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**Mild hypothermia reduces per-ischemic reactive oxygen species production
and preserves mitochondrial respiratory complexes**

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Short title: Mild hypothermia, cardioprotection and mitochondria

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1 **Abstract**

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

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

12 **Results:** In isolated rat cardiomyocytes, per-ischemic ROS generation was dramatically
13 decreased at 32 vs 38°C (e.g., $-55\pm 8\%$ after 140 min of hypoxia). In oxygenated
14 mitochondria isolated from intact rabbit hearts, hypothermia also improved respiratory control
15 ratio ($+22\pm 3\%$) and reduced H_2O_2 production ($-41\pm 1\%$). Decreased oxidative stress was
16 further observed in rabbit hearts submitted to hypothermic vs normothermic ischemia (CAO-
17 H vs CAO-N), using thiobarbituric acide-reactive substances as a marker. This was
18 accompanied by a preservation of the respiratory control ratio as well as the activity of
19 complexes I, II and III in cardiac mitochondria.

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

1 Introduction

2 Mitochondria are well known to mediate ischemic injuries through complex events
3 involving reactive oxygen species (ROS) generation ^{1,2}, alteration of electron transfer activity
4 ^{2,3}, opening of the mitochondrial permeability transition pore (mPTP) ⁴ and cytochrome c
5 release ^{1,2}. This has been ubiquitously observed in multiple ischemic insult models such as
6 regional ⁴ or global ischemia and cardiac arrest ^{2,3}. Mitochondrial dysfunction has been
7 mostly investigated during the post-ischemic reperfusion phase ^{4,5}. Targeting mitochondria is
8 then often considered as a relevant approach to prevent reperfusion injury through, *e.g.*,
9 direct inhibition of mPTP after myocardial infarction ⁴. However, electron microscopy studies
10 have clearly showed that mitochondrial injuries start during the ischemic phase ^{3,6}.
11 Alterations in mitochondrial respiratory complexes activities, cytochrome c release and ROS
12 generation were also demonstrated to occur during the hypoxic phase prior to reoxygenation
13 ¹⁻³.

14 Despite the role of mitochondria has been extensively investigated during ischemia,
15 its exact participation to the cardioprotective effect of per-ischemic mild hypothermia (32-
16 34°C) remains unclear. We previously showed that mitochondrial ultrastructure and
17 respiratory function were improved by mild hypothermia in rabbits submitted to 30 min of
18 coronary artery occlusion ⁶. The calcium-induced opening of mPTP was also ultimately
19 inhibited by per-ischemic hypothermia ⁶. The cascade of events leading to this per-ischemic
20 protection remains however still unknown. It is currently proposed that mild hypothermia
21 could involve its own survival signalling cascade with protein kinase C- ϵ ⁷, nitric oxide
22 synthase ⁸, extracellular-regulated kinase (ERK) ⁹, Akt pathway ⁸ and mammalian target of
23 rapamycin (mTOR) ¹⁰. Here, we propose to investigate whether mild hypothermia could act
24 through a direct effect of temperature on mitochondrial function and ROS generation. Our
25 hypothesis is that hypothermia could act through a reduction of per-ischemic oxidative stress.
26 This would explain why its beneficial effect is maximal when instituted early during ischemia
27 while less efficient when started after reperfusion. For this purpose, we first evaluated *in vitro*

1 the effect of mild hypothermia on ROS generation in a model of rat cardiomyocytes
2 submitted to simulated ischemia. Second, we tested *ex vivo* the direct effect of temperature
3 on respiratory parameters and ROS production in mitochondria isolated from intact rabbit
4 heart. Since these experiments showed a dramatic decrease in ROS production at 32 vs
5 38°C, we further investigated *in vivo* the overall oxidative stress induced by myocardial
6 ischemia under hypothermic vs normothermic conditions. This was performed in
7 anesthetized rabbits submitted to 30 min of coronary artery occlusion. We also assessed *ex*
8 *vivo* the respiratory function and complex activities of the mitochondria extracted from the
9 ischemic territory. In all *in vivo* experiments, hypothermia was induced using total liquid
10 ventilation with temperature-controlled perfluorocarbons that can fastly and accurately control
11 per-ischemic cardiac temperature in anesthetized rabbits. We previously showed that this
12 strategy can use the lung as a heat exchanger to quickly change the cardiac temperature
13 while maintaining appropriate gas exchange and ultimately inducing a potent anti-infarct
14 effect ^{6, 11-13}.

15

1 **Methods**

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

5 *Simulated ischemia in adult rat cardiomyocytes and measurement of ROS generation*

6 Adult rat ventricular cardiomyocytes were obtained from hearts of male Wistar rats
7 (260-300g), as described previously¹⁴. They were plated in 35 mm Petri dishes which were
8 mounted on a heated perfusion chamber and perfused with a Tyrode's modified solution. The
9 temperature of this solution was continuously monitored. The chamber was connected to a
10 gas bottle diffusing a constant stream of O₂ (21%), N₂ (74%) and CO₂ (5%) maintaining a
11 partial O₂ pressure at 21%. pO₂ within the chamber was measured continuously using a fiber
12 optic sensor system. The cardiomyocytes were paced to beat by field stimulation (5ms;
13 0.5Hz). To simulate ischemia, myocytes were exposed for 140 min to a hypoxic medium
14 (pO₂<1%) consisting in a glucose-free Tyrode's modified solution (pH=7.4) supplemented
15 with 20mM of 2-deoxyglucose and to a constant stream of N₂ (100%).

16 To evaluate ROS formation at 32 or 38°C (Figure 1, Panel A), myocytes were loaded
17 with the fluorescent probe 2',7'-dichlorofluorescein-diacetate (5 μM) for 15 min before
18 induction of simulated ischemia. Fluorescence was measured with an Olympus IX-81
19 motorized inverted fluorescent microscope equipped with a mercury lamp as a source of light
20 for epifluorescent illumination, using 495 nm and 520 nm wavelengths for excitation and
21 emission, respectively¹⁴.

22 *Experimental protocols in rabbits*

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

1 (Figure 1, Panel B), the heart was removed after a period of stabilization. Samples from the
2 left ventricular free wall were rapidly minced, homogenized and centrifuged to obtain a
3 mitochondrial suspension for *ex vivo* investigation of the direct effect of temperature (32 vs
4 38°C) on mitochondrial ROS production and respiration. In the second set of experiments
5 (Figure 1, Panel C), rabbits were anesthetized and randomly submitted to 30 min of follow-up
6 (Sham group) or to 30 min of coronary artery occlusion (CAO groups), as previously
7 described^{11,13}. In the CAO groups, animals underwent at random either normothermic
8 ischemia (CAO-N group) or hypothermia induced by liquid ventilation from the 5th min to the
9 end of CAO (CAO-H group). The latter approach allows a rapid and accurate control of the
10 cardiac temperature. As previously described^{6,11,13}, liquid ventilation was instituted by filling
11 the lungs with perfluorocarbons (Fluorinert, 3M, Cergy, France) and connecting the
12 endotracheal tube to a liquid ventilator. The ventilator was set to an initial tidal volume of ~8-
13 10 ml/kg of body weight (respiratory rate ~ 6 breaths/min). This was increased as needed in
14 order to maintain blood gases within usual values. The temperature of the perfluorocarbon
15 mixture was adjusted to maintain left atrial temperatures at a target of ~32°C throughout
16 ischemia. At the end of the 30 min CAO, the hearts were removed and a mitochondrial
17 suspension was prepared from the ischemic territory for assessment of lipid peroxidation and
18 further *ex vivo* experiments in extracted mitochondria (evaluation of respiratory parameters
19 and ROS generation).

20 *Measurement of mitochondrial respiration and ROS production*

21 Oxygen consumption was measured as previously described⁶ with a Clark type
22 electrode in a respiration buffer (50 mM sucrose, 100 mM KCl, 10 mM HEPES, 5 mM
23 KH₂PO₄) containing 0.4 mg/ml of mitochondria prepared from rabbit myocardium (Figure 1,
24 Panel B and C). Substrate-respiration rate (state 4) and ATP synthesis (state 3) were
25 investigated by addition of 5 mM pyruvate/malate and 1 mM of ADP, respectively. The
26 corresponding respiratory control ratio (state 3/ state 4) was calculated. Similar

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

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

6 *Assessment of mitochondrial respiratory complex activities*

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

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

16 *Assessment of lipid peroxidation*

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

21 *Statistical analysis*

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

1 Results

2 *ROS production is attenuated during simulated ischemia in isolated cardiomyocytes*

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

8 *Mitochondrial respiration and ROS production are directly affected by temperature in isolated* 9 *mitochondria*

10 Since we showed that hypothermia could dramatically attenuate per-ischemia ROS
11 generation in rat cardiomyocytes, we aimed at deciphering the direct effect of temperature
12 (32 vs 38°C) in intact mitochondria. This was investigated in a suspension of cardiac
13 mitochondria isolated from normal rabbit hearts (n=4). As shown in Figure 3, oxygen
14 consumption significantly decreased when the temperature decrease from 38°C to 32°C.
15 This was observed both at state 4 (substrate-dependent, Panel A) and state 3 (ADP-
16 dependent, Panel B) respiration. State 3 decreased by $36\pm 2\%$ at 32°C vs 38°C whereas
17 state 4 decreased by $47\pm 1\%$. This resulted in a significant increase in the respiratory control
18 ratio ($+22\pm 3\%$) when the temperature dropped from 38 to 32°C (Figure 3, Panel C). ROS
19 production assessed by H₂O₂ production in pyruvate/malate energized mitochondria was also
20 highly temperature-sensitive since it decreased by $41\pm 1\%$ at 32°C vs 38°C (Figure 3, Panel
21 D).

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

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

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

3 *Per-ischemic hypothermia preserves the mitochondrial respiratory chain*

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

20 To further investigate the effect of per-ischemic hypothermia on activity of the
21 respiratory chain, we assessed ROS production using H_2O_2 release as an indirect marker of
22 superoxide anion production by complexes I and III. When pyruvate and malate were used
23 as substrates, ROS production was similar among the 3 groups, as illustrated in Figure 6A.
24 When ROS production by complex I was enhanced by addition of rotenone, it remained
25 similar among groups, suggesting that complex I-induced ROS production was not affected
26 by ischemia in our experimental conditions (Figure 6B). Conversely, when succinate was
27 used as a substrate, the mechanism of ROS production was related to a production by

1 complex III but also by complex I through a reverse electron flow from complex II to I. In this
2 situation, H₂O₂ production was significantly affected by ischemia as compared to Sham and
3 this was completely preserved by hypothermia (Figure 6C). When the reverse electron flow-
4 induced ROS production was inhibited by rotenone, ROS production was only due to
5 complex III and was similar among groups (Figure 6D). This demonstrates that the difference
6 observed between groups with succinate alone was related to an alteration in reverse
7 electron flow-induced ROS production by ischemia and to its preservation by hypothermia.
8

1 Discussion

2 The present study demonstrates that mild hypothermia potently inhibits ischemia-
3 induced ROS production through a direct temperature-dependent mechanism. This resulted
4 in preservation of the electron transfer chain in the rabbit model of myocardial ischemia.
5 Indeed, mild hypothermia restored the capacity of mitochondria to consume oxygen and to
6 synthesise ATP, as observed in a previous study⁶. It also abolished the decreases in
7 complexes I, II and III activities following ischemia and improved the interaction between
8 complexes I and II as evidenced by restoration of the reverse electron flow. In the rabbit
9 model, hypothermia was permitted by an accurate control of per-ischemic myocardial
10 temperature through total liquid ventilation^{6, 11-13}. This was previously shown to result in a
11 dramatic decrease in infarct size following coronary artery occlusion and reperfusion^{11, 13, 17}.

12 An important originality of this study was to investigate mild hypothermia (32°C) in the
13 beating hearts while many previous studies devoted to hypothermia were performed at lower
14 temperature with cold cardioplegia in arrested hearts. With deep hypothermia, an increase in
15 ROS formation occurred during normoxia while it was conversely decreased after ischemia
16 and reperfusion¹⁸. In the same way, the use of electron spin resonance spectroscopy in
17 isolated rat heart allowed to evidence a reduced free-radical generation at reperfusion
18 following ischemia at 4°C¹⁹. The present study on mitochondria isolated at the end of an
19 ischemic episode without any reperfusion strongly suggested that the benefit offered by mild
20 hypothermia was directly related to the modulation of per-ischemic sensitivity to ischemia
21 rather than alterations occurring during reperfusion.

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

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

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

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

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

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6

7 **Conflict of interest:** none declared.

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1

2 **Figure legends**

3

4 Figure 1

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

8

9

10 Figure 2

11 Reactive oxygen species (ROS) production in isolated cardiomyocytes subjected to 140 min
12 of simulated ischemia. Cardiomyocytes were loaded with dichlorofluoresin diacetate (DCFH)
13 and ROS production was evaluated by measuring dichlorofluorescein (DCF) fluorescence at
14 32 and 38 °C (n=4 at each temperature).

15 *, $p < 0.05$ vs 38°C.

16

17 Figure 3

18 Respiratory activity (Panels A to C) and H₂O₂ production (Panel D) in mitochondria isolated
19 from normal rabbit hearts. States 4 and 3 oxygen consumptions represent substrate- and
20 ADP-dependent (ATP-synthesis) respiration rates, respectively. H₂O₂ production was
21 assessed in pyruvate/malate energized mitochondria.

22 *, $p < 0.05$ vs 38°C.

23

24 Figure 4

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

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

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

5

6 Figure 5

7 Activity of the respiratory complexes I to V of mitochondria isolated from rabbits submitted to
8 30 min of coronary artery occlusion (CAO) under normothermic conditions (CAO-N group,
9 $n=6$) or with mild hypothermia (CAO-H group, $n=6$). A third group was investigated with a
10 Sham procedure ($n=6$).

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

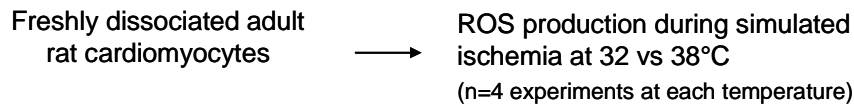
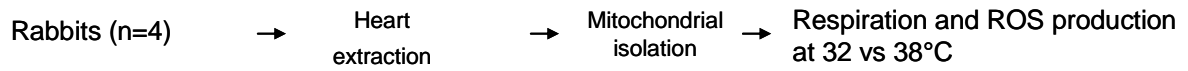
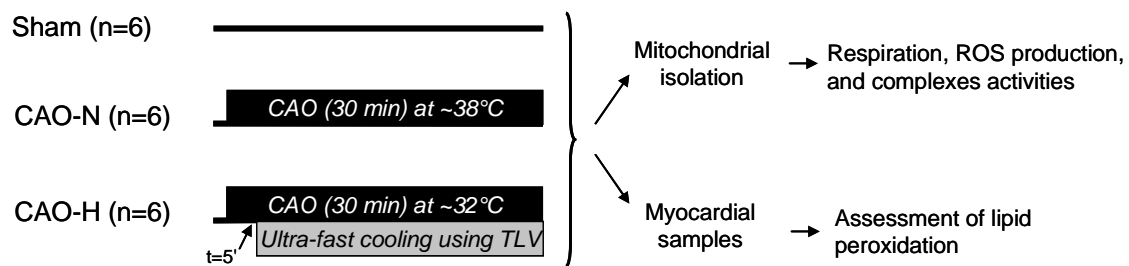
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13 Figure 6

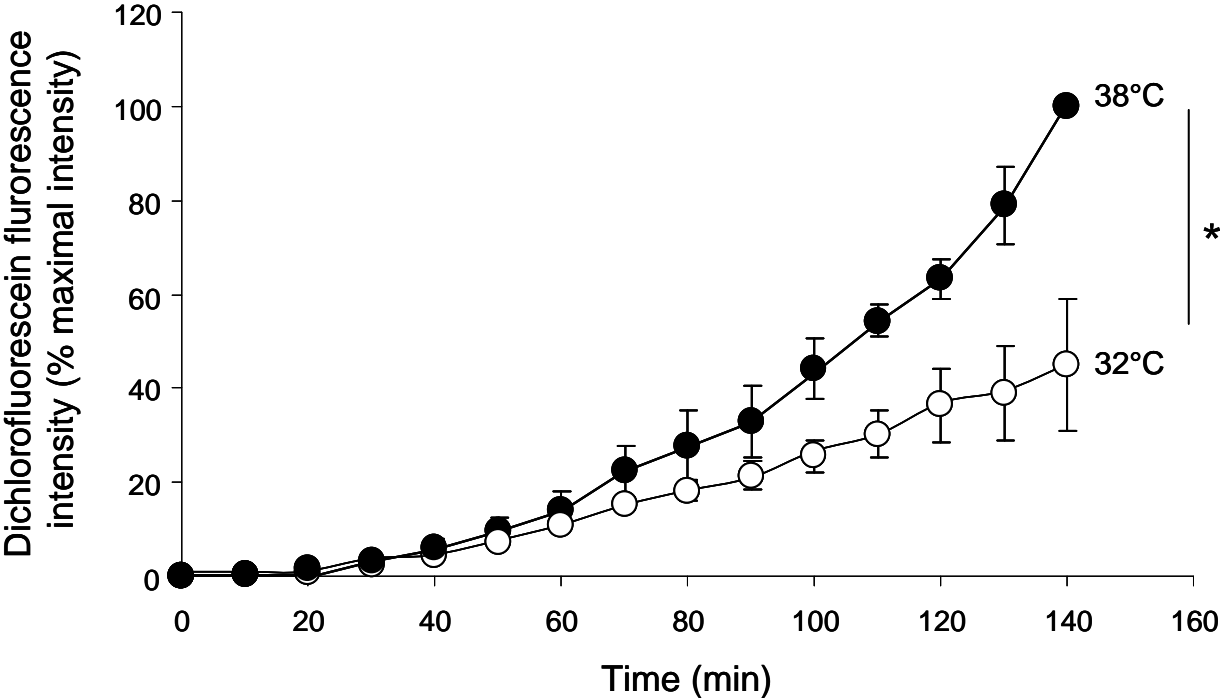
14 H_2O_2 production in mitochondria isolated from rabbits submitted to 30 min of coronary artery
15 occlusion (CAO) under normothermic conditions (CAO-N group, $n=6$ or with mild
16 hypothermia (CAO-H group, $n=6$). A third group was investigated with a Sham procedure
17 ($n=4$). Experiments were repeated in the presence of different substrates (pyruvate/malate,
18 succinate) with or without rotenone.

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

1

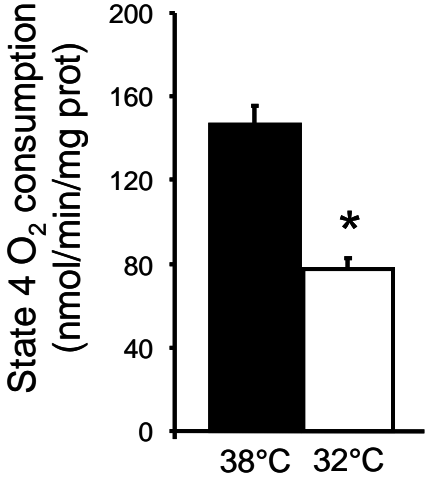
A- In vitro evaluation of ROS production during simulated ischemia**B- Ex vivo evaluation of respiration and ROS production in isolated mitochondria****C- Ex vivo evaluation after myocardial ischemia in rabbits**

1

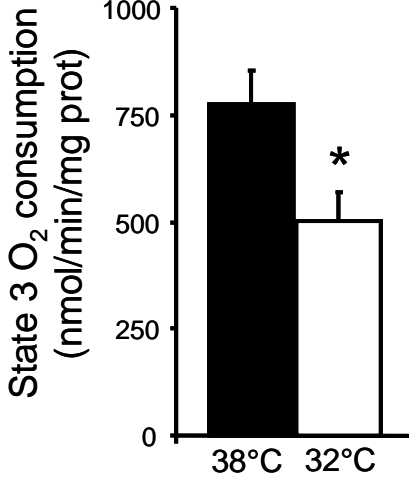


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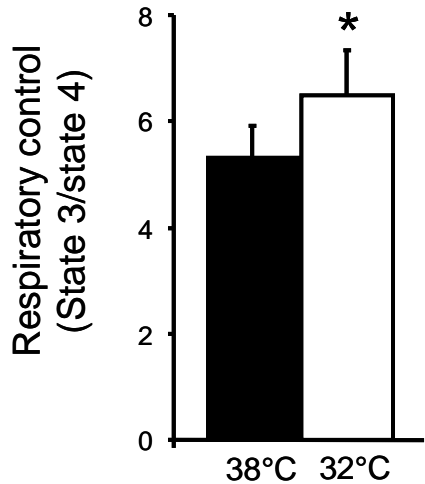
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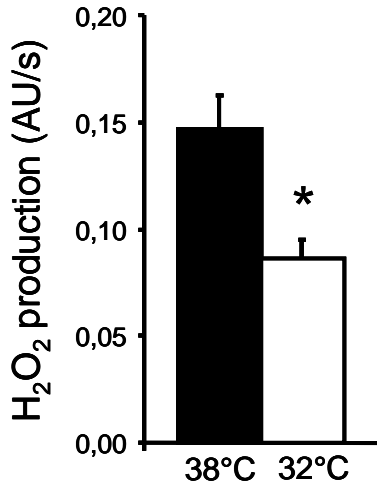
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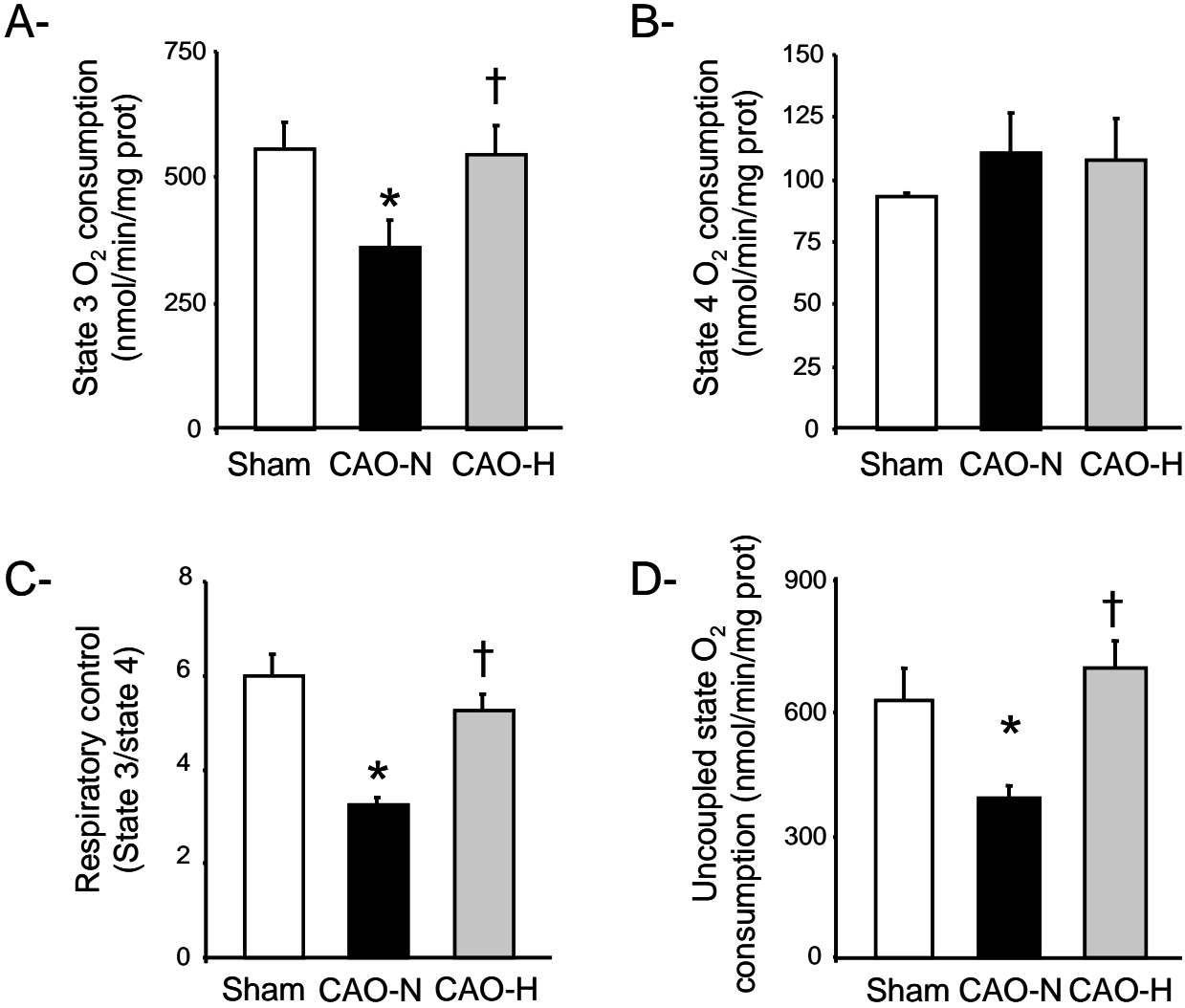
C-



D-



1



1

