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Metformin does not Enhance Insulin-Stimulated Vasodilation in Skeletal Muscle Resistance Arteries of the OLETF Rat

JACQUELINE M. CRISSEY^{*,†}, JAUME PADILLA^{*}, NATHAN T. JENKINS^{*}, JEFFREY S. MARTIN^{*}, RANDY SCOTT RECTOR^{†,‡,§}, JOHN P. THYFAULT^{†,‡,§}, and MAURICE HAROLD LAUGHLIN^{*,¶,**}

^{*}Biomedical Sciences, University of Missouri, Columbia, Missouri, USA

[†]Nutrition & Exercise Physiology, University of Missouri, Columbia, Missouri, USA

[‡]Internal Medicine, University of Missouri, Columbia, Missouri, USA

[§]Research Service, Harry S Truman Memorial VA Hospital, Columbia, Missouri, USA

[¶]Medical Pharmacology and Physiology, University of Missouri, Columbia, Missouri, USA

^{**}Dalton Cardiovascular Research Center, University of Missouri, Columbia, Missouri, USA

Abstract

Objective—To test the hypothesis that chronic metformin treatment enhances insulin-induced vasodilation in skeletal muscle resistance arteries and arterioles.

Methods—We assessed the effect of metformin treatment (from 20 to 32 weeks of age) of obese Otsuka Long Evans Tokushima Fatty (OLETF) rats and lean LETO rats (300 mg/kg) on insulin-stimulated vasodilation in isolated skeletal muscle feed arteries and arterioles.

Results—Metformin treatment significantly lowered food intake, body weight, percent body fat, and HbA1c in OLETF rats. Metformin resulted in a ~30% reduction in insulin-induced vasodilation of soleus feed arteries (SFA) from OLETF rats. Inhibition of endothelin-1 (ET-1) signaling produced 20% dilation and eliminated the difference between metformin-treated and untreated OLETF rats in insulin-induced dilation of SFA. In contrast to the SFA, metformin did not alter insulin-stimulated vasodilation in gastrocnemius feed arteries (GFA), or second-order arterioles in the red (G2A-R) or white (G2A-W) portions of the gastrocnemius muscle of OLETF rats. Metformin had no effects on vasomotor responses of arteries from LETO.

Conclusions—Although metformin exerts favorable effects on body composition and HbA1c, it does not enhance the vasodilatory responses to insulin in the skeletal muscle feed arteries or arterioles of the obese OLETF rat.

Keywords

metformin; type 2 diabetes; insulin resistance; microvascular disease

INTRODUCTION

Insulin resistance is characterized by a blunted ability of peripheral tissues to regulate glucose homeostasis in response to insulin. The metabolic effects of insulin, including suppression of hepatic glucose production and stimulation of skeletal muscle glucose uptake are impaired in T2DM [14]. In addition to insulin's metabolic effects, insulin also stimulates dilation of skeletal muscle resistance arteries. Current evidence indicates that abnormalities in the vascular reactivity (vasodilation) to insulin in T2DM contribute to decreased glucose disposal by limiting perfusion, and consequently, delivery of glucose and insulin to skeletal muscle [9,31,58]. Therefore, it is becoming clear that impairments in both the vascular and metabolic actions of insulin are interrelated and play key roles in insulin resistance with obesity and T2DM.

Insulin's vascular actions are complex and are balanced by endothelial signaling pathways controlling the production of the vasodilator, nitric oxide (NO) [10,57], and the vasoconstrictor endothelin-1 (ET-1) [43,44]. Insulin-stimulated vasodilation enhances skeletal muscle perfusion and increases delivery of insulin and glucose to skeletal muscle. In healthy individuals, insulin-mediated increases in skeletal muscle blood flow account for as much as 40–50% of total insulin-stimulated glucose uptake in skeletal muscle [4,29,46]. Conversely, with obesity, insulin resistance and T2DM, there is a reduction in insulin-stimulated NO-mediated vasodilation, and enhanced insulin-induced ET-1-mediated vasoconstriction. This imbalance in the vascular actions of insulin is thought to contribute to impairments in insulin-stimulated glucose uptake by target tissues [3,8,11,30,31,37,43,44]. Indeed, our laboratory and others have shown that the vascular reactivity to insulin, and the resulting increase in skeletal muscle perfusion, is impaired in obese and physically inactive T2DM humans and animals, ultimately attenuating nutritive blood flow and contributing to skeletal muscle insulin resistance [26,31,36,41,42,58].

Most treatment and prevention therapies for T2DM involve the use of antihyperglycemic drugs targeting whole body insulin resistance; however, the vascular effects of these drugs remain largely unknown. Metformin has long been used to treat T2DM and, based on current American Diabetes Association recommendations, is a first line treatment along with diet and exercise [16,51]. It is thought that metformin decreases blood-glucose concentration by reducing basal hepatic glucose production and enhancing skeletal muscle insulin sensitivity [21,47]. In addition to being widely used in the treatment of T2DM, metformin is also increasingly prescribed as a preventative therapy in individuals at risk for the development of overt T2DM [5,6]. In fact, results from the Diabetes Prevention Program show that treatment with metformin reduced the incidence of T2DM by 31% in nondiabetic impaired glucose-tolerant individuals [28].

Several studies have reported that metformin reverses vascular dysfunction in conduit, large arteries, and in the mesenteric circulation [22,24,38,39]; however, the ability of metformin to ameliorate or reverse vascular dysfunction in skeletal muscle resistance vasculature has not been well studied. Furthermore, while we have demonstrated that soleus muscle feed arteries are protected against insulin-resistance related vascular dysfunction, relative to gastrocnemius muscle feed arteries [5], it is unclear whether metformin impacts insulin-

induced vasoreactivity uniformly throughout the skeletal muscle vasculature and whether these effects are modulated by obesity and insulin resistance. Thus, the purpose of this study was to test the hypothesis that chronic metformin treatment enhances vascular reactivity to insulin in feed arteries and second-order arterioles of the gastrocnemius (primarily mixed/fast twitch) and in the feed artery of the soleus (primarily slow twitch) muscles of insulin-resistant pre-diabetic OLETF rats, and has no effects on these arteries and arterioles in healthy lean rats. The OLETF rat is a model of T2DM characterized by a mutated cholestykinin-1 receptor that results in hyperphagia and obesity [25]. These rats exhibit early increases in serum insulin, have hyperglycemia and advanced insulin resistance by 12–20 weeks of age, and continue to progress to overt T2DM between 20 and 40 weeks [7,25,33].

METHODS

Animals

Four-week-old male obese OLETF ($n = 29$) and healthy lean (LETO; $n = 29$) rats (Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan) were individually housed in cages maintained in temperature (21°C) and light-controlled, 12:12-hour light–dark cycle (lights off at 18:00 hours), animal quarters. All rats were provided *ad libitum* access to water and standard rodent chow (Formulab 5008; Purina Mills, St. Louis, MO, USA) comprised of ~26% protein, 18% fat, and 56% carbohydrate. Body weights and food intakes were recorded on a weekly basis. The University of Missouri Institutional Animal Care and Use Committee approved all experimental protocols.

Experimental Design

At 20 weeks of age, LETO and OLETF rats were randomly assigned to untreated or metformin-treated groups forming four groups: (i) LETO ($n = 15$), (ii) LETO + metformin (LETO + MET, $n = 14$), (iii) OLETF ($n = 15$), and (iv) OLETF + MET ($n = 14$). It should be noted that some data appear in recently published reports from our laboratory addressing fundamentally different research questions from that of this study, that is, the functional adaptation of the skeletal muscle microvasculature to exercise training [36] and the effects of training and metformin on adipose tissue inflammation [23]. Metformin treatment was initiated at 20 weeks of age, as this is the age at which the OLETF rat is known to begin progressing from advanced insulin resistance to overt T2DM [7,25,33]. We administered metformin (Bosche Scientific, New Brunswick, NJ, USA) at a dose of 300 mg/kg in the rats' drinking water for three months (20–32 weeks of age), based on previous rodent studies utilizing chronic metformin administration [6,39]. Rats were started on half a dose of metformin (150 mg/kg/day) for one week for drug acclimatization, and then increased to 300 mg/kg/day, with no noticeable side effects. We carefully monitored the water intake of the rats so that the maximum dose of metformin was not exceeded. The animals had *ad libitum* access to their water (with metformin) until anesthesia was given the morning of sacrifice. The rationale for adopting this approach of treating the animals with metformin until anesthesia is that we wanted to be consistent with what might occur in clinical settings (human patients are not normally instructed to avoid their medications prior to a fasting blood draw).

At 32 weeks of age, we anesthetized the rats with an intraperitoneal injection of sodium pentobarbital (100 mg/kg) in the morning following an overnight fast, and measured the animals' body weight and percent body fat (described below). The soleus and gastrocnemius muscles were then harvested for feed artery and arteriole isolation. Subsequently, we collected blood samples for analysis and killed the rats by exsanguinations.

Our rationale for this experimental design was that comparisons between LETO and OLETF rats would discriminate potential differences between the vascular reactivity to insulin due to obesity and diabetes. However, the primary focus of this study was to test the hypothesis that metformin effectively treats advanced insulin resistance, prevents the progression to overt T2DM (analogous to the administration of metformin to pre-diabetic patients in the Diabetes Prevention Program [28]), and improves insulin-mediated vascular function. This was accomplished by comparing OLETF and OLETF + MET rats. To complement these objectives, we also treated LETO animals with metformin to achieve a balanced experimental design and examine possible effects in a group of healthy control animals. Finally, we chose to study our animals at 32 weeks of age, as this is the age that obese OLETF rats exhibit pancreatic beta-cell failure, advanced insulin resistance, and T2DM [7,25,33,49]. However, it is important to note that not all OLETF rats progress to frank T2DM by 32 weeks; thus, herein we refer to our 32-week-old OLETF rats as obese and insulin resistant.

Body Composition and Blood Parameters

On the day of experiments, we measured body mass of the rats and determined body composition using a dual energy X-ray absorptiometry machine (Hologic QDR-1000; Bedford, MA, USA) calibrated for rodents. Whole blood was collected for analysis of HbA1c by the boronate-affinity HPLC method (Primus Diagnostics, Kansas City, MO, USA) in the Diabetes Diagnostics Lab at the University of Missouri. Serum samples were prepared by centrifugation and stored at -80°C until analysis. A commercial laboratory (Comparative Clinical Pathology Services, Columbia, MO, USA) analyzed all serum chemistry parameters on an Olympus AU680 automated chemistry analyzer (Beckman-Coulter, Brea, CA, USA) using commercially available assays according to the manufacturer's guidelines. Plasma insulin concentrations were determined using a commercially available, rat-specific ELISA (Alpco Diagnostics, Salem, NH, USA). Samples were run in duplicate and manufacturer's controls and calibrators were used according to assay instructions. The homeostasis model assessment-insulin resistance (HOMA-IR) was calculated according to the formula of Matthews *et al.* [40]: $[\text{fasting glucose (mg/dL)} \times \text{fasting insulin } (\mu\text{IU/mL})]/405$. Intraperitoneal glucose tolerance tests (IPGTT) were performed at 31 weeks of age ($n = 6-8$ per group), one week prior to sacrifice, as previously discussed [50]. Briefly, food was removed from the cages 12 hours before each received an intraperitoneal injection of dextrose (50% solution, 2 g/kg body weight). Venipuncture blood samples were collected from the lateral tail vein immediately before (0 minutes) dextrose administration and 15, 30, 45, 60, and 120 minutes after injection. Insulin sensitivity was estimated as the product of the area under the curve (AUC) for glucose using the trapezoidal method [53].

Isolation of Skeletal Muscle Feed Arteries and Arterioles

Gastrocnemius feed arteries (GFA), soleus feed arteries (SFA), second-order arterioles from the red portion of the gastrocnemius (G2A-Red), and second-order arterioles from the white portion of the gastrocnemius (G2A-White) were isolated, cannulated, and pressurized as previously described [5,36,60]. During dissection, muscles were placed in ice-cold MOPS-buffered physiological saline solution (PSS) containing (in mM) 145.0 NaCl, 4.7 KCl, 2.0 CaCl₂, 1.17 MgSO₄, 1.2 NaH₂PO₄, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, and 3.0 MOPS (pH 7.4). Following isolation, vessels were cannulated on glass micropipettes, pressurized, and mounted in MOPS-PSS bath (37°C) on an inverted microscope outfitted with a video camera for continuous measurement of intraluminal diameter with calibrated videocallipers, as previously described [1,32]. We adjusted the hydrostatic pressure reservoirs connected to the cannulated micropipettes, containing PSS plus albumin (1 g/100 mL), to achieve approximate *in vivo* intraluminal pressures of 90 and 67 cmH₂O, for GFA/SFA and G2A-Red/G2A-White vessels, respectively [1,59]. We treated the vessels with 80 mM KCl to verify viability, washed them with PSS, and allowed the vessels to equilibrate for one hour at 37°C. Following equilibration, arteries were pre-constricted with phenylephrine (PE; 10⁻⁵–10⁻⁴ M) to achieve ~30% tone for examination of vasomotor responses to insulin. We have found that skeletal muscle feed arteries and arterioles from OLETF rats do not uniformly develop spontaneous vascular tone; therefore after confirming vessel viability with KCl, we added PE to the vessel bath prior to the first insulin dose–response curve regardless of spontaneous tone. However, for subsequent curves, if sufficient tone (~30% of initial diameter) was present, no further PE was added.

We determined vasomotor responses to insulin in one SFA, GFA, G2A-Red, and G2A-White vessel per animal. The vasoreactivity to insulin was assessed at four concentrations (1, 10, 100, and 1000 µIU/mL) of bovine insulin (dissolved in 0.01 N HCl and diluted 1:10 with PSS containing 1% BSA). To evaluate the role of ET-1 in the vasomotor response to insulin, we incubated vessels with tezosentan (3 µM), a nonselective ET_A and ET_B receptor antagonist, for 20 minutes, and re-assessed the vasoreactivity to insulin (1–1000 µIU/mL). For all insulin curves, the four serial doses of insulin were given in 10-minute intervals, and intraluminal vessel diameters were recorded at the end of the 10 minutes, immediately prior to the introduction of the next dose of insulin. Between the insulin and insulin + tezosentan dose–response curves, we washed the vessel baths with fresh PSS and allowed a re-equilibration period of 30 minutes. Following insulin curves, we determined maximal vessel diameter, with Ca²⁺-free PSS-containing 100 µM sodium nitroprusside (SNP).

Drugs and Solutions

All drugs and solutions were obtained from Sigma (St. Louis, MO, USA), except albumin (USB Corporation, Cleveland, OH, USA). We prepared all PSS solutions and drugs prior to the study, froze them, and thawed aliquots each day of experiments. All drug solutions were prepared in PSS, except insulin, which was prepared in PSS with BSA (1 g/100 mL). We prepared SNP fresh daily and protected the serial dilutions from light.

Data Analysis and Statistics

Results are presented as mean $\pm$ SEM. Vasodilator responses are presented as percent possible (maximal) dilation, calculated as follows: $[(D_d - D_b)/(D_{\max} - D_b)] \times 100$, where D_d is diameter after insulin treatment, D_b is baseline diameter, and $D_{\max}$ is maximal vessel diameter. To detect group differences in animal (Table 1) and vessel characteristics (Table 2), we used a one-way analysis of variance (ANOVA) with Fisher's least significant differences (LSD) *post hoc* analysis. We confirmed equality of variances for all animal and vessel characteristic variables. Vasomotor responses to insulin were examined using a mixed-design repeated-measures ANOVA, with group as the between-subjects factor and insulin as a repeated factor, followed by Fisher's LSD *post hoc* analysis. To compare the vascular reactivity to insulin, and insulin in the presence of tezosentan, we performed a separate mixed-design repeated-measures ANOVA, and evaluated main effects of metformin in OLETF and LETO groups, as well as interactions between group, insulin dose, and tezosentan effects. We used IBM SPSS Statistics 19 for Windows (Chicago, IL, USA) for all statistical analyses, and established *a priori* statistical significance of $p < 0.05$.

RESULTS

Animal and Vessel Characteristics

Consistent with our previous reports [5,7], our untreated OLETF rats had greater body weight, percent body fat, and food intake), and blood lipids, and were insulin resistant (fasting hyperglycemia, hyperinsulinemia, and elevated HbA1c) compared to healthy LETO animals (all $p < 0.05$, Table 1 and Figure 1). Metformin treatment significantly reduced food intake, body mass, and percent body fat in OLETF rats ($p < 0.05$, Table 1). Moreover, metformin significantly lowered HbA1c, an index of chronic glycemic control ($p < 0.001$, Figure 1). However, metformin treatment did not lower blood lipids, glucose, insulin, or insulin resistance, as assessed by both the HOMA-IR [40] and glucose responses to an IPGTT compared to untreated OLETF rats (Table 1). While metformin did not alter glucose AUC during the IPGTT, insulin AUC was significantly reduced in metformin-treated OLETF animals. Although there were no statistically significant differences in plasma glucose, insulin, and HOMA-IR between groups, each of these parameters tended to be lower in metformin-treated OLETF rats (Table 1).

Maximal vessel diameters, pre- and post-tezosentan treatment diameters, percent dilation to tezosenan, and percent pre-constriction relative to maximal vessel diameter are presented in Table 2. Tezosentan (20 minutes incubation with 3 μ M) produced significant dilation in all resistance arteries within all groups, except for the G2A-Red and G2A-White arterioles of the LETO + MET group (Table 2). These results suggest the presence of basal ET-1 constrictor tone. The% dilation produced by tezosenan was significantly greater in the GFA and G2A-Red of OLETF rats than in the same arteries from LETO rats (Table 2). In addition, the SFA of OLETF + MET tended to have a greater dilation to tezosenan compared to SFA of OLETF ($p = 0.09$, Table 2). Between-group comparisons (within vessel) of the magnitude of pre-constriction indicated no significant differences among groups in SFA, GFA, and G2A-White. Also, there were no differences among groups in percent pre-constriction prior to the insulin + tezosenan dose-response experiments, except

that pre-constriction of G2A-Red was significantly lower in OLETF compared to LETO rats ($p < 0.05$). Within vessels, there were no significant differences among groups in diameter prior to the insulin dose-response experiments. It has been shown that metformin can blunt contraction to PE [34,52]. However, in our experiments this does not appear to be an issue, reflected in the lack of change in percent pre-constriction between the LETO and LETO + MET groups (Table 2).

Effects of Metformin on Microvascular Reactivity to Insulin

Although insulin produced a dose-related dilation of SFA of healthy LETO rats, it only produced a significant dilation (16%) from baseline in the SFA of healthy LETO rats at the highest supra-physiological dose of 1000 $\mu\text{IU/mL}$ insulin (Figure 2A). There were no differences in the vasoreactivity to insulin between LETO and LETO + MET animals. In contrast, in SFA of OLETFs insulin produced significant vasodilation above baseline at all insulin doses, and greater vasodilation at each successive dose, indicating a progressive vasodilatory response to insulin. No statistically significant vasodilatory effects of insulin were observed in the GFA (Figure 3A and C), G2A-Red (Figure 4A and C), or G2A-White (Figure 5A and C) vessels in LETO or OLETF animals.

Metformin treatment did not significantly alter the vascular reactivity to insulin in SFA or gastrocnemius arteries of healthy LETO animals (Figures 2A, 3A, 4A and 5A). Conversely, the vasodilatory response to insulin in SFA of OLETF rats treated with metformin was decreased at all insulin doses (Figure 2C). Metformin treatment of OLETF rats did not appear to affect insulin responses of GFA, G2A-Red, or G2A-White arteries of OLETF rats (Figures 3C, 4C and 5C).

Role of Endothelin-1 in Vasomotor Response to Insulin

Tezosentan treatment dilated all arteries in all groups. In the presence of tezosentan, arteries/arterioles from LETO animals dilated in response to insulin to a greater extent compared to control conditions. Insulin-induced dilation of the SFA was increased at 1, 100, and 1000 $\mu\text{IU/mL}$ concentrations of insulin (Figure 2A vs. Figure 2B) after treatment with tezosentan. Tezosentan increased dilation in GFA at all insulin doses (Figure 3A vs. Figure 3B); in the G2A-Red at 10, 100, and 1000 $\mu\text{IU/mL}$ insulin (Figure 4A vs. Figure 4B); and in the G2A-White at 1000 $\mu\text{IU/mL}$ insulin (Figure 5A vs. Figure 5B).

In OLETF rat SFAs, although the insulin-stimulated dose-response curve for vasodilation appeared depressed by tezosentan treatment, there was no statistically significant difference between the effects of insulin after ET-1 blockade in the SFA (Figure 2C vs. Figure 2D). Similarly, in the G2A-Red (Figure 4C vs. Figure 4D), the response after ET-1 blockade was not different from dilation to insulin alone. In the GFA, tezosentan increased insulin-induced dilation at all insulin doses (Figure 3C vs. Figure 3D). Tezosentan increased insulin-induced dilation of G2A-White vessels at 100 and 1000 $\mu\text{IU/mL}$ insulin (Figure 5C vs. Figure 5D).

Although ET-1 receptor blockade did not alter insulin-induced vasodilation in the SFA (Figure 2A and 2B) or G2A-Red arteries (Figure 5A and 5B) of LETO rats, ET-receptor

blockade increased insulin-induced vasodilation compared to insulin alone in the GFA of metformin-treated LETO at 100 and 1000 $\mu\text{IU/mL}$ insulin (Figure 3A vs. Figure 3B). Tezosentan also increased insulin-induced dilation in G2A-White of LETO + MET at 10, 100, and 1000 $\mu\text{IU/mL}$ insulin, respectively (Figure 5A vs. Figure 5B).

In OLETF-metformin-treated animals, ET-1 blockade significantly increased vasodilation to insulin in all of the arteries except the G2A-Red (Figure 4C and 4D) [SFA at 1 and 1000 $\mu\text{IU/mL}$ insulin (Figure 2C vs. Figure 2D); GFA at 100 and 1000 $\mu\text{IU/mL}$ insulin (Figure 3C vs. 3D); and G2A-White at 1000 $\mu\text{IU/mL}$ insulin (Figure 5C vs. Figure 5D)]. Blockade of ET-1 receptors by tezosentan abolished differences between insulin-induced vasodilation of SFAs from untreated and metformin-treated OLETF groups (Figure 2C and 2D), suggesting that insulin-induced ET-1 mediated vasoconstriction contributes to the reduced vasodilatory response to insulin in the SFA of metformin-treated OLETF rats.

Role of Metabolic Health in Vasomotor Responses to Insulin with Metformin

We evaluated correlations between insulin-induced vasodilation and glycemic control outcomes to better understand the vascular response to insulin with metformin treatment. We only examined insulin-induced dilation in the SFA, as this was the only vessel that significantly dilated in response to insulin. Across all groups and insulin doses, there was no significant correlation between HbA1c or HOMA-IR and insulin-induced dilation in the SFA, suggesting that insulin-induced vasodilation is not correlated with clinical parameters of insulin resistance in the OLETF rat.

DISCUSSION

Metformin is one of the most commonly used therapeutic drugs for pre-diabetic and diagnosed T2DM patients. Metformin not only reduces chronic hyperglycemia but also has been reported to have vasculoprotective effects [20,24,27,38] and is linked to reduced cardiovascular events [47]. The primary purpose of this investigation was to test the hypothesis that chronic metformin treatment enhances vascular reactivity to insulin in resistance arteries of skeletal muscle of insulin resistant, pre-diabetic OLETF rats. The major findings of our study are that (i) three months of metformin treatment in hyperphagic, obese, and insulin-resistant OLETF rats did not enhance insulin-stimulated vasodilation in skeletal muscle resistance arteries, despite improvements in one chronic measure of glycemic control (HbA1c); and (ii) paradoxically, metformin reduced insulin-stimulated vasodilation in the OLETF SFA via increased insulin-induced activation of ET-1.

It is important to note that metformin significantly lowered food intake, body weight, adiposity, and HbA1c indicating that the dose and duration of metformin treatment used were sufficient to produce whole body metabolic effects characteristic of this common antihyperglycemic drug [16,27,54–56]. The reduced adiposity induced by metformin likely contributed to the HbA1c improvements by enhancing regulation of hepatic glucose production. Importantly, however, metformin did not improve fasting glucose, glucose AUC during an IPGTT, or HOMA-IR, suggesting that skeletal muscle insulin sensitivity was likely not altered by metformin treatment in this animal model. Our speculation of unaltered insulin sensitivity is consistent with a study in humans showing unchanged insulin-

stimulated muscle glucose uptake and whole body glucose uptake following treatment with metformin [18,61]. Since we did not directly assess insulin sensitivity, the trends for improvement with HOMA-IR and glucose AUC may underestimate metformin's real effect. Further, the apparent paradox of improved HbA1c despite unchanged glucose tolerance can be explained by differences between long and short-term indices of glycemic control. HbA1c most likely reflects the regulation of hepatic glucose output, insulin-stimulated glucose uptake, and insulin production from the pancreas over the course of days to weeks, whereas fasting glucose and IPGTT only reflect glycemic control at one particular point in time. Thus, our data suggest, but do not prove, that improvements in glycemic control occurred as a result of reduced body weight and adiposity rather than from changes in glucose tolerance or insulin-induced vasodilation in skeletal muscle resistance arteries.

Metabolic and vascular studies in humans and animals utilizing metformin in T2DM have yielded conflicting results for both vascular function and changes in insulin sensitivity. With regard to metabolic outcomes, some studies report modest improvements in insulin-mediated glucose disposal [15,48], some report no change [12,13,18], while others only report effects on fasting glucose clearance [2,19,45]. Only a handful of studies have investigated vascular effects of metformin in humans and animals. Metformin is reported to increase basal and post oral glucose tolerance test forearm blood flows [22], and enhance insulin-stimulated muscle blood flow during exercise [18] in obese patients with T2DM. Also, metformin has been reported to improve acetylcholine (ACh) endothelium-dependent vasodilation in mesenteric arteries of diabetic rats [39], and improve ACh-stimulated forearm blood flows in insulin-resistant T2DM humans [38]. In the latter study, metformin improved fasting insulin sensitivity (HOMA-IR) in T2DM patients; interestingly, the degree of fasting insulin resistance following metformin treatment was the sole predictor of improved endothelial function. Given that the metformin-treated OLETF rats in this study did not exhibit improvements in fasting glucose or insulin levels, it may therefore not be surprising that they also did not display improved vascular reactivity to insulin in skeletal muscle resistance arteries. Furthermore, our finding of reduced insulin-stimulated vasodilation in the SFA of metformin-treated OLETF rats is consistent with a study that reported no effect of metformin on endothelial dysfunction (ACh-stimulated forearm blood flow) in T2DM patients [45]. In addition, insulin-induced dilation in the SFA of OLETF rats revealed no significant correlation between HbA1c or HOMA-IR and insulin-induced dilation in the SFA across all groups and insulin doses, suggesting that insulin-induced vasodilation is not correlated with clinical parameters of insulin resistance in the OLETF rat. Overall, these results suggest that the insulin-sensitizing actions of metformin primarily occur in the liver (e.g., reduced hepatic glucose production) and in skeletal muscle (e.g., enhanced insulin-stimulated glucose uptake) [21,35,47] with minimal effects on insulin-mediated vasodilation in skeletal muscle. The reported effects of metformin on muscle blood flow responses to insulin [18] do not appear to be mediated by changes in direct effects of insulin on skeletal muscle resistance arteries or arterioles.

Role of ET-1 in insulin-induced vasoregulation in SFA of OLETF rats

ET-1 receptor blockade of SFAs from OLETF rats appeared to decrease insulin-induced dilation from 30 to 40% possible dilation at doses of 10 and 100 μ IU/mL to only 20%

dilation. These results suggest that ET-1 released in response to these doses of insulin, in the absence of ET-1 receptor blockade, is signaling a net dilation. Our results do not allow determination of the mechanism for this effect but it is possible that endothelial ETB receptor effects are increased in the endothelium of OLETF rat SFA resulting in enhanced insulin-induced dilation as shown in Figure 2C.

Our observation of a reduced vasodilatory response to insulin in the SFA of metformin-treated OLETF rats appears to be partially the result of changes in ET-1 signaling. Indeed, in the presence of ET-1 inhibition, insulin-stimulated vasodilation in the SFA of metformin-treated OLETF rats was increased compared to responses to insulin alone, so that the blunted insulin-induced vasodilation produced by metformin treatment in SFA of OLETF rats disappeared after blockade of the ET-1 receptors. Thus, metformin treatment appeared to decrease insulin-induced dilation (Figure 2C) but blockade of ET-1 receptors seemed to abolish the difference between insulin-induced dilation in SFA of untreated and metformin-treated OLETF rats (Figure 2D). Furthermore, metformin tended to induce a greater dilatory response in the SFA during pre-treatment with the ET-1 inhibitor, tezosentan (prior to insulin stimulation), compared to untreated OLETF rats (Table 2), suggestive of a greater contribution of ET-1 induced constriction to basal tone in the SFA of metformin-treated OLETF rats. This enhanced dilation to ET-1 receptor block may contribute to the blunted dilation response to insulin (Figure 2C) in that it would decrease the available vasodilator reserve of these SFAs. Importantly, these effects of metformin on the SFA of OLETF rats were not observed in any of the skeletal muscle resistance arteries of healthy LETO animals treated with metformin (Figures 2–5). Collectively, our data suggest that while metformin exerts favorable effects on body composition and HbA1c, it did not improve insulin-induced vasodilation in skeletal muscle resistance arteries of obese, insulin-resistant OLETF rats. Although metformin treatment appeared to alter ET-1 signaling in SFA of OLETF rats, our results do not allow us to evaluate whether metformin increased insulin-induced ET-1 production by the endothelium or altered the relative roles of ETA and ETB receptor signaling in endothelium or vascular smooth muscle of these arteries.

Our finding that insulin did not induce vasodilation in skeletal muscle arteries and arterioles of healthy lean LETO rats (except at the highest supraphysiological dose of insulin in the SFA, Figure 2A) is not unexpected, given published data from Eringa *et al.* reporting a lack of insulin induced dilation in skeletal muscle resistance arteries of lean Zucker rats [17]. However, in the presence of ET-1 receptor blockade insulin induced 15–30% dilation in the SFA of LETO rats (Figure 2B). These findings are also consistent with the 20% dilation with ET-1 blockade in skeletal muscle resistance arteries of lean Zucker rats [17].

Metformin has been shown to decrease physical fitness and increase lactate levels, which could translate to reduced ambulation. Although we did not directly assess physical fitness or physical activity in this study, we acknowledge that it is possible that metformin-treated animals may have reduced ambulation, which could possibly explain reduced insulin-induced vasodilation of skeletal muscle resistance arteries. In future studies, we plan to measure ambulatory cage activity and physical fitness in metformin-treated animals.

In summary, our data demonstrate that 12 weeks of metformin treatment improved glycemic control, reduced adiposity and body weight, and did not improve insulin-stimulated vasodilation in skeletal muscle resistance arteries or arterioles of OLETF rats. The effect of metformin treatment on insulin-induced dilation in isolated SFA was not present in the presence of ET-1 receptor blockade. Metformin treatment did not alter insulin-induced vasodilation in gastrocnemius muscle feed arteries and arterioles of OLETF rats. Also, ET-1 receptor blockade had similar effects on insulin-induced dilation in gastrocnemius arteries of LETO and OLETF rats. Future work is needed to definitively determine the effects of metformin on *in vivo* skeletal muscle vascular responses to insulin and the role of these processes in the protection against the development of T2DM. Overall, this study provides evidence that while treatment of advanced insulin resistance in the obese OLETF rat with metformin exerts favorable effects on body composition and reverses impairments in HbA1c, it does not enhance the vasodilatory responses to insulin in isolated skeletal muscle resistance arteries.

PERSPECTIVE

Metformin is a widely used antidiabetic drug; however, its effects on insulin-stimulated dilation of skeletal muscle resistance arteries were unknown. Our study demonstrates that metformin does not enhance insulin-stimulated vasodilation of skeletal muscle resistance arteries in the hyperphagic, obese OLETF rat (an established rodent model of obesity/T2DM). Thus, metformin's beneficial effects on body weight, food consumption, and glycemic control appear to be independent of changes in the microvascular actions of insulin.

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Abbreviations used

ET-1	endothelin-1
GFA	gastrocnemius feed arteries
G2A-R	second-order arterioles in the red portion of the gastrocnemius muscle
G2A-W	second-order arterioles in the white portion of the gastrocnemius muscle
HbA1c	glycosylated hemoglobin
HDL	high-density lipoproteins
HOMA-IR	homeostasis model assessment of insulin resistance
IPGTT	Intraperitoneal glucose tolerance tests

LDL	low-density lipoprotein
LETO	Long Evans Tokushima Otsuka
MET	metformin
MOPS	3-(N-Morpholino)propanesulfonic acid
MOPS-PSS	3-(N-Morpholino)propanesulfonic acid and physiological saline solution
NO	nitric oxide
OLETF	Otsuka Long-Evans Tokushima Fatty
PE	phenylephrine
SFA	soleus feed artery
SNP	sodium nitroprusside
T2DM	type 2 diabetes mellitus
TG	triglycerides

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Figure 1. HbA1c, % in 32-week-old LETO ($n = 12$), LETO + MET ($n = 11$), OLETF ($n = 13$), and OLETF + MET ($n = 15$) rats. All data are expressed as means $\pm$ SEM. *Significantly different from OLETF rats ($p < 0.05$).

Figure 2.

Insulin-induced vascular reactivity in SFA of LETO (A, B) and OLETF (C, D) rats. Left panels (A, C) present data from insulin dose–response curves performed under control conditions, and right panels (B, D) present data from insulin dose–response curves in the presence of 3 μ M tezosentan, a nonspecific inhibitor of Endothelin-1 (ET-1) receptors. *Significant difference between metformin treatment and untreated group. ϕ Significant effect of insulin + ET-1 blockade compared to insulin alone in untreated animals. δ Significant effect of insulin + ET-1 blockade compared to insulin alone in metformin-treated animals. μ IU/mL, micro international units per milliliter. Insulin produced a significant dilation from baseline in the SFA of LETO only at 1000 μ IU/mL (2A), however insulin-induced dilation of the SFA was significantly greater than baseline at all insulin doses in OLETF (2B).

Figure 3.

Insulin-induced vascular reactivity in GFA of LETO (A, B) and OLETF (C, D) rats. Left panels (A, C) present data from insulin dose–response curves performed under control conditions, and right panels (B, D) present data from insulin dose–response curves in the presence of 3 μ M tezosentan, a nonspecific inhibitor of Endothelin-1 (ET-1) receptors. ϕ Significant effect of insulin + ET-1 blockade compared to insulin alone in untreated animals. δ Significant effect of insulin + ET-1 blockade compared to insulin alone in metformin-treated animals. μ IU/mL, micro international units per milliliter.

Figure 4.

Insulin-induced vascular reactivity in G2A-Red of LETO (**A, B**) and OLETF (**C, D**) rats. Left panels (**A, C**) present data from insulin dose–response curves performed under control conditions, and right panels (**B, D**) present data from insulin dose–response curves in the presence of 3 μ M tezosentan, a nonspecific inhibitor of Endothelin-1 (ET-1) receptors. ^φSignificant effect of insulin + ET-1 blockade compared to insulin alone in untreated animals. ^δSignificant effect of insulin + ET-1 blockade compared to insulin alone in metformin-treated animals. μ IU/mL, micro international units per milliliter.

Figure 5.

Insulin-induced vascular reactivity in G2A-White of LETO (**A, B**) and OLETF (**C, D**) rats. Left panels (**A, C**) present data from insulin dose–response curves performed under control conditions, and right panels (**B, D**) present data from insulin dose–response curves in the presence of 3 μ M tezosentan, a nonspecific inhibitor of Endothelin-1 (ET-1) receptors. §Significant effect of insulin + ET-1 blockade compared to insulin alone in untreated animals. δSignificant effect of insulin + ET-1 blockade compared to insulin alone in metformin-treated animals. μ IU/mL, micro international units per milliliter.

Table 1

Animal characteristics

Variable	LETO	LETO + MET	OETF	OETF + MET
Body weight (g)	469 ± 12	445 ± 9	687 ± 11 [*]	650 ± 7 ^{*,†}
Body fat (%)	15.5 ± 0.6	13.5 ± 0.4	35.3 ± 1.1 [*]	32.4 ± 1.0 ^{*,†}
Absolute food consumption (g/week)	152 ± 4	133 ± 4 [*]	226 ± 4 [*]	208 ± 4 ^{*,†}
Relative food consumption (g/week bw)	0.317 ± 0.004	0.295 ± 0.004 [*]	0.334 ± 0.004 [*]	0.323 ± 0.004 ^{*,†}
Total cholesterol (mg/dL)	94 ± 3	93 ± 3	153 ± 8 [*]	146 ± 8 [*]
HDL-C (mg/dL)	26.8 ± 0.6	26.4 ± 0.5	34.0 ± 1.7 [*]	35.4 ± 1.8 [*]
LDL-C (mg/dL)	58.6 ± 2.8	58.9 ± 2.7	47.2 ± 5.9	43.5 ± 5.7 [*]
Triglycerides (mg/dL)	44.3 ± 3.3	39.9 ± 2.0	359.8 ± 40.5 [*]	337.4 ± 46.1 [*]
Glucose (mg/dL)	152 ± 4	150 ± 6	300 ± 12 [*]	283 ± 21 [*]
Insulin (mg/dL)	3.1 ± 0.4	2.6 ± 0.3	9.9 ± 2.1 [*]	7.1 ± 1.0 [*]
HOMA-IR (glucose [*] insulin/405)	1.2 ± 0.1	1.0 ± 0.1	7.5 ± 1.8 [*]	5.0 ± 0.9 [*]
Glucose AUC (mg/dL*120 minutes)	38553 ± 2867	36508 ± 1798	64839 ± 2875 [*]	59821 ± 5300 [*]
Insulin AUC (ng/mL*8 hours)	505 ± 148	356 ± 53 [*]	728 ± 281 [*]	497 ± 97

Data presented as mean ± SEM (*n* = 14–15/group).

HDL, high-density lipoprotein; LDL, low-density lipoprotein; and TG, triglycerides; HOMA-IR homeostasis model assessment- insulin resistance; AUC, area under the intraperitoneal glucose tolerance curve.

^{*} Significantly different from LETO (*p* < 0.05),

[†] Significantly different from OETF (*p* < 0.05).

Table 2

Vessel characteristics

Vessel	Group	N	Vessel diameter (μm)			% Pre-constriction		
			Maximal	Before Tezosentan	After Tezosentan	% Dilation to tezosentan	Insulin	Insulin + tezosentan
SFA	LETO	13	184 $\pm$ 11	99 $\pm$ 10	114 $\pm$ 13 [*]	22.0 $\pm$ 6.8	52.0 $\pm$ 4.1	44.8 $\pm$ 3.0
	LETO + MET	13	177 $\pm$ 12	97 $\pm$ 11	115 $\pm$ 15 [*]	19.8 $\pm$ 7.3	46.7 $\pm$ 3.4	47.2 $\pm$ 3.9
	OLETf	15	209 $\pm$ 6	125 $\pm$ 12	144 $\pm$ 13 [*]	17.9 $\pm$ 5.5	50.2 $\pm$ 3.5	48.3 $\pm$ 3.7
	OLETf + MET	14	212 $\pm$ 7	97 $\pm$ 6	133 $\pm$ 8 [*]	35.9 $\pm$ 9.3 [‡]	48.2 $\pm$ 2.4	50.1 $\pm$ 2.8
GFA	LETO	14	289 $\pm$ 12	217 $\pm$ 10	225 $\pm$ 8 [*]	4.5 $\pm$ 1.4	41.4 $\pm$ 2.4	40.9 $\pm$ 3.7
	LETO + MET	14	287 $\pm$ 12	188 $\pm$ 10	202 $\pm$ 10 [*]	8.2 $\pm$ 3.3	40.9 $\pm$ 2.4	43.1 $\pm$ 3.3
	OLETf	13	289 $\pm$ 12	221 $\pm$ 18	243 $\pm$ 14 [*]	14.4 $\pm$ 6.1 [‡]	44.4 $\pm$ 3.0	41.5 $\pm$ 3.5
	OLETf + MET	13	305 $\pm$ 7	206 $\pm$ 8	225 $\pm$ 9 [*]	10.8 $\pm$ 3.2	45.0 $\pm$ 2.2	39.9 $\pm$ 3.0
G2A-Red	LETO	12	191 $\pm$ 12	124 $\pm$ 10	153 $\pm$ 10 [*]	23.5 $\pm$ 4.7	41.9 $\pm$ 4.0	49.9 $\pm$ 2.6
	LETO + MET	12	178 $\pm$ 8	129 $\pm$ 10	141 $\pm$ 8	13.3 $\pm$ 6.5	39.5 $\pm$ 2.7	48.2 $\pm$ 4.1
	OLETf	13	197 $\pm$ 10	154 $\pm$ 12	174 $\pm$ 12 [*]	8.7 $\pm$ 2.1 [‡]	44.8 $\pm$ 3.4	38.9 $\pm$ 3.5 [‡]
	OLETf + MET	12	204 $\pm$ 6	152 $\pm$ 8	182 $\pm$ 6 [*]	16.2 $\pm$ 4.0	39.4 $\pm$ 4.0	40.1 $\pm$ 4.0
G2A-White	LETO	12	178 $\pm$ 11	126 $\pm$ 11	142 $\pm$ 13 [*]	10.3 $\pm$ 4.2	38.2 $\pm$ 3.0	45.3 $\pm$ 3.4
	LETO + MET	11	181 $\pm$ 16	151 $\pm$ 14	162 $\pm$ 13	8.85 $\pm$ 4.0	36.2 $\pm$ 4.4	42.4 $\pm$ 4.7
	OLETf	13	189 $\pm$ 12	149 $\pm$ 10	167 $\pm$ 9 [*]	15.5 $\pm$ 8.0	33.4 $\pm$ 2.0	38.9 $\pm$ 3.7
	OLETf + MET	12	192 $\pm$ 12	169 $\pm$ 13	181 $\pm$ 13 [*]	3.5 $\pm$ 1.0	33.6 $\pm$ 2.4	35.6 $\pm$ 3.6

Data presented are mean $\pm$ SEM.

SFA, soleus feed artery; GFA, gastrocnemius feed artery; G2A-Red, second-order arterioles perfusing the red portion of the gastrocnemius; G2A-White, second-order arterioles perfusing the white portion of the gastrocnemius; MET, metformin.

* p 0.05 vs. before tezosentan.

‡ p 0.05 vs. LETO.

‡ p = 0.09 vs. OLETf.