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Short title: Mild hypothermia, cardioprotection and mitochondria

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Word count (text): 2999

Word count (abstract): 243

Abstract

Background: Mitochondrial dysfunction is critical following ischemic disorders. Our goal was to determine whether mild hypothermia could limit this dysfunction through per-ischemic inhibition of reactive oxygen species (ROS) generation.

Methods: First, ROS production was evaluated during simulated ischemia in *an vitro* model of isolated rat cardiomyocytes at hypothermic (32°C) vs normothermic (38°C) temperatures. Second, we deciphered the direct effect of hypothermia on mitochondrial respiration and ROS production in oxygenated mitochondria isolated from rabbit hearts. Third, we investigated these parameters in cardiac mitochondria extracted after 30-min of coronary artery occlusion (CAO) under normothermic conditions (CAO-N) or with hypothermia induced by liquid ventilation (CAO-H; target temperature: 32°C).

Results: In isolated rat cardiomyocytes, per-ischemic ROS generation was dramatically decreased at 32 vs 38°C (e.g., -55±8% after 140 min of hypoxia). In oxygenated mitochondria isolated from intact rabbit hearts, hypothermia also improved respiratory control ratio (+22±3%) and reduced H_20_2 production (-41±1%). Decreased oxidative stress was further observed in rabbit hearts submitted to hypothermic vs normothermic ischemia (CAO-H vs CAO-N), using thiobarbituric acide-reactive substances as a marker. This was accompanied by a preservation of the respiratory control ratio as well as the activity of complexes I, II and III in cardiac mitochondria.

Conclusion: The cardioprotective effect of mild hypothermia involves a direct effect on per-ischemic ROS generation and results in preservation of mitochondrial function. This might explain why the benefit afforded by hypothermia during regional myocardial ischemia depends on how fast it is instituted during the ischemic process.

Introduction

Mitochondria are well known to mediate ischemic injuries through complex events involving reactive oxygen species (ROS) generation [1, 2], alteration of electron transfer activity [2, 3], opening of the mitochondrial permeability transition pore (mPTP) [4] and cytochrome c release [1, 2]. This has been ubiquitously observed in multiple ischemic insult models such as regional [4] or global ischemia and cardiac arrest [2, 3]. Mitochondrial dysfunction has been mostly investigated during the post-ischemic reperfusion phase [4, 5]. Targeting mitochondria is then often considered as a relevant approach to prevent reperfusion injury through, *e.g.*, direct inhibition of mPTP after myocardial infarction [4]. However, electron microscopy studies have clearly showed that mitochondrial injuries start during the ischemic phase [3, 6]. Alterations in mitochondrial respiratory complexes activities, cytochrome c release and ROS generation were also demonstrated to occur during the hypoxic phase prior to reoxygenation [1-3].

Despite the role of mitochondria has been extensively investigated during ischemia, its exact participation to the cardioprotective effect of per-ischemic mild hypothermia (32-34°C) remains unclear. We previously showed that mitochondrial ultrastructure and respiratory function were improved by mild hypothermia in rabbits submitted to 30 min of coronary artery occlusion [6]. The calcium-induced opening of mPTP was also ultimately inhibited by per-ischemic hypothermia [6]. The cascade of events leading to this per-ischemic protection remains however still unknown. It is currently proposed that mild hypothermia could involve its own survival signalling cascade with protein kinase C-ε [7], nitric oxide synthase [8], extracellular-regulated kinase (ERK) [9], Akt pathway [8] and mammalian target of rapamycin (mTOR) [10]. Here, we propose to investigate whether mild hypothermia could act through a direct effect of temperature on mitochondrial function and ROS generation. Our hypothesis is that hypothermia could act through a reduction of per-ischemic oxidative stress. This would explain why its beneficial effect is maximal when instituted early during ischemia while less efficient when started after reperfusion. For this purpose, we first evaluated *in vitro*

the effect of mild hypothermia on ROS generation in a model of rat cardiomyocytes submitted to simulated ischemia. Second, we tested *ex vivo* the direct effect of temperature on respiratory parameters and ROS production in mitochondria isolated from intact rabbit heart. Since these experiments showed a dramatic decrease in ROS production at 32 vs 38°C, we further investigated *in vivo* the overall oxidative stress induced by myocardial ischemia under hypothermic vs normothermic conditions. This was performed in anesthetized rabbits submitted to 30 min of coronary artery occlusion. We also assessed *ex vivo* the respiratory function and complex activities of the mitochondria extracted from the ischemic territory. In all *in vivo* experiments, hypothermia was induced using total liquid ventilation with temperature-controlled perfluorocarbons that can fastly and accurately control per-ischemic cardiac temperature in anesthetized rabbits. We previously showed that this strategy can use the lung as a heat exchanger to quickly change the cardiac temperature while maintaining appropriate gas exchange and ultimately inducing a potent anti-infarct effect [6, 11-13].

Methods

The animal instrumentation and the ensuing experiments were conducted in accordance with French official regulations and after approval by the local ethical committee. All experiments were performed in Wistar rats or New Zealand rabbits.

Simulated ischemia in adult rat cardiomyocytes and measurement of ROS generation

Adult rat ventricular cardiomyocytes were obtained from hearts of male Wistar rats (260-300g), as described previously [14]. They were plated in 35 mm Petri dishes which were mounted on a heated perfusion chamber and perfused with a Tyrode's modified solution. The temperature of this solution was continuously monitored. The chamber was connected to a gas bottle diffusing a constant stream of O_2 (21%), N_2 (74%) and CO_2 (5%) maintaining a partial O_2 pressure at 21%. pO_2 within the chamber was measured continuously using a fiber optic sensor system. The cardiomyocytes were paced to beat by field stimulation (5ms; 0.5Hz). To simulate ischemia, myocytes were exposed for 140 min to a hypoxic medium (pO_2<1%) consisting in a glucose-free Tyrode's modified solution (pH=7.4) supplemented with 20mM of 2-deoxyglucose and to a constant stream of N_2 (100%).

To evaluate ROS formation at 32 or 38°C (Figure 1, Panel A), myocytes were loaded with the fluorescent probe 2',7'-dichlorofluorescin-diacetate (5 µM) for 15 min before induction of simulated ischemia. Fluorescence was measured with an Olympus IX-81 motorized inverted fluorescent microscope equipped with a mercury lamp as a source of light for epifluorescent illumination, using 495 nm and 520 nm wavelengths for excitation and emission, respectively[14].

Experimental protocols in rabbits

As illustrated by Figure 1 (Panels B and C), two subsequent sets of experiments were performed in rabbits. For these experiments, rabbits were anesthetized using zolazepam, tiletamine and pentobarbital (all 20-30 mg/kg i.v.). Then, they were intubated and mechanically ventilated. A left thoracotomy was performed. In the first set of experiments

(Figure 1, Panel B), the heart was removed after a period of stabilization. Samples from the left ventricular free wall were rapidly minced, homogenized and centrifuged to obtain a mitochondrial suspension for *ex vivo* investigation of the direct effect of temperature (32 vs 38°C) on mitochondrial ROS production and respiration. In the second set of experiments (Figure 1, Panel C), rabbits were anesthetized and randomly submitted to 30 min of follow-up (Sham group) or to 30 min of coronary artery occlusion (CAO groups), as previously described [11, 13]. In the CAO groups, animals underwent at random either normothermic ischemia (CAO-N group) or hypothermia induced by liquid ventilation from the 5th min to the end of CAO (CAO-H group). The latter approach allows a rapid and accurate control of the cardiac temperature. As previously described [6, 11, 13], liquid ventilation was instituted by filling the lungs with perfluorocarbons (Fluorinert, 3M, Cergy, France) and connecting the endotracheal tube to a liquid ventilator. The ventilator was set to an initial tidal volume of ~8-10 ml/kg of body weight (respiratory rate ~ 6 breaths/min). This was increased as needed in order to maintain blood gases within usual values. The temperature of the perfluorocarbon mixture was adjusted to maintain left atrial temperatures at a target of ~32°C throughout ischemia. At the end of the 30 min CAO, the hearts were removed and a mitochondrial suspension was prepared from the ischemic territory for assessment of lipid peroxidation and further *ex vivo* experiments in extracted mitochondria (evaluation of respiratory parameters and ROS generation).

Measurement of mitochondrial respiration and ROS production

Oxygen consumption was measured as previously described [6] with a Clark type electrode in a respiration buffer (50 mM sucrose, 100 mM KCl, 10 mM HEPES, 5 mM KH_2PO_4) containing 0.4 mg/ml of mitochondria prepared from rabbit myocardium (Figure 1, Panel B and C). Substrate-respiration rate (state 4) and ATP synthesis (state 3) were investigated by addition of 5 mM pyruvate/malate and 1 mM of ADP, respectively. The corresponding respiratory control ratio (state 3/ state 4) was calculated. Similar

measurements were repeated after uncoupling using 0.1 µM of carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone (FCCP).

ROS generation was assessed by measuring the rate of H_2O_2 production. This was determined fluorimetrically by oxidation of Amplex red to fluorescent resorufin, coupled to the enzymatic reduction of H_2O_2 by horseradish peroxidase (HRP) as recently described [15].

Assessment of mitochondrial respiratory complex activities

The activities of the respiratory complexes I, II, III and IV were assessed spectrophotometrically in the mitochondrial samples prepared from Sham, CAO-N and CAO-H rabbits (Figure 1, Panel C), as previously described [15].

Complex V (F1F0-ATPase) activity was assessed in the direction of ATP hydrolysis by measuring the concentration of inorganic phosphates released. Mitochondria (50 µg/ml) were incubated at 25°C in 0.5 ml of a medium containing 50 mM Tris, 5 mM $MgCl_2$, 0.5 mM EDTA and 0.1 % triton X-100 (pH=7.4). ATPase activity was started by addition of 100 µM ATP. After 5 min, the reaction was stopped by addition of 1 µM oligomycin and inorganic phosphate concentration was determined.

Assessment of lipid peroxidation

Lipid peroxidation was assessed as the generation of thiobarbituric acid-reactive substances (TBARs). Crude homogenates were prepared from the left ventricular free wall in cold homogenization buffer including 0.1 % triton X-100 and TBARs were determined as previously described [16].

Statistical analysis

Values are expressed as means ± SEM. The different parameters of mitochondrial activity were compared between groups using a one-way ANOVA followed by a Fisher PLSD test. Infarct sizes and risk zones were compared between groups using a Student's t-test. Significant differences were determined when $p < 0.05$.

Results

ROS production is attenuated during simulated ischemia in isolated cardiomyocytes

As illustrated in Figure 2, ROS production was measured in adult cardiomyocytes freshly dissociated from rat hearts and submitted to simulated ischemia. This production was clearly attenuated with hypothermia (32°C) as compared to normothermia (38°C; n=4 for each temperature). As example, ROS production was decreased by -55±8% after 140 min of simulated ischemia.

Mitochondrial respiration and ROS production are directly affected by temperature in isolated mitochondria

Since we showed that hypothermia could dramatically attenuate per-ischemia ROS generation in rat cardiomyocytes, we aimed at deciphering the direct effect of temperature (32 vs 38°C) in intact mitochondria. This was investigated in a suspension of cardiac mitochondria isolated from normal rabbit hearts (n=4). As shown in Figure 3, oxygen consumption significantly decreased when the temperature decrease from 38°C to 32°C. This was observed both at state 4 (substrate-dependent, Panel A) and state 3 (ADP-dependent, Panel B) respiration. State 3 decreased by 36±2% at 32°C vs 38°C whereas state 4 decreased by 47±1%. This resulted in a significant increase in the respiratory control ratio (+22±3%) when the temperature dropped from 38 to 32°C (Figure 3, Panel C). ROS production assessed by H_20_2 production in pyruvate/malate energized mitochondria was also highly temperature-sensitive since it decreased by 41±1% at 32°C vs 38°C (Figure 3, Panel D).

Oxydative stress is attenuated by hypothermia during in vivo myocardial ischemia in rabbits

The next goal was to confirm that attenuation of ROS generation could be also observed *in vivo* with mild hypothermia. In rabbits submitted to 30 min of CAO, cardiac samples were taken within the risk zone in order to assess the end-ischemic oxidative stress using TBARs concentration determination. These concentrations were significantly

decreased by -23% by hypothermia as compared to normothermia (0.10±0.01 vs 0.13±0.01 nmol/mg prot in CAO-H vs CAO-N groups, respectively; n=5 in each group).

Per-ischemic hypothermia preserves the mitochondrial respiratory chain

Since attenuation of ROS generation could be expected to protect the respiratory chain activity, we then attempted to investigate this function in mitochondria extracted from CAO-N and CAO-H hearts (n=6 in each group). In the CAO-N group, alteration of oxidative phosphorylation was demonstrated by a -35% decrease in state 3 oxygen consumption (ATP synthesis) as compared to Sham group (n=6, Figure 4A). This resulted in a significant decrease in respiratory control ratio in CAO-N vs Sham as state 4 oxygen consumption was not different among groups (Figure 4B and 4C). This latter point indicates that the integrity of the inner membrane of isolated mitochondria was preserved after normothermic ischemia. However, under fully uncoupled conditions, *i.e.*, in the presence of FCCP which removed the contribution of the phosphorylation system, the oxygen consumption rate was decreased, showing that ischemia limited the activity of the electron transport chain (Figure 4D). Per-ischemic hypothermia completely prevented these alterations in the CAO-H group. Such preservation was actually related to a protection of the respiratory chain since it was also observed in fully uncoupled conditions (Figure 4). As illustrated in Figure 5, this protection involved a preservation of the activity of complexes I, II and III, whereas activities of complexes IV and V were not affected by ischemia.

To further investigate the effect of per-ischemic hypothermia on activity of the respiratory chain, we assessed ROS production using H_20_2 release as an indirect marker of superoxide anion production by complexes I and III. When pyruvate and malate were used as substrates, ROS production was similar among the 3 groups, as illustrated in Figure 6A. When ROS production by complex I was enhanced by addition of rotenone, it remained similar among groups, suggesting that complex I-induced ROS production was not affected by ischemia in our experimental conditions (Figure 6B). Conversely, when succinate was used as a substrate, the mechanism of ROS production was related to a production by

complex III but also by complex I through a reverse electron flow from complex II to I. In this situation, H_20_2 production was significantly affected by ischemia as compared to Sham and this was completely preserved by hypothermia (Figure 6C). When the reverse electron flow-induced ROS production was inhibited by rotenone, ROS production was only due to complex III and was similar among groups (Figure 6D). This demonstrates that the difference observed between groups with succinate alone was related to an alteration in reverse electron flow-induced ROS production by ischemia and to its preservation by hypothermia.

Discussion

The present study demonstrates that mild hypothermia potently inhibits ischemia-induced ROS production through a direct temperature-dependent mechanism. This resulted in preservation of the electron transfer chain in the rabbit model of myocardial ischemia. Indeed, mild hypothermia restored the capacity of mitochondria to consume oxygen and to synthesise ATP, as observed in a previous study [6]. It also abolished the decreases in complexes I, II and III activities following ischemia and improved the interaction between complexes I and II as evidenced by restoration of the reverse electron flow. In the rabbit model, hypothermia was permitted by an accurate control of per-ischemic myocardial temperature through total liquid ventilation [6, 11-13]. This was previously shown to result in a dramatic decrease in infarct size following coronary artery occlusion and reperfusion [11, 13, 17].

An important originality of this study was to investigate mild hypothermia (32°C) in the beating hearts while many previous studies devoted to hypothermia were performed at lower temperature with cold cardioplegia in arrested hearts. With deep hypothermia, an increase in ROS formation occured during normoxia while it was conversely decreased after ischemia and reperfusion [18]. In the same way, the use of electron spin resonance spectroscopy in isolated rat heart allowed to evidence a reduced free-radical generation at reperfusion following ischemia at 4°C [19]. The present study on mitochondria isolated at the end of an ischemic episode without any reperfusion strongly suggested that the benefit offered by mild hypothermia was directly related to the modulation of per-ischemic sensitivity to ischemia rather than alterations occurring during reperfusion.

We also evidenced that per-ischemic hypothermia is protective against ischemia-induced oxidative phosphorylation alterations through preservation of mitochondrial complexes I, II and III activities. The importance of complex dysfunction has been well investigated in previous reports [2, 20]. All studies analysing mitochondrial dysfunctions in ischemic conditions have revealed that complex I is highly sensitive to ischemic injury [21, 22]. Dysfunctions of complexes II and III are also well known to occur following ischemia [22, 23] whereas the decline in complex IV activity is generally observed later on, mainly during

reperfusion [24]. In the present study, the protection of mitochondrial complexes could be due to the attenuation of ROS generation and inhibition of lipid peroxidation [21]. We could also hypothesize that hypothermia is protective through an initial inhibition of the mitochondrial complexes activity, as shown by the proper effect of temperature on oxidative phosphorylation. Indeed, Chen et al. [25] have shown that reperfusing the myocardium after transient inhibition of complex I during ischemia substantially decreases oxidative stress and limits infarct size.

Although we observed a clear decrease in the activity of complexes I and III, we did not observe any difference in ROS production when assessed in rabbit mitochondria fed with substrates of complex I after extraction from ischemic vs normoxic myocardium. An increase in ROS production from complexes I and III was however previously observed in an isolated rat heart model of global ischemia [26]. Such discrepancy between the present and previous studies may be due to different models of ischemia or to different conditions of evaluation of ROS production. In our study, these measurements were performed under state 4 respiration. Pasdois et al. [1] did not observe any change in ROS production after ischemia in similar conditions while it was increased when respiration was stimulated by an ADP regenerating system. When succinate was used as a substrate in our study, the production rate of mitochondrial ROS was much higher. This high rate of ROS production with succinate was abolished by rotenone showing that ROS production is due to complex I and is caused by the reverse electron transfer from complex II to complex I, as previously shown [27]. Interestingly, the reverse electron transfer was dramatically inhibited following normothermic ischemia while mild hypothermia restores reverse electron flow. This suggests that hypothermia either protects a site of ischemic damage at the level of complex I or improves the link between complexes I and II. An attractive hypothesis is therefore that the benefit offered by mild hypothermia is related to an improved interaction between complex I and II. This later point could be in line with the report of Rosca et al. [28] showing that a dramatic decrease in oxidative phosphorylation could be caused by disorganization of the

supercomplexes molecular assembly, also called respirasomes. One can imagine that hypothermia is able to maintain such organization of these supercomplexes during ischemia.

In conclusion, this study shows that the cardioprotective effect of mild hypothermia could involve a direct effect on per-ischemic ROS generation. It is associated with a protection of the mitochondrial respiratory chain in rabbits submitted to regional myocardial ischemia. This was interestingly related to an improved interaction between complexes I and II of the respiratory chain. It is reasonable to speculate that ROS production attenuation can participate to mPTP opening inhibition and ultimately to infarct size reduction with hypothermia. This might also explain why hypothermia is mostly protective when instituted early during the ischemic process.

Ackowledgements

The authors are indebted to Sandrine Bonizec for administrative assistance. This study was supported by a grant TLV-CARDAREST (R10028JS) from INSERM and a grant ET7-460 from the “Fondation de l’Avenir”. Renaud Tissier was a recipient of a “Contrat d’Interface INSERM-ENV” (2010).

Conflict of interest: none declared.

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Figure legends

Figure 1

Design of the different experiments performed either *in vitro* in rat cardiomyocytes (Panel A), *ex vivo* in mitochondria isolated from intact rabbit hearts (Panels B) or after coronary artery occlusion (CAO; Panel C).

Figure 2

Reactive oxygen species (ROS) production in isolated cardiomyocytes subjected to 140 min of simulated ischemia. Cardiomyocytes were loaded with dichlorofluoresin diacetate (DCFH) and ROS production was evaluated by measuring dichlorofluorescein (DCF) fluorescence at 32 and 38 °C (n=4 at each temperature).

**, p<0.05 vs 38°C.*

Figure 3

Respiratory activity (Panels A to C) and H_20_2 production (Panel D) in mitochondria isolated from normal rabbit hearts. States 4 and 3 oxygen consumptions represent substrate- and ADP-dependent (ATP-synthesis) respiration rates, respectively. H_20_2 production was assessed in pyruvate/malate energized mitochondria.

**, p<0.05 vs 38°C.*

Figure 4

Respiratory activity in cardiac mitochondria isolated from rabbits submitted to 30 min of coronary artery occlusion (CAO) under normothermic conditions (CAO-N group, n=6) or with mild hypothermia (CAO-H group, n=6). A third group was investigated with a Sham procedure (n=6). States 4 and 3 oxygen consumptions represent substrate- and ADP-

dependent (ATP-synthesis) respiration rates, respectively. Uncoupled state oxygen consumption was investigated using carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone (FCCP).

**, p<0.05 vs Sham; †, p<0.05 vs CAO-N.*

Figure 5

Activity of the respiratory complexes I to V of mitochondria isolated from rabbits submitted to 30 min of coronary artery occlusion (CAO) under normothermic conditions (CAO-N group, n=6) or with mild hypothermia (CAO-H group, n=6). A third group was investigated with a Sham procedure (n=6).

**, p<0.05 vs Sham; †, p<0.05 vs CAO-N.*

Figure 6

H_20_2 production in mitochondria isolated from rabbits submitted to 30 min of coronary artery occlusion (CAO) under normothermic conditions (CAO-N group, n=6 or with mild hypothermia (CAO-H group, n=6). A third group was investigated with a Sham procedure (n=4). Experiments were repeated in the presence of different substrates (pyruvate/malate, succinate) with or without rotenone.

**, p<0.05 vs Sham; †, p<0.05 vs CAO-N.*

1

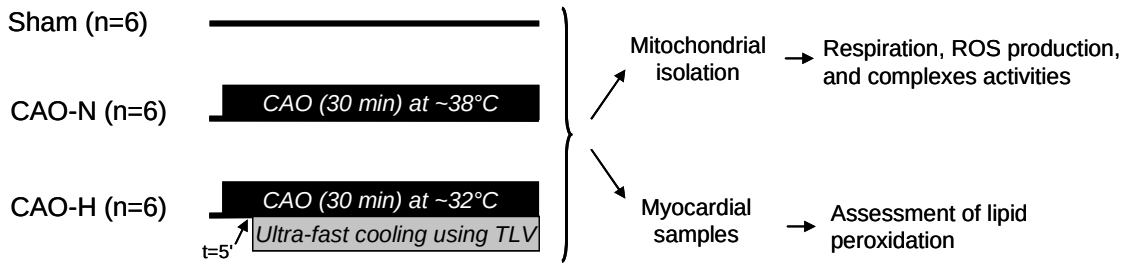
A- In vitro evaluation of ROS production during simulated ischemia

Freshly dissociated adult rat cardiomyocytes → ROS production during simulated ischemia at 32 vs 38°C (n=4 experiments at each temperature)

B- Ex vivo evaluation of respiration and ROS production in isolated mitochondria

Rabbits (n=4) → Heart extraction → Mitochondrial isolation → Respiration and ROS production at 32 vs 38°C

C- Ex vivo evaluation after myocardial ischemia in rabbits



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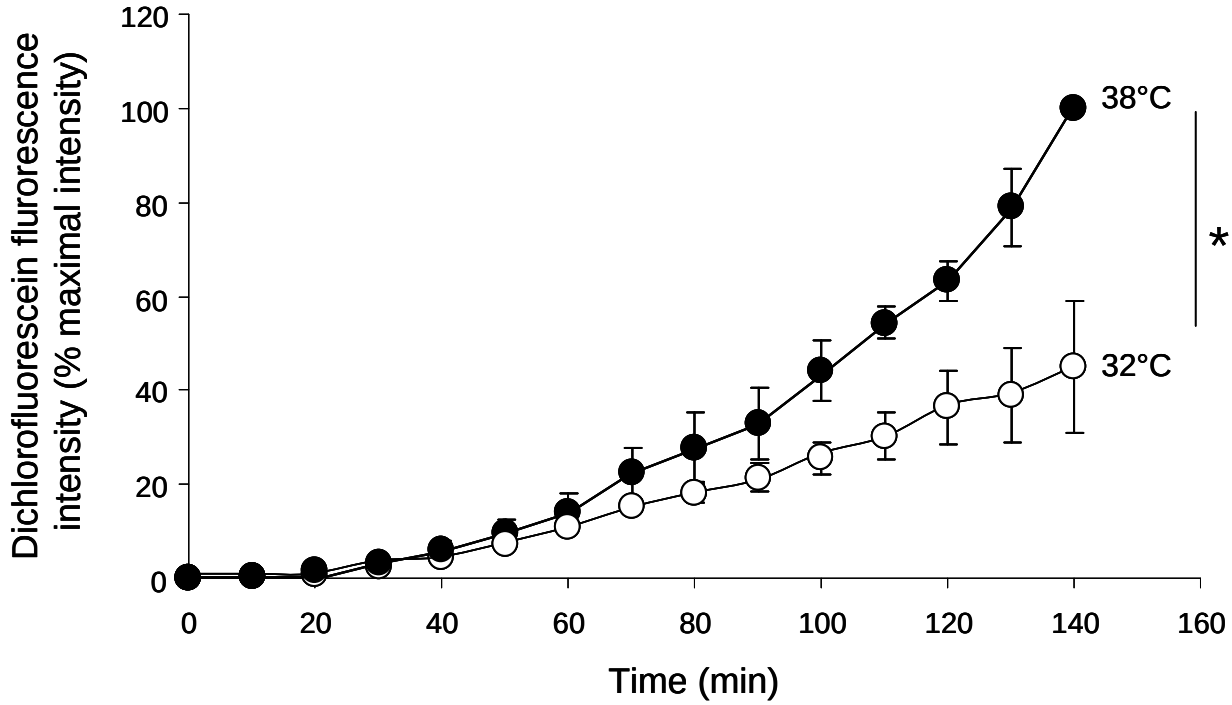
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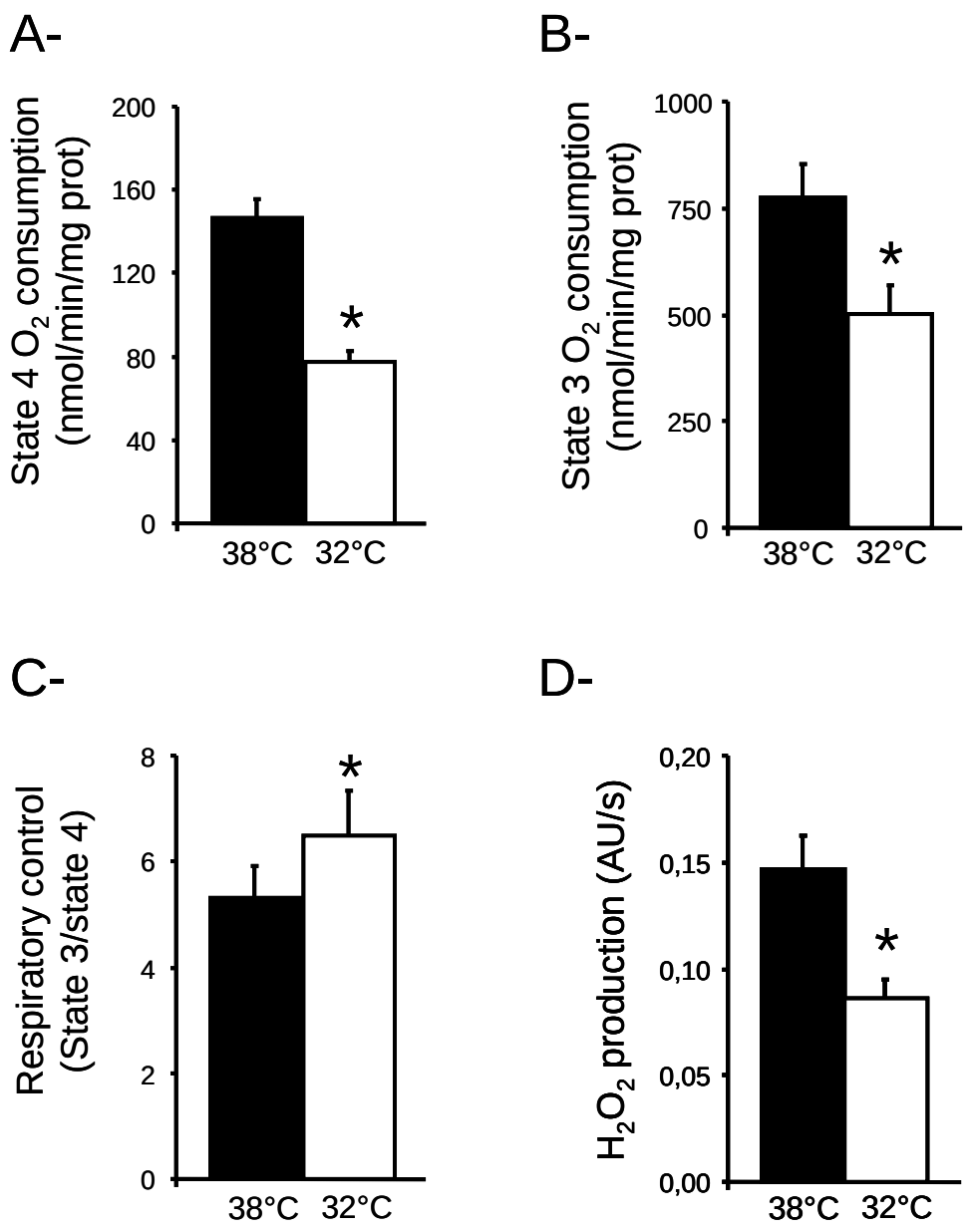
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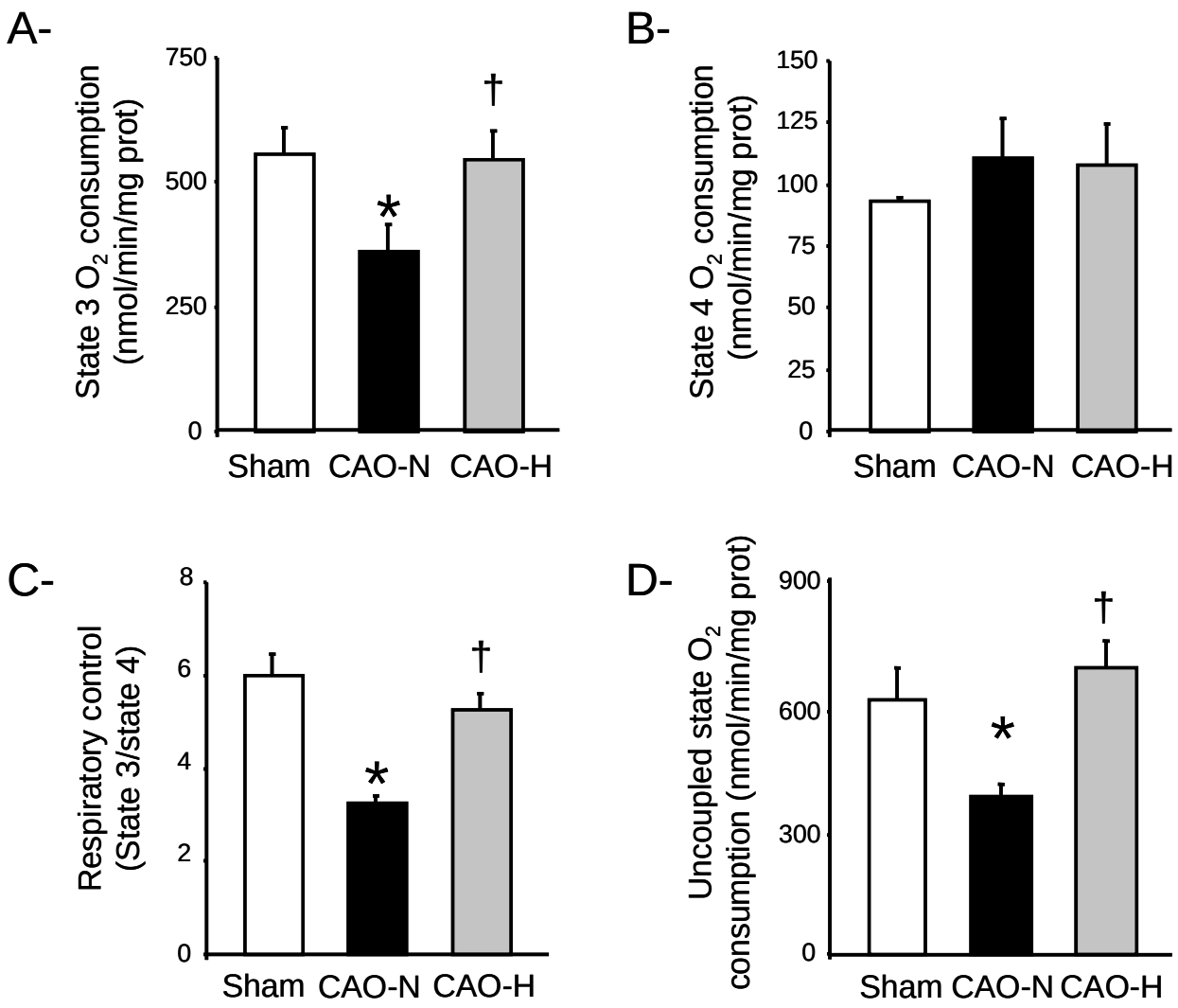
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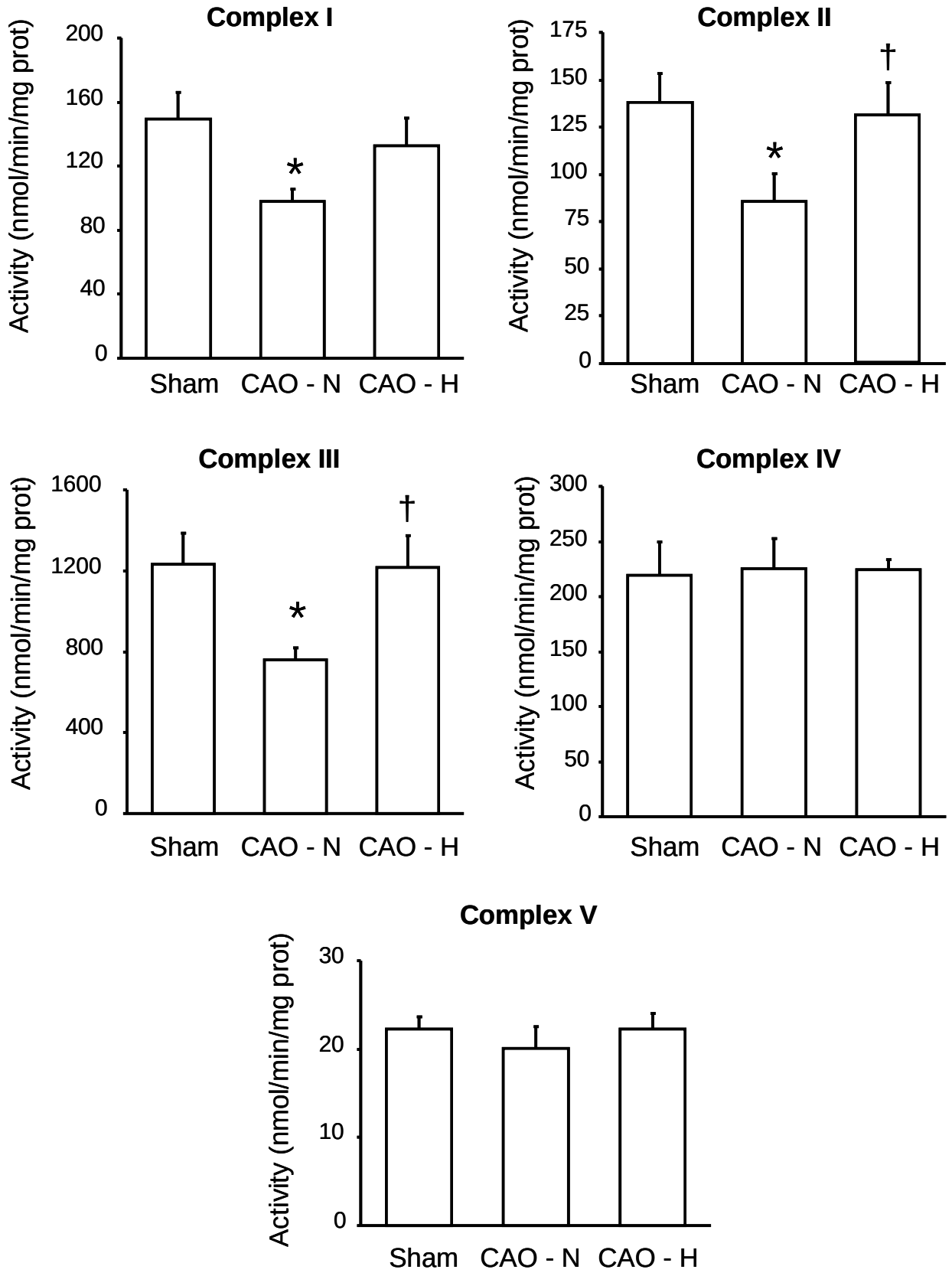
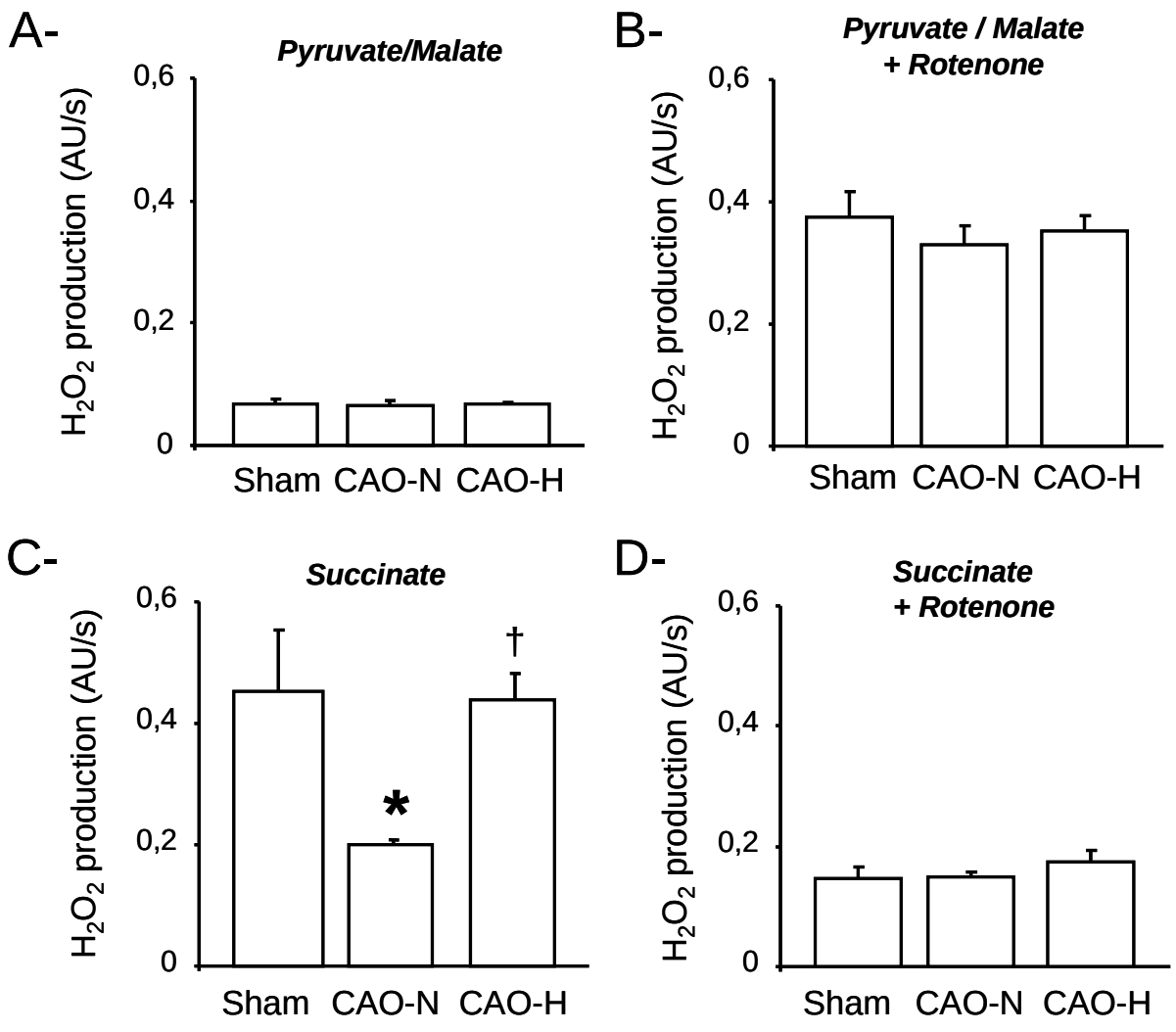
CAO-H
B-
State 4 O_2 consumption (nmol/min/mg prot)
150
125
100
75
50
25
0
Sham
CAO-N
CAO-H
C-
Respiratory control (State 3/state 4)
8
6
4
2
0
*
†
Sham
CAO-N
CAO-H
D-
Uncoupled state O_2 consumption (nmol/min/mg prot)
900
600
300
0
*
†
Sham
CAO-N
CAO-H

1


Complex I
Activity (nmol/min/mg prot)
200
160
120
80
40
0
*
Sham
CAO - N
CAO - H
Complex II
Activity (nmol/min/mg prot)
175
150
125
100
75
50
25
0
*
†
Sham
CAO - N
CAO - H
Complex III
Activity (nmol/min/mg prot)
1600
1200
800
400
0
*
†
Sham
CAO - N
CAO - H
Complex IV
Activity (nmol/min/mg prot)
300
250
200
150
100
50
0
Sham
CAO - N
CAO - H
Complex V
Activity (nmol/min/mg prot)
30
20
10
0
Sham
CAO - N
CAO - H


A-
Pyruvate/Malate
B-
Pyruvate / Malate
+ Rotenone
C-
Succinate
D-
Succinate
+ Rotenone
H2O2 production (AU/s)
0,6
0,4
0,2
0
Sham
CAO-N
CAO-H
*
†

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