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

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ABSTRACT

The cellular mechanisms whereby metformin, the first line drug for type 2 diabetes (T2DM), mediates its antidiabetic effects remain elusive, particularly as to whether metformin has a direct protective action on the vasculature. This study was designed to determine if a brief 3-h exposure to metformin protects endothelial function against the effects of hyperglycaemia. We investigated the protective effects of metformin on endothelial-dependent vasodilatation (EDV) in thoracic aortae from T2DM *db*/*db* mice and on high glucose (HG, 40 mM) induced changes in endothelial nitric oxide synthase (eNOS) signaling in mouse microvascular endothelial cells (MMECs) in culture.

Exposure of aortae from *db*+/? non-diabetic control mice to high glucose (HG, 40 mM) containing Krebs for 3-h significantly ($P < 0.05$) reduced acetylcholine (ACh)-induced EDV compared to ACh-induced EDV in aortae maintained in normal glucose (NG, 11 mM) Krebs. The reduction of EDV was partially reversed following a 3-h exposure to 50 μM metformin; metformin also improved ACh-induced EDV in aortae from diabetic *db*/*db* mice. Immunoblot analysis of MMECs cultured in HG versus NG revealed a significant reduction of the ratio of phosphorylated (p-eNOS)/eNOS and p-Akt/Akt, but not the expression of total eNOS or Akt. The 3-h exposure of MMECs to metformin significantly ($P < 0.05$) reversed the HG-induced reduction in phosphorylation of both eNOS and Akt; however, no changes were detected for phosphorylation of AMPK or the expression of SIRT1.

Our data indicate that a 3-h exposure to metformin can reverse/reduce the impact of HG on endothelial function, via mechanisms linked to increased phosphorylation of eNOS and Akt.

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 3–4 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

Abbreviations: ACh, acetylcholine; Akt, protein kinase B; AMPK, 5′ AMP-activated protein kinase; EDV, endothelium-dependent vasodilatation; eNOS, endothelial nitric oxide synthase; HG, high glucose; MMECs, mouse microvascular endothelial cells; mTOR, mammalian target for rapamycin; NG, normal glucose; peNOS, phosphorylated endothelial nitric oxide synthase.

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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 [9,14]. 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 (sirtuin 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 (BH_4), (6R)-5,6,7,8-tetrahydro-L-biopterin sulfate (6R-BH4), but not the inactive stereoisomer (6S)-5,6,7,8-tetrahydro-L-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 BH_4, 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 Habor, ME, USA): (1) The C57BLKS/J (BKS. Cg-Dock7^{m}+/+Lepr^{db}/J (*db*/*db*, 000642)) mouse that develops progressive and sustained hperglycaemia and obesity. (2) C57BL/6J (B6.BKS(D)-Lepr^{db}/J) (*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; $NaHCO_3$, 25; KCl, 4.8; NaH_2PO_4, 1.2; $MgSO_4$, 1.2; Dextrose, [either normal glucose, NG, 11.0 mM, or high glucose, HG, 40 mM]; $CaCl_2$, 1.8; bubbled with 95% O_2 and 5% CO_2. 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