Original Research

Transcriptomic effects of metformin in skeletal muscle arteries of obese insulin-resistant rats

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Impact statement

This study provides evidence that metformin treatment produces artery-specific gene expression effects. The genes whose expression was modulated with metformin do not appear to have a clear connection with its known mechanisms of action.

Abstract

We examined the effects of metformin, a commonly used antidiabetic drug, on gene expression in multiple arteries. Specifically, transcriptional profiles of feed arteries and second branch order arterioles in the soleus, gastrocnemius, and diaphragm muscles as well as aortic endothelial scrapes were examined from obese insulin-resistant Otsuka Long-Evans Tokushima Fatty rats treated with ($n = 9$) or without ($n = 10$) metformin from 20 to 32 weeks of age. Metformin-treated rats exhibited a reduction in body weight, adiposity, and HbA1c ($P < 0.05$). The greatest number of differentially expressed genes (FDR < 15%) between those treated with and without metformin was found in the red gastrocnemius 2a arterioles (93 genes), followed by the diaphragm 2a arterioles (62 genes), and soleus 2a arterioles (15 genes). We also found that two genes were differentially expressed in aortic endothelial cells (LETMD1 and HMGCS2, both downregulated), one gene in the gastrocnemius feed artery (BLNK, downregulated), and no genes in the soleus and diaphragm feed arteries and white gastrocnemius 2a arterioles. No single gene was altered by metformin across all vessels examined. This study provides evidence that metformin treatment produces distinct gene expression effects throughout the arterial tree in a rat model of obesity and insulin resistance. Genes whose expression was modulated with metformin do not appear to have a clear connection with its known mechanisms of action. These findings support the notion that vascular gene regulation in response to oral pharmacological therapy, such as metformin, is vessel specific.

Keywords: RNA sequencing, gene expression, skeletal muscle resistance arteries, diabetes

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Introduction

Based on current recommendations from the American Diabetes Association, metformin is a first-line treatment for type 2 diabetes mellitus (T2D) along with diet and exercise.[1,2] Metformin lowers blood glucose concentrations by reducing basal hepatic glucose production and increasing insulin sensitivity in skeletal muscle.[3] In addition to being extensively used in the treatment of T2D, the use of metformin as a preventative therapy in patients at risk for developing overt T2D is also becoming increasingly widespread. In this regard, data from the Diabetes Prevention Program demonstrate that use of metformin lowered the incidence of T2D by 31% in nondiabetic insulin-resistant subjects.[4]

Despite the prevalent use of metformin for the treatment and prevention of T2D, the vascular effects of metformin have not been fully elucidated. Increased knowledge in this area is important in light of the established link between diabetes and vascular complications.[5–7] Although several

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studies provide evidence that metformin improves vascular function,[8–11] this is not a universal finding.[12,13] We recently examined the effect of metformin treatment on insulin-stimulated dilation in isolated skeletal muscle feed arteries and arterioles from Otsuka Long-Evans Tokushima Fatty (OLETF) rats,[13] a well-established rodent model of obesity, insulin resistance, and T2D.[14] In contrast to our hypothesis, metformin treatment did not enhance vasodilatory responses to insulin in skeletal muscle resistance arteries despite an improvement in glycemic control.[13] As in previous studies from our group, lack of changes in vasomotor function in response to an intervention does not imply an absence of a vascular phenotypic effect at the molecular level.[15–17] As such, to gain further insights into the impact of metformin on the vasculature, we assessed transcriptional profiles in skeletal muscle resistant arteries from the OLETF rats utilized in our previous study.[13] Specifically, we performed a transcriptome-wide RNA sequencing (RNA-Seq) analysis in the feed arteries and second branch order arterioles in the soleus, gastrocnemius, and diaphragm muscles in metformin-treated (via drinking water for 12 weeks) versus untreated OLETF rats. Owing to the systemic nature of its administration, we hypothesized that the gene expression effects of metformin would be largely uniform across vascular beds examined. To more specifically evaluate the effects of metformin on the endothelium, the same RNA-Seq analysis approach was employed in aortic endothelial cell scrapes. Given that metformin's mechanisms of action appear to involve activation of AMP-activated protein kinase (AMPK),[18] mitochondrial complex 1 inhibition,[19] and inhibition of mitochondrial glycerophosphate dehydrogenase (GPD2),[20] we postulated that differentially expressed genes by metformin treatment would be largely related to these pathways.

Material and methods

Animals and experimental design

Male OLETF rats ($n = 19$) were obtained at four weeks of age (Japan SLC, Hamamatsu, Shizuoka, Japan). The OLETF rat possesses a mutated cholestykinin-1 receptor, causing hyperphagic obesity and insulin resistance.[14] Animal housing conditions, body composition measurements, food intake assessment, metformin treatment regimens, blood parameter measurements, and sacrifice/tissue harvesting procedures have been described in previous manuscripts from our laboratory.[13,21,22] The University of Missouri Animal Care and Use Committee approved all study protocols.

Isolation of arteries and RNA sequencing

The gastrocnemius–plantaris–soleus muscle complex and diaphragm were harvested and pinned down in a Petri dish containing an RNA-stabilizing agent (RNAlater; Ambion, Austin, TX, USA). A total of seven arteries of interest were prepared for RNA-Seq analysis: gastrocnemius feed artery (GFA), soleus feed artery (SFA), diaphragm feed artery (DFA), white gastrocnemius second branch order arteriole (WG2a), red gastrocnemius second branch order arteriole (RG2a), soleus second branch order arteriole (S2a), and diaphragm second branch order arteriole (D2a). The reader is referred to our recent publications for a visual of the anatomic location and structure of gastrocnemius and soleus vascular network.[17,23] RNA extraction, RNA quality control, library preparation, and RNA-seq were performed as described previously.[16,24]

Statistical analysis

The analysis of the RNA-Seq data was carried out for all eight vessel types as described previously.[24] Adjustment for multiple comparisons was performed using the false discovery rate (FDR) method of Benjamini and Hochberg.[25] We chose 15% as our FDR threshold for statistical significance. For all lists of differentially expressed genes, it should be noted that statistical evidence is not intended to prove that the gene with the highest fold change has a smaller P value than the gene with the second highest fold change. Gene ontology (GO) analyses were subsequently carried out on the gene lists for overrepresentation of biological process and molecular function, as we previously described.[15] Between-group differences for all descriptive variables were determined by independent t-test, for which statistical significance was accepted at $P < 0.05$.

Results

Figure 1 summarizes animal characteristic data from the rats used in the current RNA-Seq analysis, which was a subset of the rats used in our prior investigations.[13,21,22] As shown, compared to untreated rats, metformin-treated rats were smaller, leaner, and exhibited lower levels of HbA1c. The trend for reduction in fasting glucose with metformin did not reach statistical significance ($p = 0.08$). The results of the single-end 50bp sequencing reads yielded ~175–200 million useable reads per lane (14–17 million reads per RNA-seq sample). A full list of differentially expressed genes between metformin-treated and untreated rats for all arteries in which genes were affected is provided in Supplemental Dataset 1. Results from the GO analysis are provided in Supplemental Dataset 2.

For the eight comparisons reported involving untreated versus metformin treated across eight arteries, the average ERCC Spike-in (Set B, $n = 10$ probes) *empirical* FDR (eFDR) was 2.9% at the nominal FDR cutoff of 15% (mean fold = 1.25), while for the putative housekeeping genes the average was 6.2% (mean fold = 0.99). Collectively, these findings strongly support the methodology used because, on average, the fold changes for these controls are approximately equal to 1 and the eFDR is approximately equal to the target FDR (15%). However, there was more intercomparison variability than we have observed in past analysis of data arising from this broader set of experiments. Although we do not have a good explanation for this, it is possible that a portion of this variability is related to subtle between-animal differences in metformin dosing through drinking water. This impact appeared to be most pronounced in the RG2a group, which had an ERCC Spike-in eFDR of 23%. All other groups had an eFDR of 0% based on the spike-ins.

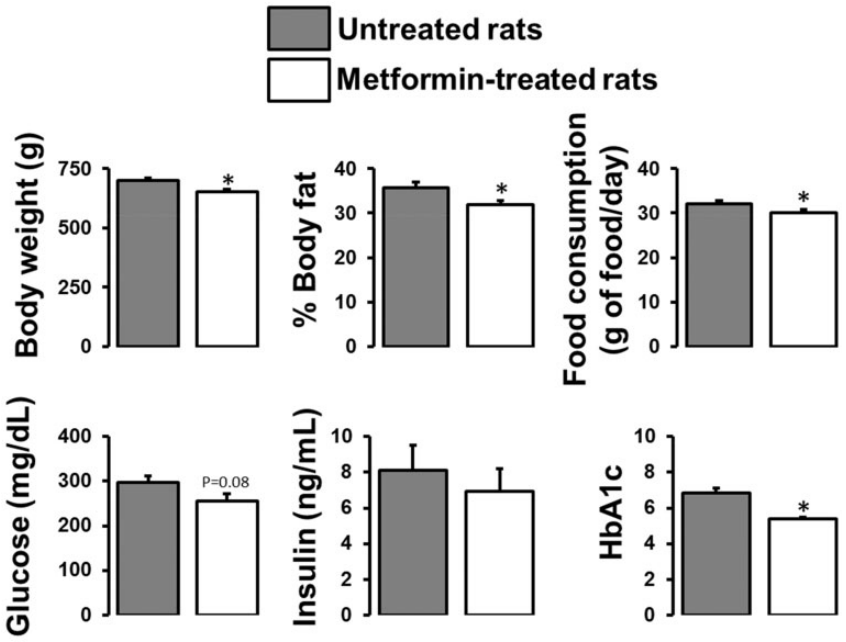

Figure 1 Effects of 12-week metformin treatment in body weight, body composition, food intake, fasting glucose, insulin, and HbA1c in OLETF rats. Food intake data represent an average of the last 10 weeks of study period. Values are expressed as mean ± SE. *denotes $P < 0.05$. OLETF: Otsuka Long-Evans Tokushima Fatty.

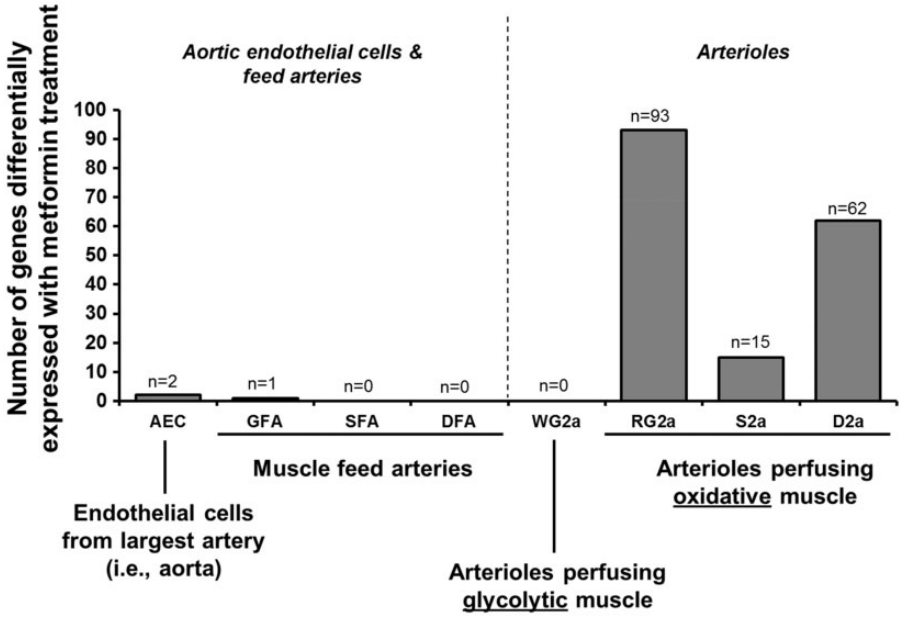

Figure 2 Number of genes significantly altered by metformin in each of the eight arteries. AEC: aortic endothelial cells; DFA: diaphragm feed artery; D2a: diaphragm second branch order arteriole; GFA: gastrocnemius feed artery; SFA: soleus feed artery; RG2a: red gastrocnemius second branch order arteriole; S2a: soleus second branch order arteriole; WG2a: white gastrocnemius second branch order arteriole. n value indicates the number of genes with significantly altered expression

Figure 2 displays the number of genes altered with metformin across all eight arteries examined. The greatest number of differentially expressed genes with metformin was found in the RG2a (93 genes; Table 1), followed by the D2a (62 genes; Table 2) and S2a (15 genes; Table 3). There were two genes differentially expressed with metformin in aortic endothelial cells (LETMD1 and HMGCS2, both downregulated), one gene differentially expressed with metformin in the GFA (BLNK, downregulated), and no genes differentially expressed with metformin in the SFA, DFA, and WG2a. No single gene was altered by metformin across all vessels examined. Expression of PCM1 was increased with metformin in both the RG2a and D2a, and JAG1 was increased in both RG2a and S2a.

Table 1 Top 20 genes differentially expressed between metformin-treated and untreated rats in RG2a, sorted by magnitude of fold change

EntrezID	Symbol	Name	FDR	Fold
Metformin-treated > untreated				
100365935	LOC100365935	rCG22129-like	0.092	4.2
64440	Syt4	Synaptotagmin IV	0.010	4.0
24525	Kras	Kirsten rat sarcoma viral oncogene	0.091	2.4
24706	Rarb	Retinoic acid receptor, beta	0.067	2.3
299799	Rab21	RAB21, member RAS oncogene family	0.108	2.3
94267	Nudt4	nudix (nucleoside diphosphate linked moiety X)-type motif 4	0.067	2.2
362176	Lmo2	LIM domain only 2	0.087	2.2
Metformin-treated < untreated				
302975	Syngr3	Synaptogyrin 3	0.108	−4.6
63839	Fhl2	Four and a half LIM domains 2	0.108	−4.2
361500	Nat14	*N*-acetyltransferase 14	0.125	−3.3
298452	Dph2	DPH2 homolog (*Saccharomyces cerevisiae*)	0.092	−3.2
300446	Zfp653	Zinc finger protein 653	0.092	−2.9
309301	Rcl1	RNA terminal phosphate cyclase-like 1	0.132	−2.7
313022	Map3k6	Mitogen-activated protein kinase kinase 6	0.108	−2.7
361857	Traf3ip2	Traf3 interacting protein 2	0.075	−2.5
363146	Dalrd3	DALR anticodon binding domain containing 3	0.075	−2.5
83725	Wfs1	Wolfram syndrome 1 (wolframin)	0.144	−2.4
287827	Armc7	Armadillo repeat containing 7	0.092	−2.4
689249	Rspry1	Ring finger and SPRY domain containing 1	0.075	−2.4
363134	Rrp9	ribosomal RNA processing 9, small subunit (SSU) processome component, homolog (yeast)	0.145	−2.2

RG2a: red gastrocnemius second branch order arteriole.

Table 2 Top 20 genes differentially expressed between metformin-treated and untreated rats in D2a, sorted by magnitude of fold change

EntrezID	Symbol	Name	FDR	Fold
Metformin-treated > untreated				
83808	Ugt2b15	UDP glucuronosyltransferase 2 family, polypeptide B15	<0.001	51.6
171521	Cyp2c13	Cytochrome P450, family 2, subfamily c, polypeptide 13	<0.001	50.3
29277	Cyp2c11	Cytochrome P450, subfamily 2, polypeptide 11	<0.001	44.0
293989	Cyp2c6v1	cytochrome P450, family 2, subfamily C, Polypeptide 6, variant 1	<0.001	29.4
292697	Apoc2	Apolipoprotein C-II	<0.001	26.3
24190	Aldob	Aldolase B, fructose-bisphosphate	<0.001	25.3
25649	Apoa2	Apolipoprotein A-II	<0.001	20.7
100360095	LOC100360095	Urinary protein 1-like	<0.001	20.2
259246	LOC259246	Alpha-2u globulin PGCL1	<0.001	18.2
287774	Apoh	Apolipoprotein H (beta-2-glycoprotein I)	<0.001	17.5
361969	Fga	Fibrinogen alpha chain	<0.001	17.1
25292	Apoc1	Apolipoprotein C-I	<0.001	17.0
252931	Cyp3a18	Cytochrome P450, family 3, subfamily a, polypeptide 18	<0.001	16.9
266682	Cyp3a2	Cytochrome P450, family 3, subfamily a, polypeptide 2	<0.001	15.6
619560	Rup2	Urinary protein 2	<0.001	14.6
25642	Cyp3a23/3a1	Cytochrome P450, family 3, subfamily a, polypeptide 23/polypeptide 1	0.001	14.5
246186	Fgl1	Fibrinogen-like 1	<0.001	14.2
83790	Cyp2c23	Cytochrome P450, family 2, subfamily c, polypeptide 23	0.001	14.2
24856	Ttr	Transthyretin	<0.001	12.6
304917	Serpinc1	Serpin peptidase inhibitor, clade C (antithrombin), member 1	0.010	11.7

D2a: diaphragm second branch order arteriole.

Table 3 Genes differentially expressed between metformin-treated and untreated rats in S2a, sorted by magnitude of fold change

EntrezID	Symbol	Name	FDR	Fold
Metformin-treated > untreated				
302975	Syngr3	Synaptogyrin 3	0.009	9.1
84405	Il12a	Interleukin 12A	0.099	4.0
503000	Ctnnbip1	Catenin, beta-interacting protein 1	0.032	3.3
29146	Jag1	Jagged 1	0.004	2.5
29480	Rgs4	Regulator of G-protein signaling 4	0.032	2.3
94174	Tinagl1	Tubulointerstitial nephritis antigen-like 1	0.104	1.9
29583	Pecam1	Platelet/endothelial cell adhesion molecule 1	0.143	1.9
362138	Rbms1	RNA binding motif, single stranded interacting protein 1	0.134	1.8
Metformin-treated < untreated				
29557	Myh7	Myosin, heavy chain 7, cardiac muscle, beta	0.046	−6.2
309374	Ankrd2	Ankyrin repeat domain 2 (stretch responsive muscle)	0.032	−5.9
25265	P2ry1	Purinergic receptor P2Y, G-protein coupled, 1	0.134	−3.8
117557	Tpm3	Tropomyosin 3	0.004	−3.6
361879	Wdr41	WD repeat domain 41	0.093	−2.7
311903	Mrrf	Mitochondrial ribosome recycling factor	0.032	−2.1
373544	Ermp1	Endoplasmic reticulum metallopeptidase 1	0.093	−2.1

S2a: soleus second branch order arteriole.

Expression of SYNGR3 was also altered by metformin in both the RG2a and S2a but in opposite directions (decrease in RG2a and increase in S2a).

Discussion

Metformin treatment for prediabetes and T2D is widespread. Metformin reduces blood glucose concentrations by lowering basal hepatic glucose production and exerting insulin-sensitizing effects in skeletal muscle.[3] In addition, metformin has beneficial effects on the vasculature[8–11] and its use is associated with decreased cardiovascular events.[3] The purpose of this study was to gain a better understanding of the effects of metformin treatment on vascular gene expression using the obese insulin-resistant OLETF rat model in which we provide evidence of its beneficial metabolic effects. RNA-Seq gene expression analysis was carried out on resistance vessels of the soleus, gastrocnemius, and diaphragm muscles as well as on aortic endothelial cell scrapes. Contrary to our hypothesis, the major finding of this study is that metformin produced markedly divergent gene expression effects across the different vascular beds examined and these effects appeared to be unrelated to the mechanisms thought to be responsible for metformin's therapeutic effects. Results from GO analysis confirmed this observation.

The heterogeneous vascular effects of metformin were most evident in the number of genes differentially expressed across vessels as well as the magnitude of changes. As illustrated in Figure 2, a noted pattern is that the effects of metformin on gene expression were largely absent in aortic endothelial cells and feed arteries (i.e. larger arteries), but apparent in the downstream arterioles that lie within the epimysium of the muscle (i.e. smaller arteries). Within the arterioles examined, another intriguing observation was that only those arterioles perfusing oxidative muscle (i.e. RG2a, S2a, D2a), and not glycolytic muscle (i.e. WG2a), appeared to manifest the effect of metformin on gene expression. However, despite metformin altering expression of genes in arterioles perfusing oxidative muscle, the amount of overlap in genes with altered expression among these three second branch order arterioles was minimal. Indeed, none of the genes differentially expressed with metformin were altered in all three arterioles of high oxidative skeletal muscle. Expression of one gene (PCM1) was increased with metformin in both the RG2a and D2a, and another gene (JAG1) was increased in both RG2a and S2a. Expression of a third gene (SYNGR3) was also modulated by metformin in both the RG2a and S2a but in opposite directions (decrease in RG2a and increase in S2a). The protein encoded by PCM1 is a component of centriolar satellites, which are electron dense granules scattered around centrosomes. This protein is thought to be required for centrosome assembly and function. The jagged 1 protein encoded by JAG1 is the ligand for the receptor notch 1. Jagged 1 signaling through notch 1 has been shown to play a role in hematopoiesis and seems to be involved in early and late stages of mammalian cardiovascular development. SYNGR3 encodes an integral membrane protein whose exact function is unclear but appears to be involved in the regulation of dopamine transporter activity. Thus, none of these three genes whose expression was ''commonly'' altered with metformin treatment appear to be linked to its established mechanisms (AMPK activation and inhibition of mitochondrial complex 1 and GPD2[18–20]), and this apparent absence of link between genes affected by metformin and its known mechanisms was also true for the remaining differentially altered genes. It should be noted that consistent with findings from others,[26] we also found that metformin altered expression of several CYP isozymes primarily in the D2a.

Table 4 List of genes differentially expressed with obesity and metformin

Artery	Symbol	Name	Altered with Obesity	Altered with Metformin
AEC	Hmgcs2	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	Decrease	Decrease
RG2a	Zfp36l1	Zinc finger protein 36, C3H type-like 1	Decrease	Increase
S2a	Syngr3	Synaptogyrin 3	Decrease	Increase
	Wdr41	WD repeat domain 41	Increase	Decrease
D2a	Rup2	Urinary protein 2	Increase	Increase
	Fabp1	Fatty acid binding protein 1, liver	Increase	Increase
	Cyp4a2	Cytochrome P450, family 4, subfamily a, polypeptide 2	Increase	Increase
	Cyp3a2	Cytochrome P450, family 2, subfamily c, polypeptide 23	Increase	Increase
	Ahsg	Alpha-2-HS-glycoprotein	Increase	Increase
	Ttr	Transthyretin	Increase	Increase
	LOC100360095	Urinary protein 1-like	Increase	Increase
	Serpinc1	Serpin peptidase inhibitor, clade C (antithrombin), member 1	Increase	Increase
	LOC259246	Alpha-2u globulin PGCL1	Increase	Increase
	Fga	Fibrinogen alpha chain	Increase	Increase
	Apoa2	Apolipoprotein A-II	Increase	Increase
	Fetub	Fetuin B	Increase	Increase
	Fgb	fibrinogen beta chain	Increase	Increase
	Vsnl1	Visinin-like 1	Increase	Decrease
	Bhmt	Betaine-homocysteine S-methyltransferase	Increase	Increase
	Cyp3a18	Cytochrome P450, family 3, subfamily a, polypeptide 18	Increase	Increase
	Creb5	cAMP responsive element binding protein 5	Decrease	Increase

AEC: aortic endothelial cells; D2a: diaphragm second branch order arteriole; RG2a: red gastrocnemius second branch order arteriole; S2a: soleus second branch order arteriole.
Effects of obesity were published in Jenkins et al.[24] (AEC) and Padilla et al.[29] (RG2a, S2a, and D2a). (A color version of this table is available in the online journal.)

We were recently surprised, although to a lesser extent, by the profound heterogeneous vascular effects of exercise training.[15–17,27] Nevertheless, the notion that the effects of exercise are not the same among skeletal muscle arteries can be reconciled with the understanding that during exercise different muscle fiber recruitment patterns exist among these skeletal muscles, thus likely contributing to this differing vascular effect of training between muscles and even within muscles.[17,27] Perhaps more surprising was our recent finding that obesity, viewed as a condition with systemic complications, also resulted in striking heterogeneous effects among arteries examined.[24,28,29] Indeed, we identified 20 genes whose expression was consistently altered among 15 arteries, which represented only a ~9% overlapping effect of obesity among all arteries examined.[29] In keeping with the theme that systemic interventions can produce heterogeneous spatial distribution of vascular transcriptomic effects throughout the arterial vasculature, the findings from the present study are especially remarkable. Herein we provide evidence that pharmacological treatment also evokes heterogeneous effects across the arterial tree with contrasting vascular effects not only observed between muscles but also within muscle, thus resembling our prior findings on the effects of exercise[15–17,27] and obesity.[24,28,29] Fundamentally, these data support the idea that regulation of vascular gene expression in response to a given insult (e.g. obesity) or treatment (e.g. exercise, metformin) is not uniform among arteries. Although we believe findings in one artery cannot be extrapolated to other arteries, this is not yet universally accepted in the field of cardiovascular biology and medicine. It is for this reason that we expect the present findings will contribute to our increasing, but complex, understanding that arteries differentially respond to a given stimulus, even when the stimulus is pharmacological and systemically administered.

We also sought to examine the extent of overlap between the genes affected by obesity (previously published[24,29]) and metformin across arteries. As summarized in Table 4, to our surprise, we noted that of the 21 genes whose expression was affected by both obesity and metformin, the effects were in the same direction on 16 of the genes. Differently put, metformin only reversed the expression of five out of 2296 genes affected by obesity across the arteries examined in the present study. We also contrasted the effects of metformin to the effects of endurance exercise previously published.[16,17,27] We found that nine genes were affected by both metformin and exercise and these effects were all in the same direction (Table 5).

Despite these novel findings, some considerations should be acknowledged. First, because we studied mRNA levels from whole artery homogenates (except for scraped aortic endothelial cells), it remains unknown if differences in vascular gene expression reported herein are originating from the endothelium, smooth muscle, or adventitia. Second, protein content was not assessed in the present study; thus, it is unknown the extent to which the present vascular transcriptomic effects of metformin translate to changes at the protein level.

Table 5 List of genes differentially expressed with endurance exercise and metformin

Artery	Symbol	Name	Altered with Endurance Exercise	Altered with metformin
AEC	Hmgcs2	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	Decrease	Decrease
S2a	Syngr3	Synaptogyrin 3	Increase	Increase
	Tpm3	Tropomyosin 3	Decrease	Decrease
	Jag1	Jagged 1	Increase	Increase
	Rgs4	Regulator of G-protein signaling 4	Increase	Increase
	Rbms1	RNA binding motif, single stranded interacting protein 1	Increase	Increase
D2a	Aldob	Aldolase B, fructose-bisphosphate	Increase	Increase
	Cyp2c6v1	Cytochrome P450, family 2, subfamily C, polypeptide 6, variant 1	Increase	Increase
	Cyp2c13	Cytochrome P450, family 2, subfamily c, polypeptide 13	Increase	Increase

AEC: aortic endothelial cells; D2a: diaphragm second branch order arteriole; S2a: soleus second branch order arteriole.
Effects of endurance exercise were published in Padilla et al.[16] (AEC), Laughlin et al.[17] (S2a), and Laughlin et al.[27] (D2a). (A color version of this table is available in the online journal.)

We conclude that metformin treatment produces distinct gene expression effects throughout the arterial tree in a rat model of obesity and insulin resistance. The genes whose expression was modulated with metformin do not appear to have a clear connection with its known mechanisms of action and are not the same genes affected by obesity. While we cannot offer explanations for metformin's vascular effects based on our data, we have identified a new catalog of genes that may play a role in the cardiovascular benefits of metformin. These genes and the functional consequences of their responsiveness to metformin treatment should be the focus of future studies. Furthermore, findings from this investigation support the provocative notion that vascular gene regulation in response to oral metformin is vessel specific.

Authors' contributions: All authors participated in the design, data collection, interpretation of the data, and review of the manuscript; JP wrote the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

SUPPLEMENTARY MATERIAL

Supplementary material for this paper can be found at http://journals.sagepub.com/doi/suppl/10.1177/1535370216689825.

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