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▶ **To cite this version:**

Matthias Kohlhauer, Mathieu Panel, Marine Vermot Des Roches, Estelle Faucher, Yara Abi Zeid Daou, et al.. Brain and myocardial mitochondria follow different patterns of dysfunction after cardiac arrest. Shock, 2021, Online ahead of print. 10.1097/SHK.0000000000001793 . inserm-03279846

HAL Id: inserm-03279846
https://inserm.hal.science/inserm-03279846

Submitted on 6 Jul 2021

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Brain and myocardial mitochondria follow different patterns of dysfunction after cardiac arrest

Matthias Kohlhauer [1,2], Mathieu Panel [1,2,3], Marine Vermot des Roches [1,2], Estelle Faucher [1,2], Yara Abi Zeid Daou [1,2]; Emilie Boissady [1,2], Fanny Lidouren [1,2], Bijan Ghaleh [1,2], Didier Morin [1,2], Renaud Tissier [1,2]

1) Univ Paris Est Créteil, INSERM, IMRB, F-94010 Créteil, France

2) Ecole nationale vétérinaire d'Alfort, IMRB, F-94700 Maisons-Alfort, France

* **Corresponding author**: Renaud Tissier
Ecole Nationale Vétérinaire d'Alfort
7 avenue du Général de Gaulle
94700 Maisons-Alfort, France
Tel : +33143967302
Email: renaud.tissier@vet-alfort.fr.
Orcid: https://orcid.org/0000-0001-6602-939X.

Running head: Mitochondria and cardiac arrest

Conflicts of Interest: M Kohlhauer and R Tissier are shareholders of a start-up company dedicated to the clinical research on therapeutic hypothermia through total liquid ventilation (Orixha).

Funding source: This study was supported by the Agence Nationale pour la Recherche (COOLIVENT Grant).

Abstract

Mitochondria is often considered as the common nexus of cardiac and cerebral dysfunction after cardiac arrest. Here, our goal was to determine whether the time course of cardiac and cerebral mitochondrial dysfunction is similar after shockable vs non-shockable cardiac arrest in rabbits. Anesthetized rabbits were submitted to 10 min of no-flow by ventricular fibrillation (VF group) or asphyxia (non-shockable group). They were euthanized at the end of the no-flow period or 30 min, 120 min or 24h after resuscitation for *in vitro* evaluation of oxygen consumption and calcium retention capacity. In the brain (cortex and hippocampus), moderate mitochondrial dysfunction was evidenced at the end of the no-flow period after both causes of cardiac arrest *vs* baseline. It partly recovered at 30 and 120 min after cardiac arrest, with lower calcium retention capacity and higher substrate-dependant oxygen consumption after VF vs non-shockable cardiac arrest. However, after 24h of follow-up, mitochondrial dysfunction dramatically increased after both VF and non-shockable cardiac arrest, despite greater neurological dysfunction after the latter one. In the heart, mitochondrial dysfunction was also maximal after 24h following resuscitation, with no significant difference among the causes of the cardiac arrest. During the earlier timing of evaluation, calcium retention capacity and ADP-dependant oxygen consumption were lower and higher, respectively, after non-shockable cardiac arrest vs VF. In conclusion, the kinetics of cardiac and cerebral mitochondrial dysfunction suggests that mitochondrial function do not play a major role in the early phase of the post-resuscitation process but is only involved in the longer pathophysiological events.

Key words: Mitochondria; Cardiac arrest; Ischaemia; Reperfusion

Introduction

Out-of-hospital cardiac arrest is a major cause of mortality in western countries. Following resumption of spontaneous circulation (ROSC), a “post-cardiac arrest syndrome” is characterized by neurological, cardiac and multi-visceral failure. The severity of this syndrome depends on the initial cardiac rhythm identified during cardiopulmonary resuscitation (CPR), with worst outcome after non-shockable vs shockable rhythms **(1, 2)**. Neurological dysfunction is responsible for 65% of the overall mortality while acute heart failure is the predominant cause of death during the first 3 days following resuscitation **(3)**.

It is often assumed that the pathophysiology of ischemic disorders shares common mechanisms in the brain and heart after cardiac arrest, considering that the mitochondria is a common nexus for both organs **(4–6)**. In the absence of oxygen, the mitochondrial respiratory chain is indeed a major source of reactive oxygen species (ROS), which leads to massive oxidation and cellular death **(7)**. For instance, ischaemia induces a reversion of the succinate dehydrogenase (complex II) leading to alteration of tricarboxylic acid cycle with dramatic reduction of fumarate into succinate **(8, 9)**. At reperfusion, succinate dehydrogenase resumes its normal function and converts back the succinate into fumarate. However, the large stock of succinate accumulated during ischaemia leads to an electron overflow in the mitochondrial respiratory chain and induces a reverse electronic flow from complex II to complex I. This contributes to a massive production of superoxide anion at reperfusion **(8)**. Additionally, the cytosolic calcium overload induced after ischaemia-reperfusion promotes the formation of a mitochondrial Permeability Transition Pore (mPTP), which is responsible for mitochondrial swelling and release of pro-apoptotic factors **(10)**. Experimentally, its pharmacological inhibition has been widely shown to provide cardioprotection after focal or generalized ischaemia **(11, 12)**. However, the pathophysiology of post-cardiac arrest disorders probably differs between brain and heart, and may depends upon the cause of cardiac arrest (shockable vs non shockable rhythms).

Accordingly, the goal of the present study was to compare the time course of mitochondrial dysfunction in brain and heart after cardiac arrest. We used two different conditions of cardiac arrest in rabbits, using ventricular fibrillation vs asphyxia models of cardiac arrest. In both conditions, we investigated the time course of the mPTP formation and mitochondrial respiration dysfunction. We also compared the neurological and cardiovascular outcomes in both conditions in order to assess putative links between mitochondrial kinetics disorders and related end-organ injuries.

Materials and Methods

Experimental procedure

Experiments were approved by the institutional review board for animal research (ComEth AnSES/ENVA/UPEC n°16; Project 18-069), in accordance with French regulations for animal use for research purpose. Male New-Zealand rabbits (2.7±0.2 kg) were anesthetised with tiletamine and zolazepam (25 mg/kg i.m. each). After intubation and mechanical ventilation, anaesthesia was maintained with sodium pentobarbital (5–10 mg/kg/h i.v.). Methadone (0.5 µg/kg i.m.) was administered for analgesia. An arterial pressure probe was inserted into the medial ear artery for mean arterial pressure (MAP) continuous recording. Electrocardiogram was evaluated throughout the experiment. After paralysis with vecuronium bromide (0.1 mg/kg i.v.), mechanical ventilation was stopped and animals were randomly submitted to 10 min of no-flow induced by either ventricular fibrillation (VF group) or asphyxia (Non-shockable CA group).

In the VF group, cardiac arrest was induced by applying an alternative current (10 V, 0.4 mA) between two electrodes applied upon the chest and inserted into the oesophagus, respectively. In the non-shockable group, cardiac arrest was induced by asphyxia through endotracheal tube clamping. Blood pressure rapidly decreased and no-flow was defined as a mean blood pressure drop below 20 mmHg.

In both groups, cardiopulmonary resuscitation (CPR) was performed after 10 min of no-flow by continuous chest compression and adrenaline administration every two minutes (15 µg/kg i.v.). Cardiac massage was performed manually at the rate of 200 beat per min. In the VF group, electric shocks were also delivered every two minutes (10 J/kg in VF group) until electrical conversion of the heart rhythm. Resumption of spontaneous circulation (ROSC) was considered as a mean blood pressure remaining above 60 mmHg during at least one minute without chest compression. After ROSC, rabbits remained under mechanical ventilation (FiO_2=30%) with adrenaline infusion at a dose adjusted to achieve a mean blood pressure of 70 mmHg.

Animals were euthanized at the end of the no-flow period or 30 min, 120 min or 24 h after ROSC. We also isolated mitochondria from additional healthy animals with no cardiac arrest for baseline values determination. In the group followed during 24h, animals were awaken 6 h after ROSC. Before euthanasia, the neurological status was assessed according to a specific score, as previously described (13). This scoring method is evaluating the normal physiological function of the rabbits (feeding and grooming) as well as impairment of locomotor function and conscious state.

Isolation of mitochondria

In order to assess mitochondrial dysfunction at different time points, animals were euthanized at the end of the no-flow period or 30 min, 120 min or 24 h after ROSC, using a lethal dose of pentobarbital (60 mg/kg i.v.). We also isolated mitochondria from additional healthy animal only submitted to anaesthesia, mechanical ventilation and surgical preparation for baseline values. After euthanasia, we rapidly sampled 1 g of the left ventricle, right brain cortex and hippocampus.

Isolation and purification of mitochondrion

Cardiac samples were minced and homogenized with an Ultra-Turrax T25 (set at position 5; IKA-Werke GmbH & Co. KG, Staufen, Germany) for 5 s in 15 mL of buffer containing 300 mM sucrose, 10 mM HEPES and 2 mM EGTA, pH 7.4, at 4°C. Cortex and hippocampus were simply minced and placed in 10 mL of the same 4°C buffer. Preparations were repeatedly passed in a Potter-Elvehjem glass homogenizer (motor-driven Teflon pestle set at 1500 rpm) with additional 25 µL of Bovine Serum Albumin. Preparations were centrifuged at 3000 g during 5 min at 4°C and supernatant containing the mitochondria were recovered and centrifuged at 10 000 g for 10 min. After the second centrifugation, we discarded the supernatant and resuspended the pellet in 200 µL of buffer for the heart and 600 µL of buffer for the brain tissues. In each solution, we determined the protein concentrations by the Advanced Protein Assay Reagent (57697, Sigma-Aldrich, St. Louis, MO, USA) in order to quantify the extraction yield.

Mitochondrial permeability transition pore

The formation of mPTP was assessed by monitoring mitochondrial calcium retention capacity. Mitochondrion were submitted to increasing concentrations of calcium (10 µM) until reaching a threshold at which mitochondria release calcium. This calcium release signs the formation of mPTP as previously described (14). Accordingly, mitochondria (1 mg/ml) were energized with 5 mM pyruvate/malate and incubated in the respiration buffer supplemented with 1mM Calcium Green-5N fluorescent probe (C3737, Invitrogen, Eugene, OR, USA). The concentration of calcium in the extramitochondrial medium was monitored by means of a JascoFP-6300 spectrofluorimeter (Jasco, Bouguenais, France) at excitation and emission wavelengths of 506 and 532 nm, respectively. The calcium signal was calibrated with addition of known calcium amount.

Mitochondrial oxygen consumption

Mitochondrial oxygen consumption was measured at 30°C with a Clark-type electrode (Hansatech Instruments Ltd, Norfolk,UK). Mitochondria (0.4 mg protein/ml) were incubated in a respiration buffer containing 100 mM KCl, 50 mM sucrose, 10 mM HEPES and 5 mM KH_2PO_4, pH 7.4 at 30°C. Respiration was initiated by addition of 2.5 mM pyruvate/2.5 mM malate (state 2 respiration rate). After 1 min, ATP synthesis was induced by addition of 1 mM ADP (state 3 respiration rate) and 1 mmol/L carboxyatractyloside was added to measure the substrate-dependent respiration rate (state 4). The respiratory control ratio (state 3/state 4) was then calculated. Finally, uncoupling state was measured by administrating 1 µM of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone in the incubation solution (state 3').

Statistical analysis

All results were expressed as mean ± SEM. Individual values are illustrated in Supplemental Figures 1, 2 and 3. Data normality was evaluated using a Shapiro-Wilk test. Normality was confirmed for all parameters except state 3 oxygen consumption in the cortex and state 4 oxygen consumption in both cortex and hippocampus. Data distribution were therefore normalized for the latter parameters prior to statistical analysis. Then, respiratory

control and mitochondrial retention capacity were analysed using a two-way ANOVA followed by Holm-Sidak test, with investigation of the group effect (VF vs non-shockable cardiac arrest) and the time effect (baseline, *i.e.*, baseline conditions, end of no-flow, 30 min, 120 min and 24 h after CPR initiation). Neurological function was compared using a Kruskall-Wallis tests, respectively. Statistical significance was set at $p<0.05$.

Results

Fifty animals successfully underwent the entire protocol before mitochondrial isolation, *i.e.*, 6 rabbits for each type of cardiac arrest with sampling at baseline, end of the no-flow period or 30 or 120 min after CPR, and 4 rabbits for each type of cardiac arrest with sampling at 24 h after neurological evaluation. In the non-shockable cardiac arrest group, MAP was dropping below the value of 20 mmHg in 5.5±0.2 min after disconnecting the ventilator. ROSC was achieved in 2.1±0.3 and 1.9±0.7 min in VF and non-shockable groups, respectively. The total dose of adrenaline administered during CPR to obtain ROSC was 23.3±4.6 and 25.1±8.1µg/kg in the non-shockable cardiac arrest and VF groups, respectively. In the VF group, the total number of electrical defibrillation shocks was 3.2±1.3 per animal. During the follow-up of the animals, hemodynamic parameters and blood gases values were not different among animals submitted to VF or non-shockable cardiac arrest (Table 1).

In the animals followed during 24 h, the amount of epinephrine administered after ROSC to prevent hypotension was higher after VF vs non-shockable cardiac arrest, with no significant difference (Figure 1, upper panel). At the timing of neurological dysfunction evaluation, corresponding dysfunction scores were significantly higher after non-shockable cardiac arrest vs VF (87 % [81 – 100] vs 66 % [52 – 87], respectively; Figure 1, lower panel).

Mitochondrial extraction yield

As illustrated by Figure 2, mitochondrial extraction yields from brain cortex and hippocampus were reduced at 24 h after cardiac arrest as compared to baseline, but not at the end of the no-flow period and 30 or 120 min after CPR. At each time point, extraction ratio remained similar among both causes of cardiac arrest. In the heart, a significant decrease in the mitochondria extraction ratio was conversely observed in all conditions after cardiac arrest as compared to baseline, suggesting an increased rate of damaged mitochondria after cardiac arrest. However, extraction ratio again remained similar among both causes of cardiac arrest.

Mitochondrial calcium retention capacity

Mitochondrial calcium retention capacity was evaluated by the calcium concentrations inducing mPTP formation. As illustrated by Figure 3 (left panel), those concentrations were significantly decreased in the hippocampus and cortex at the end of the no-flow period *vs* baseline after both VF and non-shockable cardiac arrest. After 120 min following CPR, those concentrations were lower in animals submitted to VF as compared to non-shockable cardiac arrest. At the later time-point of the follow-up (24 h), a maximal dysfunction was observed after both causes of cardiac arrest as compared to baseline, with no difference among VF and non-shockable cardiac arrest.

In the heart, the calcium retention capacity was significantly altered since the end of the no-flow period in animals submitted to non-shockable cardiac arrest as compared to baseline, but not in animals submitted to VF. In the latter group, such alteration was only observed at the later time-point of evaluation (24 h after cardiac arrest).

Mitochondrial respiration

As illustrated by Figure 3 (right panel), a strong dysfunction in the mitochodrial respiration assessed by the respiratory control ratio was evidenced in the cortex at the end of the no-flow period as compared to baseline. After a transient recovery at 30 and 120 min after CPR, a strong dysfunction was again observed 24 h after cardiac arrest. In the heart, respiratory control ratio was significantly decreased since the end of the no-flow period in animals submitted to VF but not in those submitted to non-shockable cardiac arrest. However, a maximal and similar decrease in respiratory control ratio was observed 24 h after both causes of cardiac arrest as compared to baseline.

As illustrated by Figure 4, oxygen consumption at state 3 (ATP synthesis in the presence of ADP), state 4 (substrate-dependant oxygen consumption independently from ATP synthesis) and state 3’ (oxygen consumption during mitochondrial uncoupling) progressively increased from the end of the no-flow period to 120 min after CPR in the cortex and hippocampus of the animals submitted to VF. It was not the case in the animals submitted to

non-shockable cardiac arrest, with lower oxygen consumption in the brain as compared to VF after 120 following CPR. This demonstrates an over-consumption of oxygen after VF but not non-shockable cardiac arrest at this early time-point. However, after 24 h following cardiac arrest, a strong increase in state 4 oxygen consumption was observed *vs* baseline after both causes of cardiac arrest, along with state 3 and state 3' oxygen consumption decrease. The difference was not significant among the cause of the cardiac arrest, despite a trend toward lower state 3 and state 3' oxygen consumption after VF vs non-shockable cardiac arrest. Conversely, in the heart, states 3 and 3' oxygen consumption were reduced after VF as compared to baseline and non-shockable cardiac arrest, before a strong increase in states 3, 4 and 3' oxygen consumption after both causes of cardiac arrest after 24 h.

Discussion

In our study, mitochondrial dysfunction demonstrated different patterns between the brain and the heart. In both organs, a severe mitochondrial dysfunction was observed at 24 h after cardiac arrest and was similar between the two models of cardiac arrest. In comparison, mitochondrial dysfunction was rather mild during the first 2 hours following cardiac arrest, with different time courses between organs. To date, the implication of mitochondria in post-cardiac arrest physiopathology has often been extrapolated from results obtained in selective and longer period of ischaemia than cardiac arrest, including stroke or myocardial infarction (15, 16). In those conditions, mitochondrial dysfunction is expected to be very severe and early after reperfusion. In the context of prolonged cardiac arrest (i.e. > 15 min of no-flow), it has also been shown that mitochondrial dysfunction is immediately very severe and stable over time (17, 18). To our knowledge, our study is the first to demonstrate an early phase with relatively mild mitochondrial dysfunction followed by a late phase (after 24 h) with dramatic impairment of mitochondrial dysfunction with different profiles between brain and myocardium.

Regarding the early phase of dysfunction in the heart, we observed an absence of mitochondrial dysfunction at the end of the no-flow period whereas early reperfusion was associated with impairment of the mitochondrial respiration and calcium retention capacity. This underlines the effect of reperfusion in the promotion of mitochondrial dysfunction, which is consistent with the literature on regional myocardial ischaemia showing that reactive oxygen species produced at reperfusion are responsible for mPTP opening (8, 19). This has also been demonstrated in a mouse model of cardiac arrest with a progressive alteration of myocardial electronic flow between 30 and 60 min of reperfusion but not during ischaemia (20). This is thought to be due to the specific sensitivity of complex I and IV of the mitochondrial respiratory chain, whereas other complex remain quite insensitive to ischaemia-reperfusion during post-cardiac arrest syndrome (20). However, a recent study investigated the mitochondrial function in post-cardiac arrest patients and demonstrated that succinate-driven respiration is actually more impaired that glutamate/malate-driven respiration in skeletal muscle after cardiac arrest

(21). Yet, in our model, we did not specifically assess the complex II activity. Different subpopulation of cardiac mitochondria could also be associated with differential response to myocardial ischemic-reperfusion injury. It is indeed well demonstrated that mitochondrial mechanical isolation is associated with over representation of subsarcolemmal as compared to interfibrillar mitochondrial population (22). The modest cardiac dysfunction that we observed here at the early time-points after cardiac arrest could then be related to this phenomenon, as interfibrillar mitochondria are thought to be responsible for providing most of the energy of the contractile apparatus.

Regarding the time course of dysfunction in the brain, mitochondrial function appeared to be strongly impaired during no-flow period with a transient reversion after CPR and then a delayed deterioration the day after cardiac arrest. In the literature, data obtained after 10 min of VF in rats demonstrates that 5 min after ROSC are sufficient to partially restore the mitochondrial function as compared to the one measured during ischaemia (23). Similarly, after 10 min of VF in dogs, a study demonstrated that the decrease in mitochondrial respiratory control and calcium retention capacity were reversed within 100 min of reperfusion (24). To our knowledge, there is only one study showing that mitochondrial dysfunction is severe and stable after ROSC (25). However, in this study, the authors used a very long duration of cardiac arrest (15 min) and no vasopressor were administered during follow-up (25). In another study in rats, it was demonstrated that cardiac arrest was associated with sustained mitochondrial dysfunction up to 1 h after cardiac arrest (26). In this study, the authors used a prolonged no-flow period of 8 min of CPR. Both study then used a more severe model of cardiac arrest that the one used in our study. Species difference could also explain some discrepancies since rabbits have a slower heart rate than rodents. The relatively modest mitochondrial dysfunction that we evidenced in the brain as compared to the heart at the early stage after cardiac arrest could also be related to a mechanism of energetic preservation in the brain. For example, the so-called “Astrocyte-Neuron Lactate Shuttle” could protect the brain by neurons fuelling with lactates by astrocytes in ischemic conditions (27). Additionally, the main source of energy in the brain is glucose whereas heart is mostly using fatty acid as a primary metabolic substrate.

This could explain the differences between organs in our study. However, literature on brain mitochondrial dysfunction after cardiac arrest is still sparse and sometimes contradictory, which could be explained by different experimental conditions.

Importantly, we also observed that the early cerebral mitochondrial dysfunction (from no-flow to 120 min after CPR) was more severe after VF than non-shockable cardiac arrest, despite the fact that the ultimate neurological function was worse after non-shockable cardiac arrest. Importantly, the latter observation is consistent with clinical data showing that non-shockable cardiac arrest is usually associated with more severe neurological dysfunction than shockable cardiac arrest (2). Additionally, we demonstrated that mitochondrial dysfunction during the early phase is not predictive of the mitochondrial dysfunction observed after 24h. Altogether, those results suggest that early brain mitochondrial dysfunction is not correlated to the severity of the ultimate neurological injury and that delayed mitochondrial dysfunction is more probably secondary to other pathophysiological events such as brain oedema or post-cardiac arrest myocardial dysfunction rather than direct ischemic injury during the initial no-flow. This also suggests that ideal mitochondrial protective strategies should not be administered immediately after ROSC but rather be adapted to the windows of susceptibility of mitochondria. However, the very large majority of clinical or experimental strategy to protect mitochondria target the firsts minutes of mitochondrial dysfunction. As an illustration, administration of inhibitor of the succinate dehydrogenase during CPR in rats, was not associated with any improvement of the survival rate after 3 days (28). Another example is the recent CYRUS clinical trial, which also failed to demonstrate any benefit following the selective inhibition of mPTP formation by cyclosporine when administered during CPR to prevent reperfusion-induced injury in cardiac arrest patients (10).

Conclusion

Differences between early and late mitochondrial dysfunction after VF or non-shockable cardiac arrest suggest that mitochondrial function might not play a unique role in the pathophysiology of the post-cardiac arrest syndrome but could rather be a secondary pathophysiological event.

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Legend to figures

Figure 1: Dose of epinephrrine administered after resuscitation and neurological dysfunction scores in rabbits followed during 24 h shockable (VF, n=4) or non-shockable cardiac arrest (CA, n=4).

**, p<0.05 between groups*

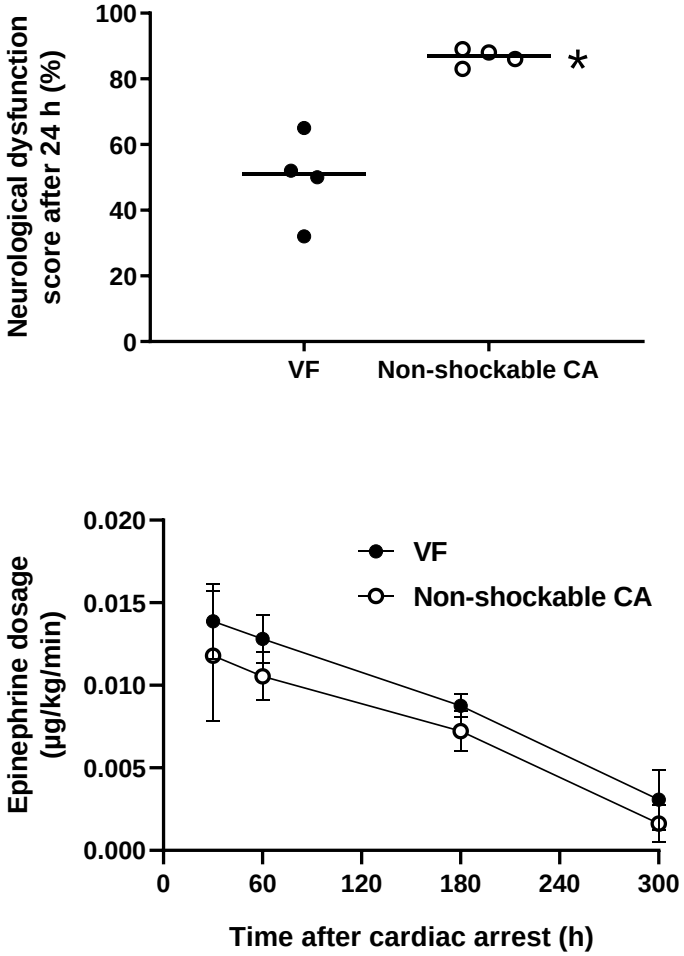

Figure 2: Mitochondrial extraction ratio assessed by mitochondrial protein dosage in hippocampus, brain cortex or heart samples of rabbits submitted to ventricular fibrillation (VF) or non-shockable cardiac arrest (CA) followed by cardiopulmonary resuscitation (CPR).

*Values are expressed as mean±SEM; *, p<0.05 vs corresponding baseline value*

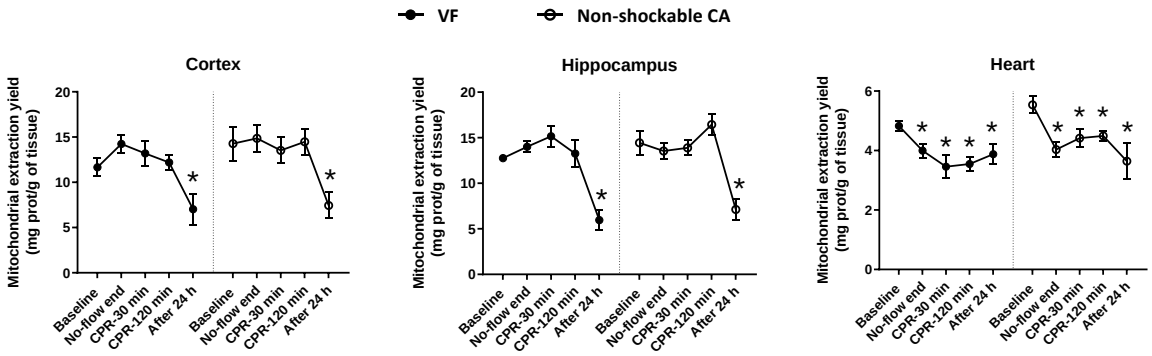

Figure 3: Calcium concentration leading to the formation of the mitochondrial permeability transition pore (mPTP, left panel) and respiratory control ratio (right panel) of mitochondria isolated in the hippocampus, brain cortex or heart of rabbits submitted to ventricular fibrillation (VF) or non-shockable cardiac arrest (CA) and subsequent resuscitation. Mitochondria were isolated from tissues withdrawn in either baseline conditions or at the endof the no-flow period or after 30 min, 120 min or 24 h following cardiopulmonary resuscitation (CPR). Control ratio has been calculated as the ratio between state 3 and state 4 oxygen consumptions, i.e., ADP- and substrate-dependent respiration rates, respectively.

*Values are expressed as mean±SEM; *, p<0.05 vs corresponding baseline value; †, p<0.05 vs corresponding VF value; mPTP, mitochondrial permeability transition pore.*

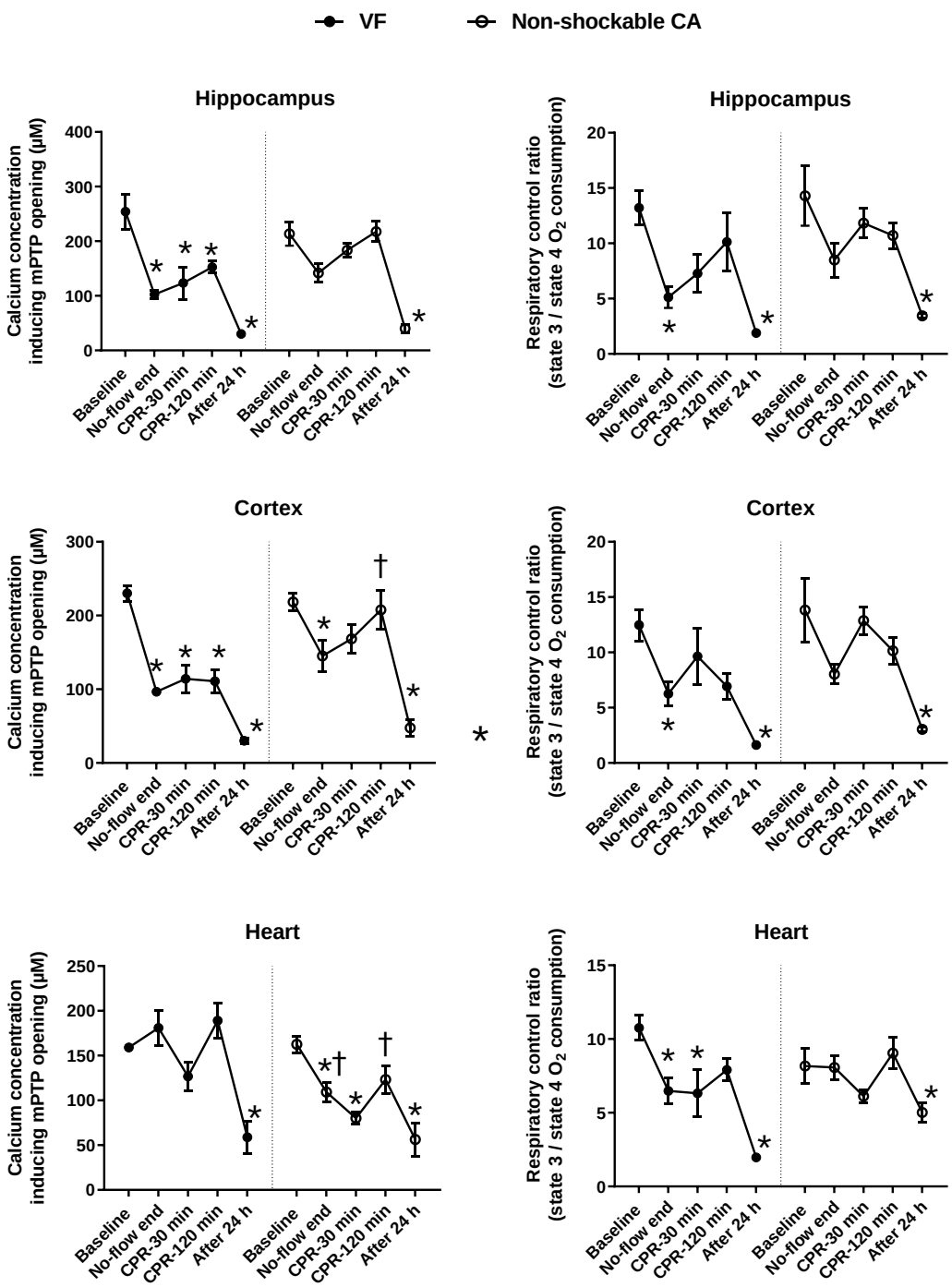
VF
Non-shockable CA
Hippocampus
Hippocampus
Cortex
Cortex
Heart
Heart
Calcium concentration inducing mPTP opening (µM)
Respiratory control ratio (state 3 / state 4 O2 consumption)
Baseline
No-flow end
CPR-30 min
CPR-120 min
After 24 h

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Figure 4: Respiratory activity in mitochondria isolated from the hippocampus, brain cortex or heart of rabbits submitted to ventricular fibrillation (VF) or non-shockable cardiac (CA) with subsequent resuscitation. Mitochondria were isolated from tissues withdrawn in either baseline conditions or at the end-of the no-flow period or after 30 min, 120 min or 24 h following cardiopulmonary resuscitation (CPR). States 3, 4 and 3' oxygen consumptions represent ADP-, substrate-dependent (pyruvate/malate) and uncoupled respiration rates, respectively.

*Values are expressed as mean±SEM; *, p<0.05 vs corresponding baseline value; †, p<0.05 vs corresponding VF value.*

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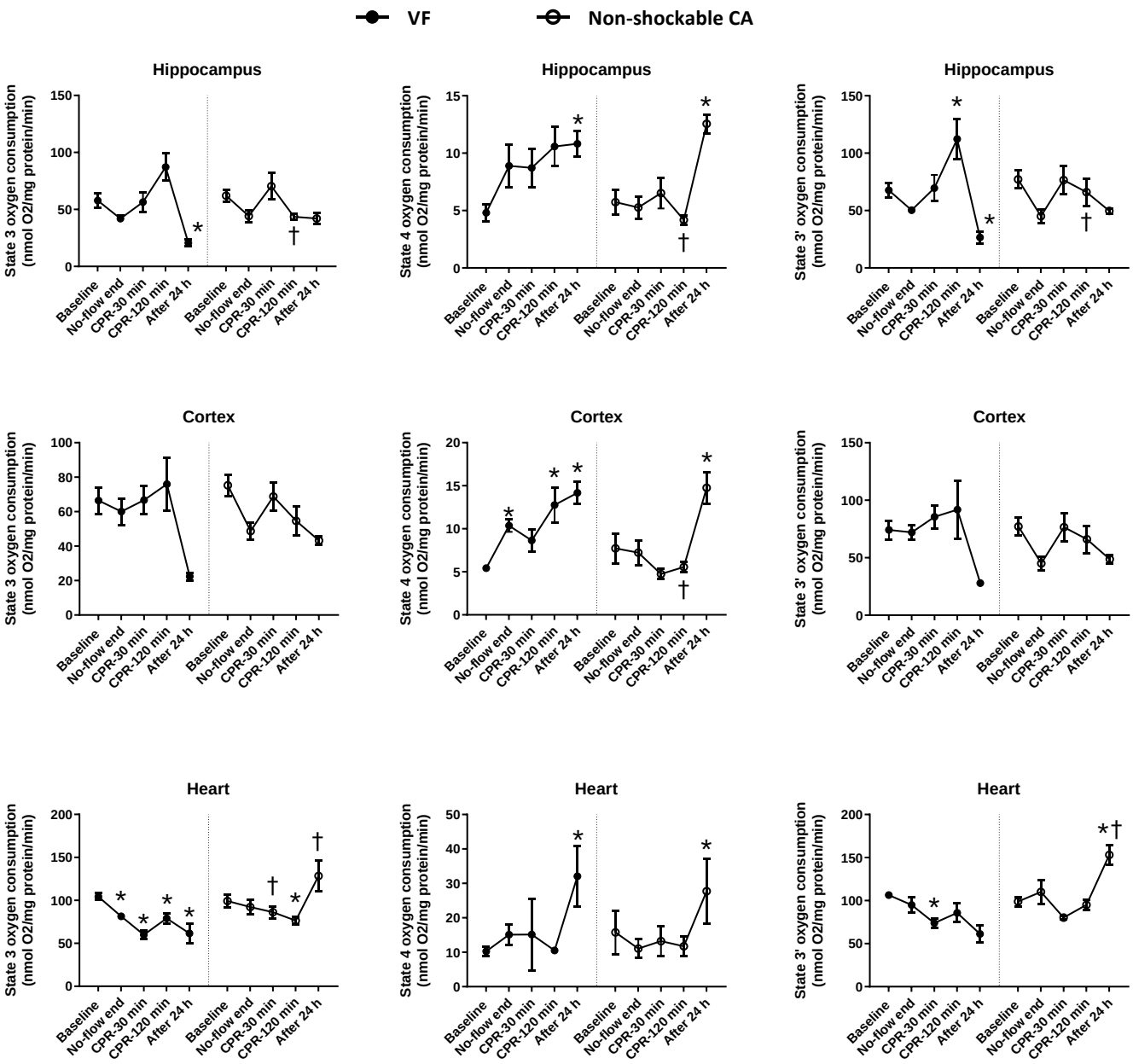
VF
Non-shockable CA
Hippocampus
Cortex
Heart
State 3 oxygen consumption (nmol O2/mg protein/min)
State 4 oxygen consumption (nmol O2/mg protein/min)
State 3' oxygen consumption (nmol O2/mg protein/min)
Baseline
No-flow end
CPR-30 min
CPR-120 min
After 24 h