Acute, local infusion of angiotensin II impairs microvascular and metabolic insulin sensitivity in skeletal muscle

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Aims

Angiotensin II (AngII) is a potent vasoconstrictor implicated in both hypertension and insulin resistance. Insulin dilates the vasculature in skeletal muscle to increase microvascular blood flow and enhance glucose disposal. In the present study, we investigated whether acute AngII infusion interferes with insulin's microvascular and metabolic actions in skeletal muscle.

Methods and results

Adult, male Sprague-Dawley rats received a systemic infusion of either saline, AngII, insulin (hyperinsulinaemic euglycaemic clamp), or insulin (hyperinsulinaemic euglycaemic clamp) plus AngII. A final, separate group of rats received an acute local infusion of AngII into a single hindleg during systemic insulin (hyperinsulinaemic euglycaemic clamp) infusion. In all animals' systemic metabolic effects, central haemodynamics, femoral artery blood flow, microvascular blood flow, and skeletal muscle glucose uptake (isotopic glucose) were monitored. Systemic AngII infusion increased blood pressure, decreased heart rate, and markedly increased circulating glucose and insulin concentrations. Systemic infusion of AngII during hyperinsulinaemic euglycaemic clamp inhibited insulin-mediated suppression of hepatic glucose output and insulin-stimulated microvascular blood flow in skeletal muscle but did not alter insulin's effects on the femoral artery or muscle glucose uptake. Local AngII infusion did not alter blood pressure, heart rate, or circulating glucose and insulin. However, local AngII inhibited insulin-stimulated microvascular blood flow, and this was accompanied by reduced skeletal muscle glucose uptake.

Conclusions

Acute infusion of AngII significantly alters basal haemodynamic and metabolic homeostasis in rats. Both local and systemic AngII infusion attenuated insulin's microvascular actions in skeletal muscle, but only local AngII infusion led to reduced insulin-stimulated muscle glucose uptake. While increased local, tissue production of AngII may be a factor that couples microvascular insulin resistance and hypertension, additional studies are needed to determine the molecular mechanisms responsible for these vascular defects.

Keywords

Insulin • Blood flow • Angiotensin II

Introduction

Collectively, skeletal muscles are a major site for insulin-mediated glucose uptake following a meal, thus playing an important role in postprandial glucose homeostasis.¹ A characteristic feature of insulin resistance is a reduction in skeletal muscle glucose uptake in response to insulin, and this metabolic defect is often accompanied by endothelial dysfunction.^{2–4} While multiple mechanisms contribute to the development and

progression of skeletal muscle insulin resistance,^{5,6} we have recently identified a loss of microvascular insulin sensitivity as a key and early contributor.^{7,8}

Insulin's vascular actions comprise two effects on muscle blood flow. Firstly, insulin dilates terminal arterioles (microvascular) to increase capillary blood flow (microvascular recruitment) to promote increased nutrient exchange by muscle cells.^{9,10} Secondly, insulin dilates large arteries (macrovascular) to increase bulk flow to muscles.^{9,10} It is important to

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note that these two vasodilatory actions of insulin can occur independently of each other, and it is the extent of microvascular recruitment, and not total limb blood flow, that determines muscle glucose uptake in response to insulin.^{9,10} Indeed, we have demonstrated that both insulin resistant humans and animal models display attenuation of insulin-stimulated microvascular recruitment, and that this defect is strongly associated with reduced glucose uptake by skeletal muscles.^{7,8,11} Furthermore, we have recently reported that insulin-stimulated microvascular recruitment is lost prior to a change in myocyte insulin sensitivity, suggesting that loss of insulin's ability to dilate the microvascular network in skeletal muscle may be one of the earliest events in the aetiology of insulin resistance.^{7,8} Notably, we found that the loss of microvascular insulin sensitivity could be prevented with the ACE inhibitor quinapril⁸ thus positioning angiotensin II (AngII) as a possible contributor to the development of skeletal muscle insulin resistance.

AngII has important central nervous system and systemic actions to maintain cardiovascular and blood pressure homeostasis.¹² Unlike many systemically acting hormones, such as insulin and glucagon, the majority of AngII is generated locally within tissues from circulating precursors by ACE expressed at the site.¹³ Thus, alterations in local AngII generation have been implicated in a number of vascular pathologies including heart disease, hypertension, and insulin resistance.^{14–17} In the vascular endothelium, cross-talk between the AngII and insulin-signalling pathways has been strongly linked with both endothelial dysfunction and insulin resistance.^{16–21} Studies have shown that AngII directly inhibits the vascular insulin-signalling pathway at multiple levels including the insulin receptor, IRS1, PI3-kinase, and Akt.^{18,22–27} We have recently demonstrated that AngII also activates the mTOR/p70S6-kinase pathway to directly inhibit insulin-mediated activation of endothelial nitric oxide synthase (eNOS),²⁸ a key enzyme necessary for nitric oxide (NO) production that determines the extent of insulin-stimulated microvascular recruitment.²⁹ The demonstration that AngII is generated locally in the hindlimb vasculature itself,³⁰ raises the possibility that increased local AngII might selectively alter skeletal muscle insulin sensitivity. However, these effects may not be demonstrable using systemic AngII infusion alone, where effects of AngII in other tissues (e.g. heart, brain) may have multiple, indirect effects on local insulin action within muscle. Given that insulin resistance is associated with increased activity of both local and systemic AngII,^{16,19,20} the primary aim of the present study was distinguish the local effects of AngII on insulin sensitivity and blood flow dynamics in skeletal muscle from its systemic effects.

Methods

Animal care

Male Sprague-Dawley rats (10–12 weeks old) were obtained from the University of Tasmania Central Animal Facility. Rats were housed at 21 ± 2°C with a 12 h–12 h light–dark cycle. All experimental procedures in this investigation were approved by the University of Tasmania animal ethics committee and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes–2013, 8th Edition.

Surgical procedure

Rats were anaesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Once fully unresponsive to tail and foot pinch, surgery was performed to cannulate carotid and jugular blood vessels. Briefly, polyethylene cannulas (PE-50; B&D, NJ, USA) were implanted into the carotid artery

and both jugular veins. Cannulation of the carotid artery enabled arterial blood sampling and direct measurement of mean arterial pressure (Transpac IV; Abbott Critical Care Systems, CA, USA) and heart rate. Cannulation of the left jugular vein enabled a dedicated, continuous infusion of pentobarbital sodium (0.6 mg/min/kg) to maintain steady state anaesthesia throughout the protocol. Level of anaesthesia was routinely monitored by assessing tail/foot pinch responsiveness via respiration rate, blood pressure, and heart rate. Cannulation of the right jugular vein enabled intravenous infusion of insulin, glucose, and AngII. In all instances where AngII was infused, saline was used as the vehicle. After making a small incision in the skin overlying the femoral vessels of both hindleg's, the femoral artery, vein, and nerve were carefully separated. For local infusions, the epigastric arteries in both hindleg's were cannulated for retrograde infusion of AngII (test leg) or saline (contralateral leg). Ultrasonic flow probes (Transonic Systems, VB series, NY, USA) were positioned around femoral arteries and connected to flow meters (T402, Transonic systems). Femoral blood flow, heart rate, and mean arterial pressure were monitored continuously using WINDAQ data acquisition software (DATAQ Instruments, OH, USA). Femoral vascular resistance was calculated by dividing the mean arterial blood pressure by the femoral blood flow at the same time-point. Body temperature was kept constant at 37°C using a heated platform and lamp.

Experimental protocols

Protocol A: effect of systemic AngII on insulin's haemodynamic and metabolic actions

Following surgical stabilization, systemic saline or AngII was initiated through a dedicated jugular cannula 15 min prior to saline or insulin infusion (Figure 1A). A fresh solution of AngII was prepared immediately prior to each experiment and infused systemically at a constant rate of 10 µL/min to a final concentration of 100 ng/kg/min. This systemic dose of AngII was chosen based on previous work by our colleagues³¹ and increases circulating concentrations of AngII by approximately two-fold (Supplementary material online, Figure S1). At time = 0 min, a 2 h infusion of saline or insulin (10 mU/min/kg; Humulin, Eli Lilly, West Ryde, NSW, Australia) was initiated through a dedicated jugular vein. Since insulin stimulates glucose disposal, a glucose solution (30% wt/vol) was infused intravenously at a variable rate to maintain fasting blood glucose concentrations. Hence, blood glucose was assessed every 10–15 min using a glucose analyser (YSI 2300, Yellow Springs Instruments, OH, USA). The effect of systemic infusions on hepatic glucose output, skeletal muscle glucose uptake, and microvascular blood flow were assessed using 3-[³H]-glucose (Perkin Elmer, IL, USA), 2-deoxy-D-[1-¹⁴C]-glucose (Perkin Elmer), and 1-methylxanthine (1-MX; Sigma) respectively, as outlined below. Plasma insulin (Mercodia AB, Uppsala, Sweden) and AngII (Phoenix Pharmaceuticals Inc., CA, USA) concentrations were determined by ELISA.

Protocol B: effect of local AngII infusion on insulin's haemodynamic and metabolic actions

The dose of local AngII (plasma concentration 5 nmol/L) was based on previous experiments⁸ (found in Supplementary material online, Figure S1 in that publication). Preliminary experiments in the current *in vivo* preparation showed that local infusion of 5 nmol/L AngII decreased femoral blood flow by ~20% but did not cause any systemic changes in mean arterial pressure and heart rate (data not shown). After post-surgical stabilization, rats were subjected to the protocol in Figure 1B in which AngII was infused locally (retrograde via the epigastric artery into one leg) 15 min prior to and throughout systemic saline or insulin infusion (10 mU/min/kg). The rate of AngII and saline infusions was regularly adjusted to match femoral blood flow in the relevant hindlimb so that a constant local blood concentration of 5 nmol/L AngII was maintained. Metabolic and vascular measures were identical as in the systemic protocol with the exception that skeletal muscle glucose uptake, femoral blood flow, and microvascular blood flow were measured in both the test (AngII) and contralateral (saline) legs in these experiments.

Figure 1 Experimental protocols tested. Anaesthetized rats were assessed for systemic (A) or local hindleg (B) haemodynamic and metabolic responses to AngII in combination with either saline or hyperinsulinemic euglycaemic insulin clamp. A standard blood sampling protocol was observed in all test conditions to ensure steady state glucose levels from time = 0 min. Blood pressure, heart rate, and femoral artery blood flow was continuously monitored throughout the protocol. Hepatic glucose production ($3\text{-}[^3\text{H}]\text{-glucose}$) and muscle specific glucose uptake ($^{14}\text{C-2-DG}$) in each set of experiments were determined using glucose tracers. Microvascular blood flow was determined using the 1-methylxanthine (1-MX) method. To facilitate local delivery of AngII to a single hindleg, the epigastric artery was cannulated and AngII was infused to a final concentration of 5nmol/L for the duration of the protocol. The epigastric artery on the contralateral leg received saline infusion at the same infusion rate as the AngII leg.

Hepatic glucose output

The rates of hepatic glucose output (R_a ; rate of glucose appearance) and whole body glucose disappearance (R_d) were determined using $3\text{-}[^3\text{H}]\text{-D-glucose}$ ($0.1\text{ }\mu\text{Ci/min}$; Perkin Elmer) incorporated in the saline/insulin solutions (Figure 1A,B) and calculated as previously described.³²

Skeletal muscle glucose uptake

Skeletal muscle glucose uptake (R'_g) was determined using 2-deoxy-D- $[1\text{-}^{14}\text{C}]\text{-glucose}$ (2DG; 0.1 mCi/mL ; Perkin Elmer). The isotopic glucose tracer was administered as an intravenous bolus 45 min prior to the end of each experiment. Plasma samples were collected at regular intervals (see Figure 1) to determine plasma 2DG clearance rate. At the end of the experiment, the calf muscles (combination of gastrocnemius, plantaris, and soleus) from the test and contralateral hindlimbs were quickly freeze clamped with liquid nitrogen and stored at -80°C until analysed as previously described.^{7,8}

Skeletal muscle microvascular blood flow

Microvascular blood flow in skeletal muscle was assessed using the 1-MX method as previously published.^{7,8,10,33} Briefly, a bolus of allopurinol

($10\text{ }\mu\text{mol/kg}$; Sigma) was administered 5 min prior to 1-MX infusion to partially inhibit the activity of xanthine oxidase. This is necessary to reduce the arterial concentration of 1-MX required for enzyme saturation. Since xanthine oxidase is located on the vascular endothelium, this step ensures that the metabolism of 1-MX thus reflects differences in endothelial surface area (microvascular blood flow) and not total blood flow. Infusion of 1-MX (0.4 mg/min/kg ; Sigma) occurred during the last 60 min of the experiment. At 120 min, arterial and venous plasma were collected and 1-MX concentrations were assessed by reverse-phase-HPLC as previously described.³³ Metabolism of 1-MX across the hindlimb was determined from the arterio-venous 1-MX difference multiplied by the total femoral artery blood flow.

Statistical analysis

Data are means $\pm$ standard error of the mean (SEM) and statistics were performed using Sigma Plot (Systat Software Inc., 2011). Comparisons between groups were made using two-way ANOVA. Comparisons between local AngII and saline infused legs were performed using paired Student's *t*-test. When data were not normally distributed, a Signed-Rank test was performed. Comparisons of time-series and within group measurements were performed by two-way repeated measures analysis of variance (ANOVA).

When a significant difference of $P < 0.05$ was detected, Student–Newman–Keuls *post hoc* was used to assess treatment differences.

Results

Haemodynamic effects of systemic AngII and insulin

To interrogate the impact of AngII on insulin actions, we first investigated whether systemic infusion of AngII (100 ng/min/kg) altered insulin's systemic haemodynamic effects (Figure 2). Compared to saline infusion, insulin alone did not affect mean arterial blood pressure (Figure 2A) or heart rate (Figure 2B) across the 120 min infusion period. However, insulin infusion caused a significant decrease in femoral artery vascular resistance (Figure 2D) leading to an increase in femoral artery blood flow at 60 min that remained higher than saline until 120 min (Figure 2C). As expected, systemic AngII infusion alone caused a marked increase in mean arterial blood pressure (Figure 2A) that remained elevated over the course of the experiment. Heart rate was reduced by systemic AngII infusion (Figure 2B) while femoral artery blood flow (Figure 2C) was highly variable and not significantly different from any other groups. Femoral

artery vascular resistance increased by 40 min of systemic AngII infusion and remained elevated until the conclusion of the experiment (Figure 2D). Infusion of insulin in combination with systemic AngII did not alter mean arterial blood pressure (Figure 2A) or heart rate (Figure 2B) compared with systemic AngII infusion alone. In the presence of systemic AngII, insulin infusion still increased femoral artery blood flow toward the end of the experiment (Figure 2C) but did not alter femoral artery vascular resistance relative to saline infusion (Figure 2D).

Whole body metabolic effects of systemic AngII and insulin

Next, we investigated the impact of systemic AngII infusion on insulin's whole body metabolic effects (Figure 3). Systemic infusion of AngII alone stimulated a rapid and sustained increase in plasma glucose concentrations within 15 min of infusion (Figure 3A). The increase in plasma glucose was accompanied by a robust stimulation of hepatic glucose output (Figure 3D), a marked increase in plasma insulin concentrations across the 120 min infusion (Figure 3B) and a small increase in whole body glucose uptake (Figure 3F) relative to saline. Insulin infusion alone suppressed hepatic glucose output (Figure 3D) and increased whole body glucose disposal (Figure 3F). For these reasons, we infused exogenous glucose at

Figure 2 Systemic AngII infusion alters central haemodynamics but does not affect the insulin-mediated increase in femoral artery blood flow. Time-courses are shown for mean arterial pressure (A), heart rate (B), femoral artery flow (C), and femoral artery vascular resistance (D) for systemic saline (black circles; $n = 10$), systemic AngII only (blue circles; $n = 5$), insulin only (green circles; $n = 10$), and INS+Systemic AngII (red circles; $n = 5$). Values are means $\pm$ SEM. $*P < 0.05$ vs. systemic SAL as assessed by two-way repeated measures ANOVA. $***P < 0.05$ for all points under the line for both AngII infused groups vs. both systemic SAL and INS groups as assessed by two-way repeated measures ANOVA.

Figure 3 Systemic AngII infusion increases circulating glucose, lactate, and insulin concentrations and stimulates increased hepatic glucose output. Time-courses are shown for circulating glucose (A), lactate (C), and glucose infusion rate (E) for systemic saline (black circles; $n = 10$), systemic AngII only (blue circles; $n = 5$), insulin only (green circles; $n = 10$), and INS+Systemic AngII (red circles; $n = 5$). Basal (0 min; open bars) and post-infusion (120 min; closed bars) plasma insulin (B). Liver glucose output (D) and rate of whole body glucose uptake (F) were assessed at the end of each infusion protocol. Open symbols indicate individual data points and closed symbols indicate means $\pm$ SEM for each group. * $P < 0.05$ vs. systemic SAL as assessed by two-way ANOVA or two-way repeated measures ANOVA as appropriate. † $P < 0.05$ vs. all other groups as assessed by two-way ANOVA or two-way repeated measures ANOVA as appropriate. *** $P < 0.05$ for all points under the line for both AngII infused groups vs. both systemic SAL and INS groups as assessed by two-way repeated measures ANOVA.

a variable rate (Figure 3E) to maintain basal glucose concentrations in all insulin infused animals (Figure 3A). Because AngII infusion alone increases plasma glucose, we clamped plasma glucose levels at approximately 8.0 mmol/L in the systemic AngII plus insulin infused group (Figure 3A). Plasma glucose was clamped at the AngII-induced glucose concentration

to see whether insulin would still elicit an increase in muscle glucose uptake in the presence of AngII. The alternative, clamping glucose to the lower concentration (i.e. that for control or insulin alone) would have reduced muscle glucose uptake, and potentially overestimated the inhibitory effects of AngII on muscle glucose uptake. No difference in glucose

infusion rate (Figure 3E) or rate of glucose uptake (Figure 3F) was detected between insulin only and insulin plus AngII groups. However, plasma insulin concentrations were significantly elevated in the AngII plus insulin group compared to the insulin only group (Figure 3B). Insulin-mediated suppression of hepatic glucose output was partially inhibited in the presence of increased systemic AngII (Figure 3D), although it is not clear whether systemic AngII infusion directly induced insulin resistance in liver. Infusion of systemic AngII alone and insulin alone both increased plasma lactate concentrations relative to saline (Figure 3C) and the combination of systemic AngII plus insulin caused a far greater and sustained increase in plasma lactate.

Skeletal muscle effects of systemic AngII and insulin

Systemic infusion of AngII alone did not alter hindlimb glucose extraction (Figure 4A), hindlimb glucose uptake (Figure 4B) or microvascular blood flow but caused a modest increase in muscle specific glucose uptake (Figure 4C) relative to saline. As expected, insulin infusion alone stimulated a robust increase in skeletal muscle microvascular blood flow (Figure 4D) and this was accompanied by increased hindlimb (Figure 4B) and skeletal muscle specific glucose uptake (Figure 4C). The infusion of

systemic AngII did not alter insulin-mediated hindlimb (Figure 4B) or skeletal muscle glucose uptake (Figure 4C) but significantly inhibited insulin-stimulated microvascular blood flow in skeletal muscle (Figure 4D).

Effects of local, hindleg infusion of AngII on insulin systemic vascular, and metabolic actions

In the final experiment, we utilized a retrograde infusion of AngII via the epigastric artery to investigate the effect of local AngII on insulin's vascular and metabolic actions in skeletal muscle during a systemic insulin infusion (10 mU/kg/min). Local AngII did not affect mean arterial pressure or heart rate, indicating no significant systemic spill-over of AngII was occurring during systemic insulin infusion. Local AngII infusion significantly decreased femoral artery blood flow (Figure 5C) in the first 20 min of infusion relative to the saline infused leg. This reduction in femoral artery blood flow by AngII was evident across the first 40 min of systemic insulin infusion after which the AngII-mediated reduction in femoral blood flow was less evident. Similarly, femoral artery vascular resistance was increased by local AngII in the first 20 min compared with the contralateral leg (Figure 5D), but returned to a similar level in the second hour.

Figure 4 Systemic AngII infusion inhibits insulin-stimulated microvascular blood flow but has no impact on glucose uptake in skeletal muscle. End of experiment measures are shown for hindlimb A-V glucose difference (A), hindlimb glucose uptake (B), skeletal muscle specific glucose uptake (C), and skeletal muscle microvascular blood flow (D). Open symbols indicate individual data points systemic saline (black circles; $n = 10$), systemic AngII only (blue circles; $n = 5$), insulin only (green circles; $n = 10$), and INS+Systemic AngII (red circles; $n = 5$). Closed symbols indicate means $\pm$ SEM for each group. * $P < 0.05$ vs. saline as assessed by two-way ANOVA. † $P < 0.05$ vs. systemic INS as assessed by two-way ANOVA.

Figure 5 Local AngII infusion does not alter central haemodynamics or the insulin-mediated increase in femoral artery blood flow. Time-courses are shown for mean arterial pressure (A) and heart rate (B) for saline only (black circles; $n = 10$), insulin only (green circles; $n = 10$), and systemic INS+Local AngII (red triangles; $n = 8$). Time course measurements are also shown for femoral artery blood flow (C) and femoral artery vascular resistance (D) for saline only (black circles; $n = 10$), insulin only (green circles; $n = 10$), systemic INS+Local AngII (red triangles; $n = 8$), and systemic INS+Local SAL (green triangles; $n = 8$). Values are means $\pm$ SEM. $^{\dagger}P < 0.05$ vs. systemic INS+Local SAL infusion as assessed by two-way repeated measures ANOVA.

Local hindlimb AngII infusion did not alter circulating plasma glucose (Figure 6A) or lactate (Figure 6C). In contrast to the systemic AngII infusion described above, local AngII infusion did not affect circulating insulin concentrations (Figure 6B). Insulin-mediated suppression of hepatic glucose output was also unaffected by local AngII infusion (Figure 6D) and no difference in glucose infusion rate (Figure 6E) or rate of glucose uptake (Figure 6F) was detected between insulin only and insulin plus local AngII groups.

Skeletal muscle effects of local AngII and insulin

Finally, we investigated the effect of local AngII infusion on the microvascular and metabolic effects of insulin in skeletal muscle (Figure 7). Compared to the local saline infusion, local AngII infusion had little effect on A-V glucose extraction (Figure 6A) but significantly reduced insulin-stimulated hindlimb glucose uptake (Figure 7B). The rate of insulin-mediated skeletal muscle specific glucose uptake was similarly reduced by local AngII infusion (Figure 7C), and this was strongly associated with inhibition of insulin-stimulated microvascular blood flow (Figure 7D).

Discussion

Accumulating evidence implicates AngII in development of hypertension and metabolic disturbances such as obesity, insulin resistance, and type 2 diabetes.^{34–36} In humans, ACE inhibitors and AT1 receptor blockers delay the onset of type 2 diabetes and/or decrease the incidence of type 2 diabetes.^{34,37–40} Transgenic rodents overexpressing AngII develop hypertension, glucose intolerance and cardiovascular disease early in life.⁴¹ Chronic AngII infusion (2 weeks) alone decreases whole body glucose disposal and reduces insulin-mediated muscle glucose uptake in rats.⁴² It should be noted, however, that some studies show improvements in insulin sensitivity with systemic AngII infusion in humans and rodents^{43–46} and others suggest that AngII has insulin-mimetic actions on microvascular blood flow and glucose disposal in rats.³¹ A notable complication of the latter studies is that systemic infusion of AngII may not accurately reproduce the regional heterogeneity of local AngII production that might occur in hypertension and obesity, for example. The primary goal of the current study was to distinguish the local effects of AngII in skeletal muscle from the effects of a global infusion on insulin's metabolic and vascular actions.

We found three major effects in the current study. Firstly, while systemic AngII infusion alone led to expected changes in systemic

Figure 6 Local AngII infusion does not alter the insulin's systemic metabolic effects. Time-courses are shown for circulating glucose (A), lactate (C), and glucose infusion rate (E) for systemic saline (black circles; $n = 10$), insulin only (green circles; $n = 10$), and insulin plus local AngII (red triangles; $n = 8$). Basal (0 min; open bars) and post-infusion (120 min; closed bars) plasma insulin (B). Liver glucose output (D) and rate of whole body glucose uptake (F) were assessed at the end of each infusion protocol. Open symbols indicate individual data points and closed symbols indicate means $\pm$ SEM for each group. * $P < 0.05$ vs. systemic SAL as assessed by two-way ANOVA. *** $P < 0.05$ for all points under the line vs. systemic SAL only as assessed by two-way repeated measures ANOVA.

haemodynamics we also noted a significant shift in basal glucose and insulin homeostasis in otherwise healthy rats. Secondly, under hyperinsulinaemic euglycaemic conditions, systemic AngII infusion inhibited insulin-stimulated microvascular blood flow in skeletal muscle, but this effect did not appear to alter muscle glucose uptake. However, it should be noted that systemic co-infusion of AngII also stimulates a robust increase in

circulating insulin (to a level far higher than the insulin clamp alone), and hence likely impacts our findings regarding AngII effects on muscle glucose uptake in this setting. Thirdly, under hyperinsulinaemic euglycaemic conditions we found that local, hindlimb infusion of AngII reduced both insulin-stimulated microvascular blood flow and muscle glucose uptake without affecting systemic metabolism or haemodynamics. Therefore,

Figure 7 Local AngII infusion inhibits insulin-stimulated microvascular blood flow and glucose uptake in skeletal muscle. End of experiment measures are shown for hindlimb A-V glucose difference (A), hindlimb glucose uptake (B), skeletal muscle specific glucose uptake (C), and skeletal muscle microvascular blood flow (D). Open symbols indicate individual data points for systemic INS+Local SAL (green triangles; $n = 8$) and systemic INS+Local AngII (red triangles; $n = 8$). Closed symbols indicate means $\pm$ SEM for each group. $^{\dagger}P < 0.05$ vs. systemic INS+Local SAL as assessed by paired Student's t -test. When data were not normally distributed, a Signed-Rank test was performed.

we conclude that even acute increases in AngII are sufficient to inhibit insulin action at both the systemic and local levels, and that in skeletal muscle AngII interferes with insulin's microvascular actions leading to a state of acute muscle insulin resistance. To our knowledge, this is the first study to delineate the systemic vs. local effects of AngII on insulin's vascular and metabolic actions in muscle. While our data add to the notion that AngII is a likely contributor to development of insulin resistance systemically, we also highlight a novel role for increased local AngII in the development of microvascular and skeletal muscle insulin resistance. The outcomes of this work further indicate that regulation of AngII at a local level has important physiological consequences and that more careful consideration is warranted when investigating the various pathophysiological settings involving AngII.

Systemic AngII infusion alone increases circulating glucose and insulin concentrations

In our hands, systemic infusion of AngII alone caused a rapid and sustained increase in plasma glucose concentrations, likely through direct stimulation of hepatic glucose output. It is important to note that the hepatic glucose output measure is derived from the plasma [3 H]-glucose

specific activity achieved over the 2-h time course, and so reflects hepatic output at 2 h, when plasma glucose concentration is not changing, rather than the first 20 min of AngII infusion. Thus hepatic glucose output may have been much higher in this 20 min period and may have preceded a subsequent insulin release that would bring hepatic output and whole body glucose uptake back into balance (i.e. constant plasma glucose concentration). The ability of AngII to stimulate hepatic glucose output has been reported previously,⁴⁷ and our data support the notion for AngII in the development of hepatic insulin resistance. Not surprisingly, the increase in plasma glucose following systemic AngII infusion was also associated with increased plasma insulin levels, which reached a similar level to insulin-only infused animals by 120 min. Whether this is the net result of increased plasma glucose or a direct effect of AngII on pancreatic beta cells is not clear from the current study. Indeed, the effects of AngII on beta cells remains controversial in the literature where some studies report a stimulation of insulin release and some report inhibition of insulin release in response to AngII.^{48,49} These varied pancreatic effects of AngII may be dependent on the length of exposure (i.e. acute or chronic) and/or source of AngII (systemic vs. local) and clearly need to be addressed in future work.

Systemic AngII infusion during hyperinsulinaemic euglycaemic clamp led to a striking increase in plasma insulin concentrations relative to

insulin-only or AngII-only infused animals. These data tentatively support the notion of a direct effect of systemic AngII on insulin release from beta cells. However, we cannot rule out the possibility of reduced insulin clearance contributing to the elevation in plasma insulin in the AngII-infused animals. Intriguingly, even though insulin concentrations were significantly higher in the AngII plus insulin infused rats, the extent of hepatic glucose output remained above that of insulin infusion alone. This finding is in agreement with previous work using chronic AngII infusion⁴² and indicates a potent inhibitory effect of acute increases in AngII on insulin's classic ability to reduce hepatic glucose output. From the current study, we cannot identify how AngII inhibits insulin action in hepatocytes; however, previous work strongly suggests direct AngII-mediated attenuation of the hepatic insulin signalling at multiple sites including IRS-1 and Akt.⁴²

Insulin's microvascular and not macrovascular actions on blood flow are inhibited by acute AngII infusion

Systemic AngII infusion led to the classic and expected increase in mean arterial blood pressure and compensatory drop in heart rate. Interestingly, the effect of systemic AngII-only infusion on femoral artery blood flow was highly variable in the first 60 min, but became more stable in the final 60 min. The exact reason for initial variability in femoral flow remains unclear but is likely linked to the lower number of animals (five) studied in this particular group. Co-infusion of insulin did not alter AngII's effects on mean arterial blood pressure or heart rate. In contrast, femoral artery blood flow increased with insulin co-infusion, reaching a similar level observed in the insulin-only infused animals. In addition, while femoral artery vascular resistance was increased by AngII infusion, co-infusion of insulin reduced this to a similar level as the insulin-only infused group. Whether these changes in femoral resistance are exclusively linked with femoral artery diameter alone or also due to downstream vessel diameter changes in the hindlimb is difficult to decipher in the present experiment. Nevertheless, our data suggest that insulin infusion can overcome the AngII-mediated reduction in femoral artery blood flow to stimulate increased femoral blood flow. Indeed, this was also apparent in our local AngII infused experiments where insulin appears to have completely overcome the initial AngII-mediated reduction in femoral artery blood flow to stimulate an increase. While we are lacking an additional control group demonstrating the longer-term effects of local AngII alone on the femoral artery flow, the vasodilatory effect of insulin on larger blood vessels appears to be intact during co-infusion of AngII. However, previous work indicates that an insulin-stimulated increase in femoral artery blood flow is not necessarily associated with an increase in skeletal muscle metabolism.^{50,51} Rather, it is the extent of insulin-stimulated microvascular blood flow that determines nutrient exchange in skeletal muscle.^{7,8,11,33} Chai *et al.*³¹ have reported that systemic infusion of AngII alone increases microvascular blood flow in skeletal muscle. While the reasons for the opposing findings are unclear at this stage, these may be due to differences in the animals used and their relative balance of AT1 (vasoconstriction—decreasing microvascular blood flow) and AT2 (vasodilation—increasing microvascular blood flow) receptors—with the animals in the present study appearing to be more AT1 dominant.

In our experiments, the ability of insulin to increase microvascular blood flow was almost completely inhibited by both systemic and local AngII infusion. These data suggest that AngII exerts a more potent effect on smaller blood vessels than large vessels such as the femoral artery.

Interestingly, only the local AngII infusion was associated with a decrease in insulin-stimulated skeletal muscle glucose uptake. However, it should be noted that animals receiving systemic AngII infusion also exhibited a significant and sustained rise in both plasma glucose and insulin relative to the insulin-only infused animals. Whether this AngII-mediated increase in plasma insulin contributes to a proportionally greater stimulation of glucose uptake by skeletal muscles in the systemic AngII infused animals is not clear. Nevertheless, even in the presence of much higher plasma insulin concentrations, systemic AngII infusion still inhibited the insulin-stimulated increase in microvascular blood flow, a finding that confirms with a previous report in skin microvasculature.⁴⁶ To explore the findings regarding local AngII further, we assessed the extent of insulin-stimulated Ser⁴⁷³Akt phosphorylation in muscle and discovered no difference vs. the saline infused leg (Supplementary material online, Figure S1). These data suggest that even when insulin-stimulated microvascular flow is reduced by the presence of local AngII, insulin signalling in muscle cells can remain intact. These findings therefore indicate that the limiting factor for insulin-stimulated glucose uptake in the setting of increased local AngII is the delivery of glucose to myocytes, rather than direct effects of AngII on muscle cells. Taken together, our systemic and local data indicate that AngII has a potent vasoconstrictor effect on the microvasculature and that local increases in AngII are sufficient to inhibit insulin-stimulated microvascular blood flow thus limiting glucose uptake by skeletal muscle. While the current study highlights the potential of AngII to impair insulin-mediated microvascular flow, more definitive evidence is required to determine if the actions of local AngII account for impaired microvascular blood flow in an insulin resistant model.

The insulin-signalling pathway in vascular endothelial cells has been extensively characterized involving IRS-1/PI3-kinase/Akt/eNOS and ultimately leading to production of NO (a potent vasodilator).^{18,22–27} Strong evidence demonstrates that AngII directly inhibits insulin signalling in endothelial cells at a number of levels by: directly blocking insulin binding to the insulin receptor²⁴; inhibiting IRS-1¹⁸; inhibiting Akt and eNOS.²⁸ Unfortunately, it is exceedingly difficult to determine if the entire vascular insulin-signalling pathway is similarly inhibited by AngII in our current *in vivo* study. Nevertheless, when considered in the context of previous research, it would be reasonable to conclude that AngII inhibits insulin-stimulated microvascular blood flow by limiting vascular production of NO in response to insulin.^{18,22–28} This conclusion is further supported by our recent findings demonstrating that inhibition of ACE in high-sodium fed animals restores both microvascular and metabolic sensitivity to insulin in skeletal muscle *in vivo*.⁸ Therefore, we propose that within skeletal muscle, AngII impairs insulin-mediated NO production to limit insulin-mediated microvascular blood flow thus leading to a state of acute muscle cell insulin resistance.

As mentioned above, both local and systemic AngII infusion had marked effects on femoral artery blood flow and femoral vascular resistance that were mostly but not completely overcome by insulin. This suggests that mechanisms of vascular insulin resistance differ between larger vessels and the microvessels further down the vascular tree where insulin is suspected to act. From these data alone, we cannot explicitly rule out the involvement of these large blood vessel effects of AngII from the alteration of insulin-mediated microvascular and metabolic responses. However, our previous work indicates a clear dissociation between insulin's microvascular and metabolic actions in muscle from its actions on larger vessels, such as the femoral artery.^{9,10} Therefore, it is reasonable to conclude that the detrimental effects of AngII on muscle insulin sensitivity are due to alterations in microvascular, rather than macrovascular blood flow. It is also important to note that all

experimentation regarding AngII, insulin and muscle blood flow dynamics in the current study has been performed in anaesthetized animals. This imposes some limitations on understanding the exact role of AngII alone on both central and local regulation of muscle blood flow and needs to be addressed in future work.

Conclusions

In conclusion, acute infusion of AngII has potent inhibitory effects on insulin's microvascular actions in skeletal muscle and only local AngII infusion leads to a concurrent impairment of skeletal muscle glucose uptake. The discordant effects of systemic vs. local AngII infusion on skeletal muscle insulin sensitivity may be attributable to the stimulation of glucose and insulin release during systemic AngII infusion. Nevertheless, our data suggest that increasing the local circulating concentration of AngII is sufficient to interfere with normal microvascular and metabolic insulin action in skeletal muscle leading to a state of acute muscle insulin resistance. Additional studies are required to fully identify the precise molecular mechanisms of AngII-mediated microvascular insulin resistance and whether these mechanisms are associated with the pathological changes in AngII reported during hypertension, insulin resistance, and type 2 diabetes.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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D.P. performed or oversaw all experimental work, analysis and writing of the manuscript. E.A. performed the western blotting experiments provided in the [Supplementary material online](#), Figures. D.P., S.R., S.M.R., and M.A.K. were involved in design, analysis and writing of the manuscript. J.K. was involved in analysis and writing of the manuscript. M.A.K. and D.P. had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest: none declared.

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