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**MITOCHONDRIAL REACTIVE OXYGEN SPECIES ENHANCE AMPK
ACTIVATION IN THE ENDOTHELIUM OF PATIENTS WITH CORONARY
ARTERY DISEASE AND DIABETES**

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Abstract

The aim of this study was to determine whether the endothelial dysfunction associated with coronary artery disease (CAD) and type 2 diabetes (T2D) is concomitant with elevated mitochondrial reactive oxygen species (mtROS) production in the endothelium and establish if this, in turn, regulates the activity of endothelial AMP-activated protein kinase (AMPK). We investigated endothelial function, mtROS production and AMPK activation in saphenous veins from patients with advanced CAD. Endothelium-dependent vasodilation was impaired in patients with CAD and T2D relative to those with CAD alone. Levels of mitochondrial hydrogen peroxide and activity of AMPK were significantly elevated in primary saphenous vein endothelial cells (HSVECs) from patients with CAD and T2D compared to those from patients with CAD alone. Incubation with the mitochondria-targeted antioxidant, MitoQ₁₀ significantly reduced AMPK activity in HSVECs from patients with CAD and T2D but not in cells from patients with CAD alone. Elevated mtROS production in the endothelium of patients with CAD and T2D increases AMPK activation, supporting a role for the kinase in defence against oxidative stress. Pharmacological activators of AMPK may therefore have enhanced potential in prevention and treatment of endothelial dysfunction in these patients.

Introduction

The prevalence of coronary artery disease (CAD) in patients with diabetes is notably higher than in the general population. Up to a third of patients requiring coronary intervention have diabetes, and outcome is poorer in these patients than in patients without diabetes [1;2]. With the worldwide incidence and prevalence of diabetes increasing, more individuals will be affected by CAD and related complications and further pressure on health systems is expected [3]. The relationship between diabetes and vascular disease is complex and multifactorial [1]. Increased vascular oxidative stress has been proposed as a mechanism responsible for impaired endothelial function in patients with diabetes [4], but it is unknown whether these findings persist in the light of the more aggressive primary and secondary prevention strategies to which these vulnerable patients are subject [3].

Mitochondria are a major source of reactive oxygen species (ROS) production in the vasculature and contribute to oxidative stress and endothelial dysfunction in a range of cardiovascular pathologies, including CAD and type 2 diabetes mellitus (T2D) [5;6]. However, mitochondrial ROS (mtROS) are also of importance in cellular signalling and, at low oxygen (O₂) concentrations, mitochondria of human umbilical vein endothelial cells (HUVECs) have been shown to generate ROS for activation of enzymes such as AMP-activated protein kinase (AMPK) [7].

Human AMPK is a heterotrimeric serine/threonine kinase, consisting of a catalytic α -subunit and regulatory β - and γ -subunits, each of which has two or more isoforms which are encoded by distinct genes and differentially expressed in various tissues [8]. Involved in the regulation of cellular and whole body energy status [9], activation of AMPK requires phosphorylation at Thr172 by an AMPK kinase. Two AMPK kinases have been isolated to date, LKB1 [10] and Ca²⁺/calmodulin-dependent kinase kinase (CaMKK) [11]. It has been proposed that LKB1 activity is constitutive, such that stimuli which increase the AMP/ATP ratio, including hypoxia, hypoglycemia and ischemia, inhibit dephosphorylation at Thr172, permitting phosphorylation and activation of AMPK by LKB1 [10]. In contrast, activation of AMPK by Ca²⁺ is AMP-independent and mediated by CaMKK [11;12]. More recently, AMPK activation via a ROS-mediated mechanism has been described [7;13-18].

Proposed as a candidate target for therapeutic intervention in endothelial dysfunction, AMPK stimulates endothelial nitric oxide (NO) synthase (eNOS), leading to NO production in cultured endothelial cells [19]. Furthermore, stimulation of endothelial AMPK is reported to attenuate proinflammatory signalling [20;21] and there is accumulating evidence to suggest a role for the kinase in defence against oxidative stress in the endothelium [8;22;23].

The current study was designed to test the hypothesis that the endothelial dysfunction associated with CAD and T2D occurs in parallel with increased mtROS production in the endothelium and that this, in turn, regulates endothelial AMPK activity.

Methods

Detailed methods can be found in Supplemental Material.

Subjects

We recruited 79 volunteers with severe CAD from cardiothoracic pre-operative clinics. A blood sample was taken after three hours of fasting on the day of admission for coronary artery bypass graft (CABG) surgery. Twenty three volunteers had a history of T2D. Type 2 diabetes was defined as having fasting venous blood glucose ≥ 6.1 mmol/L or ≥ 10 mmol/L two hours post oral glucose load (75 g). We also recruited 19 control volunteers free of evidence of CAD who were undergoing surgery for the removal of varicose veins. For these volunteers, a blood sample was taken following three hours of fasting one to two weeks after surgery. The study complies with the Declaration of Helsinki and was approved by the local ethics committee. All participants gave written informed consent.

Preparation of vascular tissue

Surplus portions of freshly harvested saphenous veins from volunteers undergoing CABG surgery or elective varicose vein removal were stored in sterile saline solution. Maximum storage time was 2 hours. Only non-varicosed portions of veins from control patients, as identified by the surgical team, were utilised. Samples were taken to the laboratory and cleaned of excess connective tissue. Endothelial cells were isolated from sections of samples on the day of surgery and the remainder of samples stored at 4°C in a Krebs Hepes buffer (118 mmol/L NaCl, 10 mmol/L Hepes-NaOH, pH 7.4, 25 mmol/L NaHCO₃, 4.7 mmol/L KCl, 1.2 mmol/L MgSO₄, 1.2 mmol/L KH₂PO₄, 11 mmol/L glucose, 10 µmol/L indomethacin, 50 µmol/L EDTA) for study of endothelial function the following day.

Assessment of Endothelial Function

Three millimeter rings of saphenous vein were studied in organ bath chambers as previously described [24]. Vessels were constricted with phenylephrine (3 µmol/L) and relaxation in response to the calcium ionophore A23187 (0.01-10 µmol/L) was studied. Maximum relaxation was calculated and expressed as a percentage of constriction to phenylephrine.

Cell Culture

Human saphenous vein endothelial cells (HSVECs) were isolated on the day of surgery by standard collagenase digestion based on a modified version of the protocol described by Jaffe and colleagues [25]. Cells were cultured in complete Large Vessel Endothelial Cell Basal Medium (TCS Cellworks Ltd), supplemented with 20% (v/v) fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mmol/L L-glutamine. Cells were used at passage 3 and all experimental procedures were carried out when cells were ~80% confluent.

For immunofluorescent staining of von Willebrand factor (vWF), passage 3 HSVECs were harvested and plated onto sterile coverslips before being fixed in 4% (w/v) paraformaldehyde. Cells were then incubated with mouse anti-vWF primary antibody (1:50 in 20% (v/v) goat serum/PBS) followed by goat-anti-mouse IgG-fluorescein isothiocyanate conjugate secondary antibody (Dako, 1:200 in 20% (v/v) goat serum/PBS). Coverslips were subsequently mounted in Vectashield (Vector Laboratories Inc.), containing propidium iodide

for nucleic counter-staining, and cells visualized under a fluorescence microscope (Olympus BX40).

Assessment of Mitochondrial Hydrogen Peroxide Production

Measurement of HSVEC mitochondrial hydrogen peroxide (H₂O₂) production was carried out using the mitochondria-targeted H₂O₂ mass spectrometry probe, MitoB, as previously described for cell culture experiments [26]. Where required, HSVECs were incubated at 37°C in medium supplemented with 1 µmol/L MitoQ₁₀ for 1 hour prior to washing in PBS and incubation in medium supplemented with 5 µmol/L MitoB for 6 hours.

mRNA Expression

Total RNA was extracted from HSVECs using the RNeasy[®] Mini Kit (QIAGEN) and quantified (NanoDrop ND-100 Spectrophotometer). cDNA was synthesized from 1 µg of DNase-treated (TURBO DNA-free[™], Ambion) total RNA (TaqMan[®] Reverse Transcription Reagents, Applied Biosystems). Relative quantitation of *PRKAA1* expression, relative to *GAPDH*, was calculated using the comparative ($\Delta\Delta C_t$) method [27].

AMPK Activity Assay

HSVECs were serum-starved overnight before being incubated for 1 hour at 37°C in Krebs Ringer Hepes buffer (119 mmol/L NaCl, 20 mmol/L Hepes, pH 7.4, 5 mmol/L NaHCO₃, 4.7 mmol/L KCl, 1.3 mmol/L CaCl₂, 1.2 mmol/L MgSO₄, 1 mmol/L KH₂PO₄, 0.1 mmol/L l-arginine, 5 mmol/L glucose) in the presence of 1 µmol/L MitoQ₁₀, 1 µmol/L decyl triphenylphosphonium (TPP) bromide (DTPP) and 2 mmol/L 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) where required. Cell lysates were prepared and AMPK immunoprecipitated and assayed using the SAMS peptide as previously described [28].

Statistical Analyses

For clinical data and measurements in whole vessels, continuous data are given as mean ± standard deviation or median (interquartile range) unless otherwise indicated. Values stated are means ± standard error of the mean (SEM) for cellular data. For comparisons of a continuous variable between 2 experimental groups, paired and unpaired Student's t-test and Mann-Whitney U-tests were applied as appropriate. For comparisons of a continuous variable in data sets with more than 2 groups, analysis of variance (ANOVA) was applied, followed by the Tukey's post-hoc test for all possible pairwise comparisons. Categorical data were analyzed by Fisher's exact test. A *P*-value of less than 0.05 (two tailed) was considered significant.

Results

Characteristics of Study Participants

Demographic and clinical characteristics of patients and control subjects are given in Table 1. As expected, patients with CAD were older and more likely to be on cardiovascular medication than control subjects. Total and LDL cholesterol levels were lower in patients with CAD compared to control subjects, consistent with lipid lowering therapy in the patient

group. HDL cholesterol levels were significantly greater in control subjects compared to patients. Patients with T2D had greater body mass index and greater percentage of glycosylated hemoglobin A_{1c} but no other significant differences to patients without diabetes were observed. Ten of the twenty three patients with T2D (43%) were treated with metformin.

Endothelial Function

Endothelium-dependent relaxation was impaired in vessels of patients with CAD compared to those obtained from control subjects (maximum relaxation to A23187, 43±16 vs 62±16%; *P*=0.001; Figure 1A and C). Patients with CAD and T2D had significantly reduced endothelium dependent relaxation compared to those with CAD alone (maximum relaxation to A23187, 34±11 vs 47±16%; *P*=0.008; Figure 1B and C). Data concerning vasorelaxation to carbachol (Supplementary Figure 1) and endothelium-dependence of the relaxation to carbachol and A23187 (Supplementary Figures 1 and 2) in saphenous vein are available in the online supplement.

Mitochondrial Hydrogen Peroxide Production and Expression of Mitochondrial Superoxide Dismutase in Primary HSVECs

It has been demonstrated that mtROS contribute to the oxidative stress and endothelial dysfunction characteristic of CVD [5;6]. To establish if increased levels of mitochondrially-produced H₂O₂ were concomitant with the endothelial dysfunction observed in vessels from patients with CAD, we investigated mitochondrial H₂O₂ production in primary HSVECs (Figure 2A) isolated from these patients and control subjects.

Significantly greater mitochondrial H₂O₂ production was noted in HSVECs from CAD patients with T2D relative to those from patients with CAD alone (MitoP/MitoB ratio, 0.054±0.003 vs 0.042±0.003; *P*=0.02; Figure 2B). This finding was independent of mitochondria number (Supplementary Figure 3). The mitochondria-targeted antioxidant, MitoQ₁₀ had no significant effect on basal mitochondrial H₂O₂ production in HSVECs from patients with CAD alone (MitoP/MitoB ratio, 0.042±0.003 vs 0.044±0.004; *P*=0.61; Figure 2B), patients with CAD and T2D (MitoP/MitoB ratio, 0.054±0.003 vs 0.052±0.004; *P*=0.35; Figure 2B) and control subjects (MitoP/MitoB ratio, 0.043±0.006 vs 0.045±0.003, *P*=0.58; Figure 2B).

AMPK Activation in Primary HSVECs

AMPK has recently been implicated in attenuation of endothelial oxidative stress [8;22;23]. In addition, the kinase has been shown to be activated in response to a variety of ROS, including H₂O₂ [13;18;29].

As shown in Figure 3A, activity of AMPK was increased in cells from CAD patients as compared to those from control subjects (0.045±0.007 vs 0.022±0.006 nmol/min/mg; *P*=0.05). On comparison with control subjects, a greater increase in AMPK activity was observed in HSVECs from patients with CAD and T2D (0.062±0.011 vs 0.022±0.006 nmol/min/mg; *P*=0.01; Figure 3A) than in cells from patients with CAD alone (0.031±0.005 vs 0.022±0.006 nmol/min/mg; *P*=0.26; Figure 3A). AMPK activity was significantly higher in HSVECs from patients with CAD and T2D than in those from patients with CAD alone (0.062±0.011 vs 0.031±0.005; *P*=0.01; Figure 3A).

No change in mRNA expression of *PRKAA1*, encoding the AMPK- α 1 catalytic subunit, was observed between patient groups (Figure 3B), consistent with modulation of AMPK activity via post-translational modification. In addition, there was no significant difference in protein expression of AMPK α 1 (Supplementary Figure 4) or the upstream AMPK kinase, LKB1 (Supplementary Figure 5), as assessed by immunoblotting.

AMPK Substrate Phosphorylation in Primary HSVECs

Given the increased AMPK activity observed in HSVECs from patients with CAD and the poorer endothelial function of these subjects, phosphorylation of eNOS, an AMPK substrate, was investigated in cells from these CAD patients by means of immunoblotting (Figure 4A). Densitometric analysis revealed significantly lower basal eNOS phosphorylation in HSVECs from CAD patients with T2D (Figure 4B) despite the increased basal AMPK activity in these cells (Figure 3A). Incubation of HSVECs with AICAR, an artificial activator of AMPK [30], appeared to result in increased eNOS phosphorylation in cells from patients with CAD alone, although results were not significant (Figure 4B). However, AICAR treatment failed to stimulate eNOS phosphorylation in HSVECs from those CAD patients with T2D (Figure 4B).

Mitochondrial ROS-Mediated AMPK Activation in Primary HSVECs

Vascular endothelial cells have been reported to be highly glycolytic [7]. In support of this, HSVEC ATP synthesis was largely the result of glycolysis, as assessed by the relative effects of 2-deoxy-D-glucose (2DG) and rotenone (Supplementary Figure 6).

An alternative, potentially significant role for endothelial mitochondria is the generation of ROS for signaling purposes [7]. The contribution of mtROS to AMPK activation was investigated by treating HSVECs from CAD patients with MitoQ₁₀ and subsequently assaying AMPK activity. To control for non-specific effects of MitoQ₁₀, cells were treated with the non-active control compound, DTPP [31]. Exposing HSVECs to MitoQ₁₀ resulted in a reduction in AMPK activity (Figure 5). The MitoQ₁₀-mediated decrease in AMPK activity was greater in HSVECs from CAD patients with T2D (0.056 ± 0.004 vs 0.009 ± 0.01 nmol/min/mg; $P=0.02$; Figure 5B) than in those from patients with CAD alone (0.035 ± 0.005 vs 0.014 ± 0.0002 nmol/min/mg; $P=0.06$; Figure 5A).

Treating HSVECs from CAD patients with AICAR resulted in a significant increase in kinase activity in cells from patients with CAD alone (0.035 ± 0.005 vs 0.095 ± 0.01 nmol/min/mg; $P=0.05$; Figure 5A).

Discussion

Increased vascular oxidative stress has been proposed as one potential mechanism underlying endothelial dysfunction in patients with CAD and T2D [4]. The present study involved investigation of endothelial function and molecular determinants of oxidative stress in human saphenous veins. Previous studies have demonstrated human arterial and venous levels of oxidative stress are closely related [32;33], such that results should not be affected by the choice of vessel. Herein we report impaired endothelium-dependent relaxation in vessels from patients with advanced CAD, with poorer endothelial function was observed in those CAD patients with the additional cardiovascular risk factor, T2D. To investigate the

molecular basis for the impaired endothelium-dependent relaxation in vessels from patients with CAD and T2D, endothelial cells were isolated from vascular tissue. Endothelial dysfunction was observed to be maintained in culture with significantly lower levels of basal eNOS Ser1177 in cells from CAD patients with T2D as compared to those with CAD alone. As isolated cells were maintained in culture for several weeks prior to investigation, it seemed likely that the effects of pharmacological treatments (including metformin, a known activator of AMPK [8]) would be lost, allowing more accurate insight into molecular determinants of impaired endothelial function and potentially causative oxidative stress.

The mitochondrial electron transport chain has been identified as a major source of ROS in the vasculature, contributing to the oxidative stress and endothelial dysfunction characteristic of CAD and T2D [5;6]. As such, following isolation and characterization of HSVECs, we investigated cellular mitochondrial H₂O₂ production and observed an increase in cells from patients with CAD and T2D which was not due to an increase in mitochondria number. Known to induce endothelial dysfunction [34], increased levels of mitochondrially-produced H₂O₂ may therefore have a causal role in the significantly impaired vasorelaxation of CAD patients with T2D.

Vascular endothelial cells are recognised as being highly glycolytic and we have confirmed this to be the case for HSVECs. A potential reason for the favouring of glycolysis by endothelial cells has been proposed by Quintero and colleagues [7], whereby mitochondria are not preferentially used bioenergetically in these cells, allowing them to function primarily in the generation of ROS for signalling purposes, resulting in activation of enzymes, including AMPK.

Traditionally associated with maintenance of cellular energy homeostasis, it is well documented that AMPK is activated in response to the increased AMP/ATP ratio characteristic of hypoxic stress. With regard to endothelial AMPK specifically, phosphorylation of the kinase has been observed at low O₂ concentrations in HUVECs but is undetectable at 21% O₂ or 'normoxia' [7]. However, it has been suggested that kinase activation at low O₂ concentrations may occur via a mechanism that is independent of altered nucleotide levels but is mtROS-mediated [7;13]. Interestingly, we were able to assay AMPK activity in HSVECs isolated from patients with CAD and cultured under normoxic conditions, indicating cardiovascular disease phenotype could be linked to enzyme activation. Indeed, at 21% O₂, HSVEC AMPK activity was significantly greater in cells from patients with CAD relative to control subjects. On stratifying CAD patients according to the presence of T2D, we found AMPK activity to be significantly increased in the endothelium of patients with CAD and T2D as compared to that of patients with CAD alone, despite no change in AMPK α 1 expression or difference in levels of the upstream AMPK kinase, LKB1. Incubating HSVECs with AICAR, an artificial, ROS-independent activator of AMPK, known to stimulate the kinase in endothelial cells [19], resulted in a significant increase in AMPK activity in cells from patients with CAD alone but not in cells from those patients with CAD and T2D whose basal AMPK activity approached maximal levels.

Given the elevated mitochondrial H₂O₂ production in HSVECs from patients with CAD and T2D, in addition to the glycolytic nature of the cells, it seemed likely that enhanced endothelial AMPK activation was occurring in an mtROS-mediated manner in these patients. In order to test this hypothesis, we treated HSVECs isolated from CAD patients with the mitochondria-targeted antioxidant, MitoQ₁₀ which has been shown to prevent oxidative damage in endothelial cells *in vitro* [35]. Our findings demonstrated a significant decrease in

AMPK activation on treatment with MitoQ₁₀ in cells from those patients with T2D. The non-antioxidant control for MitoQ₁₀, DTPP, had no effect on AMPK activity in a parallel experiment, indicating results can be attributed to the antioxidant action of MitoQ₁₀ specifically. The same effect was not seen in cells from CAD patients without T2D.

Taken together, our findings indicate a novel, mtROS-mediated activation of AMPK in the endothelium of patients with CAD and T2D. In terms of mtROS likely to be involved in activation of the kinase, in concordance with results presented here, a role for H₂O₂ has been reported [13;18;29]. However, MitoQ₁₀ does not act by directly lowering H₂O₂ production [36], confirmed via investigation of HSVEC mitochondrial H₂O₂ levels in the presence and absence of the antioxidant. Therefore, the signal emanating from mitochondria and activating AMPK in the endothelium of patients with CAD and T2D is unlikely to be H₂O₂ itself, but rather a downstream radical with which MitoQ₁₀ reacts. Such radicals include lipid peroxidation products, generated on oxidation of mitochondrial lipids by H₂O₂.

Recent studies suggest AMPK activation improves endothelial function by counteracting oxidative stress in the endothelium. Indeed, the kinase suppresses NAD(P)H oxidase and ROS production in endothelial cells [23] and stimulates NO production by eNOS, inducing endothelium-dependent vasodilation [37]. In addition, AMPK activation attenuates proinflammatory signalling and monocyte adhesion to the endothelium [20]. Furthermore, metformin, known to exert a portion of its effect through AMPK, has been reported to decrease intracellular production of mtROS in aortic endothelial cells [38], while activation of AMPK has been observed to reduce hyperglycemia-induced mtROS production by induction of the endogenous mitochondrial antioxidant, superoxide dismutase 2 (SOD2) in HUVECs [39]. Similarly, Colombo and Moncada [22] have demonstrated that endothelial AMPK α 1 is responsible for the expression of a number of genes involved in antioxidant defence, including *SOD2*.

However, our observation that eNOS Ser1177 phosphorylation is significantly reduced in cells from CAD patients with T2D implies that elevated AMPK activity alone against a background of type 2 diabetes is not sufficient to increase eNOS phosphorylation at this residue. It could perhaps be the case that a phosphatase is activated in these patients or that this additional CVD risk factor results in eNOS being regulated in an alternative manner, rendering it much more difficult to phosphorylate. Interestingly, AMPK has recently been shown to phosphorylate eNOS at the additional residue, Ser633 and ablation of AMPK α 2 was observed to be sufficient to inhibit atorvastatin-stimulated eNOS phosphorylation at both this residue and Ser1177, despite AMPK α 1 being the principle isoform in terms of total cellular activity [40]. Consequently, it is feasible that the increased AMPK activity observed in CAD patients with T2D reflects increased AMPK α 1 activity alone and that AMPK α 2 activity is required for eNOS Ser1177 phosphorylation. In accordance with our findings, Wang *et al.* [41] have reported that HUVECs subject to low glucose concentrations demonstrate reduced NO bioavailability associated with increased mtROS production and AMPK activation, yet this fails to stimulate eNOS phosphorylation.

In the endothelium of patients with CAD and T2D, AMPK may be part of a feedback or adaptive mechanism, wherein elevated mtROS production results in activation of AMPK which, in turn, stimulates protective responses which may contribute towards, but are not solely responsible for, increasing NO bioavailability and attenuating endothelial dysfunction. Although not investigated here, the mt-ROS-activated AMPK may also induce SOD2

activity, thus counteracting mitochondrial oxidative stress and generating H₂O₂ for further kinase activation and perpetuation of the cycle (Figure 6).

In summary, our results demonstrate elevated mtROS production in the endothelium of patients with CAD and T2D, suggesting mitochondria contribute to the more severe endothelial dysfunction observed in these patients. In addition, we have shown a novel, mtROS-mediated mechanism for AMPK activation in the endothelium of patients with CAD and T2D. While this novel AMPK activation supports a role for the kinase in counteraction of oxidative stress, we demonstrate that increased AMPK activity does not simply translate to increased eNOS phosphorylation in these subjects and is therefore not sufficient to attenuate the more severe endothelial dysfunction characteristic of diabetic patients.

Clinical Perspectives

Increasing evidence exists to suggest that elevated mitochondrial ROS (mtROS) production may contribute to poorer endothelial function in patients with CAD and diabetes as compared to non-diabetic patients. We have demonstrated significantly increased levels of mtROS in primary endothelial cells from patients with CAD and diabetes and a concomitant increase in endothelial AMPK activity. We show that this enhanced kinase activity is, however, insufficient to increase eNOS phosphorylation. As such, it seems unlikely that treatment with well-tolerated pharmacological activators of AMPK will prove adequate in attenuating endothelial dysfunction in T2D patients.

Author Contributions

Patients were recruited by CD and JAD. RMM, IPS, WHM, AL and CAH performed the experiments. All authors analysed and interpreted the data. The paper was drafted by RMM, IPS and CD with critical input from all authors.

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Conflicts of Interest

One of the authors (MPM) is a consultant for, and holds stock in a company (Antipodean Pharmaceuticals Inc.) commercialising MitoQ₁₀.

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Figure Legends

Figure 1. Vasorelaxation in human saphenous veins. Maximum relaxation of saphenous vein to the endothelium-dependent vasodilator, calcium ionophore A23187 (A, B). Panel (A) shows comparisons between patients with CAD (n = 49) and control subjects (n = 10) and panel (B) comparisons between patients with CAD and type 2 diabetes (CAD T2D, n = 14) and patients with CAD alone (CAD ND, n = 35). Panel (C) shows the dose-response curves to A23187 for the three groups (means and standard errors).

Figure 2. Mitochondrial hydrogen peroxide production in HSVECs. (A) Representative photomicrographs showing HSVECs in primary culture. Endothelial cells were successfully isolated, as determined by their characteristic cobblestone morphology (left hand panel) and positive staining (green) for von Willebrand factor (middle and right hand panels). (B) HSVEC mitochondrial hydrogen peroxide production was determined using the mitochondria-targeted mass spectrometry probe, MitoB, in both the presence and absence of the mitochondria-targeted antioxidant, MitoQ₁₀. * $P < 0.05$ vs CAD ND. CAD ND, CAD patients without type 2 diabetes (n = 4); CAD T2D, CAD patients with type 2 diabetes (n = 4); controls, control subjects (n = 3).

Figure 3. AMPK activation in HSVECs. (A) Total AMPK activity in immunoprecipitates from HSVEC lysates cultured at 21% O₂. * $P < 0.05$ and † $P < 0.01$ vs control subjects, ‡ $P < 0.01$ vs CAD ND. (B) HSVEC *PRKAA1* mRNA expression relative to *GAPDH*. RQ, relative quantitation; CAD ND, CAD patients without type 2 diabetes; CAD T2D, CAD patients with type 2 diabetes.

Figure 4. Comparison of eNOS Ser1177 phosphorylation in HSVECs. HSVECs were isolated from CAD patients with (CAD T2D, n=5) and without (CAD ND, n=5) type 2 diabetes and eNOS Ser1177 phosphorylation in the presence or absence of AICAR assessed by western blotting of cell lysates. (A) Representative immunoblots from 2 CAD patients with and 2 CAD patients without T2D. (B) Densitometric analysis of the phosphorylated eNOS (P-eNOS S1177):eNOS ratio relative to an internal standard lysate. * $P < 0.05$ vs CAD T2D (vehicle), † $P < 0.05$ vs CAD T2D (AICAR).

Figure 5. Effect of mitochondrial-ROS on AMPK activation in HSVECs. (A) Cells from CAD patients without type 2 diabetes (CAD ND, n=5) and (B) with type 2 diabetes (CAD T2D, n=5) were incubated in the presence (+) of MitoQ₁₀, DTPP and AICAR. Total AMPK was immunoprecipitated from lysates which were then assayed for AMPK activity. * $P = 0.05$ vs basal value for CAD ND, † $P < 0.05$ vs basal value for CAD T2D, ‡ $P < 0.01$ vs + DTPP value for CAD T2D.

Figure 6. Proposed mechanism of mitochondrial reactive oxygen species-mediated AMPK activation in endothelial cells. Results suggest a potentially mitochondrial reactive oxygen species (mtROS)-mediated increase in AMPK activity in patients with coronary artery disease and type 2 diabetes. The mtROS in question are likely to be downstream derivatives of hydrogen peroxide (H₂O₂), such as lipid peroxidation products. AMPK may therefore be part of a feedback or adaptive mechanism with a role in defence against oxidative stress in the endothelium, attenuating proinflammatory signaling and regulating expression of antioxidant genes, including *SOD2*. However, increased kinase activity does not appear to be sufficient to stimulate activation of endothelial nitric oxide synthase (eNOS), in patients with type 2 diabetes.

Table 1. Characteristics of the study cohort.

	CAD		<i>P</i> -value Diabetes vs no diabetes	Controls n=19	<i>P</i> -value CAD vs controls
	Diabetes n=23	No diabetes n=56			
Age (years)	65±11	65±9	0.961	44±19	<0.001
Sex (m:f)	20/3	45/11	0.747	8/11	0.001
BMI (kg/m ²)	31.7±5.2	28.6±4.7	0.026	26.5±1.9	0.004
SBP (mmHg)	138±22	138±26	0.897	132±23	0.471
DBP (mmHg)	73±13	80±11	0.065	84±12	0.162
Total cholesterol (mmol/L)	3.95±1.10	4.11±0.99	0.542	4.96±0.99	0.029
LDL cholesterol (mmol/L)	1.80±0.83	2.00±0.78	0.314	2.69±1.11	0.024
HDL cholesterol (mmol/L)	1.11±0.22	1.17±0.27	0.366	1.72±0.30	<0.001
Triglycerides (mmol/L)	2.54±1.97	2.04±0.97	0.262	1.17±0.47	0.052
CRP (mg/L)	2.5±2.9	5.0±10.5	0.280	3.5±4.2	0.843
HbA _{1c} (%)	7.2±1.3	5.6±0.4	<0.001	5.4±0.2	0.111
Active smoking (y/n)	2/22	2/54	0.579	2/17	0.324
ACEI/ARB (y/n)	18/5	32/24	0.122	1/18	<0.001
Statin (y/n)	21/2	53/3	0.625	2/17	<0.001
Metformin (y/n)	10/13	0/56	<0.001	0/19	<0.001

Continuous data are given as mean ± standard deviation irrespective of distribution or skewness. *P*-values, however, derive from Student's t-test or Mann-Whitney U-test as appropriate. Comparison between categorical data was performed using Fisher's exact test. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoprotein; HDL, high density lipoprotein; CRP, C-reactive protein; HbA_{1c}, glycosylated haemoglobin; A_{1c}, ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.

Figure 1

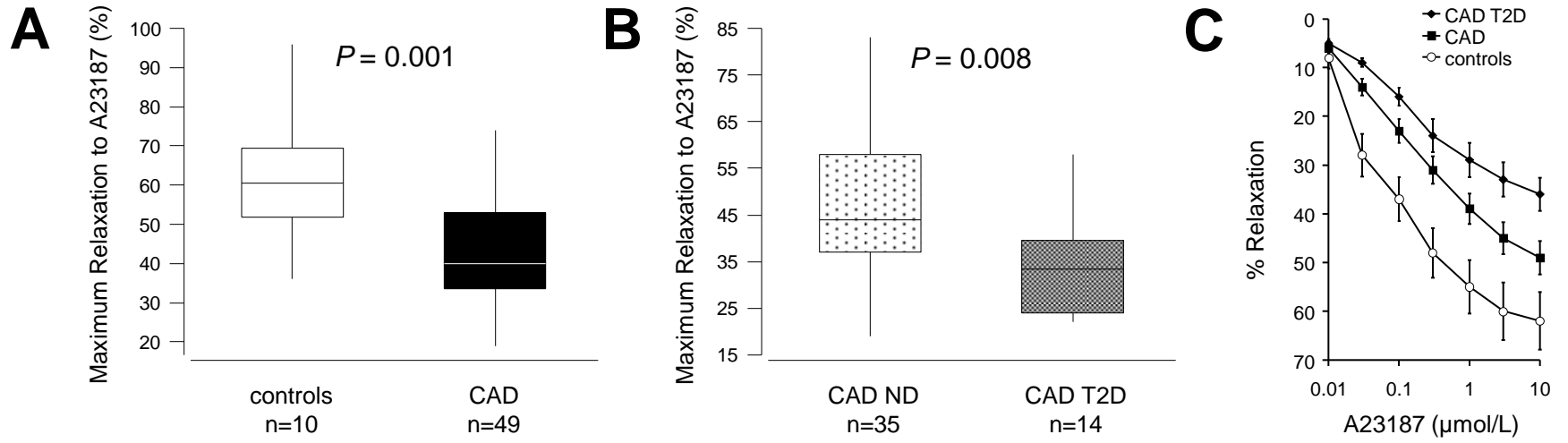


Figure 2

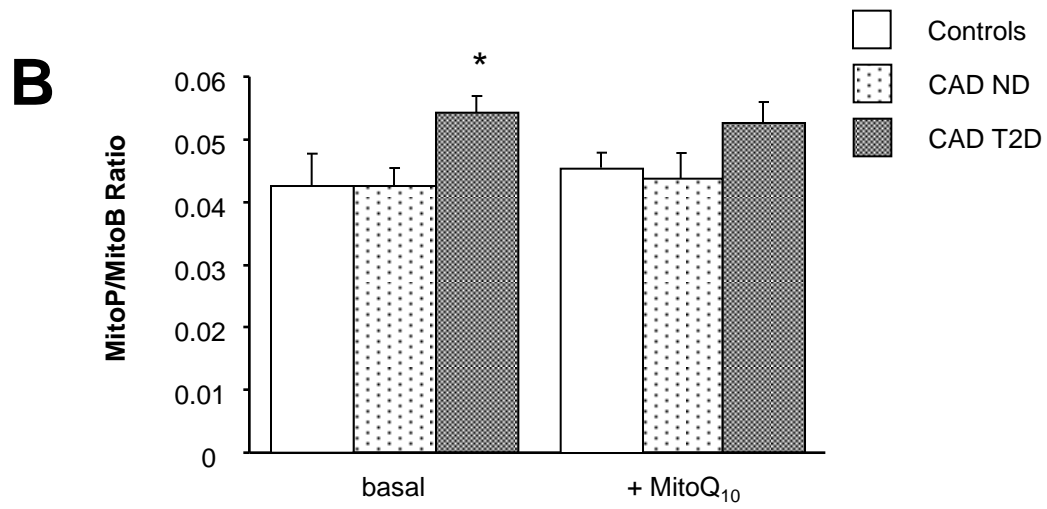
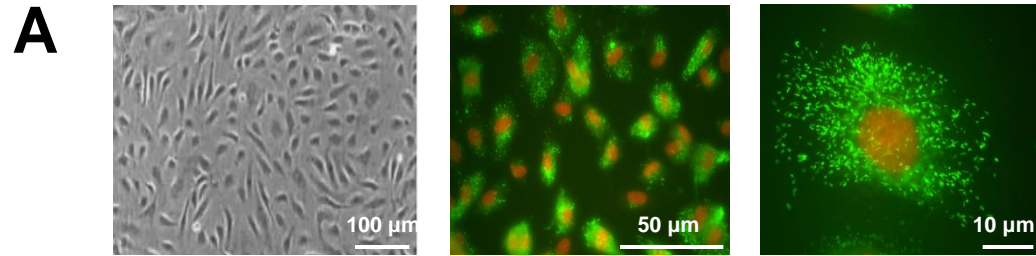


Figure 3

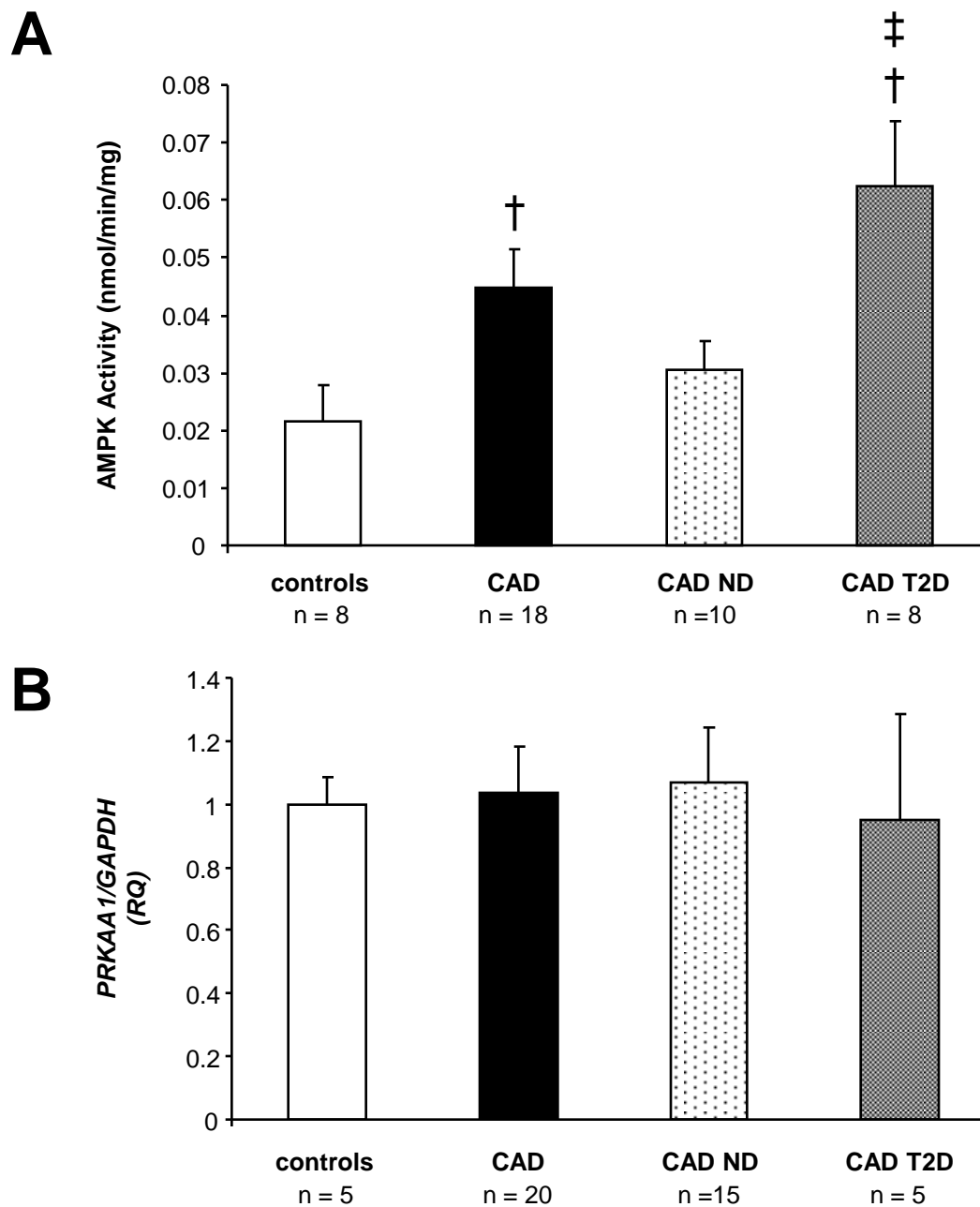


Figure 4

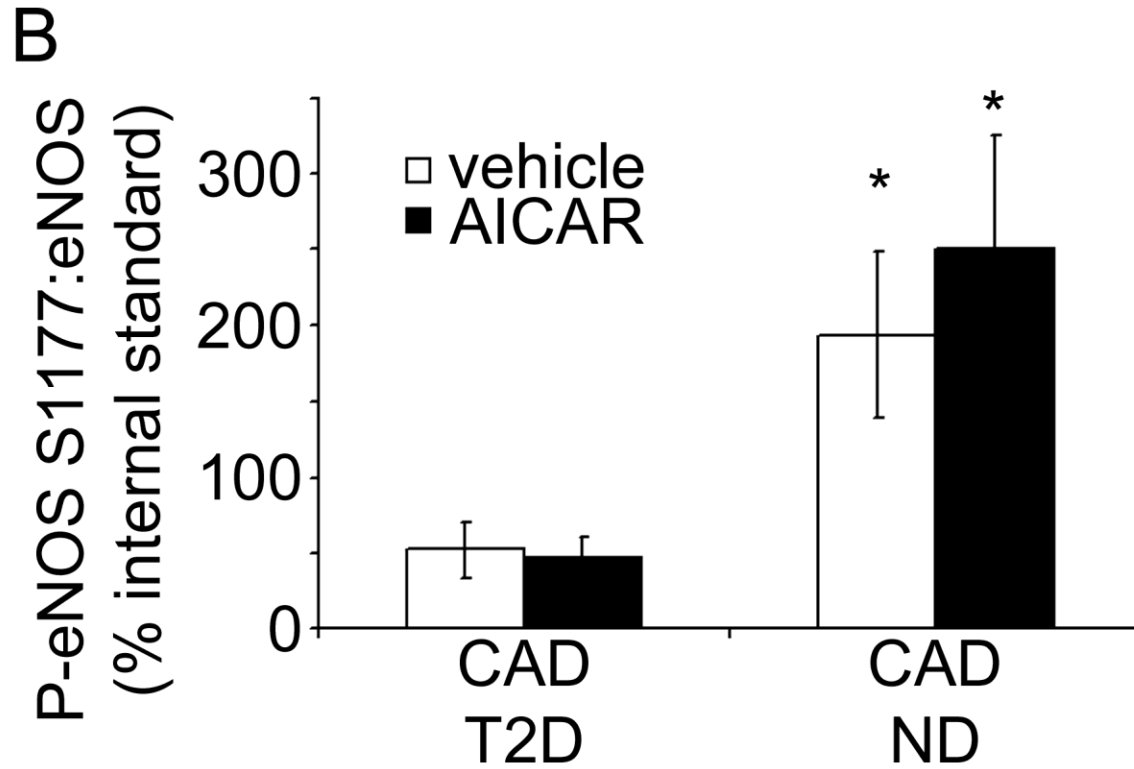
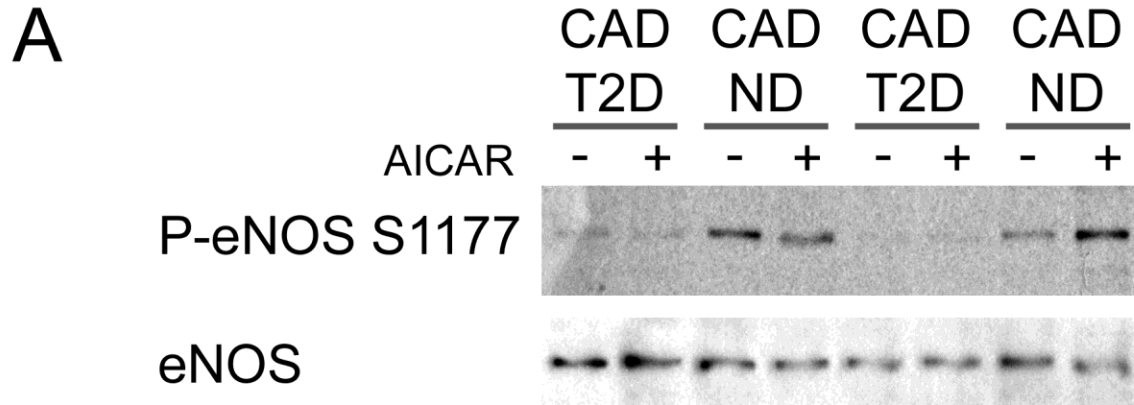
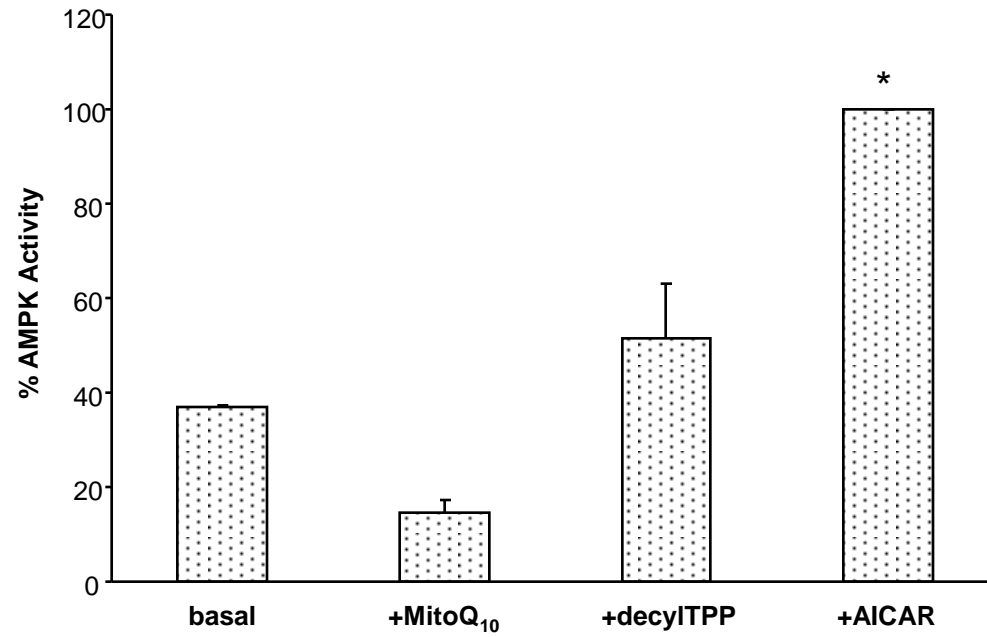


Figure 5

A



B

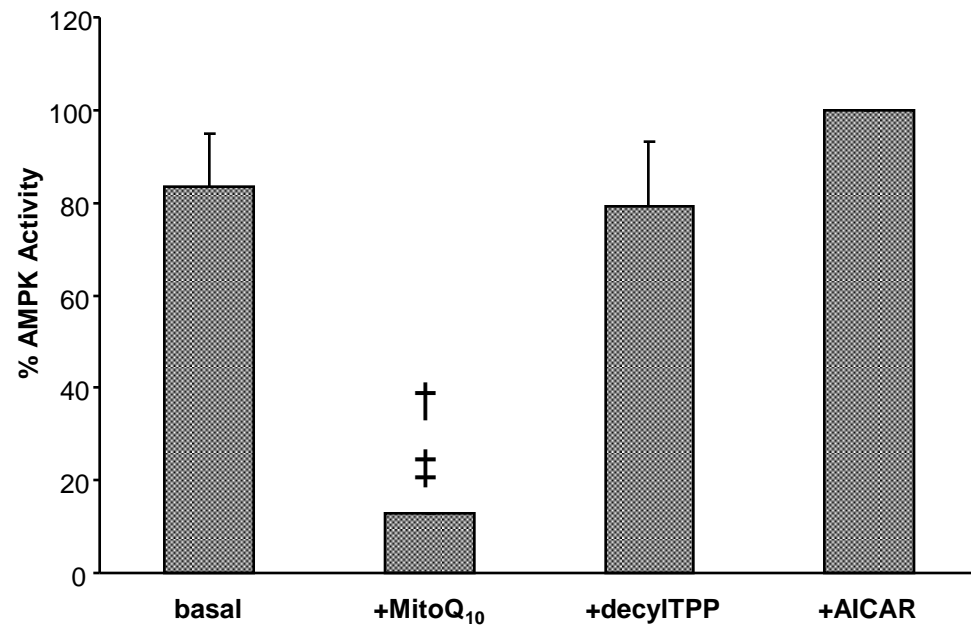
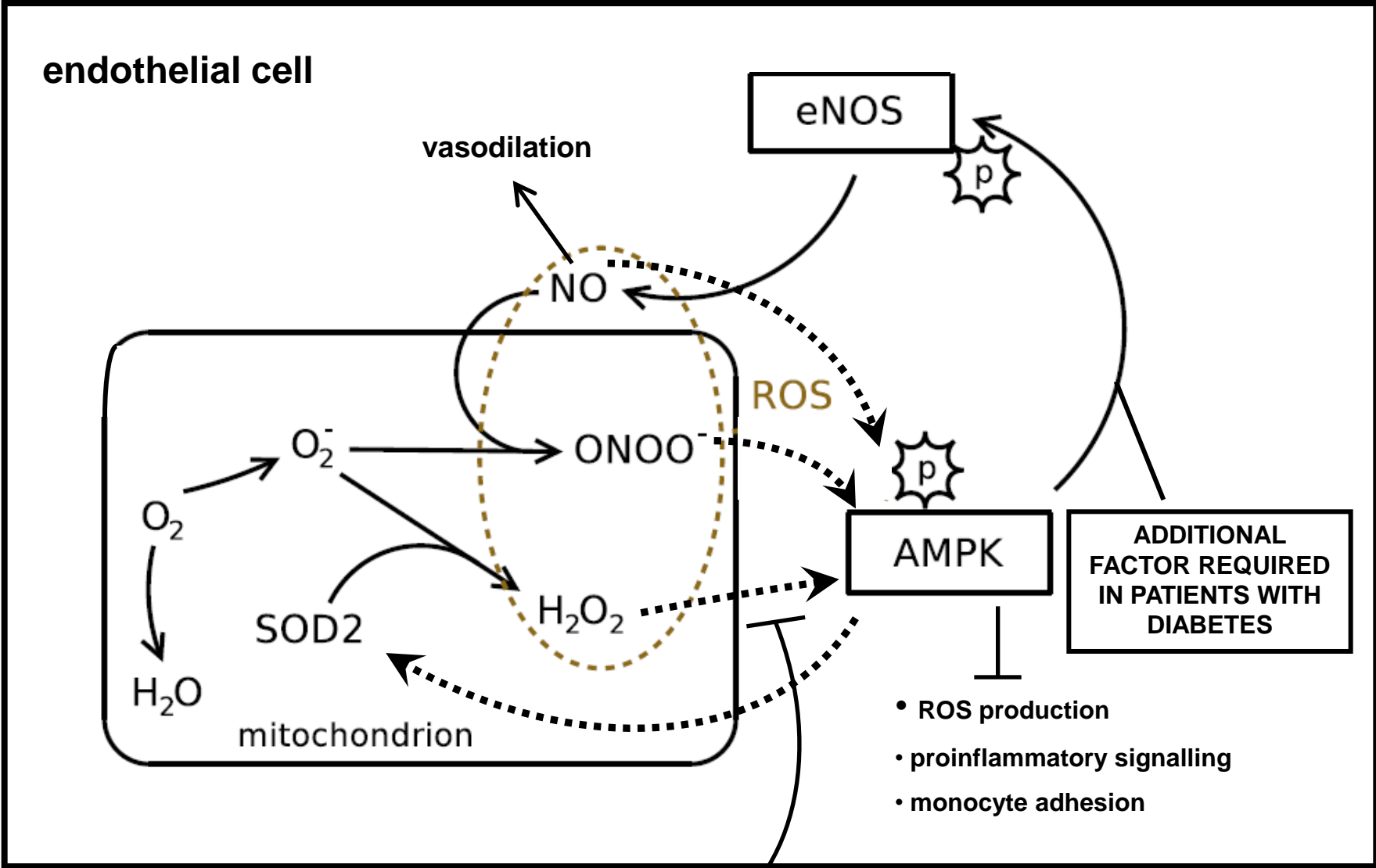


Figure 6



.....➔ suggested mechanism

MITOCHONDRIAL REACTIVE OXYGEN SPECIES ENHANCE AMPK ACTIVATION IN THE ENDOTHELIUM OF PATIENTS WITH CORONARY ARTERY DISEASE AND DIABETES

Supplemental Material

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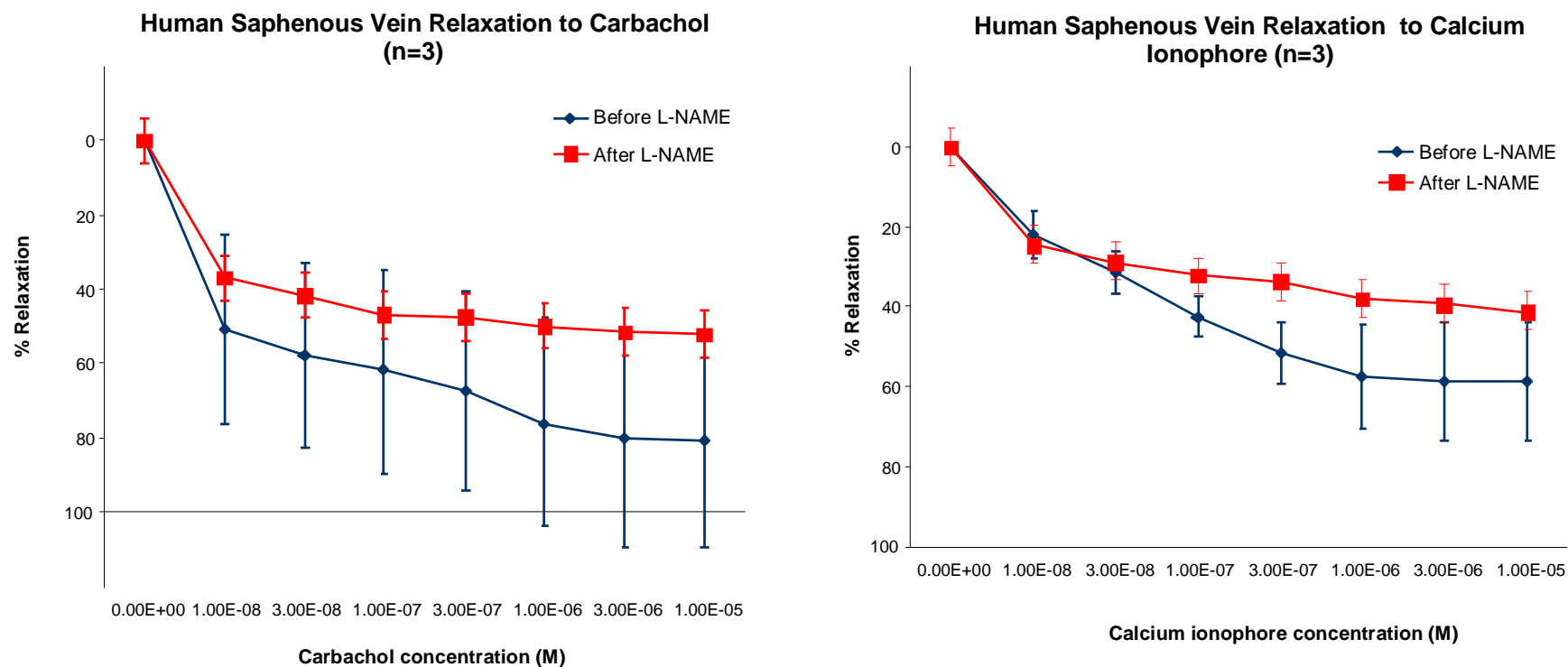
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Detailed Methods

Reagents

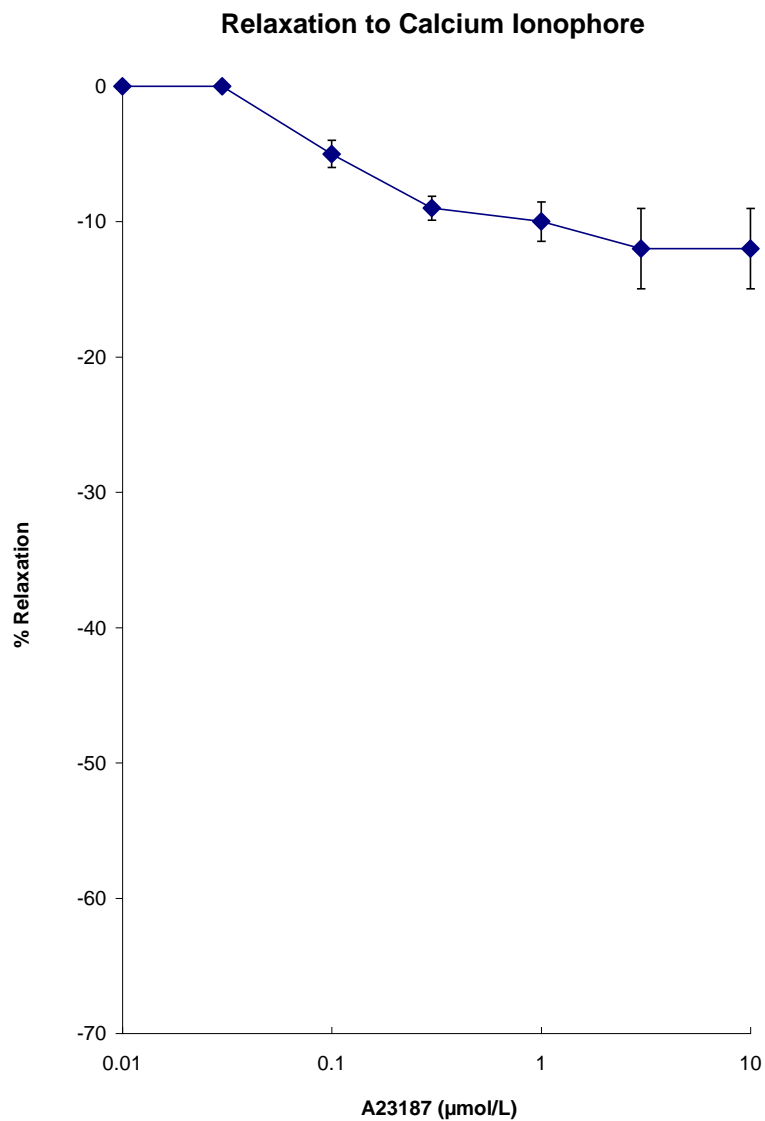
Endothelial cell culture medium was obtained from TCS Cellworks (Botolph Claydon, Bucks, U.K.) and PromoCell (Heidelberg, Germany). Mouse anti-von Willebrand factor (vWF) antibody was obtained from Dako. Rabbit anti-phospho-AMPK- α_1 -Thr-172 and anti-LKB1 antibodies were from Cell Signaling Technology (Beverly, MA), and sheep anti-AMPK α_1 antibody was a generous gift from Professor D.G. Hardie, University of Dundee, Dundee, U.K. 2-deoxy-D-glucose (2DG) and rotenone were purchased from Sigma. Custom TaqMan[®] Gene Expression Assays for *PRKAA1* (Hs01562315_m1), *MT-CYB* (Hs02596867_s1) and *HBB* (Hs00758889_s1), and TaqMan[®] Endogenous Control, *GAPDH* (4326317E) were from Applied Biosystems. 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) was purchased from Toronto Research Chemicals (Toronto, Canada) and MitoB, MitoQ₁₀ mesylate and decyl triphenylphosphonium (TPP) bromide (decyl TPP) were from Prof. Robin Smith, Department of Chemistry, University of Otago, Dunedin, New Zealand. SAMS peptide (HMRSAMSGHLHLVKRR) was synthesized by Pepceuticals Ltd (Nottingham, U.K.).

Supplementary Figure 1



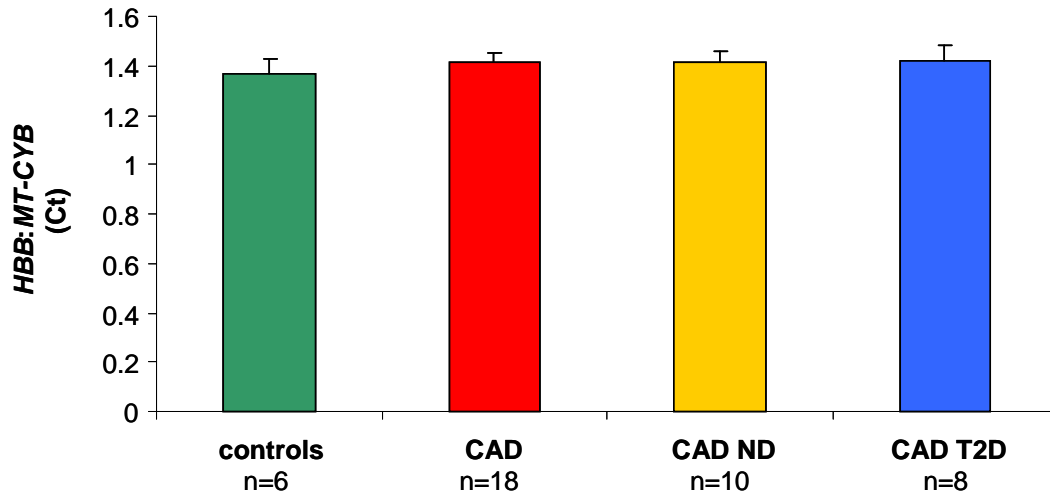
Experiments were performed in saphenous vein samples from 3 patients with CAD but without diabetes. Vasodilation was induced by carbachol (left panel) or calcium ionophore A23187 (right panel). There were no significant differences in maximum relaxation to either compound (blue symbols and lines; 80% vs 58%) but significant blunting of the response after pre-incubation (30 minutes) with L-NAME (0.1 mmol/L; red symbols and lines) in both sets of experiments.

Supplementary Figure 2



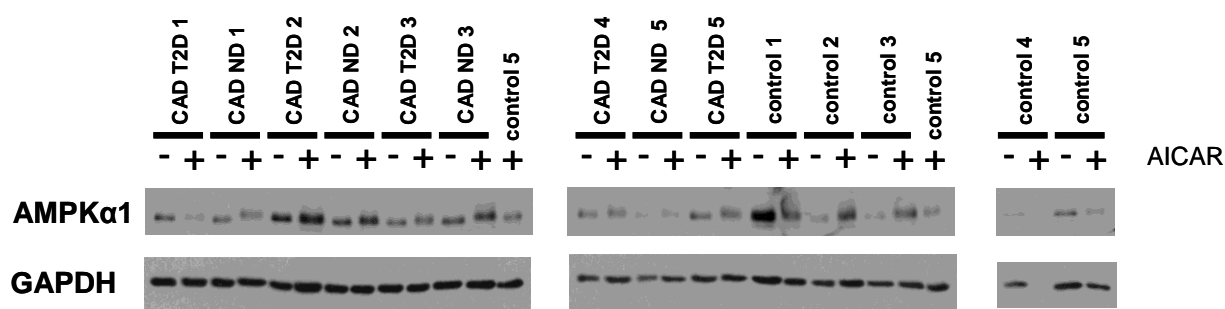
Relaxation to calcium ionophore A23187 in endothelium-denuded rings of saphenous vein from patients (n=3) with CAD but without diabetes. Response to A23187 is significantly blunted compared to experiments in endothelium-intact vessels (Supplementary Figure 1). Means and standard errors are displayed.

Supplementary Figure 3



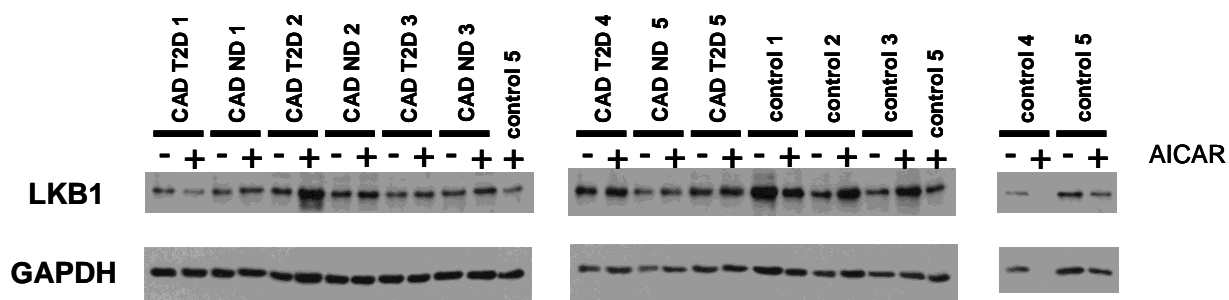
Determination of mitochondrial number in HSVECs by PCR. Total DNA was isolated from HSVECs using the QIAamp[®] DNA Mini Kit (Qiagen) and 25ng used for PCR reactions. Using TaqMan[®] Gene Expression Assays (Applied Biosystems) for single copy nuclear β -haemoglobin (*HBB*) and mitochondrially encoded cytochrome b (*MT-CYB*), abundance of mitochondrial DNA relative to nuclear DNA was determined by calculating Ct (cycle threshold value) ratios for each patient. No difference in Ct ratio between patient groups was observed, indicating HSVEC mitochondrial numbers do not vary across the groups. CAD ND, CAD patients without type 2 diabetes; CAD T2D, CAD patients with type 2 diabetes.

Supplementary Figure 4



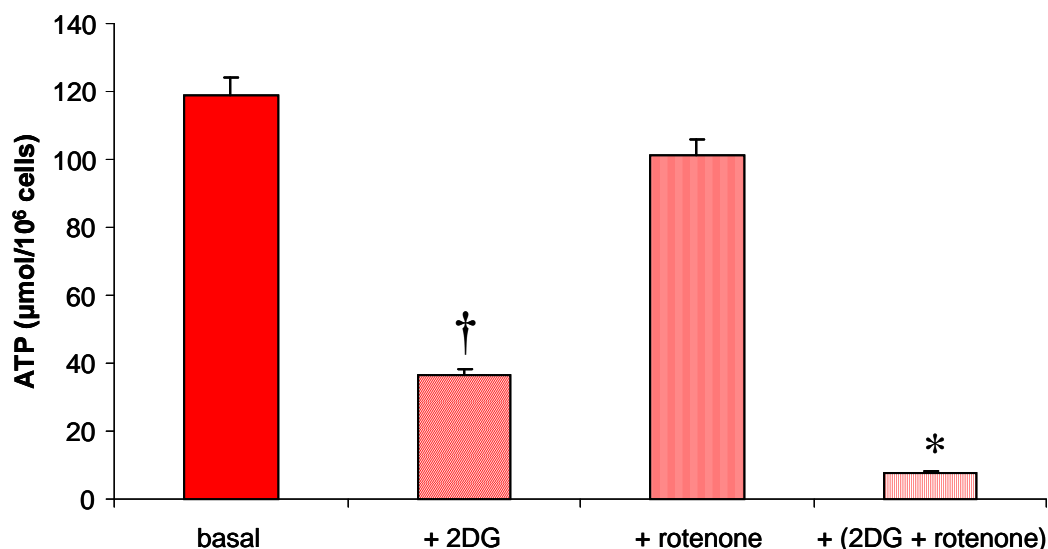
AMPK α 1 expression in HSVECs. Cells were incubated in the presence (+) or absence (-) of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR, 2 mmol/L), an artificial, ROS-independent activator of AMPK known to stimulate the kinase in intact cells [1]. Lysates were then prepared from HSVECs of control subjects (n=5), and CAD patients without type 2 diabetes (CAD ND, n=5) and with type 2 diabetes (CAD T2D, n=5) in the same way as previously described [2]. Lysates were subjected to western blotting and probed with sheep anti-AMPK α 1 antibody (1:1000) before incubation with donkey anti-rabbit IgG (1:1000, GE Healthcare). Resultant immunoblots demonstrate no change in AMPK α 1 expression between patient groups under basal conditions or conditions stimulating AMPK phosphorylation.

Supplementary Figure 5



LKB1 expression in HSVECs. Cells were incubated in the presence (+) or absence (-) of AICAR (2 mmol/L) and lysates prepared from HSVECs of control subjects (n=5), and CAD patients without type 2 diabetes (CAD ND, n=5) and with type 2 diabetes (CAD T2D, n=5) in the same way as previously described [2]. Lysates were subjected to western blotting and probed with sheep anti-LKB1 antibody (1:500) before incubation with protein G-peroxidase from *streptococcus* sp. (1:1000, Sigma). Resultant immunoblots demonstrate no change in LKB1 activity between patient groups under basal conditions or conditions stimulating AMPK phosphorylation.

Supplementary Figure 6



ATP production in HSVECs. Determination of the contribution of oxidative phosphorylation and glycolysis to the generation of ATP in HSVECs was carried out in Professor Moncada's laboratory, using the method of Quintero and colleagues [3]. HSVECs isolated from CAD patients were grown for 24 hours in 96-well plates [PerkinElmer (Waltham, MA) 3603 clear bottom, black walls, seeding density 10,000 cells per well] in phenol-red free ECM2 supplemented with 2% (v/v) FCS. On the day of the experiment, fresh medium was added and cells pretreated with 20 mmol/L 2-deoxy-D-glucose (2DG) and 0.5 µmol/L rotenone (both Sigma). 2DG is a glycolytic pathway inhibitor and rotenone, an inhibitor of the mitochondrial respiratory chain [3]. ATP was measured by the luciferin/luciferase method with the PerkinElmer ATPlite™ Luminescence Assay System, following the manufacturer's protocol. Chemiluminescence was determined in a TopCount (Packard Biosciences). The addition of 2DG causes a greater decrease in intracellular ATP production than the addition of rotenone and there is an additive effect when inhibitors are used in combination. Results support findings that endothelial cells are highly glycolytic [3]. *, † p < 0.0001 relative to basal values for untreated cells.

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