Losartan increases muscle insulin delivery and rescues insulin’s metabolic action during lipid infusion via microvascular recruitment

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Wang N, Chai W, Zhao L, Tao L, Cao W, Liu Z. Losartan increases muscle insulin delivery and rescues insulin’s metabolic action during lipid infusion via microvascular recruitment. Am J Physiol Endocrinol Metab 304: E538–E545, 2013. First published January 8, 2013; doi:10.1152/ajpendo.00537.2012.—Insulin delivery and transendothelial insulin transport are two discrete steps that limit muscle insulin action. Angiotensin II type 1 receptor (AT1R) blockade recruits microvasculature and increases glucose use in muscle. Increased muscle microvascular perfusion is associated with increased muscle delivery and action of insulin. To examine the effect of acute AT1R blockade on muscle insulin uptake and action, rats were studied after an overnight fast to examine the effects of losartan on muscle insulin uptake (protocol 1), microvascular perfusion (protocol 2), and insulin’s microvascular and metabolic actions in the state of insulin resistance (protocol 3). Endothelial cell insulin uptake was assessed, using 125I-insulin as tracer. Systemic lipid infusion was used to induce insulin resistance. Losartan significantly increased muscle insulin uptake (~60%, P < 0.03), which was associated with a two-to threefold increase in muscle microvascular blood volume (MBV; P = 0.002) and flow (MBF; P = 0.002). Losartan increased muscle microvascular perfusion, associated with increased muscle delivery and action of insulin. Angiotensin II had no effect on insulin internalization in cultured endothelial cells. Lipid infusion abolished insulin-mediated increases in muscle MBV and MBF and lowered insulin-stimulated whole body glucose disposal (P = 0.0001), which were reversed by losartan administration. Inhibition of nitric oxide synthase abolished losartan-induced muscle insulin uptake and reversal of lipid-induced metabolic insulin resistance. We conclude that AT1R blockade increases muscle insulin uptake mainly via microvascular recruitment and rescues insulin’s metabolic action in the insulin-resistant state. This may contribute to the clinical findings of decreased cardiovascular events and new onset of diabetes in patients receiving AT1R blockers.

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MUSCLE MICROVASCULATURE provides endothelial surface area for the exchanges of oxygen, nutrients, and hormones between the plasma compartment and muscle interstitium. In the insulin-sensitive state, insulin enhances its own delivery to muscle interstitium by relaxing precapillary arterioles to recruit microvasculature, thus expanding endothelial exchange surface area, and transendothelial transport, two discrete steps that limit insulin’s action (1, 2, 12, 45). Insulin’s microvascular action is coupled with its metabolic action since inhibition of insulin-mediated microvascular recruitment with nitric oxide (NO) synthase inhibitor decreases insulin-stimulated glucose disposal by ~40% (39, 40).

Insulin resistance is clearly present in the skeletal muscle microcirculation in patients with or animal models of obesity and/or diabetes, and microvascular insulin resistance is closely associated with metabolic insulin resistance in diabetes (1, 23, 24, 37). Both obese Zucker rats (41) and Zucker diabetic fatty rats (14) have decreased basal muscle microvascular blood volume (MBV) and impaired insulin-mediated glucose disposal and microvascular recruitment. In humans with moderate insulin resistance as seen in simple obesity, basal muscle MBV is not decreased, but insulin fails to induce muscle microvascular recruitment (15). In experimental insulin-resistant states, both inflammatory cytokines and high concentrations of plasma free fatty acids are able to decrease insulin-mediated whole body glucose disposal along with blunted insulin-mediated microvascular recruitment (13, 21, 49, 50).

We and others have shown that factors that increase muscle microvascular recruitment, such as low-intensity muscle contraction (22) and systemic administration of glucagon-like peptide 1 (8), increase muscle insulin uptake. On the other hand, microvascular recruitment induced by PD-123319 is associated with decreased muscle insulin delivery and attenuated insulin action in muscle (9). Thus, modulation of muscle microvascular perfusion could actively alter muscle insulin action, and as such, precapillary arterioles could be a therapeutically targetable site for insulin resistance.

Microvessels express both angiotensin II type 1 (AT1R) and type 2 (AT2R) receptors (5, 6, 34). Whereas AT1Rs mediate vasoconstriction, AT2R activities promote vasodilation (3, 27, 30). AT1R blockers have been widely used clinically in patients with various cardiovascular conditions and have been shown to decrease cardiovascular morbidity and mortality and new onset of diabetes in patients on chronic treatment (30, 36). We have reported recently that acute AT1R blockade potently recruits muscle microvasculature and increases glucose use and that AT2R antagonism yields exactly the opposite (10). Although microvascular recruitment associated with AT1R blockade reduces muscle insulin uptake (9), it remains unknown whether AT1R blockade-induced microvascular recruitment contributes to increased muscle delivery of insulin and hence, muscle insulin action.

In the current study, we hypothesized that acute AT1R blockade increases muscle insulin delivery and improves insulin sensitivity in the insulin-resistant state. Our results indicate that administration of losartan both increases muscle uptake of insulin via increased microvascular recruitment and prevents lipid-induced metabolic insulin resistance via a NO-dependent pathway.
RESEARCH DESIGN AND METHODS

Animal Preparations and Experimental Protocols

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 220–320 g were studied after an overnight fast. Rats were housed at 22 ± 2°C on a 12:12-h light-dark cycle and fed standard laboratory chow and water ad libitum prior to study. After being anesthetized with pentobarbital sodium (50 mg/kg ip; Abbott Laboratories, North Chicago, IL), rats were placed in a supine position and intubated to maintain a patent airway. A heating pad was used to ensure euthermia. 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- to 45-min baseline period to assure hemodynamic stability and a stable level of anesthesia, rats were studied under the following three protocols (Fig. 1).

**Protocol 1.** Three groups of rats were studied under this protocol (Fig. 1, top). Group 1 received a continuous infusion of saline (10 μl/min) for 30 min. Group 2 received a bolus injection of losartan (AT1R blocker, 0.3 mg/kg iv) at time 0 and then saline infusion for 30 min. Group 3 received losartan injection and then systemic infusion of Nω-nitro-l-arginine methyl ester (l-NAME; 50 μg·kg⁻¹·min⁻¹; Sigma-Aldrich, St. Louis, MO) for 30 min. Blood samples were collected at 25 min for insulin measurement, and then each rat received a bolus intravenous (iv) injection of ¹²⁵I-insulin (1.5 μCi; PerkinElmer, Boston, MA). Rats were euthanized at 30 min. Blood and gastrocnemius were obtained for determination of muscle ¹²⁵I-insulin uptake.

**Protocol 2.** Two groups of rats were studied under this protocol (Fig. 1, middle). One group received a continuous infusion of saline (10 μl/min) for 30 min, whereas the other received a bolus injection of losartan (0.3 mg/kg iv) at time 0 and then saline infusion for 30 min. Skeletal muscle MBV, MFV, and microvascular blood flow (MBF) (MBF = MBV × MFV) were determined using contrast-enhanced ultrasound at time 0 and 30 min, as described previously (8, 10, 21, 22, 40).

**Protocol 3.** Four groups of rats were studied under this protocol (Fig. 1, bottom). Each rat received either saline (group 1, 10 μl/min) or intralipid plus heparin (3.3% and 30 U/ml at 10 μl/min, groups 2–4) infusion for 3 h, with a euglycemic hyperinsulinemic clamp (3 mU·kg⁻¹·min⁻¹) superimposed in the last 2 h (time 0 to 120 min). Groups 3 and 4 received a bolus iv injection of losartan (0.3 mg/kg) 5 min before the initiation of insulin clamp. Group 4 rats also received a simultaneous infusion of l-NAME in the last 2 h. Arterial blood glucose was determined every 10 min using an Accu-Chek Advantage glucometer (Roche Diagnostics, Indianapolis, IN), and 30% dextrose (30% wt/vol) was infused at a variable rate to maintain blood glucose within 10% of basal (11, 44). Skeletal muscle MBV, MFV, and MBF were determined at times 0, 30, 60, and 120 min. Plasma NO concentrations were determined at times 0, 30 and 60 min, as described below. Rats were then euthanized, and gastrocnemius muscle was freeze-clamped for later measurement of Akt and ERK1/2 phosphorylation using Western blotting, as described previously (11, 44).

Throughout the study, mean arterial blood pressure (MAP) and heart rate were monitored via a sensor connected to the carotid arterial catheter (Harvard Apparatus, Holliston, MA, and ADInstruments, Colorado Springs, CO). Pentobarbital sodium was infused at a variable rate to maintain steady levels of anesthesia and blood pressure throughout the study. Insulin infusion at 3 mU·kg⁻¹·min⁻¹ has been shown to potently recruit muscle microvasculature in rats (40). Losartan was obtained from Sigma Chemicals (St. Louis, MO) and at the doses selected does not significantly alter systemic blood pressure (10, 48) but significantly recruits muscle microvasculature in the postabsorptive state (10). l-NAME at the dose selected (50 μg·kg⁻¹·min⁻¹) raises MAP by 20–30 mmHg above baseline without affecting heart rate and completely inhibits insulin-mediated increases in muscle MBV (39).

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication no. 85-23, revised 1996). The study protocols were

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**Fig. 1.** Experimental protocols. l-NAME, Nω-nitro-l-arginine methyl ester; CEU, contrast-enhanced ultrasound.
approved by the Animal Care and Use Committee at the University of Virginia.

Muscle 125I-Insulin Uptake

Muscle insulin uptake was determined in protocol 1 studies, using 125I-insulin as tracer to track the uptake of native insulin, as described previously (8, 9, 22). Blood samples were collected for insulin determination, and each rat received a bolus iv injection of 1.5 μCi 125I-insulin 5 min prior to the end of the study. Protein-bound 125I-iodide in blood and muscle samples was precipitated with 30% trichloroacetic acid, and radioactivity was measured. Skeletal muscle insulin uptake was calculated using the following formula: muscle insulin uptake (fmol·g muscle−1·5 min−1) = 125I-insulin in muscle (DPM·g dry wt−1·5 min−1)/blood 125I-insulin (DPM/ml) × plasma insulin (fmol/ml).

Measurement of Plasma NO Levels

Plasma NO levels were measured using a 280i Nitric Oxide Analyzer (GE Analytical) according to the manufacturer’s instructions. In brief, ice-cold ethanol was added into plasma samples at a ratio of 2:1. The mixture was vortexed, kept at 0°C for 30 min, and then centrifuged at ~14,000 rpm for 5 min. The supernatant was then used for NO analysis.

Culture of Endothelial Cells and Determination of Insulin Uptake

Endothelial cell insulin uptake was assessed using 125I-insulin, as reported previously (4, 17–19). In brief, bovine aortic endothelial cells in primary culture were purchased from Lonza (Walkersville, MD). Cells between passages 3 and 6 were cultured in six-well plates until 80% confluence, serum starved for 18–22 h, and then incubated with prewarmed N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-binding buffer (0.1 M HEPES, 0.12 M NaCl, 5 mM KCl, 1.2 mM MgSO4, 8 mM glucose, and 1% bovine serum albumin, pH 7.8) containing 200 pM 125I-insulin in the presence or absence of unlabeled regular insulin (2 μM), angiotensin II (1 μM), angiotensin II (1 μM) + losartan (10 μM), or losartan (10 μM) at 37°C for 15 min. The reaction was stopped by transferring the culture plates onto ice and washed with ice-cold HEPES-binding buffer. Cells were then washed twice with ice-cold acid solution (0.5 M NaCl and 0.2 M acetic acid, pH 3.0) to remove surface-bound 125I-insulin and lysed with 0.5 ml 1 M NaOH on ice for 1 h. Aliquots of cell lysate were used for protein content determination and radioactivity quantification using a γ-counter. After the nonspecific binding was subtracted, insulin uptake was calculated and expressed as counts per minute per microgram of protein.

Statistical Analysis

All data are presented as means ± SE. Statistical analyses were performed with SigmaStat 3.1.1 software (Systat Software), using Student’s t-test or ANOVA with post hoc analysis where appropriate. A P value of <0.05 was considered statistically significant.

RESULTS

Losartan Acutely Increases Muscle Uptake Via NO-Dependent Pathway

We have reported previously that systemic administration of losartan markedly recruits muscle microvasculature (9, 10) and that microvascular recruitment is associated with increased muscle delivery of insulin (1, 2). To assess whether losartan indeed increases muscle insulin delivery/uptake, we used 125I-insulin to trace native insulin movement in vivo. Plasma insulin concentrations did not differ significantly among any of the three groups at either baseline (83 ± 19 vs. 93 ± 8 vs. 81 ± 4 pM, saline vs. losartan vs. losartan + L-NAME, respectively, P = 0.687, ANOVA) or 25 min (86 ± 14 vs. 116 ± 11 vs. 76 ± 6 pM, P = 0.06, ANOVA). As shown in Fig. 2, losartan did not alter insulin degradation rates in either blood or in muscle, as reflected by stable fraction of intact 125I-insulin in each compartment. Muscle clearance of blood insulin trended up, but this increase was not statistically significant (P = 0.158, ANOVA). However, losartan injection potently increased muscle insulin uptake (by ~60%, P < 0.03), and this effect was completely abolished by systemic infusion of L-NAME, suggesting that losartan-mediated muscle uptake of insulin is NO dependent.

Losartan Acutely Recruits Muscle Microvasculature but Does Not Affect Endothelial Internalization of Insulin

To assess whether the increased muscle uptake of insulin was secondary to increased insulin delivery to the microcircu-

Fig. 2. Angiotensin II type 1 receptor (AT1R) blockade increases skeletal muscle 125I-insulin uptake. Five minutes after bolus injection of 125I-insulin (1.5 μCi iv), blood and skeletal muscle samples were collected, and intact 125I-insulin was determined after TCA precipitation. A: fraction of blood and muscle intact 125I-insulin. B: muscle clearance of 125I-insulin. C: muscle 125I-insulin uptake; n = 5–9. *P < 0.03 (ANOVA).
lation and/or increased insulin transportation through the vascular endothelium, we first reconfirmed in the current study that losartan injection indeed acutely recruited muscle microvasculature (Fig. 3). Consistent with our prior report, losartan increased muscle MBV ad MBF two- to threefold \( (P = 0.002 \) for both) within 30 min without muscle MFV being affected. We next determined whether losartan altered insulin internalization by the endothelial cells, a key step in the process of transendothelial insulin transport. In the presence of excess unlabeled regular insulin, \( {^{125}}\text{I}-\text{insulin} \) internalization decreased by 43\% \( (Fig. 4, P = 0.0005) \). On the contrary, losartan, in the presence or absence of angiotensin II, had no effect on \( {^{125}}\text{I}-\text{insulin} \) internalization \( (Fig. 4) \). This suggests that the increased muscle uptake of insulin induced by losartan was secondary to increased insulin delivery via microvascular recruitment, and endothelial uptake of insulin is not the rate-limiting step in this process.

Losartan Prevents Lipid-Induced Metabolic Insulin Resistance Via NO-Dependent Mechanism

We next examined whether the increases in muscle microvascular recruitment and insulin uptake help prevent lipid-induced metabolic insulin resistance. Systemic lipid infusion potently inhibited insulin-stimulated whole body glucose disposal \( (by \sim 40\%, P < 0.0001) \), but this inhibitory effect was completely reversed with one dose of losartan injection before the initiation of insulin clamp \( (Fig. 5) \). In the presence of systemic infusion of \( \text{i-NAME} \), this salutary effect of losartan disappeared.

Effect of Losartan on Insulin-Stimulated Akt and ERK1/2 Phosphorylation During Lipid Infusion

Figure 6 shows insulin-stimulated phosphorylation of muscle Akt \( (Fig. 6, \text{top}) \) and ERK1/2 \( (Fig. 6, \text{bottom}) \) in the presence or absence of lipid infusion and losartan. There was no statistically significant difference among three groups in insulin-stimulated Akt phosphorylation. Lipid infusion decreased insulin-stimulated ERK1/2 phosphorylation in muscle, and this effect was prevented by systemic administration of losartan prior to initiation of insulin infusion \( (P < 0.03) \).

Lipid Infusion Inhibits Insulin-Mediated but not Losartan Plus Insulin-Mediated Microvascular Recruitment

As shown in Fig. 7, insulin infusion potently recruited muscle microvasculature by increasing both muscle MBV \( (P < 0.02) \) and MBF \( (P < 0.04) \) without affecting muscle MFV. This effect was completely abolished by systemic lipid infusion. Administration of losartan prior to insulin infusion recruited muscle microvasculature in the presence of lipid infusion, as evidenced by two- to threefold increases in both MBV \( (P < 0.02) \) and MBF \( (P < 0.04) \). As with insulin alone, MFV did not change. Coinfusion of \( \text{i-NAME} \) completely abolished losartan-induced increases in both MBV and MBF. Consistent with the changes in MBV and MBF, lipid infusion ablished insulin-mediated increases in plasma NO concentrations, which were restored back to insulin-alone levels in the presence of losartan \( (Fig. 8, \text{top}) \). As expected, lipid infusion did not alter MAP values, but \( \text{i-NAME} \) infusion did increase MAP by 20–30 mmHg \( (Fig. 8, \text{bottom}) \), which was similar to our prior report \( (10) \).

DISCUSSION

The current study demonstrated for the first time that acute \( \text{AT}_{1}\text{R} \) blockade with losartan potently increased muscle insulin uptake and prevented lipid-induced metabolic insulin resistance in vivo via a NO-dependent mechanism. As in cultured
endothelial cells, losartan in the presence or absence of AT1R ligand angiotensin II did not alter insulin internalization, a surrogate index of insulin uptake by the endothelial cells, and the increased muscle insulin uptake is likely secondary to microvascular recruitment and expanded endothelial surface area. Our findings thus strongly suggest that AT1R blockade improves insulin action via microvascular insulin delivery, which is a major rate-limiting step in skeletal muscle insulin action (1, 12, 32, 33, 45).

Although we have reported previously that losartan administration acutely increases muscle microvascular recruitment and glucose use in the postabsorptive state (10), losartan administration did not significantly alter insulin-mediated glucose disposal in the hyperinsulinemic euglycemic state (9). It is very likely that, in the insulin-sensitive state, insulin per se acted to increase its own delivery by recruiting muscle microvasculature and enhancing its own transendothelial transport via activation of insulin-signaling pathways (1, 2, 42, 43). Because both steps are impaired in the insulin-resistant states, our observation that losartan treatment acutely increases muscle microvascular recruitment and restores insulin’s metabolic sensitivity during lipid infusion is of particular significance. Indeed, numerous clinical trials have confirmed a salutary effect of AT1R blockade on decreasing cardiovascular morbidity and mortality and on decreasing the incidence of new onset of diabetes in patients who are chronically on AT1R blockers (30, 36). Thus, chronic therapy with AT1R blocker could enhance insulin action via increased muscle delivery of insulin, as evidence has strongly suggested that it is the insulin con-

Fig. 5. AT1R antagonism prevents lipid-induced metabolic insulin resistance. Each rat received 3 h of saline or intralipid + heparin infusion with insulin clamp (3 mU·kg⁻¹·min⁻¹) superimposed in the last 2 h with or without losartan injection (0.3 mg·kg⁻¹ iv) at time = 5 min. A: time course of glucose infusion rates (GIR). B: steady-state GIR; n = 6-11. *P < 0.001 (ANOVA).

Fig. 6. Effect of AT1R blockade on insulin-stimulated skeletal muscle Akt (top) and ERK1/2 (bottom) phosphorylation. *P < 0.03 compared with lipid + insulin.

Fig. 7. AT1R blockade recruits muscle microvasculature during lipid infusion via a nitric oxide-dependent mechanism. A: changes in muscle MBV. *P < 0.02 (ANOVA). B: changes in muscle MFV. C: changes in muscle MBF. **P < 0.04 (ANOVA); n = 5-6.
centrations in the muscle interstitium, not plasma, that correlate with insulin’s metabolic effects (7).

We and others have reported previously that insulin potently increases muscle MBV and thus expands microvascular endothelial surface area (13, 15, 16, 29, 31, 35). In the current study, insulin again increased muscle MBV and MBF significantly, and these effects were abolished in the presence of systemic lipid infusion, which is consistent with prior reports in both laboratory animals (13, 21) and humans (28, 31). Administration of losartan prior to the initiation of insulin infusion in the presence of lipid infusion increased muscle MBV by approximately two- to threefold (Fig. 5) to the levels seen with losartan alone (10). This was associated with an almost complete restoration in insulin-mediated glucose disposal. Although addition of losartan to insulin did not further increase muscle insulin action beyond what we saw with insulin alone (9), our current results strongly suggest that increased microvascular recruitment must have contributed to this complete restoration in insulin’s metabolic action in the insulin-resistant state and strongly suggest that pharmacological manipulation of microvascular AT1R and AT2R activity ratio could be an important therapeutic target for the prevention and management of obesity and diabetes.

In conclusion, acute AT1R blockade with losartan increases muscle insulin uptake mainly via microvascular recruitment and rescues insulin’s metabolic action in the insulin-resistant state induced by systemic lipid infusion. This may contribute to the clinical findings of decreased cardiovascular events and new onset of diabetes in patients receiving AT1R blockers. It is likely that AT2R may have also played an important role in this process since AT1R blockade leads to unopposed stimulation of AT2R, whose antagonism recruits microvasculature, decreases insulin delivery, and attenuates insulin action in muscle.

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DISCLOSURES
The authors have no conflicts of interest to disclose, financial or otherwise.

AUTHOR CONTRIBUTIONS
N.W., W. Chai, and L.Z. performed the experiments; N.W., W. Chai, L.Z., and Z.L. analyzed the data; N.W., W. Chai, L.T., W. Cao, and Z.L. interpreted the results of experiments; N.W., W. Chai, L.Z., and Z.L. prepared the figures; N.W., W. Chai, L.Z., L.T., W. Cao, and Z.L. approved the final version of the manuscript; Z.L. contributed to the conception and design of the research; Z.L. drafted the manuscript; Z.L. edited and revised the manuscript.
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