Endurance training prevents negative effects of the hypoxia mimetic dimethyloxalylglycine on cardiac and skeletal muscle function

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Running title
Cardiac and skeletal muscle function after PHD inhibition

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Abstract

Hypoxic preconditioning is a promising strategy to prevent hypoxia-induced damages on several tissues. This effect is related to prior stabilization of the Hypoxia-Inducible Factor-1α via inhibition of the prolyl-hydroxylases (PHD) which are responsible for its degradation under normoxia. Although PHD inhibition has been shown to increase endurance performance in rodents, potential side effects of such a therapy have not been explored. We investigated here the effects of 1-wk dimethylloxalylglycine (DMOG) treatment (150 mg/kg) on exercise capacity as well as on cardiac and skeletal muscle function in sedentary and endurance trained rats. DMOG improved maximal aerobic velocity and endurance in both sedentary and trained rats. This effect was associated with an increase in red blood cells without significant alteration of skeletal muscle contractile properties. In sedentary rats, DMOG treatment resulted in enhanced left ventricle (LV) weight together with impairment in diastolic function, LV relaxation and pulse pressure. Moreover, DMOG decreased maximal oxygen uptake (state 3) of isolated mitochondria from skeletal muscle. Importantly, endurance training reversed the negative effects of DMOG treatment on cardiac function and restored maximal mitochondrial oxygen uptake to the level of sedentary placebo-treated rats. In conclusion, we provide here evidence that the PHD inhibitor DMOG has detrimental influence on myocardial and mitochondrial function in healthy rats. However, one may suppose that the deleterious influence of PHD inhibition would be potentiated in patients with already poor physical condition. Therefore, the present results prompt us to take into consideration the potential side effects of PHD inhibitors when administrated to patients.

Key words

2-oxoglutarate; exercise; heart; mitochondrial respiration; prolyl hydroxylase inhibitor
Hypoxia is a condition encountered during ascent to high altitude or in pathologies such as heart failure or chronic obstructive pulmonary disease. Prolonged reduction in O\textsubscript{2} pressure leads to cellular adaptations to cope with the energetic challenge imposed by O\textsubscript{2} rarefaction. Hypoxia-related effects are mainly due to activation of the Hypoxia-Inducible Factor (HIF)-1. Indeed, HIF-1 transcription factor triggers the expression of more than one hundred genes notably involved in glycolysis, vascular remodeling or iron metabolism and erythropoiesis (40). HIF-1 is a heterodimer composed by a β subunit, that is constitutively expressed, and a α subunit whose expression is O\textsubscript{2}-dependent. Under normoxia, HIF-1α protein is quickly addressed for proteasomal degradation. This mechanism is triggered by prolyl-hydroxylases (PHD) that require Fe\textsuperscript{2+}, 2-oxoglutarate and oxygen to hydroxylate proline residues on HIF-1α (16, 17). Under hypoxia, PHD activity decreases due to co-factors (Fe\textsuperscript{2+}) and substrate depletion (O\textsubscript{2}) leading to HIF-1α accumulation and dimerization with the β subunit. Moreover, HIF-1α hydroxylation on asparagine residue via factor inhibiting HIF-1 represses its transcriptional activity under normoxia (37).

Therefore, inhibition of PHD has been proposed to mimic cellular adaptations to hypoxia and PHD inhibitors (PHI) have been successfully used to increase expression of HIF-1 targets, notably erythropoietin (EPO) (15) or vascular endothelial growth factor (3). HIF-1α hydroxylation can be inhibited by iron chelators, such as cobalt chloride (a widely used hypoxia mimetic) and ethyl-3,4-dihydroxybenzoate (EDHB) or 2-oxoglutarate antagonists like dimethyloxalylglycine (DMOG). Since severe hypoxia, as encountered during cardiac ischemia, can lead to apoptosis (24), PHI can be of particular interest in the prevention of hypoxic damages. Indeed, previous reports showed that PHI-mediated hypoxic preconditioning is a promising way to protect tissues from severe hypoxia during ischemic episodes (8, 31). Importantly, PHI administration few hours after myocardial infarction is still efficient to reduce infarct size (46), and DMOG has beneficial effects in pathologies related to other tissues such as bone (48) or retina (44). In addition to this protective action in pathological models, iron-dependent PHI have been shown to increase aerobic performance under normoxia (39) or severe hypoxia (18).

In this context, the ergogenic effect of 2-oxoglutarate antagonists still needs to be explored.

Nevertheless, HIF-1-related effects of hypoxia can be detrimental for oxidative metabolism (6, 41). For example, hypoxia decreases mitochondrial function via reduction of mitochondrial enzymes activity, respiration rate and content (10, 14, 36). This, in turn, will result in reduced muscle endurance and consequently higher
muscle fatigability. In addition, hypoxia induces cardiac remodeling (34, 35), and prolonged HIF-1α stabilization causes cardiomyopathy with impairment of heart function in genetic models (4, 21, 29, 30). In just a few months, three professional athletes have been tested positive to 2-oxoglutarate antagonist. Taken as a whole, these data stress the need to identify potential deleterious effects of PHI.

In this study, we determined whether 2-oxoglutarate antagonist DMOG enhances exercise endurance performance in rats. We also depict DMOG-induced adaptations on hematological parameters (polycythemia) and on skeletal as well as cardiac muscle function to define the central and/or peripheral effects of DMOG on performance. Lastly, due to the potential side effects of PHI, we examined whether regular aerobic exercise optimizes PHI-mediated adaptations.
Materials and methods

Animals

Twelve-week-old male Wistar rats (Charles River, L’Arbresle, France) were used for all experiments except for erythropoietin (EPO) determination where 10-week-old C57 Black/6J mice were obtained from our animal facility. Animals were housed under standard conditions with a 12h light/dark cycle and access to food and water ad libitum. All procedures were approved by the Ethic Committee of the Languedoc-Roussillon for animal experiment (project: CE-LR-11007).

Protocol

Forty rats were divided into four groups: sedentary control (SED CTRL), sedentary DMOG-administrated (SED DMOG), trained control (TRAIN CTRL) or trained DMOG-administrated (TRAIN DMOG; n=10/group). Training consisted in treadmill running 5 days a week during 6 weeks. The duration and the speed were progressively increased during the first week to reach 40 min and 25 m/min, respectively. The 5 next weeks were performed at 25 m/min for 30 min after a 10 min warm up. During the last week of the training, rats were intraperitoneally (i.p.) administrated with either 150 mg/day/kg body wt of DMOG in NaCl 0.9 % (DMOG groups) or a similar volume of NaCl 0.9 % (CTRL groups).

Hematologic parameters

Blood sampling was performed on rats to measure total hemoglobin (Avoximeter 4000, A-VOX Systems, Inc., San Antonio, Texas) and hematocrit (HAEMATOKRIT 210, Hettich) at the end of the protocol. Due to the kit compatibility, C57 Black/6J mice received i.p. injection of DMOG or EDHB (100 and 200 mg/kg body wt) and were killed 8 h after administration for plasma EPO determination using a mouse EPO ELISA kit (R&D Systems, Minneapolis, MN, USA). Previous study on EPO induction with PHI showed that EPO level was close to the maximal concentration 8 h after injection (15).

Running performance
We determined the maximal aerobic velocity (MAV), a marker of skeletal muscles oxidative metabolism, for every rats during a treadmill running test as previously described (34). Briefly, speed was set to 10 m/min for 3 min, after which it was increased by 4 m/min every 90 s until \( \approx 80\% \) of the expected MAV was reached. Then, the speed (26 and 34 m/min for sedentary and trained groups, respectively) was increased by 1 m/min every 60 s until exhaustion. We used a different schedule between sedentary and trained rats to keep exercise duration in a similar range (15 to 20 min) for all animals. The test took place 24h after the last training session for TRAIN groups. Sedentary rats were acclimatized to the treadmill on the 3 days preceding the test (10 min at 15 m/min).

Aerobic endurance was assessed 24h after MAV determination. Rats were asked to run until exhaustion at 85% of their MAV both in normoxia and moderate hypobaric hypoxia (simulated altitude of 3,000m). Rats were randomly assigned to normoxic or hypoxic session, each test being separated from the preceding by 24h.

Skeletal muscle contractile properties

Twenty-four hours after the last time trial session, rats were anesthetized with pentobarbital sodium (100 mg/kg) and EDL muscle was removed for determination of contractile properties. Briefly, the distal tendon of the muscle was tied to a fixed pin in the organ bath, while the proximal tendon was attached to the lever arm of a dual-mode servomotor (305-LR, Aurora Scientific, Aurora, Ontario, Canada). Muscles were stimulated along their entire length with platinum wire electrodes at their optimum muscle length (L0), i.e. the muscle length producing maximal twitch tension. All subsequent measurements were made at L0. Muscles were preincubated for 15min in Krebs-Ringer buffer (containing in mM: 120 NaCl, 4.8 KCl, 25 NaHCO3, 2.5 CaCl2, 1.2 KH2PO4, and 1.2 MgSO4; pH 7.4) supplemented with 5 mM HEPES, 5 mM glucose and saturated with 95% O2-5% CO2 gas mixture, maintained at 30°C. A computer generated the signal for electrical stimulation and force/length control of the levers during the contractions through commercial software (DMC v. 4.1.6, Aurora scientific). The tension-frequency response was then determined (701B Stimulator, Aurora Scientific Inc, Ontario, Canada) using stimulation trains of 500 ms, with pulse duration of 0.5 ms at frequencies of 25, 75, 100, 150 and 200 Hz. Stimulus trains were separated by 1 min interval. The maximum isometric tetanic tension (P0) was then determined. Five minutes after the tension-frequency determination, the resistance to fatigue was evaluated using repeated contractions (200 Hz trains of 120 ms, evoked twice per s) until the muscle lost 50%
of its initial force. The muscle fatigue index (Tlim) was defined as the time taken to reach 50% of P0. After all measurements, muscles were removed from the bath, trimmed of the connective tissue, blotted dry and weighed.

**Skeletal muscle mitochondrial respiration**

Gastrocnemius muscles were quickly excised and immediately placed into ice-cold buffer (100 mM KCl, 5 mM MgSO4, 5 mM EDTA, 50 mM Tris-HCl, pH = 7.4). Mitochondria were fractionated by differential centrifugation as described previously (20). Briefly, muscles were freed of connective tissues, minced, homogenized with an Ultra-turax homogenizer and treated with Subtilisin A (0.1 mg/g wet muscle). Mitochondria were separated by centrifugation at 8000g, then at 800g. Finally, mitochondria were pelleted from the supernatant at 9000g. Mitochondria were resuspended in 100 mM KCl, 10 mM MOPS, pH 7.4. Mitochondrial protein content was determined using the Bradford assay.

Mitochondria oxygen consumption was measured using the high-resolution Oxygraph-2k (OROBOROS Instruments, Innsbruck, Austria). Mitochondria were incubated in two sealed thermostated chambers (37°C) containing 2 ml of MIRO5 respiration medium (0.5 mM EGTA, 3 mM MgCl2, 6H2O, 65 mM KCl, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose and 1 g/l BSA, pH 7.1). Resting rate (state 4) was evaluated in the presence of 2.5 mM malate, 5 mM glutamate; ADP-stimulated rate (state 3) was determined after addition of 0.5 mM ADP. Data acquisition and analysis was performed using Oxygraph-2k-DatLab software version 4.3. The Respiratory Control Ratio (RCR) was set as the ratio of oxygen consumption at state 3 over oxygen consumption at state 4.

**Skeletal muscle biochemical analyzes**

*LDH isoforms.* LDH isoenzymes from plantaris muscle were separated as previously described (28). Briefly, ≈20mg of muscle was homogenized in 210 mM sucrose, 2 mM EGTA, 5 mM EDTA, 40 mM NaCl, 30 mM HEPES, pH 7.4 with protease inhibitors and samples were centrifuged at 10,000g for 5 min. Twenty five µg of protein from the supernatant were separated by agarose gel electrophoresis and gels were stained with a solution containing 208 mM Li-L-lactate, 5.6 mM NAD+, 2.4 mM p-nitro blue tetrazolium chloride and 0.33 mM
phenazine methosulfate. The reaction was blocked in a 10 % acetic acid and 5 % methanol solution. Lastly, LDH isoenzymes bands were scanned and optical density was quantified.

**Glycogen content.** Muscle glycogen content was measured as previously described (25). Briefly, 30-50 mg of plantaris muscle was dissolved in 30 % KOH saturated with Na$_2$SO$_4$ at 100°C for 20 min. Glycogen was precipitated by addition of 1.2 vol of 95 % ethanol and the sample was centrifuged at 840 g (20°C) for 20 min. The glycogen precipitate was dissolved in 3 ml of distilled water. One ml of 5 % phenol solution and 5 ml of H$_2$SO$_4$ were added to 200 μl of the obtained glycogen solution, which was then incubated at room temperature for 10 min. Glycogen concentration was determined spectrophotometrically at 490 nm.

**HIF-1α immunoblotting.** HIF-1α accumulation was measured in the nuclear fraction of gastrocnemius muscle 5h after administration of DMOG (150 mg/kg). Muscle was homogenized in STM buffer (250mM sucrose, 50mM Tris HCl (pH 7.4), 5mM MgCl$_2$, protease/phosphatase inhibitor cocktail) and lysate was centrifuged at 800g to separate nuclear fraction (pellet) from others (supernatant). Nuclear fraction was washed one time in STM buffer and the pellet was resuspended in NET buffer (20mM HEPES (pH 7.9), 1.5mM MgCl$_2$, 0.5M NaCl, 0.2mM EDTA, 20% Glycerol, 1% triton X100, protease/phosphatase inhibitor cocktail). Protein concentrations were determined using the BCA assay (Interchim, Montluçon, France). Fifty micrograms of protein were subjected to SDS-PAGE as previously described (7). Membranes were probed with primary antibody against HIF-1α (NB100-123, Novus Biologicals, Littleton, CO).

**mRNA expression analysis.** mRNA extraction and gene expression analysis were performed as previously described (7). Briefly, total RNA was extracted from the plantaris muscle using the RNeasy fibrous tissue kit (Qiagen, Venlo, The Netherlands). cDNA was then synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystem) from 1 µg of total mRNA. Quantitative PCR was performed using KAPA 2 SYBR Green Master Mix on a Miniopticon thermocycler (Bio-Rad). Cycling conditions were one cycle at 98°C for 30 s followed by 40 cycles at 95°C for 1 s and 60°C for 15 s. Fusion index was measured by increments of 0.5°C every 5 s (starting at 65°C, finishing at 95°C). Each sample was run in duplicate. Sequences of the mouse forward and reverse primers are listed in Table 1. Results were expressed using the comparative cycle threshold (CT) with ribosomal protein S9 as the control gene.
Cardiac morphology and function

Doppler echocardiography. Cardiac morphology and function were evaluated via a noninvasive Doppler-echocardiography method as previously described (35). Rats were anesthetized with i.p. injection of pentobarbital sodium (120 mg/kg). A two-dimensional view of the left ventricle (LV) was obtained at the level of the papillary muscles with a Mylab 30 ultrasound apparatus (ESAOTE, Italy). M-mode tracings were recorded through the anterior and posterior walls. End-diastolic anterior and posterior wall thicknesses (AWTd and PWTd, respectively) and LV internal diastolic (LVEDd) and systolic (LVEDs) dimensions were measured from at least three consecutive cardiac cycles on the M-mode tracings. Diastolic relative wall thickness (RWT) was calculated as [(AWTd + PWTd)/LVEDd] × 100 and was used as an index of LV geometry. Pulsed-wave Doppler spectra of mitral inflow were recorded from an apical four-chamber view. Peak velocities of early diastolic rapid inflow (peak E), atrial contraction filling (peak A), as well as their ratio (E/A) were recorded and served as indexes of diastolic function. Pulsed-wave Doppler spectra of the RV outflow were also recorded from a parasternal view of the pulmonary artery obtained at the level of the aorta. Measurement of the pulmonary peak flow velocity (Vpulm) was recorded as a negative index of pulmonary artery pressure. For all variables, measurements represent the mean of at least three consecutive cardiac cycles.

LV pressure. Systolic and diastolic LV pressures were performed on intact closed-chest anesthetized rats, via intraventricular catheterization (Mikro-Tip® Pressure Catheter, Millar Instruments, Houston, TX) as previously described (1). Pulse pressure is the difference between the systolic and diastolic pressure. This hemodynamic evaluation was conducted under basal conditions and during β-adrenergic challenging obtained by isoproterenol infusion (1 mg/kg i.p.).

Statistical analysis

EPO concentration was analyzed using one-way ANOVA to determine the effect of PHI dose (0, 100 or 200 mg/kg). Endurance performance was analyzed using three-way ANOVA to determine the main statistical effect of treatment, training and ambiance (normoxia vs. hypoxia) and interactions between these factors. All others parameters were analyzed using a two-way ANOVA to determine the main statistical effect of treatment (DMOG vs. CTRL) and training (sedentary vs. trained) and interactions between these factors. Fisher’s least
significant difference post hoc analysis was used to determine differences between groups when ANOVA was significant. Statistical significance was set at p<0.05. Data are expressed as mean ± SEM.
Results

HIF-1α stabilization and hematological parameters

We first ensured that DMOG administration induced HIF-1α stabilization in skeletal muscle tissue. Fig. 1A shows HIF-1α accumulation in the nuclear fraction of gastrocnemius muscle 5h after a single injection of DMOG. We next assessed whether this accumulation was associated with an increase in HIF-1 transcriptional activity by measuring plasma EPO, a direct target of HIF-1. Acute DMOG administration resulted in a robust and dose-dependent increase in EPO concentration 8h after injection (12 and 24 fold with 100 and 200 mg/kg, respectively; Fig. 1B). We next checked whether repeated DMOG administration led to erythropoiesis in sedentary and trained rats. Consistently, 7-d treatment with 150 mg/kg of DMOG caused a significant increase in hematocrit and blood hemoglobin in both sedentary and trained rats (Fig. 1C and D). In sharp contrast, the iron-dependent PHI EDHB induced a small increase in EPO concentration only with 200 mg/kg and did not alter hematocrit levels after a 7-d treatment (not shown). We thus focused on DMOG treatment.

Exercise performance and muscle contractile properties

Training significantly increased maximal aerobic velocity (MAV) in both DMOG and CTRL-treated rats (training effect p<0.001; Fig. 2A). In addition, 150 mg/kg of DMOG during 7 d improved MAV (DMOG effect p<0.001). Interestingly, training and DMOG had synergic effects since DMOG had greater effects on MAV in trained rats (training x DMOG interaction P<0.001). We next determined aerobic endurance in normoxia and mild hypoxia during running test until exhaustion at 85% of normoxic MAV (Fig. 2B). Endurance was positively affected by treatment (DMOG vs. NaCl 0.9%; p=0.03) and training (p=0.025). In contrast, ambiance had a negative effect on performance (hypoxia vs. normoxia; p=0.005). There was no significant interaction between these three factors. Finally, we measured contractile properties of isolated EDL muscles. Neither maximal force nor resistance to fatigue during intermittent contractions was altered by either training or DMOG administration (Fig. 2C).

Cardiac morphology
DMOG treatment significantly increased heart (p=0.004) and LV weights (p=0.007) in SED rats (Fig. 3). In contrast, no effect of DMOG on these variables was observed in trained animals. Endurance training had no effect on either heart or LV mass. Similarly, RV weight was not altered by either DMOG treatment or endurance training (not shown). Expression of heart and LV weight relative to body weight showed a positive effect of both DMOG and training (not shown). However, the training effect resulted from a lower body weight (-10%) in exercised rats. Lastly, echocardiographic exploration revealed no significant alteration of LV morphology whatever the group considered (Table 2).

Cardiac function

We assessed cardiac function under basal and β-adrenergic stress conditions. On the one hand, we founded no effect of DMOG treatment and/or endurance training on pulmonary peak flow velocity (not shown), LV pulse pressure, maximal and minimal LV derivatives pressures under basal conditions (Table 3). However, the E/A ratio, an index of diastolic function, was significantly impaired by DMOG treatment (p=0.04; Fig. 4A). Importantly, post hoc analysis revealed significant differences between DMOG-treated and CTRL animals in SED (p=0.01) but not in trained groups. On the other hand, DMOG had greater effects under β-adrenergic stimulation. Indeed, LV pulse pressure was decreased by DMOG administration in sedentary rats (p=0.04; Fig. 4B). As for E/A ratio, the negative effect of DMOG on pulse pressure was prevented by chronic exercise (significant differences between DMOG-treated and CTRL in SED but not trained rats). Similarly, the minimum derivative of LV pressure over time, an index of myocardial relaxation, was impaired by DMOG administration in SED but not in trained rats (p=0.03; Fig. 4C). However, maximum derivative of LV pressure over time was not altered by either DMOG or training (Fig. 4D).

Muscle metabolism

Oxidative capacity of muscle cells is tightly coupled to the mitochondrial activity. Respiration experiments on isolated mitochondria from gastrocnemius muscles revealed opposite effects of endurance training and DMOG administration (Fig. 5 A and B). Indeed, training had a main positive effect on ADP-stimulated maximal oxygen uptake (state 3; p=0.002) and on the respiratory control ratio (RCR=state 3/state 4; p<0.001), while it tended to decrease the basal oxygen uptake (state 4; p=0.07). On the contrary, DMOG had a main negative influence on
state 3 (p=0.004) whereas it increased state 4 (p<0.001). DMOG administration thus led to a decrease in RCR (p<0.001). There was no significant interaction between training and DMOG treatment. DMOG did not alter gene expression of PGC-1α, NRF and Tfam (Fig. 5 C), three transcription factors involved in mitochondrial biogenesis, while training tended to increase PGC-1 α mRNA level (p=0.08). Besides, glycolytic metabolism is dependent on glycogen stores as well as on the distribution of LDH isoforms which promote either lactate production or oxidation. Glycogen content of plantaris muscle was not altered between the four groups (Fig. 6A). Similarly, we did not record any effect of training on LDH isoforms distribution (Fig. 6B). However, DMOG increased the activity of the LDH-4 (HM3) isoenzyme (main effect p=0.05), although post hoc analysis revealed no differences between groups. Lastly, we measured gene expression of two glycolytic enzymes up-regulated by HIF-1. Again, hexokinase II and phosphofructokinase mRNA level was not altered by either DMOG or training (Fig. 6C).
Discussion

Skeletal and cardiac muscles are metabolically active tissues that are highly dependent on oxygen availability. Therefore, a reduction in O₂ availability will induce phenotypic modifications of muscle cells. HIF-1 is essential for hypoxia acclimatization as it regulates 89% of genes induced after exposure of mouse embryonic fibroblasts to 1% O₂ (12). Hypoxia adaptations can be mimicked by HIF-1α stabilization through PHD inhibition. In this study we investigated the effects of DMOG, a 2-oxoglutarate antagonist PHI, on cardiac and skeletal muscle function. We previously showed that 5 wk of moderate hypoxia (equivalent to 2,800m) reduced LV filling (35). Here, only 1 wk of DMOG administration, which increased nuclear HIF-1α protein content in skeletal muscle, resulted in significant decrement in heart function as attested by a decrease in LV filling rate (E/A), pulse pressure and relaxation (dP/dt min). These data illustrate an alteration in diastolic function, which is potentiated under stress conditions, together with a slight impairment in systolic function. Interestingly, several studies reported that long term HIF-1α stabilization via genetic manipulation caused cardiomyopathy with LV dysfunction (4, 21, 29, 30). Bekeredjian et al. notably observed that HIF-1α overexpression induced a decrease in the sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA)-2a together with a reduction in calcium reuptake (4). These mechanisms could explain the decrease in myocardial relaxation observed after HIF-1α stabilization in a previous (21) and the present study. We observed that DMOG administration also caused an increase in heart and LV weight. Again, this phenomenon has been reported in models with genetic HIF-1α stabilization (4, 21). Altogether, this strongly suggests that 1 wk of DMOG treatment initiates HIF-1-dependent cardiac remodeling that would be deleterious for cardiac function if administration was prolonged. However, we showed that chronic exercise prevented the induction of DMOG-mediated side effects on cardiac muscle. Indeed, the increase in heart weight was observed in sedentary but not in trained rats after DMOG administration. Moreover, the E/A ratio, the LV pulse pressure and dP/dt min were not altered by treatment in trained rats. These results demonstrate that endurance training maintains myocardial function despite DMOG administration. The beneficial role of aerobic exercise on cardiac function is recognized, especially in patients with heart failure, (45). Endurance training notably induces the expression of heat shock protein and strengthens defenses against oxidative stress (reviewed in (2)). But, it has also been shown that endurance training improved SERCA-2a-mediated calcium uptake in mice, and this was associated to a significant increase in SERCA-2a protein content (19). This mechanism could therefore explain the absence of DMOG-related
decrease in LV relaxation with endurance training. Collectively, our findings pointed the importance of regular exercise to prevent the induction of deleterious effects consecutive to DMOG treatment.

In line with our results on cardiac muscle, we found similarities between DMOG treatment and hypoxia exposure on skeletal muscle. For example, hypoxia negatively regulates mitochondrial mass and function in skeletal muscle (10, 11, 14, 26). In agreement, we observed a significant reduction in maximal \(O_2\) uptake (relative to mitochondrial mass) and in mitochondrial coupling (RCR) in the gastrocnemius of DMOG treated rats. Moreover, we did not detect variation of PGC-1 or Tfam gene expression with DMOG administration, suggesting that mitochondrial biogenesis was not altered. Therefore, DMOG effect is rather related to a modification of mitochondrial efficiency rather than mitochondrial content. In contrast, Saxena et al. reported an increase in the expression of mitochondrial biogenesis markers and in mitochondrial density after treatment with CoCl\(_2\). Dose and treatment duration are probably of key importance since cobalt has also been shown to cause respiration deficiency in yeast (22). This points that different types of PHI such as 2-oxoglutarate antagonist (DMOG) or iron chelator (CoCl\(_2\)) could have distinct effects on muscle oxidative metabolism. The finding of negative effect of DMOG on mitochondrial respiration observed in isolated mitochondria (present study) or in primary cardiomyocytes (42) has recently been related to HIF-1-independent metabolic disturbance (49). Indeed, once de-esterified, DMOG quickly reduced \(O_2\) consumption of rat liver mitochondria likely because of competitive inhibition mitochondrial enzymes (such as mitochondrial \(\alpha\)-ketoglutarate dehydrogenase or isocitrate dehydrogenase). Hypoxia and DMOG can thus impair mitochondrial metabolism but through distinct pathways. Glycolytic metabolism was not markedly altered by DMOG since glycogen stores, HKII and PFK gene expression remained unaltered. The absence of induction of these known HIF-1 target genes may be surprising however the time delay between the last DMOG injection and the measures (96h) could explain the absence of significant modifications. While it has been proposed that hypoxia would enhance anaerobic metabolism, a recent review highlighted that skeletal muscle presents only subtle modifications of this metabolism after chronic hypoxia (13).

Besides, endurance training partly reversed side effects of DMOG treatment. Muscle specific HIF-1\(\alpha\) deletion clearly demonstrates that HIF-1\(\alpha\) and endurance training have opposite effects on skeletal muscle metabolism (27). Moreover, endurance trained muscle displays enhanced expression of HIF-1 negative regulators (23). Therefore, it is not surprising that endurance training antagonized the HIF-1 dependent effects of DMOG on.
mitochondrial respiration. However, DMOG treatment led to a similar decrease in state 3 between sedentary and trained rats and RCR of DMOG-treated trained rats remained slightly but significantly lower than in SED CTRL animals. This probably resulted from HIF-1-independent metabolic competition due DMOG de-esterification. Nonetheless, it should be kept in mind that chronic exercise restored maximal O₂ uptake of DMOG-treated muscles to the level of sedentary CTRL rats. As oxidative metabolism capacity is tightly coupled with resistance to fatigue, this could have functional consequences on muscle and subject’s fatigability.

Previous studies reported that CoCl₂ increased endurance performance during swimming test in sedentary and trained rats under normoxia (38, 39). Similarly, iron chelation-mediated PHD inhibition by EDHB administration resulted in endurance improvement during running test under severe hypoxic environment (equivalent to 7,400m) in sedentary mice (18). Accordingly, we observed that DMOG has positive effect on aerobic performance in sedentary and trained rats under both normoxia and moderate hypoxia (equivalent to 3,000m). While others showed that this ergogenic effect occurs during endurance exercises (time to exhaustion at a set speed), we showed that DMOG also improved performance during intense aerobic exercise (incremental test for MAV determination). Cardiac output and O₂ delivery are major limiting factors during such efforts (47). However, it had never been investigated whether PHI-induced increase in performance is due to central (O₂ delivery) or peripheral (skeletal muscle) effects. We reported here that DMOG treatment led to a 12.7% increase in MAV that was associated to a similar increase (11.3%) in hemoglobin content. We observed that EDHB was less potent than DMOG to promote erythrocytosis. Our results are in total agreement with the data of Kasiganesan et al. which reported a slight effect on plasma EPO concentration with no change in hematocrit after EDHB treatment (18). While both EDHB and DMOG stabilize HIF-1α, DMOG is more efficient for suppressing HIF-1α hydroxylation on asparagine 803 (43). Since this residue is crucial for HIF-1 transactivation, DMOG appears as a powerful activator of HIF-1 by enhancing stabilization and transcriptional activity of HIF-1α. Despite this, ex-vivo muscle contractile properties, and particularly resistance to fatigue, were unaltered by DMOG administration. Collectively, these results strongly suggest that PHI-mediated increase in aerobic performance is mainly due to central, via an increase in O₂ delivery, rather peripheral effect.

Conclusions and perspectives
Hypoxic preconditioning is of clinical interest for the prevention of ischemic complications. We confirm that PHI treatment activates erythrocytosis, although all molecules are not equally potent for such activation. Moreover, DMOG has recently been used to promote bone regeneration (48) and odontoblast maturation (33) or to prevent oxygen-induced retinopathy (44). Therefore, DMOG would be clinically relevant for a broad range of patients and some of PHI have already been used in humans (32). Endurance athletes may also be tempted to use DMOG in order to improve their performances as recently revealed. However, the potential side effects of such therapy remain poorly considered. For example, CoCl₂ was used to improve exercise performance (38, 39) and limit myocardial infarction (5) whereas it could have toxic effects in animals (9). We provide here evidence that the 2-oxoglutarate antagonist DMOG has negative effect on myocardial function in healthy rats. Efforts are made to develop more selective PHI compared to DMOG that may also inhibit other 2-oxoglutarate-dependent dioxygenases including the collagen-modifying hydroxylases. However, alteration of heart function with DMOG appears to be dependent upon HIF-1α activation and specific PHI would probably lead to same effects. One may also suppose that the deleterious influence of PHI would be potentiated in patients with already poor physical condition. Fortunately, these detrimental effects can be reversed by regular aerobic exercise. In conclusion, our work underlines the need to take into consideration the potential side effects of PHI when administrated to patients and the interest of physical activity to prevent these effects.

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**Figure legend**

**Fig. 1. Effects of acute and chronic DMOG administration on HIF-1 stabilization and hematological parameters.** A) HIF-1α protein content in the nuclear fraction of rat gastrocnemius muscle 5h after DMOG (150 mg/kg) or vehicle injection (CTRL). B) Plasma EPO concentration in mice receiving 100 or 200 mg/kg of DMOG (n=3-4/group). ***p<0.001 vs. control, ††p<0.01 and †††p<0.001 vs. 100 mg/kg. C) and D) hematocrit and hemoglobin concentration in sedentary (SED) or trained (TRAIN) rats subjected to one-wk DMOG (DMOG) or NaCl 0.9% (CTRL) treatment (n=8-10/group). ***p<0.001 vs. corresponding CTRL.

**Figure 2. Effect of DMOG administration on exercise performance and muscle contractile properties.** A) Maximal aerobic velocity of sedentary (SED) or trained (TRAIN) rats subjected to one-wk DMOG (DMOG) or NaCl 0.9% (CTRL) treatment (n=8-9/group). B) endurance time in sedentary (SED) or trained (TRAIN) rats subjected to one-wk DMOG (DMOG) or NaCl 0.9% (CTRL) treatment. Rats were subjected to treadmill running until exhaustion at 85% of their normoxic MAV in both normoxic and hypoxic environment. C) Maximal force and resistance to fatigue of isolated EDL in sedentary (SED) or trained (TRAIN) rats subjected to one-wk DMOG (DMOG) or NaCl 0.9% (CTRL) treatment. ***p<0.001 vs. corresponding CTRL, †††p<0.001 vs. corresponding SED group, ‡p<0.05 vs. corresponding normoxic group.

**Fig. 3: Effects of DMOG treatment on heart and left ventricular mass.** A) heart and B) left ventricular (LV) weights in sedentary (SED) or trained (TRAIN) rats subjected to one-wk DMOG (DMOG) or NaCl 0.9% (CTRL) treatment. *p<0.05 vs. corresponding CTRL.

**Fig. 4: Effects of DMOG treatment on cardiac function.** A) on E/A ratio in basal condition, B) LV pulse pressure, C) minimum and D) maximum derivative of LV pressure under β-adrenergic stimulation (Isoproterenol, 1 mg/kg/min i.p.) in sedentary (SED) or trained (TRAIN) rats subjected to one-wk DMOG (DMOG) or NaCl 0.9% (CTRL) treatment. **p<0.01 vs. corresponding CTRL.
Figure 5. Effect of DMOG treatment on mitochondrial respiration. A) oxygen uptake of state 4 and 3, B) respiratory control ratio (RCR = state 3/state 4) and C) gene expression of transcription factors involved in mitochondrial biogenesis in sedentary (SED) or trained (TRAIN) rats subjected to one-wk DMOG (DMOG) or NaCl 0.9% (CTRL) treatment. NRF1: nuclear respiratory factor 1; PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha; Tfam: transcription factor A mitochondrial. *p<0.05 and ***p<0.001 vs. corresponding CTRL. †p<0.05, ††p<0.01 and †††p<0.001 vs. corresponding SED.

Figure 6. Effect of DMOG treatment on anaerobic metabolism in plantaris muscle. A) glycogen content, B) quantification of LDH isoenzymes activity and C) gene expression of hexokinase (HK)II and phosphofructokinase (PFK) in sedentary (SED) or trained (TRAIN) rats subjected to one-wk DMOG (DMOG) or NaCl 0.9% (CTRL) treatment.
Table 1. Primers used for real-time quantitative PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>HKII</td>
<td>GTTTCAAGCGGCTGAACCTG</td>
<td>ATTGGTCACCTTCTGACCTT</td>
</tr>
<tr>
<td>NRF1</td>
<td>TTATTCTGCTGGCTGATGG</td>
<td>CCTCTGATGCTGCTGCTCT</td>
</tr>
<tr>
<td>PFK</td>
<td>TCAGTGAGACAAGACCAGTT</td>
<td>CGTTTTTGTCGATTGACCCC</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>ATGTGTCGCTTTCTTGCTCT</td>
<td>ATCTACTGCTGGGACCTT</td>
</tr>
<tr>
<td>RPS9</td>
<td>GAAGCTGGGTTGTCGCAAAA</td>
<td>CGGAGGCCATACTCTCAAAT</td>
</tr>
<tr>
<td>Tfam</td>
<td>GCTAAACACCGATGCAAAA</td>
<td>CGAGGTCTTTTGAGTTTCCA</td>
</tr>
</tbody>
</table>

HKII, hexokinase II; NRF1, nuclear respiratory factor 1; PFK, phosphofructokinase; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; RPS9, ribosomal protein S9; Tfam, transcription factor A mitochondrial.

Table 2. Effects of DMOG treatment on left ventricular morphology in sedentary and trained rats.

<table>
<thead>
<tr>
<th></th>
<th>SED</th>
<th>TRAINED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTRL DMOG</td>
<td>CTRL DMOG</td>
</tr>
<tr>
<td>LVEDs (mm)</td>
<td>0.312 ± 0.02</td>
<td>0.313 ± 0.023</td>
</tr>
<tr>
<td>LVEDd (mm)</td>
<td>0.617 ± 0.016</td>
<td>0.63 ±0.013</td>
</tr>
<tr>
<td>AWTd (mm)</td>
<td>0.165 ±0.006</td>
<td>0.175 ± 0.006</td>
</tr>
<tr>
<td>PWTd (mm)</td>
<td>0.145 ± 0.006</td>
<td>0.16 ± 0.007</td>
</tr>
<tr>
<td>RWT (%)</td>
<td>48.56 ± 1.76</td>
<td>53.29 ± 1.86</td>
</tr>
</tbody>
</table>

LVEDs, left ventricular end-systolic diameter; LVEDd, left ventricular end-diastolic diameter; AWTd, end-diastolic anterior wall thickness; PWTd, end-diastolic posterior wall thickness; RWT, relative wall thickness in sedentary (SED) or trained (TRAIN) rats subjected to one-wk DMOG (DMOG) or NaCl 0.9% (CTRL) treatment (n=8-10/group).
Table 3. Effects of DMOG treatment on cardiac function at baseline in sedentary and trained rats.

<table>
<thead>
<tr>
<th></th>
<th>SED</th>
<th>DMOG</th>
<th>TRAIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV pulse pressure (mmHg)</td>
<td>135.3 ± 7.2</td>
<td>125.6 ± 4.4</td>
<td>123.4 ± 10.4</td>
</tr>
<tr>
<td>dP/dt min (mmHg/s)</td>
<td>−6386 ± 454</td>
<td>−5920 ± 282</td>
<td>−5780 ± 540</td>
</tr>
<tr>
<td>dP/dt max (mmHg/s)</td>
<td>6806 ± 404</td>
<td>6450 ± 293</td>
<td>5937 ± 545</td>
</tr>
</tbody>
</table>

LV: left ventricle; dP/dt min and max: minimum and maximum derivative of left ventricle pressure in sedentary (SED) or trained (TRAIN) rats subjected to one-wk DMOG or NaCl 0.9% (CTRL) treatment (n=8-10/group).
A. Glycogen content (mg/g)

B. LDH isoform activity (% of total activity)

C. mRNA (relative to SED CTRL)