Metformin improves endothelial function in aortic tissue and microvascular endothelial cells subjected to diabetic hyperglycaemic conditions

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1. Introduction

Type 2 diabetes (T2DM) is increasingly recognized as a disease of the cardiovascular system and diabetes-associated vascular complications are the major clinical problem responsible for 75% of the deaths with diabetic patients also having a mortality rate 4 times that of the general population [1]. Numerous studies, notably the Diabetes Control and Complications Trial (DCCT) for type 1 diabetes (T1DM) and the UK Prospective Diabetes Study (UKPDS) for T2DM, link hyperglycaemia to the development of cardiovascular disease [2–4]. Based on a metaregression analysis of approximately 96,000 people it has also been established that there is a progressive relationship between blood glucose levels and cardiovascular risk [5]. There is also a very strong association between endothelial dysfunction and the development of cardiovascular disease in humans with diabetes, and one of the key factors that contribute to the development of diabetes-related vascular disease is hyperglycaemia, a common feature of both T1DM and T2DM [6]. Published data based on studies of vascular function in blood vessels isolated from rodent models of diabetes and blood vessels from patients with diabetes indicate that diabetes is associated with a profound reduction in endothelium-dependent vasodilatation (EDV) that can be linked
to a dysregulation of endothelial nitric oxide synthase (eNOS) [7–9].

Metformin, a biguanide, is an orally effective synthetic antidiabetic drug with an estimated current worldwide usage of 120 million people [10,11]. It is the drug of choice for the treatment of T2DM and the majority of patients with T2DM are first treated with metformin with other drugs added to their therapeutic regimen as required. Vascular disease and associated complications are all frequent co-morbidities and precipitated by the loss of good glycaemic control. Thus, for the optimal treatment of diabetes it is recommended to not only restore glycaemic control, but also seek therapeutic protocols that protect the vasculature and specifically the endothelium against the damaging effects of glucose toxicity and hence reduce the development of cardiovascular disease. Despite being in clinical use for over 50 years, the precise cellular mode(s) of action of metformin remains unclear; however, clinical data suggests that treatment with metformin does reduce the impact of diabetes-associated macro- and microvascular disease and this protective action may be mediated via a direct action(s) on the endothelium [3,12,13].

A reduction in EDV is termed “endothelial dysfunction” and contributes to the development of insulin resistance as blood flow and, in consequence, glucose disposal is reduced [8,14]. Results from studies with endothelial cells in culture also indicate that elevated glucose raises oxidative stress resulting in an “uncoupling” of eNOS and promotes a pro-apoptotic state [15,16].

A general consensus in the literature argues that the antidiabetic actions of metformin are mediated secondarily to a mild inhibition of mitochondrial complex 1 and a subsequent reduction of the ATP/AMP ratio and activation of AMP kinase (AMPK); nonetheless whether the activation of AMPK is an absolute requirement and whether metformin can access mitochondria to a sufficiently high concentration to inhibit complex 1 continue to be debated [11,17,18]. Metformin has also been shown to protect the expression of the deacetylase protein product of SIRT1 (siruin 1) from hyperglycaemia-induced downregulation in mouse microvascular endothelial cells (MMEC) [19]. SIRT1 activation enhances the activity of eNOS and thus should promote EDV via enhancing the generation of nitric oxide (NO) [20]. Thus, by directly, or indirectly, enhancing the expression and/or activation of SIRT1, metformin may improve endothelial function via the increasing eNOS activity.

During clinical use of metformin for the treatment of patients with T2DM the drug is provided orally in doses of 500–850 mg/ three times day (tid) with meals and plasma concentrations are reported to be between 1 and 50 μM [21]. In contrast the greater majority of the in vitro studies have used metformin at a concentration of <100 μM [22]. Furthermore, it is well known that an oral glucose test (OGT), as used to assess glucose tolerance, results, within a 1–2 h time period, in a reduction of flow-mediated EDV (FMD) that can be measured by brachial artery plethysmography/Doppler techniques [23]. This glucose-induced reduction in EDV, which is comparable to what might be expected following a high glycaemic meal, has been shown to be linked to the uncoupling of eNOS as it can be prevented by pre-treatment of the patient with the active isomer of the eNOS co-factor, tetrahydrobiopterin (BH₄), (6R)-5,6,7,8-tetrahydro-L-β-biopterin sul fate (6R-BH₄), but not the inactive stereoisomer (6S)-5,6,7,8-tetrahydro-L-β-biopterin sulfate (6S-BH₄) [24]. Of particular relevance to the current investigation is that the acute treatment of patients with a single dose of 500 mg of metformin also offsets the negative effects of an OGT on FMD [25]. Collectively these clinical data suggest that metformin has an endothelial protective action that is mediated via eNOS; however, the specific cellular mechanisms involved remain unknown.

In order to determine whether metformin at a clinically relevant concentration can protect the endothelium and eNOS function from a diabetic hyperglycaemic milieu we designed protocols comparable to that described above by Zhang et al. [25] for their clinical study. Thus, we investigated the effects of an acute 3-h exposure to metformin on EDV in aortic blood vessels from control and diabetic mice and in a parallel cell culture protocol studied the effects of metformin on eNOS phosphorylation in MMECs. Mouse aortic tissue was chosen as we have previously reported that acetylcholine-mediated EDV in this vessel is entirely mediated by nitric oxide (NO) [26]. In addition, our previous studies with MMECs have shown that exposure to high glucose results in the dysregulation of eNOS that can be prevented by providing a precursor of BH₄, sepiapterin, thus comparable to that reported following an OGT in humans [15,16,24].

2. Materials and methods

2.1. Animals

The research protocol is approved by the Animal Care Committee of Weill Cornell Medical College. Two strains of db/db diabetic mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA): (1) The C57BLKS/J [BKS. Cg-Dock7tm1(+)/Leprtm1(Sj)/] (db/db, 000642)) mouse that develops progressive and sustained hyperglycaemia and obesity. (2) C57BL/6J (B6.BKS(D-Leprtm1)[db/db] (db, 000697) mice as a model of obesity that show early hyperglycaemia peaking between 6 and 10 weeks, but with blood glucose levels reported to decline to the normal range by 12 weeks. Male mice (16–24 weeks of age) of either the C57BLKS/J or C57BL/6J strain, or age-matched control litter-mates (db/+?) were used in this study.

2.2. Wire myograph experiments

Aortic ring segments of 3 mm in length were dissected from the ascending thoracic aorta toward the diaphragm after the mice (16–24 weeks of age) were sacrificed. Aortae were removed and kept in Krebs solution (composition, mM): NaCl, 120; NaHCO₃, 25; KCl, 4.8; NaH₂PO₄, 1.2; MgSO₄, 1.2; Dextrose, [either normal glucose, NG, 11.0 mM, or high glucose, HG, 40 mM]; CaCl₂, 1.8; bubbled with 95% O₂ and 5% CO₂. Each aortic segment was mounted through the lumen on two parallel 200-μm stainless steel pins in a Mulvany–Halpern myograph for isometric force recording. Each vessel preparation was gradually stretched according to the normalisation procedures of Mulvany and Halpern [44] to determine optimum resting tension and was equilibrated for 1 h before commencement of the experimental protocol. Preparations were contracted with phenylephrine (PE) (0.1–1 μM) and allowed to stabilise before constructing cumulative concentration–response curves to the endothelium-dependent vasodilator acetylcholine (ACh, 100 nM–10 μM). ACh-mediated concentration-dependent relaxation in the absence or presence of 50 μM of metformin was investigated.

2.3. Cell culture

MMECs were obtained from American Type Culture collection (catalog # CRL2 2279, Manassas, VA, USA) and were cultured on extracellular matrix coated plates in Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Culture media consisted of either NG or HG (11 or 40 mM glucose) up to 3 h. These glucose concentrations were chosen as representing non-fasting blood glucose levels previously reported for non-diabetic C57BL mice and C57BLKS/J db/db diabetic mice [7]. 29 mM monosaccharide mannitol added to
NG (11 mM) to reach the same osmotic concentration as for the HG protocol (40 mM) was also used as an osmotic control. Metformin was added to the culture media with either 11 or 40 mM glucose (NG+met or HG+met) for 3 h. Cell cultures were maintained in an incubator at 37 °C, with saturating humidity and an atmosphere of 5% carbon dioxide to 95% air. At the end of 3 h MMECs were collected for Western immunoblot analysis of proteins.

2.4. AMPK siRNA transfection

MMECs were transfected with either control or AMPKα1/2 siRNA according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Briefly MMECs were grown in antibiotic-free DMEM for 24 h, then transfected with 75 pM AMPKα1/2 siRNA or control siRNA with Lipofectamine 2000 for 6 h in OPTI-MEM medium. After 24–36 h of incubation cells were cultured in high glucose media (40 mM), with either 50 μM metformin or solvent for 3 h.

2.5. Antibodies and Western blotting

Immunoblotting was used to detect the expression of proteins with antibodies for SIRT1, eNOS, p-eNOS, Akt, pAkt, AMPK and pAMPK (Cell Signaling Technology, Inc., Beverly, MA, USA). MMECs were homogenized in lysis buffer RIPA (Sigma). Equal amounts of protein samples (30 μg per lane) were separated by SDS-PAGE in the presence of dithiothreitol and transferred to nitrocellulose. The nitrocellulose was blocked with 5% milk in Tris-buffered saline and then probed with the appropriate primary antibody for phospho-ylated proteins overnight at 4 °C. The nitrocellulose membranes were washed with Tris-buffered saline-Tween (0.05%) solution and incubated with horseradish peroxidase-conjugated second antibody for 1 h. The reaction was visualized by chemiluminescence. Membranes were stripped with Stripping Buffer from Thermo Scientific (Waltham, MA, USA) and re-probed with the appropriate primary antibody for total protein.

2.6. Materials

All chemicals were of analytical grade and purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.7. Data analysis

Data are expressed as pD2 values and pD2 is defined as the negative logarithm to base 10 of the EC50 values. In all experiments, n equals the number of animals. Relaxation is expressed as percentage of PE-induced tone (plateau phase) ± s.e. mean. The significance of differences between mean values was calculated by Student’s t-test. Statistical significance of differences between the means of data groups was performed using ANOVA for curve analysis. Significance was assumed if P < 0.05.

The data are expressed as means ± s.e.mean. In all experiments, n equals the number of animals, or individual experiments (not replicates), performed. For the quantification of changes in protein expression individual immunoblots were quantified by densitometry and expressed as a ratio relative to β-actin. In the histograms presented in Figs. 3–8, the data depsects either changes in total protein expression (eNOS, SIRT1, AMPK, or Akt) as a ratio of β-actin and normalised to protein expression under control, HG, conditions for each individual experiment, or expressed as a ratio of phosphorylated to non-phosphorylated protein and again normalised to the ratio obtained under NG conditions. The significance of differences between mean values was calculated by Student’s t-test. Statistical significance of differences between the means of data groups was performed using one-way ANOVA and followed by Tukey’s Multiple Comparison tests. Significance was assumed if P < 0.05.

**Fig. 1.** Acetylcholine-mediated vasodilatation in obese db/db mice. C57BL/6J db/db mice and age matched db+/? controls (a) body weights, plasma glucose levels; (b) acetylcholine (ACH)-mediated dose-dependent vasodilatation of phenylephrine (1 μM) contracted aortae isolated from db/db and db+/? mice. ACH concentration response curves in aortae from the db+/? mice (c) and db/db mice (d) incubated in normal 11 mM glucose Krebs, 40 mM glucose (HG) Krebs for 3 h, and HG plus 50 μM metformin (Met) for 3 h. Significantly different (db/db vs. db+/? *P < 0.05). n = 6.
3. Results

3.1. 3-h exposure to metformin can partially correct depressed acetylcholine-mediated vasodilatation in obese and diabetic db/db mouse aorta

We compared the body weights and blood glucose levels for the C57BL/6J db/db mice (B6.BKS(D)-Lepr<sup>db</sup>/J, 000697) and age matched controls at the time of sacrifice (16–24 weeks). C57BL/6J db/db mice were significantly obese (35.5 g ± 0.1 vs. 62.2 g, P < 0.001) but euglycaemic (9.7 ± 0.1 vs. 9.7 ± 0.1, P > 0.05) when compared to control lean db+/? mice (Fig. 1a).

We next determined the effects of metformin on ACh-mediated endothelium-dependent vasodilatation in partially contracted mouse thoracic aortae from C57BL/6J db/db and db+/? mice. ACh induced a concentration-dependent relaxation in the aortae from both db/db and control mice. ACh-mediated relaxation was impaired in the aortae from obese but non-hyperglycaemic db/db mice (Fig. 1b; db/db group: pD<sub>2</sub> = 6.0 ± 0.2; E<sub>max</sub> = 70.3 ± 7.6%; control group: pD<sub>2</sub> = 7.0 ± 0.1; E<sub>max</sub> = 82.6 ± 3.8%; n = 6, P < 0.05). Comparing to those incubated in NG Krebs the ACh-mediated EDV was significantly reduced (P < 0.05) when aortic segments from control db+/? mice were exposed to HG Krebs for 3 h. The reduction was partially reversed in the presence of 50 μM metformin (Fig. 1c; db+/?: NG: pD<sub>2</sub> = 7.0 ± 0.2; E<sub>max</sub> = 82.7 ± 6.0%; HG: pD<sub>2</sub> = 6.3 ± 0.1; E<sub>max</sub> = 65.3 ± 3.8%; HG + met: pD<sub>2</sub> = 6.5 ± 0.1; E<sub>max</sub> = 75.3 ± 3.8%; n = 6, P < 0.05). Similar data were collected with obese euglycaemic db/db mice, ACh-mediated EDV of the aorta was reduced by a 3 h incubation in HG incubation, but partially corrected by co-incubation with metformin (Fig. 1d; db/db: NG: pD<sub>2</sub> = 7.0 ± 0.2; E<sub>max</sub> = 80.7 ± 7.6%; HG: pD<sub>2</sub> = 6.5 ± 0.1; E<sub>max</sub> = 59.3 ± 3.8%; n = 6; HG + met: pD<sub>2</sub> = 6.8 ± 0.1; E<sub>max</sub> = 70.3 ± 3.8%; n = 6, P < 0.05).

The body weight and blood glucose of the diabetic C57BLKS/J (BKS. Cg-Dock7<sup>/+</sup>+Lepr<sup>db</sup>/J) db/db, 000642) mice were compared with their age matched controls. The C57BLKS/J db/db mice showed both increased body weight (47.1 g ± 0.3 g vs. 31.7 ± 0.3 g, P < 0.05) and blood glucose level (32.6 mM ± 0.1 vs. 8.5 mM, P < 0.001) as compared to control (Fig. 2a).

Following the same protocol as for the C57BL/6J mice we determined the effects of metformin on ACh-mediated EDV in partially contracted mouse thoracic aortae from C57BLKS/J db/db and db+/? mice. ACh-induced relaxation in aortae from these db/db mice was significantly impaired when compared to that recorded in control mice (Fig. 2b; db/db group: pD<sub>2</sub> = 5.8 ± 0.2; E<sub>max</sub> = 75.3 ± 7.6%; control group: pD<sub>2</sub> = 7.9 ± 0.1; E<sub>max</sub> = 98.0 ± 3.8%; n = 6, P < 0.05). In addition, the reduction in ACh-mediated EDV that resulted from a 3 h exposure to HG was partially reversed by the presence of 50 μM metformin (Fig. 2c; db+/?: NG: pD<sub>2</sub> = 7.9 ± 0.2; E<sub>max</sub> = 98.0 ± 7.6%; HG: pD<sub>2</sub> = 6.40 ± 0.1; E<sub>max</sub> = 78 ± 3.8%; HG + met: pD<sub>2</sub> = 7.3 ± 0.1; E<sub>max</sub> = 82.6 ± 3.8%; n = 6, P < 0.05). The 3-h exposure to metformin also improved ACh-mediated EDV in aortae from diabetic db/db mice (Fig. 2d; db/db: pD<sub>2</sub> = 5.8 ± 0.1; E<sub>max</sub> = 75.8 ± 3.8%; db/db + met: pD<sub>2</sub> = 6.4 ± 0.1; E<sub>max</sub> = 85.0 ± 3.8%; n = 6, P < 0.05).

3.2. 3-h exposure to metformin significantly increases eNOS phosphorylation in microvascular endothelial cells exposed to HG

In order to investigate the cellular basis of the protective action of metformin on EDV we determined the effects of metformin on endothelial function using cell culture protocols designed to study the effects of metformin in the presence of NG, HG or the osmotic control, mannitol, on the expression of SIRT1 and phosphorylation status of eNOS, AMPK and Akt in MMECs. eNOS protein expression was assessed by Western blot in MMECs in NG, HG and HG with...
either 50 or 100 μM metformin, as well as in NG plus 50 μM metformin, and in 11 mM glucose plus 29 mM mannitol as an osmotic control for 3 h. Exposure to HG resulted in no change in total eNOS expression, but a significant 20 ± 0.5% decrease in the ratio of phosphorylated (p)-eNOS Ser1177 to total eNOS in MMECs. The presence of 50 or 100 μM metformin rescued MMECs from the HG-induced decrease in p-eNOS/eNOS and returned the ratio to control levels (P < 0.05) (Fig. 3a,b). In contrast the p-eNOS/eNOS ratio was not changed in MMECs exposed to NG and treated with 50 μM metformin versus MMECs not treated with metformin, or versus the MMECs exposed to the osmotic control, mannitol. In addition, there were no significant differences in the expression levels of SIRT1 (Fig. 3a and c), total AMPK and the ratio of p-AMPK to total AMPK (Fig. 3a and d) in MMECs when compared HG, and HG with metformin to NG (P > 0.05).

3.3. 3-h exposure to metformin significantly increases Akt phosphorylation in endothelial cells

However, although total Akt expression was not affected in the presence of HG the ratio of p-Akt Ser473 to total Akt, but not the ratio of p-Akt Thr308 to total Akt, was significantly decreased in the presence of HG and reversed in the presence of 50 or 100 μM metformin (P < 0.05, Fig. 4). Furthermore, no significant difference was observed for total Akt protein expression after HG, or in the presence of metformin. In addition, for the mannitol control, no significant difference was apparent in the p-Akt Ser473 to total Akt ratio compared to control (Fig. 4).

3.4. Inhibition of Akt phosphorylation blocks metformin induced increase of eNOS phosphorylation in endothelial cells

Both the p-Akt Ser473 and p-Akt Thr308 to total Akt ratio were significantly decreased after addition of 10 μM of the Akt inhibitor, A6730, and, in the presence of A6730, 50 μM metformin no longer reversed the HG-induced decrease of p-Akt Ser473 to total Akt ratio in MMECs (P > 0.05, Fig. 5). Similar results were observed for the p-eNOS Ser1177 to total eNOS ratio and, after treatment with 10 μM A6730, 50 μM metformin no longer reversed the HG-induced decrease of eNOS phosphorylation (Fig. 5).

3.5. Knock down of AMPK indicates a role for AMPK in mediating effects of metformin

The role of AMPK in mediating the effects of metformin was further investigated using an AMPK siRNA knockdown technique. The total AMPK protein expression levels in MMECs cultured in HG without and with metformin treatment were 68.0 ± 6.0% and 69.0 ± 4.0% after transfection with AMPK siRNA in comparison to cells transfected with control siRNA (P < 0.05, Fig. 6b). However, no significant difference was observed for both the ratio of p-AMPK to total AMPK and p-eNOS to eNOS (P > 0.05, Fig. 6c and d).
molecular basis for the results from clinical studies that have also shown that hyperglycaemia reduces FMD [23–25]. In summary, our data indicates that 50 μM metformin prevents the HG-induced reduction in EDV in aorta from control non-diabetic mice and improves EDV observed in aorta from both 16–24-week-old type 2 diabetic db/db and obese normoglycaemic db/db mice. The persistence of endothelial dysfunction in the aortae of the normoglycaemic db/db mice suggests that the effects of the early onset, but not sustained, hyperglycaemia has a long lasting epigenetic, “memory” effect on endothelial function; comparable data has been presented for endothelial cells cultured in HG and then restored to normoglycaemia [27].

Furthermore, our data indicates that, since EDV in the mouse aorta is entirely dependent on eNOS-generated NO, the beneficial effects of metformin are mediated, directly or indirectly, via the modulation of eNOS function [26]. Support for this conclusion is provided by the data from cell culture protocols with MMECs wherein the brief 3-h exposure to metformin although not affecting total protein expression for either eNOS, SIRT1, AMPK or Akt, results in post-translational changes in phosphorylation of eNOS, and Akt. The failure of a 3-h exposure to affect total protein expression is not surprising; however, the presence of 50 μM metformin for 3 h does enhance levels of p-eNOS and reverse the negative effects of a pre-incubation with HG on phosphorylation of Ser1177 eNOS. It is well known that phosphorylation of eNOS at Ser1177 is a key regulatory process for enhancing the generation of NO and is mediated, in vivo, by shear stress and activation of the serine/threonine protein kinase Akt/PKB [28]. Akt itself is also activated by phosphorylation via Ser473 and Thr308, but these phosphorylation processes are mediated by different kinases: phosphorylation of Thr308 via phosphoinositide-dependent kinase (PKD)-1 [24] and phosphorylation of Ser473 via the rapamycin-insensitive companion of mammalian target of rapamycin (mTOR rictor or mTORC2), namely PD2K [29,30]. In our study the presence of HG lowered Akt phosphorylation of Ser473, but not phosphorylation of Thr308. Furthermore, the reduction in the ratio of pSer473/Akt was reversed in the presence of metformin and also insulin.

Collectively these data suggest that metformin may target the mTORC2 pathway in MMECs and this results in the phosphorylation of Akt. Although high concentrations of metformin, >1 mM, are known to inhibit signaling through mTORC1 [31] and may also modulate mTORC2, albeit at a concentration of 4 mM [32], the effects of concentrations of metformin that are within the anticipated therapeutic range in the blood, 1–50 μM, have not, to the best of our knowledge, been previously reported. Additional studies are required to further elucidate the contribution of the mTOR pathway as a target whereby metformin can modulate endothelial cell function.

The benefits of treatment with metformin include not only a reduction in hepatic gluconeogenesis, but also a reduction in insulin resistance and thus an improvement of endothelial function could be secondary to improved glucose metabolism and utilization [4]. A 12-week treatment of patients with type 2 diabetes with metformin reduced insulin resistance and, as based on blood flow responses to intraarterial administration of the endothelium-dependent vasodilator ACh, improved endothelial function in situ, but did not affect the vasodilatation responses to the endothelium-independent vasodilators, sodium nitroprusside, or verapamil [12]. Mather et al. reported that metformin improved insulin resistance and it was therefore concluded that the beneficial effects of metformin were most likely secondary to improved insulin signaling [12]. Our previously reported data with MMECs in cell culture and a 72-h exposure to metformin indicated a direct action of metformin to reduce hyperglycaemia-induced endothelial senescence and apoptosis via a SIRT1-dependent

Fig. 4. Effect of a 3-h exposure to metformin on protein levels of Akt and phosphorylated-Akt. Representative gel of protein expression for total Akt, phosphorylated p-Akt Ser473, T308 and β-actin in MMECs cultured with NG, NG plus 50 μM metformin, HG, HG plus 50 μM metformin, HG plus 100 μM metformin and HG plus 29 mM mannitol for 3 h. Bar graph shows quantified densitometric analysis from Western blots. The graph reflects changes in total Akt/β-actin ratio relative to that normalised to 1.0 for NG (upper); and relative p-AktSer473/Akt ratio (middle); p-AktThr308/Akt ratio (lower). n = 5 from five independent experiments. Significantly different (NG vs. HG, *P < 0.05; HG vs. HG+50, **P < 0.05). n = 5 from five independent experiments.

3.6. There are no additive effects of insulin together with metformin treatment

We also investigated whether the presence of insulin modulated the same cell signaling pathways. As illustrated in Fig. 7 the presence of insulin (50 mM) for 3-h reversed the HG-induced decrease in the p-eNOS/eNOS ratio (P < 0.05) and similar to that shown for metformin in Fig. 3, however, there was no additive effect when insulin was combined with metformin (P > 0.05, Fig. 7). Although there was no significant change in total Akt protein expression after HG, metformin, or insulin treatment, the p-Akt Ser473, but not the p-Akt Thr308, to total Akt ratio was significantly decreased in the presence of HG, but reversed following treatment with metformin. There were also a significant insulin-evoked robust increase of 50 ± 0.5% in both p-Akt Ser473 and p-Akt Thr308 to total Akt ratios (Fig. 7, P < 0.05).

4. Discussion

The novel findings of the present study are that a brief 3-h exposure to metformin can both prevent and reverse hyperglycaemia-induced endothelial dysfunction via offsetting the negative effects of high glucose on eNOS. These data provide a
Fig. 5. Effect of a 3-h exposure to metformin on protein levels of Akt and phosphorylated-Akt in the presence of the Akt inhibitor, A6730. Representative gel of protein expression for (a) total Akt, p-Akt Ser473, p-Akt Thr308 and β-actin; (b) total eNOS, p-eNOS and β-actin in MMECs cultured with NG, HG, HG plus 50 μM metformin, and HG plus 10 μM Akt1/2 inhibitor (A6730) with or without 50 μM metformin for 3 h. Bar graphs show quantified densitometric analysis from Western blots relative to data for NG normalised to 1.0. Significantly different (NG vs. HG, *P < 0.05; HG + A6730 and HG + A7630 + Met vs. HG + Met, **P < 0.05) n = 5 from five independent experiments.

Fig. 6. Effect of metformin associated increase of p-eNOS after AMPK inhibition by siRNA. Representative gel of protein expression for total eNOS, phospho-eNOS, total AMPK, phospho-AMPKα and β-actin (a) in MMEC with HG knockdown MMECs using and cultured with HG plus control siRNA, HG plus AMPKα/1/2 siRNA, and HG plus combination of AMPKα/1/2 siRNA and 50 μM metformin for 3 h. Bar graph shows quantified densitometric analysis from Western blots. Values of T-AMPK (b); p-AMPKα (Thr172)/T-AMPK (c), p-eNOS/eNOS ratio (d) are expressed as the relative ratio to β-actin. Significantly different (AMPKα/1/2 siRNA vs control siRNA *P < 0.01) n = 6 from six independent experiments.
process [19]. In that study a 72-h exposure to HG reduced SIRT1 expression, whereas in the current study we have demonstrated that although a 3-h exposure to HG does not affect expression of SIRT1 there is, nonetheless, an improvement in endothelial function as evident by the results of the myograph study that correlates with the cell culture data indicating that metformin reverses the negative effect of HG on p-eNOS. Previously we reported that the protective effects following a longer 72-h exposure of MMECs to metformin against HG-induced endothelial cell senescence were lost when SIRT1 levels were significantly reduced using an siRNA protocol [19]. Thus, collectively, these data suggest time-dependent effects of metformin and that the rapid effects of metformin are mediated by changes in eNOS signaling and an improvement in NO mediated EDV that are independent of SIRT1.

Despite over 50 years of clinical use the cellular mode(s) of action of metformin remain elusive. The role of AMPK, possibly secondarily to an inhibition of mitochondria complex 1 and a subsequent decrease in the ATP/AMP ratio, has been inferred [33,34]. However, as indicated in a report by Madiraju et al., metformin only inhibits complex 1 at concentrations >1 mM and a more likely target for metformin-mediated inhibition of hepatic gluconeogenesis is mitochondrial glycero phosphate dehydrogenase with a \( K_r \) for human recombinant of approximately 55 \( \mu \)M [17]. In our study we have shown that even though there is no change in the protein level of AMPK after metformin treatment, the decreased ratio of phosphorylation to total eNOS can no longer be reversed by metformin after partial knockdown of total AMPK using siRNA. These data suggest that the ability of metformin to phosphorylate eNOS is at least partially dependent on AMPK. However, decreased AMPK has been associated with increased levels of reactive oxygen species (ROS) and hydrogen peroxide and therefore indirectly a knockdown in AMPK will increase oxidative stress and reduce levels of p-eNOS. For instance, Schuhmacher et al. demonstrated that basal mitochondrial ROS production was increased in AMPK knockout mice compared to WT mice [35]. Oxidative stress and a shift in the cellular redox balance are also associated with a decrease in the bioavailability of NO and in human umbilical vein endothelial cells in which AMPKAlpha1 has been silenced, manganese superoxide dismutase (SOD), catalase and eNOS are reduced [36]. Furthermore, metformin itself may possess anti-oxidative stress activity and in our previous study with MMECs we did report that 50 \( \mu \)M metformin reduced HG-induced increased dihydroethidium fluorescence; however, we did not determine whether this was a direct action of metformin to reduce the generation of ROS [19]. Metformin has also been reported to improve FMD in type 1 diabetic patients, despite an increase in oxidative stress [37]. In contrast, as previously discussed, Zhang et al. demonstrated that a single dose of 500 mg metformin prevented the transient OGT-induced reduction in FMD in non-diabetic patients with primary hypertension thus indicating the effects of metformin are independent of a reduction in insulin resistance [25]. Furthermore, the positive effects of metformin on FMD were accompanied by improved indices of oxidative stress as evidenced by increased levels of superoxide dismutase (SOD) and total antioxidant capacity [25].

Metformin may also have other targets including the peroxisome proliferator-activated receptor \( \delta \), PPAR\( \delta \), and data from studies of ACh-mediated EDV in aortae from mice on a high-fat diet, who were then fed metformin (100 mg/kg) for 7 days, have indicated an improved EDV in the group treated with metformin via cellular mechanisms that were dependent on AMPK and PPAR\( \delta \) [38].
Concentration/dose-dependent, time-dependent as well as tissue-dependent differences may contribute to some of the controversies concerning the cellular mechanisms of action of metformin. For instance, in a study with rat primary hepatocytes the threshold following a 1-h incubation for metformin-mediated enhancement of AMPK activity was 500 μM; at 7 h 50 μM and at 39 h 10 μM [39]. Foret et al. [40] have also presented data that infers an AMPK-independent action of metformin to inhibit hepatic glucoseogenesis. Collectively these findings indicate that the ability of metformin to activate AMPK is both time and concentration dependent and, furthermore, metformin also has cellular actions that are at least partially independent of AMPK.

A potential limitation of our study is that the effects of metformin on EDV were derived from studying mouse aortic tissue whereas the molecular data concerning the effects of high glucose on eNOS were determined in cell culture protocols using MMECs. EDV in mouse microvessels is less dependent on eNOS than is EDV in conduit vessels such as the aorta [41]. However, we have previously reported that it is the NO-mediated component of EDV that is compromised in microvessels from the db/db mouse model of diabetes [42]. Collectively these data suggest that although the cellular mechanism that we have described for the effects of high glucose on EDV in the aorta are comparable to those seen in the microcirculation additional investigations of the effects of metformin on endothelial function in both the macro- and microvasculature are warranted.

In conclusion, although there is clinical data suggesting that metformin improves endothelial function in patients with type 2 diabetes it is, nonetheless, unclear as to whether this is due to a direct action on the endothelium, or due to an indirect action linked to the ability of metformin to reduce insulin resistance [12]. Our study, based on data obtained using the wire myograph technique, demonstrates that metformin, at clinically relevant concentrations, improves EDV in isolated aortae from a mouse model of T2DM as well as reverses the negative effects of HG on EDV in aortae from normal non-diabetic mice that were exposed to HG for 3 h. The time course of the protective effects of metformin on endothelial function that we have observed in these in vitro protocols is comparable to the time course expected for the effects of metformin on OGt, or post-prandial, induced reductions in FMD that have been reported in clinical studies [24,25]. Furthermore, the data from the cell culture protocols with MMECs indicate that the beneficial effects of metformin are mediated by phosphorylation of Akt, but not by changes in the expression of the deacetylase SIRT1. The role of AMPK in mediating the effects of metformin in MMECs remains to be further investigated as, on the one hand, no change in the phosphorylation of AMPK could be detected in MMECs in the presence of metformin, but, on the other hand, a partial knockdown of AMPK resulted in a reduction in the ability of metformin to elevate p-eNOS. Because of the multiple cellular functions of AMPK [43] it is apparent that any modulation of AMPK activity will have an affect on multiple cellular pathways; however, such effects do not necessarily infer a direct link between, as in this study, metformin and AMPK.

Conflict of interest

The authors declare no conflict of interest.

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References


