

RESEARCH

Metformin improves vascular and metabolic insulin action in insulin-resistant muscle

Eloise A Bradley^{1,*}, Dino Premilovac^{1,2,*}, Andrew C Betik³, Donghua Hu¹, Emily Attrill², Stephen M Richards^{1,2}, Stephen Rattigan¹ and Michelle A Keske^{1,3}

¹Menzies Institute for Medical Research, University of Tasmania, Hobart, Tasmania, Australia

²School of Medicine, University of Tasmania, Hobart, Tasmania, Australia

³Institute for Physical Activity and Nutrition (IPAN), School of Exercise and Nutrition Sciences, Deakin University, Geelong, Victoria, Australia

Correspondence should be addressed to M A Keske: Michelle.Keske@deakin.edu.au

*(E A Bradley and D Premilovac contributed equally to this work)

Abstract

Insulin stimulates glucose disposal in skeletal muscle in part by increasing microvascular blood flow, and this effect is blunted during insulin resistance. We aimed to determine whether metformin treatment improves insulin-mediated glucose disposal and vascular insulin responsiveness in skeletal muscle of insulin-resistant rats. Sprague–Dawley rats were fed a normal (ND) or high-fat (HFD) diet for 4 weeks. A separate HFD group was given metformin in drinking water (HFD + MF, 150 mg/kg/day) during the final 2 weeks. After the intervention, overnight-fasted (food and metformin removed) anaesthetised rats underwent a 2-h euglycaemic–hyperinsulinaemic clamp (10 mU/min/kg) or saline infusion. Femoral artery blood flow, hindleg muscle microvascular blood flow, muscle glucose disposal and muscle signalling (Ser⁴⁷³-AKT and Thr¹⁷²-AMPK phosphorylation) were measured. HFD rats had elevated body weight, epididymal fat pad weight, fasting plasma insulin and free fatty acid levels when compared to ND. HFD-fed animals displayed whole-body and skeletal muscle insulin resistance and blunting of insulin-stimulated femoral artery blood flow, muscle microvascular blood flow and skeletal muscle insulin-stimulated Ser⁴⁷³-AKT phosphorylation. Metformin treatment of HFD rats reduced fasting insulin and free fatty acid concentrations and lowered body weight and adiposity. During euglycaemic–hyperinsulinaemic clamp, metformin-treated animals showed improved vascular responsiveness to insulin, improved insulin-stimulated muscle Ser⁴⁷³-AKT phosphorylation but only partially restored (60%) muscle glucose uptake. This occurred without any detectable levels of metformin in plasma or change in muscle Thr¹⁷²-AMPK phosphorylation. We conclude that 2-week metformin treatment is effective at improving vascular and metabolic insulin responsiveness in muscle of HFD-induced insulin-resistant rats.

Key Words

- ▶ insulin resistance
- ▶ glucose metabolism
- ▶ metformin
- ▶ nutrition
- ▶ muscle

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Introduction

Metformin is the most common first-line oral glucose-lowering pharmacological therapy for people with type 2 diabetes (Bailey & Turner 1996, Gunton & Twigg 2003,

Inzucchi *et al.* 2015a,b). It is primarily known for its role in suppressing hepatic glucose production (Bailey & Turner 1996, Hundal *et al.* 2000). More recently, metformin has

been demonstrated to promote the secretion of incretins such as glucagon-like peptide-1 (DeFronzo *et al.* 2016, Preiss *et al.* 2017) and increase glucose disposal to skeletal muscle (Magalhaes *et al.* 2006, Kristensen *et al.* 2014). Thus, the actions of metformin to lower blood glucose are multifactorial and despite its clinical use for the past 60 years, the mechanisms involved are still not fully understood (Marshall 2017, Rena *et al.* 2017).

Glucose and insulin delivery via the vasculature is important in regulating skeletal muscle glucose uptake (Barrett *et al.* 2009, Kubota *et al.* 2013, Keske *et al.* 2016). In this regard, one of the mechanisms by which insulin increases muscle glucose disposal is through greater blood flow through capillaries in skeletal muscle to facilitate delivery of glucose and insulin to myocytes (Barrett *et al.* 2009, Kubota *et al.* 2013, Keske *et al.* 2016). Insulin's ability to increase microvascular blood flow in muscle is responsible for ~40% of insulin-stimulated glucose disposal by skeletal muscle using physiological relevant doses of insulin and exposure times (similar to postprandial levels) during a euglycaemic-hyperinsulinaemic clamp in both animals and humans (Vincent *et al.* 2004, Zhang *et al.* 2004, Eggleston *et al.* 2013). The ingestion of a mixed meal (which stimulates endogenous insulin production) increases microvascular blood flow to a similar extent as exogenously infused insulin (Vincent *et al.* 2006, Keske *et al.* 2009, Liu *et al.* 2009, Russell *et al.* 2018). Acute inhibition of this important physiological action of insulin with agents such as tumour necrosis factor alpha and vasoconstrictors is accompanied by a corresponding reduction in insulin-stimulated muscle glucose uptake (Rattigan *et al.* 1999, Youd *et al.* 2000, Clark *et al.* 2003, Vincent *et al.* 2003, Zhang *et al.* 2003, Bradley *et al.* 2013, Premilovac *et al.* 2018). The reliance of muscle glucose uptake and/or whole-body glucose homeostasis on appropriate microvascular blood flow responses also holds true in animal models of insulin resistance (St-Pierre *et al.* 2010, Kubota *et al.* 2011, Sjoberg *et al.* 2015), human obesity (Clerk *et al.* 2006, Keske *et al.* 2009, Ketel *et al.* 2011, Meijer *et al.* 2015) and type 2 diabetes (Wallis *et al.* 2002, Russell *et al.* 2017). Moreover, recent data strongly suggest that loss of microvascular insulin responsiveness is an early event in the pathogenesis of insulin resistance (Kubota *et al.* 2011, Premilovac *et al.* 2013, 2014, Zhao *et al.* 2015). Consequently, improving muscle microvascular flow also improves insulin-mediated skeletal muscle glucose uptake in muscle (Rattigan *et al.* 2001, Fu *et al.* 2013, Premilovac *et al.* 2014, Bradley *et al.* 2015, Sjoberg *et al.* 2017). While insulin-stimulated microvascular blood flow is markedly impaired in insulin-resistant states

(Wallis *et al.* 2002, St-Pierre *et al.* 2010, Russell *et al.* 2017), these deficits do not impede the ability of muscle contraction to stimulate microvascular blood flow and glucose uptake in muscle (Wheatley *et al.* 2004, St-Pierre *et al.* 2012, Russell *et al.* 2017). Therefore, microvascular blood flow in insulin-resistant skeletal muscle has the capacity to be increased, but not in response to insulin. This dichotomy suggests that the mechanisms of contraction and insulin-stimulated increases in microvascular blood flow are distinct from each other and indicate that targeting improved microvascular insulin action in skeletal muscle may be a novel mechanism for restoring skeletal muscle glucose uptake. Whether metformin impacts on muscle microvascular blood flow to decrease glucose concentrations in pre-diabetes and type 2 diabetes is not known and warrants investigation given its widespread clinical use.

Previous work has shown that metformin improves brachial artery endothelial function (flow-mediated dilation) in people with type 1 diabetes (Pitocco *et al.* 2013) and polycystic ovarian syndrome (Jensterle *et al.* 2008). Metformin has also been reported to augment forearm blood flow and muscle glucose disposal in people with type 2 diabetes in response to a 75 g oral glucose load (Magalhaes *et al.* 2006), although it is not clear whether this restores glucose disposal to values found in healthy muscle. However, it is now clear that although insulin increases total muscle blood flow, the extent of insulin-mediated glucose uptake is more closely related to increased microvascular blood flow than total blood flow (Rattigan *et al.* 1997, Vincent *et al.* 2004, Zhang *et al.* 2004). To our knowledge, there are no reports of metformin's effects on the microvasculature in skeletal muscle. Given the dependence of insulin-stimulated glucose uptake on the delivery of glucose and insulin to the myocyte (Laakso *et al.* 1990, Clark *et al.* 2003, Keske *et al.* 2016, 2017), the aim of the current study was to determine whether metformin improves skeletal muscle insulin responsiveness via augmentation of metabolic and microvascular insulin action in insulin-resistant high-fat diet-fed rats.

Materials and methods

Animal care

All procedures were approved by the University of Tasmania Animal Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of

Animals for Scientific Purposes – 2013, 8th Edition. Male Sprague–Dawley rats (4–5 weeks of age) were randomly assigned into two groups and provided either a normal diet (ND) (4.8% fat wt/wt.; Specialty Feeds, Glen Forest, VIC, Australia) or high-fat diet (HFD) (22% fat wt/wt.; Specialty Feeds) *ad libitum* for 4 weeks. A subset of the HFD group was treated with metformin hydrochloride (HFD+MF) (LKT Laboratories Inc., St. Paul, MN, USA) during the final 2 weeks of the HFD intervention. Metformin was dissolved in drinking water, with the concentration adjusted according to rat water consumption and body weight three times per week, to achieve a dose of ~150mg/kg/day per rat. Animals were housed at $21 \pm 2^\circ\text{C}$ with a 12 hour light/darkness cycle.

Surgical procedure

Rats fasted overnight (no food or metformin) and were anaesthetised with an intraperitoneal injection of pentobarbital sodium (50mg/kg body wt.). Complete surgical details are described previously (Rattigan *et al.* 1997, St-Pierre *et al.* 2010, Bradley *et al.* 2015). An ultrasonic flow probe (VB series 0.5mm; Transonic Systems, Ithaca, NY, USA) was positioned around the femoral artery. Mean arterial blood pressure (MAP), femoral artery blood flow (FBF) and heart rate (HR) were recorded continuously using WINDAQ data acquisition software (DATAQ Instruments, Akron, OH, USA). The rats were maintained under anaesthesia by continuous infusion of pentobarbital sodium (0.6mg/min/kg) via the jugular vein and the body temperature was maintained at 37°C using a heated pad.

Experimental protocol

The experimental protocol is shown in Fig. 1. Following postsurgical stabilisation (~60min) a 2-h infusion of either saline or insulin (10mU/min/kg without a primed infusion; Humulin R, Eli Lilly) was administered via the jugular vein. Blood glucose concentration was monitored every 10–15min using a glucose analyser (YSI Model 2300, Yellow Springs Instruments, OH, USA). In the experiments where rats received insulin, 30% (wt./vol.) glucose solution was co-infused at a variable rate to maintain basal blood glucose.

Plasma biochemistries

Fasting blood and plasma glucose concentrations were assessed using a glucose analyser (YSI, Yellow Springs

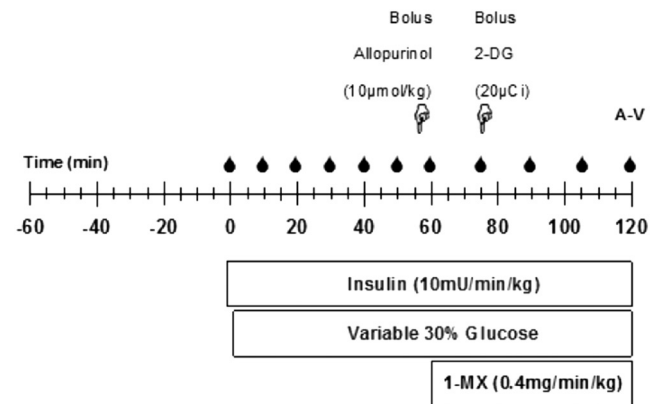


Figure 1

Experimental protocol. Following surgical preparation and ~60 min of equilibration, a constant infusion of saline or insulin (10 mU/min/kg) was commenced at 0 min and continued for 120 min. Glucose (30% wt./vol.) infusion, initiated shortly after the commencement of insulin infusion, was administered at a rate required to maintain euglycaemia as determined from arterial blood glucose assessment (black teardrops). At 55 min, a bolus injection of allopurinol (10 $\mu\text{mol/kg}$) was made followed by infusions of 1-methylxanthine (1-MX, 0.4 mg/min/kg) for 60 min. Constant infusions are indicated by the bars. At 75 min a bolus of 2-DG (20 μCi) was administered and radioactive plasma samples were collected at 80, 85, 90, 105 and 120 min to determine the plasma 2-DG clearance. Arterial and femoral vein samples (A-V) were taken 120 min to determine 1-MX metabolism. Calf muscles were freeze clamped and stored at -80°C at the end of the experiment for subsequent determination of muscle glucose uptake (R'g) and AKT and AMPK phosphorylation.

Instruments). Fasting and end point plasma insulin concentrations were determined by ELISA (Mercodia, Uppsala, Sweden). Fasting plasma free fatty acid (FFA) concentrations were determined using an enzymatic assay kit (Wako Pure Chemical Industries, Osaka, Japan).

Homeostatic Model Assessment of Insulin Resistance (HOMA-IR, index of insulin resistance) was calculated using the following formula:

$$\text{HOMA-IR} = \frac{\text{fasting plasma glucose (mmol/L)} \times \text{fasting plasma insulin } (\mu\text{U/mL})}{22.5}$$

Muscle microvascular blood flow

Microvascular blood flow was determined by measuring metabolism of infused 1-methylxanthine (1-MX, Sigma-Aldrich), a substrate targeted for xanthine oxidase, that is located predominantly in capillary endothelium in rat leg muscle (Jarasch *et al.* 1986). This is a technique we have used extensively in the past to measure microvascular blood flow (Rattigan *et al.* 1997, 1999, Youd *et al.* 2000, Clerk *et al.* 2002, Vincent *et al.* 2003, Zhang *et al.* 2003, 2004, Wheatley *et al.* 2004, Wallis *et al.* 2005, St-Pierre *et al.* 2010, 2012, Premilovac *et al.* 2013, 2014). A bolus of

allopurinol (10 $\mu\text{mol/kg}$, Sigma-Aldrich) was administered 5 min prior to 1-MX infusion. Allopurinol is required to partially inhibit the activity of xanthine oxidase (reduce the K_m and V_{max} of this enzyme), which enables a steady-state arterial level of 1-MX ($\sim 20 \mu\text{mol/L}$). At the end of the experiment, arterial and hindleg venous plasma (100 μL) were collected, added to 2M perchloric acid (20 μL) to precipitate proteins and centrifuged for 10 min. The supernatant was used to determine 1-MX concentrations by reverse phase high-performance liquid chromatography (HPLC) as previously described (Rattigan *et al.* 1997). Microvascular blood flow expressed as 1-MX metabolism was calculated from arterio-venous plasma 1-MX difference multiplied by FBF at the end of the experiment and expressed as nmol/min .

Skeletal muscle glucose uptake

At 45 min before the completion of the experiment, a 20 μCi bolus dose of [$1\text{-}^{14}\text{C}$]2-deoxy-D-glucose (2-DG) (specific activity 1.85-2.29 GBq/mmol; American Radiolabeled Chemicals, Inc., Saint Louis, MO, USA) was administered via the venous cannula. The clearance of 2-DG from the blood was determined from plasma samples (25 μL) collected at 5, 10, 15, 30 and 45 min after administration of the 2-DG bolus. At the conclusion of the experiment, the calf muscle group (soleus, plantaris, gastrocnemius) was freeze clamped and stored at -80°C . Frozen muscles were powdered under liquid nitrogen and homogenised. Free and phosphorylated 2-DG were separated using an anion exchange column (AG-1X8; Bio-Rad Laboratories). Biodegradable counting scintillant (Amersham) was added to each sample and radioactivity was measured using a scintillation counter (Tri-Carb 2800TR; Perkin Elmer). From this measurement and the specific activity of 2-DG in the plasma, muscle glucose uptake ($R'g$) was calculated as previously described by others (Kraegen *et al.* 1985).

Skeletal muscle AKT and AMPK signalling

The extent of muscle Ser 473 AKT and Thr 172 AMPK α phosphorylation in all groups was assessed using Western blot. Calf muscles were ground into a fine powder under liquid nitrogen and sonicated (3 \times 30s cycles at 360W; Bioruptor, Diagenode, Liege, Belgium) in ice-cold solubilising buffer (1:6wt/vol). The homogenate was centrifuged at 16,500g for 10 min at 4°C and supernatant protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories).

Aliquots containing 10 μg of protein were added to bolt LDS sample buffer (catalogue id: B0008; Thermo Fischer Scientific) and heat-treated at 70°C for 5 min. Following this, 20 μL of each sample was subjected to gel electrophoresis (4-12% Bis-Tris Protein Gel; catalogue id: NP0321BOX; Thermo Fischer Scientific) for 45 min at 180 volts (constant voltage). Proteins were transferred from gel to nitrocellulose membranes overnight at 30 volts (constant voltage) at 4°C . Following transfer, membranes were blocked with 70mM Tris-buffered saline (pH 7.6), 5% non-fat dry milk powder, 0.1% Tween 20. Primary antibodies were diluted 1:1000 in blocking buffer and membranes were probed overnight at 4°C for either Ser 473 -AKT (catalogue id: #9272; Cell Signalling Technology) or Thr 172 AMPK α (catalogue id: #2535; Cell Signalling Technology). The following day, membranes were probed with a horse radish peroxidase-linked secondary antibody (1:1000; catalogue id: #7074, Cell Signalling Technology) for 60min at room temperature. The membranes were then bathed in West Pico Chemiluminescent Substrate (Thermo Fischer Scientific) for 5 min and band intensity densities were quantified using ImageJ (National Institutes of Health). After imaging, membranes were exposed to stripping buffer (catalogue id: 46430; Thermo Fischer Scientific) for 15 min at room temperature to remove the phosphorylated antibodies. After stripping, the membranes were again blocked with 70mM Tris-buffered saline (pH 7.6), 5% non-fat dry milk powder, 0.1% Tween 20 for 60 min. After blocking, membranes were incubated overnight at 4°C with primary antibodies for total AKT (diluted 1:1000 in blocking buffer; catalogue id: #9271 Cell Signalling Technology) or total AMPK α (diluted 1:1000 in blocking buffer; catalogue id: #2531 Cell Signalling Technology). The following day, membranes were probed with a horse radish peroxidase-linked secondary antibody (1:1000; catalogue id: #7074, Cell Signalling Technology) for 60min at room temperature and then bathed in West Pico Chemiluminescent Substrate for 5 min. As described earlier, band intensity densities were quantified using ImageJ.

HPLC: analysis of metformin in plasma

Plasma samples at time=0min, prior to beginning infusion of insulin for the insulin clamps were used for HPLC. Following deproteinisation with 2M perchloric acid and centrifugation at 10,000g, 50 μL of un-neutralised supernatant was subjected to HPLC analysis for metformin. Details of the separation column and HPLC equipment were as used previously (Rattigan *et al.* 1997) except that the

mobile phase consisted of 75 mM ammonium dihydrogen orthophosphate (pH 5.5), 4 mM sodium heptanesulphonate and 13% v/v acetonitrile isocratic pumped at 1.5 ml/min. Metformin was identified by elution time (8.0 min) and quantified by absorbance at 233 nm.

Statistical analysis

All data are presented as means \pm s.e.m. Comparisons between the ND, HFD and HFD+MF for the physical and biochemical characteristics were made using a one-way ANOVA. Comparisons of time course measurements in each group were performed using a two-way repeated-measures ANOVA. Comparisons of end point measurements in each group were made using a two-way ANOVA. When a significant difference ($P < 0.05$) was found, pairwise comparisons by the Student–Newman–Keuls (SNK) test was used to assess treatment differences. Pearson bivariate correlations were used to evaluate associations. All tests were performed using SigmaStat™ (Systat Software, Inc., San Jose, CA, USA).

Results

Physical and biochemical characteristics

The physical characteristics and plasma biochemistries of ND, HFD and HFD+MF rats are shown in Table 1. At the conclusion of the 4-week dietary intervention, body weight, epididymal fat pad weight, fasting plasma FFA and fasting plasma insulin were significantly ($P < 0.05$) elevated in the HFD group compared with the ND group. The calculated HOMA-IR was higher in HFD compared with the ND group (11.0 ± 1.1 vs 5.3 ± 0.5 , $P < 0.001$). Body weight, fasting plasma FFA and fasting plasma insulin were significantly reduced by metformin treatment in the HFD+MF group. Thus, the HOMA-IR was lower in rats treated with MF compared with the HFD group

(8.4 ± 0.8 vs 11.0 ± 1.1 , $P < 0.05$). The fasting blood and plasma glucose values were not statistically different between any of the three dietary intervention groups (Table 1).

Whole-body glucose metabolism

Blood glucose concentrations were clamped to basal fasting levels during the 2-h insulin infusion in all groups (Fig. 2B). The glucose infusion rate (GIR) required to maintain euglycaemia during insulin infusion was significantly lower (~23%) over the last hour of the experiment in HFD compared with ND (Fig. 2A; 18.1 ± 0.8 vs 23.3 ± 0.6 mg/kg/min, $P < 0.001$). The HFD+MF group displayed a significantly higher (46%) GIR compared with HFD (26.4 ± 0.9 vs 18.1 ± 0.8 mg/kg/min, $P < 0.001$), which was also 13% higher than the ND group. These differences in GIR occurred despite equivalent plasma insulin concentrations between the ND, HFD or HFD+MF groups at the conclusion of the 2-h insulin infusion (1300 ± 70 vs 1440 ± 70 vs 1270 ± 70 pmol/L, respectively, Fig. 3).

Skeletal muscle microvascular blood flow and glucose uptake

Microvascular blood flow was not significantly different between any of the groups during saline infusion (Fig. 4A). Insulin increased microvascular blood flow in the ND group (8.2 ± 0.6 vs 4.4 ± 0.4 nmol/min; $P < 0.001$) to a similar extent as reported previously (Premilovac *et al.* 2013). In the HFD group, the insulin-stimulated increase in microvascular blood flow was blocked compared with the ND group (5.6 ± 0.5 vs 8.2 ± 0.6 nmol/min; $P < 0.005$). Conversely, the HFD+MF had significantly higher insulin-stimulated microvascular blood flow compared to HFD alone group (7.2 ± 0.5 vs 5.6 ± 0.5 nmol/min; $P < 0.05$). ND was not significantly different from HFD+MF (8.2 ± 0.6 vs 7.2 ± 0.5 nmol/min; $P = 0.208$).

Table 1 Characteristics of rats treated with normal diet (ND), high-fat diet (HFD) or high-fat diet + metformin (HFD + MF) for 4 weeks and then fasted overnight.

	ND (n = 25)	HFD (n = 27)	HFD + MF (n = 25)
Body weight (g)	269 \pm 8	299 \pm 6 ^a	276 \pm 6 ^{a,b}
Epididymal fat pad (g)	0.66 \pm 0.04	1.14 \pm 0.05 ^a	1.05 \pm 0.05 ^a
Plasma FFA (mmol/L)	0.55 \pm 0.02	0.62 \pm 0.01 ^a	0.54 \pm 0.02 ^b
Plasma glucose (mmol/L)	7.3 \pm 0.1	7.3 \pm 0.2	7.4 \pm 0.2
Blood glucose (mmol/L)	3.7 \pm 0.1	3.7 \pm 0.2	3.8 \pm 0.1
Plasma insulin (pmol/L)	110 \pm 10	230 \pm 20 ^a	170 \pm 10 ^{a,b}

Data are means \pm s.e.m. One-way ANOVA and SNK *post hoc* test.

^a($P < 0.05$) vs ND, ^b($P < 0.05$) HFD vs HFD + MF.

FFA, free fatty acids.

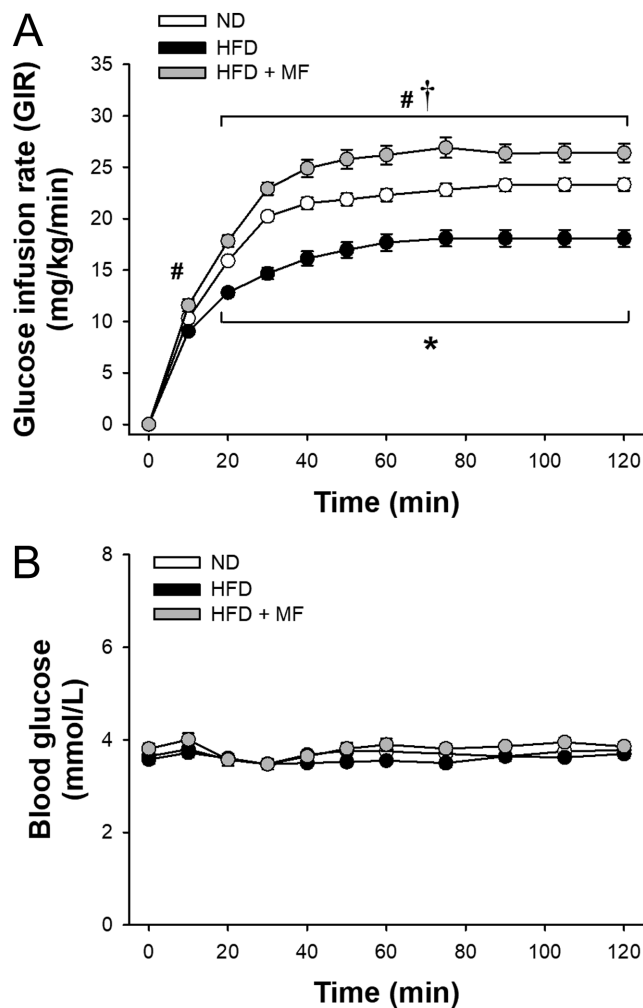


Figure 2 Glucose infusion rate (GIR) (panel A) required to maintain fasting blood glucose levels (panel B) during a euglycaemic-hyperinsulinaemic clamp (10 mU/min/kg) for normal diet (ND), high-fat diet (HFD) and high-fat diet + metformin (HFD + MF)-treated rats. Data are means \pm s.e.m. for $n = 14$ –17 rats in each group. * $P < 0.05$ ND vs HFD, # $P < 0.05$ HFD vs HFD + MF, † $P < 0.05$ ND vs HFD + MF. Two-way repeated-measures ANOVA and SNK *post hoc* test.

Muscle specific glucose uptake ($R'g$) was not different between groups during saline infusion (Fig. 4B). Insulin infusion significantly ($P < 0.001$) increased $R'g$ in all groups compared with their respective saline controls. Compared with ND, insulin-stimulated $R'g$ was significantly impaired (42% reduction) in the HFD group (27.2 ± 1.6 vs 15.7 ± 1.1 $\mu\text{g/g/min}$, $P < 0.001$ respectively). Insulin-stimulated $R'g$ in the HFD+MF rats was markedly increased (45% improvement) in comparison to the HFD alone group (22.8 ± 1.7 vs 15.7 ± 1.1 $\mu\text{g/g/min}$, $P < 0.001$); however, this was not fully restored to ND values (60% restored). Interestingly, $R'g$ and microvascular blood flow correlated positively

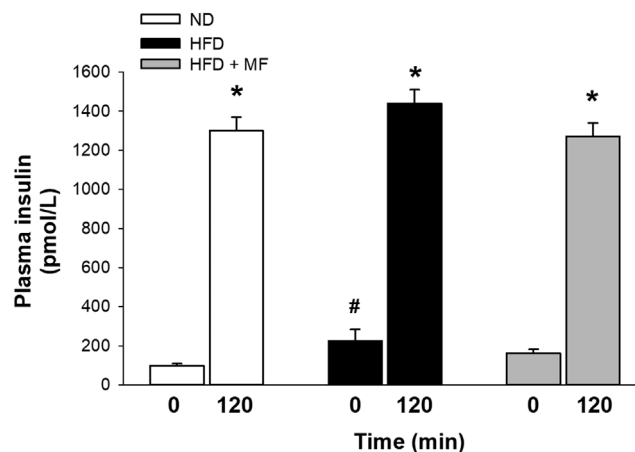


Figure 3 Plasma insulin levels before (0 min) and at the end (120 min) of the euglycaemic-hyperinsulinaemic clamp (10 mU/min/kg) for normal diet (ND), high-fat diet (HFD) and high-fat diet + metformin (HFD + MF)-treated rats. Data are means \pm s.e.m. for $n = 14$ –17 rats in each group. * $P < 0.05$ vs 0 min, # $P < 0.05$ ND at 0 min vs HFD at 0 min. Two-way ANOVA and SNK *post hoc* test.

when all three insulin-treated groups were combined ($R = 0.413$, $P = 0.004$).

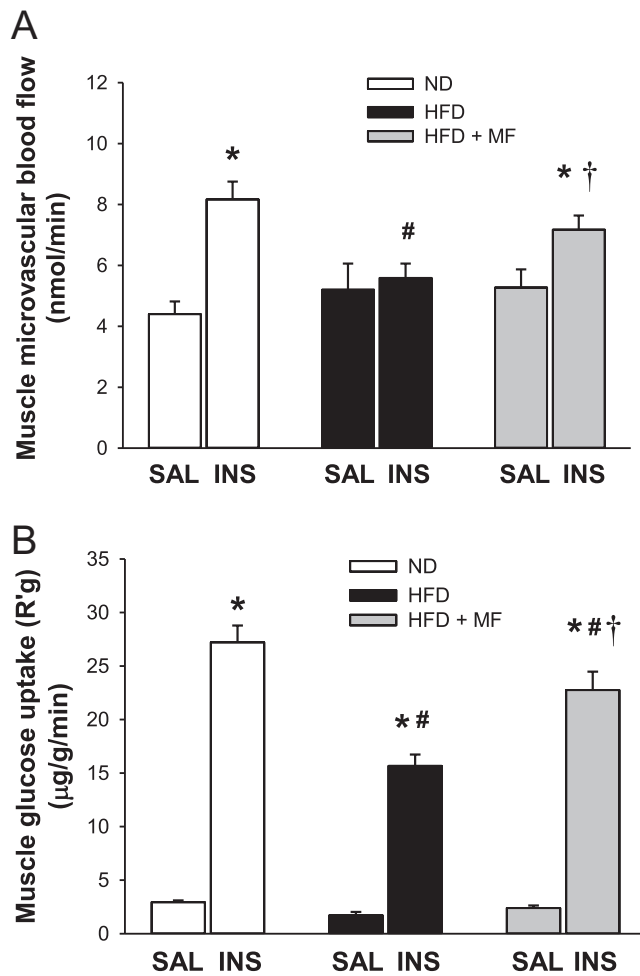
Muscle AKT and AMPK signalling

Somewhat reflecting $R'g$, skeletal muscle Ser⁴⁷³-AKT phosphorylation was not different between groups following saline infusion but increased predictably in all groups following insulin infusion (Fig. 5A). Compared with ND, the HFD group had lower Ser⁴⁷³-AKT phosphorylation following insulin infusion (1.52 ± 0.21 vs 1.00 ± 0.13 AU, $P < 0.01$). In contrast, insulin-stimulated Ser⁴⁷³-AKT phosphorylation in the HFD+MF group was higher than that in the HFD group (1.00 ± 0.13 vs 1.48 ± 0.16 AU, $P < 0.05$) and returned to levels seen in the ND group. An example immunoblot is shown in Supplementary Fig. 1, see section on [supplementary data](#) given at the end of this article.

Skeletal muscle phosphorylation of Thr¹⁷²-AMPK was not different between groups (whether metformin treated or not) (Fig. 5B). This is not surprising given that metformin was removed from the drinking water overnight and metformin was not detectable in rat plasma (assessed by HPLC) at the start of the euglycaemic-hyperinsulinaemic clamp. An example immunoblot is shown in Supplementary Fig. 2.

Femoral artery blood flow

Insulin infusion in the ND group resulted in a significant increase ($P < 0.05$) in FBF by 105 min compared to saline

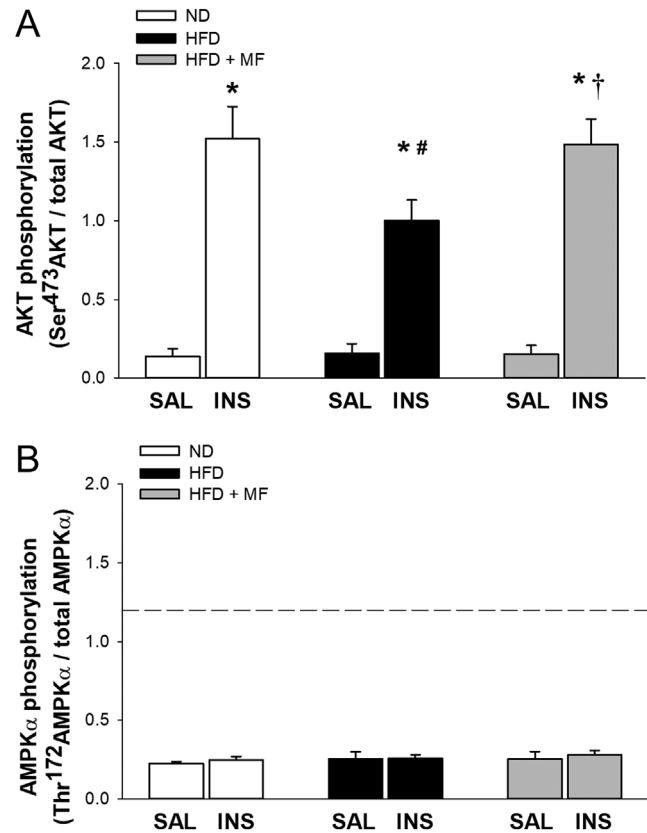
**Figure 4**

Skeletal muscle microvascular blood flow (panel A) and muscle specific glucose uptake (R'g) (panel B) at the conclusion of a 2-h euglycaemic-hyperinsulinaemic clamp (10 mU/min/kg) or saline infusion for normal diet (ND), high-fat diet (HFD) and high-fat diet + metformin (HFD + MF) treated rats. Data are means \pm s.e.m. for $n = 14$ – 17 rats in each group. * $P < 0.05$ vs saline, # $P < 0.05$ ND, † $P < 0.05$ HFD. Two-way-ANOVA and SNK *post hoc* test.

(Fig. 6A). Insulin-stimulated increases in FBF were absent in the HFD treatment group (Fig. 6B). Importantly, insulin increased FBF in the HFD+MF group by the 60-min time point compared to saline and this remained significant until the end of the 120-min experiment (Fig. 6C). Insulin-mediated increases in FBF were not significantly different between HFD+MF and ND by 120 min (Fig. 6D; 1.56 ± 0.11 vs 1.38 ± 0.08 mL/min; $P = 0.220$). Interestingly, there was no correlation between R'g and FBF when all three insulin-treated groups were combined ($R = 0.222$, $P = 0.134$).

Mean arterial blood pressure and heart rate

MAP was not affected by insulin or any of the dietary interventions (Supplementary Fig. 3A, B and C). MAP was

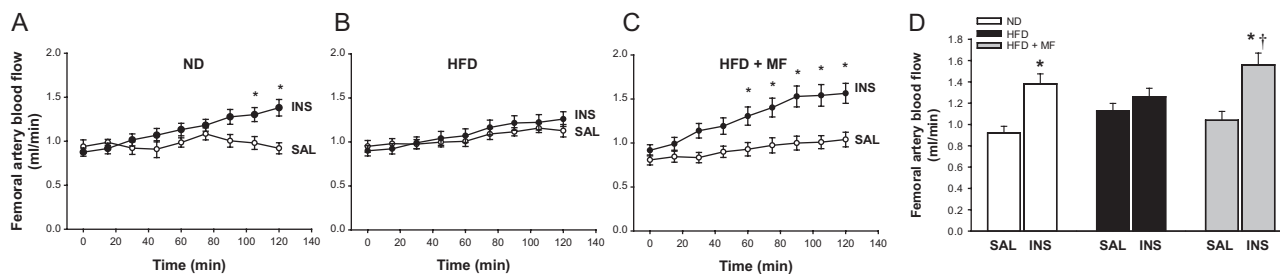
**Figure 5**

Skeletal muscle Western blot analysis of Ser⁴⁷³-AKT phosphorylation (panel A) and Thr¹⁷²-AMPK (panel B) at the conclusion of a 2-h euglycaemic-hyperinsulinaemic clamp (10 mU/min/kg) or saline infusion for normal diet (ND), high-fat diet (HFD) and high-fat diet + metformin (HFD + MF) treated rats. Data expressed as means \pm s.e.m. for $n = 7$ rats for panel A and $n = 3$ – 4 rats in panel B. * $P < 0.05$ vs saline, # $P < 0.05$ ND, † $P < 0.05$ HFD. Two-way ANOVA and SNK *post hoc* test. Dotted line represents the extent of AMPK activation with contracted muscle.

not significantly different between groups at the end of the 120-min infusion of saline or insulin (Supplementary Fig. 3D). Likewise, HR was not affected by insulin or any of the dietary interventions (Supplementary Fig. 4A, B and C). HR was not significantly different between groups at the end of the 120-min infusion of saline or insulin (Supplementary Fig. 4D).

Discussion

Delivery of glucose and insulin to myocytes through the microvasculature is important for regulating skeletal muscle glucose uptake. Impairment of this important physiological process in skeletal muscles can compromise normal metabolic activity and manifests as insulin resistance (Kubota *et al.* 2011, Premilovac *et al.* 2013, 2014).

**Figure 6**

Femoral artery blood flow time course during a 2-h euglycaemic-hyperinsulinaemic clamp (10 mU/min/kg) or saline infusion in normal diet (ND, panel A), high-fat diet (HFD, panel B) and high-fat diet + metformin (HFD + MF, panel C)-treated rats. Femoral artery blood flow at the conclusion of the 2 h experiment in ND, HFD and HFD + MF rats (panel D). Data are means \pm s.e.m. for $n = 14$ –17 rats in each group. * $P < 0.05$ vs saline, † $P < 0.05$ HFD. Two-way ANOVA and SNK *post hoc* test.

In the current study we sought to determine if 2 weeks of metformin treatment improves microvascular insulin responsiveness in skeletal muscle of HFD-fed, insulin-resistant rats. To our knowledge, this is the first study to demonstrate that metformin increases both insulin-mediated muscle microvascular blood flow and glucose uptake in skeletal muscle. Therefore, the present study highlights a previously unknown insulin-sensitising action of metformin that may partly explain its long-lived success as an anti-hyperglycaemic agent; however, other adjunct therapies may need to be considered to fully reverse the metabolic defect in insulin-resistant skeletal muscle.

It is well established that metformin helps regulate blood glucose levels by lowering hepatic glucose output in those with type 2 diabetes (Bailey & Turner 1996, Hundal *et al.* 2000). More recently, metformin has also been shown to promote the secretion of incretins (DeFronzo *et al.* 2016, Preiss *et al.* 2017) and to increase skeletal muscle glucose uptake (Sarabia *et al.* 1992, Magalhaes *et al.* 2006, Kristensen *et al.* 2014), both of which likely contribute to the glucose-lowering effects of the drug. Since normal insulin-mediated skeletal muscle glucose uptake is in part dependent on microvascular blood flow (Rattigan *et al.* 1997, Vincent *et al.* 2004, Zhang *et al.* 2004), we hypothesised that metformin would restore glucose uptake in insulin-resistant skeletal muscle in part by improving microvascular insulin action. We show that 4 weeks of HFD feeding leads to an obese, insulin-resistant phenotype associated with markedly reduced insulin-stimulated AKT phosphorylation, microvascular blood flow and glucose uptake in skeletal muscle. After only 2 weeks of treatment, metformin restored insulin-stimulated Ser⁴⁷³-AKT phosphorylation in skeletal muscle, microvascular blood flow and femoral artery flow of HFD rats, and partially reversed insulin-mediated glucose disposal. We conclude that metformin treatment increases

vascular insulin responsiveness, skeletal muscle glucose uptake and insulin signalling in skeletal muscle.

Kristensen *et al.* showed that 2 weeks of metformin treatment markedly increases insulin-stimulated skeletal muscle glucose uptake in healthy mice (Kristensen *et al.* 2014). The authors used an *ex vivo* muscle preparation and suggested this improvement was due to augmentation of glucose transport at the myocyte itself. Normalisation of insulin-mediated AKT phosphorylation in the present study would support this assertion given that one of the end products of the AKT signalling pathway is increased GLUT4 translocation to the cell membrane. Importantly, however, the *ex vivo* muscle preparation depends on simple diffusion of insulin and glucose from the surrounding fluid into the myocyte and does not require delivery and transport of glucose and insulin through the microvasculature as occurs *in vivo*. Unfortunately, whether metformin similarly improved insulin's microvascular actions in their study is not known. In the present study, we show that metformin improves insulin-stimulated microvascular blood flow in insulin-resistant muscle. These data suggest that metformin does have effects on the vasculature and these actions help to improve both vascular and consequently muscle insulin sensitivity.

It is well established that insulin stimulates skeletal muscle microvascular blood flow via a mechanism that is nitric oxide synthase (NOS) dependent and this vascular action contributes to 40% of muscle glucose disposal (Vincent *et al.* 2003, 2004). We have demonstrated that this occurs via local muscle NOS rather than a central (brain) process (Bradley *et al.* 2010, 2013). In addition, we have previously demonstrated that the AMPK activator AICAR (at a low dose that does not increase muscle glucose uptake on its own) augments insulin-stimulated microvascular blood flow and insulin-mediated muscle glucose uptake (Bradley *et al.* 2015). Others have also demonstrated that in the vascular endothelium AMPK can activate

eNOS (de Boer *et al.* 2016). Metformin is well known for activating AMPK to improve liver insulin sensitivity (Bailey & Turner 1996, Hundal *et al.* 2000) and promoting myocyte glucose uptake *ex vivo* (Kristensen *et al.* 2014). In the current study, metformin was removed overnight and there was no detectable levels of metformin in plasma or activation of muscle AMPK; therefore, we propose the chronic benefits of metformin involve improving insulin action (or responsiveness) rather than a direct effect of AMPK. It is well known that both insulin (Montagnani *et al.* 2001) and metformin (An *et al.* 2016) increase eNOS phosphorylation in cultured endothelial cells. The novel aspect of the current project is the metformin-mediated augmentation of vascular insulin action in skeletal muscle *in vivo* and its association with improvement of muscle insulin signalling and glucose disposal.

Insulin alone is known to increase total blood flow in skeletal muscle, primarily by NOS-mediated vasodilation (Steinberg *et al.* 1994, Baron 1996, Rattigan *et al.* 1997, Vincent *et al.* 2003, Sjöberg *et al.* 2017). In the present study, insulin did not increase either total leg blood flow or microvascular blood flow in the HFD group. While there are no previous reports regarding metformin's ability to improve microvascular insulin sensitivity, metformin has been reported to improve flow-mediated dilation in the brachial artery of those with type 1 diabetes (Pitocco *et al.* 2013) and polycystic ovarian syndrome (Jensterle *et al.* 2008). Interestingly, in the current study metformin treatment improved insulin-stimulated total leg blood flow in the HFD group to a similar magnitude observed in the ND group. This improvement in large artery blood flow following metformin treatment was matched by an improvement in microvascular blood flow in the HFD+MF group. It is noteworthy that glucose disposal was correlated to microvascular blood flow, but not to FBF. This would not be totally unexpected given our previous work demonstrating that increased total limb blood flow is preceded by increased microvascular blood flow in response to insulin (Vincent *et al.* 2004, Zhang *et al.* 2004). Importantly, it is the extent of the microvascular blood flow and not total flow that impacts on muscle glucose disposal (Vincent *et al.* 2004, Zhang *et al.* 2004). Therefore, it is not overly surprising that although large blood vessel insulin sensitivity has improved to a similar extent as ND, this did not translate to a full recovery of insulin-stimulated glucose disposal in muscle and the latter is more closely paralleled by the degree of microvascular blood flow.

There are some other notable (but expected) effects of metformin in the current study. As has been shown

elsewhere, the 4 weeks of HFD compared to ND caused phenotypic and metabolic alterations indicative of insulin resistance (Storlien *et al.* 1986, St-Pierre *et al.* 2010, Kubota *et al.* 2011, Premilovac *et al.* 2013, Turner *et al.* 2013). The HFD increased body weight, adiposity, fasting plasma FFA and plasma insulin levels, and these were (with the exception of adiposity) reduced by metformin treatment. During the insulin clamp, whole-body glucose disposal in the HFD group was lower than ND, and metformin treatment in the HFD group improved whole-body glucose disposal above that seen in the ND group. The fact that muscle glucose uptake was not fully restored to ND values (60% restored) suggests that the marked sensitisation in whole-body insulin sensitivity was due partly to improved hepatic insulin sensitivity – a well-known benefit of metformin (Bailey & Turner 1996, Hundal *et al.* 2000).

There are several limitations in the current study. Firstly, we only investigated one dose of metformin and the supplementation was relatively short term (2 weeks) in the HFD rat model. Whether a higher dose of metformin or a longer supplementation would fully reverse the muscle metabolic impairment in the HFD rat would be important to follow-up. Secondly, metformin treatment caused a reduction in body weight in HFD-fed rats so it is not known the extent that weight loss played in the insulin sensitising action of metformin. A pair-fed HFD group would help answer this question in future studies. Thirdly, whether metformin produces similar microvascular and metabolic benefits in other models of insulin resistance such as the high salt diet-fed rat or overt type 2 diabetes is not known. Fourthly, whether metformin can augment microvascular blood flow in a postprandial state (rather than the insulin clamp condition) is of clinical importance and requires further investigation. Finally, the mechanism of action of metformin on microvascular insulin sensitivity was not investigated but is important to follow-up to help target therapies for microvascular insulin resistance.

In conclusion, this study highlights a novel mechanism whereby metformin improves HFD-induced muscle insulin resistance in part by improving microvascular insulin action. These novel findings may have real-world implications for people with insulin resistance and type 2 diabetes given that metformin remains a first-line therapy. Given the clinical importance and wide spread use of metformin, understanding where and how metformin improves insulin responsiveness is of importance for the development of adjunct therapies to target mechanisms additional to those affected by metformin alone.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0067>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

MA K and S R were responsible for the conception and design of research; E A B and D P performed the *in vivo* experiments. D P, E A and D H performed the Western blot analysis. S M R conducted the HPLC analysis on plasma. E A B and M A K analysed the data; E A B, D P, M A K, S M R, A C B and S R interpreted the results of experiments; E A B, D P and M A K prepared the figures; D P, E A B, M A K and A C B drafted the manuscript; E A B, D P, S M R, D H, A C B, E A, M A K and S R edited the revised manuscript; M A K approved the final version of the manuscript.

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