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ARTICLE

Reduced skeletal-muscle perfusion and impaired ATP release during hypoxia and exercise in individuals with type 2 diabetes

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Abstract

Aims/hypothesis Plasma ATP is a potent vasodilator and is thought to play a role in the local regulation of blood flow. Type 2 diabetes is associated with reduced tissue perfusion. We aimed to examine whether individuals with type 2 diabetes have reduced plasma ATP concentrations compared with healthy control participants (case–control design).

Methods We measured femoral arterial and venous plasma ATP levels with the intravascular microdialysis technique during normoxia, hypoxia and one-legged knee-extensor exercise (10 W and 30 W) in nine participants with type 2 diabetes and eight control participants. In addition, we infused acetylcholine (ACh), sodium nitroprusside (SNP) and ATP into the femoral artery to assess vascular function and ATP signalling.

Results Individuals with type 2 diabetes had a lower leg blood flow (LBF; 2.9 ± 0.1 l/min) compared with the control participants (3.2 ± 0.1 l/min) during exercise ($p < 0.05$), in parallel with lower venous plasma ATP concentration (205 ± 35 vs 431 ± 72 nmol/l; $p < 0.05$). During systemic hypoxia, LBF increased from 0.35 ± 0.04 to 0.54 ± 0.06 l/min in control individuals, whereas it did not increase (0.25 ± 0.04 vs 0.31 ± 0.03 l/min) in the those with type 2 diabetes and was lower than in the control individuals ($p < 0.05$). Hypoxia increased venous plasma ATP levels in both groups ($p < 0.05$), but the increase was higher in control individuals (90 ± 26 nmol/l) compared to those with type 2 diabetes (18 ± 5 nmol/l). LBF and vascular conductance were lower during ATP (0.15 and 0.4 μmol min^{-1} [kg leg mass]$^{-1}$) and ACh (100 μg min^{-1} [kg leg mass]$^{-1}$) infusion in individuals with type 2 diabetes compared with the control participants ($p < 0.05$), whereas there was no difference during SNP infusion.

Conclusions/interpretation These findings demonstrate that individuals with type 2 diabetes have lower plasma ATP concentrations during exercise and hypoxia compared with control individuals, and this occurs in parallel with lower blood flow. Moreover, individuals with type 2 diabetes have a reduced vasodilatory response to infused ATP. These impairments in the ATP system are both likely to contribute to the reduced tissue perfusion associated with type 2 diabetes.

Trial registration ClinicalTrials.gov NCT02001766.

Keywords Exercise · Human · Metabolic physiology in vivo · Microvascular disease

Abbreviations

ACh	Acetylcholine
Kir	Inwardly rectifying potassium channels
LBF	Leg blood flow
LVC	Leg vascular conductance
MAP	Mean arterial pressure
NO	Nitric oxide
SNP	Sodium nitroprusside

Introduction

In the USA, 15% of the adult population (>20 years of age) have type 2 diabetes, while another 37% have impaired

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Research in context

What is already known about this subject?

- Individuals with type 2 diabetes have reduced tissue perfusion
- Plasma ATP is a potent vasodilator that contributes to blood flow regulation

What is the key question?

- Is type 2 diabetes associated with reduced levels of plasma ATP?

What are the new findings?

- Individuals with type 2 diabetes had lower plasma ATP levels during exercise and hypoxia, compared with healthy control participants. This was observed in parallel with lower blood flow in these individuals

How might this impact on clinical practice in the foreseeable future?

- These findings suggest that therapies to restore ATP release in those with diabetes (e.g. phosphodiesterase 3- and phosphodiesterase 5-inhibitors) may help to restore tissue perfusion in these individuals

glucose tolerance [1]. Type 2 diabetes is associated with endothelial dysfunction and risk of cardiovascular complications, affecting organs such as the heart, kidneys and eyes, as well as the extremities, especially the lower limbs [2–4].

Skeletal muscle blood flow is tightly regulated to match O_2 delivery with metabolic demand. This regulation mainly relies on locally derived vasoactive substances and neural vasoconstrictor activity [5, 6]. Individuals with type 2 diabetes have impaired endothelial function and a reduced vasodilatory reserve compared with healthy individuals [2]. Moreover, these individuals have been reported to have a lower blood flow at rest and during exercise [7, 8], potentially resulting in a mismatch between the rate of supply and demand of O_2 and metabolites. Erythrocytes have been proposed to not only function as O_2 carriers, but also as regulators of blood flow by release of ATP into the blood [9, 10]. Intraluminal ATP is a potent vasodilator that can evoke vasodilation in the leg vasculature to a similar extent as that observed during intense exercise [11]. In vitro studies have demonstrated that erythrocytes release ATP in response to stimuli, including hypoxia with and without hypercapnia [9, 12], reduced pH levels [9], mechanical deformation [13], shear stress [14] and an increased temperature [15]. In vitro studies also suggest that, in contrast with erythrocytes from healthy individuals, erythrocytes from those with type 2 diabetes do not release ATP when exposed to low oxygen tension, despite similar levels of intracellular ATP [16, 17].

Intraluminal ATP can induce vasodilation by activating endothelial P2Y receptors [18, 19], resulting in an increased formation of vasoactive substances, such as nitric oxide (NO) and prostacyclin [18, 20], and by activation of inwardly rectifying potassium channels (Kir) [21]. Moreover, intraluminal ATP can blunt sympathetic vasoconstriction (functional sympatholysis) [11, 22, 23]. Individuals with type 2 diabetes have been shown to have a reduced vasodilatory response to arterially infused ATP [24], although this finding has not been consistent [25]. A reduced vasodilatory response to infused ATP does not appear to be related to an altered receptor distribution [24], but could be caused by altered receptor sensitivity and/or endothelium dysfunction, resulting in impaired formation of downstream signalling substances.

In healthy individuals, plasma ATP levels increase during exercise and hypoxia [10, 26] and this increase has been shown to be attenuated in the elderly [27]. Human studies have reported large variations in plasma ATP levels [28], which are likely due to differences in the treatment of blood samples, the short half-life of ATP and the continuous release and degradation of ATP in blood samples. To avoid these confounding factors, we have developed a method using intravascular microdialysis, which allows for in vivo separation of ATP from plasma by diffusion [26]. Given that the occurrence of vascular complications in type 2 diabetes is most common in the lower limbs, it is of clinical relevance to determine ATP release and signalling in the leg vasculature.

The purpose of this study was to: (1) examine if individuals with type 2 diabetes have altered plasma ATP levels during resting conditions and in response to increased metabolic demand, compared with healthy individuals; and (2) investigate whether ATP release in response to hypoxia is altered in those with type 2 diabetes, compared with healthy individuals. To accomplish this, we combined the intravascular microdialysis technique with the one-legged knee-extensor exercise model and reduced inspiratory O_2 fraction and performed arterial infusions of known vasodilators. We hypothesised that ATP release in response to hypoxia and exercise would be impaired

in participants with type 2 diabetes, in parallel with reduced leg blood flow (LBF), as compared with healthy individuals.

Methods

Participants

Nine individuals diagnosed with type 2 diabetes and eight healthy control participants were included in this study (Table 1). Participants were recruited by advertisements in local newspapers (individuals with type 2 diabetes and control participants) and from the Rigshospitalet and Odense University Hospital patient database (patients with type 2 diabetes only). Exclusion criteria were known ischaemic/non-ischaemic heart disease, claudicatio intermittens, unstable angina pectoris, unstable diabetes (change in medication during study period), diabetic retinopathy, nephropathy, severe neuropathy (severely affected sensation), kidney disease, angiopathy (previous or current foot ulcers), hypertension (>140/90 mmHg), use of β-blockers, pregnancy or giving birth within the last 3 months, surgery within the last 6 months, excessive alcohol intake, or smoking. After initial screening, 22 individuals (11 healthy and 11 with type 2 diabetes) were invited to the hospital for the pre-experimental day. Three healthy control participants and two individuals with type 2 diabetes were subsequently excluded as they failed to meet the inclusion criteria (tobacco use, $n = 1$; hypertension, $n = 3$; unstable diabetes, $n = 1$).

Participants were informed verbally and in writing of the potential risks and discomforts associated with the experiments before giving informed, written consent to participate. The study was approved by the Ethics Committee of the Capital Region (H-2011-070) and conducted in accordance with the Declaration of Helsinki. The study is registered with ClinicalTrials.gov as NCT02001766, and the part of the study reported here is described as 'project 1' on the ClinicalTrials.gov record.

Pre-experimental day

The pre-experimental day consisted of an ECG (MAC800, GE Medical systems, Milwaukee, WI, USA), blood screening (glucose, HbA_{1c}, lipids, markers of haematology, thyroid, kidney and liver function; BD, REF 368654, Franklin Lakes, NJ, USA), blood pressure measuring (Omron M6 comfort, Kyoto, Japan), exercise testing and a dual-energy x-ray absorptiometry (DEXA)-scan (Lunar Prodigy Advance; GE Healthcare, Madison, WI, USA). To accustom participants to the exercise model used in this study and to determine the maximum workload, an incremental test to exhaustion (10 min at 6 W and 6 W min^{-1} thereafter) was performed using the one-legged knee–extensor exercise model. Peak pulmonary oxygen uptake ($\dot{V}O_{2peak}$) was also measured (Cosmed, Rome, Italy) during an incremental cycling (Lode Excalibur, Groningen, the Netherlands) test (10 min at 50–100 W and 25 W min^{-1} thereafter) to exhaustion.

Experimental protocol

Participants arrived at the laboratory at 08:30 h after a light standardised breakfast. Participants refrained from caffeine, exercise and glucose-lowering medication 24 h prior to the experiment. Catheters were inserted at a level just proximal to the bifurcation of the common femoral artery (ultrasound guided) using the Seldinger technique [29] under local anaesthesia (Xylocain, 10 mg/ml, AstraZeneca, Mölndal, Sweden; electronic supplementary material [ESM] Fig. 1). Three catheters were placed in the experimental leg. One catheter (20GA, Arrow International, Reading, PA, USA) was placed in the femoral artery and advanced 10 cm in the proximal direction. This catheter was used for blood pressure measurement, blood sampling and pharmacological infusions. A second catheter (20GA, Arrow International Inc. Reading, PA, USA) was placed in the femoral vein and advanced 10 cm in the proximal direction and was used for blood sampling and blood pressure measurement. A third catheter (18GA, Arrow International) was placed in the femoral vein and advanced 10 cm in the distal direction. A fourth catheter (18GA, Arrow International) was placed in the femoral artery of the non-experimental leg and advanced 10 cm in the proximal direction. In the distal femoral venous catheter and the arterial catheter in the non-experimental leg, a microdialysis probe (M Dialysis AB, Solna, Sweden) with a 10 mm membrane (20 kDa cut-off) was inserted. After 30 min of rest, the participants completed the following trials: (1) two bouts of 7 min one-leg knee-extensor exercise (60 repetitions per min) at 10 W and 30 W, respectively; (2) 7 min of rest during hypoxia (fraction of inspired oxygen [F_{IO_2}] = 12.5%); (3) acetylcholine (ACh) infusion (Miochol-E; Bausch + Lomb, Berlin, Germany): three stepwise 2.5 min infusions at 10, 25 and 100 μg min^{-1} [kg leg mass]$^{-1}$; (4) ATP infusion (A7699; Sigma-Aldrich, St Louis, MO, USA diluted in isotonic saline [154 mmol/l NaCl]): three stepwise 2.5 min infusions at 0.04, 0.15 and 1.4 μmol min^{-1} [kg leg mass]$^{-1}$; and (5) sodium nitroprusside (SNP) infusion (Nitropress, Hospira, Lake Forrest, IL, USA): 2.5 min infusion at 1.0 μg min^{-1} [kg leg mass]$^{-1}$(ESM Fig. 2). The trials were separated by 20–30 min of rest. The exercise and hypoxia trials were always performed first (order not randomised), whereas the order of the infusion trials was randomised (by sealed envelope).

Arterial and venous microdialysis Arterial and venous microdialysate samples were collected for 7 min before and during each trial, as well as during the 7 min recovery period

Table 1 Participant characteristics

Variable	Control	Type 2 diabetes	*p*
Participants (*n*)	8	9	
Male	5	7	
Female	3	2	
Age (years)	47 ± 6	51 ± 4	0.17
Time since diagnosis (years)	–	8 ± 3	
Body composition			
Weight (kg)	77 ± 11	84 ± 13	0.27
Height (m)	1.73 ± 0.08	1.74 ± 0.09	0.87
BMI (kg/m^2)	26 ± 3	27 ± 2	0.21
Total body fat (%)	29.9 ± 10.2	27.5 ± 7.4	0.63
$\dot{V}O_{2peak}$			
Absolute (l/min)	2.74 ± 0.99	2.80 ± 0.65	0.89
Relative (ml kg^{-1} min^{-1})	35 ± 10	34 ± 8	0.74
Peak workload (W)[a]	48 ± 17	45 ± 17	0.71
Lipids (mmol/l)			
Total cholesterol	4.9 ± 1.1	4.4 ± 1.1	0.42
HDL-cholesterol	1.6 ± 0.2	1.1 ± 0.4	0.01
LDL-cholesterol	2.9 ± 0.9	2.7 ± 1.0	0.59
Triacylglycerols	1.1 ± 0.8	1.6 ± 0.8	0.18
Glycaemic control			
HbA_{1c} (mmol/mol)	34 ± 2	51 ± 10	<0.001
HbA_{1c} (%)	5.3 ± 0.2	6.8 ± 0.9	<0.001
Plasma glucose (mmol/l)	5.2 ± 0.3	8.8 ± 3.7	0.01
Blood pressure (mmHg)			
Systolic	126 ± 13	139 ± 11	0.06
Diastolic	79 ± 10	86 ± 11	0.19
Glucose-lowering medication (*n*)			
Metformin	–	6	
DDP-4 inhibitor	–	2	
Sulfonylurea	–	1	
SGLT-2-inhibitor	–	1	
GLP-1 analogues	–	1	

Values are expressed as means ± SD or *n*

[a] Measured on one-legged knee-extensor

DDP-4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide-1; SGLT-2, sodium–glucose cotransporter 2; $\dot{V}O_{2peak}$, peak pulmonary oxygen uptake

after the exercise and hypoxia trial. The microdialysis probes were perfused at a rate of 5 μl/min with Ringer's solution (Fresenius Kabi, Uppsala, Sweden). Dalteparin (25 U/ml; Fragmin; Pfizer, Ballerup, Denmark) was added to the perfusate to avoid blood clotting in the membrane. To determine the relative exchange of ATP over the dialysis membrane and to calculate the probe recovery, a small amount (2.7 nmol/l) of [2-^{3}H]ATP (<300 Bq/ml) was added to the perfusate. Measurement of the [2-^{3}H]ATP activity and ATP concentrations in perfusate (inflow) and dialysate (outflow), as well as calculation of probe recovery (relative loss) and plasma ATP concentration, was performed as previously described [26]. After the hypoxia trial, the arterial microdialysis probe was removed. Due to technical difficulties, microdialysis data was obtained in only 14 participants ($n = 7$ in the control group and $n = 7$ in the type 2 diabetes group). The analyser was blinded toward group assignment.

Leg haemodynamics and O_2 variables Leg blood flow was measured with Doppler ultrasound (Logic E9, GE Healthcare, Pittsburgh, PA, USA), as previously described [30]. The analyser was blinded toward group assignment. Heart rate and arterial and venous pressure were recorded via a data acquisition system (PowerLab 16/30, ADInstruments, Bella Vista, NSW, Australia) for later software analysis (Labchart 8, ADInstruments), and transducers positioned at the level of the heart (pressure monitoring kit, ref. T450217A; Edwards Lifesciences, Irvine, CA, USA). Blood samples were drawn: (1) before each trial; (2) during the 7 min of exercise and hypoxia (2.3 and 4.7 min); (3) during each step of ATP/ACh/SNP infusion (at 2 min after infusion); and (4) during the recovery period of the exercise and hypoxia trials (at 3.5 min). Blood samples were analysed using an ABL835 FLEX analyser (Radiometer, Copenhagen, Denmark). Mean arterial pressure (MAP) was calculated from the area under the arterial pressure curve over 8–16 heart cycles. Leg vascular conductance (LVC) was calculated as the quotient between LBF and MAP. Arterial and venous blood O_2 content was calculated as content $O_2 = ([Hb] \times O_2 \text{ saturation} \times 1.34) + (PaO_2 \times 0.003)$. Leg O_2 delivery was calculated as the product of LBF and arterial O_2 content. Leg O_2 uptake ($\dot{V}O_2$) was calculated as the product of LBF and the difference in O_2 content between corresponding arterial and venous blood samples.

Statistical analysis

A one-way repeated measures ANOVA was performed to test significance of treatments within control participants and those with type 2 diabetes. The difference between the groups was tested using a two-way ANOVA. If found to be significant using an F test, pair-wise differences were identified using Tukey's honestly significant difference (HSD) post hoc test. The significance level was set at $p < 0.05$ and a tendency was noted if $0.05 \leq p < 0.10$. Data are presented as means ± SEM unless otherwise stated.

Results

Participant characteristics

There were no differences in baseline characteristics between groups, except for HDL-cholesterol, which was lower in

individuals with type 2 diabetes compared with controls, and variables of glycaemic control were higher in these participants (Table 1).

Leg haemodynamics and plasma ATP concentration during exercise

LBF and LVC increased in both the control and type 2 diabetes groups during the 10 W and 30 W exercise, but were higher in the control group compared with the type 2 diabetes group during both workloads (LBF: 2.1 ± 0.0 vs 1.8 ± 0.0 l/min at 10 W and 3.2 ± 0.1 vs 2.9 ± 0.1 l/min at 30 W in the control and the type 2 diabetes group, respectively. LVC: 19 ± 1 vs 14 ± 1 ml min^{-1} $mmHg^{-1}$ at 10 W and 28 ± 2 vs 20 ± 2 min^{-1} $mmHg^{-1}$ at 30 W, respectively; Fig. 1a, b; $p < 0.05$). Compared with baseline, MAP increased during the 10 W exercise in individuals with type 2 diabetes and during 30 W exercise in both groups ($p < 0.05$). During the 10 W exercise, MAP was higher in the individuals with type 2 diabetes compared with the control participants; although not significant, it also tended to be higher at baseline, during the 30 W exercise and in the recovery period ($p = 0.07$–0.09; ESM Table 1). Leg O_2 delivery was lower at 30 W ($p < 0.05$) and tended to be (non-significantly) lower at 10 W ($p = 0.09$) in individuals with type 2 diabetes compared with control participants (ESM Table 2). There was no difference between groups in partial pressure of oxygen (Po_2), haemoglobin, O_2 saturation, $\dot{V}O_2$ or lactate, whereas glucose was higher in all conditions

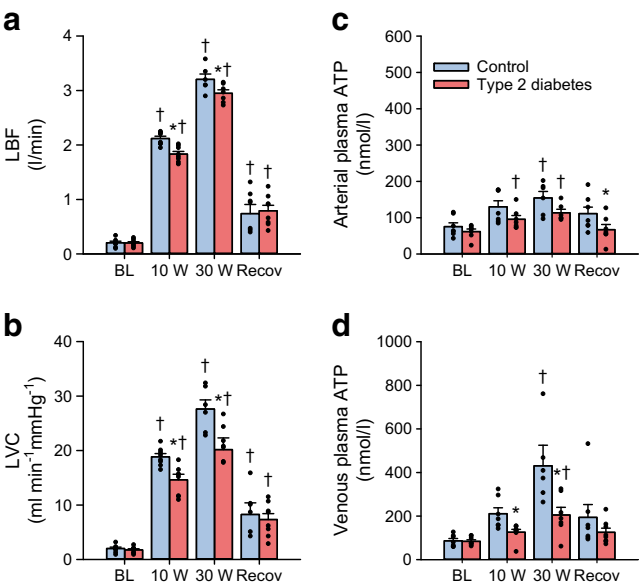

Fig. 1 LBF (**a**), LVC (**b**), femoral arterial plasma ATP (**c**) and femoral venous plasma ATP (**d**) at baseline (BL), and during exercise (10 W and 30 W) and the recovery (Recov) from exercise in the type 2 diabetes and control groups. Data are means ± SEM. The key in (**c**) applies to all figure parts. *$p < 0.05$ vs control; †$p < 0.05$ vs baseline within group

in those with type 2 diabetes, as compared with controls ($p < 0.05$).

Compared with baseline, arterial plasma ATP levels increased during the 10 W and 30 W exercise in the control participants (62 ± 7, 96 ± 10 and 114 ± 10 nmol/l at baseline, 10 W and 30 W, respectively; $p < 0.05$) and during the 30 W exercise in those with type 2 diabetes (76 ± 10 and 155 ± 18 nmol/l at baseline and 30 W, respectively; $p < 0.05$), returning to baseline during the recovery period in both groups (Fig. 1c). Venous plasma ATP levels increased from baseline to 30 W in both the participants with type 2 diabetes (84 ± 7 nmol/l at baseline to 205 ± 35 nmol/l at 30 W) and the control (87 ± 10 nmol/l at baseline to 431 ± 72 nmol/l at 30 W; $p < 0.05$) group, returning to baseline during the recovery period (Fig. 1d). Individuals with type 2 diabetes had lower venous ATP during the 10 W and 30 W exercise compared with control individuals and lower arterial plasma ATP during the recovery period ($p < 0.05$) (Fig. 1c, d). They also had a non-significant tendency towards lower arterial plasma ATP during the 10 W and 30 W exercise ($p = 0.07$ and $p = 0.05$, respectively) compared with control participants.

Leg haemodynamics, heart rate and plasma ATP concentration during hypoxia

Compared with baseline, LBF increased during hypoxia in control participants (0.35 ± 0.04 to 0.54 ± 0.06 l/min at baseline and hypoxia, respectively; $p < 0.05$), whereas it did not change in those with type 2 diabetes (0.25 ± 0.04 vs 0.31 ± 0.03 l/min, respectively; Fig. 2a). In both groups, hypoxia did not change LVC from baseline levels (Fig. 2b), whereas MAP and heart rate increased (ESM Table 1; $p < 0.05$). LBF and LVC were lower in those with type 2 diabetes, both during baseline conditions and during hypoxia, compared with control participants (LVC: 2.3 ± 0.4 vs 3.7 ± 0.4 ml min^{-1} $mmHg^{-1}$ $[kg\ leg\ mass]^{-1}$ at baseline and 2.7 ± 0.4 vs 5.3 ± 0.6 ml min^{-1} $mmHg^{-1}$ $[kg\ leg\ mass]^{-1}$ during hypoxia in the type 2 diabetes and control group, respectively; $p < 0.05$). There were no differences between groups in blood gas variables before, during or after hypoxia (ESM Table 2). Leg O_2 delivery tended to be higher (although not significant) in the control individuals, as compared with the type 2 diabetes group during baseline conditions ($p = 0.09$) and was higher in the control participants during hypoxia (ESM Table 2; $p < 0.05$).

Arterial plasma ATP levels did not change from baseline to during hypoxia in both groups and there was no difference between groups (Fig. 2c). On the other hand, venous plasma ATP levels increased from baseline in both the type 2 diabetes (83 ± 6 nmol/l at baseline to 101 ± 5 nmol/l during hypoxia) and control (104 ± 9 nmol/l to 194 ± 23 nmol/l) groups during hypoxia ($p < 0.05$) (Fig. 2d). However, this increase was smaller in those with type 2 diabetes (18 ± 5 nmol/l) compared with control (90 ± 26 nmol/l) participants and the level of

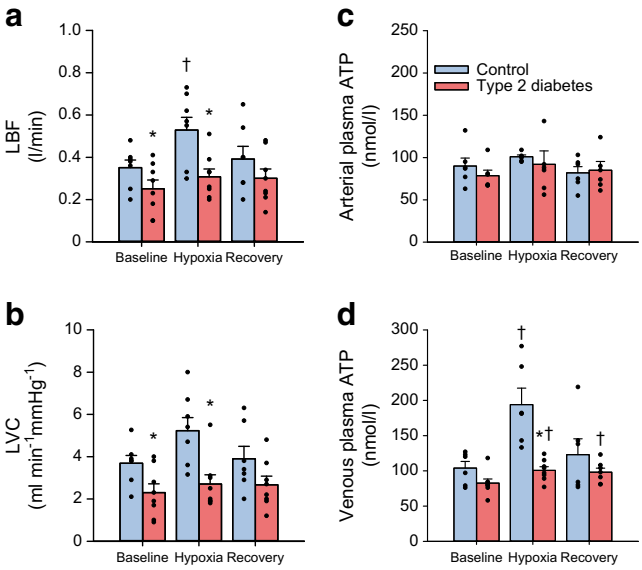

Fig. 2 LBF (**a**), LVC (**b**), femoral arterial plasma ATP (**c**) and femoral venous plasma ATP (**d**) during baseline, hypoxia and in the recovery from hypoxia in the type 2 diabetes and control groups. Data are means ± SEM. The key in (**c**) applies to all figure parts. *$p < 0.05$ vs control; †$p < 0.05$ vs baseline within group

venous plasma ATP during hypoxia was different between the two groups ($p < 0.05$). In the recovery period, venous plasma ATP in the control group tended to be non-significantly higher compared with baseline values ($p = 0.06$) and was significantly higher in the type 2 diabetes group ($p < 0.05$; Fig. 2d).

Leg haemodynamics during ACh, SNP and ATP infusion

During ACh infusion, LVC increased in both groups vs baseline (ESM Table 3), but tended to be reduced (although non significantly; $p = 0.06$) at 25 μg min^{-1} [kg leg mass]$^{-1}$ (the middle dose) and was significantly reduced at 100 μg min^{-1} [kg leg mass]$^{-1}$ in the type 2 diabetes group, compared with control individuals ($p < 0.05$; Fig. 3a; ESM Table 3). During SNP infusion, LVC increased in both groups ($p < 0.05$; ESM Table 3) and there was no difference between groups (ESM Fig. 3b). During ATP infusion, LVC increased in both groups vs baseline (ESM Table 4), but values were lower in those with type 2 diabetes compared with control participants at the two highest doses (0.15 and 0.4 μmol min^{-1} [kg leg mass]$^{-1}$; $p < 0.05$; ESM Table 4; Fig. 3c).

Discussion

The main findings of the present study were: (1) during exercise, individuals with type 2 diabetes had lower LBF and LVC in the exercising leg in parallel with a lower femoral venous plasma ATP compared with healthy participants; (2) during hypoxia, femoral venous plasma ATP was lower in individuals with type 2 diabetes compared with control individuals and hypoxia increased LBF in control individuals only; (3) the type 2 diabetes group had a lower vasodilatory response to exogenous ATP and ACh. Collectively, these findings suggest that reduced plasma ATP levels in response to exercise and hypoxia in the type 2 diabetes group, compared with the control group, could contribute to the impaired skeletal muscle perfusion and O_2 delivery that occur in individuals with type 2 diabetes.

Haemodynamic response to exercise in type 2 diabetes

LBF and O_2 delivery during exercise were lower in the individuals with type 2 diabetes compared with the control individuals, which is in agreement with previous findings [7, 31]. The lower LBF in the individuals with type 2 diabetes was accompanied by a reduced plasma ATP during exercise. In comparing the increase in venous plasma ATP during exercise at 30 W between the two groups, an approximate 2.5-fold increase was observed in individuals with type 2 diabetes, whilst a ~5-fold increase was observed in control participants. The increase in venous plasma ATP from rest to 30 W is in accordance with our previous findings from studies that used the intravascular microdialysis technique to determine plasma ATP [26], as well as other [10, 27, 32–35], but not all [11, 36], previous studies of the exercising leg and forearm.

In contrast to our previous findings [26], we also observed a small increase in arterial plasma ATP levels during exercise in both groups, as compared with baseline. This increase in arterial plasma ATP could be due to increased temperature during exercise [34, 37] resulting in ATP release from erythrocytes [15].

Our results demonstrate that individuals with type 2 diabetes have impaired regulation of LBF coupled with reduced plasma ATP, compared with control individuals, when presented with an increased metabolic demand.

Haemodynamic response to hypoxia in type 2 diabetes

During hypoxia, venous plasma ATP was increased in both groups relative to baseline, but in the participants with type 2 diabetes, this increase was markedly lower (~20% increase) vs control participants (~85% increase). While the observed increase in venous plasma ATP during hypoxia is in line with some in vivo studies [26, 27], not all studies have found increased plasma ATP levels during systemic hypoxia in resting healthy participants [10]. It has been shown that endothelial cells can release ATP when exposed to increased shear stress [38] and that they release a larger amount of ATP when concomitantly exposed to shear stress and hypoxia, but not when

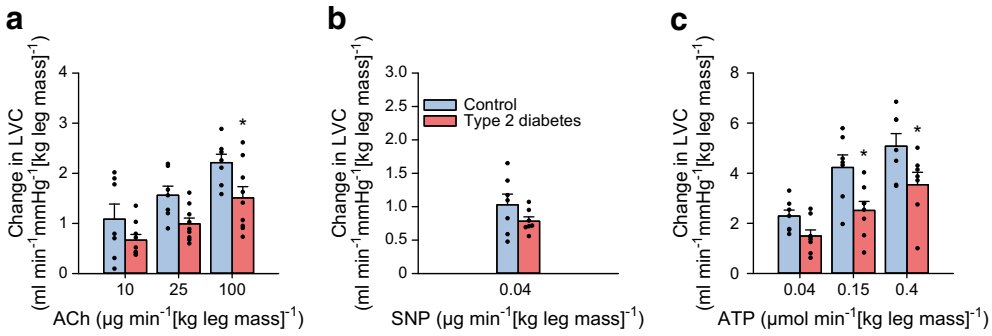

Fig. 3 Changes from baseline in LVC during infusion of ACh (**a**), SNP (**b**) and ATP (**c**) compared with baseline. Data are means ± SEM. The key in (**b**) applies to all figure parts. *$p < 0.05$ vs control

they are exposed to hypoxia alone [39]. We did not observe an increase in LBF in the individuals with type 2 diabetes during hypoxia; therefore, increased endothelial release of ATP in response to shear stress is unlikely to contribute to the increase from baseline in venous plasma ATP observed in this group. Both groups showed a similar decrease in arterial oxygen saturation from baseline during hypoxia, with no increase in arterial plasma ATP, suggesting that ATP release from erythrocytes was evoked by the offloading of O_2 from haemoglobin [9, 10, 40].

Haemolysis has been suggested to be a mechanism for ATP release in erythrocyte suspensions [41], but given that ATP and erythrocytes were separated in vivo in the present study, this cannot explain the observed differences between the groups. An alternative possibility for the lower venous plasma ATP concentration during hypoxia (and exercise) in the type 2 diabetes group is increased ATP degradation and reuptake, but this seems unlikely given that baseline ATP levels were not different between the groups. These observations, therefore, suggest that the lower femoral venous ATP levels in individuals with type 2 diabetes was associated with impaired ATP release in response to hypoxia compared with control participants [16, 42, 43].

ATP responsiveness

The vasodilator response to arterially infused ATP was lower in individuals with type 2 diabetes compared with the control group. In line with this observation, a previous study found that a higher dose of ATP was needed to achieve the same hyperaemic response in individuals with type 2 diabetes compared with healthy participants [24]. ATP can induce vasodilation both through activation of P2Y receptors on the endothelium, resulting in the formation of vasodilators, including NO and prostacyclin [18, 20], by activation of Kir [21] and by reducing the effect of sympathetic activity [11]. Although it is not possible to discern which vasodilator pathway(s) caused the reduced ATP responsiveness in type 2 diabetes, the finding that ACh-, but not SNP-induced vasodilation was lower in the type 2 diabetes vs control group suggests that a reduced formation of endothelial relaxation factors upon ATP stimulation could play a role in the lower vasodilator response observed in the individuals with type 2 diabetes. In line with this, a similar ATP response was observed in participants with type 2 diabetes without endothelial dysfunction as with control individuals [25].

The findings from this study suggest that impaired ATP release in individuals with type 2 diabetes during exercise and hypoxia, combined with the attenuated vasodilatory response of intraluminal ATP, is likely to contribute to the impaired hyperaemic response observed in the individuals with type 2 diabetes [7, 31]. During hypoxia, LBF was ~50% lower in the individuals with type 2 diabetes compared with control participants whereas it was 10–12% lower during exercise. In contracting skeletal muscle, blood flow is regulated by a complex interaction between multiple vasodilator substances, including ATP, NO and prostacyclin [6]; hence, the less pronounced impairment in blood flow during exercise vs hypoxia most likely reflects compensatory upregulation of other vasodilator systems [5]. A unique property of intraluminal ATP is that it can also override local sympathetic vasoconstrictor activity (termed 'functional sympatholysis') [11] and this mechanism is thought to play an important role in matching local O_2 supply with demand [23]. Given the lower intraluminal ATP levels in the participants with type 2 diabetes, this mechanism may also be impaired in these individuals.

Conclusions and clinical perspective

In conclusion, type 2 diabetes is associated with a lower increase in venous plasma ATP in response to exercise and hypoxia compared with control participants, in parallel with a lower blood flow during these conditions. Moreover, individuals with type 2 diabetes have a reduced vasodilatory response to infused ATP. The association between lower plasma ATP levels, lower ATP sensitivity and impaired blood flow regulation suggests that these impairments in ATP release and signalling are likely to contribute to the reduced tissue perfusion associated with type 2 diabetes.

Vascular complications have a major impact on the quality of life of those with type 2 diabetes and, taking into account the increased prevalence of type 2 diabetes, it contributes significantly to the burden on the healthcare systems. Considering the

potency of ATP as a vasodilator, the impaired ATP responsiveness and signalling observed in individuals with type 2 diabetes in this study is likely to have major clinical implications. Using erythrocytes from individuals with type 2 diabetes, in vitro studies have demonstrated that diabetes-associated deficiencies in erythrocytes to release ATP when exposed to low O_2 tension can be rescued by pretreatment with a phosphodiesterase 3-inhibitor [43] or by achieving specific ratios of insulin:C-peptide [42]. Investigations into whether these, or other treatments, can rescue the impaired ATP release and normalise tissue perfusion in patients with type 2 diabetes are, therefore, warranted.

Data availability The datasets generated during the current study are not publicly available due to local legislation, but are partially available from the corresponding author on reasonable request.

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Contribution statement MBG was responsible for acquisition of data, analysis and interpretation of the data and drafting the manuscript. TAK and SHF were responsible for acquisition of data, analysis and interpretation of the data and revision of the manuscript. BKP analysed and interpretated the data and revised the manuscript. YH was responsible for conception and design, acquisition of data, analysis and interpretation of the data and revision of the manuscript. SPM was responsible for conception and design, acquisition of data, analysis and interpretation of the data and drafting the manuscript. All authors approved the final version of the manuscript. SPM is responsible for the integrity of the data.

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