Evidence for a direct effect of the NAD\(^+\) precursor Acipimox on muscle mitochondrial function in humans

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Abstract

Recent preclinical studies showed the potential of nicotinamide adenine dinucleotide (NAD+) precursors to increase oxidative phosphorylation and improve metabolic health, but human data is lacking. Here, we hypothesized that the nicotinic acid derivative Acipimox, a NAD⁺ precursor, would directly affect mitochondrial function, independent of reductions in non-esterified fatty acid (NEFA) concentrations. In a multi-center randomized cross-over trial, 21 patients with type 2 diabetes (age 57.7±1.1 years, BMI, 33.4±0.8 kg/m²) received either placebo or 250 mg Acipimox thrice daily for 2 weeks. Acipimox treatment increased plasma NEFA (759±44 vs. 1135±97 µmol/L, p<0.01 for placebo vs. Acipimox), due to a previously described rebound effect. As a result, skeletal muscle lipid content increased and insulin sensitivity decreased. Despite the elevated plasma NEFA levels, ex vivo mitochondrial respiration in skeletal muscle increased. Subsequently, we showed that Acipimox treatment resulted in a robust elevation in expression of nuclear-encoded mitochondrial gene-sets and by presence of a mitonuclear protein imbalance, which may indicate activation of the mitochondrial unfolded protein response (UPR⁷). Further studies in C2C12 myotubes confirmed a direct effect of Acipimox on NAD+ levels, mitonuclear protein imbalance and mitochondrial oxidative capacity. To the best of our knowledge, this is the first demonstration that NAD+ boosters can also directly impact skeletal muscle mitochondrial function in humans.
Introduction

Acipimox is a nicotinic acid analogue used to treat hyperlipidemia (1; 2). Besides its effects on cholesterol and triglycerides, Acipimox treatment acutely decreases plasma NEFA concentrations by 46-57%, which may lower ectopic fat accumulation in type 2 diabetes mellitus (T2D)(3; 4) and thereby improve whole body insulin sensitivity(5). Furthermore, elevated plasma NEFA concentrations have been shown to deteriorate mitochondrial function (for review see (6)), and lowering plasma NEFA levels may therefore restore mitochondrial function. The latter is important as several features of muscle mitochondrial function can be impaired in patients with T2D and may be related to insulin sensitivity (7-9). Indeed, some studies showed sustained beneficial effects of Acipimox treatment on plasma NEFA and insulin sensitivity (3; 10). A major short coming of these studies, however, is the fact that the last dosage of Acipimox was given on the morning of the test days, making it impossible to separate sustained effects from the acute effect of the last Acipimox dosage. Indeed, even after a treatment with Acipimox dosed four-times daily for several weeks, a rebound increase in plasma NEFAs can be observed (11). Next to this rebound effect, Acipimox is also known to induce flushing in a large number of patients (12), which will limit the clinical relevance of Acipimox for the treatment of insulin resistance and mitochondrial dysfunction, and urge for the development of alternative treatments that can improve mitochondrial oxidative capacity. In that respect, NAD⁺ precursors, such as nicotinamide riboside (NR) (13; 14) or nicotinamide mononucleotide (NMN) (15) have been reported to improve oxidative metabolism in animal models. NR or NMN supplementation to mice resulted in elevated NAD⁺ levels, sirtuin 1 (SIRT1) activation and beneficial adaptation in mitochondrial gene expression profiles (13; 15). Longer term NR or NMN supplementation to mice fed a high-fat diet further improved mitochondrial function and metabolic health (13; 15).
Furthermore, NR did not activate the GPR109A receptor, which is responsible for the flushing observed with Acipimox treatment (16).

So far, NR and NMN have not been tested for efficacy or safety in human clinical trials nor for their effects on mitochondrial metabolism. However, besides the known effects of Acipimox on lipolysis, Acipimox is also a NAD+ precursor. Since both NR/NMN and Acipimox are NAD+ precursors and Acipimox is already available for clinical use in humans, we here examined the potential for such NAD+ precursors in improving mitochondrial function in humans. To this end, in a proof-of-concept study we treated T2D patients with Acipimox for two weeks and examined the sustained effects on mitochondrial metabolism in detail. To rule out the acute lipid-lowering effects of the last dosage of Acipimox, patients were measured after an overnight fast, omitting Acipimox in the morning.

We found that - despite an anticipated rebound effect on plasma NEFA levels - Acipimox has pronounced effects both in vivo, ex vivo and in cell culture experiments on mitochondrial function that are comparable with the effects of other NAD+ precursors such as NR and NMM as found in animals. These results suggest a strong potential for novel, safe NAD+ precursors to boost mitochondrial function in humans.
Materials and Methods

In the present multi-center, randomized, double-blind, placebo-controlled cross-over trial, 21 patients with T2D were included (10 T2D patients in the Maastricht University Medical Center and 11 T2D patients in the German Diabetes Center in Düsseldorf). Figure 1 depicts the flow chart of this trial. All T2D patients were treated once with placebo (Cellulosum microcryst PH102) and once with Acipimox (250 mg 3dd) for 2 weeks each, in random order. Between interventions, a 4-week washout period was maintained. During both 2-week intervention periods, T2D patients were asked to stop their oral glucose lowering medication. At the end of both 2-week periods, patients were provided with a standardized meal the day prior to the clamp. Furthermore, patients were advised to refrain from physical exercise 3 days before the test days. After 2 weeks of Acipimox and placebo treatment, measurements were performed to assess mitochondrial function, ectopic lipid accumulation and insulin sensitivity.

Subjects

Both male and post-menopausal female humans were included (see table 1). Before inclusion in the study, participants underwent physical examination and anthropometry measurements and completed a medical history questionnaire, including history of cardiovascular, renal and pulmonary disease, cancer and duration of diabetes. Also routine medical laboratory tests including hematology, and a maximal aerobic capacity test with concurrent ECG were performed as previously described (17). Body composition was determined using hydrostatic weighing in Maastricht according to the method of Siri et al. (18). In Dusseldorf, body composition was measured with a Dual-energy X-ray absorptiometry (DEXA) scan. T2D patients had well-controlled diabetes (HbA1C 7.08 ± 0.16%, 54 ± 1.51 mmol/mol) and were either on monotherapy with metformin (n=16), on metformin combined with sulfonylurea (n=4) or on diet only (n=1). Patients were allowed to take
other medications, such as the statins rosuvastin \( (n=2) \), atorvastatin \( (n=1) \), simvastatin \( (n=1) \), anti-hypertensive \( (n=10) \) and uricostatic drugs \( (n=6) \). Other medications used, included omeprazole, acetylsalicylic acid, anti-migraine medication and anti-depressant drugs (also see table 1 of the supplementary materials).

Patients were included when diagnosed with T2D for at least one year. None of the participants were following a weight-loss dietary program and all patients had stable body weight for the last 6 months. The Maastricht University Medical Ethical Committee (The Netherlands) and the Medical Association North Rhine in Düsseldorf (Germany) approved the study, and written informed consent was obtained from all participants prior to screening. The study was performed according to the principles expressed in the most recent version of the Declaration of Helsinki.

**Hyperinsulinemic-euglycemic clamp**

All participants underwent a 2-step 6-h hyperinsulinemic-euglycemic clamp \( (10 \text{ and } 40 \text{ mU/m}^2/\text{min}) \) \( (19) \). After an overnight fast, participants received a primed-continuous infusion of \( [6,6-^2\text{H}_2] \) glucose \( (0.04 \text{ mg/kg/min}) \) to determine rates of endogenous glucose production (EGP) and whole body glucose disposal rates (WGD) as previously described \( (20) \). After 180 minutes, low insulin infusion was started \( (10 \text{ mU }/\text{m}^2/\text{min}) \) with the co-infusion of \( 0.1 \mu\text{g/kg/min} \) of somatostatin \( (21) \) for 3.5 hours until a steady state was reached, after which blood sampling and indirect calorimetry was performed during 30 minutes. Thereafter, high insulin infusion was started \( (40 \text{ mU/m}^2/\text{min}) \) with the co-infusion of \( 0.1 \mu\text{g/kg/min} \) somatostatin for 1.5 hours, after which steady state was reached and blood sampling and indirect calorimetry were repeated. During the clamp, oxygen consumption and carbon dioxide production were measured with daily-calibrated automated respiratory gas analyzer using a ventilated hood system \( (\text{Omnical, IDEE, Maastricht, The Netherlands}) \). Whole body glucose and fat oxidation rates were calculated using
stoichiometric equations based on measured oxygen consumption and carbon dioxide concentrations (22) with the assumption that protein oxidation was negligible.

**Muscle biopsies and analysis**

Muscle biopsies were taken from the *m. vastus lateralis*, prior to the clamp under local anesthesia (2% lidocaine), according to the Bergstrom technique (23). Any visible non-muscle material was dissected from muscle tissue. One portion was immediately frozen in liquid nitrogen for biochemical analyses, one portion was frozen in nitrogen-cooled isopentane and embedded in Tissue-Tek for immunohistochemical analyses, and both samples were stored at -80ºC until further use. The remainder muscle tissue was used for mitochondrial respiration analysis.

In the muscle tissue obtained, lipid accumulation was assessed histochemically in cross-sections using a modified oil red O staining for fluorescence microscopy (24). In addition, ~30 mg of the muscle tissue was used for high-resolution respirometry to determine ex vivo mitochondrial function. ATP content was measured in muscle tissue as described (14; 25). Furthermore, immunoblotting was used to detect protein levels of mitochondrial-encoded cytochrome C oxidase I (MTCO1), succinate dehydrogenase complex, subunit A (SDHA) and heat-shock protein 60 (HSP60) (14; 25). MTCO1 and SDHA antibodies were from Abcam and Hsp60 antibody was from Santa Cruz Inc. Coomassie staining of proteins was used as a loading control and was performed using a standard protocol.

**Mitochondrial respiration in permeabilized muscle fibers**

A small portion of the muscle biopsy sample (~30 mg) was immediately placed in ice-cold biopsy containing preservation medium (BIOPS; OROBOROS Instruments, Innsbruck, Austria). Muscle fibers were permeabilized with saponin as previously described (7). After completion of the permeabilization protocol, muscle fibers were transferred into ice-cold mitochondrial respiration buffer (MiRO5; OROBOROS
Instruments, Innsbruck, Austria). Subsequently, mitochondrial function ex vivo was determined by measuring oxygen consumption polarographically using a two-chamber Oxygraph (OROBOROS Instruments). Oxygen consumption, or oxygen flux, reflects the first derivative of the oxygen concentration (nmol/ml) in the respiration chambers, expressed as (pmol/(s*mg)), corrected for wet weight muscle tissue (2–5 mg). To evaluate mitochondrial oxidative capacity, different substrate protocols were applied. In every protocol, 4.0 mmol/l malate was added to obtain state 2 respiration followed by addition of 8.0 mmol/l glutamate as a substrate for complex I, which was combined with or without 40.0 µmol/l palmitoyl-carnitine. In addition, an excess of 1.6 mmol/l adenosine diphosphate (ADP) was added to evaluate state 3 respiration of complex I (state 3 respiration reflects substrate oxidation coupled to energy production). Then 8.0 mmol/l succinate was added to obtain state 3 respiration from complex I and II. Finally, titrations (in steps of 0.5 µl of 1.0 mmol/l) of the chemical uncoupler fluoro-carbonyl cyanide phenylhydrazone (FCCP) were added to evaluate maximal respiratory capacity, state U.

**Measures of mitochondrial density**

Mitochondrial DNA (mtDNA) copy number was determined as a marker for mitochondrial density using quantitative real-time PCR, based on the TaqMan probe method. mtDNA copy number was calculated from the ratio of NADH dehydrogenase subunit 1 (ND1) to lipoprotein lipase (LPL) (mtDNA/nuclear DNA), as described previously described (7).

**Plasma assays**

Blood collected in tubes containing EDTA was immediately centrifuged and plasma stored at -80°C until assayed. Plasma non-esterified fatty acids (NEFAs) and glucose were measured with enzymatic assays on a Cobas Fara/Mira (NEFA: Wako Nefa C test kit; Wako Chemicals, Neuss, Germany; Glucose: hexokinase method; Roche,
Basel, Switzerland). Insulin concentration was determined by radioimmunoassay (Linco Research, St. Charles, MO). Cholesterol, LDL and triglycerides were measured colorimetrically (Roche, Vienna, Austria).

RNA analysis

Total RNA was isolated from human vastus lateralis muscle biopsies using the acidic guanidinium thiocyanate method and purified with RNAeasy RNA isolation kit (Qiagen) and prepared simultaneously for array analysis, with all RNA integrity numbers ≥8.0. Microarrays were analyzed using the Human Gene 2.0 ST platform (Affymetrix). Gene Set Enrichment Analysis (GSEA, by the Broad Institute) was performed using ontology sets designed by the Reactome consortium (26). These general gene sets were supplemented with specific, custom NAD$^+$ booster gene sets designed and detailed in previous publications (13; 14; 27; 28).

In vitro cell culture study

C2C12 myoblasts were differentiated into myotubes as described (29) in 6-well plates. Four-day-differentiated myotubes were treated with Acipimox (10 mol/l) in normal glucose (4.5 g/L) Dulbecco’s Modification of Eagle’s Medium (DMEM) containing 1% albumin-bound oleic acid (Sigma), 10% Foetal Calf Serum (FCS), 2% HEPES, 1% non-essential amino acids and 1% penstrep. NAD$^+$ was extracted 3 hours after the Acipimox incubation and measured using HPLC as described (30). Western blot analysis was performed 24 hours after the Acipimox incubation. Mitochondrial respiration was measured 24 hours after the Acipimox incubation using high-resolution respirometry as described above (Oroboros Instruments). Respirometry was corrected for cell count and viability of the cells loaded.

Calculations

Determination of atom percent enrichment (APE) of $^2$H was done as previously
described (31) after de-proteinization. Steele’s single-pool non-steady-state equations were used to calculate WGD and EGP (32). Volume of distribution was assumed to be 0.160 l/kg for glucose. Non-oxidative glucose disposal (NOGD) was calculated as WGD minus carbohydrate oxidation.

**Statistics**

Data are reported as means ± SE. Statistical analyses were performed using SPSS version 16.0.2 for MacOSX (SPSS Inc., NC, USA). Differences between the interventions were analyzed with a 2-tailed, paired Student’s *t*-test. Statistical significance was set *a priori* at *p*<0.05.
Results

Effect of Acipimox on plasma metabolite concentrations

Patients were treated with Acipimox or placebo for 2 weeks. To test compliance and efficacy of the treatment, we determined plasma cholesterol and triglyceride levels. Despite the short duration of the intervention, cholesterol and triglyceride levels tended to decrease upon Acipimox treatment compared to placebo, indicating that administration of Acipimox was effective in our patients (total cholesterol; 5.6±0.3 vs. 5.1±0.3 mmol/L p=0.08, and TG; 2.6±0.4 vs. 1.7±0.3 mmol/L, p=0.08, for placebo vs. Acipimox). We next determined the effect of Acipimox treatment on plasma non-esterified fatty acid (NEFA) levels. We observed that plasma NEFA levels increased rather than decreased upon Acipimox treatment, consistent with the previously described rebound effect of Acipimox (11; 33). Fasting plasma NEFA concentrations increased from 0.77±0.05 mmol/L with placebo, to 1.13±0.10 mmol/L in Acipimox treated condition (p<0.01, Figure 2A). Notably, plasma NEFA levels remained even significantly elevated throughout the insulin-stimulated period of a hyperinsulinemic clamp (Figure 2A).

Insulin sensitivity

Acipimox has been suggested to improve insulin sensitivity; however, elevations of plasma NEFA levels are generally associated with an induction of insulin resistance (34). Therefore, we examined whether sustained Acipimox treatment influenced insulin sensitivity when the acute effect of Acipimox was omitted. In non-insulin stimulated and at low insulin concentrations, we observed no significant differences in insulin sensitivity and metabolic flexibility between the Acipimox and placebo-treated T2D patients. However, consistent with elevated plasma NEFA, at high insulin concentrations (40 mU/m²/min) whole body glucose disposal (WGD) and non-oxidative glucose disposal (NOGD) were lower upon Acipimox treatment (WGD 20.0±2.6 vs. 13.5±2.1, p=0.03, NOGD 9.9±2.6 vs. 2.5±3.1 µmol/kg/min, p=0.02, for
placebo vs. Acipimox, see Figure 2B), whereas insulin-stimulated oxidative glucose disposal was not affected (figure 2B). Also EGP was less suppressed by insulin upon Acipimox treatment (1.7±0.8 vs. 3.4±0.5 µmol/kg/min, p=0.05, for placebo vs. Acipimox, see Figure 2C).

The effect of high NEFA levels on insulin sensitivity has been previously ascribed to fat accumulation in skeletal muscle (34). Therefore, we examined the effect of Acipimox treatment on skeletal muscle lipid content and found that intramyocellular lipid (IMCL) content was increased from 2.8±0.5 to 3.8±0.8% of total fiber area upon Acipimox treatment (p<0.05, Figure 2D).

**Mitochondrial capacity**

Although Acipimox induced a rebound increase of plasma NEFA levels, which was associated with the development of insulin resistance, we hypothesized that Acipimox may have a direct effect on mitochondrial oxidative capacity, independent of its confounding effects on NEFA levels. Therefore, we first examined if 2 weeks of Acipimox treatment resulted in increases in mitochondrial content. mtDNA copy number was unaffected by Acipimox treatment (2305±218 AU vs. 2353±202 AU, p=0.84, in placebo vs. Acipimox). To investigate functional improvements in mitochondrial respiration, we next performed high-resolution respirometry in permeabilized muscle fibers. Ex vivo mitochondrial respiration increased upon Acipimox treatment for both state 3 and maximal uncoupled respiration on malate/glutamate/succinate (Figure 3A). Upon addition of octanoyl-carnitine, only maximal uncoupled respiration was significantly higher in the Acipimox treated condition (Figure 3B). As mtDNA content was not different between the interventions, a similar tendency with increased mitochondrial respiration upon Acipimox treatment was observed after normalization for mitochondrial respiration to mtDNA content (data not shown).
To find further evidence for a direct effect of Acipimox on mitochondrial metabolism, we performed microarray analysis on vastus lateralis muscle biopsies of a small and randomly chosen subset of patients (n=3). Unbiased gene set enrichment analysis showed a robust and significant enrichment for energy metabolism in Acipimox-treated patients. In particular, Acipimox treatment resulted in a highly significant enrichment of genes involved in the TCA cycle and electron transport chain, suggesting a specific effect of Acipimox on mitochondrial metabolism (Figure 4A). In fact, of all pre-defined gene sets, only those directly related to mitochondrial metabolism were significantly enriched (FWER, p<0.05). However, no pre-defined gene sets specifically address NAD$^+$ metabolism, so we examined a custom-designed gene set composed of genes known to be induced upon treatment with other compounds that boost cellular NAD$^+$ content, such as NR (13; 14) and Poly(ADP-ribose) polymerase (PARP) inhibitors (13; 14; 27; 28). This custom gene set is highly enriched in the Acipimox-treated patients, with particular similarity to gene sets targeted by PARP inhibitors or other NAD$^+$ precursors (Figure 4B). Furthermore, ATP content was markedly increased in the Acipimox-treated patients (35.2±21.3 vs. 70.0±24.5 RLU/mg protein for placebo vs. Acipimox, p<0.01; Figure 4C).

Recently, it has been hypothesized that part of the beneficial effects of NAD$^+$ boosting on mitochondrial metabolism may involve the presence of a stoichiometric imbalance between mitochondrial proteins encoded by the mtDNA and nDNA, i.e. a mitonuclear protein imbalance (25). This imbalance will lead to the induction of the UPR$^\text{mt}$, an adaptive and hermetic signaling pathway that ultimately repairs and improves mitochondrial function and metabolic health in animal models, ranging from the nematode C. elegans to the mouse (14; 25; 35). In a subset of subjects, we examined the presence of mitonuclear protein imbalance and UPR$^\text{mt}$. Acipimox treatment tended to increase the ratio of MTCO1 (a mtDNA-encoded ETC protein)


and SDHA (a nDNA-encoded ETC component) protein content (MTC01/SDHA: 0.26±0.19 vs. 0.75±0.30 in placebo vs. Acipimox treatment, p=0.07) and increased protein content of the mitochondrial chaperone HSP60, which is induced during UPR\textsuperscript{mt} (0.54±0.36 vs. 1.14±0.15 for placebo vs. Acipimox, p<0.05, Figure 4D). To further investigate whether Acipimox has a direct effect on muscle mitonuclear protein imbalance, we used differentiated C2C12 cells incubated with Acipimox or an empty vehicle. First, we confirmed that Acipimox is able to increase NAD+ levels in muscle cells (Figure 5A). Furthermore, 24-hour incubation of C2C12 cells with 10 mmol/l Acipimox increased HSP60 (Figure 5B) and the MTCO1/SDHA ratio (Figure 5C). Finally, to evaluate functional effects of Acipimox in C2C12 cells, we performed mitochondrial respiration assays in C2C12 cells 24 hours after Acipimox (10 mmol/l) incubation. FCCP-driven maximal state U respiration was increased upon Acipimox, mimicking the results observed in the muscle biopsies obtained from the type 2 diabetic subjects (Figure 5D). Together, the data obtained in C2C12 cells strengthen the concept that Acipimox – as a NAD+ precursor – has direct effects on skeletal muscle mitochondrial respiration and metabolism, independent of plasma NEFA levels.
Discussion

We showed that treatment for 2 weeks with a standard clinical dosage of Acipimox — a NAD$^+$ precursor — caused a rebound effect in plasma NEFA concentrations when measured after omission of the morning dose of Acipimox and following overnight fasting, which was paralleled by reduced insulin sensitivity. Despite this rebound effect, Acipimox still improved mitochondrial oxidative capacity and specifically activated a set of mitochondrial genes, which overlaps with the gene-sets induced by NAD$^+$ boosters, such as nicotinamide riboside, nicotinamide mononucleotide, or PARP inhibitors (13-15; 27; 35), in animal studies. These changes were accompanied by a significant mitonuclear protein imbalance and the activation of the UPR$^{\text{mt}}$ in human muscle biopsies and in cultured myotubes. To the best of our knowledge, this is the first demonstration that, like in animals treated with NAD$^+$ boosters (14; 35), UPR$^{\text{mt}}$ is also present in humans. These data hence show that NAD$^+$ precursors activate mitochondrial metabolism in humans using similar signaling pathways as elucidated in a variety of model organisms, ranging from worms to mice. Furthermore, our data urge for testing of new NAD$^+$ precursors in human clinical trials that are devoid of the side effects observed after sustained Acipimox treatment, such as flushing and a rebound rise in NEFA levels.

The observation of elevated NEFA levels and impaired insulin action is in contrast to some other studies that did show a long-term reduction of plasma NEFA concentrations by Acipimox (3; 10). There are two major differences between these studies and our study. First, we administered Acipimox 3 times a day, i.e. after every meal, according to clinical and medicinal guidelines, whereas other studies used a 4 times daily dosing. Second, in all the previous studies, the last dose of Acipimox was given on the test-day just prior to blood sampling and experimental testing, which may obscure the sustained effects of Acipimox and result in acute effects of a morning dosage of Acipimox. Of note, it has been shown previously that Acipimox
treatment can lead to a rebound effect with plasma NEFA rising in the morning, even with a 4 times daily dosage (11). Thus, it was shown that the administration of Acipimox resulted in a sustained daytime rise in plasma NEFA, but did not change plasma NEFA concentrations over a 24 hour period (11). It was suggested that this compensatory free fatty acid rise may be necessary to maintain energy production. This notion is of interest, as it is known that mainly the nocturnal concentrations of plasma NEFAs are elevated in T2D patients (36), with insulin resistance being more pronounced in the morning than in the afternoon (37; 38). Hence, the increase in plasma NEFA concentrations upon Acipimox is most likely a reflection of a nocturnal rebound effect, which in our study was more pronounced, as we did not administer Acipimox in the morning of the experimental test days. Thus, it is likely that previous reports on the effect of Acipimox on plasma NEFA concentrations and insulin sensitivity actually reflect the remnant effect of the last dose of Acipimox taken, rather than being a reflection of prolonged Acipimox treatment. Interestingly, the reduction in insulin sensitivity upon Acipimox treatment in our study, was completely accounted for by a reduction in insulin-stimulated non-oxidative glucose disposal, whereas insulin-stimulated glucose oxidation was not affected by the elevated plasma NEFA levels upon Acipimox treatment, suggesting that Acipimox prevented the decline in oxidative capacity that is normally observed upon high NEFA levels.

In that respect, the rebound rise in plasma NEFA provides a model to study the direct effects of Acipimox on mitochondrial function, independent of reduced plasma NEFA levels. We and others have previously shown that acute elevation of plasma NEFA may actually decrease mitochondrial biogenesis and function (21; 39; 40). In concert with a direct effect of the NAD⁺ precursor Acipimox on mitochondrial function, we observed that in humans Acipimox increased ex vivo mitochondrial oxidative capacity and ATP production despite the potential deleterious elevated plasma NEFA concentrations. A direct effect of Acipimox on muscle mitochondrial oxidative
capacity and ATP production was confirmed in C2C12 myotubes. Acipimox is a nicotinic acid analogue and can thereby increase intracellular NAD\(^+\) concentrations in skeletal muscle. Recent reports show that stimulation of the NAD\(^+\) biosynthesis pathway, via NR or NMN, has beneficial effects on mitochondrial function (13; 15; 41). Indeed, in C2C12 myotubes, Acipimox had a direct effect on enhancing NAD\(^+\) levels. Using an unbiased micro-array approach, we also observed that Acipimox had clear and significant effects on mitochondrial gene expression in skeletal muscle biopsies from type 2 diabetic subjects. Most striking was the observation that Acipimox induced a gene expression signature that was similar to the signature that we observed previously when animals were treated long term with the NAD\(^+\) boosters, such a NR (13) or PARP inhibitors (27; 35). Furthermore, we show that 2 weeks of Acipimox treatment induced a mitonuclear protein imbalance, both in cell culture as in ex vivo analysis, which is indicative of an activation of the mitochondrial unfolding protein response (UPR\(^{\text{mt}}\)), a pathway that we recently showed to be involved in mediating the beneficial effects of NAD\(^+\) on metabolic health (14).

Taken together, our results show for the first time in humans that an NAD\(^+\) precursor like Acipimox activates mitochondrial metabolism in human skeletal muscle and induces a mitonuclear protein imbalance and UPR\(^{\text{mt}}\). This beneficial impact of Acipimox on mitochondrial metabolism is all the more striking as it occurs despite the negative effects of Acipimox on plasma NEFA levels and insulin sensitivity. These findings suggest that, although Acipimox itself may not be a suitable candidate, new NAD\(^+\) precursors that are devoid of such side effects, such as nicotinamide riboside or nicotinamide mononucleotide, may have the potential to act as mitochondrial boosters. Human clinical trials with such agents are urgently needed, given that interventions that target mitochondrial function, such as caloric restriction (42), exercise (43), and Resveratrol (44), have previously been shown to be effective in improving metabolic health.
In conclusion, a two-week intervention with clinical dosages of Acipimox led to a rebound rise in plasma NEFA, which negatively impacted insulin sensitivity. Regardless of this negative effect, skeletal muscle mitochondrial oxidative capacity and ATP production improved, most likely via alterations in NAD$^+$ levels and activation of mitochondrial metabolism and the UPR$^{mt}$. Future research is needed to investigate the safety and efficacy of NAD$^+$ precursors to boost mitochondrial function and improve metabolic health in human subjects such as T2D patients.

**Author contributions**

The corresponding authors take full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript. All authors were involved in one or more aspects of this manuscript, T.W., E.P., L.B., P.S. including writing, P.S., M.R., M.H., J.A. revising, P.S., M.H., M.R., J.S., T.W. designing the study protocol, obtaining, analysing and or processing data E.P., T.W., L.B., P.N., E.P., N.M., E.R., V.S., S.P., L.S., B.H.

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Conflict of interest

The authors declare that they have no conflict of interest.
References

Figure legends

**Figure 1: Study design flow chart**

After screening and subject characterization, patients were randomly assigned to either 2 weeks of Acipimox treatment or 2 weeks of Placebo treatment. At the end of both treatments mitochondrial function, ectopic lipid accumulation and insulin sensitivity were assessed. After a wash-out period of 4 weeks, patients entered in the other intervention arm, such that all patients serve as their own control.

**Figure 2: metabolic effects of 2 weeks of Acipimox treatment or placebo in type 2 diabetes patients**

Effect of Acipimox treatment for 2 weeks on A) plasma NEFA concentrations in T2D patients, both in the fasted state as during a hyperinsulinemic euglycemic clamp. First and second arrows indicate the start of low (10mU/m2/min) and resp. high (40mU/m2/min) infusion of insulin, B) whole body glucose disposal rates, divided into oxidative (black) and non-oxidative (grey) glucose disposal, C) endogenous glucose production, and D) skeletal muscle lipid content as measured by ORO staining in m. vastus lateralis. Significant differences $p<0.05$ are indicated by $\ast$.

**Figure 3: two weeks of Acipimox treatment improves mitochondrial respiration**

Effect of Acipimox treatment for 2 weeks on ex vivo mitochondrial function measured in permeabilized fibers upon addition of complex I and II substrates without (A) and with (B) addition of octanoyl-carnitine as a substrate. Data are uncorrected for mtDNA. $M$=malate, $G$=glutamate, $S$=succinate, $O$=octanoyl-carnitine, $3$=state 3 after addition of ADP, $cytC$=cytochrome C, FCCP=maximal respiration upon chemical uncoupling with FCCP. Significant differences with $p<0.05$ are indicated by $\ast$. 

**Figure 4: NAD⁺ signature after two weeks of Acipimox treatment**

Gene Set Enrichment of muscle biopsies from humans, before and after 2 weeks of Acipimox treatment. In the Reactome gene set list, only one set is significantly changed between cohorts: TCA Cycle & Electron Transport Chain, which is upregulated in treated samples. B) We added a custom-designed gene set more directly related to NAD⁺ metabolism. The NAD⁺ booster gene set is significantly enriched in treated samples. C) ATP content measured by CellTiter-Glo® chemiluminescence kit in muscle biopsies, corrected for protein loading, of placebo and Acipimox treated T2D patients. **, p<0.01. D) Protein levels of mtDNA-encoded MTCO1 and nuDNA-encoded SDHA show a significant mitonuclear imbalance, leading to the induction of UPR⁰mt, as reflected by the induction of HSP60 expression in muscle biopsies of T2D patients after placebo or Acipimox treatment. Coomassie blue staining served as a loading control.

**Figure 5: effect of Acipimox on mitochondrial function in C2C12 cells.**

NAD⁺ levels after 3 hours of Acipimox (10mM) incubation in myotubes (n=6). B) Evaluation of ATP5A, Uqcr10, MTCO1, SDHA and HSP60 protein levels in C2C12 cells 24 hours after Acipimox (10mM) incubation. HSP90 and ponceau staining served as a loading control. C) MTCO1/SDHA ratio after Acipimox (10mM) incubation. D) Evaluation of mitochondrial respiration at the basal condition or after oligomycin and FCCP administration in C2C12 cells 24 hours after Acipimox (10mM) incubation (n=6). Significant differences p<0.05 are indicated by *. 
<table>
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<th>Subject characteristic</th>
<th>Value</th>
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<tr>
<td>Male/female (n)</td>
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<td>Age (years)</td>
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<tr>
<td>Weight (kg)</td>
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<tr>
<td>BMI (kg/m²)</td>
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<td>VO₂max (ml/min/kg)</td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
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<td>Diastolic blood pressure (mmHg)</td>
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<td>Fasting plasma glucose (mmol/L)</td>
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<tr>
<td>HbA1c (%)</td>
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<td>HbA1c (mmol/mol)</td>
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<td>ASAT (U/L)</td>
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<td>ALAT (U/L)</td>
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<td>y-GT (U/L)</td>
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<td>Triglycerides (mmol/L)</td>
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<td>NEFA (μmol/L)</td>
<td>672 ± 70</td>
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**B.** Diabetes

- Placebo
- Acipimox

**Whole body glucose disposal (µmol/kg/min)**

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**Notes:**
- Non-oxidative glucose disposal
- Oxidative glucose disposal

---

**C.**

**Endogenous Glucose Production (µmol/kg/min)**

- Placebo
- Acipimox

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**Notes:**
- * indicates statistical significance

---

**D.**

**ORO staining (% of total fiber area)**

- Placebo
- Acipimox

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**Example of ORO staining in 1 patient**

- Placebo
- Acipimox
A. TCA Cycle & ETC

Enrichment Score

119 genes in set
FWER p = .021

Up in Control | Up in Acipimox

B. NR & PARPi Gene Set

Enrichment Score

18 genes in set
FWER p = .034

Up in Control | Up in Acipimox

C. ATP Content

Control | Acipimox

ATP Level
RLU/mg Protein

D. Control | Acipimox

Gene

Control
Acipimox

Gene

Control
Acipimox

Gene

Control
Acipimox

Gene

Control
Acipimox

Gene

Control
Acipimox

Gene
## Supplement 1: Table with medication use of participants

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