Dissociation of Hyperglycemia from Altered Vascular Contraction and Relaxation Mechanisms in Caveolin-1 Null Mice

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ABSTRACT

Hyperglycemia and endothelial dysfunction are associated with hypertension, but the specific causality and genetic underpinning are unclear. Caveolin-1 (cav-1) is a plasmalemmal anchoring protein and modulator of vascular function and glucose homeostasis. Cav-1 gene variants are associated with reduced insulin sensitivity in hypertensive individuals, and cav-1^{-/-} mice show endothelial dysfunction, hyperglycemia, and increased blood pressure (BP). On the other hand, insulin-sensitizing therapy with metformin may inadequately control hyperglycemia while affecting the vascular outcome in certain patients with diabetes. To test whether the pressor and vascular changes in cav-1 deficiency states are related to hyperglycemia and to assess the vascular mechanisms of metformin under these conditions, wild-type (WT) and cav-1^{-/-} mice were treated with either placebo or metformin (400 mg/kg daily for 21 days). BP and fasting blood glucose were in cav-1^{-/-} > WT and did not change with metformin. Phenylephrine (Phe)- and

KCl-induced aortic contraction was in cav-1^{-/-} < WT; endothelium removal, the nitric-oxide synthase (NOS) blocker L-NAME (*N*^ω-nitro-L-arginine methyl ester), or soluble guanylate cyclase (sGC) inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) enhanced Phe contraction, and metformin blunted this effect. Acetylcholine-induced relaxation was in cav-1^{-/-} > WT, abolished by endothelium removal, L-NAME or ODQ, and reduced with metformin. Nitric oxide donor sodium nitroprusside was more potent in inducing relaxation in cav-1^{-/-} than in WT, and metformin reversed this effect. Aortic eNOS, AMPK, and sGC were in cav-1^{-/-} > WT, and metformin decreased total and phosphorylated eNOS and AMPK in cav-1^{-/-}. Thus, metformin inhibits both vascular contraction and NO-cGMP-dependent relaxation but does not affect BP or blood glucose in cav-1^{-/-} mice, suggesting dissociation of hyperglycemia from altered vascular function in cav-1-deficiency states.

Introduction

Hypertension is a multifaceted disorder that involves abnormalities in the vascular, renal, nervous, and endocrine systems (Williams, 1994; Hall et al., 1996; Lohmeier et al., 2005). Vascular dysfunction occurs in many forms of hypertension and involves changes in different regulatory proteins and signaling pathways. Caveolin-1 (cav-1) is an anchoring protein in plasma membrane caveolae of many cell types, including vascular smooth muscle (VSM) (Ishizaka et al., 1998) and endothelial cells (Feron et al., 1998; Minshall et al., 2003). In endothelial cells, cav-1 anchors endothelial nitric oxide (NO) synthase (eNOS) in plasma membrane caveolae, which limits its translocation and phosphoactivation (Feron et al., 1998; Batova

et al., 2006) and thereby reduces its capacity to generate NO and activate the NO-cGMP vasorelaxation pathway. Endothelial cell dysfunction and abnormalities in NO-cGMP are important factors in hypertension.

Metabolic disorders such as obesity, hyperglycemia, and diabetes mellitus are often detected in association with hypertension (Brands and Hopkins, 1996). Abnormal vascular function may play a role in obesity-related hypertension (Lastra et al., 2010). Interestingly, cav-1 may modulate the metabolic pathways in adipose cells. Indeed, cav-1 expression in human adipose tissue is altered in obesity-associated type 2 diabetes (Catalan et al., 2008). In addition, our studies in humans have shown that cav-1 gene variants are associated with reduced insulin sensitivity in hypertensive, but not normotensive, individuals (Pojoga et al., 2011). Furthermore, cav-1-deficient mice show hyperglycemia, adipose tissue defects in insulin receptor expression and mitochondrial function (Asterholm et al., 2012), as well as dramatic changes in vascular function and blood pressure (BP) (Pojoga et al., 2010, 2011). Although cav-1 has been linked to regulation of both vascular and glycemic

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ABBREVIATIONS: Ach, acetylcholine; AMPK, AMP-activated protein kinase; BP, blood pressure; cav-1, caveolin-1; eNOS, endothelial nitric-oxide synthase; L-NAME, *N*^ω-nitro-L-arginine methyl ester; NO, nitric oxide; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; Phe, phenylephrine; PKC, protein kinase C; PRA, plasma renin activity; sGC, soluble guanylate cyclase; SNP, sodium nitroprusside; VSM, vascular smooth muscle; WT, wild-type.

control mechanisms, the relation between hyperglycemia and altered vascular function and BP in cav-1 deficiency states is poorly defined. For example, whereas insulin-sensitizing compounds such as metformin are the first-line therapy for type 2 diabetes and glycemic control in prediabetic states and metformin improves hyperglycemia in several experimental models (Heishi et al., 2006; Matsumoto et al., 2008), certain patients with diabetes show inadequate glycemic control with metformin monotherapy (Bailey et al., 2013). On the other hand, metformin may have pleiotropic vascular effects that are independent of its effects on glycemic control. For instance, in women with polycystic ovary syndrome characterized by hyperinsulinemia, hyperandrogenism, dyslipidemia, dysadipocytokinemias, and central obesity, metformin improves cardiovascular outcomes while having minimal effects on glucose levels (Agarwal et al., 2010). Interestingly, some of the cellular actions of metformin require cav-1 (Salani et al., 2012). Thus, it is conceivable that in cav-1-deficiency states metformin may not affect glycemic control; however, whether it could still exert vascular actions under these conditions is unclear.

The purpose of this study was to determine whether the pressor changes and altered vascular function associated with cav-1 deficiency are related to or independent from hyperglycemia. By use of cav-1 knockout (cav-1^{-/-}) mice, which show hyperglycemia and altered vascular reactivity (Cohen et al., 2003; Pojoga et al., 2010, 2011; Asterholm et al., 2012) and metformin treatment at a dose that improved hyperglycemia in other models (Heishi et al., 2006; Matsumoto et al., 2008), we hypothesized that if the vascular changes in cav-1 deficiency states are related to hyperglycemia, then metformin improvement of hyperglycemia should be paralleled with improved vascular function. Conversely, if the vascular changes in cav-1 deficiency states are independent of hyperglycemia and metformin changes vascular outcome despite inadequate glycemic control as shown clinically in certain patients with diabetes (Bailey et al., 2013), metformin then should affect vascular function without modifying hyperglycemia. We used WT and cav-1^{-/-} mice chronically treated with placebo or metformin to test whether metformin affects glycemic control, BP, and vascular contraction and relaxation mechanisms and whether these effects are different in cav-1 deficiency states. We also tested whether the metformin-induced changes in vascular function involve changes in the NO-cGMP pathway.

Materials and Methods

Animals. Cav-1^{-/-} (strain 004585) and genetically matched WT (strain 101045) male mice (16 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). The genotypes were confirmed by polymerase chain reaction. Animals were housed in 12-hour/12-hour light/dark cycle at an ambient temperature of 22 ± 1°C and were maintained on ad libitum rodent chow and tap water. After 3 days of acclimatization, mice were treated for 21 days with placebo or metformin (400 mg/kg daily in drinking water), a dose reported to improve hyperglycemia in other models (Heishi et al., 2006; Matsumoto et al., 2008). All procedures were approved by the Animal Care and Use Committee at Harvard Medical School.

Blood Pressure. Systolic BP was measured in conscious mice using tail-cuff plethysmography (CODA; Kent Scientific, Torrington, CT) (Pojoga et al., 2010). Mice were kept warm at 30°C for 10 minutes and allowed to rest quietly before BP measurement. BP measurements were taken in a quiet room, and mice were kept calm and handled by the same person.

Fasting Blood Glucose. Animals were fasted overnight. The next morning at 7:00 AM, blood samples were collected via tail vein, and glucose levels were determined using a Freestyle glucometer (Abbott Laboratories, North Chicago, IL).

Plasma Renin Activity. Blood was collected in purple-top BD Microtainer tubes (EDTA). The plasma was separated by centrifugation, and plasma renin activity (PRA) levels were determined in duplicate using a GammaCoat RIA kit (DiaSorin Inc., Stillwater, MN).

Tissue Preparation. Mice were killed with isoflurane overdose, and the thoracic and abdominal aortas were rapidly excised. The thoracic aorta was cleaned of connective tissue and cut into 3-mm-wide rings. The rest of thoracic aorta and all abdominal aortas were placed in liquid nitrogen for protein analysis.

Isometric Contraction and Relaxation. Aortic segments were suspended between two tungsten wire hooks, one hook was fixed at the bottom of tissue bath, and the other hook was connected to Grass force transducer (FT03; Astro-Med, Inc, West Warwick, RI). Aortic segments were stretched under 0.5-g resting tension for 45 minutes in temperature-controlled tissue bath, filled with 50 ml of Krebs' solution continuously bubbled with 95% O₂, 5% CO₂ at 37°C. Changes in isometric contraction were recorded on Grass polygraph (Model 7D, Astro-Med).

A control aortic contraction to 96 mM KCl was first elicited followed by rinsing in Krebs' 3 × 10 minutes. Aortic segments were then stimulated with phenylephrine (Phe, 10⁻⁹–10⁻⁵ M), concentration-contraction curves were constructed, and maximal Phe contraction was measured. Individual Phe concentration-response curves were further analyzed using a nonlinear regression curve (best-fit sigmoidal dose-response curve; Sigmaplot, La Jolla, CA), and the effective concentration that produced half maximal contraction (ED₅₀) was measured and presented as pED₅₀ (–log M). In other experiments, vessels were precontracted with Phe (10⁻⁵ M), acetylcholine (ACh, 10⁻⁹–10⁻⁵ M) was added, and the percent of relaxation of Phe contraction was measured. Parallel contraction and relaxation experiments were performed in aortic rings pretreated with the NOS inhibitor N^ω-nitro-L-arginine methyl ester (L-NAME, 3 × 10⁻⁴ M) or guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10⁻⁵ M) for 10 minutes. In some experiments, the endothelium was removed by scraping the vessel interior around the tip of fine forceps, and the responses to Phe and ACh were compared with those in endothelium-intact vessels. The relaxation of Phe-precontracted aortic rings to the exogenous NO donor sodium nitroprusside (SNP) was also measured. Individual ACh and SNP concentration-relaxation curves were further analyzed using a nonlinear regression best-fit sigmoidal dose-response curve, and ACh and SNP ED₅₀ values were calculated.

Western Blots. Aortic tissues were homogenized in RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA). Protein extracts (40 µg) were combined with an equal volume of 2× Laemmli loading buffer, boiled for 5 minutes, and size-fractionated by electrophoresis on 7.5% SDS-polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane by electroblotting. Membranes were incubated with 5% nonfat dried milk in Tris-buffered saline-Tween (USB Corporation, Santa Clara, CA) for 1 hour and then incubated overnight at 4°C with the primary antibody anti-eNOS (1:2500) (BD Transduction Laboratories, Franklin Lakes, NJ), anti-peNOS (1:1000), anti AMP-activated protein kinase (AMPK) (1:400), anti-pAMPK (1:400) (Cell Signaling Technology, Danvers, MA) and antisoluble guanylate cyclase (sGC) (1:200) (Cayman Chemical, Ann Arbor, MI). Membranes were then washed, incubated with peroxidase-conjugated secondary antibody, and analyzed using enhanced chemiluminescence (PerkinElmer Life and Analytical Sciences, Waltham, MA). Blots were then re probed for β-actin (1:20,000; Sigma-Aldrich), immunoreactive bands were quantitated by optical densitometry, and the results were normalized to β-actin to correct for loading.

Solutions and Drugs. Krebs' solution contained (in mM): NaCl 120, KCl 5.9, NaHCO₃ 25, NaH₂PO₄ 1.2, dextrose 11.5, CaCl₂ 2.5, MgCl₂ 1.2, at pH 7.4, and 96 mM KCl was prepared as Krebs' with equimolar substitution of NaCl with KCl. Stock solutions of Phe, ACh, SNP, and

L-NAME (10^{-1} M; Sigma-Aldrich) were prepared in distilled water. Stock solution of ODQ (10^{-1} M; EMD Bioscience, Darmstadt, Germany) was prepared in DMSO. Final concentration of DMSO in solution was $<0.1\%$. All other chemicals were of reagent grade or better.

Statistical Analysis. Data were presented as means $\pm$ S.E.M., with n value representing the number of mice. Data were first analyzed using one-way analysis of variance for comparison of different groups (cav-1 status vs. metformin treatment). When a statistical difference was observed, the data were further analyzed using Student-Newman-Keuls post hoc test for multiple comparisons. Also, pairwise comparisons were performed when a significant effect was noted in analysis of variance, and unpaired Student's t test was used for comparison of two means. Differences were considered significant if $P < 0.05$. In all studies, experiments were performed blinded to animal genotype and treatment.

Results

Cav-1 $^{-/-}$ mice had lower body weights and higher BP and fasting blood glucose levels compared with WT mice (Fig. 1, A, B, and C). Metformin treatment did not significantly affect body weight, BP, or blood glucose levels in cav-1 $^{-/-}$ or WT. PRA levels were not significantly different in cav-1 $^{-/-}$ versus WT, and metformin did not modify PRA levels (Fig. 1D).

In endothelium-intact aortic rings, Phe caused concentration-dependent contraction that reached a maximum at 10^{-5} M. Phe contraction was less in cav-1 $^{-/-}$ than WT and in cav-1 $^{-/-}$ + metformin than WT + metformin. Metformin caused insignificant reduction of Phe contraction in WT and had no effect in cav-1 $^{-/-}$ mice (Fig. 2A; Table 1).

To test whether the reduced contraction in cav-1 $^{-/-}$ versus WT is due to changes in α -adrenergic receptor sensitivity, Phe contraction was presented as the percent of maximum, and Phe ED₅₀ was calculated. Although the Phe concentration-response curve showed a shift to the right in cav-1 $^{-/-}$ (Fig. 2B), the Phe ED₅₀ was not significantly different between cav-1 $^{-/-}$ and WT (Table 1). Although the Phe ED₅₀ was not significantly different between WT + metformin and WT, Phe was more potent in cav-1 $^{-/-}$ + metformin than cav-1 $^{-/-}$ (Fig. 2B; Table 1).

To test whether the changes in aortic contraction in cav-1 $^{-/-}$ are specific to a particular agonist or receptor, we measured contraction to 96 mM KCl. KCl causes membrane depolarization and stimulates Ca²⁺ entry through voltage-gated channels via receptor-independent mechanisms (Murphy and Khalil, 2000). KCl contraction was reduced in cav-1 $^{-/-}$ versus WT, and metformin treatment reduced KCl contraction in WT, but not cav-1 $^{-/-}$ (Fig. 2C; Table 1).

Relative to maximal KCl contraction, the maximal Phe contraction was ~ 1.4 -fold in WT, but it was almost similar to KCl contraction in cav-1 $^{-/-}$ (Fig. 2D; Table 1). Assuming that KCl contraction is due mainly to Ca²⁺ entry through voltage-gated channels (Murphy and Khalil, 2000), the greater maximal Phe contraction relative to maximal KCl in WT could be due to additional activation of other Ca²⁺ channels (Lee et al., 2001; Park et al., 2008) or Ca²⁺-sensitization pathways (Salamanca and Khalil, 2005; Dimopoulos et al., 2007), and these contraction enhancement mechanisms are inactive or obscured in cav-1 $^{-/-}$. Metformin did not significantly affect Phe contraction relative to KCl in WT or cav-1 $^{-/-}$ (Fig. 2D; Table 1).

Fig. 1. Body weight (A), blood pressure (B), fasting blood glucose (C), and plasma renin activity (PRA) (D) in WT and cav-1 $^{-/-}$ mice with or without metformin treatment. Data represent means $\pm$ S.E.M. of measurements in six to nine mice. *Measurements in cav-1 $^{-/-}$ are significantly different ($P < 0.05$) from corresponding measurements in WT.

Fig. 2. Phe- and KCl-induced contraction in aortic segments of WT and *cav-1*^{-/-} mice with or without metformin treatment. Endothelium-intact aortic rings were stimulated with increasing concentrations of Phe, and the contractile response was measured and presented in grams (A) or as percentage of maximum Phe contraction (B). Vascular contraction to KCl (95 mM KCl) was also measured (C), and the contractile response to Phe was presented as % of KCl contraction (D). Data represent means $\pm$ S.E.M. of measurements in 9–17 mice. *Measurements in *cav-1*^{-/-} are significantly different ($P < 0.05$) from corresponding measurements in WT mice. #Measurements in metformin-treated mice are significantly different ($P < 0.05$) from corresponding measurements in mice of the same genotype without metformin treatment.

To test the role of endothelium in the altered vascular responses, the magnitude of Phe contraction was enhanced in endothelium-denuded compared with intact aortas, particularly in *cav-1*^{-/-}, but not in WT, WT + metformin, or *cav-1*^{-/-} + metformin (Fig. 3A; Table 1). To test for a potential role of endothelium-derived NO in the reduced aortic contraction in *cav-1*^{-/-}, the NOS inhibitor L-NAME caused insignificant enhancement of Phe contraction in *cav-1*^{-/-} and no significant change in Phe contraction in WT, WT + metformin or *cav-1*^{-/-} + metformin. Pretreatment with the guanylate cyclase inhibitor ODQ minimally affected Phe contraction in WT, but it significantly enhanced Phe contraction in WT + metformin, *cav-1*^{-/-}, and to a lesser extent in *cav-1*^{-/-} + metformin (Fig. 3A; Table 1).

In all groups, when Phe contraction was presented as the percent of the maximum, the Phe concentration-response curve appeared to be shifted to the left in endothelium-denuded and L-NAME- or ODQ-treated aortas compared with control intact and nontreated aortas (Fig. 3B), suggesting a role of endothelium-dependent NO-cGMP pathway in reducing the vascular responsiveness to Phe. Further analysis of Phe ED₅₀ showed that Phe was more potent in endothelium-denuded versus intact aortas of WT + metformin. Phe was

equally potent in L-NAME-treated and nontreated vessels of WT or *cav-1*^{-/-} with or without metformin treatment. ODQ significantly increased Phe potency in aortas of *cav-1*^{-/-} and WT + metformin, but not WT, and *cav-1* + metformin (Table 1).

Relative to KCl contraction, Phe contraction was dramatically enhanced in endothelium-denuded versus intact aortas of *cav-1*^{-/-}, and this enhancement was absent in WT, WT + metformin, and *cav-1*^{-/-} + metformin (Fig. 3C; Table 1). Treatment of endothelium-intact aortas with L-NAME enhanced Phe contraction in *cav-1*^{-/-}, but not WT, WT + metformin, or *cav-1*^{-/-} + metformin. ODQ caused minimal effects on Phe contraction in aorta of WT but significantly enhanced Phe contraction in WT + metformin, *cav-1*^{-/-}, and *cav-1*^{-/-} + metformin (Fig. 3C; Table 1).

ACh caused concentration-dependent aortic relaxation that was greater in *cav-1*^{-/-} than in WT and *cav-1*^{-/-} + metformin than in WT + metformin. ACh relaxation was not different in WT + metformin and WT but was reduced in *cav-1*^{-/-} + metformin versus *cav-1*^{-/-} (Fig. 4). When ACh response was measured as the percentage of maximum relaxation and ED₅₀ was calculated, ACh was more potent in *cav-1*^{-/-} than in WT and *cav-1*^{-/-} + metformin than in WT + metformin. ACh was equally potent in inducing relaxation in WT + metformin and

TABLE 1

Phenylephrine (Phe) and potassium chloride (KCl) contraction, and acetylcholine (ACh) and sodium nitroprusside (SNP) relaxation in aortic rings of wild-type (WT) and caveolin-1 (cav-1)^{-/-} mice with and without metformin treatment

Data represent means ± S.E.M. of cumulative measurements in aortic segments of 9 to 17 mice.

	WT	+ Metformin	Cav-1 ^{-/-}	+ Metformin
Phe max (10 ⁻⁵ M)				
Contraction (g)	0.94 ± 0.15	0.65 ± 0.07	0.40 ± 0.10*	0.42 ± 0.07*
+ L-NAME (3 × 10 ⁻⁴ M)	1.21 ± 0.14	0.64 ± 0.11 [#]	0.69 ± 0.10*	0.64 ± 0.10
+ ODQ (10 ⁻⁵ M)	1.10 ± 0.16	1.31 ± 0.17 [†]	0.89 ± 0.13 [†]	0.76 ± 0.13 [†]
- Endo	1.05 ± 0.19	0.58 ± 0.08 [#]	0.72 ± 0.11 [†]	0.54 ± 0.07
Phe pED ₅₀ (-log M)	7.11 ± 0.18	7.00 ± 0.10	6.66 ± 0.13	7.47 ± 0.10 ^{##}
+ L-NAME	7.36 ± 0.17	7.36 ± 0.14	6.91 ± 0.18	7.03 ± 0.14
+ ODQ	7.43 ± 0.14	7.53 ± 0.10 [†]	7.05 ± 0.10 ^{*†}	7.10 ± 0.09*
- Endo	7.36 ± 0.16	7.36 ± 0.13 [†]	6.94 ± 0.13	7.07 ± 0.11
KCl contraction (g)	0.70 ± 0.04	0.51 ± 0.05 [#]	0.47 ± 0.05*	0.42 ± 0.04
- Endo	0.65 ± 0.06	0.42 ± 0.06 [#]	0.37 ± 0.04*	0.45 ± 0.06
Phe max contraction (% 96 mM KCl)	141.18 ± 20.46	133.83 ± 7.39	108.43 ± 23.30	111.46 ± 17.93
+ L-NAME	186.21 ± 22.88	176.26 ± 22.35	206.67 ± 38.31 [†]	156.58 ± 21.08
+ ODQ	166.76 ± 20.95	202.41 ± 8.24 [†]	188.93 ± 19.56 [†]	187.41 ± 27.87 [†]
- Endo	161.98 ± 19.75	145.55 ± 15.54	223.32 ± 42.08 [†]	131.62 ± 15.59 [#]
ACh (10 ⁻⁵ M) % relaxation	23.51 ± 5.33	24.36 ± 5.63	85.20 ± 8.15*	64.36 ± 5.74 ^{##}
pED ₅₀ (-log M)	6.32 ± 0.14	6.23 ± 0.13	7.45 ± 0.27*	6.70 ± 0.11 ^{##}
SNP (10 ⁻⁸ M) % relaxation	57.66 ± 4.60	53.03 ± 7.59	73.75 ± 4.93*	50.08 ± 5.11 [#]
pED ₅₀ (-log M)	8.12 ± 0.09	8.08 ± 0.13	8.52 ± 0.09*	7.98 ± 0.15 [#]

*Measurements in cav-1^{-/-} are significantly different ($P < 0.05$) from corresponding measurements in WT.

[#]Measurements in metformin-treated mice are significantly different ($P < 0.05$) from corresponding measurements in mice without metformin treatment.

[†]Measurements in L-NAME or ODQ-treated or endothelium-denuded arteries are significantly different from corresponding measurements in control nontreated arteries.

WT, but it was less potent in cav-1^{-/-} + metformin than in cav-1^{-/-} (Table 1).

Consistent with the vasorelaxation results, Western blots revealed an increase in aortic eNOS in cav-1^{-/-} versus WT (Fig. 5A). Metformin increased total eNOS levels in WT but reduced total eNOS in cav-1^{-/-}. Activated and phosphorylated peNOS levels were not different in cav-1^{-/-} and WT (Fig. 5C). Metformin treatment did not affect aortic peNOS level in WT, but it significantly decreased it in cav-1^{-/-} (Fig. 5C). We also measured AMPK as a potential modulator of eNOS activity (Morrow et al., 2003; Osuka et al., 2009; Tanano et al., 2013) and a known target for metformin (Hauton, 2011). Western blots revealed that the amounts of total aortic AMPK (Fig. 5B) and activated pAMPK (Fig. 5D) were significantly greater in cav-1^{-/-} than in WT. Total AMPK and pAMPK were significantly increased with metformin treatment in WT but were significantly reduced in metformin-treated cav-1^{-/-} (Fig. 5, B and D). These observations could be explained by possible interaction between the cav-1 genotype and metformin treatment in the regulation of eNOS and AMPK protein levels or activity. Importantly, ratiometric analysis of the data suggested that peNOS/eNOS and pAMPK/AMPK ratios were not different in cav-1 and WT (Fig. 5, E and F). In addition, metformin treatment did not change the ratio of phosphorylated and total eNOS or AMPK in either genotype (Fig. 5, E and F), suggesting that the observed effects of cav-1 deficiency and chronic metformin treatment involve long-term changes in protein levels of eNOS and AMPK rather than effects on phosphorylation and activation.

In all groups, ACh relaxation was abolished by endothelium removal, the NOS blocker L-NAME, or guanylate cyclase inhibitor ODQ (Fig. 6), supporting a role of endothelium-dependent NO-cGMP relaxation pathway.

The exogenous NO donor SNP caused concentration-dependent relaxation of aortic segments of all groups. SNP

produced similar maximal relaxation, but it was more potent in cav-1^{-/-} than in WT (Table 1). Metformin reduced SNP-induced relaxation and reversed the enhanced SNP potency in cav-1^{-/-} but had no significant effect in WT (Fig. 7, A and B; Table 1). Aortic sGC protein amount was greater in cav-1^{-/-} than in WT, and metformin increased sGC in WT but did not significantly affect sGC levels in cav-1^{-/-} (Fig. 7C).

Discussion

The following are the main findings of this study: 1) cav-1^{-/-} mice show altered vascular function and increased BP and glucose levels; 2) metformin does not affect blood glucose level or BP but affects vascular function; 3) vascular contraction to Phe and KCl is less in cav-1^{-/-} than in WT, endothelium-denudation or inhibition of NO-cGMP enhances Phe contraction, particularly in cav-1^{-/-}, and this enhancement is blunted with metformin; 4) endothelium-dependent ACh relaxation and eNOS and AMPK levels are enhanced in cav-1^{-/-} versus WT, and metformin reduces ACh relaxation and both total and phosphorylated eNOS and AMPK in cav-1^{-/-}; and 5) endothelium-independent SNP relaxation and sGC levels are enhanced in cav-1^{-/-} versus WT, and metformin reverses the enhanced SNP relaxation.

Cav-1 is an anchoring protein and a regulator of signal transduction in VSM and endothelial cells (Feron et al., 1998; Ishizaka et al., 1998; Minshall et al., 2003; Shakhmurova et al., 2006; Ushio-Fukai and Alexander, 2006). We and others used cav-1^{-/-} mice to examine the role of cav-1 in mechanotransduction, vascular remodeling, and cardiovascular function (Drab et al., 2001; Yu et al., 2006; Pojoga et al., 2010). Cav-1^{-/-} mice show hyperproliferative and vascular abnormalities (Razani et al., 2001). Consistent with our previous reports (Pojoga et al., 2010), the present study showed that Phe contraction was reduced and ACh-induced relaxation was

Fig. 3. Effect of endothelium removal or blockade of NO-cGMP pathway on Phe-induced contraction (A), sensitivity to Phe (B), and Phe-induced contractile response relative to control KCl contraction (C) in aorta of WT and *cav-1*^{-/-} mice with or without metformin treatment. Aortic rings from WT, WT + metformin, *cav-1*^{-/-}, and *cav-1*^{-/-} + metformin mice were either kept endothelium-intact (open circles), pretreated with the NOS inhibitor L-NAME (3×10^{-4} M) (closed circles) or the guanylate cyclase inhibitor ODQ (10^{-5} M) (open triangles) for 10 minutes, or endothelium-denuded (closed triangles). After measuring contraction to KCl (96 mM), the vessels were stimulated with increasing concentrations of Phe, and the contractile response was presented in grams (A), as percentage of maximum Phe contraction (B) or as percentage of KCl contraction (C). Data represent mean $\pm$ S.E.M. ($n = 9-17$ mice). *Measurements in L-NAME-treated arteries are significantly different ($P < 0.05$) from corresponding measurements in control nontreated arteries. #Measurements in ODQ-treated arteries are significantly different ($P < 0.05$) from corresponding measurements in control nontreated arteries. †Measurements in endothelium-denuded arteries are significantly different ($P < 0.05$) from corresponding measurements in intact arteries.

enhanced in *cav-1*^{-/-} versus WT. The reduced Phe contraction in *cav-1*^{-/-} is not due to changes in α -adrenergic receptor sensitivity as Phe ED₅₀ was not significantly different between *cav-1*^{-/-} and WT. To further examine the causes of reduced contraction in *cav-1*^{-/-} mice, we measured contraction to high KCl, which is due mainly to Ca²⁺ influx (Murphy and Khalil, 2000). Similar to Phe, KCl-induced contraction was reduced in *cav-1*^{-/-} versus WT, supporting that the reduced contraction is not specific to a particular agonist/receptor coupling mechanism in VSM. In addition, endothelium removal enhanced Phe contraction, particularly in *cav-1*^{-/-}, suggesting a role of endothelium-derived vasodilators in the reduced contraction in *cav-1*^{-/-} mice.

NO is a major endothelium-derived vasodilator. Under basal conditions, eNOS is bound to cav-1 in endothelial cell caveolae. An increase in endothelial cell Ca²⁺ by agonists such as ACh induces the release of eNOS from cav-1 to the cytosol (Feron et al., 1998; Minshall et al., 2003), where it is phosphorylated and fully activated by protein kinases that reside in caveolae, such as p38 mitogen-activated protein kinase (Anter et al., 2004) phosphatidylinositol 3-kinase-Akt

(Anter et al., 2004; Uruno et al., 2005), cAMP-dependent protein kinase A, and AMPK (Tanano et al., 2013). Activated eNOS converts L-arginine to L-citrulline and increases NO release. NO diffuses into VSM, where it activates sGC (Venema et al., 2003) and increases cGMP production. The increased cGMP activates cGMP-dependent protein kinase, which promotes vascular relaxation by decreasing [Ca²⁺]_i and inhibiting myosin light chain kinase or Ca²⁺ sensitization pathways of VSM contraction, such as protein kinase C (PKC) and Rho-kinase (Salamanca and Khalil, 2005). The enhanced vascular relaxation in *cav-1*^{-/-} mice is likely due to increased eNOS activity and NO-cGMP because 1) Phe contraction was enhanced by the NOS inhibitor L-NAME or guanylate cyclase inhibitor ODQ; 2) ACh relaxation was blocked by endothelium removal, L-NAME, or ODQ; 3) NO donor and guanylate cyclase activator SNP was more potent in *cav-1*^{-/-} than in WT; and 4) vascular levels of eNOS, AMPK, and sGC were greater in *cav-1*^{-/-} than in WT. These observations suggest that *cav-1* may limit the expression or activity of vascular eNOS, AMPK, and sGC and that the absence of *cav-1* is associated with constitutively larger

Fig. 4. ACh-induced relaxation in aortic rings of WT and *cav-1*^{-/-} mice with and without metformin treatment. Aortic rings were precontracted with Phe (10^{-5} M), increasing concentrations of ACh were added, and the percent of relaxation of Phe contraction was measured. Data represent means $\pm$ S.E.M. ($n = 9$ –17 mice). *Measurements in *cav-1*^{-/-} are significantly different ($P < 0.05$) from corresponding measurements in WT mice. #Measurements in metformin-treated mice are significantly different ($P < 0.05$) from corresponding measurements in control mice without metformin treatment.

amounts of eNOS, AMPK, and sGC and upregulation of NO-cGMP.

Given the important role of vascular mechanisms in BP regulation, the reduced vascular contraction and enhanced NO-cGMP relaxation pathway in *cav-1*^{-/-} are predicted to decrease BP. This is not the case, however, as BP was increased in *cav-1*^{-/-} versus WT, consistent with our previous reports

(Pojoga et al., 2010). The increase in BP despite increased eNOS activity in *cav-1*^{-/-} mice has been partly explained by partial eNOS uncoupling, whereby increased NO production would lead to increased formation of peroxynitrites and decreased NO bioavailability (Pojoga et al., 2008). The increased BP in *cav-1*^{-/-} is less likely due to increased renin-angiotensin-aldosterone system because PRA levels were not different in *cav-1*^{-/-} and WT. On the other hand, hyperglycemia has been associated with hypertension and vascular dysfunction in both humans and animal models (Schulman and Zhou, 2009; Bender et al., 2013). Importantly, *cav-1* is expressed in adipose cells and is an important modulator of glucose homeostasis (Cohen et al., 2003; Catalan et al., 2008). Moreover, *cav-1* gene variants are associated with reduced insulin sensitivity in hypertensive, but not normotensive individuals (Pojoga et al., 2011), suggesting an interplay between *cav-1*, BP, and glucose homeostasis. Consistent with previous reports, the total body weight was different in *cav-1*^{-/-} and WT, possibly because of altered peripheral glucose uptake (Pojoga et al., 2011). In addition, blood glucose was greater in *cav-1*^{-/-} than in WT, supporting a link between *cav-1* deficiency and hyperglycemia. These findings are consistent with previous reports (Cohen et al., 2003; Pojoga et al., 2011; Asterholm et al., 2012) and made it important to investigate possible relationship between hyperglycemia and BP in *cav-1*^{-/-} mice.

Previous studies showed that metformin treatment (100 mg/kg) for 4 weeks decreased BP in spontaneously hypertensive rats (Bhalla et al., 1996). Although we used metformin at greater doses (400 mg/kg daily) that were reported to improve glycemic control in other models (Heishi et al., 2006; Matsumoto et al., 2008), it did not normalize glucose levels

Fig. 5. Western blot analysis of total eNOS (A), total AMPK (B), peNOS (C), pAMPK (D), peNOS/total eNOS (E), and pAMPK/total AMPK (F) in the aortas of WT and *cav-1*^{-/-} mice with and without metformin treatment. Data represent means $\pm$ S.E.M. ($n = 6$ –8 mice). *Measurements in *cav-1*^{-/-} are significantly different ($P < 0.05$) from corresponding measurements in WT. #Measurements in metformin-treated mice are significantly different ($P < 0.05$) from corresponding measurements in control mice without metformin treatment.

Fig. 6. Effect of endothelium removal or blockade of the NO-cGMP pathway on ACh-induced relaxation in aortic segments of WT and *cav-1*^{-/-} mice with and without metformin treatment. Aortic rings from WT (A), WT + metformin (B), *cav-1*^{-/-} (C), and *cav-1*^{-/-} mice treated with metformin (D) were either kept endothelium-intact (open circles), pretreated with L-NAME (3×10^{-4} M) (closed circles) or ODQ (10^{-5} M) (open triangles), or endothelium-denuded (closed triangles). The tissues were precontracted with Phe (10^{-5} M), increasing concentrations of ACh were added, and the percent of relaxation of Phe contraction was measured. Data represent means $\pm$ S.E.M. ($n = 9$ –17 mice). *Measurements in L-NAME or ODQ-treated or endothelium-denuded arteries are significantly different from corresponding measurements in control nontreated arteries.

in *cav-1*^{-/-} mice. This is consistent with reports that *cav-1* is needed to mediate the acute effects of metformin on AMPK phosphorylation (Salani et al., 2012), an essential step in gluconeogenesis (Viollet et al., 2012). Also, metformin treatment did not change body weight or BP in *cav-1*^{-/-} or WT. These results are consistent with the clinical observations that certain patients with diabetes show inadequate glycemic control with metformin monotherapy (Bailey et al., 2013), although metformin could still affect cardiovascular outcomes (Agarwal et al., 2010). These data also suggest a more complex interaction between hyperglycemia and the altered vascular function and BP and prompted us to examine methodically examine the vascular mechanisms of metformin.

Our data suggest that metformin inhibits the mechanisms of vascular contraction. Metformin reduced KCl contraction in both intact and endothelium-denuded vessels of WT mice (Table 1). Assuming that KCl contraction is due mainly to Ca^{2+} influx (Murphy and Khalil, 2000), the reduced KCl contraction in metformin-treated WT mice is likely due to decreased Ca^{2+} entry. This assumption is consistent with reports that metformin causes repolarization and decreases Ca^{2+} influx, $[\text{Ca}^{2+}]_i$ and isometric force in rat tail artery (Chen et al., 1997).

In comparison with KCl, α -adrenergic receptor agonists such as Phe stimulate Ca^{2+} entry through voltage-gated as well as ligand-gated and store-operated Ca^{2+} channels (Lee et al., 2001; Park et al., 2008). Phe also activates Ca^{2+} -sensitization pathways such as PKC and Rho-kinase (Salamanca and Khalil, 2005; Dimopoulos et al., 2007). We found that in WT mice metformin caused insignificant reduction of Phe contraction in intact aortas and significantly reduced Phe contraction in endothelium-denuded vessels (Table 1), likely as a result of metformin-induced decrease in Ca^{2+} entry in VSM. Interestingly, relative to KCl contraction, Phe contraction was slightly greater in both intact (1.4-fold) and endothelium-denuded (1.6-fold) vessels of WT mice. Whereas Phe contraction was similar to that of KCl in endothelium-intact vessels of *cav-1*^{-/-} mice (1.08-fold), endothelium removal almost doubled Phe contraction (2.23-fold) (Table 1). Again, assuming that KCl contraction is due to Ca^{2+} influx, the enhanced Phe contraction in endothelium-denuded vessels of *cav-1*^{-/-} mice is likely due to activation of other Ca^{2+} channels or Ca^{2+} sensitization by PKC or Rho-kinase (Salamanca and Khalil, 2005). Whether these increases in Ca^{2+} sensitivity of VSM contraction in *cav-1*^{-/-} mice are related to hyperglycemia is unclear; however, studies

Fig. 7. SNP-induced vascular relaxation and sGC protein levels in WT and *cav-1*^{-/-} mice. Aortic rings from WT (A) and *cav-1*^{-/-} mice (B) with (closed symbols) and without metformin treatment (open symbols) were precontracted with Phe (10⁻⁵ M), increasing concentrations of SNP were added, and the percent of relaxation of Phe contraction was measured. Data represent means ± S.E.M. (*n* = 9–17 mice). In other experiments, aortic tissue homogenates were prepared for Western blot analysis, and sGC protein levels were assessed and normalized to β-actin levels (C). *Measurements in *cav-1*^{-/-} are significantly different (*P* < 0.05) from corresponding measurements in WT mice. #Measurements in metformin-treated mice are significantly different (*P* < 0.05) from corresponding measurements in control mice without metformin treatment.

have shown distinct dysfunctions of mesenteric artery contraction in diabetic mice, possibly as a result of changes in Rho-kinase signaling (Nobe et al., 2012). Also, Rho-kinase and PKC promote Ca²⁺ sensitization in arteries from diabetic rats (Kizub et al., 2010). The effects of Ca²⁺ sensitization were not observed in endothelium-intact vessels of *cav-1*^{-/-} mice, likely because they were obscured by increased NO-cGMP, and only with endothelium removal or blockade of NO-cGMP these enhanced contraction mechanisms could be manifested. This finding is supported by reports that NO and cGMP reduce

[Ca²⁺]_i (Cornwell and Lincoln, 1989), and cGMP-dependent protein kinase causes phosphorylation and inhibition of myosin light-chain kinase and Ca²⁺ sensitization pathways such as PKC and Rho-kinase (Salamanca and Khalil, 2005). The observation that metformin caused significant reduction in Phe contraction relative to KCl contraction in endothelium-denuded vessels of *cav-1*^{-/-} mice (Table 1) is consistent with the possibility that metformin not only reduces Ca²⁺ entry (Sharma and Bhalla, 1995; Bhalla et al., 1996) but could also inhibit Ca²⁺ sensitization pathways such as PKC (Gallo et al., 2005; Mahrouf et al., 2006) and Rho-kinase (Agard et al., 2009).

Whereas our data suggest an effect of metformin on vascular contraction mechanisms, some observations point to additional effects of metformin on vascular relaxation pathways. Metformin increased the sensitivity to Phe in intact vessels of *cav-1*^{-/-} mice. Also, endothelium denudation and inhibition of NO-cGMP enhanced Phe contraction in *cav-1*^{-/-} mice, and this enhancement was blunted with metformin. These findings can be explained by possible interaction of metformin with NO-cGMP, and these interactions may be augmented during increased NO-cGMP activity in *cav-1*^{-/-} mice. This is supported by the observations that metformin reduced ACh and SNP induced relaxation and decreased total and phosphorylated eNOS and AMPK levels in *cav-1*^{-/-} mice. Another possible interaction of metformin with the NO pathway is that during *cav-1* deficiency the increased eNOS activity could lead to partial eNOS uncoupling, thus producing excess reactive oxygen species and peroxynitrites, and resulting in decreased NO bioavailability. Under these conditions, metformin could act by reducing reactive oxygen species, thus increasing NO bioavailability and reducing vascular contraction.

Other observations or limitations include the following. 1) The glucose data should be interpreted with caution as overnight fasting may represent a potential limitation given the higher metabolic demands of the mouse. However, the terminology, time course, and metabolic effects of fasting versus starvation in mice have not been clearly defined in the literature. For instance, some studies have used food deprivation in mice for periods ranging from 24 to 40 hours (Li et al., 2008; Fernandez-Rojo et al., 2013), which are longer than the overnight time period used in the present study, and still referred to this intervention as fasting. Nevertheless, all the present experiments were performed under the same conditions and using the same overnight fasting protocol for all the animal groups before performing the glucose tolerance test as previously described by other studies (Heikkinen et al., 2007; Muniyappa et al., 2008). 2) In contrast with decreasing AMPK activity in *cav-1*^{-/-}, metformin appeared to increase AMPK in WT mice, and AMPK activation could affect the vascular relaxation and contraction mechanisms. Our observation that metformin increased aortic total and pAMPK in WT mice is consistent with reports that AMPK and pAMPK levels are increased in the heart of rats treated with metformin long term (Hauton, 2011) and that AMPK causes phosphorylation of eNOS (Chen et al., 1999; Tanano et al., 2013). However, the ability of metformin to enhance eNOS and AMPK appears to be compromised in *cav-1*^{-/-} mice, supporting that *cav-1* may be necessary for the actions of metformin on eNOS and AMPK. 3) In WT mice, metformin appeared to increase the vascular dependence on cGMP, as shown by the enhanced Phe contraction in vessels treated with the guanylate cyclase inhibitor ODQ and the increased aortic sGC levels with metformin. 4) Metformin

appeared to dampen the enhanced total and phosphorylated eNOS and AMPK, but it did not alter the increased vascular sGC levels or reverse the enhanced Phe contraction by ODQ in cav-1^{-/-} mice. Interestingly, sGC activation and cGMP production can be stimulated not only by NO but also by NO-independent mechanisms (Evgenov et al., 2006). One possibility is that in the absence of cav-1, metformin switches sGC and cGMP production from an eNOS/AMPK-dependent to an NO-independent mechanism. The observed decrease in sensitivity to SNP in cav-1^{-/-} mice is consistent with a switch to a sGC pathway less sensitive to NO. 5) Whether the observed changes in conduit vessels also occur in resistance vessels that control BP need to be examined in future studies.

In conclusion, cav-1 deficiency is associated with increased BP, hyperglycemia, enhanced NO-cGMP-dependent relaxation, and reduced vascular contraction. Metformin does not change BP in cav-1^{-/-} mice, likely because of its dual inhibitory effects on both vascular contraction mechanisms and vascular relaxation via NO-cGMP. Metformin does not affect blood glucose levels or body weight in cav-1^{-/-} mice, consistent with its reported inadequate effects on glycemic control in certain patients with diabetes (Bailey et al., 2013). The data support the notion of partial independence of the vascular changes from hyperglycemia in cav-1 deficiency states. The data also suggest that hypertensive individuals with reduced insulin sensitivity, such as minor allele carriers of cav-1 gene variants (Pojoga et al., 2011), may benefit from the vascular effects of metformin, even in the absence of improvements in BP or glucose homeostasis.

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Authorship Contributions

Participated in research design: Pojoga, Williams, Khalil.

Conducted experiments: Pojoga, Yao, Reslan, Khalil.

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