Chronic Angiotensin-(1–7) Improves Insulin Sensitivity in High-Fat Fed Mice Independent of Blood Pressure

Ian M. Williams, Yolanda F. Otero, Deanna P. Bracy, David H. Wasserman, Italo Biaggioni, Amy C. Arnold

Abstract—Angiotensin-(1–7) improves glycemic control in animal models of cardiometabolic syndrome. The tissue-specific sites of action and blood pressure dependence of these metabolic effects, however, remain unclear. We hypothesized that Ang-(1–7) improves insulin sensitivity by enhancing peripheral glucose delivery. Adult male C57BL/6J mice were placed on standard chow or 60% high-fat diet for 11 weeks. Ang-(1–7) (400 ng/kg per minute) or saline was infused subcutaneously during the last 3 weeks of diet, and hyperinsulinemic–euglycemic clamps were performed at the end of treatment. High-fat fed mice exhibited modest hypertension (systolic blood pressure: 137±3 mmHg versus 123±5 mmHg; P=0.001), which was not altered by Ang-(1–7) (141±4 mmHg; P=0.574). Ang-(1–7) did not alter body weight or fasting glucose and insulin in chow or high-fat fed mice. Ang-(1–7) increased the steady-state glucose infusion rate needed to maintain euglycemia in high-fat fed mice (31±5 mg/kg per minute; P=0.017) reflecting increased whole-body insulin sensitivity, with no effect in chow-fed mice. The improved insulin sensitivity in high-fat fed mice was because of an enhanced rate of glucose disappearance (34±5 mg/kg per minute; P=0.049). Ang-(1–7) enhanced glucose uptake specifically into skeletal muscle by increasing translocation of glucose transporter 4 to the sarcolemma. Our data suggest that Ang-(1–7) has direct insulin-sensitizing effects on skeletal muscle, independent of changes in blood pressure. These findings provide new insight into mechanisms by which Ang-(1–7) improves insulin action, and provide further support for targeting this peptide in cardiometabolic disease.

Key Words: hypertension • insulin resistance • metabolism • obesity • renin-angiotensin system

Over activation of the renin–angiotensin system (RAS) is a potential mechanism linking hypertension and the development of insulin resistance. Angiotensin (Ang) II promotes hypertension by inducing AT1 receptor–mediated vasoconstriction, renal fluid and salt retention, and sympathetic activation.1 Enhanced systemic RAS activity is also observed in obese and diabetic patients and closely correlates with insulin resistance.2 Ang II promotes insulin resistance by decreasing insulin-stimulated glucose uptake in peripheral tissues, inhibiting insulin-mediated suppression of hepatic glucose production, and impairing insulin signaling.2 Therapies that block Ang II activity (angiotensin-converting enzyme [ACE] inhibitors and AT1 receptor antagonists) reduce incidence of new-onset type II diabetes mellitus in randomized clinical trials.3 These drugs also increase levels of Ang-(1–7), a peptide that counter-regulates Ang II actions, which may contribute to their beneficial cardiometabolic effects.4

Ang-(1–7) binds the G-protein–coupled receptor mas to produce vasodilatory, antihypertensive, sympatholytic, and cardioprotective effects.4 Accumulating evidence suggests that activation of the Ang-(1–7)/mas axis also improves glucose homeostasis. Chronic peripheral or central Ang-(1–7) infusion lowers blood pressure (BP) and improves glucose tolerance in fructose-fed rats.5–7 Conversely, global mas receptor and ACE2 deletion, both of which reduce Ang-(1–7) activity, induce a metabolic syndrome–like phenotype in mice.5,9 These studies support the potential for targeting Ang-(1–7) in cardiometabolic disease. The precise mechanisms and BP dependence of Ang-(1–7) effects on insulin action, however, remain unclear.

We hypothesized that Ang-(1–7) would improve insulin sensitivity during progression of cardiometabolic syndrome, when levels of the peptide are deficient,10 by enhancing peripheral glucose delivery. To test this, we performed hyperinsulinemic–euglycemic clamps in high-fat diet (HFD) and chow-fed mice after chronic peripheral Ang-(1–7) administration. We demonstrate that Ang-(1–7) produces BP-independent improvement in whole-body insulin sensitivity in HFD mice by enhancing insulin-stimulated muscle glucose uptake (MGU). In contrast to our hypothesis, this
improvement was not because of changes in glucose delivery but instead was associated with increased glucose transporter 4 (GLUT4) levels at the myocyte plasma membrane (sarcolemma).

Methods
These studies were approved by the Vanderbilt Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Detailed Methods are available in the online-only Data Supplement.

Mouse Models
Six-week-old C57BL/6J male mice (Jackson Laboratory) were fed standard chow (5001 Laboratory Rodent Diet) or 60% HFD (BioServ F3282) ad libitum for 11 weeks. After 8 weeks of diet, subcutaneous osmotic minipumps (Alzet Model 2004) were implanted to deliver Ang-(1–7) (400 ng/kg per minute; Bachem) or saline vehicle for 3 weeks.

Assessment of Body Composition, Hemodynamics, and Cardiac Function
Body composition was determined after 2 weeks of treatment using an mq10 nuclear magnetic resonance analyzer (Bruker Optics). Transthoracic echocardiography was also performed at this time point using a Sonos 5500 system (Agilent). BP and heart rate were measured via an indwelling carotid artery catheter connected to a transducer and a BPA-400 analyzer (Digi-Med).

Hyperinsulinemic–Euglycemic Clamps (Insulin Clamps)
Insulin clamps were performed in conscious, unstressed, chronically catheterized mice as previously described.2 Mice were fasted for 5 hours before starting the insulin clamp. A $[^3H]$glucose (0.04 μCi/min; Perkin Elmer) infusion was starting at t=0 to determine rates of plasma glucose appearance ($R_s$) and disappearance ($R_d$). At t=0 minutes, continuous insulin (4 μU/kg per minute; Humulin R; Eli Lilly) and variable glucose (D50+50 μCi/$[^3H]$glucose) infusions were initiated. From time t=0 to 120 minutes, arterial glucose was measured every 10 minutes, with the exogenous glucose infusion rate adjusted to maintain euglycemia ($130–150$ mg/dL). Steady-state glucose infusion rate and $[^3H]$glucose kinetics were determined from t=80 to 120 minutes. Arterial blood samples were taken at baseline (basal) and during steady state (clamp) to measure insulin and nonesterified fatty acid (NEFA) levels. At t=120 minutes, mice received an intravenous bolus of 2$[^4C]$deoxyglucose (2$[^4C]$DG; 13 μCi; Perkin Elmer) to determine the tissue-specific glucose metabolic index ($R_g$).

Analysis of Plasma and Tissue Samples From Insulin Clamp
Insulin was measured by double antibody radioimmunoassay12 and NEFA by enzymatic colorimetric assay (NEFA C Kit; Wako Chemicals). $[^3H]$glucose, $[^3C]$2DG, and $[^3C]$2DG-6-phosphate radioactivity were measured using liquid scintillation counting. EndoR and $R_g$ were calculated using non–steady-state equations.13 $R_g$ was calculated as previously described.14

Immunoblotting
Gastrocnemius homogenates were resolved on SDS–PAGE gels, transferred to polyvinylidene fluoride membranes, and probed with phosphorylated Akt (Ser1$,;$ Cell Signaling), total Akt (Cell Signaling), phosphorylated AS160 (Ser1$,;$ Cell Signaling), and total AS160 (Millipore) antibodies.

Microspheres
In a separate cohort of HFD mice ($n=4$ vehicle, $n=3$ Ang-[1–7$]$), 100 μL of yellow 15-μm microspheres (Dye-Trak, Triton Technology) were injected into the carotid artery after the last sampling time point of the insulin clamp to measure regional blood flow to the hindlimb.

Circulating Ang Peptide Levels
Plasma Ang-(1–7) and Ang II levels were measured in a separate cohort of mice that did not undergo insulin clamps ($n=6$ chow-fed vehicle; $n=6$ chow-fed Ang-(1–7$); $n=8$ HFD vehicle; and $n=8$ HFD Ang-(1–7$)) by radioimmunoassay.

Statistical Analysis
Data are presented as mean±SEM. Analyses were performed using Prism (Version 6.0, GraphPad), with a 2-tailed $P$ value of <0.05 defined as statistical significance. Differences in outcomes were compared using unpaired $t$ test or 2-way ANOVA with Tukey post hoc tests.

Results
Effect of Ang-(1–7) on Cardiovascular Indices
Plasma Ang-(1–7) was reduced in HFD versus chow-fed mice (Table 1; $P=0.045$ diet effect). Chronic Ang-(1–7) infusion restored HFD-induced reductions in the peptide to $\approx 70\%$ of levels seen in chow-fed mice. There were significant diet ($P=0.015$) and interaction ($P=0.004$) effects for plasma Ang II, which were driven entirely by increased Ang II levels in chow-fed mice after Ang-(1–7) treatment. HFD mice had increased systolic ($P=0.001$), diastolic ($P=0.039$), and mean ($P=0.001$) BP, with no difference in heart rate ($P=0.129$). There was no effect of Ang-(1–7) on BP or heart rate in chow-fed or HFD mice (Table 1).

Effect of Ang-(1–7) on Metabolic Indices
Body weight and adiposity were higher in HFD mice ($P=0.001$ diet effect; Table 2). Ang-(1–7) did not alter body weight in HFD or chow-fed mice; however, there was a small reduction in adiposity ($P=0.040$ drug effect). HFD mice had higher basal arterial glucose and insulin ($P=0.018$ and $P=0.001$ diet effect, respectively), with no difference in NEFA. Ang-(1–7) tended to lower basal insulin ($P=0.055$ drug effect), but did not alter fasting glycemia or NEFA. During steady-state hyperinsulinemia, glucose was clamped at $140$ mg/dL in both HFD and chow-fed mice (Figure 1A and 1B). HFD mice maintained higher insulin levels during the clamp ($P=0.002$ diet effect; Table 2), compared with chow-fed mice. Ang-(1–7) did not alter clamp glucose and insulin levels, but significantly enhanced insulin-mediated suppression of NEFA ($P=0.001$ drug effect; Table 2).

Ang-(1–7) Reverses HFD-Induced Skeletal Muscle Insulin Resistance
The glucose infusion rate required to maintain euglycemia during steady-state hyperinsulinemia, a measure of whole-body insulin sensitivity, was similar after Ang-(1–7) versus saline treatment in chow-fed mice (Figure 1C and 1E;
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As expected, HFD mice had lower whole-body insulin sensitivity compared with chow-fed mice (P=0.001). Ang-(1–7) doubled the glucose infusion rate required to maintain euglycemia in HFD mice (Figure 1D and 1F; P=0.017). This enhanced whole-body insulin sensitivity was because of increased insulin-stimulated peripheral glucose disposal (Rd; Figure 2A; P=0.049). There was no effect of Ang-(1–7) on insulin-mediated suppression of endogenous glucose production (EndoRa; Figure 2B; P=0.476). Consistent with effects on peripheral glucose disposal, Ang-(1–7) augmented insulin-stimulated glucose uptake (Rg) in soleus, gastrocnemius, and vastus muscles in HFD mice, with no effect in epididymal adipose or cardiac tissue (Figure 2C and 2D). Ang-(1–7) did not alter Rd, EndoRa, or Rg in chow-fed mice (Figure S1).

Ang-(1–7) Does Not Improve Measures of Perfusion in HFD Mice

To determine if the Ang-(1–7)–mediated improvement in insulin sensitivity was because of improved vascular glucose delivery, we measured indices of skeletal muscle (SkM) perfusion in HFD mice. Levels of a capillary density (CD31) marker were not different in gastrocnemius muscle after Ang-(1–7) treatment (Figure S2A and S2B). Hindlimb perfusion, measured by deposition of colored microspheres at the end of the insulin clamp, was not different between treatment groups (Figure S2C). Finally, Ang-(1–7) did not alter cardiac function (Table S1).

Ang-(1–7) Enhances Sarcolemmal Glut4 Translocation in HFD Mice

A critical step in insulin-stimulated MGU is translocation of Glut4 from intracellular vesicles to the sarcolemma. To determine if Ang-(1–7) improves SkM insulin sensitivity by increasing sarcolemmal Glut4 levels, we measured Glut4 abundance within the region demarcated by the sarcolemmal marker Cav3 in gastrocnemius muscle (Figure 3A). Ang-(1–7) increased mean Glut4 fluorescence at the muscle plasma membrane in HFD mice (Figure 3B; P=0.011). This increased Glut4 trafficking was

Table 1. Hormonal and Cardiovascular Effects of Chronic Ang-(1–7) Administration

<table>
<thead>
<tr>
<th>Parameter, Unit</th>
<th>Chow Vehicle</th>
<th>Chow Ang-(1–7)</th>
<th>HFD Vehicle</th>
<th>HFD Ang-(1–7)</th>
<th>P_Diet</th>
<th>P_Drug</th>
<th>P_Int</th>
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</thead>
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<tr>
<td>n</td>
<td></td>
<td>9</td>
<td>10</td>
<td>6</td>
<td>7</td>
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<tr>
<td>Ang-(1–7), ng/mL</td>
<td>2.94±1.29</td>
<td>1.72±0.90</td>
<td>0.45±0.15</td>
<td>1.97±1.31</td>
<td>0.045</td>
<td>0.267</td>
<td>0.191</td>
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<tr>
<td>Ang II, ng/mL</td>
<td>0.16±0.06</td>
<td>0.51±0.14</td>
<td>0.18±0.02</td>
<td>0.09±0.03</td>
<td>0.015</td>
<td>0.074</td>
<td>0.004</td>
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<td>SBP, mmHg</td>
<td>123±5</td>
<td>128±3</td>
<td>143±4</td>
<td>143±3</td>
<td>0.001</td>
<td>0.574</td>
<td>0.566</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>97±5</td>
<td>98±4</td>
<td>109±3</td>
<td>104±3</td>
<td>0.039</td>
<td>0.658</td>
<td>0.515</td>
</tr>
<tr>
<td>MBP, mmHg</td>
<td>110±5</td>
<td>113±3</td>
<td>126±3</td>
<td>123±2</td>
<td>0.001</td>
<td>0.949</td>
<td>0.454</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>647±41</td>
<td>701±24</td>
<td>618±21</td>
<td>648±19</td>
<td>0.129</td>
<td>0.119</td>
<td>0.652</td>
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</table>

Data are mean±SEM and were analyzed by 2-way ANOVA for diet effect (P_Diet), drug effect (P_Drug), and their interaction (P_Int). Ang indicates angiotensin; DBP, diastolic blood pressure; HFD, high-fat diet; MBP, mean BP; and SBP, systolic BP.

Table 2. Metabolic Effects of Chronic Ang-(1–7) Administration

<table>
<thead>
<tr>
<th>Parameter, Unit</th>
<th>Chow Vehicle</th>
<th>Chow Ang-(1–7)</th>
<th>HFD Vehicle</th>
<th>HFD Ang-(1–7)</th>
<th>P_Diet</th>
<th>P_Drug</th>
<th>P_Int</th>
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<tr>
<td>n</td>
<td></td>
<td>9</td>
<td>10</td>
<td>6</td>
<td>7</td>
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<tr>
<td>Body mass, g</td>
<td>29±1</td>
<td>28±1</td>
<td>39±2</td>
<td>37±1</td>
<td>0.001</td>
<td>0.321</td>
<td>0.981</td>
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<tr>
<td>Adiposity, %</td>
<td>6.7±0.7</td>
<td>6.5±0.2</td>
<td>35.6±2.6</td>
<td>30.1±1.4</td>
<td>0.001</td>
<td>0.040</td>
<td>0.056</td>
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<tr>
<td>Glucose, mg/dL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Basal</td>
<td>132±7</td>
<td>131±7</td>
<td>152±13</td>
<td>153±7</td>
<td>0.018</td>
<td>0.969</td>
<td>0.923</td>
</tr>
<tr>
<td>Clamp</td>
<td>141±3</td>
<td>142±4</td>
<td>133±3</td>
<td>137±3</td>
<td>0.078</td>
<td>0.555</td>
<td>0.603</td>
</tr>
<tr>
<td>Insulin, pmol/L</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Basal</td>
<td>226±40</td>
<td>136±19</td>
<td>648±100</td>
<td>533±47</td>
<td>0.001</td>
<td>0.055</td>
<td>0.843</td>
</tr>
<tr>
<td>Clamp</td>
<td>585±59</td>
<td>569±58</td>
<td>1518±430</td>
<td>1245±290</td>
<td>0.002</td>
<td>0.549</td>
<td>0.480</td>
</tr>
<tr>
<td>NEFA, mmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.81±0.12</td>
<td>0.62±0.13</td>
<td>0.57±0.10</td>
<td>0.37±0.08</td>
<td>0.055</td>
<td>0.129</td>
<td>0.947</td>
</tr>
<tr>
<td>Clamp</td>
<td>0.25±0.06</td>
<td>0.05±0.03</td>
<td>0.25±0.05</td>
<td>0.11±0.06</td>
<td>0.677</td>
<td>0.001</td>
<td>0.472</td>
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</table>

Data are mean±SEM and were analyzed by 2-way ANOVA for diet effect (P_Diet), drug effect (P_Drug), and their interaction (P_Int). Metabolic hormones were measured in arterial blood at baseline (basal), and during steady state of hyperinsulinemic–euglycemic clamps (clamp). Ang indicates angiotensin; HFD, high-fat diet; and NEFA, nonesterified fatty acids.
associated with reduced total AS160 protein, a negative regulator of Glut4 translocation (Figure 3C; \( P = 0.034 \)), with no effect on phosphorylated AS160 (\( P = 0.999 \)). Ang-(1–7) did not alter phosphorylated or total Akt in HFD mice (Figure S4).

**Discussion**

The main finding of this study is that chronic systemic Ang-(1–7) administration reverses HFD-induced whole-body insulin resistance by enhancing insulin-stimulated MGU. These insulin-sensitizing effects of Ang-(1–7) on SkM were associated with increased sarcolemmal Glut4 levels and downregulation of AS160, a negative regulator of Glut4 translocation. Importantly, these effects occurred independent of changes in body weight, BP, and measures of systemic and regional perfusion. Taken together, these findings provide evidence for BP-independent effects of Ang-(1–7) on insulin action, and further support targeting this peptide to treat insulin resistance in cardiometabolic disease.

Numerous previous studies using intraperitoneal glucose and insulin tolerance tests have demonstrated that activation of the Ang-(1–7)/mas axis improves whole-body glucose homeostasis.\(^5\)-\(^7\),\(^15\) These tests involve several variables, including intestinal glucose absorption, glucose-stimulated insulin secretion, insulin sensitivity, glucose effectiveness, and counter-regulatory responses. Here, we specifically measured the contribution of insulin sensitivity to Ang-(1–7)-induced improvements in glucose tolerance using insulin clamps. Moreover, by combining the insulin clamp with isotopic glucose tracers, as in this study, we are able to assess tissue-specific insulin action. Using this gold standard method, we provide evidence that restoration of physiological Ang-(1–7) levels improves whole-body insulin sensitivity in a well-established animal model of cardiometabolic syndrome that exhibits mild hyperglycemia, hyperinsulinemia, insulin resistance, and modest hypertension.\(^16\) We did not observe an effect of chronic Ang-(1–7) in chow-fed mice, in contrast to reports
showing acute intravenous Ang-(1–7) enhances insulin sensitivity in lean anesthetized rats. This may reflect differences in species and methodology, as well as compensatory mechanisms to prolonged Ang-(1–7) elevation, including mas receptor internalization.

Interestingly, Ang-(1–7) did not alter the mild fasting hyperglycemia induced by HFD feeding in this study. The first response, however, to increased insulin action is compensation by the endocrine pancreas. This is evidenced by an initial reduction in insulin, without changes in blood glucose or hemoglobin A1c levels. In our study, Ang-(1–7)–treated mice had ≈20% lower fasting insulin levels than vehicle-treated mice. Similarly, short-term Ang-(1–7) treatment (2–4 weeks) lowers plasma insulin levels in fructose fed rats, but does not lower fasting glycemia. Longer-term Ang-(1–7) treatment (8–24 weeks) lowers both fasting insulinemia and glycemia in animal models of cardiometabolic syndrome. Thus, an improvement in insulin sensitivity precedes and contributes to normalization of glycemia. In our study, it is likely glycemia would have been lowered with more prolonged Ang-(1–7) treatment.

The reason for maintained hyperglycemia after short-term Ang-(1–7) treatment is unclear. It is conceivable that an increase in basal glucose transport and delivery would increase MGU and normalize fasting glycemia. In this study, both sarcolemmal Glut4 levels and SkM perfusion were measured under the insulin-stimulated conditions. It is not likely that the lack of effect of Ang-(1–7) on SkM perfusion in our study would contribute to the preserved basal hyperglycemia, given that an increase in glucose delivery in the absence of hyperinsulinemia has no impact on glucose uptake. Importantly, an improvement in insulin sensitivity alone has beneficial effects on cardiometabolic health. For example, insulin sensitivity is a stronger predictor of type 2 diabetes development compared with fasting glucose or hemoglobin A1c levels. Furthermore, insulin sensitivity is a stronger risk factor for cardiovascular disease than hyperglycemia.

Over activation of the RAS, and in particular Ang II, is associated with development of SkM and hepatic insulin resistance. We found that chronic Ang-(1–7) infusion improves insulin-stimulated MGU in HFD mice. This corroborates in vitro studies showing that Ang-(1–7) potentiates insulin-stimulated MGU in normal rodents and reverses Ang II–induced muscle insulin resistance. Ang-(1–7) did not alter basal or insulin-stimulated hepatic glucose output in HFD mice, which was normal in HFD compared with chow-fed mice. The lack of hepatic insulin resistance in HFD mice is consistent with previous reports from our group. This is probably because of the high physiological insulin dose infused during clamps, which could mask more subtle effects of Ang-(1–7) on hepatic insulin sensitivity. Ang-(1–7) reduced adiposity and improved insulin-stimulated suppression of lipolysis in our study, consistent with reports showing improved adipose insulin action. Although there was no effect of Ang-(1–7) on adipose glucose uptake during insulin clamps, one cannot rule out an indirect contribution of reduced adiposity to improved insulin sensitivity.

It is unclear to what extent the vascular effects of Ang-(1–7) contribute to its metabolic effects. Ang-(1–7) promotes endothelial-dependent vasodilation in macro-
Figure 3. Angiotensin-(1–7) increases sarcolemmal glucose transporter 4 (Glut4) in high-fat diet (HFD) mice by reducing levels of AS160, a negative regulator of Glut4 trafficking. A, Representative 500× magnification micrographs of gastrocnemius sections from HFD mice treated with vehicle versus Ang-(1–7). Sections were stained for Cav3 (green) and Glut4 (white), and images were obtained by confocal microscopy. B, Ang-(1–7) increased sarcolemmal Glut4, measured as the mean Glut4 fluorescence intensity in the region demarcated by Cav3 staining. C, Immunoblot of gastrocnemius extracts from clamped HFD vehicle and Ang-(1–7)–treated mice. Ang-(1–7) decreased total AS160 protein levels, with no effect on phosphorylated AS160.
microvessels.4 The improved glucose tolerance in fructose-fed rats with Ang-(1–7) was associated with BP lowering,5,6 which could indicate vasodilation and manifest as improved glucose delivery and insulin sensitivity. Ang-(1–7) did not alter BP or cardiac function, however, in HFD mice. The lack of a depressor effect in our model is consistent with accumulating evidence showing that the antihypertensive effect of Ang-(1–7) is not apparent in all research models. There are several potential reasons for varying BP effects with Ang-(1–7), including species differences, diet, and dose, duration, and route of treatment. The discrepancy between the 2 previous studies and our results most likely reflects differences in animal models. Fructose-fed rats do not gain weight during the first 8 weeks feeding and, for the most part, develop isolated systolic hypertension.30 In contrast, HFD-fed mice have ≈5x more adiposity than their lean counterparts and develop both systolic and diastolic hypertension. The hypertension in these 2 models may, therefore, be driven by different mechanisms with varying sensitivity to Ang-(1–7).

Ang-(1–7) could also influence regional perfusion to improve insulin sensitivity. Indeed, acute Ang-(1–7) increases muscle microvascular perfusion in normal rats.31 We were unable to detect differences in insulin-stimulated muscle perfusion after Ang-(1–7) in HFD mice. There are several possible explanations: (1) previous studies used acute intravenous Ang-(1–7) and were done under anesthesia;5,34 which alone has hemodynamic effects; (2) the ability of acute Ang-(1–7) to promote peripheral vasodilation could be lost after prolonged exposure; (3) although microsphere deposition is the gold standard for assessing microvascular perfusion in conscious animals, it may not give the resolution necessary to detect small changes; (4) the mild hyperglycemia in HFD mice may contribute to endothelial dysfunction, thus preventing Ang-(1–7) vasodilatory actions; and (5) Ang-(1–7) may improve other aspects of endothelial function that enhance SkM insulin action, such as transendothelial insulin transport from capillaries to the myocyte surface.

Given that we did not observe changes in perfusion, we examined direct effects of Ang-(1–7) on SkM. A critical component of SkM insulin action is partitioning of Glut4 to the sarcolemma to enhance glucose transport.31 Chronic Ang-(1–7) potentiated insulin-stimulated Glut4 translocation in HFD mice, thereby increasing glucose transport into myocytes. This is consistent with previous reports demonstrating effects of Ang-(1–7) on Glut4. Namely, ACE2 deletion in mice reduces SkM Glut4 expression, an effect reversed by Ang-(1–7) infusion.9 Furthermore, ACE inhibition, which increases Ang-(1–7) levels, improves insulin sensitivity by enhancing Glut4 translocation.32 Insulin stimulates Glut4 translocation via activation of the PI3K/Akt pathway. Ultimately, Akt phosphorylates and inactivates AS160 (a Rab GTPase-activating protein) to allow Glut4-containing vesicles to fuse with the sarcolemma.33 In normal and fructose-fed rats, Ang-(1–7) stimulates Akt phosphorylation in insulin-sensitive tissues.3,34,35 In addition, Ang-(1–7) increases AS160 phosphorylation in normal and diabetic rats.34,36 In our study, Ang-(1–7) reduced AS160 protein levels, which functionally promotes increased Glut4 at the myocyte plasma membrane,37 independent of changes in classical Akt signaling. The precise mechanism suppressing AS160 levels is unclear, but may involve transcriptional regulation.

There are some potential limitations to these studies. We did not examine receptor and related intracellular signaling mechanisms involved in Ang-(1–7) metabolic effects. The literature suggests that most, if not all, of Ang-(1–7) actions in vivo are mediated by mas receptors.38 Indeed, the ability of Ang-(1–7) to augment insulin-mediated glucose disposal is mas-dependent in anesthetized rats.18 There is an evidence, however, that AT1 receptors participate in complex vascular effects of Ang-(1–7). Furthermore, because of limitations in blood volume sampling in mice, we were unable to measure all components of the circulating RAS. The few studies examining Ang-(1–7) effects on RAS components showed no effect on renin activity in lean rats39 and reduced aldosterone in fructose-fed rats.7 We observed significant plasma Ang II elevations after Ang-(1–7) infusion in chow-fed mice, with no effect in HFD mice. A similar elevation was observed in lean rats and thought to reflect a homeostatic feedback response.29 Finally, it should be noted that the HFD had lower sodium content compared with the control diet. Despite lower sodium, HFD mice exhibited a cardiometabolic syndrome–like phenotype and did not have increased RAS components. Furthermore, all HFD mice were fed the same diet, indicating the improved insulin sensitivity with Ang-(1–7) was sodium independent.

In summary, these studies show that chronic Ang-(1–7) reverses diet-induced muscle insulin resistance by enhancing insulin-stimulated Glut4 translocation. Importantly, these effects occurred even when giving a dose of Ang-(1–7) that does not lower BP. These findings provide evidence for divergence between Ang-(1–7) effects on BP and glucose metabolism, and support the growing notion that the RAS influences whole-body physiology beyond control of BP.40

**Perspectives**

The worldwide prevalence of insulin resistance secondary to obesity is alarmingly high. Thus, there is an urgent need to understand molecular mechanisms contributing to obesity-associated insulin resistance and related cardiovascular comorbidities. Therapies that block Ang II actions have emerged as an important strategy to lower BP in obese patients because of their positive metabolic profile.1 The positive metabolic effects of these therapies, however, may also reflect elevated Ang-(1–7) levels.4 Animal models of cardiometabolic syndrome have reduced circulating levels of Ang-(1–7),40 and modulation of the Ang-(1–7)/ACE2/mas receptor axis improves glucose metabolism. We extend these findings by showing that Ang-(1–7) has direct insulin-sensitizing effects on SkM in obese mice, which are BP independent. These findings enhance our understanding of RAS mechanisms involved in regulation of insulin action, and provide new insight into the potential for targeting Ang-(1–7) in cardiometabolic disease.
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Disclosures

None.

References


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### Novelty and Significance

**What Is New?**

- Chronic angiotensin-(1–7) administration reverses diet-induced muscle insulin resistance by enhancing insulin-stimulated glucose transporter 4 translocation. Importantly, these insulin-sensitizing effects are independent of changes in body weight and blood pressure.

**What Is Relevant?**

- These findings provide new insight into tissue-specific mechanisms by which angiotensin-(1–7) improves insulin action in an animal model of cardiometabolic syndrome, and enhance our understanding of renin–angiotensin mechanisms involved in glucose homeostasis.

### Summary

This study provides new evidence for blood pressure-independent effects of angiotensin-(1–7) to improve skeletal muscle insulin action in obese mice. These findings provide further support for targeting angiotensin-(1–7) in treatment of cardiometabolic disease.
Chronic Angiotensin-(1–7) Improves Insulin Sensitivity in High-Fat Fed Mice Independent of Blood Pressure

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ONLINE SUPPLEMENT:

CHRONIC ANGIOTENSIN-(1-7) IMPROVES INSULIN SENSITIVITY IN HIGH-FAT FED MICE INDEPENDENT OF BLOOD PRESSURE

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Short Title: Insulin-Sensitizing Effects of Angiotensin-(1-7)

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Supplemental Methods

Mouse Models:
Mice were housed at 23°C in a humidity-controlled room on a 12:12-hour light: dark cycle. Male 6 week-old C57BL/6J mice (Jackson Laboratory) were fed a standard chow (5001 Laboratory Rodent Diet) or 60% high fat (Bioserv F3282) diet ad libitum for 11 weeks. After 8 weeks of diet, subcutaneous osmotic mini-pumps (Alzet Model 2004) were implanted to deliver Ang-(1-7) [400 ng/kg/min; Bachem] or saline vehicle for 3 weeks. This dose, route of administration, and time course of Ang-(1-7) was chosen as it has been shown to improve endothelial function in HFD mice independent of changes in body weight.¹

Assessment of Blood Pressure and Heart Rate:
Blood pressure and heart rate were measured in conscious mice on the morning of insulin clamps, after approximately 2.5 hours of fasting. The indwelling carotid artery catheter was connected to a strain-gauge transducer and a stand-alone BPA-400 analyzer connected to a printer (Digi-Med). The analyzer recorded blood pressure and heart rate data at 10-second intervals. After a 15-minute acclimation period, we recorded the first five measurements and took the average of these to report blood pressure and heart rate. This method was used since: (1) direct arterial blood pressure measurement is more sensitive and accurate than tail cuff methods; and (2) it is not possible to measure blood pressure by gold standard radiotelemetry methods in the clamped animals given the need for carotid artery catheterization for both procedures.

Hyperinsulinemic-Euglycemic Clamps (Insulin Clamp):
Insulin clamps were performed in conscious, unstressed mice as previously described.²⁻⁴ At least 5 days before experiments, carotid artery and jugular vein catheters were implanted for blood sampling and infusions, respectively. Mice were fasted for 5 hours prior to starting the insulin clamp. A primed, continuous infusion of [3-³H]glucose (0.04μCi·min⁻¹; Perkin Elmer) was started at t=−90 min to determine rates of plasma glucose appearance (Ra) and disappearance (Rd). Basal arterial samples were collected to measure glucose specific activity (t=−5 and -5 min), insulin (t=−5 min), and non-esterified fatty acids (NEFA; t=−15 and -5 min; average value reported). At t=0 min, continuous insulin (4mU·kg⁻¹·min⁻¹; Humulin R; Eli Lilly) and variable glucose (D50 + 50μCi[3⁻³H]glucose) infusions were initiated. Donor erythrocytes were infused throughout the insulin clamp to prevent a fall in hematocrit. From time t=0 to 120 min, arterial glucose was measured every 10 min, with the exogenous glucose infusion rate (GIR) adjusted to maintain euglycemia (130-150 mg/dl). The steady-state GIR and [3⁻³H]glucose kinetics were determined from t=80 to 120 min. During the clamp, insulin was measured at t=100 and 120 min and NEFA at t=80 and 120 min (average value reported). At t=120 min, mice received an intravenous bolus of 2[¹⁴C]deoxyglucose (2[¹⁴C]DG; 13μCi; Perkin Elmer) to determine the glucose metabolic index (Rg), a measure of tissue-specific glucose uptake.⁵ Blood samples were obtained at 2, 7, 15, 25, and 35 min after the bolus to measure plasma 2[¹⁴C]DG disappearance. At t=155 min, mice were sacrificed and tissues were immediately freeze-clamped and stored at -80°C for biochemical analysis or incubated in 10% neutral buffered formalin followed by 70% ethanol for histological analysis.

Determination of Plasma 3[³H]glucose and 2[¹⁴C]2deoxyglucose:
To measure plasma and infusate levels of the isotopic tracers, $^{3}$H-glucose and $^{2}$H$_{2}$O, samples were first diluted twofold in 0.9% NaCl and then deproteinized by sequential treatment with barium hydroxide [Ba(OH)$_{2}$, 0.3N] and zinc sulfate (ZnSO$_{4}$, 0.3N). Deproteinized plasma samples were centrifuged for 5 minutes at 13,000 rpm and 100 µl of the resulting supernatant was transferred to a glass vial. For $^{3}$H-glucose samples, the supernatant was dried in a vacuum oven for 1 hour at 60°C followed by addition of 1 ml ddH$_{2}$O. For $^{2}$H$_{2}$O samples, 990 µl ddH$_{2}$O was added directly to 100ul of supernatant. Finally, 10 ml of Ultima Gold scintillation cocktail (Perkin Elmer) was added to all vials and radioactivity was measured using a Tri-Carb 2900TR liquid scintillation counter (Packard).

**Calculation of $R_{a}$, $R_{d}$, and Endo$R_{a}$:**

The rates of whole-body plasma glucose appearance ($R_{a}$) and disappearance ($R_{d}$) were determined from plasma $^{3}$H-glucose levels and calculated using non-steady state equations$^{6}$:

\[
R_{a} = \frac{I}{V_{d}A} \frac{SA}{t} \quad R_{d} = \frac{R_{a}}{V_{d}} \frac{A}{t}
\]

where $I$ is the tracer infusion rate (dpm•kg$^{-1}$•min$^{-1}$), $V_{d}$ is the volume of distribution (dl•kg), $A$ is the arterial glucose (mg•dl$^{-1}$), $\partial SA/\partial t$ is the rate of change in glucose specific activity over 30 minutes (dpm•mg$^{-1}$•min$^{-1}$), $SA$ is glucose specific activity (dpm•mg$^{-1}$), and $\partial A/\partial t$ is the rate of change in arterial glucose over 30 minutes. These equations describe the turnover of glucose in a single, well-mixed compartment. Arterial glucose and glucose specific activity used in the equations were calculated using a moving average over three consecutive time points.$^{7}$ $R_{a}$ and $R_{d}$ were calculated at $t =$ 90, 100, and 110 min and averaged. To calculate Endo$R_{a}$, the exogenous glucose infusion rate was subtracted from total $R_{a}$. Non-steady state equations were used for all calculations; however, steady state calculations gave nearly identical rates, suggesting that steady state conditions were present.

**Determination of Tissue $^{2}$H$_{2}$O and Glucose Metabolic Index ($R_{g}$):**

To determine levels of $^{2}$H$_{2}$O and $^{2}$H$_{2}$O-6-phosphate ($^{2}$H$_{2}$DG), tissues were first homogenized (Bullet Blender, Next Advance) in 0.5% perchloric acid to extract metabolites. After centrifugation at 13,000 rpm for 5 minutes, the homogenate pH was adjusted to 7.5 with KOH. Radioactivity was counted in one aliquot of the neutralized homogenate to determine tissue levels of combined $^{2}$H$_{2}$DG and $^{2}$H$_{2}$DGP. A separate aliquot was treated with Ba(OH)$_{2}$ and ZnSO$_{4}$ to remove $^{2}$H$_{2}$DG and any glycogen-incorporated radioactivity. This sample was then counted to measure $^{2}$H$_{2}$DG. The difference in radioactivity between the first aliquot ($^{2}$H$_{2}$DG + $^{2}$H$_{2}$DGP) and second aliquot ($^{2}$H$_{2}$DG) is equal to $^{2}$H$_{2}$DGP. Glucose metabolic index ($R_{g}$) was calculated using the equation$^{5}$:

\[
R_{g} = \frac{2^{14}C_{DGP_{tissue}}}{AUC2^{14}C_{DG_{plasma}}} \cdot [arterial glucose]
\]
where \(2[14C]DGP_{\text{tissue}}\) is the accumulated \(2[14C]DGP\) in tissue (dpm•mg\(^{-1}\)), AUC\(2[14C]DG_{\text{plasma}}\) is the area under the plasma \(2[14C]DG\) disappearance curve (dpm•ml\(^{-1}•\text{min}\)), and [arterial glucose] is the average blood glucose (mmol•L\(^{-1}\)) from \(t=120\)-155 minutes of the clamp period.

**Immunoblotting:**
Gastrocnemius muscle was homogenized in buffer containing 50mM Tris-HCl (pH 7.5), 100mM NaCl, 1mM EDTA, 1mM EGTA, 1% TritonX-100, 1mM DTT, 10% glycerol, and Halt Protease and Phosphatase Inhibitor Cocktail (Life Technologies) and centrifuged at 13,000 rpm for 20 min at 4°C. Homogenates were run on SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. Membranes were probed with the following antibodies: phosphorylated Akt (Ser\(^{473}\); Cell Signaling), total Akt (Cell Signaling), phosphorylated AS160 (Ser\(^{588}\); Cell Signaling), and total AS160 (Millipore). Appropriate fluorophore-conjugated secondary antibodies were used (LI-COR). Band densities were imaged (Odyssey, LI-COR) and quantified by densitometry (ImageJ).

**Microsphere Recovery:**
In a separate cohort of high-fat fed mice \([n=4 \text{ saline and } n=3 \text{ Ang-(1-7)}]\), 100 µl of yellow 15-µm microspheres (Dye-Trak, Triton Technology) were injected into the carotid artery after the last sampling time point of the insulin clamp to measure regional blood flow to the hind limb. At 5 minutes following microsphere administration, the hind limb (gastrocnemius, soleus, and vastus muscles) was excised, weighed and incubated in 1M KOH at 60°C overnight. Blue microspheres (10,000) were added to each sample to control for loss of microspheres during processing. The following day, samples were vortexed, 50°C ddH\(_2\)O was added, and samples were centrifuged for 15 minutes at 2623 rpm at room temperature. The supernatant was then aspirated and the pellet resuspended in TritonX-100 by sonication. This centrifugation, supernatant aspiration, resuspension sequence was repeated with 0.2% HCl in ethanol followed by ethanol alone. After aspirating the ethanol, pellets were allowed to dry overnight. To elute the dye from microspheres, pellets were incubated in \(N,N\)-dimethylformamide for 15 minutes. Following centrifugation for 5 min at 2623 rpm, supernatants were added to 96 well polypropylene plates and Abs\(_{440}\) and Abs\(_{670}\) were measured on a spectrophotometer. Absorbance readings were converted to microsphere numbers based on a known standard curve from the manufacturer. Arterial microsphere mixing was considered adequate if the difference in the number of microspheres deposited in the left and right kidneys was less than 15%.

**CD31 Immunostaining and Quantification:**
CD31 staining was assessed by immunohistochemistry (IHC) in paraffin-embedded gastrocnemius sections (5µm) using the Leica Bond Max IHC stainer. Epitopes were retrieved by heat-induced antigen retrieval using Epitope Retrieval 2 solution (Leica) for 20 minutes. Slides were incubated with an anti-CD31 primary antibody (1:100; Dianova) for 60 minutes followed by a biotinylated anti-rat antibody (1:200; Vector) for 15 minutes. Slides were then lightly counterstained with Mayer hematoxylin solution. The EnVision+HRP/DAB System (DakoCytomation) was used to produce localized, visible staining. Images were captured at 175x total magnification using a Nikon DS-Ri2 camera mounted on a Nikon AZ100M upright, wide-field microscope. Quantification of capillary density was performed by counting CD31+ structures using a custom, automated macro in ImageJ. An investigator blind to diet and treatment performed all image acquisition and analyses.
**Glut4/Cav3 Immunofluorescent Staining:**
Following hyperinsulinemic-euglycemic clamps, the gastrocnemius muscle was excised, fixed in 10% neutral-buffered formalin, and paraffin-embedded. 5μm sections were cut using a microtome and mounted on charged slides. Sections were deparaffinized in xylene and rehydrated through graded alcohol solutions. To retrieve epitopes masked by formalin fixation, sections were incubated in a 10mM Tris buffer (pH 9) for 50 minutes at 90°C. After a brief wash in phosphate-buffered saline (PBS), non-specific secondary antibody binding was blocked for 1 hour in 5% normal goat serum (NGS; Jackson ImmunoResearch) and ‘Mouse on Mouse’ blocking reagent (M.O.M.; Vector) at room temperature. Then, sections were incubated overnight at 4°C in rabbit anti-Glut4 (1:200; Abcam) and mouse anti-Cav3(1:200; Santa Cruz) in 5%NGS/PBS. The next day sections were washed in PBS followed by incubation in an AlexaFluor 647 conjugated goat anti-rabbit (1:500; Life Technologies) and an AlexaFluor 555 conjugated goat anti-mouse secondary antibody (1:500; Life Technologies) in 5% NGS/PBS. Finally, sections were rinsed, mounted in 50% glycerol/H2O, cover slipped, and stored at 4°C in the dark. Control sections stained in the same manner as described above, but lacking primary antibodies, were used to demonstrate specificity of the secondary antibodies (Figure S3). Similarly, sections stained with only one primary antibody were used to ensure that secondary antibodies only recognized the correct primary antibody (Figure S3).

**Glut4/Cav3 Image Acquisition:**
Images were acquired using an inverted Zeiss LSM 510 confocal microscope equipped with two helium-neon (HeNe) lasers (543nm and 633nm) and a 40X 1.3NA oil immersion Plan Neofluar objective (Zeiss). Alexa Fluor 555 was excited with the 543nm laser line of the HeNe laser and emitted light was collected using a 560-615nm bandpass emission filter. Alexa Fluor 647 was excited with the 633 nm laser line of the HeNe laser and emitted light was collected using a longpass 650nm filter. Sampling was performed at a resolution fulfilling the Nyquist criteria and with the pinhole set to give an optical section of 1.6μm. The two fluorophores were excited sequentially to prevent bleed-through and frame averaged 2 times. Detector settings were kept constant throughout imaging to allow for quantitative comparisons. At least 2 images were collected per section from 3 serial sections per mouse.

**Glut4/Cav3 Image Analysis:**
All image analysis was performed using ImageJ software (NIH). Initially, images were background corrected using the rolling ball radius background subtraction method. Then, automatically thresholded Cav3 immunostaining was used to create a mask demarcating the myofiber plasma membrane. This mask was applied to the Glut4 (AlexaFluor 647) channel and the mean Glut4 fluorescence intensity within this mask was measured. An investigator blinded to the study performed image acquisition data analysis.

**Circulating Angiotensin Peptide levels:**
Plasma Ang-(1-7) and Ang II levels were measured in a separate cohort of mice that did not undergo insulin clamps [n=6 chow-fed, vehicle; n=6 chow-fed Ang-(1-7); n=8 HFD, vehicle; n=8 HFD Ang-(1-7)]. Blood was collected in an EDTA tube containing peptidase inhibitor cocktail to prevent in vitro metabolism. Plasma was harvested and sent to the Hypertension Core Laboratory at Wake Forest University for analysis as previously described. Ang-(1-7) was
measured by radioimmunoassay using a custom antibody. The minimum detectable level is 0.8 pg/tube with 8% intra-assay coefficient of variation. Ang II was measured using a commercially available radioimmunoassay kit (ALPCO Diagnostics, RK-A22). The minimum detectable level is 2.5 pg/tube with 12% intra-assay coefficient of variation.

**Antibodies:**

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WB = western blot; IHC = immunohistochemistry; IF = immunofluorescence.
Reference List:

Table S1: Effect of Ang-(1-7) on Cardiac Function in High-Fat Fed Mice

<table>
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<tr>
<th>Parameter (unit)</th>
<th>Vehicle</th>
<th>Ang-(1-7)</th>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>7</td>
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<tr>
<td>Cardiac output (ml/min)</td>
<td>23.2 ± 1.9</td>
<td>24.1 ± 1.6</td>
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<td>Stroke volume (μl)</td>
<td>35.6 ± 2.9</td>
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<td>Ejection fraction (%)</td>
<td>78.1 ± 1.3</td>
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<td>Fractional shortening (%)</td>
<td>45.8 ± 1.2</td>
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<td>Left ventricular mass (mg)</td>
<td>102.8 ± 9.3</td>
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<td>1.32 ± 0.04</td>
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</table>

Data are mean ± SEM. Parameters of cardiac function were calculated from M-mode echocardiographic tracings. Ang, angiotensin; LVIDd, left ventricular internal dimension, diastolic; LVIDs, left ventricular internal dimension, systolic; LVPWd, left ventricular posterior wall, diastolic; LVPWs, left ventricular posterior wall, systolic; IVSd, interventricular septum, diastolic; IVSs, interventricular septum, systolic.
Chronic Ang-(1-7) infusion does not alter glucose turnover or tissue-specific glucose uptake in chow-fed mice. A and B: There were no differences in the basal and insulin-stimulated clamp rates of (A) endogenous glucose production (EndoRa) or (B) peripheral glucose disposal (Rd) following Ang-(1-7) versus vehicle in chow fed mice. C: The glucose metabolic index (Rg) in various insulin-sensitive tissues was also not different between groups.
Ang-(1-7) infusion does not change capillary density or insulin-stimulated muscle perfusion in high fat diet-fed (HFD) mice. **A**: Representative 175X magnification micrographs of gastrocnemius sections from clamped mice stained immunohistochemically for CD31 to visualize blood vessels. **B**: The number of CD31+ structures per field of view, an index of capillary density, was not altered by Ang-(1-7) treatment in chow or HFD mice. **C**: Colored microspheres (15μm-diameter) were administered into the carotid artery at the end of hyperinsulinemic-euglycemic clamps, with their deposition measured in soleus, gastrocnemius, and vastus skeletal muscles, to obtain an index of hind limb perfusion. There was no difference in hind limb perfusion following vehicle versus Ang-(1-7) treatment in HFD mice.
Figure S3

Labeling controls for CAV3/GLUT4 immunofluorescence staining. Representative micrographs of gastrocnemius sections obtained following hyperinsulinemic-euglycemic clamps in high-fat fed mice. Images were obtained by confocal microscopy to demonstrate specificity of secondary antibodies for the appropriate primary antibody. All control sections were treated with both AlexaFluor555-conjugated goat anti-mouse and AlexaFluor647-conjugated goat anti-rabbit secondary antibodies. The left column represents sections not treated with any primary antibodies (no primary control). The sections in the middle and right columns were treated with either CAV3 or GLUT4 primary antibodies, respectively; to ensure that secondary antibodies only recognized the correct primary antibody.
Figure S4

Chronic Ang-(1-7) treatment does not alter skeletal muscle Akt phosphorylation or protein levels. Immunoblot of gastrocnemius extracts from high-fat diet fed (HFD) mice treated with antibodies against total Akt, phosphorylated Akt [pAkt(S473)], and the loading control GAPDH. There was no effect of chronic Ang-(1-7) infusion on Akt or phosphorylated Akt protein levels in HFD mice.