Mitochondrial uncoupling reduces exercise capacity despite several skeletal muscle metabolic adaptations

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ABSTRACT

The effects of mitochondrial uncoupling on skeletal muscle mitochondrial adaptation and maximal exercise capacity are unknown. In this study, rats were divided into a control group (CTL, n=8) and a group treated with 2-4-dinitrophenol, a mitochondrial uncoupler, for 28 days (DNP, 30 mg/kg/day in drinking water, n=8). The DNP group had a significantly lower body mass (p<0.05) and a higher resting oxygen uptake ($\dot{V}O_2$, p<0.005). The incremental treadmill test showed that maximal running speed and running economy (p<0.01) were impaired but that $\dot{V}O_2$max was higher in the DNP-treated rats (p<0.05). In skinned gastrocnemius fibers, basal respiration ($V_0$) was higher (p<0.01) in the DNP-treated animals, whereas the acceptor control ratio (ACR, $V_{max}/V_0$) was significantly lower (p<0.05), indicating a reduction in OXPHOS efficiency.

In skeletal muscle, DNP activated the mitochondrial biogenesis pathway, as indicated by changes in the mRNA expression of PGC1α&β, NRF-1&2, and TFAM, and increased the mRNA expression of cytochrome oxidase 1 (p<0.01). The expression of two mitochondrial proteins (Prohibitin and Ndufs 3) was higher after DNP treatment. Mitochondrial fission 1 protein (Fis-1) was increased in the DNP group (p<0.01), but Mitofusin-1 and -2 were unchanged. Histochemical staining for NADH dehydrogenase and succinate dehydrogenase activity in the gastrocnemius muscle revealed an increase in the proportion of oxidative fibers after DNP treatment.

Our study shows that mitochondrial uncoupling induces several skeletal muscle adaptations, highlighting the role of mitochondrial coupling as a critical factor for maximal exercise capacities. These results emphasize the importance of investigating the qualitative aspects of mitochondrial function in addition to the amount of mitochondria.

Keywords: 2-4 Dinitrophenol; mitochondrial uncoupling; exhaustive exercise; maximal running speed; gas exchanges; $\dot{V}O_2$max

Abbreviations:
ACR, acceptor control ratio; CO$_2$, carbon dioxide; CS, citrate synthase; CTL, control group; DNP, 2-4-dinitrophenol; FCS–DMEM, fetal calf serum–Dulbecco’s modified Eagle’s medium; NO, nitric oxide; O$_2$, oxygen; RER, respiratory exchange ratio; S, skinning solution; TIF2, transcriptional mediators/intermediary factor 2; UCP, mitochondrial uncoupling protein; $V_0$, basal mitochondrial respiration; $\dot{V}CO_2$, carbon dioxide.
dioxide output; $V_{\text{max}}$, maximal mitochondrial respiration; $\dot{V}O_2$, oxygen uptake; $\dot{V}O_2\text{max}$, maximal oxygen uptake.
INTRODUCTION

Exercise capacity depends to a large extent on the efficiency of the cardiorespiratory, vascular and muscle metabolic systems. Limiting factors for maximal oxygen uptake ($\dot{V}O_2\text{max}$) are currently under debate, and a consensus has not been reached. Some authors have concluded that it is the heart’s ability to pump blood that imposes limits on maximal oxygen uptake in a physiologically normal individual at sea level (4); for a review, see (49). In that scenario, the capacity of the circulatory system to transport oxygen to muscle mitochondria is the limiting factor defining the upper limit of $\dot{V}O_2\text{max}$ in humans (8, 10). In contrast, others have claimed that each step in the O$_2$ cascade is important and, in particular, that the mitochondrial level in skeletal muscle is crucial for the determination of endurance performance, and $\dot{V}O_2\text{max}$ (52, 57, 58). Indeed, mitochondria are the primary subcellular structures that use O$_2$ to produce the ATP required for contractile work (29), and the improvement of muscle oxidative capacity (i.e., mitochondrial amount) allows humans and animals to increase their exercise capacity before fatigue occurs (7, 60).

Some in vivo studies using magnetic resonance spectroscopy (3) and ex vivo studies (55, 60, 61) have shown that endurance capacity requires a close coupling between mitochondrial oxidation and phosphorylation. When metabolic demand increases, it has been suggested that both quantitative (i.e., mitochondrial number) and qualitative (i.e., improvement of mitochondrial function) adaptations occur at the level of the skeletal muscle mitochondria to adjust energy conversion (i.e., ATP production) as a function of ATP consumption, thereby allowing an increase in exercise capacity (34, 60, 61). However, the importance of mitochondrial qualitative characteristics, notably the efficiency of oxidative phosphorylation (OXPHOS) coupling, for determining $\dot{V}O_2\text{max}$ and maximal exercise capacity are largely unknown (60).

In addition to muscle contractions, mitochondrial respiratory activity can be increased using different strategies, including the enhanced availability of substrates, increased oxidative stress, NO production, and caloric restriction (13, 14, 39). Another strategy could be to trigger mitochondrial uncoupling, resulting in respiration that proceeds without phosphorylation (47, 48). This strategy could help us better understand the importance of mitochondrial OXPHOS efficiency for VO$_2\text{max}$ and maximal running speed. Several natural substances have been described to cause mitochondrial uncoupling (6). Of these, fatty acids are effective uncouplers, but the protein component of the mitochondrial membrane is necessary to facilitate their translocation.
It has been suggested that the proteins in question are the so-called uncoupling proteins (UCPs). In brown adipose tissue, UCP1 uncouples respiration from ATP production, reducing the efficiency of mitochondrial coupling but increasing heat production. UCP3 is specifically expressed in skeletal muscle and may play a major role in energy expenditure. It also seems to participate in the determination of mitochondrial efficiency (5, 12). However, the overexpression or knock-out mice models used to study mitochondrial uncoupling by UCP3 are controversial (5). Recently, it has been shown in transgenic mice with ectopic expression of UCP1 in skeletal muscle that skeletal muscle mitochondrial uncoupling increases fatty acid oxidation, delays the development of obesity, and increases the median lifespan in mice fed a high-fat diet (32, 33). In addition to the UCPs, Duteil et al. showed that TIF2^{ijskm-/-} mice, in which TIF2 (a p160 transcriptional co-regulator family member) was selectively ablated in skeletal muscle myofibers at adulthood, have greater energy expenditure due to low skeletal muscle mitochondrial uncoupling, which allows for a reduction in the adverse effects of being sedentary, such as reduced muscle oxidative capacities and type 2 diabetes (21).

Despite these findings, no effective pharmacological uncoupling protein agonists have been identified, but artificial uncouplers that efficiently uncouple mitochondria exist. For example, 2,4-dinitrophenol (DNP) allows protons to cross the inner mitochondrial membrane uncoupled to oxidative phosphorylation (26, 41), resulting in increased electron transport and oxygen consumption rates. DNP represents a useful tool for investigating the effects of mild mitochondrial uncoupling on animal energy metabolism, muscle mitochondrial adaptations, and exercise capacities. One study showed that DNP treatment increased mouse longevity, as well as mitochondrial biogenesis, in skeletal muscle following 6 months of treatment at a very low dose. However, that study did not measure the mitochondrial respiratory parameters or the exercise capacities of the animals (15).

This study was designed to determine whether mitochondrial uncoupling induces metabolic stress (qualitative mitochondrial alterations), thus allowing quantitative mitochondrial adaptations in skeletal muscle that alter maximal oxygen consumption and/or running speed in rats.
MATERIALS AND METHODS

Animals

Experiments were performed on adult male Wistar rats (Depré, France) weighing approximately 400 g. They were housed at a density of 1 individual per cage in a neutral temperature environment (20° ± 2°C) on a 12:12 hour photoperiod and were provided food and water ad libitum. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and were approved by our local ethics committee (CREMEAS). After 2 weeks of acclimation and habitation, 12-week-old male Wistar rats were randomly divided into 2 groups as follows: the control group (CTL, n=8) and rats treated with the mitochondrial uncoupler 2,4-dinitrophenol (DNP group, n=8) added to the drinking water. DNP was prepared biweekly and stored in light-protected bottles. Based on water ingestion, the DNP doses were 30 mg/kg/day, in accordance with the review of Harper et al. (26). DNP did not alter water ingestion at any time point (data not shown). The animals underwent daily weight, and temperature measurements, and food and water consumption was recorded daily. A summary of the study design is shown in Fig. 1.

Body temperatures

Rectal temperature was measured daily using a digital thermometer (Beurer, FT 14, Germany). The animals were adapted to rapid and comfortable immobilization and measurements. The temperatures were recorded between 1:00 and 2:00 pm. The room temperature was 20±2°C. The data shown are the average of 5 days of measurements per animal from D24 to D28.

Body weight and efficiency of energy conversion

The average body weight of the animals was given every 7 days. Weight gain/ingestion was calculated over the last 7 days of the protocol for each rat.

Gas exchange measurements

To perform the respiratory measurements, a facemask was put over the nose and mouth of each animal and was held in place with a necklace at rest and/or during treadmill
running. The incoming airflow rate was maintained at a constant 3 L/min for measurements at rest and 5 L/min during treadmill running. The outflowing O₂ concentration and outflowing CO₂ concentration were monitored continuously with O₂ and CO₂ analyzers (Ergocard with Exp’Air software version 1.26.35, Medi-Soft, Dinant, Belgium). The environmental temperature and the barometric pressure were continuously recorded, and the gas values were corrected to STPD (standard temperature and barometric pressure, dry). The signals from the gas analyzers were entered into a computer to calculate VO₂ and VCO₂ (every 10 seconds) using standard gas exchange equations. The metabolic rate data were converted to units of ml/min/kg.

Gas exchange measurements at rest

Two weeks before starting the experiment, the animals were acclimated to the facemask used for respiratory measurements. The animals were placed in a small cage to prevent any movement. Resting gas exchange was measured by averaging the measurements taken over 20 min. The room temperature was 20±2°C.

Gas exchange measurements during maximal incremental test

Two weeks before starting the experiment, the animals were acclimated to the treadmill exercise with the facemask used for respiratory measurements. Acclimatization consisted of running on the treadmill at 25 cm/sec with a 5° incline for 5 min for 3 days/week. The maximal incremental tests were conducted after 21 days of treatment. After measuring the rectal temperature, the animals were placed on a treadmill (Treadmill Control, Letica, Spain) to measure O₂ consumption and CO₂ production. The incline was set at + 5°, and the speed was set at 30 cm/s. The speed was maintained at that rate for 2 min. The speed was then increased by 3 cm/s every 90 sec until exhaustion of the animal. The respiratory exchange ratio (RER) was calculated as the ratio of VCO₂/VO₂. The VO₂ data shown are averages of the last 60 sec at each running speed. The criterion for exhaustion was a time of 5 sec spent on the electrical grid without running. VO₂max was defined as a VO₂ value after which an increase in speed did not result in an increase in VO₂ (22, 23). A respiratory exchange ratio above 1.1 and a blood lactate concentration exceeding 8 mmol/l validated the VO₂max value. The rectal temperature was measured immediately after exhaustion. Blood samples from the tip of
the tail were obtained immediately at the end of exercise to measure blood lactate using a lactate pro-LT device (Lactate Pro LT-1710, ARKRAY®). To assess running economy, oxygen uptake was measured at a submaximal exercise intensity obtained from the maximal incremental test results. The submaximal exercise speed was 23.4 m/min (39 cm/sec). At this speed, the animals were in a steady state during the last 60 seconds. Running economy was expressed in ml/kg/min. One rat in each group was removed from the results because it did not run correctly with the mask and did not reach the maximal running speed or the VO2max (maximal lactate <6 mM).

Gas exchange measurements during maximal incremental exercise

Oxygen uptake (VO2) and carbon dioxide uptake (VCO2) were measured during the maximal incremental test with the same methods as described for the basal condition. The respiratory exchange ratio (RER) was calculated as the ratio of VCO2/VO2. The VO2 data shown are the averages of the last 60 sec at each running speed. The maximum value obtained during the maximal test for VO2 was defined as the maximal oxygen uptake (VO2max, ml/min/kg). VO2max was defined as the maximal oxygen uptake measured during the incremental test. The criteria for reaching VO2max were a leveling off of oxygen uptake despite an increased workload, a respiratory exchange ratio above 1.1 and a blood lactate concentration exceeding 8 mmol/l.

Tissue processing

The animals were euthanized at D28, 7 days after the exhaustive exercise, to avoid the acute effects of the exercise test. The rats were anesthetized via an intraperitoneal injection of sodium pentobarbital (0.1 ml/100 g body weight). The superficial part of the gastrocnemius was excised and cleaned of adipose and connective tissues. The muscle was immediately used to study the respiratory parameters.

Glycogen, triglyceride content and citrate synthase (CS) activity

Muscle samples were prepared for glycogen extraction as previously described (38). Briefly, the samples were homogenized on ice using 10-30 mg/ml tissue in 0.025 M citrate (pH 4.2) containing 2.5 g/l NaF. After centrifugation (15,000 rpm for 5 min), the glycogen content was measured using the EnzyChrom glycogen assay kit (Bioassay...
To determine the triglyceride content, 30 mg of tissue was prepared on ice by adding extraction buffer containing 5 volumes of isopropanol, 2 volumes of water and 2 volumes of Triton X-100. In a 2 mL eppendorf tube, 50 μl of the extraction buffer was added per mg of tissue. The samples were vortexed for at least 30 sec. After centrifugation (15000 rpm for 5 min), 10 μl of the clear supernatant was removed for quantifying triglycerides using the commercially available colorimetric-based EnzyChrom triglyceride assay kit (Bioassay Systems). The data are expressed in mg/dL of muscle.

Citrine synthase activity was determined according to the method of Zoll et al. (60).

**Study of muscle mitochondrial respiration**

Thin muscle fibers were isolated in the skinning (S) solution containing (in mol/l): 2.77 CaK$_2$EGTA, 7.23 K$_2$EGTA, 6.56 MgCl$_2$, 5.7 Na$_2$ATP, 15 phosphocreatine (PCr), 20 taurine, 0.5 DTT, 50 K methanesulphonate, and 20 imidazole (pH 7.1), and they were incubated for 30 min in a solution containing 50 g/ml saponin. Permeabilized fibers were transferred to the respiration (R) solution (the same as the S solution, but containing 3 mmol/l K$_2$HPO$_4$ instead of PCr and ATP) for 10 min to wash out adenine nucleotides and PCr. All steps were performed at 4°C with continuous stirring. The respiration of permeabilized muscle fibers was measured by high-resolution respirometry with an Oxygraph-2k respirometer (OROBOROS INSTRUMENTS, Innsbruck, Austria) at 37°C, using 3-7 mg tissue (wet weight) in each 2 ml glass chamber (9). This technique ensured the determination of global mitochondrial function, reflecting both the density and the functional properties of the muscle mitochondria (61). Mitochondrial respiration was studied in R solution that contained 2 mg/ml bovine serum albumin (51). Basal mitochondrial respiration ($V_0$) was measured in the presence of fibers with the substrates glutamate-malate (5 mM and 2 mM, respectively) and succinate (25 mM). After the determination of $V_0$, the maximal fiber respiration rates were measured at 37°C under continuous stirring in the presence of a saturating amount of adenosine diphosphosphate (ADP) as a phosphate acceptor (2 mM; $V_{max}$). Complex I was stimulated with glutamate-malate and complex II was stimulated with succinate. At the end of the experiment, 50 μM of DNP was added to verify that the maximal respiration rate was obtained with ADP in both groups. The acceptor control ratio (ACR) is defined as $V_{max}/V_0$ and represents the degree of coupling between
oxidation and phosphorylation (OXPHOS). After $V_0$ and $V_{\text{max}}$ measurements, the fibers were dried for 15 minutes at 150°C. Respiration rates are expressed as pmol/(s*mg) dry weight.

**Cell culture and Intracellular ATP measurement**

To study the effect of DNP *in vitro*, myoblasts (L6 Woody, ATCC (American Type Culture Collection, Rockville, MD, USA) were used. To simulate exercise, cells were incubated with a high concentration of ADP (100 µM), which activates mitochondrial respiration. Cells were grown in monolayers at 37°C in a humidified atmosphere at 5% CO$_2$ in 20% FCS–DMEM (fetal calf serum–Dulbecco’s modified Eagle’s medium). At 70% confluence, the cells were divided into four groups: (1) control cells (CTL); (2) cells incubated with 50 µM DNP for 24 hours (DNP); (3) cells incubated with 100 µM ADP for 24 hours (ADP); and (4) cells incubated with 100 µM ADP and 50 µM DNP for 24 hours (DNP/ADP). ATP production was measured at the end of the 24-hour period using an ATPlite kit according to the manufacturer’s instructions (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA). Luminescence was detected using a Victor3 Wallac 1420 multilabel counter (PerkinElmer).

**Histochemical staining of NADH dehydrogenase and succinate dehydrogenase activities**

Gastrocnemius muscle tissue was frozen in liquid nitrogen-cooled isopentane immediately after dissection. For NADH-tetrazolium reductase staining, 10 µm cryosections were incubated in 0.2 M Tris-HCl (pH 7.4), containing 1.5 mM NADH and 1.5 mM nitroblue tetrazolium (NBT) for 15 min at 55°C and washed with three exchanges of deionized H$_2$O. The unbound NBT was removed from the sections with three washes each of 30, 60 and 90 % acetone solutions in increasing and then decreasing concentration. The sections were then rinsed several times with deionized water and mounted with aqueous mounting medium (25).

For SDH staining, 10 µm cryosections were incubated for 1 hour in 20 mM potassium dihydrogen phosphate, 76 mM di- sodium hydrogen phosphate, 5.4 % sodium succinate and 0.02 % nitroblue tetrazolium and washed in Dulbecco’s Phosphate Buffered Saline (DPBS) for 5 min, three times. The sections were then postfixxed in 10 % buffered
formalin solution for 10 min, rinsed twice in 15% ethanol for 5 min, and mounted with aqueous mounting medium.

Protein preparation and analysis

Quadriceps muscles were ground in RIPA buffer [50 mM tris pH 7.5, 1% nonident P40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethanesulphonylfluoride (PMSF) and protease inhibitor cocktail (45 g/mL, 11 873 580 001, Roche)] in a mortar at 4°C. Homogenates (50 μg of protein) were electrophoresed on 12% polyacrylamide gels. The proteins were electroblotted to Hybond nitrocellulose membranes (Amersham Biosciences) and immunodetected using primary antibodies directed against Ndufs3 (439200, Invitrogen, 1/1000), Prohibitin (ab28172, Abcam, 1/5000), GAPDH (MAB374, MILLIPORE UPSTATE CHEMICON, 1/10000). Secondary antibodies conjugated to horseradish peroxidase (Amersham Biosciences) were detected using an enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA, 1/10000).

Quantitative Real-Time Polymerase Chain Reaction (q-RT-PCR)

Total RNA was obtained from quadriceps muscle using Trizol reagent™ (Invitrogen Life Technologies, Rockville, MD, USA), as previously described (56) and following the manufacturer’s instructions. RNA was stored at −80°C until the reverse transcription reaction was performed. cDNA was synthesized from total RNA with the SuperScript First-Strand Synthesis System (Invitrogen) and random hexamer primers. For the real time PCR reaction, 2 μL of cDNA was used in a final volume of 12 μL containing 10 μM of each primer (sense and antisense), SYBR green (Invitrogen Life Technologies, Rockville, MD, USA) as a fluorescent dye and H₂O. The real-time PCR measurement of individual cDNAs was performed in triplicate using SYBR green dye to measure duplex DNA formation with the LightCycler System (Roche Diagnostics, Meylan, France). The sequences of the primers were designed using information obtained from the public database GeneBank (National Center for Biotechnology Information: NCBI). The sequences of the primer sets are listed in Table 1. The quantification of gene expression was conducted using the method described by Liu and Saint ((36), using β-actin as an internal control because it is a stable gene for RT-PCR measurements in
muscles. The amplification efficiency of each sample was calculated as described by Ramakers et al. (43).

Statistical analysis

The data are presented as the mean±SEM. Statistical analyses were carried out using one-way ANOVA. The effects of DNP were evaluated by comparing the data from control rats to DNP rats. The variables for which the one-way ANOVA test indicated a significant difference were also analyzed using a two-way repeated-measures ANOVA, followed by a Tukey post hoc test for intergroup comparisons, to determine changes in body mass, oxygen uptake at rest, VO2 and VCO2 kinetics (GraphPad Prism 5, GraphPad Software, Inc., San Diego, CA, USA). Statistical significance is shown as * P<0.05, ** P < 0.01 and *** P<0.001.
RESULTS

The animals were continuously treated with the uncoupler DNP from D0 to D28 at a concentration of 30 mg/kg/day (in drinking water). Our preliminary results have shown that this treatment promotes a mild uncoupling, with an augmentation of baseline $\overline{V}O_2$ (data not shown). Although hyperthermia can occur with high DNP doses (24), the dosage did not affect body temperature in the CTL or DNP groups (37.4±0.1°C vs. 37.8±0.1°C, respectively), water consumption (30.4 ± 2.6 ml vs. 24.8± 1.8 ml, respectively) or food intake (24.3± 0.5 g vs. 23.8± 0.3 g, respectively, Figs. 2a-2c). It is important to note that because the animals were housed at 20°C, extra heat generated due to the uncoupling promoted by DNP could dissipate, thereby preventing hyperthermia. The body mass of the DNP group was significantly lower than the CTL group from D14 (-4.5 %; p<0.05) to D28 (-5.2 %, p<0.05; Fig. 2d). Indeed, weight gain/food ingestion was lower in DNP-treated animals (19.2±1.6 % vs. 9.2±2.2 % in the CTL and DNP rats, respectively, p<0.05, Fig. 2e). These results suggest that the dosage of DNP used in this study induce a mild mitochondrial uncoupling effect (26).

DNP treatment increases oxygen uptake at rest without any decrease in glycogen and triglyceride content

Oxygen uptake was measured in an open-circuit flow-through system at D0, D14 and D28. Oxygen uptake was significantly higher in DNP rats than in CTL rats from D14 (35.9±1.1 vs. 30.8±1.8 ml/min/kg, p<0.05) to D28 (35.8±2.2 vs. 27.0±1.3 ml/min/kg in DNP and CTL rats, respectively, p<0.005, Fig. 3a). In absolute values, the difference persisted, with a significantly higher oxygen uptake in DNP rats than in CTL rats from D14 (15.4±0.6 vs. 13.2±0.7 ml/min, p<0.05) to D28 (15.4±0.8 vs. 12.9±0.7 ml/min in DNP and CTL rats, respectively, p<0.05). CTL rats had an overall reduction in resting $\overline{V}O_2$, which increased over the duration of the experiment in the DNP rats, as shown by the $\overline{V}O_2$ gain calculation ($\overline{V}O_2$ gain=$\overline{V}O_2$J0- $\overline{V}O_2$J28, Fig. 3b). Indeed, the $\overline{V}O_2$ gain was higher in DNP rats than in CTL rats (7.3±2.4 vs. -2.9±2.0 ml/min/kg, respectively, p<0.01; Fig. 3b). This augmentation of basal oxygen uptake suggested the uncoupling effect of DNP. Basal glycogen concentration was higher in the muscles from DNP-treated animals (94.8±14.7 vs. 139.1±16.1 μg/ml; p=0.06, Fig. 3c), whereas the triglyceride (TG) content was not different between both groups (50.3±1.2 vs. 50.9±1.4 mg/dl in CTL and DNP rats, respectively, Fig. 3d).
DNP treatment decreases maximal exercise capacities but increases VO$_2$\textsuperscript{max}

At D21, we administered a maximal incremental treadmill test to both groups to test the effects of muscle mitochondrial uncoupling on the maximal velocity and parameters of VO$_2$\textsuperscript{max}. Body temperatures were higher after exercise in both CTL and DNP animals (Fig. 4a). The lactate levels were also higher after exercise in both groups, to similar levels (11.6±0.8 mmol/L and 10.8±1.1 mmol/L in CTL and DNP rats, respectively; Fig. 4b), indicating that both groups of animals underwent exhaustive exercise.

The maximal running speed was reduced in DNP compared to CTL rats (42.4±1.7 vs. 47.6±1.4 cm/sec, respectively, -11%, p<0.05; Fig. 4c), whereas the maximal oxygen uptake was higher in the DNP (+9%) than in the CTL group (79.6±1.9 and 73.3±1.6 ml/min/kg, respectively, p<0.05; Fig. 4d). As shown in Fig. 4e, the ratio of VCO$_2$/VO$_2$ was higher in the DNP compared to the CTL group (1.2±0.1 vs. 1.0±0.1 ml/min/kg, respectively, p<0.01). At submaximal speed (39 cm/sec), the energy expenditure was higher in the DNP group, indicating impaired running economy for DNP-treated animals (3.1±0.1 vs. 3.8±0.2 ml/kg/min in CTL and DNP animals, respectively; p<0.01; Fig. 4f). This result indicates that mitochondrial uncoupling impairs the work economy of treated animals.

VO$_2$ and VCO$_2$ kinetics

The measurement of VO$_2$ every ten seconds during the incremental test allowed us to calculate the kinetics of O$_2$ uptake and CO$_2$ uptake as a function of the running speed (Figs. 5a-5b) as in humans (18). Oxygen uptake increased as a function of the running speed in both groups. During the first block of the exercise test, the oxygen uptake was higher in DNP (+12.9%) than in CTL rats (58.2±1.3 and 65.7±1.7 ml/min/kg in CTL and DNP animals, respectively; p<0.01; Fig. 5a). Moreover, oxygen uptake remained higher in the DNP group until exhaustion (+11.7% at 42 cm/sec; 74.2±1.2 and 82.9±2.1 ml/min/kg in CTL and DNP animals, respectively; p<0.01). With a running speed of 42 cm/sec, the VO$_2$ of the DNP group reached a plateau, whereas it increased until exhaustion in the CTL group. The VCO$_2$ also increased from the beginning of the exercise session in the DNP group, whereas the VCO$_2$ in the CTL group only increased in the final blocks of the incremental test (Fig. 5b). Then, VCO$_2$ remained higher in the DNP group from the beginning at 30 cm/sec (+12.7%; 64.9±2.7 and 73.1±3.3 ml/min/kg in the CTL and DNP groups, respectively) until the 45 cm/sec point of the
protocol (+24.5 %; 79.2±1.6 and 98.5±3.4 ml/min/kg in the CTL and DNP groups, respectively; p=0.005).

Respiration rates of skinned skeletal muscle fibers
We measured the basal (V₀) and maximal (Vₘₐₓ) respiration rates in skinned fibers from the superficial part of the gastrocnemius muscle. After 4 weeks of treatment, the V₀ rate was higher in DNP rats compared to CTL rats (86.9±6.7 and 62.4±2.3 pmol/(s*mg dry weight) respectively, +38 %; p<0.01; Fig. 6a). Vₘₐₓ tended to be higher in DNP animals (137.3±9.7 and 119±6.9 pmol /s*mg dry weight) in DNP and CTL, respectively, +15 %; ns; Fig. 6b). The addition of DNP after Vₘₐₓ measurement did not increase the respiration rates in the DNP and CTL groups (148.8±10.7 and 122.7±18.3 pmol /s*mg dry weight) in DNP and CTL, respectively, ns). The acceptor control ratio (ACR) is defined as Vₘₐₓ/V₀ and represents the coupling between oxidation and phosphorylation. ACR is a good index of mitochondrial respiration efficiency. This parameter was significantly lower (-19 %) in DNP animals (1.6±0.1 vs. 1.9±0.1 in the DNP and CTL groups, respectively; p<0.05; Fig. 6c). These results suggest a reduction in oxidative phosphorylation coupling after the DNP treatment. The citrate synthase activity measured in the gastrocnemius muscle was not significantly different between the CTL and DNP rats (5.3±1.1 vs. 3.3±1.0 Uī/g dry weigh, respectively; ns), whereas the ratio between Vₘₐₓ (pmol/(s*mg))/ CS activity (Uī/gPF) was not greater in the DNP group (47.9± 12.3 vs. 30.10 ± 5.9 (pmol/(s*(Uī/gPF))) in the DNP and CTL rats, respectively).
In addition, a strong correlation was observed between the maximal running speed of the rats and the skeletal muscle ACR (r=0.78; p<0.001; Fig. 6d), suggesting the importance of this qualitative mitochondrial parameter for exercise capacity. There was a low correlation between VO₂ max and running speed (r=0.44; p=0.11; Fig. 6e) when the CTL and DNP rats were pooled. This correlation between VO₂ max and running speed became highly significant with the CTL animals alone (r=0.82; p<0.05; Fig. 6f), but not with the DNP animals alone (r=0.58; p=0.20 Fig. 6g), showing that maximal running speed is strongly linked to VO₂ max in CTL rats but not in animals treated with DNP.
Mitochondrial uncoupling following DNP treatment induces skeletal muscle mitochondrial adaptations

The expression of several genes implicated in mitochondrial biogenesis was analyzed by q-RT-PCR in the quadriceps muscle. The peroxisome proliferator-activated receptor gamma co-activator-1α (PGC-1α) and PGC-1β mRNA expression levels were significantly higher after DNP treatment (+330% for PGC-1α; p<0.05 and +452% for PGC-1β; p<0.01; Fig. 7). The expression of nuclear respiratory factor 1 (NRF-1), NRF2a&2b and mitochondrial transcription factor A (TFAM) was significantly higher in the DNP group (+89% for NRF-1; p<0.01, +293% for NRF-2a; p<0.05, +511% for NRF-2b; p<0.01, +304% for TFAM; p<0.01, Fig. 7). For mitochondrial proteins, the mRNA expression of citrate synthase (CS), cytochrome oxidase 4i-1 (COX4i-1) and COX4i-2 were not significantly increased in DNP-treated rats, whereas cytochrome oxidase 1 (COX-1) mRNA expression was clearly augmented in the muscle of DNP animals (+257%; p<0.01; Fig. 7). The expression of uncoupling protein 3 (UCP-3) was lower in the DNP group (-55%; ns). Regarding the mitochondrial dynamic, mitochondrial fission 1 protein (Fis-1), which promotes mitochondrial fission, clearly increased in the DNP group (+507%; p<0.01; Fig. 7), without any significant difference in both groups for Mitofusin-1 and -2 (MFN-1 and MFN-2), mitochondrial membrane proteins that participate in mitochondrial fusion.

To quantify the increase in mitochondrial amount in the skeletal muscle, we quantified two mitochondrial proteins in the quadriceps muscle using Western blots (Fig. 8). Prohibitin, NADH dehydrogenase (ubiquinone) iron-sulfur protein 3, and mitochondrial (Ndufs 3) were higher in the DNP group (+27%; p<0.05 and +27%; p=0.17 for Prohibitin and Ndufs 3, respectively).

Histochemical staining of the reduced form of NADH dehydrogenase (mitochondrial respiratory complex I) and succinate dehydrogenase (complex II) activities in the gastrocnemius muscle revealed that the proportion of darkly stained oxidative fibers increased after DNP treatment, suggesting the presence of more mitochondria in the DNP group (Fig. 9).

Muscle mitochondrial uncoupling in L6 woody myoblasts

Contractile activity is augmented during exercise testing, thereby increasing ATP consumption. This phenomenon (i.e., the augmentation of cellular and mitochondrial
ADP concentration) activates the mechanisms of oxidative phosphorylation to trigger ATP synthesis. To further understand the mechanisms explaining how skeletal muscle mitochondrial uncoupling affects exercise capacity and oxygen uptake, we used a cell culture model. We used L6 woody myoblasts to determine whether muscle mitochondrial uncoupling could decrease ATP formation under conditions of increased ADP concentration, which mimics the cellular consequences of physical exercise. In the unstimulated condition (without ADP), DNP did not impair ATP production by L6 woody cells (Fig. 10). In the stimulated condition, ATP production was increased in CTL+ADP cells compared to CTL cells (2.06 ± 0.17 µM vs. 1.54 ± 0.09 µM, respectively; p<0.01; +33.8 %), showing the stimulation of mitochondrial ATP synthesis when the ADP concentration increases. ATP production was lower in cells exposed to DNP and ADP (DNP+ADP cells) in comparison to CTL+ADP cells (1.45 ± 0.10 vs. 2.06 ± 0.17 µM, respectively; p<0.01; -29.4 %), showing that mitochondrial uncoupling decreases mitochondrial capacity to produce ATP in conditions of high ADP concentration.
DISCUSSION

The results of this study show that (1) DNP treatment induces a mild mitochondrial uncoupling that result in the augmentation of basal oxygen uptake and a reduction in weight gain; (2) mild mitochondrial uncoupling induces important skeletal muscle mitochondrial adaptations to compensate for this qualitative mitochondrial impairment; and (3) despite these mitochondrial adaptations, the maximum running speed is reduced, whereas the $\dot{V}O_2$\textsubscript{max} of DNP-treated rats increases.

Altogether, this study demonstrates the importance of the functional properties of mitochondria (i.e., mitochondrial OXPHOS coupling) in addition to the amount of mitochondria for the determination of maximal exercise capacity and maximal oxygen uptake.

2,4-Dinitrophenol (DNP) treatment induced a mild mitochondrial uncoupling

The chronic treatment of rats with the protonophore DNP enables the direct promotion of mitochondrial uncoupling. To our knowledge, no effective pharmacological uncoupling protein agonists have been identified, and the uncoupling activity of UCP3 in muscle remains controversial (17, 46). We delivered the DNP in the drinking water (30 mg/day/kg) to decrease the efficiency of energy conversion. Indeed, we observed that basal oxygen uptake increased while the weight gain was lower, suggesting that the higher tissue oxygen consumption enhanced substrate oxidation. No hyperthermia in the basal state was observed. Glycogen and triglyceride stored within striated muscle cells represent a large energy source used during exercise. The results showed no difference in triglyceride content, whereas glycogen content had a tendency to be higher in the skeletal muscle of DNP rats. This could represent an adaptation following DNP treatment, as it is the case after an exercise training period in response to the increase in energy demand (28). The small difference in glycogen and TG content between groups and the fact that the incremental test was of very short duration suggest that these biological parameters are unlikely to play a role in the difference in maximal running speed between the groups during the incremental exercise test.

Taken together, these results show that the DNP dose induces moderate and non-toxic mitochondrial uncoupling in Wistar rats, in agreement with previous studies (16, 31, 40).
Mitochondrial uncoupling following DNP treatment induced skeletal muscle mitochondrial adaptations

Our laboratory has previously shown that in skeletal muscle, the coupling between oxidation and phosphorylation (OXPHOS), as calculated from the ACR (V\text{max}/V_0), is higher in athletes than in sedentary people and that endurance training increases this parameter in rats (60, 61). These results suggest that skeletal muscle mitochondrial coupling is an important factor for exercise performance. Recently, it has been shown that the efficiency of ATP production is diminished in the absence of the inner mitochondrial membrane solute transporter (SLC25A25), resulting in a reduction of endurance capacity in animals (1). That work suggests the importance of mitochondrial ATP production for maintaining endurance capacity. In our study we showed that DNP treatment reduced skeletal muscle OXPHOS efficiency. In response to this mitochondrial uncoupling, the mechanisms of mitochondrial biogenesis were clearly activated in muscle, as shown by the increase in mRNA expression of several transcription factors (PGC1α, PGC1β, NRF1&2, TFAM). This transcription activation was associated with an increase in the expression of two mitochondrial proteins, suggesting an increase in mitochondrial amount in the skeletal muscle following DNP treatment. These results were confirmed by the histochemical staining of NADH dehydrogenase and succinate dehydrogenase activities in gastrocnemius muscle, which clearly increased after DNP treatment. The augmentation of maximal mitochondrial respiration in gastrocnemius muscle corroborates these results.

Mitochondria continually fuse and divide in physiological conditions. Several studies have shown that these processes have important consequences for the morphology, function and distribution of mitochondria (19, 20). The increase in FIS-1 without any change in mitofusin-1&2 at the mRNA level suggests an increase in mitochondrial fragmentation after uncoupling treatment. This finding is in line with an in vitro study showing that ionophores, by reducing the mitochondrial membrane potential, cause mitochondrial fragmentation because of an inhibition of mitochondrial fusion (35).

Then, our study clearly demonstrated for the first time that a mitochondrial uncoupling triggered important quantitative mitochondrial adaptations in skeletal muscle, enabling the cells to counteract the qualitative impairments at the level of OXPHOS efficiency.

Moreover, these results showed that chronic mitochondrial uncoupling effects mimics the “classical” metabolic effects of exercise training (27, 42, 59), highlighting the importance of mitochondrial coupling for the regulation of muscle metabolism.
Exercise capacity of rats was reduced following DNP treatment

Studies exploring the effects of mitochondrial uncoupling on exercise capacity are scarce. One study showed that DNP exposure for 24 h decreased the swimming endurance of adult zebrafish (37). In this study, we show that DNP treatment reduces the maximal running speed of rats despite the muscle mitochondrial adaptations and the lower weight of DNP animals. Interestingly, the maximal running speed positively correlates with the ACR, suggesting that an impairment of mitochondrial OXPHOS efficiency in skeletal muscle participates in the reduction of the maximal exercise capacities of DNP rats. To better understand the mechanisms implicated in this impairment, we used an in vitro cell culture model, and showed that at high ADP concentrations, DNP clearly reduced the mitochondrial capacity of L6 myoblasts to produce ATP. These results suggest that the decrease in mitochondrial capacity to produce ATP following OXPHOS uncoupling could be directly responsible for the impairments of maximal exercise capacity, independent of oxygen availability.

This in vitro model had some significant limitations. Indeed, an increase of ADP concentration in the medium of myoblasts in culture does not reflect the complexity of an acute exercise in vivo. On the other hand, such an experimental system allows the visualization of the maximal capacity of mitochondria to produce ATP in uncoupling condition, independently of others factors which could influence the mitochondrial function. In the future, it could be interesting to use the combination of electric pulse stimulation as well as mechanical stretch or temporary hypoxia, which might further help to approximate the environment that a fiber of skeletal muscle is exposed to (11).

Maximal oxygen uptake is increased after DNP treatment

During exercise in rats, VO$_2$ and VCO$_2$ were higher, but VCO$_2$ increased more than VO$_2$ (higher RER), suggesting that CO$_2$ production by the working muscles was high from the beginning of the exercise period and indicating a large activation of anaerobic glycolysis due to physical exercise and stress. Indeed, even after habituation, the rats were stressed from the beginning of the exercise to the end, increasing VCO$_2$. Even if DNP treatment increased the whole-body VO$_2$, during exercise, the skeletal muscle mitochondria were activated. When the animals ran at a submaximal speed, running economy (VO$_2$/running speed), which is a good indicator of exercise performance (2, 44, 45), was impaired in DNP animals. Therefore, DNP animals consumed more
oxygen while running at a given velocity, showing for the first time that chronic treatment with a chemical uncoupler impaired exercise performance by reducing the running economy of rats. The higher $\dot{V}O_2max$ of DNP treated-rats could be attributed to a greater arterio-venous difference in oxygen content which has been shown by others in work that indicates that the capacity of a portion of the oxygen transport system outside the heart is increased above normal with the same cardiac output (4). Some authors have suggested that the heart’s maximal pumping performance imposes the limit on maximal oxygen uptake in humans at sea level (49, 54), especially during exhaustive treadmill exercise that engages large muscle groups. Conversely, others consider that every step of the oxygen pathway contributes to determining maximal oxygen transport, with each step affecting transport almost equally (30, 49, 53, 54). Our results suggest that a higher amount of skeletal muscle mitochondria as well as the uncoupling state of these organelles increased oxygen demand during exercise, enhancing the $\dot{V}O_2max$ of DNP rats. Interestingly, we found that maximal running speed was better correlated with $\dot{V}O_2max$ in CTL rats than in DNP rats. Thus, whereas in normal conditions $\dot{V}O_2max$ is a major parameter for maximal running speed, after DNP treatment, the limiting factor for exercise capacity seems to be at the muscle level and is less dependent on maximal oxygen uptake.

CONCLUSION

Our results showed for the first time that a reduction in OXPHOS efficiency (qualitative impairment) induced muscle mitochondrial adaptations (quantitative adaptations) to compensate for the reduction in ATP synthesis capacity. However, despite these skeletal muscle adaptations, as well as the improvement in $\dot{V}O_2max$, mitochondrial uncoupling reduced maximal exercise capacity, showing the importance of this qualitative parameter for exercise performance. Thus, even if the oxygen transport system is an important parameter that participates in setting the upper limit for exercise performance, this work shows that mitochondrial OXPHOS efficiency significantly participates in this process by altering running economy.
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The manuscript was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English-speaking editors at American Journal Experts.

Author contributions

Zoll J and Schlagowski A-I contributed to the conception and the design of the experiment. Schlagowski A-I, Singh F, Charles A-L, and Gali Ramamoorthy T collected, analyzed and interpreted the data. Zoll J, and Schlagowski A-I drafted the article and Favret F, Piquard F and Geny B revised it critically for important intellectual content. All authors read, edited and approved the final version of the manuscript.

Disclosures

The authors declare that they have no conflict of interest.
FIGURE LEGENDS

Figure 1. Study design. Oxygen uptake (VO₂) evolution and maximal incremental tests were studied in rats. Before beginning the study (D-14), the rats were acclimated to the treadmill work and wore a facemask for respiratory measurements. “Start” indicates the beginning of the DNP treatment, and “End” indicates the end of the protocol (28 days after the beginning). VO₂: Oxygen uptake at rest. VO₂ max was measured during the maximal incremental exercise and represents the maximal oxygen consumption of the rats.

Figure 2. Rats treated for 4 weeks with 2,4-dinitrophenol (DNP) demonstrate less efficient energy conversion. (a) Average water ingestion (mL/animal/day), (b) food ingestion (g/animal/day) and (c) rectal temperature (°C) in the control and DNP groups. From D14 to D28, body mass (d) was lower in the DNP than the CTL group; *p<0.05. (e) Efficiency of energy conversion was determined by calculating weight gain/ingestion over the final 7 days of the experiment for each animal. Weight gain/ingestion was lower in DNP-treated animals vs. the CTL group; (*p<0.05). These results demonstrated the efficiency of DNP treatment as a mild mitochondrial uncoupling agent. CTL (control group; empty bars/symbols) and DNP (DNP-treated group; full bars/symbols). The data represent the means ±SEM.

Figure 3. 2,4-Dinitrophenol (DNP) treatment increases oxygen consumption at rest without a decrease in glycogen and triglyceride content in muscle. Oxygen uptake (VO₂) was measured in an open-circuit flow-through system. (a) VO₂ at rest was higher in DNP rats than CTL rats after 2 weeks (*p<0.05) and after 4 weeks (**p<0.01). (b) The increase in the VO₂ was higher in DNP than in CTL rats after 4 weeks of treatment (**p<0.01). This augmentation of basal oxygen uptake clearly showed the uncoupling effect of DNP. (c) Glycogen was higher in the DNP group, whereas (d) the triglyceride content of the quadriceps muscles after 4 weeks of treatment was not different. CTL (control group; empty bars/symbols) and DNP (DNP-treated group; full bars/symbols). The data represent the means ±SEM.
Figure 4. 2,4-Dinitrophenol (DNP) treatment reduces maximal exercise capacities but increases maximal oxygen uptake ($\dot{V}O_{2\text{max}}$). After 21 days, animals treated or not with DNP performed an incremental treadmill exercise to test the effects of muscle mitochondrial uncoupling on maximal velocity as well as $\dot{V}O_{2\text{max}}$ parameters. (a) Rectal temperature as well as blood lactate (b) increased after exercise to a similar extent in control and DNP animals (**$p<0.001$). Lactate values after exercise showed that animals underwent exhaustive exercise. (c) The maximal velocity was higher in control than in DNP animals (*$p<0.05$), whereas the $\dot{V}O_{2\text{max}}$ was higher in the DNP than in the CTL rats (*$p<0.05$). (e) The ratio of VCO$_2$/VO$_2$ was higher in the DNP group (*$p<0.01$). (f) At 39 cm/sec, the energy expenditure was higher in the DNP group, indicating an impaired running economy in DNP-treated animals (**$p<0.01$). These figures demonstrated the importance of an efficient mitochondrial function to reach maximal exercise capacity. CTL (control group; empty bars/symbols) and DNP (DNP-treated group; full bars/symbols). The data represent the means ±SEM.

Figure 5. 2,4-Dinitrophenol (DNP) treatment alters oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) kinetics during exercise. At D21, animals treated or not treated with DNP were subjected to the maximal incremental treadmill test. The $\dot{V}O_2$ measurement every ten seconds allowed for the calculation of the kinetics of oxygen uptake relative to the running speed. (a) $\dot{V}O_2$ uptake was higher in DNP than CTL rats running at the speed of 30 cm/sec (**$p<0.01$) to 42 cm/sec (**$p<0.01$). (b) $\dot{V}CO_2$ was higher in the DNP group running at 30 cm/sec (*$p<0.05$) to 42 cm/sec (**$p<0.01$). CTL (control group; empty bars/symbols) and DNP (DNP-treated group; full bars/symbols). The data represent the means ±SEM.

Figure 6. 2,4-Dinitrophenol (DNP) treatment increases basal ($V_0$) respiration rates of skinned fibers and reduces the acceptor control ratio (ACR, $V_{\text{max}}/V_0$) of the gastrocnemius muscle. (a) Basal mitochondrial respiration ($V_0$) in skinned gastrocnemius fibers from untreated and DNP-treated rats (**$p<0.001$). (b) Maximal mitochondrial respiration ($V_{\text{max}}$) in skinned gastrocnemius fibers from untreated and DNP-treated rats. (c) The acceptor control ratio (ACR, $V_{\text{max}}/V_0$), representing the degree of coupling between oxidation and phosphorylation (*$p<0.05$). (d) The correlation between the running speed of rats and the ACR of the gastrocnemius muscle ($r=0.78$; **$p<0.001$). (e) The correlation between $\dot{V}O_{2\text{max}}$ and running speed with
untreated and DNP-treated rats ($r=0.44; \ p=0.11$). (f) The correlation between $\dot{V}O_2\text{max}$ and running speed in CTL rats ($r=0.82; \ *p<0.05$). (g) The correlation between $\dot{V}O_2\text{max}$ and running speed in DNP-treated rats ($r=0.52; \ p=0.20$). CTL (control group; empty bars/symbols) and DNP (DNP-treated group; full bars/symbols). The data represent the means ±SEM.

**Figure 7.** 2,4-Dinitrophenol (DNP) treatment increases mitochondrial biogenesis and mitochondrial fission. PGC-1α, PGC-1β, NRF-1, NRF-2a, NRF-2b, Tfam, CS, COX-1, COX4i-1, COX4i-2, UCP-3, FIS-1, MFN-1, and MFN-2 mRNA expression levels determined by real-time PCR in the quadriceps muscle. PGC-1α and -1β, peroxisome proliferator-activated receptor gamma co-activator 1 alpha and beta; NRF-1, -2a and -2b; nuclear respiratory factor 1, 2a and 2b; Tfam, transcription factor A, mitochondrial; CS, citrate synthase; COX-1, 4i-1 and 4i-2, cytochrome c oxidase mitochondrial-1, subunit 4 isoform -1 and -2; UCP-3, mitochondrial uncoupling protein 3; FIS-1, mitochondrial fission 1; MFN-1 and -2, mitofusin-1 and -2. (*$p<0.05$; **$p<0.01$). CTL (control group; empty bars/symbols) and DNP (DNP-treated group; full bars/symbols). The data are presented as % of CTL.

**Figure 8.** 2,4-Dinitrophenol (DNP) treatment increases mitochondrial proteins. Proteins used as mitochondrial biogenesis markers were quantified using Western blots in quadriceps muscle. Prohibitin was significantly increased in the DNP group (*$p<0.05$), Ndufs 3, and mitochondrial NADH dehydrogenase (ubiquinone) iron-sulfur protein 3. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control. CTL (control group; empty bars/symbols) and DNP (DNP-treated group; full bars/symbols). The data are presented as % of CTL.

**Figure 9.** 2,4-Dinitrophenol (DNP) treatment increases mitochondrial enzymatic activities. Histochemical staining of NADH dehydrogenase and succinate dehydrogenase activity in gastrocnemius muscle. Three different fiber-types are distinguished: oxidative and intermediate fibers are darkly and moderately stained, respectively; glycolytic fibers are unstained. CTL (control group) and DNP (2,4-dinitrophenol-treated group).
Figure 10. DNP treatment reduces mitochondrial capacity to produce ATP in the presence of high ADP concentrations in L6 woody myoblasts. In normal conditions (without DNP), ATP production was increased when ADP (100 µM) was added to the sample (**p<0.01). When DNP (50 µM) was co-incubated with ADP, ATP production decreased compared to the condition without DNP (**p<0.01). CTL: cells in normal conditions (without ADP and DNP); DNP: cells incubated with DNP for 24 hours (without ADP); ADP: cells incubated with ADP for 24 hours (without DNP); ADP/DNP: cells exposed to both ADP and DNP for 24 hours.
REFERENCES


FIGURES AND TABLES

Table 1. List of oligonucleotides used for PCR analyses

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Fig. 1

Fig. 2

a. Water ingestion, ml/kg/day

b. Food ingestion, ml/kg/day

c. Rectal temperature (°C)

d. Body mass (g)

Age (days)

Weight gain (%)

CTL

DNP
Fig. 7

![Graph showing mRNA Relative expression levels (% of Control) for different proteins and genes related to mitochondrial biogenesis, proteins, and dynamics.](image)

Fig. 8

![Western Blots showing protein expression levels for Ndufs3 and Prohibitin under control and DNP conditions.](image)
Fig. 9

**Succinate dehydrogenase**

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**NADH dehydrogenase**

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Fig. 10

![Graph showing ATP production (μmol/L) for different conditions.](image)

- **CTL**
- **DNP**
- **AOP**
- **ADP/DNP**

**A** 

Comparison of ATP production under different conditions.
## FIGURES AND TABLES

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Fig. 10

ATP production (μmol/L)

CTRL  DNP  ADP  ADP/DNP

**  **  **