion (time, 30–150 minutes). Interestingly, there is a tendency for Ang-(1–7), at 100 ng/kg per minute, to synergize with insulin after 30 minutes to increase MBV and MBF, despite the presence of A-779 (P<0.05 at 120 minutes for MBF). These were coupled with a loss of the previously seen Ang-(1–7) enhancement of insulin-mediated glucose disposal (Figure 7). Neither mean arterial blood pressure nor blood glucose levels changed during the entire procedure (Tables 1 and 2).

Effects of Ang-(1–7) on Muscle Akt and ERK1/2 Phosphorylation

Because Ang-(1–7) increased the metabolic action of insulin via an Mas-dependent signaling pathway, we measured the phosphorylation of muscle Akt and ERK1/2, 2 key signal intermediates in the insulin signaling pathways, in rats studied under all 3 protocols. As shown in Figure 8, insulin infusion significantly increased muscle Akt and ERK1/2 phosphorylation. Although Ang-(1–7) infusion alone did not affect either Akt or ERK1/2 phosphorylation, it did further enhance insulin-mediated phosphorylation of these 2 proteins in a dose-dependent fashion. A-779 pretreatment completely abolished the effect of Ang-(1–7) without affecting insulin’s actions on either Akt or ERK1/2 phosphorylation.

Discussion

In the current study, we provide experimental evidence using both ex vivo arterial myographic and in vivo animal study...
approaches that Ang-(1–7) potently recruits muscle microvasculature and enhances insulin’s metabolic action, and these actions are mediated by Mas in the endothelium.

Previous studies have confirmed that Ang-(1–7) exerts a potent vasodilatory action in various arterial beds. Ang-(1–7) counteracts the vasoconstrictor actions of Ang II and lowers blood pressure in transgenic (mRen2)27 hypertensive rats and in spontaneously hypertensive rats. However, in the current study we did not observe a significant change in the mean arterial blood pressure during Ang-(1–7) infusion, likely secondary to the fact that our animals were not hypertensive and that the RAS was not upregulated. Indeed, even chronic infusion of Ang-(1–7) failed to produce a significant change in blood pressure in the presence of high dietary salt intake and in normal rats. Ang-(1–7) likely is more important in the regulation of regional as opposed to systemic vascular resistance. Acute infusion of Ang-(1–7) leads to a significant change in regional blood flow distribution with a decrease in total peripheral resistance in Wistar rats. Similarly, expression of an Ang-(1–7)–producing fusion protein in rats elevates plasma Ang-(1–7) levels and reduces total peripheral resistance, which is associated with increased stroke volume and cardiac index. Although we did not assess cardiac parameters and peripheral vascular resistance, our findings that Ang-(1–7) significantly increased microvascular recruitment suggests that Ang-(1–7) is able to vasodilate muscle precapillary arterioles directly.
arterioles that control microvascular perfusion and are major contributors to total peripheral resistance.

The increased muscle microvascular recruitment seen with Ang-(1–7) infusion provides a physiological explanation for numerous study findings that Ang-(1–7) administration improves insulin sensitivity and action because muscle microvasculature plays a critical role in the regulation of muscle insulin action by providing the endothelial exchange surface area and actively regulating insulin delivery to the muscle interstitium. It is the insulin concentrations in the muscle interstitium, not in plasma, that best correlate with insulin’s muscle metabolic action and whole-body glucose disposal. A recent study demonstrates that in rats Ang-(1–7) by itself does not promote glucose transport but significantly increases insulin-stimulated glucose disposal. This lends further support to our study findings. We and others have previously demonstrated that factors that are able to recruit microvasculature, such as low intensity exercise, AT1R blockade, adiponectin, ranolazine, and glucagon-like peptide-1 all significantly increase muscle insulin delivery and action. That Ang-(1–7)–induced microvascular recruitment is associated with increased whole-body glucose disposal and muscle Akt and ERK1/2 phosphorylation during insulin clamp is certainly consistent with increased muscle delivery and action of insulin.

Our observations that the combination of Ang-(1–7) and insulin (1 nmol/L) exerted an enhanced vasodilatory action compared with Ang-(1–7) or insulin alone ex vivo and that the MBV increase in vivo was also greater with combined insulin plus Ang-(1–7) infusion compared with either agent alone suggest that Ang-(1–7) and insulin exert additive or synergistic vasodilatory actions on the precapillary arterioles. This is not surprising because insulin and Ang-(1–7) each engenders vasorelaxation via an NO-dependent pathway, and high NO production, as seen in the case of glucagon-like peptide-1 and losartan, can increase MBV by 2- to 3-fold.

Although previous studies have reported that Ang-(1–7) stimulated muscle Akt phosphorylation and this was completely inhibited by the coadministration of Mas inhibitor A-779, Ang-(1–7) alone did not increase muscle Akt phosphorylation in the current study. This discrepancy likely resulted from the difference in the timing of muscle sample collection for Akt phosphorylation analysis (5 minutes in previous reports versus 150 minutes in the current study). Similarly, Ang-(1–7) did not alter ERK1/2 phosphorylation. It is certainly possible that Ang-(1–7) could acutely phosphorylate muscle Akt and ERK1/2, but this effect is not sustained. The lack of Ang-(1–7) enhancement of insulin-mediated Akt and ERK1/2 phosphorylation during A-779 infusion is

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**Table 1. Blood Glucose Levels (mg/dL)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>−60 Min</th>
<th>−30 Min</th>
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<th>30 Min</th>
<th>60 Min</th>
<th>90 Min</th>
<th>120 Min</th>
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<tr>
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<td>NA</td>
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<tr>
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<td>112±1.94</td>
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<td>102±3.58</td>
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Ang-(1–7) indicates angiotensin-(1–7); Ang-(1–7)_10, Ang-(1–7) at 10 ng/kg per minute; and Ang-(1–7)_100, Ang-(1–7) at 100 ng/kg per minute.
consistent with the findings that Mas inhibition blunts Ang-(1–7)–mediated microvascular recruitment and enhancement in insulin-stimulated whole-body glucose disposal. Thus, our study results strongly suggest that Ang-(1–7) recruits muscle microvasculature and increases microvascular endothelial surface area that lead to increased muscle delivery, thus interstitial concentrations and action of insulin. Although the Western blot results should be interpreted with caution because we did not separate myocytes from other cell types such as endothelial cells, vascular smooth muscle cells, and fibroblasts, our results most likely reflect the findings in myocytes, given its dominance in number in muscle.

Although expression of Mas in endothelial cells is well documented and Mas has been repeatedly shown to mediate the vasodilatory actions of Ang-(1–7), several studies have suggested that Ang-(1–7) may exert vasorelaxation via AT2R activation or by antagonizing AT1R. While we have previously reported that basal AT1R tone restricts and basal AT2R

Table 2. Mean Arterial Pressure (mm Hg)

<table>
<thead>
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<th>Treatment</th>
<th>−60 Min</th>
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<th>30 Min</th>
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<th>90 Min</th>
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Ang-(1–7) indicates angiotensin-(1–7); Ang-(1–7)_10, Ang-(1–7) at 10 ng/kg per minute; and Ang-(1–7)_100, Ang-(1–7) at 100 ng/kg per minute.

Figure 4. Effects of angiotensin-(1–7) [Ang-(1–7)] on muscle microvascular recruitment during insulin clamp. Each rat received a continuous intravenous infusion of Ang-(1–7) or saline (control) for 150 minutes (−30 to 120 minutes), with an insulin clamp (3 mU/kg per minute) superimposed on the last 120 minutes. A, Microvascular blood volume (MBV). B, Microvascular flow velocity (MFV). C, Microvascular blood flow (MBF). n=5 each. *P<0.05 vs baseline (−30 minutes). #P<0.01 vs insulin alone. Ang-(1–7)_10 indicates Ang-(1–7) at 10 ng/kg per minute; and Ang-(1–7)_100, Ang-(1–7) at 100 ng/kg per minute.

Figure 5. Angiotensin-(1–7) [Ang-(1–7)] dose-dependently augments insulin-stimulated whole-body glucose disposal. Each rat received a continuous intravenous infusion of Ang-(1–7) or saline (control) for 150 minutes, with an insulin clamp (3 mU/kg per minute) superimposed on the last 120 minutes. A, Time course of glucose infusion rate (GIR) during insulin clamp. B, GIR area under the curve (AUC) during insulin clamp. n=5 each. *P<0.05 vs control. Ang-(1–7)_10 indicates Ang-(1–7) at 10 ng/kg per minute; and Ang-(1–7)_100, Ang-(1–7) at 100 ng/kg per minute.
activity enhances muscle MBV, it is unlikely that Ang-(1–7) acted directly on the AT_R to exert its biological effects in our study because of its extremely low affinity for AT_R. It remains possible that Ang-(1–7) regulates AT_R function via Mas that is a physiological antagonist of AT_R. Our finding that pretreatment with Mas antagonist A-779 blocks both Ang-(1–7)’s vasodilatory actions on isolated arterioles ex vivo and precapillary arterioles in vivo confirms that Mas plays a key role in this process. However, our observations that Mas inhibition completely abolished the Ang-(1–7)–mediated increase in MBV and its potentiation on insulin’s microvascular action at 10 but not 100 ng/kg per minute are somewhat puzzling. This may reflect that microvessels undergo a nonspecific loss of tension mediated by NO or non–Mas-mediated effects of Ang-(1–7) at high concentrations. Ang-(1–7) is capable of binding to AT_R directly, although at much lower affinity than Ang II.

Further study is warranted.

Perspectives
Muscle microvasculature plays a critical role in the regulation of muscle delivery of nutrients and hormones including insulin by providing endothelial exchange surface area. Ang-(1–7) has been shown to cause arterial dilation and increase insulin sensitivity. Our current results demonstrate that acute administration of Ang-(1–7) potently recruits muscle microvasculature via its receptor Mas in the endothelium that expands the microvascular exchange surface area and thus enhances insulin’s metabolic action in muscle. These effects...
may contribute to the cardiovascular protective responses associated with Mas activation and explain the insulin-sensitizing action of Ang-(1–7).

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Disclosures

None.

References


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