

Article

JAK and MEK Pathway Regulation of Mitochondrial Activity as Possible Targets for Saphenous Vein Smooth Muscle Cell Dysfunction in Diabetes

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Highlights

What are the main findings?

- We have identified JAK and MEK pathways as targets to regulate specific aspects of mitochondrial function in human saphenous vein smooth muscle cells (HSVSMCs).
- We have also shown how these pathways are impaired in HSVSMCs from type 2 diabetic patients (T2DM), a condition linked to poorer outcomes following coronary artery bypass graft surgery.

What are the implications of the main findings?

- These pathways represent potential targets for regulation of HSVSMC function that can be explored for drug development to limit saphenous vein graft failure in T2DM.

Abstract

While glucose-driven mitochondrial dysfunction has been proposed to promote vascular dysfunction responsible for saphenous vein graft failure (VGF) following bypass surgery, the impact of type 2 diabetes mellitus (T2DM) on mitochondrial function in human saphenous vein smooth muscle cells (HSVSMCs) responsible for maladaptive remodelling is unknown. Our aim was to identify signalling pathways that mediate any mitochondrial dysfunction in HSVSMCs in vitro and assess the impact of T2DM. HSVSMCs explanted from surplus HSV tissues from consenting T2DM and non-diabetic patients undergoing coronary artery bypass graft surgery were treated with known activators and inhibitors of the JAK/STAT and MAPK/ERK pathways. Following this, real-time oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measures of mitochondrial function were then determined. Our findings revealed that both IL-6/sIL-6R α trans-

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signalling complexes and platelet-derived growth factor-BB (PDGF-BB) significantly increased OCR in HSVSMCs from T2DM patients but not non-diabetic controls. Meanwhile, only PDGF-BB increased ECAR in HSVSMCs from T2DM patients but not in non-diabetic controls. The observed increases in OCR and ECAR were abolished by JAK1/2-selective inhibitor ruxolitinib. Furthermore, thrombin caused a significant increase in OCR, specifically in HSVSMCs from T2DM patients, and this effect was abolished by the MEK1/2-selective inhibitor trametinib. Both ruxolitinib and trametinib significantly reduced basal OCR and ECAR in HSVSMCs from both T2DM and non-diabetic patients. Together, these findings demonstrate a JAK/STAT- and MAPK/ERK-mediated regulation of mitochondrial function in HSVSMCs. As such, they represent potential targets for regulation of HSVSMC function that can be explored for drug development to limit saphenous VGF in T2DM.

Keywords: Type 2 diabetes mellitus; oxygen consumption rate; extracellular acidification rate; JAK/STAT; MAPK/ERK1,2

1. Introduction

Glucose-dependent alterations in cellular function have been suggested to promote proliferation and migration of vascular smooth muscle cells (VSMCs), which are linked to vein graft failure (VGF) [1–3]. VSMCs from type 2 diabetes mellitus (T2DM) patients have distinct functional properties [4,5], including significantly greater migratory activity than those from non-diabetic controls [5,6]. Additionally, T2DM is known to trigger changes in mitochondrial function [7], and together, these findings hint at a link between T2DM, mitochondrial dysfunction, and VGF. Metabolic reprogramming, adaptive oxygen consumption, and glucose utilisation are now appreciated as key mechanisms by which cells such as arterial endothelial and malignant cancer cells sustain high proliferative rates even in hostile environments in which the supplies of oxygen and essential nutrients are limited [8–10]. However, the impact of T2DM and possible downstream signalling pathways that regulate these processes in human saphenous vein smooth muscle cells (HSVSMCs), a crucial cell type implicated in the vascular dysfunction responsible for saphenous VGF, remains unclear [2,3].

Mitochondrial oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) have been described as gold standard measures for the determination of mitochondrial function in vascular endothelial cells (ECs), VSMCs, and platelets [11–13]. Since oxidative phosphorylation measured by OCR accounts for most of the increase in cellular ATP demand, the activity of this pathway is a major indicator of metabolic function [14,15]. Measurement of OCR in the presence of certain inhibitors can offer valuable information on mitochondrial bioenergetic profiles. The components of oxygen consumption that are linked to ATP production can be determined by inhibitors that target the various complexes in the electron transport chain [15]. These components include the so-called coupled OCR, as well as the contribution made to OCR by passive or active proton leakage across the inner mitochondrial membrane, the difference between maximal oxygen consumption and basal (spare capacity), and non-mitochondrial oxygen consumption. Together, these tests offer a detailed picture of mitochondrial function [16].

In contrast, ECAR provides a measure of the lactic acid produced by cells whilst in culture. This is typically excreted by cells when lactate dehydrogenase converts pyruvate to lactate to replenish NAD⁺, which is necessary to sustain glycolysis [17,18]. The flow through catabolic pathways necessary to produce ATP is, therefore, connected to a cell's ECAR. The ECAR is also linked to ATP turnover, as the rates of ATP synthesis and

consumption are equivalent in steady state [19]. Even though it is technically difficult to quantify ATP turnover, however, it may be demonstrated that fluctuations in extracellular fluxes are correlated with changes in ATP turnover rates [19]. For these reasons, mitochondrial OCR and ECAR are now appreciated as key parameters in assessing altered metabolic homeostasis in pathological conditions [11].

JAK/STAT and ERK 1/2 signalling pathways are important in numerous human physiological processes [20,21]. Importantly, activating these pathways with interleukin-6 (IL-6), platelet-derived growth factor-BB (PDGF-BB), Angiotensin II (Ang II) or thrombin can promote either vascular cell migration or proliferation events which are responsible for vascular remodelling [22–25]. However, their involvement in the modulation of mitochondrial function of vascular cells is poorly understood. Of importance is the fact that in patients with coronary artery disease (CAD) with or without T2DM, coronary artery bypass graft (CABG) using the autologous saphenous vein remains a major procedure for restoration of blood supply to the heart [26,27]. Patients with T2DM who have CAD, however, are more susceptible to neointimal hyperplasia (NIH), vascular remodelling, and stenosis that result in VGF [28]. While the underlying mechanisms responsible for this are unclear, activities of the activators of the JAK/STAT and MAPK/ERK signalling pathways are upregulated in T2DM patients [21]. To prevent VGF, it may therefore be possible to identify novel therapeutic targets by identifying characteristic metabolic modifications mediated by these downstream signalling pathways. Therefore, in this study, we assessed the roles of JAK/STAT and MAPK/ERK in regulating mitochondrial functions (OCR and ECAR), as well as the potential impact of T2DM on these processes in HSVSMCs in vitro.

2. Materials and Methods

2.1. Materials

HSV samples were collected between January 2020 and May 2022 from patients undergoing elective coronary artery bypass graft procedure. Table 1 summarises the information on the tissue donors. Other materials used in this study and further details on the tissue donors are listed in the Supplementary Materials Tables S1 and S2.

Table 1. Patient information for HSV samples.

	Description	T2DM	Non-T2DM
1	Number of samples	17	38
2	Age	Range: 56–80 Mean $\pm$ SD: 69.2 $\pm$ 6.2	Range: 47–84 Mean $\pm$ SD: 66.4 $\pm$ 8.9
3	Sex	Male—15 Female—2	Male—36 Female—2
4	Ethnicity	White British—17	White British—37 Asian—1
5	Reason for surgery	CAD	CAD

CAD: coronary artery disease; T2DM: type 2 diabetes mellitus; SD: standard deviation.

2.2. Methods

2.2.1. HSVSMC Isolation

As shown in Table 1, surplus HSV tissues were obtained from consenting (written) T2DM and non-diabetic patients undergoing coronary artery bypass graft (CABG) surgery at the Hull University Teaching Hospitals NHS Trust Department of Cardiothoracic Surgery, Castle Hill Hospital, Cottingham, UK, under UK Health Research Authority ethical approval (NHS REC:15/NE/0138). The procedures we used to obtain and process

surplus HSV tissues adhered to the guidelines outlined in the 2024 revision of the Declaration of Helsinki [29]. HSV tissues (length 0.5–4 cm) were stored at 4 °C for a maximum of 48 h in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL fungizone. VSMCs were explanted from HSV as previously described [30]. Briefly, HSV tissue was placed in a 10 cm dish with enough medium to cover it, and a sterile blade was used to remove the perivascular fat, vein branches, connective tissue, and adventitia. The vein was then dissected longitudinally, and the endothelial and tunica intima layers gently removed with sterile surgical scissors and forceps. Then, HSV tissue was dissected into small fragments (~1 mm³) which were transferred into a 25 cm² tissue culture flask with 2 mL of SmGM2 and cultured at 37 °C in 5% (v/v) CO₂ in humidified atmosphere. Once HSVSMCs had migrated from the explant tissue, after approximately two weeks, the growth medium was topped up with 0.5 mL fresh SmGM2 twice weekly. Addition of 0.5 mL fresh SmGM2 continued until a total of 5 mL of growth medium had been added; then, 2.5 mL of growth medium was removed and replenished with fresh 2.5 mL SmGM2 until 90% confluence. Thereafter, HSVSMCs were transferred to a 75 cm² tissue culture flask and fed with 10 mL of SmGM2, which was replaced twice weekly until cells reached 90% confluency and expanded for either cryopreservation in liquid nitrogen or downstream experimental applications. The integrity of HSVSMC preparations was confirmed by the presence of SMC markers and absence of fibroblast and endothelial cell markers by immunofluorescence staining and confocal microscopy as we have previously described [31].

2.2.2. Cell Culture, SDS-PAGE and Immunoblotting

HSVSMCs were maintained with SmGM2 at normal glucose concentration (5 mM) in a T75 flask and kept in a cell culture incubator maintained at 37 °C and 5% (v/v) CO₂ in a humidified atmosphere. For all experiments, cells were allowed to be 80–90% confluent before subculturing. All experiments were done with Passage 4 of HSVSMCs, and all cells were free of mycoplasma contamination. SDS-PAGE and Immunoblotting were performed as we have previously described [31].

2.2.3. HSVSMC Mitochondrial Stress Analysis of OCR and ECAR

HSVSMCs were seeded in Seahorse XFp 8-well plates at a density of 10,000 cells/well and allowed to grow over 24 h. Cells were then treated with the respective inhibitors of the downstream signalling pathways. To evaluate the contribution of JAK/STAT signalling pathways, cells were treated with 0.1 µM JAK1/2-selective inhibitor ruxolitinib [32] for 90 min, after which IL-6 (5 ng/mL) and sIL-6Rα (25 ng/mL) (IL-6/sIL-6Rα) or 10 ng/mL PDGF-BB was added for a further 24 h. To examine a role for ERK1,2 signalling pathways, cells were treated with 10 nM MEK1,2-selective inhibitor trametinib [33] for 90 min after which 100 nM Ang II or 1 U/mL thrombin were used to treat cells over 24 h. Also, the probes of the XFp 8-well plate were hydrated overnight by adding 200 µL/well of Seahorse calibrant and 400 µL into the ridges of the plate to prevent evaporation [34]. A humidified incubator was used to incubate this hydrated sensor containing XFp 8-well plates overnight at 37 °C.

On the day of assay, modulators of mitochondrial respiration, 20 µL oligomycin (1.5 µM), 22 µL carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) (5 µM), and 25 µL rotenone (5 µM) and antimycin A (5 µM) complex were added to the injection ports of the pre-hydrated Seahorse XFp 8-well plate [34]. The Seahorse XFp 8-well plate was kept in the non-CO₂ incubator for 30 min to allow the drugs to equilibrate. Each treatment was done in duplicate. Following this, plates were mounted in the Agilent (Santa Clara, CA, USA) Seahorse XFp Analyzer (model number 102745-100) and allowed to calibrate. While calibrating, the growth media on the pre-treated cells were withdrawn and 180 µL

of the XF DMEM medium was added to each well. Following calibration, the cells were mounted in the Agilent Seahorse XFp Analyzer (model number 102745-100), and real-time OCR and ECAR of the pre-treated cells were determined [34]. After the assay, the treated cells were lysed, and protein concentrations were determined using a BCA assay. Peak OCR and ECAR values, with or without treatments, following the injection of each modulator of mitochondrial respiration were normalised with the determined protein concentrations. Four biological replicates were performed using samples from different patients. Samples from these same donors were used across treatments.

2.2.4. Real-Time Quantitative PCR

qPCR was used to determine the mtDNA expression of the mitochondrially encoded gene for cytochrome c oxidase subunit I (COI), a key protein in the mitochondrial respiratory chain [35,36]. A total volume of 20 μ L PCR mixture, comprising 1 ng of cDNA, 10 μ L of SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA), 1 μ L of the appropriate primer (Table 2), and 7.4 μ L of RNase-free H₂O, was added to each well of a 96-well PCR plate. The 96-well plate was then installed in the StepOne machine after being sealed with optical adhesive film. StepOne software v2.3 was used to perform the real-time quantitative PCR (qPCR) following the manufacturer's recommendations. The cycle conditions for real-time qPCR were optimised and set at 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s. Every assay was carried out twice, and the average was determined. RNase-free water served as the negative control. Each sample's average C_T values were computed and normalised to 18S RNA controls.

Table 2. Two PCR primer sequences.

Gene	Primer Sequence (5'-3')
MT-COI	Forward—CCT ACT CCT GCT CGC ATC TG
	Reverse—AGA GGG GCG TTT GGT ATT GG
18S	Forward—GTA ACC CGT TGA ACC CCA TT
	Reverse—CCA TCC AAT CGG TAG TAG CG

2.2.5. Statistical Analysis

Statistical analysis was carried out using GraphPad Prism® 6, San Diego, CA, USA. Data are presented as mean $\pm$ standard error of mean (SEM) and were analysed using an independent *t*-test and both one-way and two-way analysis of variance, followed by both Dunnett's and Holm–Šidák's post hoc tests to determine significant differences between means, where $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of IL-6/sIL-6R α on OCR in HSVSMCs from T2DM and Non-Diabetic Patients

Studies have shown that T2DM is associated with elevated levels of pleiotropic cytokines such as IL-6, which can cause a number of adverse cellular effects, including mitochondrial dysfunction [20]. However, the mechanism(s) and impact on mitochondrial function of VSMCs are unknown. First, we determined the concentration of ruxolitinib sufficient to significantly inhibit IL-6/sIL-6R α -mediated phosphorylation of STAT3 on Tyr705 (Figure 1). Then, as shown in Figure 2A, an IL-6/sIL-6R α trans-signalling complex was found to significantly increase OCR in HSVSMCs from T2DM patients at maximal respiration ($p < 0.05$ versus unstimulated cells, $n = 4$), but not in those from non-diabetic HSVSMCs (Figure 2B). The IL-6/sIL-6R α -stimulated increase in OCR found in HSVSMCs from T2DM patients was abolished by pre-treatment with JAK1/2-selective inhibitor ruxolitinib (Figure 2A), at the predetermined concentration (0.1 μ M) (Figure 1). Furthermore,

ruxolitinib significantly decreased OCR in HSVSMCs from non-diabetic patients at basal and maximal respiration with or without IL-6/sIL-6R α stimulation ($p < 0.05$ versus unstimulated cells, $n = 4$; Figure 2B). No significant differences in OCR between HSVSMCs from T2DM and non-diabetic patients were observed (Figure 2C).

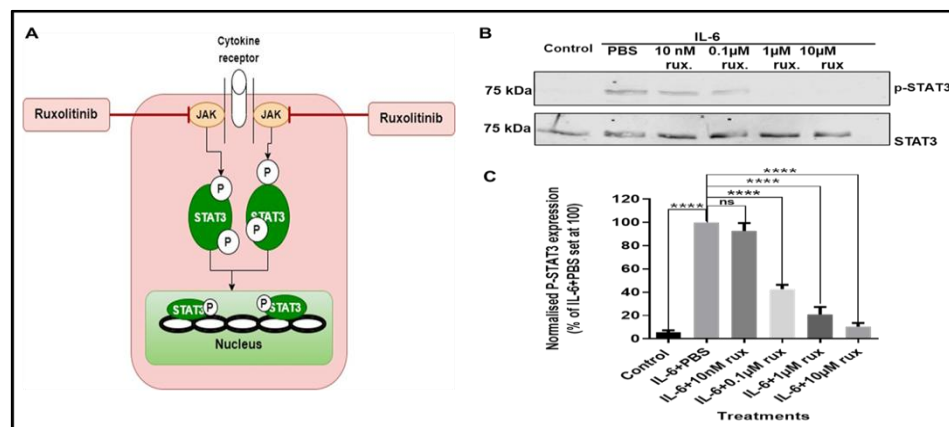


Figure 1. Downstream activation and inhibition of the JAK/STAT signalling pathway. (A) Schematic of downstream activation and inhibition of the JAK/STAT signalling pathway. The JAK/STAT pathway transmits information from chemical signals outside of a cell to the cell nucleus, where it activates genes through transcription. This is mediated by activation of receptors such as IL-6R and proteins, Janus kinases (JAKs), and signal transducer and activator of transcription (STAT). Ruxolitinib (JAK 1/2-selective inhibitor) blocks this signalling pathway. (B) Representative Western blot of downstream inhibition of IL-6/sIL-6R α -stimulated JAK1/2-mediated phosphorylation of STAT3 (98 kDa) on Tyr705 by ruxolitinib. Lower panel: expression of total STAT3. (C) Densitometric analysis of p-STAT3 normalised to total STAT3. Data are expressed as mean $\pm$ SEM and from $n = 4$ biological replicates using HSVSMC samples from different non-diabetic patients. Statistical significance was assessed using one-way ANOVA followed by Dunnett's post hoc test to determine significant differences between means. IL-6: IL-6/sIL-6R α ; PBS: phosphate-buffered saline; rux: ruxolitinib. **** $p < 0.0001$; ns: not significant.

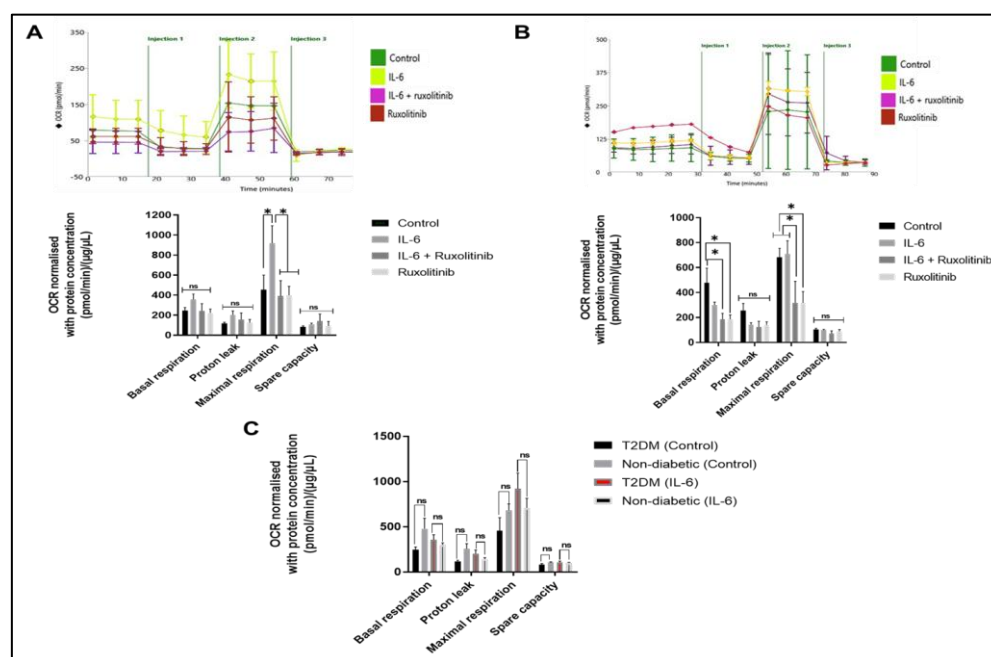


Figure 2. Mitochondrial stress analysis to determine the OCR of HSVSMCs from T2DM and non-T2DM patients after stimulation with IL-6/sIL-6R α +/- ruxolitinib. (A) **Upper panel:** representative

time course curve of OCR of HSVSMCs from T2DM patients treated with IL-6/sIL-6R α (IL-6 (5 ng/mL) and sIL-6R α (25 ng/mL)) +/- ruxolitinib (0.1 μ M) and untreated control from T2DM patients. **Lower panel:** comparative analysis of normalised peak OCR between treatment groups and untreated control at basal glycolysis, and after addition of inhibitors of mitochondrial respiration and the uncoupler. Normalised data are presented as mean $\pm$ SEM from $n = 4$ biological replicates using HSVSMC samples from different T2DM patients. **(B) Upper panel:** representative time course curve of OCR of HSVSMCs from non-diabetic patients treated with IL-6/sIL-6R α (IL-6 (5 ng/mL) and sIL-6R α (25 ng/mL)) +/- ruxolitinib (0.1 μ M) and untreated control from non-diabetic patients. **Lower panel:** comparative analysis of normalised peak OCR between treatment groups and untreated control at basal glycolysis, and after addition of inhibitors of mitochondrial respiration and the uncoupler. Normalised data are presented as mean $\pm$ SEM from $n = 4$ biological replicates using HSVSMC samples from different non-diabetic patients. **(C)** Comparison of normalised peak OCR of unstimulated and IL-6/sIL-6R α -stimulated HSVSMCs from T2DM patients versus non-diabetic control. Normalised data are presented as mean $\pm$ SEM and statistical significance was assessed using an independent t -test, $n = 4$ biological replicates using HSVSMC samples from different T2DM and non-diabetic patients. In the lower panels of Figures (A,B), normalised data from different treatment conditions were compared with data from the normalised untreated control. Statistical significance was assessed using an independent t -test and both one-way and two-way ANOVA, followed by Dunnett's and Holm-Šidák's post hoc tests to determine significant differences between means. * $p < 0.05$. IL-6: IL-6/sIL-6R α ; OCR: oxygen consumption rate.

3.2. Effect of IL-6/sIL-6R α on the ECAR in HSVSMCs from T2DM and Non-Diabetic Patients

The effect of pro-inflammatory cytokines such as IL-6 on ECAR in vascular cells is currently unclear. ECAR serves as a proxy measure of glycolysis [17]. We therefore determined the ECAR of HSVSMCs from T2DM and non-diabetic patients after treatment with IL-6/sIL-6R α , following pre-treatment with or without ruxolitinib. ECAR was measured simultaneously during mitochondrial stress tests. Consequently, ECAR values are interpreted here as an index of non-mitochondrial acidification and the compensatory glycolytic response to mitochondrial inhibition. Our findings suggest that IL-6/sIL-6R α did not cause any significant change in the ECAR of HSVSMCs from either T2DM or non-diabetic patients (Figure 3A,B). However, with or without IL-6/sIL-6R α stimulation, ruxolitinib significantly ($p < 0.05$ versus unstimulated cells, $n = 4$) decreased resting ECAR and maximal ECAR compensation, and ($p < 0.01$ versus unstimulated cells, $n = 4$) decreased non-mitochondrial ECAR in HSVSMCs from non-diabetic patients (Figure 3B). No significant differences in the ECAR of HSVSMCs from T2DM and non-diabetic patients after stimulation with IL-6/sIL-6R were observed (Figure 3C).

3.3. Effect of PDGF-BB on the OCR in HSVSMCs from T2DM and Non-Diabetic Patients

Given that the OCR is proportional to mitochondrial respiration [17], it is unclear how growth factors like PDGF alter this cellular metabolic index (OCR) in HSVSMCs. To investigate this, the OCRs of HSVSMCs from T2DM patients and non-diabetic controls were assessed after treatment with PDGF-BB in the presence or absence of ruxolitinib. As shown in Figure 4A, PDGF-BB significantly increased OCR in HSVSMCs from T2DM patients ($p < 0.05$ versus unstimulated cells, $n = 4$) and maximum respiration ($p < 0.01$ versus unstimulated cells, $n = 4$). However, PDGF-BB had no significant effect on the OCR of HSVSMCs from non-diabetic patients (Figure 4B). This increase in OCR in T2DM patient HSVSMCs was abolished by pre-treatment with ruxolitinib (Figure 4A). Ruxolitinib also significantly reduced OCR at maximum respiration ($p < 0.05$ versus unstimulated cells and $p < 0.01$ versus PDGF-BB-stimulated cells, $n = 4$) in HSVSMCs from T2DM patients (Figure 4A)—a pattern also seen in HSVSMCs from non-diabetic controls (Figure 4B) ($p < 0.05$ versus unstimulated and PDGF-BB-stimulated, $n = 4$). In contrast, there were no

significant differences between OCRs in HSVSMCs from T2DM and non-diabetic patients (Figure 4C).

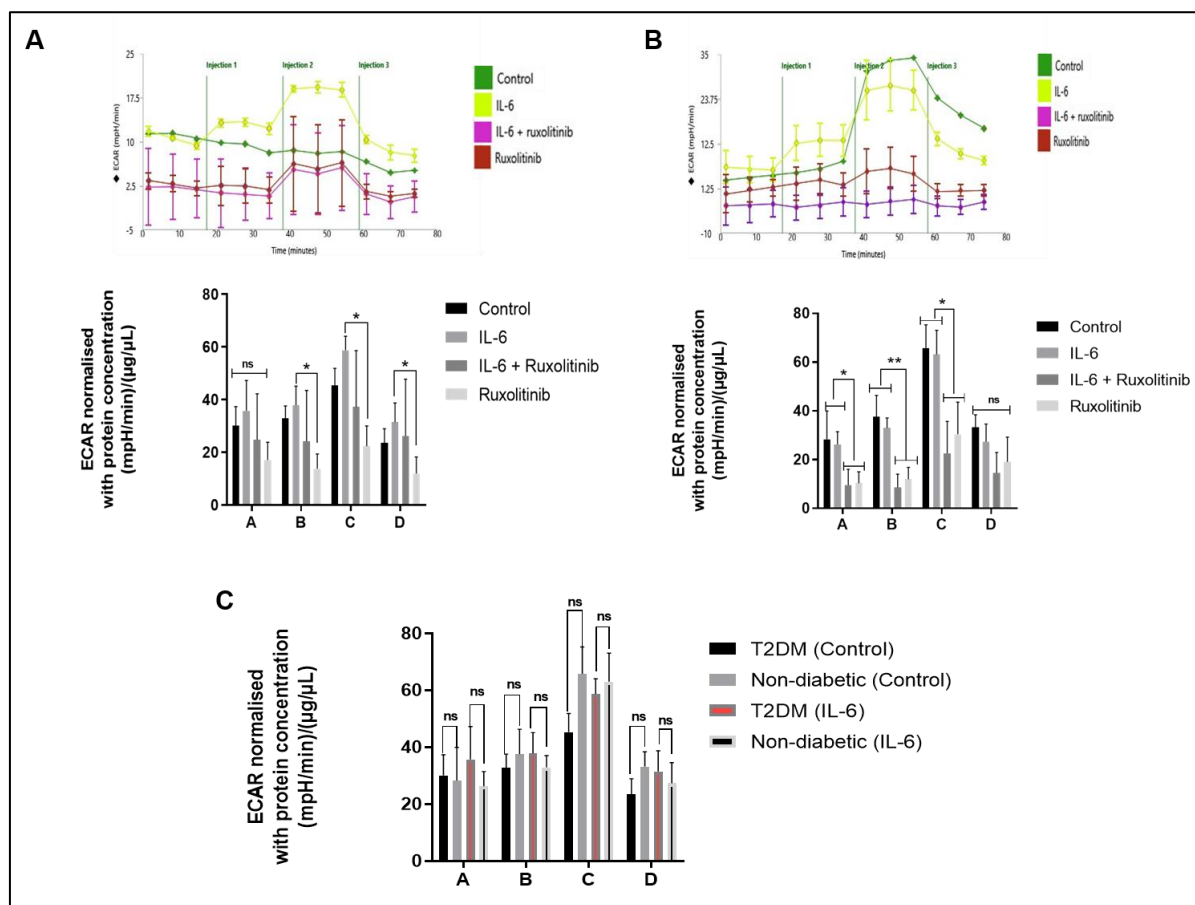


Figure 3. Mitochondrial stress analysis to determine the ECAR of HSVSMCs from T2DM and non-T2DM patients after stimulation with IL-6/sIL-6Rα +/- ruxolitinib. **(A) Upper panel:** representative time course curve of ECAR of HSVSMCs from T2DM patients treated with IL-6/sIL-6Rα (IL-6 (5 ng/mL) and sIL-6Rα (25 ng/mL)) +/- ruxolitinib (0.1 μM) and untreated control from diabetic patients. **Lower panel:** comparative analysis of normalised peak ECAR between treatment groups and untreated control at basal glycolysis, and after addition of inhibitors of mitochondrial respiration and the uncoupler. Normalised data are presented as mean ± SEM from $n = 4$ biological replicates using HSVSMC samples from different T2DM patients. **(B) Upper panel:** representative time course curve of ECAR of HSVSMCs from non-diabetic patients treated with IL-6/sIL-6Rα (IL-6 (5 ng/mL) and sIL-6Rα (25 ng/mL)) +/- ruxolitinib (0.1 μM) and untreated control from non-diabetic patients. **Lower panel:** comparative analysis of normalised peak ECAR between treatment groups and untreated control at basal glycolysis, and after addition of inhibitors of mitochondrial respiration and the uncoupler. Normalised data are presented as mean ± SEM from $n = 4$ biological replicates using HSVSMC samples from different non-diabetic patients. **(C)** Comparison of normalised peak ECAR of unstimulated and IL-6/sIL-6Rα-stimulated HSVSMCs from T2DM patients versus non-diabetic control. Normalised data are presented as mean ± SEM and statistical significance was assessed using an independent t -test, $n = 4$ biological replicates using HSVSMC samples from different T2DM and non-diabetic patients. In the lower panels of Figure 3 (A,B), normalised data from different treatment conditions were compared with data from the normalised untreated control. Statistical significance was assessed using an independent t -test and both one-way and two-way ANOVA, followed by Dunnett's and Holm–Šidák's post hoc tests to determine significant differences between means. * $p < 0.05$; ** $p < 0.01$. ns: not significant; IL-6: IL-6/sIL-6Rα; A: resting ECAR; B: oligomycin-induced ECAR; C: ECAR response to uncoupling; D: non-mitochondrial ECAR; ECAR: extracellular acidification rate.

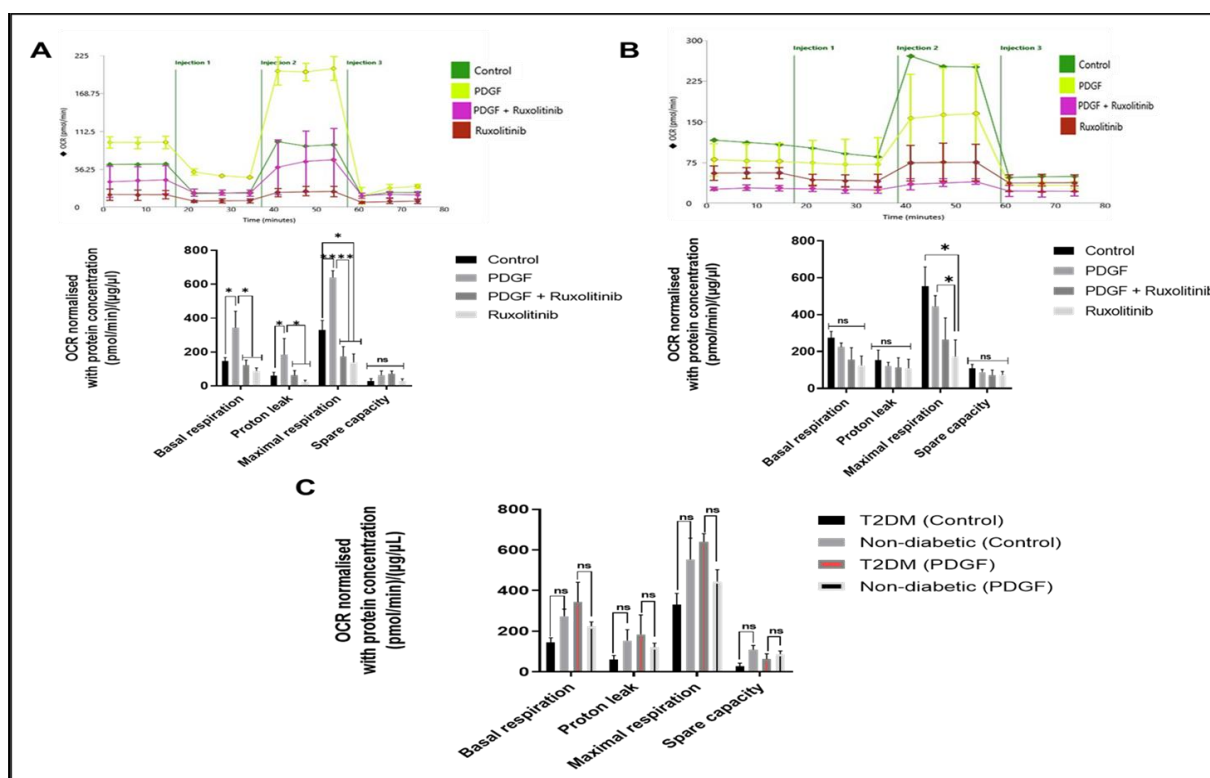


Figure 4. Mitochondrial stress analysis to determine the OCR of HSVSMCs from T2DM and non-T2DM patients after stimulation with PDGF-BB +/- ruxolitinib. **(A) Upper panel:** representative time course curve of OCR of HSVSMCs from T2DM patients treated with PDGF-BB (10 ng/mL) +/- ruxolitinib (0.1 μ M) and untreated control from diabetic patients. **Lower panel:** comparative analysis of normalised peak OCR between treatment groups and untreated control at basal glycolysis, and after addition of inhibitors of mitochondrial respiration and the uncoupler. Normalised data are presented as mean $\pm$ SEM from $n = 4$ biological replicates using HSVSMC samples from different T2DM patients. **(B) Upper panel:** representative time course curve of OCR of HSVSMCs from non-diabetic patients treated with PDGF-BB (10 ng/mL) +/- ruxolitinib (0.1 μ M) and untreated control from non-diabetic patients. **Lower panel:** comparative analysis of normalised peak OCR between treatment groups and untreated control at basal glycolysis, and after addition of inhibitors of mitochondrial respiration and the uncoupler. Normalised data are presented as mean $\pm$ SEM from $n = 4$ biological replicates using HSVSMC samples from different non-diabetic patients. **(C)** Comparison of normalised peak OCR of unstimulated and PDGF-BB-stimulated HSVSMCs from T2DM patients versus non-diabetic control. Normalised data are presented as mean $\pm$ SEM and statistical significance was assessed using an independent t -test, $n = 4$ biological replicates using HSVSMC samples from different T2DM and non-diabetic patients. In the lower panels of Figure 4 (A,B), normalised data from different treatment conditions were compared with data from the normalised untreated control. Statistical significance was assessed using an independent t -test and both one-way and two-way ANOVA, followed by Dunnett's and Holm-Šidák's post hoc tests to determine significant differences between means. * $p < 0.05$; ** $p < 0.01$. ns: not significant; PDGF: PDGF-BB; OCR: oxygen consumption rate.

3.4. Effect of PDGF-BB on the ECAR in HSVSMCs from T2DM and Non-Diabetic Patients

We next determined the ECAR response of HSVSMCs from T2DM and non-diabetic control patients treated with or without PDGF-BB following pre-treatment with or without ruxolitinib and in response to mitochondrial disruption. As shown in Figure 5A, PDGF-BB significantly increased resting ECAR, as well as ECAR compensation, ECAR in response to uncoupling, and non-mitochondrial ECAR in HSVSMCs from T2DM patients ($p < 0.05$ versus unstimulated cells, $n = 4$) but not in non-diabetic controls (Figure 5B).

Furthermore, ruxolitinib significantly decreased resting ECAR, ECAR compensation, ECAR in response to uncoupling, and non-mitochondrial ECAR in HSVSMCs from non-diabetic patients ($p < 0.05$ versus unstimulated and PDGF-BB-stimulated cells but not in those from T2DM patients (Figure 5B). Direct comparison of the ECAR of HSVSMCs from T2DM versus non-diabetic patients also revealed a significantly higher ECAR in response to uncoupling in HSVSMCs from T2DM patients compared with non-diabetic controls ($p < 0.05$, $n = 4$; Figure 5C).

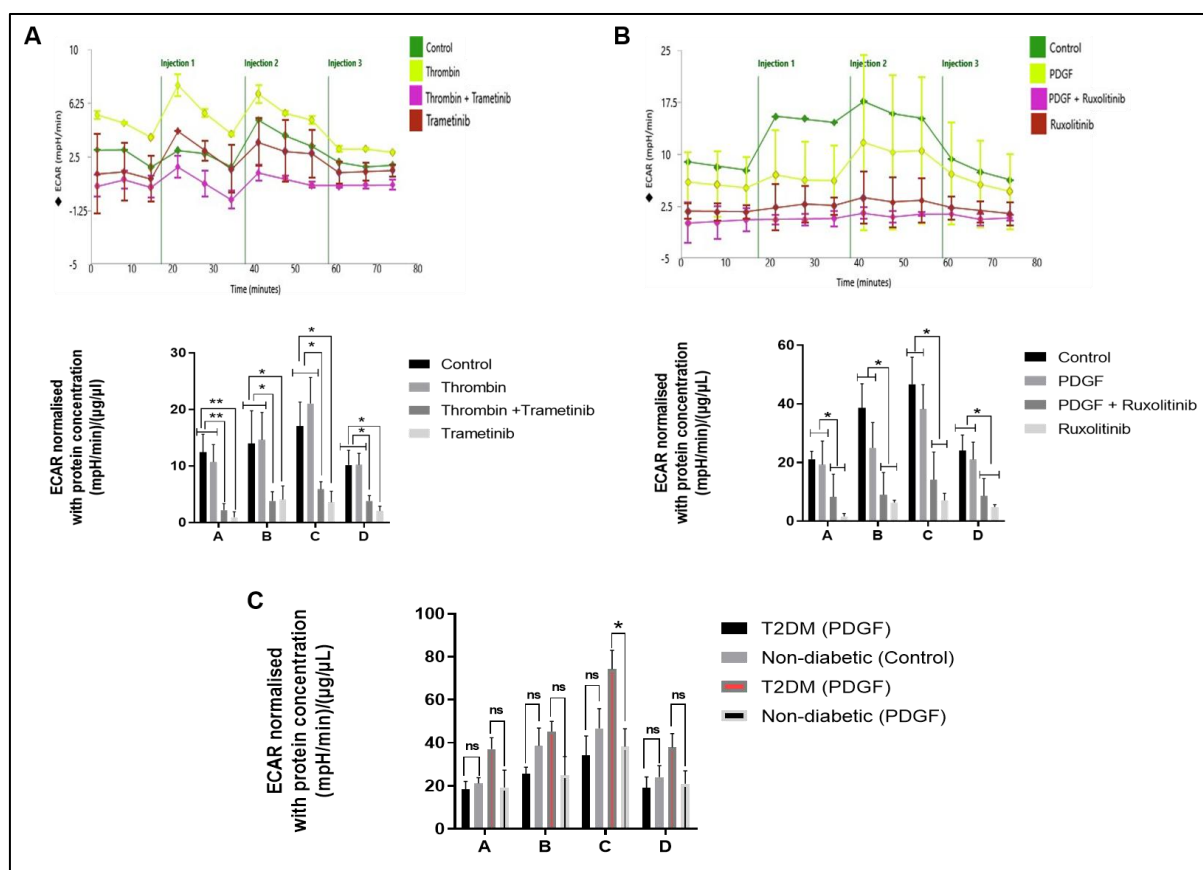


Figure 5. Mitochondrial stress analysis to determine the ECAR of HSVSMCs from T2DM and non-T2DM patients after stimulation with PDGF-BB +/- ruxolitinib. (A) **Upper panel:** representative time course curve of ECAR of HSVSMCs from T2DM patients treated with PDGF-BB (10 ng/mL) +/- ruxolitinib (0.1 μM) and untreated control from diabetic patients. **Lower panel:** comparative analysis of normalised peak ECAR between treatment groups and untreated control at basal glycolysis, and after addition of inhibitors of mitochondrial respiration and the uncoupler. Normalised data are presented as mean ± SEM from $n = 4$ biological replicates using HSVSMC samples from different T2DM patients. (B) **Upper panel:** representative time course curve of ECAR of HSVSMCs from non-diabetic patients treated with PDGF-BB (10 ng/mL) +/- ruxolitinib (0.1 μM) and untreated control from non-diabetic patients. **Lower panel:** comparative analysis of normalised peak ECAR between treatment groups and untreated control at basal glycolysis, and after addition of inhibitors of mitochondrial respiration and the uncoupler. Normalised data are presented as mean ± SEM from $n = 4$ biological replicates using HSVSMC samples from different non-diabetic patients. (C) Comparison of normalised peak ECAR of unstimulated and PDGF-BB-stimulated HSVSMCs from T2DM patients versus non-diabetic control. Normalised data are presented as mean ± SEM and statistical significance was assessed using an independent t -test, $n = 4$ biological replicates using HSVSMC samples from different T2DM and non-diabetic patients. In the lower panels of Figure 5 (A,B), normalised data from different treatment conditions were compared with data from the normalised untreated control. Statistical significance was assessed using an independent t -test and both one-way and two-way ANOVA, followed by Dunnett's and Holm-Šidák's post hoc tests to determine

significant differences between means. * $p < 0.05$; ** $p < 0.01$. ns: not significant; PDGF: PDGF-BB; A: Resting ECAR; B: Oligomycin-induced ECAR; C: ECAR response to uncoupling; D: Non-mitochondrial ECAR; ECAR: extracellular acidification rate.

3.5. mtDNA Expression in HSVSMCs from T2DM and Non-Diabetic Patients After Treatment with IL-6/sIL-6R α and PDGF-BB +/-Ruxolitinib

As JAK/STAT activators IL-6/sIL-6R α and PDGF-BB caused a significant increase in the OCR of HSVSMCs from T2DM (Figures 2A and 4A) but not non-diabetic patients (Figures 2B and 4B), it was therefore necessary to ascertain whether this increase was associated with a parallel increase in mitochondria. Therefore, qPCR of the mitochondrially encoded gene for COI was used to determine mtDNA expression [35,36].

As shown in Figure 6A, there was no significant difference in mtDNA expression in HSVSMCs from T2DM patients after treatment with IL-6/sIL-6R α +/-ruxolitinib compared with the untreated control. Similarly, in the HSVSMCs from non-diabetic patients, there was no significant difference in mtDNA expression after treatment with IL-6/sIL-6R α +/-ruxolitinib compared with the untreated control (Figure 6B). Comparison between HSVSMCs from T2DM patients versus the non-diabetic control treated with IL-6/sIL-6R α +/-ruxolitinib showed no significant difference (Figure 6C). Furthermore, as shown in Figure 6D, there was no significant difference in mtDNA expression in HSVSMCs from T2DM patients after treatment with PDGF-BB+/-ruxolitinib compared with the untreated control. Similarly, in the HSVSMCs from non-diabetic patients, there was no significant difference in mtDNA expression after treatment with PDGF-BB+/-ruxolitinib compared with the untreated control (Figure 6E). Also, comparison between HSVSMCs from T2DM patients versus the non-diabetic control treated with PDGF-BB+/-ruxolitinib showed no significant difference (Figure 6F).

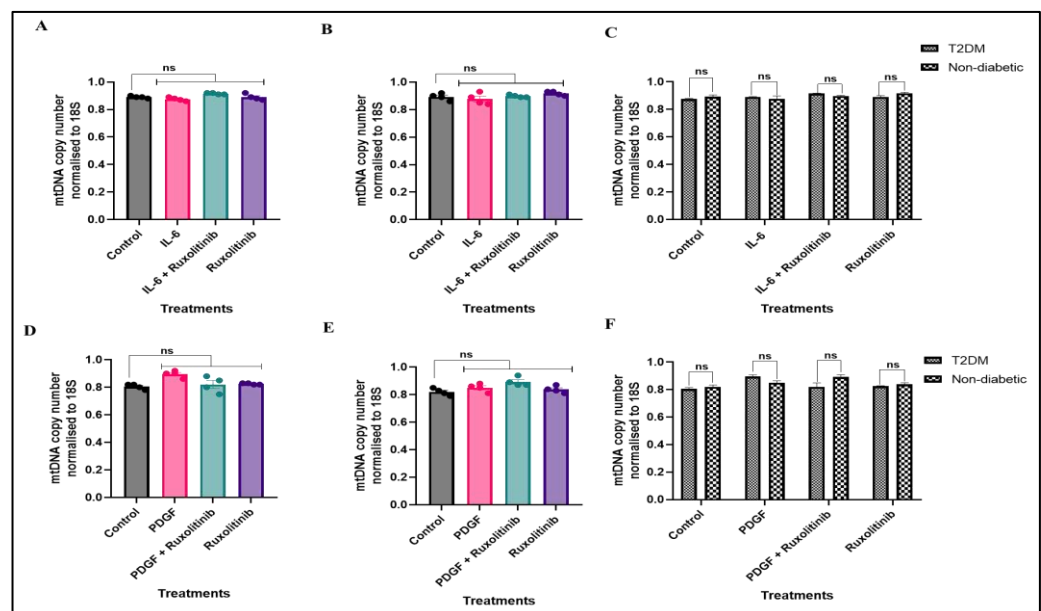


Figure 6. mtDNA expression of HSVSMCs from T2DM and non-diabetic patients after treatment with IL-6/sIL-6R α or PDGF-BB +/- ruxolitinib, along with qPCR analysis of mtDNA expression of the mitochondrially encoded gene for COI as a marker of mtDNA expression normalised to 18S. **Top panel:** (A) T2DM patients, (B) non-diabetic patients, and (C) comparison between T2DM patients versus the non-diabetic control treated with IL-6/sIL-6R α (IL-6 (5 ng/mL) and sIL-6R α (25 ng/mL)) +/- ruxolitinib (0.1 μ M). **Bottom panel:** (D) T2DM patients, (E) non-diabetic patients, and (F) comparison between T2DM patients versus the non-diabetic control treated with PDGF-BB (10 ng/mL) +/- ruxolitinib (0.1 μ M). All data were normalised to 18S, and normalised data from different

treatment conditions were compared with data from the normalised untreated control for statistical difference. Data are presented as mean $\pm$ SEM from $n = 4$ experiments using HSVSMC from different patients. Statistical significance was assessed using an independent *t*-test and both one-way and two-way ANOVA, followed by Dunnett's and Holm–Šidák's post hoc tests to determine significant differences between means. ns: not significant; IL-6: IL-6/sIL-6R α ; PDGF: PDGF-BB.

3.6. Effect of Ang II on OCR of HSVSMCs from T2DM and Non-Diabetic Patients

While Ang II has been shown to promote VSMC migration and proliferation by activating the MAP/ERK1,2 pathway [37], it is not totally clear how Ang II modulates these processes. However, we hypothesise that the increased cell proliferation and migration induced by Ang II [37] may necessitate higher ATP synthesis, which would increase oxygen consumption. Therefore, we assessed the effect of Ang II +/- MEK1/2-selective inhibitor trametinib on the OCR of HSVSMCs from patients with and without T2DM. The optimised concentration of trametinib that significantly ($p < 0.05$ versus untreated cells, $n = 4$) inhibited activation of ERK1,2 in HSVSMCs from non-diabetic patients was 10 nM trametinib (Figure 7). Hence, this concentration was used in subsequent experiments. As shown in Figure 8A,B, Ang II (100 nM) did not significantly alter the OCR of HSVSMCs from T2DM and non-diabetic patients, respectively, even though the same concentration of Ang II stimulated a transient increase in ERK1,2 phosphorylation in HSVSMCs (Figure 9). However, trametinib with or without Ang II stimulation significantly reduced OCRs at basal respiration ($p < 0.01$ versus unstimulated and Ang II-stimulated cells, $n = 4$) and maximal respiration ($p < 0.05$ versus unstimulated and Ang II-stimulated cells, $n = 4$) in HSVSMCs from T2DM patients (Figure 8A). Similarly, in HSVSMCs from non-diabetic patients, Ang II treatment did not significantly alter the OCR (Figure 8B). Furthermore, trametinib caused a significant reduction in the OCRs at basal respiration ($p < 0.01$ with Ang II stimulation; $p < 0.001$ without Ang II stimulation, both versus unstimulated and Ang II-stimulated cells, $n = 4$) in HSVSMCs from non-diabetic patients (Figure 8B). Interestingly, trametinib caused a significant reduction in the OCRs at maximal respiration ($p < 0.05$ with or without Ang II stimulation versus unstimulated and Ang II-stimulated cells, $n = 4$) in HSVSMCs from non-diabetic patients (Figure 8B). Also, direct comparison of OCRs after treatment with Ang II revealed a significant increase in the OCR of HSVSMCs from T2DM patients at maximal respiration ($p < 0.05$ versus HSVSMCs from non-diabetic patients, $n = 4$; Figure 8C).

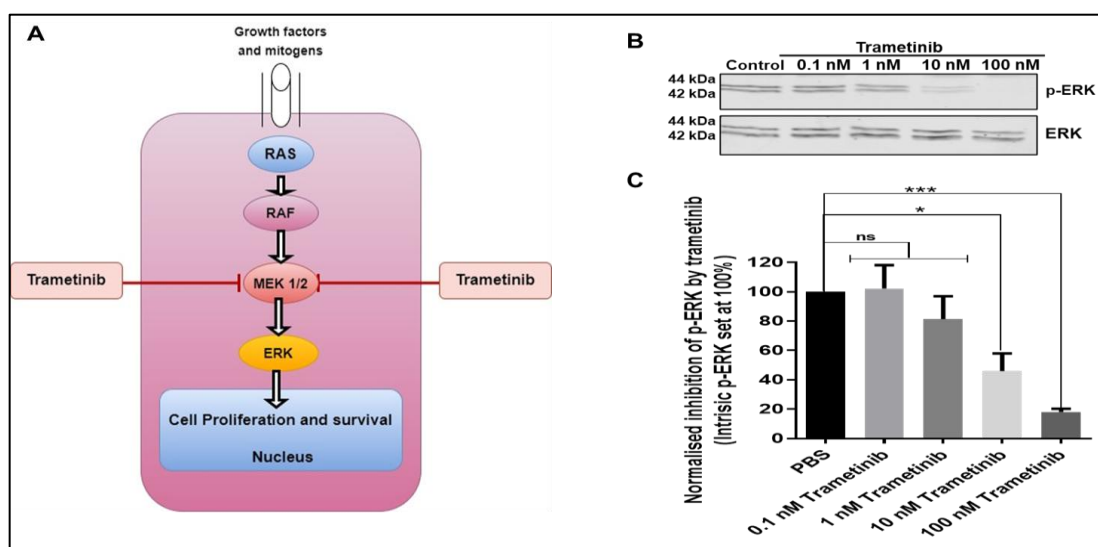


Figure 7. Downstream activation and inhibition of the MAPK/ERK signalling pathway. (A) Schematic of downstream activation and inhibition of the MEK/ERK signalling pathway. Mitogens

trigger activation of the small GTPase Ras protein, which in turn activates the Ser/Thr-directed protein kinase Raf, which phosphorylates and activates MEK 1/2. MEK1/2 then phosphorylates and stimulates ERK1,2 (Thr202/185 and Tyr204/187 in human ERK1/2 respectively). ERK further activates a panel of substrates that promote cell proliferation. **(B)** Upper panel: representative Western blot of downstream inhibition of the MEK 1/2 by trametinib. Lower panel: expression of total ERK (44/42 kDa). **(C)** Densitometric analysis of p-ERK at both 44/42 kDa normalised to total ERK at both 44/42 kDa. Data are expressed as mean $\pm$ SEM and from $n = 4$ biological replicates using HSVSMC samples from different non-diabetic patients. Statistical significance was assessed using one-way ANOVA followed by Dunnett's post hoc test to determine significant differences between means. PBS: phosphate-buffered saline. * $p < 0.05$; *** $p < 0.001$. ns: not significant.

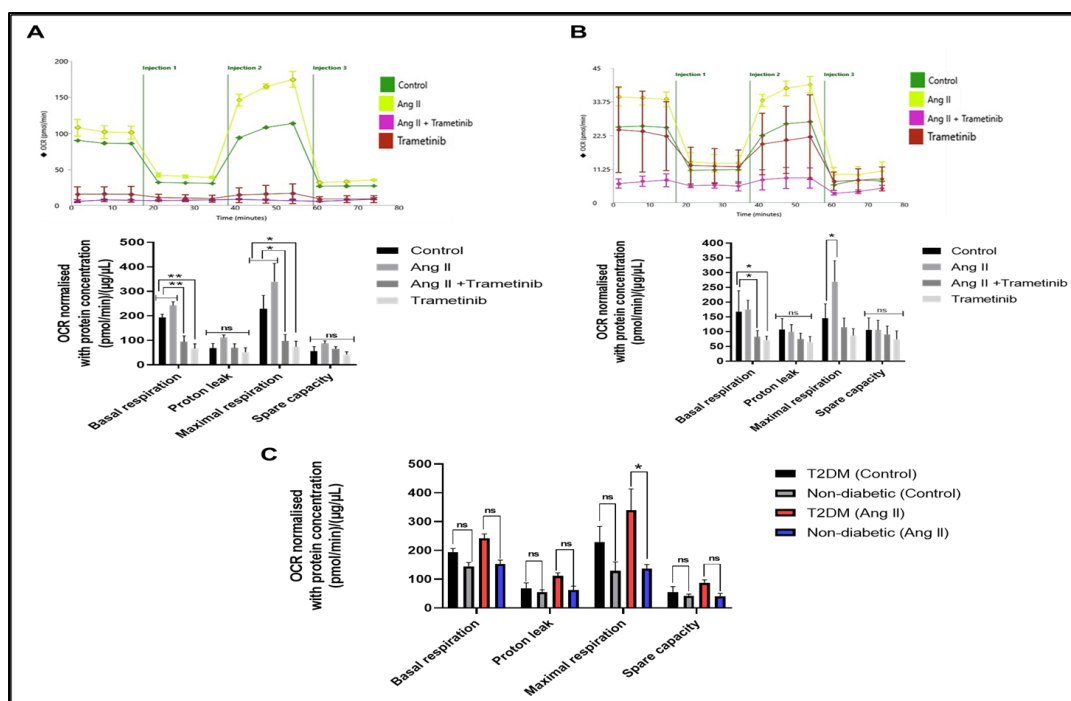


Figure 8. Mitochondrial stress analysis to determine the OCR of HSVSMCs from T2DM and non-T2DM patients after stimulation with Ang II +/- trametinib. **(A) Upper panel:** representative time course curve of the OCR of HSVSMCs from T2DM patients treated with Ang II (100 nM) +/- trametinib (10 nM) and untreated control from diabetic patients. **Lower panel:** comparative analysis of normalised peak OCR between treatment groups and untreated control at basal glycolysis, and after addition of inhibitors of mitochondrial respiration and the uncoupler. Normalised data are presented as mean $\pm$ SEM from $n = 4$ biological replicates using HSVSMC samples from different T2DM patients. **(B) Upper panel:** representative time course curve of the OCR of HSVSMCs from non-diabetic patients treated with Ang II (100 nM) +/- trametinib (10 nM) and untreated control from non-diabetic patients. **Lower panel:** biological replicates using HSVSMC samples from different non-diabetic patients. **(C)** Comparison of normalised peak OCR of unstimulated and Ang II-stimulated HSVSMCs from T2DM patients versus non-diabetic control. Normalised data are presented as mean $\pm$ SEM and statistical significance was assessed using an independent t -test, $n = 4$ biological replicates using HSVSMC samples from different T2DM and non-diabetic patients. In the lower panels of Figure 8 (A,B), normalised data from different treatment conditions were compared with data from the normalised untreated control. Statistical significance was assessed using an independent t -test and both one-way and two-way ANOVA, followed by Dunnett's and Holm-Šidák's post hoc tests to determine significant differences between means. * $p < 0.05$; ** $p < 0.01$. ns: not significant; Ang II: Angiotensin II; OCR: oxygen consumption rate.

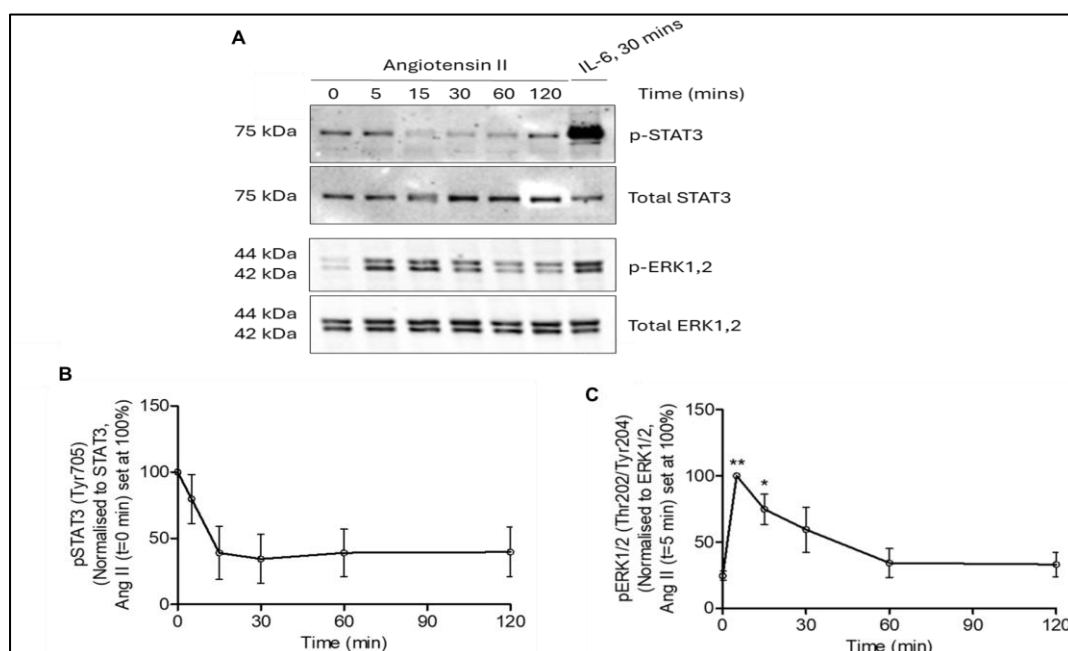


Figure 9. Time course of the effects of Angiotensin II treatment on STAT3 and ERK1/2 phosphorylation in HSVSMCs. HSVSMCs were either treated with vehicle or Angiotensin II (Ang II) (0.1 μ M) for the indicated times or siIL-6R α /IL-6 for 30 min before cell harvest. (A) Protein-equalised cell lysates were fractionated by SDS-PAGE for immunoblotting. P-STAT3 (Tyr705) (B) and P-ERK1/2 (Thr202/Tyr204) (C) levels were normalised to total STAT3 and ERK1/2, respectively, and measured relative to the maximum response (set at 100%). Data are presented as mean $\pm$ SEM. Data are expressed as mean $\pm$ SEM and from $n = 4$ biological replicates using HSVSMC samples from different non-diabetic patients. Statistical significance was assessed using one-way ANOVA followed by Dunnett's post hoc test to determine significant differences between means. * $p < 0.05$; ** $p < 0.01$ versus $t = 0$ min (min).

3.7. Effect of Ang II on ECAR of HSVSMCs from T2DM and Non-Diabetic Patients

There is evidence that Ang II may cause mitochondrial oxidative damage in vascular endothelial cells, which could reduce NO bioavailability and increase vascular oxidative stress [21]. While this finding emphasises the role of Ang II in controlling metabolic homeostasis in endothelial cells, its effect on ECAR of HSVSMCs is unknown. Therefore, we evaluated the effects of Ang II $\pm$ trametinib on the ECAR of HSVSMCs from T2DM patients and non-diabetic controls in response to mitochondrial inhibition. As shown in Figure 10A, Ang II treatment did not significantly alter the ECAR in HSVSMCs from T2DM patients. However, in HSVSMCs from T2DM patients, trametinib significantly reduced resting ECARs ($p < 0.05$ with Ang II stimulation, $p < 0.01$ without Ang II stimulation, $n = 4$), and oligomycin-induced ECAR ($p < 0.05$ with Ang II stimulation, $p < 0.01$ without Ang II stimulation, $n = 4$). Furthermore, trametinib significantly reduced ECARs in response to uncoupling ($p < 0.05$ with Ang II stimulation, $p < 0.01$ without Ang II stimulation, $n = 4$), and non-mitochondrial ECAR ($p < 0.05$ with Ang II stimulation, $p < 0.01$ without Ang II stimulation, $n = 4$) in HSVSMCs from T2DM patients. Similarly, in HSVSMCs from non-diabetic patients, there was no significant change to ECAR in response to Ang II (Figure 10B). Conversely, trametinib caused a significant reduction in resting ECARs ($p < 0.01$ with or without Ang II stimulation, $n = 4$), oligomycin-induced ECAR ($p < 0.01$ with or without Ang II stimulation, $n = 4$), ECAR in response to uncoupling ($p < 0.05$ with or without Ang II stimulation, $n = 4$), and non-mitochondrial ECAR ($p < 0.05$ with or without Ang II stimulation, $n = 4$) in HSVSMCs from non-diabetic patients (Figure 10B). However, no

significant differences were observed in the ECARs between Ang II-stimulated HSVSMCs from T2DM and non-diabetic patients (Figure 10C).

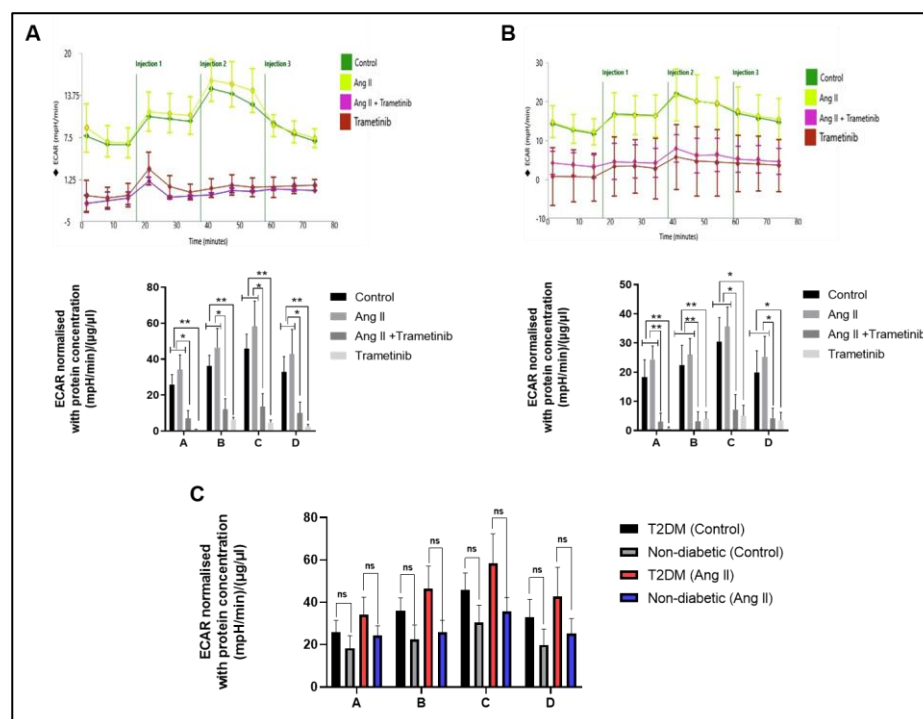


Figure 10. Mitochondrial stress analysis to determine the ECAR of HSVSMCs from T2DM and non-T2DM patients after stimulation with Ang II +/- trametinib. **(A) Upper panel:** representative time course curve of ECAR of HSVSMCs from T2DM patients treated with Ang II (100 nM) +/- trametinib (10 nM) and untreated control from diabetic patients. **Lower panel:** comparative analysis of normalised peak ECAR between treatment groups and untreated control at basal glycolysis, and after addition of inhibitors of mitochondrial respiration and the uncoupler. Normalised data are presented as mean $\pm$ SEM from $n = 4$ biological replicates using HSVSMC samples from different T2DM patients. **(B) Upper panel:** representative time course curve of ECAR of HSVSMCs from non-diabetic patients treated with Ang II (100 nM) +/- trametinib (10 nM) and untreated control from non-diabetic patients. **Lower panel:** comparative analysis of normalised peak ECAR between treatment groups and untreated control at basal glycolysis, and after addition of inhibitors of mitochondrial respiration and the uncoupler. Normalised data are presented as mean $\pm$ SEM from $n = 4$ biological replicates using HSVSMC samples from different non-diabetic patients. **(C)** Comparison of normalised peak ECAR of unstimulated and PDGF-BB-stimulated HSVSMCs from T2DM patients versus non-diabetic control. Normalised data are presented as mean $\pm$ SEM and statistical significance was assessed using an independent t -test, $n = 4$ biological replicates using HSVSMC samples from different T2DM and non-diabetic patients. In the lower panels of Figure 10 (A,B), normalised data from different treatment conditions were compared with data from the normalised untreated control. Statistical significance was assessed using an independent t -test and both one-way and two-way ANOVA, followed by Dunnett's and Holm-Šidák's post hoc tests to determine significant differences between means. * $p < 0.05$; ** $p < 0.01$. ns: not significant; Ang II: Angiotensin II; A: resting ECAR; B: oligomycin-induced ECAR; C: ECAR response to uncoupling; D: non-mitochondrial ECAR; ECAR: extracellular acidification rate.

3.8. Effect of Thrombin on OCR of HSVSMCs from T2DM and Non-Diabetic Patients

Thrombin has been associated with vascular remodelling via a number of potential mechanisms, including activation of the MAPK/ERK signalling pathway [25]. However, it is still unknown how thrombin affects mitochondrial respiration, a crucial mediator of

VSMC metabolic function. Given that understanding the metabolic homeostasis of VSMCs is crucial for targeting vascular remodelling, we therefore assessed the impact of thrombin +/- trametinib on the OCR of HSVSMCs from T2DM and non-diabetic controls. Thrombin caused a significant increase ($p < 0.05$ versus unstimulated cells) in OCR of HSVSMCs from T2DM patients at maximal respiration which was abolished by trametinib (Figure 11A). On the other hand, trametinib significantly reduced OCR at basal respiration ($p < 0.05$ with or without thrombin stimulation versus unstimulated cells, $n = 4$). Furthermore, in HSVSMCs from non-diabetic patients, thrombin did not significantly change OCR, although trametinib significantly reduced (at both basal and maximal respiration) OCRs ($p < 0.05$ with or without thrombin stimulation versus unstimulated cells, $n = 4$; Figure 11B). Meanwhile, direct comparison of OCRs in thrombin-treated HSVSMCs from T2DM and non-diabetic patients revealed there was a significant increase in OCR at maximal respiration ($p < 0.01$, $n = 4$) and spare capacity ($p < 0.05$, $n = 4$) (Figure 11C). Moreover, spare capacity was significantly higher in control T2DM HSVSMCs versus non-diabetic control cells.

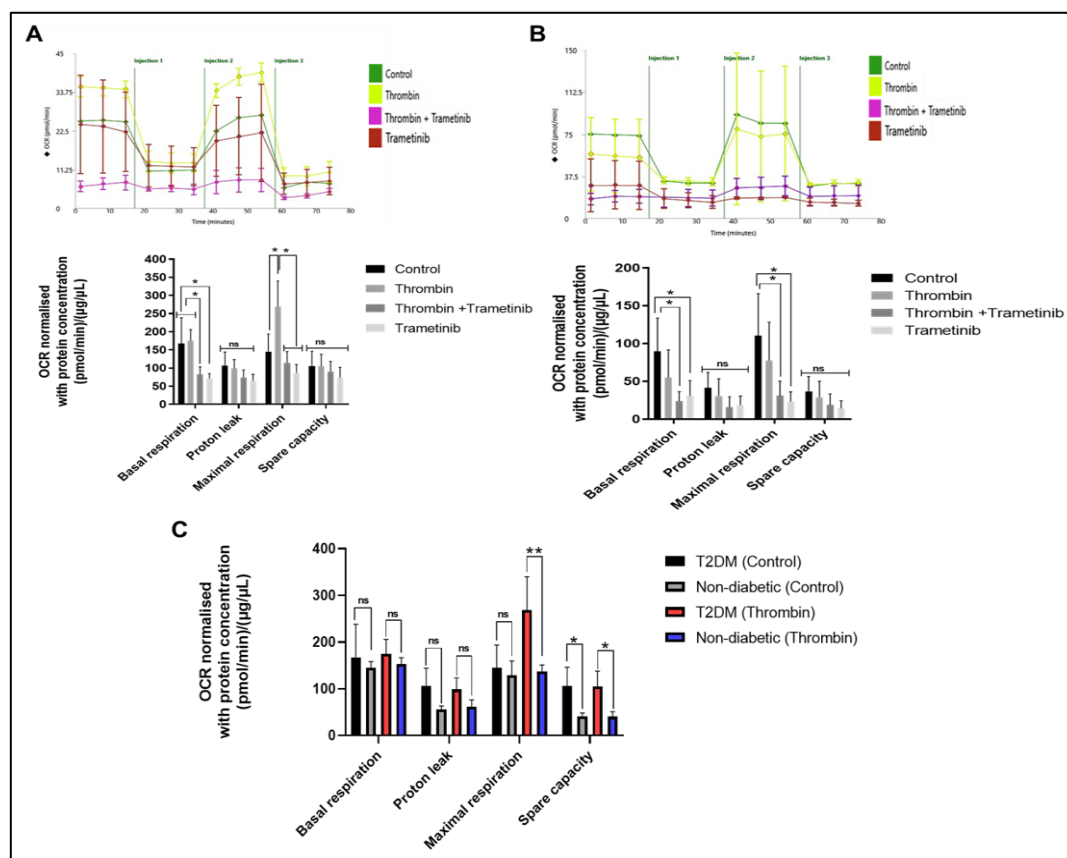


Figure 11. Mitochondrial stress analysis to determine the OCR of HSVSMCs from T2DM and non-T2DM patients after stimulation with thrombin +/- trametinib. **(A) Upper panel:** representative time course curve of OCR of HSVSMCs from T2DM patients treated with thrombin (1 U/mL) +/- trametinib (10 nM) and untreated control from diabetic patients. **Lower panel:** comparative analysis of normalised peak OCR between treatment groups and untreated control at basal glycolysis, and after addition of inhibitors of mitochondrial respiration and the uncoupler. Normalised data are presented as mean $\pm$ SEM from $n = 4$ biological replicates using HSVSMC samples from different T2DM patients. **(B) Upper panel:** representative time course curve of OCR of HSVSMCs from non-T2DM patients treated with thrombin (1 U/mL) +/- trametinib (10 nM) and untreated control from non-diabetic patients. **Lower panel:** comparative analysis of normalised peak OCR between treatment groups and untreated control at basal glycolysis, and after addition of inhibitors of mitochondrial respiration and the uncoupler. Normalised data are presented as mean $\pm$ SEM from $n = 4$ biological

replicates using HSVSMC samples from different non-diabetic patients. (C) Comparison of normalised peak OCR of unstimulated and thrombin-stimulated HSVSMCs from T2DM patients versus non-diabetic control. Normalised data are presented as mean $\pm$ SEM and statistical significance was assessed using an independent *t*-test, $n = 4$ biological replicates using HSVSMC samples from different T2DM and non-diabetic patients. In the lower panels of Figure 11 (A,B), normalised data from different treatment conditions were compared with data from the normalised untreated control. Statistical significance was assessed using an independent *t*-test and both one-way and two-way ANOVA, followed by Dunnett's and Holm–Šidák's post hoc tests to determine significant differences between means. * $p < 0.05$. ns: not significant; ECAR: extracellular acidification rate.

3.9. Effect of Thrombin on ECAR of HSVSMCs from T2DM and Non-Diabetic Patients

Currently, there are no documented research findings highlighting the effects of the MAPK/ERK pathway on ECAR of VSMCs despite the significance of ECAR as a tool for measuring glycolytic flux in cells [38]. Therefore, we assessed the effect of thrombin +/- trametinib on the ECAR of HSVSMCs from T2DM and non-diabetic controls. As shown in Figure 12A, thrombin did not cause any significant alteration in the ECAR of HSVSMCs from T2DM patients. However, trametinib significantly reduced resting ECARs, oligomycin-induced ECAR, ECAR in response to uncoupling, and non-mitochondrial ECAR ($p < 0.01$ with or without thrombin, $n = 4$) in HSVSMCs from T2DM patients (Figure 12A). Similarly, in HSVSMCs from non-diabetic patients, while there was no significant alteration in ECAR after thrombin stimulation, trametinib significantly reduced the OCRs at resting ECAR ($p < 0.01$ with or without thrombin, $n = 4$), ECAR in response to uncoupling ($p < 0.05$ with or without thrombin, $n = 4$), and non-mitochondrial ECAR ($p < 0.05$ with thrombin, $n = 4$) in HSVSMCs from non-diabetic patients (Figure 12B). Also, the ECAR in response to uncoupling in thrombin-stimulated HSVSMCs from T2DM patients was significantly higher when compared with those from non-diabetic patients ($p < 0.05$, $n = 4$; Figure 12C).

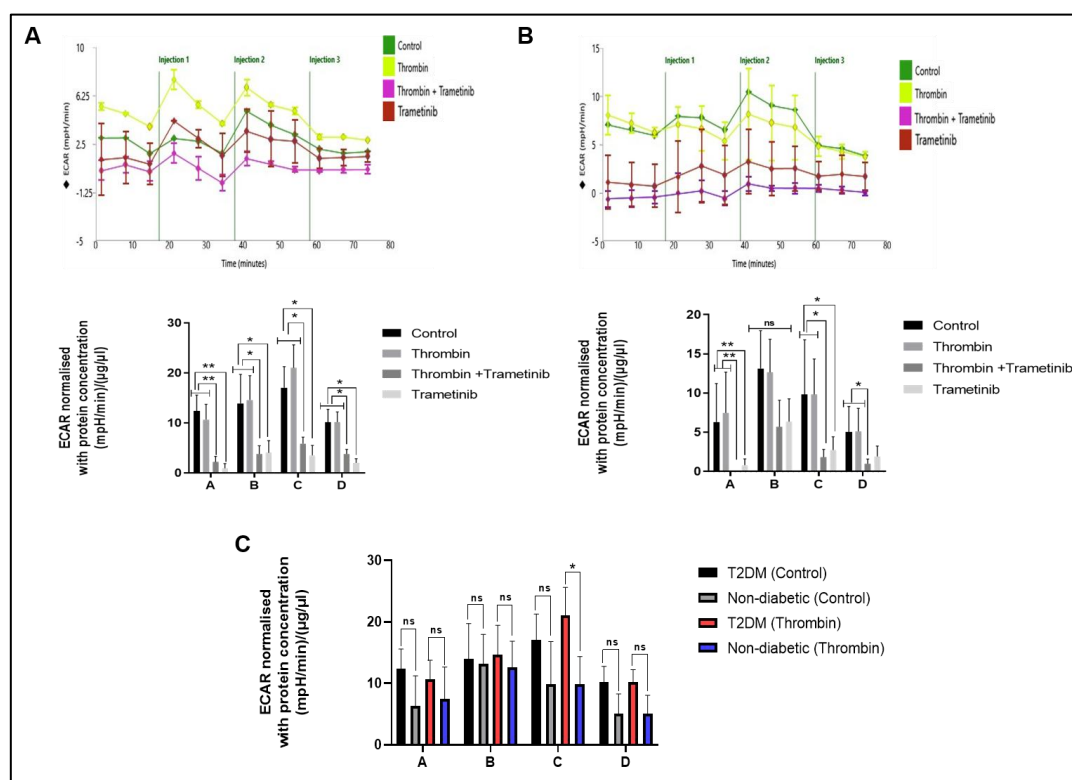


Figure 12. Mitochondrial stress analysis to determine the ECAR of HSVSMCs from T2DM and non-T2DM patients after stimulation with thrombin +/- trametinib. (A) **Upper panel:** representative time

course curve of ECAR of HSVSMCs from T2DM patients treated with thrombin (1 U/mL) +/- trametinib (10 nM) and untreated control from diabetic patients. **Lower panel:** comparative analysis of normalised peak ECAR between treatment groups and untreated control at basal glycolysis, and after addition of inhibitors of mitochondrial respiration and the uncoupler. Normalised data are presented as mean $\pm$ SEM from $n = 4$ biological replicates using HSVSMC samples from different T2DM patients. **(B) Upper panel:** representative time course curve of ECAR of HSVSMCs from non-diabetic patients treated with thrombin (1 U/mL) +/- trametinib (10 nM) and untreated control from non-diabetic patients. **Lower panel:** comparative analysis of normalised peak ECAR between treatment groups and untreated control at basal glycolysis, and after addition of inhibitors of mitochondrial respiration and the uncoupler. Normalised data are presented as mean $\pm$ SEM from $n = 4$ biological replicates using HSVSMC samples from different non-diabetic patients. **(C)** Comparison of normalised peak ECAR of unstimulated and thrombin-stimulated HSVSMCs from T2DM patients versus non-diabetic control. Normalised data are presented as mean $\pm$ SEM and statistical significance was assessed using an independent t -test, $n = 4$ biological replicates using HSVSMC samples from different T2DM and non-diabetic patients. In the lower panels of Figure 12 (**A,B**), normalised data from different treatment conditions were compared with data from the normalised untreated control. Statistical significance was assessed using an independent t -test and both one-way and two-way ANOVA, followed by Dunnett's and Holm-Šidák's post hoc tests to determine significant differences between means. * $p < 0.05$; ** $p < 0.01$. ns: not significant; A: resting ECAR; B: oligomycin-induced ECAR; C: ECAR response to uncoupling; D: non-mitochondrial ECAR; ECAR: extracellular acidification rate.

3.10. mtDNA Expression in HSVSMCs from T2DM and Non-Diabetic Patients After Treatment with Ang II and Thrombin +/- Trametinib

Ang II caused a significant increase in the OCR of HSVSMCs from T2DM at maximal respiration ($p < 0.05$ versus HSVSMCs from non-diabetic patients, $n = 4$; Figure 8C). Also, the OCR of HSVSMCs from T2DM patients, but not from non-diabetic patients, was significantly increased by thrombin (Figure 11). It was therefore important to determine whether this could be explained by an increase in mitochondria number in response to stimulation by Ang II and thrombin. Therefore, the expression of the mitochondrially encoded gene for COI after treatment with Ang II and thrombin +/- trametinib was determined using qPCR [35,36].

As shown in Figure 13A, there was no significant difference in mtDNA expression in HSVSMCs from T2DM patients after treatment with Ang II +/- trametinib compared with the untreated control. Also, in the HSVSMCs from non-diabetic patients, there was no significant difference in mtDNA expression after treatment with Ang II +/- trametinib compared with the untreated control (Figure 13B). Comparison between HSVSMCs from T2DM patients versus the non-diabetic control treated with Ang II +/- trametinib showed no significant difference (Figure 13C). As shown in Figure 13D, there was no significant difference in mtDNA expression in HSVSMCs from T2DM patients after treatment with thrombin +/- trametinib compared with the untreated control. There was also no significant difference in mtDNA expression in HSVSMCs from non-diabetic patients after treatment with thrombin +/- trametinib compared with the untreated control (Figure 13E). Also, comparison between HSVSMCs from T2DM patients versus the non-diabetic control treated with thrombin +/- trametinib showed no significant difference (Figure 13F).

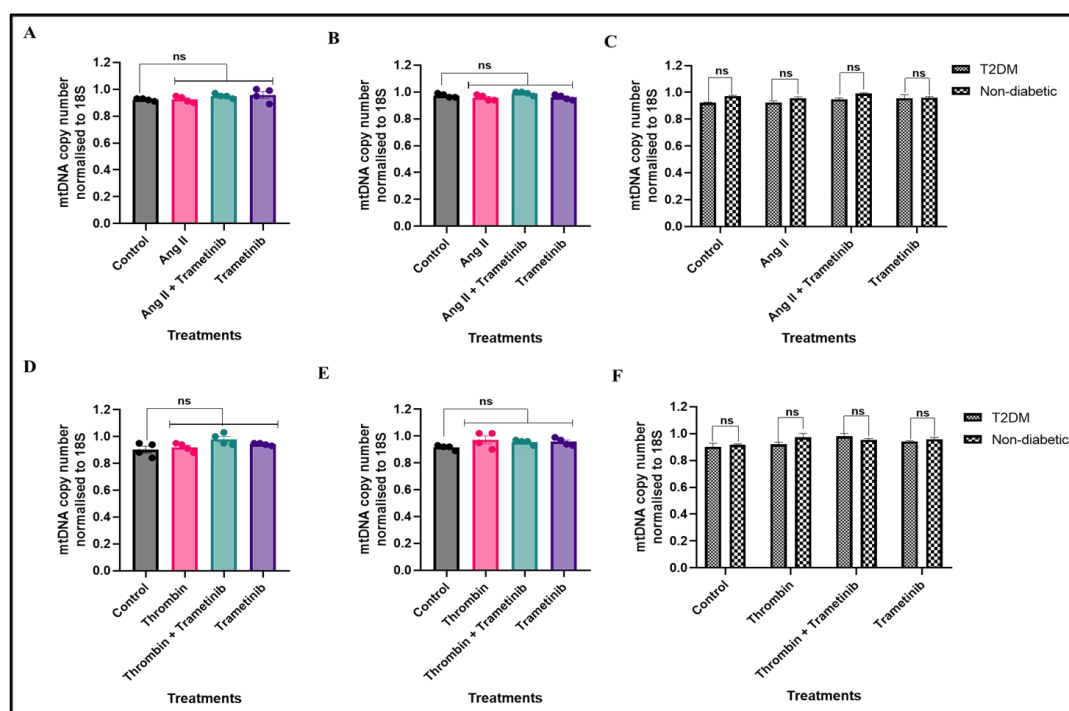


Figure 13. mtDNA expression in HSVSMCs from T2DM and non-diabetic patients after treatment with Ang II and thrombin +/- trametinib. mtDNA expression of the mitochondrially encoded gene for COI as a marker of mtDNA expression in response to stimulation by Ang II and thrombin. **Top panel:** (A) T2DM patients, (B) non-diabetic patients, and (C) comparison between T2DM patients versus non-diabetic control treated with Ang II (100 nM) +/- trametinib (10 nM). **Bottom Panel:** (D) T2DM patients, (E) non-diabetic patients, and (F) comparison between T2DM patients versus non-diabetic control treated with thrombin (1 U/mL) +/- trametinib (10 nM). All data were normalised to 18S, and normalised data from different treatment conditions were compared with data from the normalised untreated control for statistical difference. Data are presented as mean $\pm$ SEM from $n = 4$ experiments using HSVSMC from different patients. Statistical significance was assessed using an independent *t*-test and both one-way and two-way ANOVA, followed by Dunnett's and Holm-Šidák's post hoc tests to determine significant differences between means. ns: not significant; Ang II: Angiotensin II.

4. Discussion

VSMCs have garnered attention as a possible target to treat vascular pathologies due to their role in neointima hyperplasia (NIH) and venous graft stenosis [39–42]. More so, HSV wall thickening is caused by the HSVSMCs shifting from a differentiated to a dedifferentiated phenotype, resulting in uncontrolled cell migration and proliferation responsible for long-term VGF [43]. The rate of VGF after 10 years is about 50% and is suggested to be worse (70%) in T2DM patients [44,45]. Furthermore, it has been established that VSMCs from T2DM patients exhibit unique functional characteristics and are more migratory than those of non-diabetic controls [5,6]. Additionally, there is evidence that T2DM triggers changes in the mitochondrial function of VSMCs of mice [46]. However, for HSVSMCs—important cells linked to the vascular dysfunction causing saphenous VGF—the effects of T2DM on the mitochondrial function and potential downstream signalling mechanisms that regulate these processes are unknown. Hence, our findings have highlighted some of the downstream signalling pathways that are involved in regulating these mitochondrial functions alongside possible effects of T2DM on these functions.

While it is well established that the levels and activities of proinflammatory cytokines such as IL-6 are increased in T2DM [47], the exact impact on mitochondrial OCR and

ECAR in VSMCs—and, specifically, HSVSMCs—is unclear. Our study has now shown that IL-6/sIL-6R α increases mitochondrial respiration through the JAK/STAT pathway, as the IL-6/sIL-6R α -induced increase in the OCR of HSVSMCs from T2DM but not non-diabetic patients was abolished by JAK1/2-selective inhibitor ruxolitinib (Figure 2). Ruxolitinib also caused a reduction in the basal OCR of HSVSMCs from both T2DM and non-diabetic patients (Figure 2). The JAK/STAT pathway is an emerging target in inflammation, a major feature of cardiovascular diseases. Hence, anti-inflammatory drugs are increasingly being considered in the management of cardiovascular events, including those resulting from vascular dysfunction [48]. For instance, current available treatments for pulmonary hypertension (PH), which is caused by remodelling of the pulmonary arteries marked by endothelial dysfunction and proliferation of smooth muscle cells, are vasodilatory drugs, which do not stop disease progression [48]. It has been well demonstrated that the JAK/STAT molecular pathway plays an essential role in the vascular remodelling linked to pulmonary hypertension [49]. Interestingly, our results showed that stimulation of HSVSMCs with IL-6/sIL-6R α did not significantly modify the ECAR of HSVSMCs from either T2DM or non-diabetic patients, suggesting that glycolysis is unaffected by the JAK/STAT pathway. The reason for this is still unclear; however, it has been shown that IL-6/sIL-6R α shifted the control for ATP synthesis towards processes that generate the mitochondrial membrane potential [50]. This suggests that IL-6/sIL-6R α induces a metabolic state where cellular functions are limited by the mitochondrial energy supply, limiting the conversion of glucose to lactate, which determines ECAR [50]. However, it is important to acknowledge that ECAR in this assay is not a pure or calibrated readout of glycolytic flux. Nonetheless, it remains a valid indicator of the compensatory metabolic response to mitochondrial perturbation. The differences in ECAR dynamics observed therefore likely reflect underlying alterations in metabolic plasticity; specifically, the capacity to shift between oxidative and glycolytic ATP production in cells from patients with diabetes.

Similarly, PDGF-BB increased the OCR of HSVSMCs from T2DM patients at basal and maximal respiration and these were again both abolished by ruxolitinib (Figure 4), which suggests that the modulation of mitochondrial respiration by PDGF-BB is also JAK 1/2-dependent. Conversely, there was no significant increase in the OCR of HSVSMCs from non-diabetic subjects in response to PDGF-BB (Figure 4). Also, in both HSVSMCs from T2DM and non-diabetic patients, ruxolitinib caused a significant reduction in the basal and PDGF-stimulated OCR under all conditions (Figure 4). The importance of mitochondrial metabolism as a key regulator of cell growth and proliferation is becoming increasingly appreciated [51]. Increased glycolysis and glutamine usage are seen in rapidly dividing cells, which contribute to the production of energy, reducing equivalents, and the carbon and nitrogen building blocks necessary for daughter cell synthesis [51]. Growth factors like PDGF have been found to boost glycolysis and mitochondrial activity in rat aortic VSMCs [52]. Additionally, studies on pre-clinical models and human pulmonary arterial SMCs have demonstrated that VSMCs have greater rates of glycolysis [53,54]. These studies also indicated a role for mitochondrial fission in pulmonary arterial SMC proliferation, with pulmonary arterial SMC mitochondria in cellular and pre-clinical pulmonary arterial hypertension (PAH) models found to be dysmorphic and hyperpolarised [53,54]. While little is known about how mitochondrial structure controls the phenotypic and metabolic properties of HSVSMCs, it has been demonstrated that such changes in mitochondrial morphology have a direct impact on cell differentiation and proliferation of rat aortic SMCs [55,56]. Furthermore, despite numerous studies that have linked PDGF to vascular dysfunction [56–60], a role for PDGF in mitochondrial respiration and its metabolic components has not yet been investigated in HSVSMCs. The significance of our findings is highlighted by the discovery that PDGF-BB can control mitochondrial

respiration in HSVSMCs via the JAK/STAT pathway, which is less well-known for PDGF's downstream activity than other pathways such as ERK1,2 and Akt [61].

Conversely, ruxolitinib reduced ECAR in HSVSMCs from both T2DM and non-diabetic patients (Figures 3 and 4) and also abolished the PDGF-BB-induced increase in ECAR in HSVSMCs from T2DM patients (Figure 4). These findings suggest a JAK-mediated modulation of mitochondrial glycolysis of HSVSMCs. The JAK/STAT pathway is an established therapeutic target for immune and inflammatory conditions that promote several CVDs [48]. Furthermore, emerging findings have suggested that patients with PAH of different types have pulmonary arteries with overactive JAK/STAT pathways [49,62]. Additionally, several cytokines, including IL-6, IL-13, and IL-11, as well as growth factors, including PDGF, VEGF, and TGF β 1, activate the JAK/STAT pathway and trigger pulmonary remodelling, which contributes to the onset of PAH [49]. Therefore, targeting the cellular signalling pathways implicated in vascular dysfunction to prevent vascular remodelling may also be a viable option for preventing VGF.

Furthermore, our results showed that Ang II did not cause any noticeable alteration in OCR of HSVSMCs from both T2DM and non-diabetic patients. However, trametinib reduced OCR at basal and maximal respiration of HSVSMCs from both T2DM and non-diabetic patients (Figure 8). This suggests that mitochondrial respiration can be modulated through the ERK1,2 signalling pathway. Furthermore, the OCR of HSVSMCs from T2DM and non-diabetic patients did not significantly differ from each other in either the absence or presence of Ang II (Figure 8). While we have described this in HSVSMCs for the first time, it was previously demonstrated that Ang II caused a reduction in the OCR of HUVECs [63]. In this study [63], it was shown that Ang II reduced OCR at basal and maximal respiration compared with the control group. The explanation for this is unclear at this time.

In addition, the ERK1,2 pathway can indirectly modulate cellular metabolic homeostasis through tumour-suppressor liver kinase B1-induced activation of AMPK, a crucial regulator of energy homeostasis, under low-energy situations in most cellular contexts [64,65]. When activated, AMPK blocks most of the biosynthetic pathways required for cell growth, reducing ATP utilisation, and activates catabolic pathways that produce ATP, enabling cells to restore energy equilibrium [65]. Furthermore, it has been shown that Ang II decreased the OCR of mitochondria isolated from the kidney cortex of both non-diabetic controls and type 1 diabetic rats via AT2 receptor-mediated nitric oxide release [66]. It was further demonstrated in this study that whereas Ang II increases the OCR at mitochondria leak respiration in diabetic animals via AT1 receptors, AT1 receptors do not influence mitochondria function in control mice [66]. Together, these findings suggest that while Ang II can elicit its effects on mitochondrial function activity through multiple signalling pathways, in the current study we have looked specifically at the effect of Ang II as a known activator of ERK1,2 signalling in HSVSMCs, where it did not cause any significant alteration in the OCR in cells from T2DM or non-diabetic patients. Also, while there were no significant alterations in the ECAR of HSVSMCs from both T2DM and non-diabetic patients after stimulation with Ang II, trametinib caused a significant reduction in ECAR in HSVSMCs from both T2DM and non-diabetic patients (Figure 10).

We also found that thrombin significantly increased the OCR in HSVSMCs from T2DM but not non-diabetic patients, and that this was attenuated by trametinib (Figure 13). Direct comparison of the OCR of HSVSMCs from T2DM and non-diabetic patients revealed that thrombin significantly increased the OCR of HSVSMCs from T2DM patients compared to non-diabetic control subjects at maximal glycolysis and spare capacity (Figure 11). In addition, trametinib reduced basal OCR in HSVSMCs from both T2DM and non-diabetic patients (Figure 11). Previous findings have revealed that thrombin triggered a significant rise in OCR in platelets [67,68]. While these two studies used platelets from

healthy donors [67,68], similar studies using unstimulated platelets from T2DM patients indicated reduced OCR when compared with those from healthy donors [69]. We have now demonstrated an increase in the OCR of HSVSMCs from T2DM patients, but not non-diabetic patients, following thrombin stimulation. The fact that trametinib attenuated this increase in OCR suggests that thrombin elicited its effect through the ERK1,2 pathway, although the underlying mechanism is not entirely clear.

Trametinib, the MEK1/2 inhibitor employed in this study not only abolished the thrombin-induced increase in the OCR of HSVSMCs from T2DM patients but also reduced the OCR of HSVSMCs from both T2DM and non-diabetic patients. Trametinib binds to unphosphorylated MEK1/2 preferentially, blocking Raf-dependent phosphorylation and activation of MEK [70]. While it might be premature to assume that the ability of trametinib to abolish the MEK-mediated increase in OCR in HSVSMCs from T2DM patients would be of any clinical benefit, trametinib used alone or in combination with B-Raf inhibitor dabrafenib is, however, a clinical tool used to manage malignant melanoma driven by constitutively active Val600Glu- and Val600Lys-mutated B-Raf [71]. Dabrafenib binds the ATP binding site of the mutated B-Raf protein, specifically the Val600Glu mutation. This inhibits the kinase activity of B-Raf and ultimately ERK1,2, resulting in programmed cell death [72].

It has long been recognised that mitochondrial dysfunction contributes significantly to the underlying pathogenesis of a number of age-related diseases, including cancers, CVDs and neurodegenerative diseases [73–75]. Hence, mtDNA expression is increasingly used to evaluate the role of mitochondria in diseases as its measurement is a straightforward proxy for mitochondrial function [73,74]. Furthermore, mtDNA expression levels offer direct insights into a cell's metabolic activity, energy generation capacity, and general mitochondrial health because it encodes crucial protein subunits for the respiratory chain [73,74]. Additionally, mtDNA expression provides an opportunity to determine changes not only in disease conditions but also as a genetic marker [73]. In this study, we used mtDNA expression to investigate whether the observed alterations in OCR and ECAR detected in HSVSMCs from T2DM patients after the activation of the JAK/STAT and the MAPK/ERK signalling pathways were caused by an increase in mtDNA expression or by other factors that are unknown but may be related to T2DM. From our findings, there were no significant changes in mtDNA expression after treatment with the different treatment conditions, and when compared between T2DM patients and non-T2DM controls (Figures 6 and 13). These data therefore suggest that the observed changes in OCR relate to increased metabolic activity by the mitochondria. Therefore, while it is not entirely clear, the observed increase in OCR and ECAR in HSVSMCs from T2DM patients and not from non-diabetic controls could be due to the T2DM status. However, while mtDNA expression, the proxy we used in this study, is very useful as an indicator of mitochondrial function, we acknowledge that determining the mtDNA copy number would have been more appropriate [76]. This is because mitochondria are capable of producing an excess of transcripts that are controlled and maintained post-transcriptionally, and often RNA levels and final protein output do not always correlate [77].

In summary, understanding any link between T2DM and compromised vascular function, specifically in autologous saphenous veins used as conduits in bypass surgery in T2DM patients, is critical to improving vein graft patency and patient outcomes. Our study has identified downstream signalling pathways that regulate mitochondrial function in HSVSMCs and described how T2DM may modify these functions. The significance of these findings lies in the fact that proliferation and migration of vascular cells, including SMCs, have been linked to vascular remodelling and restenosis responsible for VGF. Furthermore, SMCs from T2DM patients have been shown to be more migratory than those from non-diabetic patients [5,6]. Taken together, because increased oxygen and enhanced

glycolysis are required to generate more ATP to drive these cellular processes, identifying the pathways through which this can be effectively modulated is significant for the development of novel therapeutic opportunities to limit vein graft failure. Given the clinical relevance of our findings and the potential for the identified pathways to be targets to improve vein graft potency, we acknowledge that further investigations will be required to strengthen these initial findings. For example, determining whether these variations result from modified receptor expression, increased STAT3 or ERK1/2 activation, modifications to mitochondrial priming, or other metabolic adaptations linked to diabetes would be useful. Furthermore, investigating potential downstream mediators of mitochondrial regulation, such as mitochondrial STAT3, as well as changes in ATP synthesis, electron transport chain activity, and mitochondrial membrane potential, would be useful to establish the MAPK/ERK1,2 and JAK/STAT signalling pathways as key targets to enhance vein graft patency.

5. Conclusions

Through this study, we have demonstrated a JAK- and MEK-mediated regulation of mitochondrial function (OCR and ECAR) alongside a possible effect of T2DM on these processes in HSVSMCs—key cells implicated in the vascular dysfunction that is responsible for VGF. Our findings have therefore contributed to the body of knowledge identifying possible novel glucose-dependent alterations that cause vascular dysfunction in T2DM patients. These findings therefore offer a background and rationale for future investigations to develop novel therapies to improve vein graft patency.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells15151402/s1>, Table S1: Materials; Table S2: Patients' characteristics with medication history and sample ID number.

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Data Availability Statement: Data will be made available upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AMPK	Adenosine monophosphate-activated protein kinase
Ang II	Angiotensin II
AT1	Angiotensin 1 receptor
AT2	Angiotensin 2 receptor
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CVD	Cardiovascular disease
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
FCCP	Carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HSVSMC	Human saphenous vein smooth muscle cell
HUVEC	Human umbilical vein endothelial cell; IL-6, interleukin-6
JAK	Janus kinase
MAPK	Mitogen-activated protein kinase
NAD	Nicotinamide adenine dinucleotide
NIH	Neointimal hyperplasia
NOS	Nitric oxide synthase
OCR	Oxygen consumption rate
PAH	Pulmonary arterial hypertension
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Tween® detergent
PDGF	Platelet-derived growth factor
PH	Pulmonary hypertension
sIL-6Ra	Soluble interleukin-6 receptor
STAT	Signal transducer and activator of transcription
T2DM	Type 2 diabetes mellitus
TGF	Transforming growth factor
VEC	Vascular endothelial cell
VGf	Vein graft failure
VSMC	Vascular smooth muscle cell

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