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MITOCHONDRIA AT THE HEART OF CARDIOPROTECTION

Identification of a mechanism promoting mitochondrial sterol accumulation during myocardial ischemia–reperfusion: role of TSPO and STAR

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Abstract

Hypercholesterolemia is a major risk factor for coronary artery diseases and cardiac ischemic events. Cholesterol per se could also have negative effects on the myocardium, independently from hypercholesterolemia. Previously, we reported that myocardial ischemia–reperfusion induces a deleterious build-up of mitochondrial cholesterol and oxysterols, which is potentiated by hypercholesterolemia and prevented by translocator protein (TSPO) ligands. Here, we studied the mechanism by which sterols accumulate in cardiac mitochondria and promote mitochondrial dysfunction. We performed myocardial ischemia–reperfusion in rats to evaluate mitochondrial function, TSPO, and steroidogenic acute regulatory protein (STAR) levels and the related mitochondrial concentrations of sterols. Rats were treated with the cholesterol synthesis inhibitor pravastatin or the TSPO ligand 4'-chlorodiazepam. We used *Tspo* deleted rats, which were phenotypically characterized. Inhibition of cholesterol synthesis reduced mitochondrial sterol accumulation and protected mitochondria during myocardial ischemia–reperfusion. We found that cardiac mitochondrial sterol accumulation is the consequence of enhanced influx of cholesterol and not of the inhibition of its mitochondrial metabolism during ischemia–reperfusion. Mitochondrial cholesterol accumulation at reperfusion was related to an increase in mitochondrial STAR but not to changes in TSPO levels. 4'-Chlorodiazepam inhibited this mechanism and prevented mitochondrial sterol accumulation and mitochondrial ischemia–reperfusion injury, underlying the close cooperation between STAR and TSPO. Conversely, *Tspo* deletion, which did not alter cardiac phenotype, abolished the effects of 4'-chlorodiazepam. This study reveals a novel mitochondrial interaction between TSPO and STAR to promote cholesterol and deleterious sterol mitochondrial accumulation during myocardial ischemia–reperfusion. This interaction regulates mitochondrial homeostasis and plays a key role during mitochondrial injury.

Keywords Myocardial ischemia–reperfusion · Mitochondria · Translocator protein · Steroidogenic acute regulatory protein

Abbreviations

CYP	Cytochrome P450
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
SOD2	Superoxide dismutase 2
COX-IV	Cytochrome C oxidase

Juliette Bréhat and Shirin Leick have contributed equally to this work.

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TSPO	Translocator protein
STAR	Steroidogenic acute regulatory protein
mPTP	Mitochondrial permeability transition pore
VDAC	Voltage-dependent anion channel
ROS	Reactive oxygen species
AUC	Area under the curve
AOC	Area over curve

Introduction

Ischemic heart disease is a leading cause of morbidity and mortality worldwide with a constant increasing prevalence and development of novel cardioprotective strategies remains necessary [28, 55]. Although numerous studies have shown their ability to confer cardioprotection in animal models, the translation of these promising results into clinical setting has been disappointing. The global lack of consideration of co-morbidities in preclinical studies is considered as one of the reasons for this large-scale failure [9, 29, 31].

Hypercholesterolemia is considered as a principal risk factor for coronary artery diseases. High circulating levels of cholesterol contribute to the formation of atheroma plaques and thus to the induction of ischemic pathologies. Thereby, patients with high hypercholesterolemia have an elevated risk of ischemic events [60] and lipid-lowering therapies are associated with delayed cardiovascular events and prolonged survival [43, 54]. This elevated risk is attributed to the development of atherosclerosis as a result of hypercholesterolemia. However, during the last decades, several experimental and clinic observations have shown that hypercholesterolemia per se could also exert direct negative effects on the myocardium, inducing alterations of the structure and the function of the cardiomyocytes [30, 64]. It may interfere with cardioprotective mechanisms [1]. The molecular mechanisms by which hypercholesterolemia directly alters cardiac cells are ill known. Some data indicate that hypercholesterolemia induces oxidative stress [36, 46] and apoptosis [47], and favors opening of the mitochondrial permeability transition pore (mPTP) [36, 41] which is a key event contributing to myocardial reperfusion injury [25].

The mitochondrial translocator protein (18 kDa; TSPO) is a high affinity cholesterol-binding protein primarily located in the outer mitochondrial membrane and is expressed in a wide variety of species and ubiquitously distributed with a predominantly expression in steroid-synthesizing tissues [39]. TSPO is also abundant in kidney and heart. In steroidogenic tissues, TSPO seems to be part of a dynamic complex including outer mitochondrial membrane proteins such as the voltage-dependent anion channel (VDAC) and cytosolic proteins such as the steroidogenic acute regulatory protein (STAR) which is the principal mean pathway of cholesterol transport from lipid droplets to mitochondria [37, 50]. STAR is synthesized as a 37-kDa protein, targeted to mitochondria, through its mitochondrial signal, where it is processed by proteases in the outer mitochondrial membrane to a mature 30-kDa form [2, 3]. The role of TSPO in mPTP opening was largely discussed, but its participation in mPTP formation was dismissed using a cardiac mouse *Tspo* gene deleted model [59]. However, a body of evidence suggests that TSPO plays a critical role in regulating physiological cardiac function and that TSPO ligands may represent interesting drugs to protect the heart during ischemia–reperfusion [39]. Indeed, several TSPO ligands, such as SSR180575, 4'-chlorodiazepam, or TRO40303, demonstrated cardioprotective effects by reducing infarct size in different animal models of myocardial infarction [32, 45, 58]. This effect was associated with a protection of mitochondrial function and a limitation of the increase in mitochondrial membrane permeability.

We previously demonstrated that the reperfusion of an ischemic myocardium causes a large increase in mitochondrial cholesterol content and a subsequent strong generation of auto-oxidized oxysterols resulting from the oxidation of cholesterol by reactive oxygen species (ROS). Oxysterols, whether formed endogenously or brought in certain foods, have major cytotoxic properties [65]. This mitochondrial accumulation of sterols at reperfusion was dramatically enhanced in hyper-cholesterolemic animals [41]. Interestingly, TSPO ligands inhibited cholesterol and concomitant auto-oxidized oxysterol accumulation, limited the increase in mitochondrial membrane permeability and protected mitochondrial function both in normo- and hyper-cholesterolemic animals [41, 45, 51]. Although some genetic experiments questioned the involvement of TSPO in the mitochondrial transport of cholesterol [5, 62], the inhibition of cholesterol and oxysterol accumulation could represent a promising approach to limit mitochondrial injury during myocardial ischemia–reperfusion and, thus, could participate to the cardioprotective effect of TSPO ligands.

Here, to verify this hypothesis, we examined whether the inhibition of cholesterol synthesis led to the inhibition of mitochondrial cholesterol and oxysterol accumulation and concomitantly protected mitochondrial functions during myocardial ischemia–reperfusion. Then, we investigated the mechanism by which cholesterol accumulates into mitochondria during myocardial reperfusion by determining whether this accumulation results from an increase in cholesterol influx and/or a decrease in cholesterol conversion. To do that: (1) we investigated the potential role of the proteins that convert cholesterol inside mitochondria, *i.e.*, the cytochromes P450 (CYP) 11A1 and 27A1; (2) we studied the role of the main proteins that have been described to participate in the transport of cholesterol into mitochondria of steroidogenic

organs, *i.e.,* TSPO and STAR. For this purpose, we used the TSPO ligand 4'-chlorodiazepam and TSPO global knock-out rats ($Tspo^{-/-}$).

Our data demonstrate that the inhibition of cholesterol synthesis protects mitochondria against ischemia–reperfusion injury and indicate that mitochondrial processing of STAR is required to transfer cholesterol into mitochondria during myocardial ischemia–reperfusion, a process that is modulated by TSPO.

Methods

Animals

Male Wistar and Sprague–Dawley rats (≈ 300 g) were used in this study. All animals were maintained under optimal environmental conditions (temperature 22–25 °C and a constant cycle of 12-h light/dark) in the animal house of the faculty of Medicine of the University Paris-Est (Approval No. E94028028).

Male Wistar rats were purchased from Janvier (Le Genest-St-Isle, France). A couple of heterozygous global *Tspo* knock-out Sprague–Dawley rats ($Tspo^{\pm}$) has been kindly provided by Pr Papadopoulos (University of Southern California, USA). These Sprague–Dawley rats carried out a 89 bp deletion in the *Tspo* gene resulting in the absence of protein expression [48]. These animals were crossed to generate of wild-type ($Tspo^{+/+}$) and homozygous *Tspo* deleted ($Tspo^{-/-}$) rats. $Tspo^{+/+}$ and $Tspo^{-/-}$ rats are either littermates or generated from littermates to avoid generating high percentage of pups not needed from heterozygous breeding for ethical considerations. In addition, the animals were used after height generations of back-crossing. All rats used in this study were PCR genotyped using specific primers (Fig. 1a) and no TSPO immunoreactive protein was detected in myocardium (Fig. 1b) and cardiac mitochondria (Fig. 1c) of $Tspo^{-/-}$ rats. Cardiac morphology was similar between both genotypes and $Tspo^{-/-}$ rats did not display alterations in left-ventricular function. This absence of cardiac phenotype variation persisted during aging (Online Fig. S1 and Online Fig. S2). *Tspo* deletion did not alter rat reproduction and morphology (Online Fig. S3a and S3b), although $Tspo^{-/-}$ rats tend to have a lower body weight (Online Fig. S3c). In the same way, we did not observe major differences concerning circulating steroidogenic hormones (Online Fig. S3d), basal glucose, and lipid blood levels between both genotypes during aging (Online Fig. S4a), and both genotypes respond identically when subjected to metabolic tests, *i.e.*, insulin and glucose tolerance tests (Online Fig. S4b and S4c).

In vivo coronary artery occlusion–reperfusion

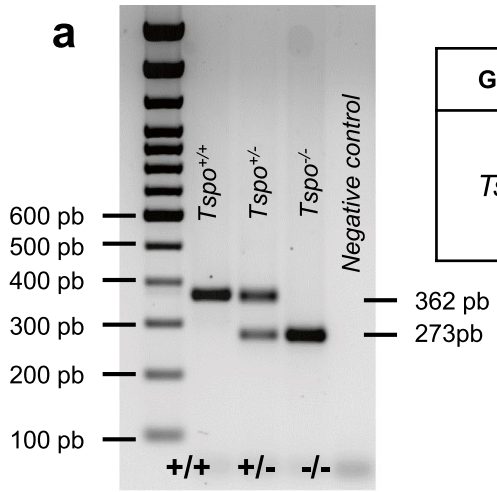
Rats were subjected to 30 min of ischemia followed by 15 min of reperfusion. Rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) given intraperitoneally. The depth of anesthesia was monitored using the tail pinching response and the pedal reflex. Once anesthetized, rats were placed on a warming pad to maintain body temperature at 37 °C (Homeothermic Blanket Control Unit, Harvard Apparatus, Les Ulis, France). Then, the animals were intubated using an endotracheal tube and artificially ventilated with a rodent ventilator model VentElite (respiratory rate, 63/min; respiratory volume, 2.20 mL; Harvard Apparatus, Les Ulis, France) with a mixture of carbogen (95% O_2, 5% CO_2).

A left thoracotomy in the fourth intercostal space was performed, followed by pericardiectomy, using a surgical microscope. A surgical needle with a 5–0 Prolene thread was passed around the left coronary artery, and the ends of the suture were passed through a polypropylene tube to form a snare. Tightening the snare induced coronary artery occlusion and its release initiated reperfusion for 15 min. This reperfusion duration was chosen, because mPTP opening was shown to occur within the first minutes of reperfusion [24]. In parallel, sham animals were subjected to same surgical procedure, but the coronary artery was not occluded.

4'-Chlorodiazepam (10 mg/kg) or its vehicle (dimethylsulfoxide) were administered intravenously through the jugular vein. This dose of 4'-chlorodiazepam was selected, because it was shown to be cardioprotective in a previous study [45]. In the ischemia-reperfused group, 4'-chlorodiazepam was injected 10 min before reperfusion during a 5 min infusion. For the sham group, the drugs were administered 25 min before heart excision.

Pravastatin (10 mg/kg) was given by gavage 3 days before surgery. We chose this dose and a short duration of treatment, because the aim was to limit cholesterol metabolism to decrease cellular cholesterol available for mitochondria and not to induce hypocholesterolemia. The molecule was dissolved in the drinking water at a concentration of 10 mg/mL and administered orally at 10 mg/kg per day. Animals underwent surgical procedures 24 h after the last administration.

At the end of 15 min of reperfusion, the heart was excised and the left-ventricular area at risk of ischemia-reperfused rats or the left ventricle for sham rats were used to prepare mitochondria and cytosols. To delineate the area at risk, both the position of the suture used for the coronary artery occlusion and the change in coloration of the myocardium at the area at risk level during the occlusion were used. This allowed avoiding any contamination of the area at risk by a dye that could impair mitochondrial functions. The weight of the area at risk varied from 190 to 220 mg according to the experiments. The same area of the myocardium was



Gene	Primer name (reference)	Sequence (5' > 3')	Molecular weight	Melting temperature
Tspo	R5CKOZFN-R (6430489)	ACT-CCT-AAA-GGG-GTT-GCA-GG	6182,1 g/mol	62°C
	R5CKOZFN-F (6430488)	AGA-GCA-TAC-TCT-TGC-CGT-CG	6093,0 g/mol	62°C

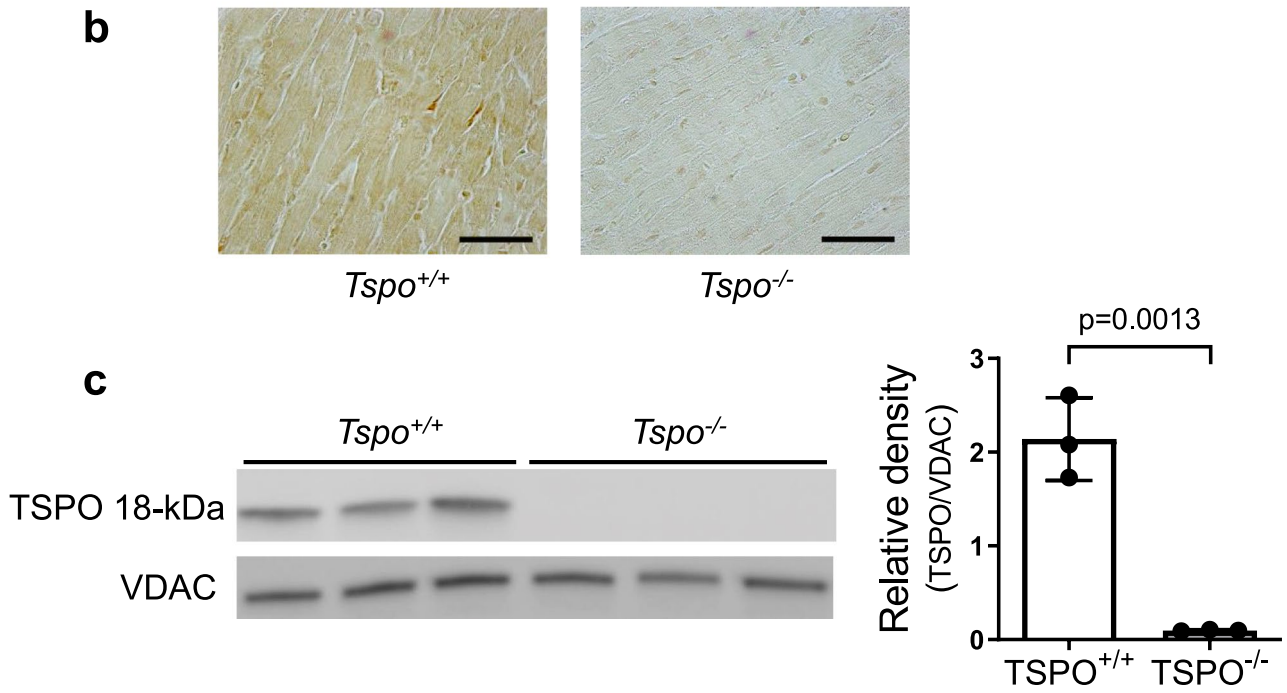


Fig. 1 *Tspo* deletion in Sprague–Dawley rats. **a**: Agarose gel image of the typical PCR products used for genotyping screening of rat with *Tspo* locus modification ($Tspo^{+/+}$, 362 bp; $Tspo^{+}$, two bands 362 and 273 bp; $Tspo^{-/-}$, 273 bp) and primers used for genotyping. **b**: Representative immunohistochemical images of TSPO in myocardium showing the lack of TSPO *in* $Tspo^{-/-}$ rats of 2 months old. Magnification: 400x, scale-bar: 50 µm. **c**: Immunoblot analysis of cardiac mitochondria extracted from $Tspo^{+/+}$ and $Tspo^{-/-}$ rats. Voltage-dependent anion channel (VDAC) was used as a loading control. Each value represents the mean ± SEM of 3 independent mitochondrial preparations. Statistical analysis was done using an unpaired two-tailed *t* test

excised from sham animals (myocardium not subjected to ischemia–reperfusion) to analyze mitochondrial functions.

To determine infarct size, the same ischemia–reperfusion procedure was applied, but the chest was closed in layers and the pneumothorax was evacuated and the animals were reperfused for 24 h. At the end of reperfusion, rats were anaesthetized. The chest was opened, the coronary artery was re-occluded at the same location than previously performed and Evan's blue solution was injected through the apex to delineate the area at risk. The heart was excised, the left ventricle was cut into 6 slices and the infarct area was identified by 2,3,5-triphenyltetrazolium chloride (TTC) staining. The area at risk was identified as the non-blue region and expressed as a percentage of the left ventricle weight. The infarcted area was identified as the TTC positive zone and expressed as a percentage of area at risk.

Isolation of cardiac mitochondria and cytosols

Rats were euthanized by intraperitoneal injection of pentobarbital (150 mg/kg). Rat left ventricles were removed and immediately immersed in ice cold 0.9% NaCl, scissor minced and homogenized using a Polytron homogenizer in a cold buffer (4 °C, pH 7.4) containing: mannitol (220 mM), sucrose (70 mM), HEPES (10 mM), and EGTA (2 mM). The samples were further homogenized for 10 consecutive times using a Potter homogenizer at 1500 rev/min. The homogenates were then centrifuged at 1000 g for 5 min at 4 °C to remove tissue debris and nuclei. The supernatants were centrifuged for 10 min at 10,000 g. The final pellets containing mitochondria were resuspended in the homogenization buffer with only 0.01 mM of EGTA and were used immediately (evaluation of mitochondrial function) or frozen at − 80 °C (western blot experiments and sterol determination). The supernatants were centrifuged at 100,000 g for 60 min at 4 °C. The pellets were discarded and the supernatants, corresponding to the cytosols, were frozen at − 80 °C after determination of the protein concentrations (Pierce™ BCA Protein Assay Kit 23225, Thermofischer Scientific, Illkirch, France). Testis and liver mitochondria were prepared using the same protocol without the Polytron homogenization step.

Evaluation of mitochondrial oxygen consumption and mitochondrial permeability transition pore opening

Oxygen consumption was measured with a Clark-type electrode (Hansatech Instruments Ltd, Norfolk, UK). Mitochondria (0.4 mg /mL) were incubated in a respiration buffer (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/malate. After 1 min, ATP synthesis was induced by addition of 1 mM ADP (ADP-stimulation respiration rate). ADP uptake was then inhibited by adding 1 µM carboxyatractyloside (substrate-dependent respiration rate). The respiratory control ratio (ADP-stimulation/substrate-dependent respiration rate) was then calculated.

mPTP opening was assessed by monitoring mitochondrial calcium retention capacity. Rat cardiac mitochondria were loaded with increasing concentrations of calcium until the load reached a threshold at which mitochondria underwent a fast process of calcium release, which was due to mPTP opening. Mitochondria (1 mg/mL), energized with 5 mM pyruvate/malate, were incubated in the respiration buffer supplemented with 1 mM Calcium Green-5N fluorescent probe (C3737, Invitrogen, Eugene, OR, USA). The reaction was started by addition of successive 10 µM Ca^{2+} pulses. The concentration of calcium in the extramitochondrial medium was monitored by means of a Jasco FP-6300 spectrofluorimeter (Jasco, Bouguenais, France) at excitation and emission wavelengths of 506 and 532 nm, respectively. The calcium signal was calibrated by addition to the medium of known calcium amounts.

Assessment of mitochondrial respiratory complex activities

The detailed methods of measurement of mitochondrial respiratory chain enzymatic activities are described in the supplementary file.

Measurement of superoxide anion production in myocardial mitochondria

Superoxide anion generation was assessed by measuring the rate of hydrogen peroxide production. This was determined fluorometrically by the oxidation of Amplex Red to fluorescent resorufin, coupled to the enzymatic reduction of hydrogen peroxide by horseradish peroxidase. Essentially, the superoxide generated in mitochondria is converted endogenously to hydrogen peroxide and then measured by the assay. Briefly, Amplex Red (10 µM) and horseradish peroxidase (1 U/mL) were added to isolated mitochondria (0.2 mg/mL protein) in respiration buffer maintained at 30 °C. The reaction was initiated by addition of the respiratory substrates (pyruvate/malate or succinate, 5 mM) in the presence or absence of 1 µM rotenone (inhibitor of complex I) and 1 µM antimycin A (inhibitor complex III). The subsequent increase in fluorescence was monitored over time using a fluorescence spectrometer (Perkin-Elmer SA LS 50B, excitation wavelength = 563 nm; emission wavelength = 587 nm).

Measurement of 4-hydroxynonenal

4-Hydroxynonenal was measured using the Elabioscience competitive ELISA kit (Euromedex, Souffelweyersheim, France) according to the manufacturer's instruction. Briefly, the samples (0.2 mg protein) were added into a microplate pre-coated with 4-hydroxynonenal. After incubation with 4-hydroxynonenal antibody and with labeled horse radish peroxidase, substrate and stop solutions were added. The absorbance was measured at a wavelength of 450 nm using Multiskan Sky (Thermo Scientific) plate reader, and the concentration of 4-hydroxynonenal was determined by comparing the optical density of the sample to the standard curve.

Isolation of primary adult rat cardiomyocytes

Left ventricular cardiomyocytes were isolated from *Tspo*$^{+/+}$ and *Tspo*$^{-/-}$ rats by an enzymatic technique. The rat was euthanized by intraperitoneal injection of pentobarbital (150 mg/kg) and the heart was excised. The heart was retrogradely perfused for 15 min at 37°C with a stock perfusion

buffer bubbled with 95%O_2/5%CO_2 containing 133 mM NaCl, 4.7 mM KCl, 0.6 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 12 mM $NaHCO_3$, 10 mM $KHCO_3$, 10 mM HEPES, 30 mM taurine, 0.032 mM phenol red, 5.5 mM glucose, 10 mM 2,3butanedionemonoxime pH 7.4 to wash out blood. After 2 min of perfusion, liberase (Blendzyme 10 mg/100 mL, Roche Applied Science, Mannheim, Germany), trypsin EDTA (14 mg/100 mL), and 12.5 µM Ca^{2+} were added to the buffer and the heart was perfused for approximately 13–15 min. The heart was placed into a beaker in the same buffer containing 10% bovine serum albumin pH 7.4 at 37 °C to stop the digestion. Left ventricles were then cut into small fragments and cells isolated by stirring the tissue and successive aspirations of the fragments through a 10 mL pipette. After 10 min, the supernatant was removed and the remaining tissue fragments were re-exposed to 10 mL of the same buffer. Then, the cells were suspended in the same buffer and Ca^{2+} was gradually added from 12.5 µM to 1 mM into an incubator at 37°C. Finally, the cardiomyocytes were suspended in culture medium M199 supplemented with 0.1% insulin–transferrin–selenium. The cells were seeded on 35 mm Petri dishes (150,000/dish) pre-coated with 10 µg/mL sterilized laminin and incubated for 90 min before being used.

Evaluation of mitochondrial reactive oxygen species (ROS) production in living cardiomyocytes

Assessment of mitochondrial superoxide production in cardiomyocytes was performed using MitoSOX™ Red fluorescent probe which targets mitochondria and is oxidized by superoxide anion. Cells were washed with a Tyrode's buffer (in mM: NaCl 130; KCl 5; HEPES 10; $MgCl_2$ 1; $CaCl_2$ 1.8, glucose 5.6, pH 7.4 at 37 °C), loaded with 1 µM MitoSOX™ (30 min at 37°C), and then washed two times with the Tyrode solution.

The cardiomyocytes were placed into a thermostated (37 °C) chamber (Warner Instruments, Hamden, CT), which was mounted on the stage of an IX-81 Olympus microscope, and were paced to beat by field stimulation (5 ms, 0.5 Hz).

Cardiomyocytes were imaged with the Olympus IX-81 motorized inverted microscope equipped with a mercury lamp as a source of light for epifluorescence illumination and with a cooled camera (Hamamatsu ORCA-ER). MitoSOX™ Red fluorescence were excited at 520–550 nm and recorded at 580 nm. Images were acquired every 10 min from 0 to 60 min and analyzed using a digital epifluorescence imaging software (XCellence, Olympus, Rungis, France). Fluorescence was integrated over a region of interest (≈80 µm2) for each cardiomyocyte and a fluorescence background corresponding to an area without cells was subtracted. The global response was analyzed by averaging the fluorescence changes obtained from all the cardiomyocytes contained in a single field (20–30 cells).

Blood sample analysis

Blood was collected from 2, 6, and 12 months *Tspo*$^{+/+}$ and *Tspo*$^{-/-}$ rats. Rats were anesthetized with isoflurane (0.2 L/min O_2, 2.5% isoflurane) and blood were collected from the jugular vein. Serum lipid profile (total cholesterol, glycemia, HDL, free fatty acid, and triglycerides) was performed with an automaton (Olympus AU 400) by Bichat Hospital's Biochemistry platform. Serum steroid hormones were measured with GC/MS by the mass spectrometry platform. Serum oxysterols measurements were carried with GC/MS.

Evaluation of CYP11A1 activity

The detailed methods of measurement of CYP11A1 activity in rat ventricular and testicular mitochondrial fractions using a cholesterol–resorufin probe and of synthesis of this probe are described in the supplementary file.

Metabolic tests

Metabolic tests were carried out according to Pitasi et al. [53]. Insulin tolerance tests were performed in 2, 6, and 12 months *Tspo*$^{+/+}$ and *Tspo*$^{-/-}$ rats. One week later, glucose tolerance tests were performed in the same rats. After 6 h of fasting, rats received an intraperitoneal injection of insulin (1 UI/kg, 3% BSA in saline solution) or D-glucose (1 g/kg in saline). Glycemia (fasting, 15, 30, 60, 90, and 120 min after the injection) was measured with a hand-held glucometer (Accu-Chek Performa, Roche, France) through blood collected from tail vein. The area under the curve (AUC) was calculated using Graphpad Prism. The AUC values for the glucose tolerance tests were normalized as follows: AUC = totalAUC—(fasting glycemia * 120). The area over curve (AOC) for the insulin tolerance tests was calculated as follows: AOC = (fasting glycemia * 120)—totalAUC.

Western blot analysis

After denaturing proteins at 90 °C for 5 min, a total of 40 µg of protein samples were loaded onto 8–16% Mini-PROTEAN® TGX™ Precast Gel (4,561,104, Bio-Rad) and separated by SDS-PAGE electrophoresis and then transferred to PVDF membranes. Next, membranes were blocked with a 5% skim milk solution for 1h30 at room temperature. After, they were probed with antibodies against CYP11A1 (1/250, sc18043, Santa Cruz Biotechnologies), CYP27A1 (1/250, sc14835, Santa Cruz Biotechnologies), TSPO (1/1000, ab154878, Abcam), STAR 37-kDa (1/1000, MBS3223061, Cliniscience), STAR

30-kDa (1/1000, ab58013, Abcam), VDAC (1/1000, 4866, Cell Signaling), superoxide dismutase 2 (SOD2; 1/1000, ab13533, Abcam), cytochrome C oxidase (COX-IV; 1/2000, 4844, Cell Signaling), β-actin (1/2000, 4970, Cell Signaling), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1/1000, 5174, Cell Signaling). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies, anti-mouse (1/5000, 7076, Cell Signaling), and anti-rabbit (1/5000, 7074, Cell Signaling). Finally, blots were revealed with ECL Western Blotting detection reagents (Amersham, GERPN2209, Sigma-Aldrich). The signal intensities for specific bands on the Western Blot were quantified using Image J software (1.30v—National Institute of Health).

Immunohistochemistry

Formalin-fixed rat left ventricles were embedded in paraffin and cross-sections (5 µm thickness) were performed using a rotary microtome. Briefly, after rehydration, antigen retrieval was performed with heated citrate buffer. After endogenous peroxidase antigen blocking (H_2O_2 3% 15 min) followed by antigen blocking (1h, 30% goat serum), sections were incubated with primary antibodies (TSPO, goat ab154878 1:180—overnight at 4 °C). The following day sections were washed and incubated with a horseradish peroxidase-conjugated secondary antibody (Abcam; ab97051 1:200) for 30 min at room temperature. The sections were washed again and incubated with 3,3'-diaminobenzidine (DAB; Vector Laboratories) under visual observation until the signal appears. The reaction was stopped by immersing slides in water. Images were acquired using an inverted optical microscope (Axioplan 2, Zeiss) at × 400 magnification.

Analysis of mRNA expression

Total RNAs were extracted from left ventricles (sham or ischemia-reperfused) with a column extraction kit (Maxwell® 16 LEV simplyRNA Tissue, Promega) following the manufacturer's instructions. Reverse transcription was performed using the AffinityScript qPCR cDNA Synthesis kit (Agilent). Quantitative polymerase chain reaction (qPCR) was performed using TaqMan Gene Expression assays (Thermo Fischer Scientific) and TaqMan primers (FAM) for *Tspo* (Rn00560892-m1), *Star* (Rn00580695-m1), *Cyp11A1* (Rn00568733-m1), *Cyp27A1* (Rn00710297-m1), and *Rplp0* (Rn03302271-gH) from Thermo Fisher Scientific. Amplification reactions were carried out using a 7000 Real-Time PCR system (Applied Biosystems). Gene expression was assessed by the comparative CT (ΔΔCT) method, with *Rplp0* as the reference gene.

Determination of cholesterol and oxysterol levels

Sterol and oxysterol measurements were performed on cytosolic and mitochondrial extracts. After extraction, they were measured by gas chromatography–isotope dilution mass spectrometry. The details are described in the online supplementary file.

Evaluation of cardiac hypertrophy

To assess cardiac hypertrophy, formalin-fixed rat left ventricles were embedded in paraffin and cross-sections (5 µm thickness) were performed using a rotary microtome. Sections were rehydrated and incubated for 45 min at room temperature with 10 µg/mL Alexa488-conjugated wheat germ agglutinin (WGA; Invitrogen). Slides were then mounted using glycerol and containing 4′,6-diamidino-2-phenylindole (DAPI). Images were acquired using an inverted fluorescent microscope at × 200 magnification (AxioImager M2, Zeiss) and analyzed with the Image J software. Cardiac hypertrophy was also assessed by measuring the ratio between the weight of the left ventricle and the body weight of the animals.

Echocardiography

Rats were lightly anesthetized with ketamine (60 mg/kg) and xylazine (6 mg/kg), given intraperitoneally, before echocardiography protocol. A 10 MHz ultrasound probe was used with a digital ultrasound system (Vivid 7, GE Medical Systems, Little Chalfont, UK). A trained operator performed echocardiograms and analysis.

The left-ventricular (LV) end-diastolic diameter (LVEDD), as the thickness of the interventricular septum (IVSd), and posterior wall (LVPWd) in the end-diastole were determined on M-mode echocardiography in the parasternal short-axis view at the level of papillary muscles. Transmitral flow velocities (E and A velocities and their ratio) were measured by pulse wave Doppler in the apical 4-chamber view.

LV fractional shortening (LV FS) was calculated as (LVEDD-LVESD)/LVEDD × 100, LVESD is LV end-systolic diameter. Heart rate (HR) was measured between two diastolic cycles on the M-mode image obtained in the parasternal short-axis view at the level of papillary muscles. LV ejection fraction (EF) was calculated using Teicholz formula: EF (%) = (EDV-ESV)/EDV*100, where EDV (end-diastolic volume) = [7/(2.4 + LVEDID)]*$LVEDID^3$ and ESV (end-systolic volume) = [7/(2.4 + LVESID)]*$LVESID^3$. LVEDID and LVESID were LV internal diameter in the end-diastole and end-systole, respectively, measured using the cine loop of the parasternal short-axis view at the level of papillary muscles.

Statistical analysis

Rats were randomly assigned to the various treatment groups and experiments were performed in parallel with rats from each strain. No formal exclusion or inclusion criteria for animals were applied (beside the inclusion of male rats only). No statistical method was used to predetermine sample size. Sample sizes were chosen based on our previous experience and correspond to standards in the field. Sample sizes are indicated in the corresponding figure legend. No animals were excluded after randomization except procedure failure.

The experimenter was blind when he analyzed histological immunostaining and performed echocardiographic experiments and sterol, steroidogenic hormone, and lipid blood level dosages.

Statistical analysis was performed with GraphPad Prism 9.1.2 (Graph Pad Software, USA). Data were presented as the means ± standard error of mean (SEM). Statistical significance of differences between two groups was determined using an unpaired two-tailed *t* test. Significant differences between more than two groups were evaluated by one-way or two-way ANOVA analysis followed by the multi-comparison post-test recommended by GraphPad Prism if ANOVA produced a significant value of F ($p < 0.05$). Statistical parameters and significance are reported in the figures and the figure legends.

Results

The inhibition of cholesterol synthesis reduces mitochondrial cholesterol accumulation and protects mitochondria against myocardial ischemia–reperfusion injury.

To reduce cholesterol biosynthesis, we used pravastatin, an inhibitor of 3-hydroxy-3-methyglutaryl-CoA reductase, which blocks the mevalonate pathway. Male Wistar rats were treated or not with pravastatin (10 mg/kg/day per os, 3 days) and submitted or not to 30 min of coronary artery occlusion followed by 15 min of reperfusion. After sacrifice, the hearts were removed and cytosols and mitochondria were isolated from the ischemic area to measure cholesterol and oxysterol concentrations.

Figure 2a shows that the reperfusion of an ischemic myocardium produced an increase in mitochondrial

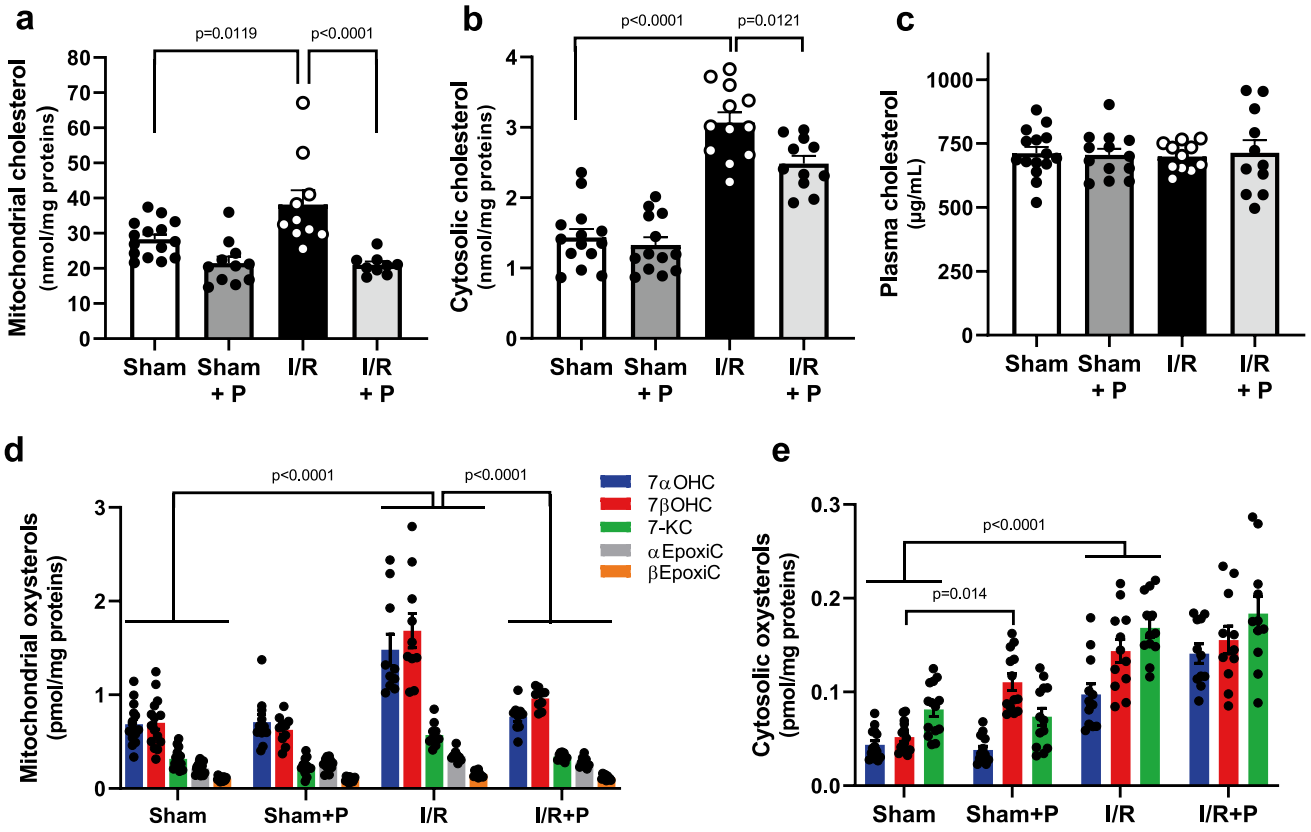


Fig. 2 Effects of pravastatin (P) treatment on the level of cholesterol and oxysterols in cardiac mitochondria (**a**, **d**), cytosols (**b**, **e**) and plasma (**c**) of rats subjected or not to cardiac ischemia–reperfusion (I/R). 7α-hydroxycholesterol (7αOHC); 7β-hydroxycholesterol (7βOHC); 7-ketocholesterol (7KC); cholesterol-5α,6α-epoxide (αEpoxiC); cholesterol-5β,6β-epoxide (βEpoxiC). Each value is the mean of 9–15 independent experiments. All the figures were analyzed by a two-way ANOVA followed by a Tukey's multi-comparison test

cholesterol concentration. A similar result was obtained in the corresponding cytosols (Fig. 2b) indicating that reperfusion promotes a global cellular accumulation of cholesterol that can result from a cellular stimulation of synthesis and/or an efflux of cholesterol from the blood compartment. This was associated with a significant increase in oxysterol contents in both mitochondria and cytosols (Fig. 2d and e).

In sham animals, pravastatin did not modify cytosolic (Fig. 2b) but slightly decreased mitochondrial cholesterol (Fig. 2a). We did not observe any effect of pravastatin on the concentrations of oxysterols measured in cardiac mitochondria isolated from sham rats (Fig. 2d). In cardiac cytosols, pravastatin treatment did not modify the concentrations of 7α-hydroxycholesterol and 7-ketocholesterol but, intriguingly, increased the concentration of 7β-hydroxycholesterol (Fig. 2e).

When rats were subjected to ischemia–reperfusion, pravastatin limited the enhancement of cholesterol in the cytosol and abolished its accumulation in the mitochondria (Fig. 2a and b).

This was associated with strong inhibition of oxysterol production in the mitochondria, whereas no decrease could be observed in the cytosols (Fig. 2d and e). It should be noted that pravastatin treatment did not lower serum cholesterol whatever the group of rats (Fig. 2c).

We next examined the concomitant effect of pravastatin treatment on mitochondrial function. Along with the reduction in mitochondrial cholesterol and oxysterols accumulation, pravastatin significantly improved oxidative phosphorylation as demonstrated by the increase in both ADP-stimulated respiration (state 3) and the respiratory control ratio (+48.4% versus ischemia–reperfusion). Pravastatin also decreased the sensitivity of mitochondria to mPTP opening as demonstrated by their increased capacity to retain calcium (+52.4% versus ischemia–reperfusion) (Table 1).

Cardiac mitochondrial cholesterol accumulation is not mediated by the inhibition of cholesterol catabolism during ischemia–reperfusion

Two CYP are responsible for the catabolism of cholesterol in mitochondria: CYP11A1 and CYP27A1. They are located to the matrix side of the inner mitochondrial membrane and convert cholesterol to pregnenolone and 27-hydroxycholesterol, respectively. In heart mitochondria, the presence and/or the role of these proteins remains a source of questions [7].

As illustrated in Fig. 3a, Western Blot experiments confirmed the presence of CYP11A1 in cardiac mitochondria, which is at the limit of detection when comparing the content observed in the testis on the same gel (Fig. 3b). Original western blots of all the figures are shown in the supplementary file. To go further, we next analyzed the activity of the enzyme in isolated mitochondria using the fluorescent probe cholesterol-resorufin. We synthesized a CYP11A1 fluorescent probe in which a resorufin molecule was conjugated to the side chain of cholesterol (see supplementary file). CYP11A1 cuts the cholesterol side chain and releases the fluorescent resorufin, which can be measured at 590 nm. In accordance with the expression of CYP11A1, the activity of the enzyme in the heart was very low compared to that observed in the testis (Fig. 3c). This is corroborated by the fact that we were unable to detect pregnenolone using isotope dilution mass spectrometry in isolated heart mitochondria (results not shown). Western blot experiments allowed also to reveal the presence of CYP27A1 in rat isolated cardiac mitochondria (Fig. 3a) which, as CYP11A1 compared to testis, was very low compared to liver (Fig. 3b). This had not been established before and this was supported by the presence of 27-hydroxycholesterol inside cardiac mitochondria (0.0442 ± 0.0073 µg/mg protein; n = 15 different preparations). Then, we examined the effect of ischemia–reperfusion on both cytochromes. Figure 3a shows that protein and mRNA expressions of both cytochromes were not affected after ischemia–reperfusion nor was the activity of CYP11A1

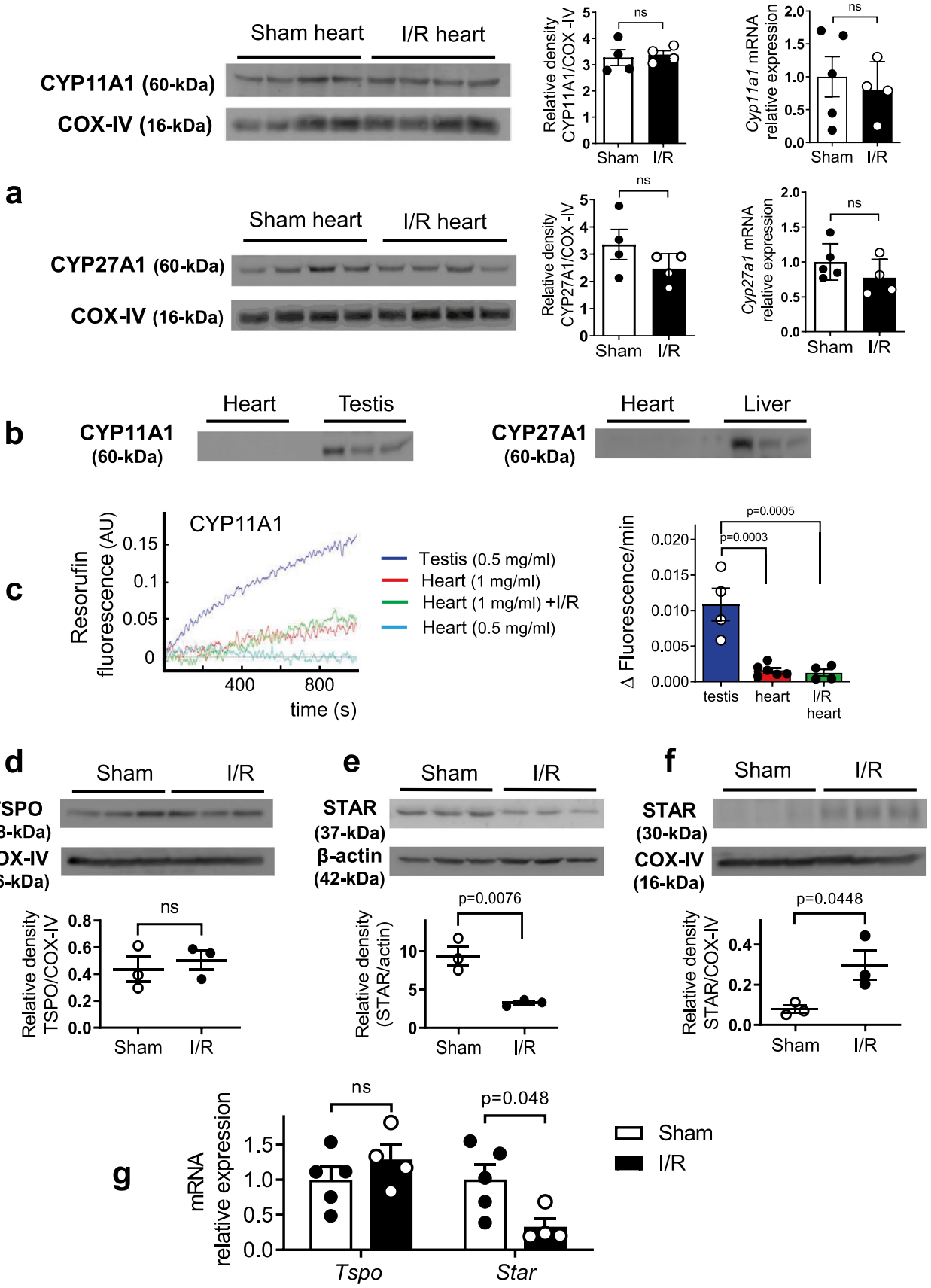
Table 1 Effects of pravastatin treatment on respiration and calcium retention capacity of cardiac mitochondria from Wistar rats subjected to 30 min ischemia and 15 min reperfusion

	Sham ($n=7$)	I/R ($n=10$)	I/R + P ($n=10$)
Respiration			
Substrate-dependent respiration	36.3 ± 2.24	43.6 ± 3.59	42.1 ± 3.4
ADP-stimulated respiration	234 ± 8	131 ± 11[§]	188 ± 11[‡]
Respiratory control ratio	6.66 ± 0.31	3.20 ± 0.39[§]	4.75 ± 0.5*
Calcium retention capacity	139 ± 11	53 ± 7[§]	81 ± 7[†]

Respiration is expressed as nmoL/min/mg protein and calcium retention capacity in nmoL/mg protein. *I/R* ischemia–reperfusion, *P* pravastatin

Each value is the mean ± SEM of 7–10 mitochondrial independent preparations. Data were analyzed by a one-way ANOVA followed by a Tukey's multi-comparison test. *$p=0.0348$, [†]$p=0.0419$ and [‡]$p=0.0017$ *vs* respective I/R

[§]$p<0.0001$ *vs* respective Sham


a
Sham heart
I/R heart
CYP11A1 (60-kDa)
COX-IV (16-kDa)
Relative density CYP11A1/COX -IV
ns
Sham
I/R
Cyp11a1 mRNA relative expression
CYP27A1 (60-kDa)
Relative density CYP27A1/COX -IV
Cyp27a1 mRNA relative expression
b
Heart
Testis
Liver
CYP11A1 (60-kDa)
CYP27A1 (60-kDa)
c
CYP11A1
Resorufin fluorescence (AU)
time (s)
Testis (0.5 mg/ml)
Heart (1 mg/ml)
Heart (1 mg/ml) +I/R
Heart (0.5 mg/ml)
Δ Fluorescence/min
p=0.0005
p=0.0003
testis
heart
I/R heart
d
TSPO (18-kDa)
COX-IV (16-kDa)
Relative density TSPO/COX-IV
e
STAR (37-kDa)
β-actin (42-kDa)
Relative density (STAR/actin)
p=0.0076
f
STAR (30-kDa)
COX-IV (16-kDa)
Relative density STAR/COX-IV
p=0.0448
g
mRNA relative expression
p=0.048
Tspo
Star

◂**Fig. 3** Effect of ischemia–reperfusion (I/R) on cardiac CYP11A, CYP27A1, TSPO, and STAR expression. **a** I/R does not alter protein level mitochondrial protein levels and mRNA left ventricle expression of CYP11A1 and CYP27A. COX-IV was used as a loading control. **b** Expression of CYP11A1 and CYP27A1 in cardiac mitochondria compared to their respective control organs (testis and liver), respectively ($n=3$, independent mitochondrial preparations). **c** Representative experiment measuring CYP11A1 enzymatic activity in testis, sham, and I/R cardiac mitochondria (left). Quantification of CYP11A1 enzymatic activity (right). Each value is the mean ± SEM of 4–6 independent mitochondrial preparations. Statistical analysis: one-way ANOVA followed by a Tukey's multi-comparison test. **d** I/R does not alter mitochondrial level of TSPO. **e** I/R decreases cytosolic level of STAR 37-kDa. **f** I/R increases mitochondria level of STAR 30-kDa. **g** I/R does not alter *Tspo* but decreases *Star* mRNA expression in the myocardium. Each value is the mean ± SEM of at least 3 independent preparations (**c**–**g**). COX-IV (**d** and **e**) and actin (**f**) were used as a loading control. Statistical analyses were done using an unpaired two-tailed *t* test (**c**–**f**) or a two-way ANOVA followed by a Sidak's multi-comparison test (**g**). *ns* non-significant

(Fig. 3c). In the same way, the mitochondrial concentration of 27-hydroxycholesterol significantly increased after ischemia–reperfusion [from 0.0442 ± 0.0073 ($n=15$) to 0.071 ± 0.004 ($n=10$) µg/mg protein] ruling out an inhibition of CYP27A1 activity. These results tend to indicate that the accumulation of mitochondrial cholesterol was not a consequence of a cholesterol metabolism deficiency during ischemia–reperfusion.

Cardiac cholesterol accumulation at reperfusion is related to an increase in mitochondrial STAR but not in TSPO levels

The accumulation of mitochondrial cholesterol at reperfusion can also be due to an enhancement of the intra-mitochondrial transport of cholesterol. Therefore, we investigated two proteins considered as critical for this transport, STAR and TSPO, even if the role of the latter in steroid organs was discussed [40, 49]. To assess the role of these proteins, we examined their mitochondrial levels in the absence or in the presence of ischemia–reperfusion in isolated cardiac cytosols and mitochondria. TSPO was expressed in cardiac mitochondria, but no significant change in protein and mRNA expression could be observed after ischemia–reperfusion (Fig. 3d and g). This is in agreement with our previous study, indicating that ischemia–reperfusion did not change the level of TSPO when it was probed with the specific TSPO ligand PK11195 [37]. As illustrated in Fig. 3e, STAR was expressed as a 37-kDa form in the cytosols isolated from rat hearts. After ischemia–reperfusion, the level of 37-kDa STAR decreased by 65% in the cytosol (Fig. 3e). This decrease was associated with a strong decrease in the expression of mRNA (Fig. 3g). Concomitantly, we observed the apparition of a 30-kDa form of STAR in mitochondria (Fig. 3f).

The effect of pravastatin on mitochondrial sterol accumulation is associated with the inhibition of mitochondrial STAR processing

As pravastatin limited the accumulation of cholesterol and oxysterols in the mitochondria at reperfusion (Fig. 2a and d), we wondered whether the limitation of mitochondrial STAR processing could be involved in this effect. Thus, according to the protocol previously described, we examined the effect of the administration of pravastatin on the induction of the mitochondrial STAR processing. Figure 4a confirms the mitochondrial accumulation of STAR 30 kDa at reperfusion. It was totally inhibited by pravastatin administration (Fig. 4a).

The TSPO ligand 4′-chlorodiazepam inhibits STAR mitochondrial processing

We previously demonstrated that TSPO ligands inhibited oxysterol formation by reducing the accumulation of cholesterol in the mitochondrial matrix at reperfusion [51]. We reasoned that this effect could be caused by the limitation of STAR mitochondrial membrane processing. Thus, we examined the effect of the administration of 4'-chlorodiazepam on mitochondrial STAR accumulation at reperfusion. When administrated before reperfusion, 4'-chlorodiazepam greatly limited STAR mitochondrial levels (Fig. 4b). As previous data have suggested that the effect of TSPO ligands could be independent from TSPO [26, 63], we wanted to ascertain the involvement of TSPO in the effect of 4'-chlorodiazepam. To do that, we used $Tspo^{-/-}$ rats.

Tspo deletion reduces mitochondrial sterols' accumulation without altering mitochondrial function in rats subjected to ischemia–reperfusion

Figure 5 shows the impact of *Tspo* deletion on mitochondrial sterol content. In sham animals, *Tspo* deletion did not alter the level of mitochondrial cholesterol (Fig. 5a) but strongly decreased the level of mitochondrial oxysterols (Fig. 5b–g). These oxysterols are known to be formed by auto-oxidation, except 25-hydroxycholesterol which has also be found to result from enzymatic conversion of cholesterol. This decrease in oxysterol content was not observed in cardiac cytosols (Table 2), indicating that it could result from a limitation of mitochondrial ROS production in $Tspo^{-/-}$ mitochondria. To ascertain this hypothesis, we compared the basal level of mitochondrial superoxide generation in cardiomyocytes isolated from $Tspo^{+/+}$ and $Tspo^{-/-}$ rats and we measured lipid peroxidation in cardiac mitochondria isolated from $Tspo^{+/+}$ and $Tspo^{-/-}$ rats. Figure 6a and b provides direct evidence of this hypothesis. *Tspo* deletion limits mitochondrial superoxide anion production over time in

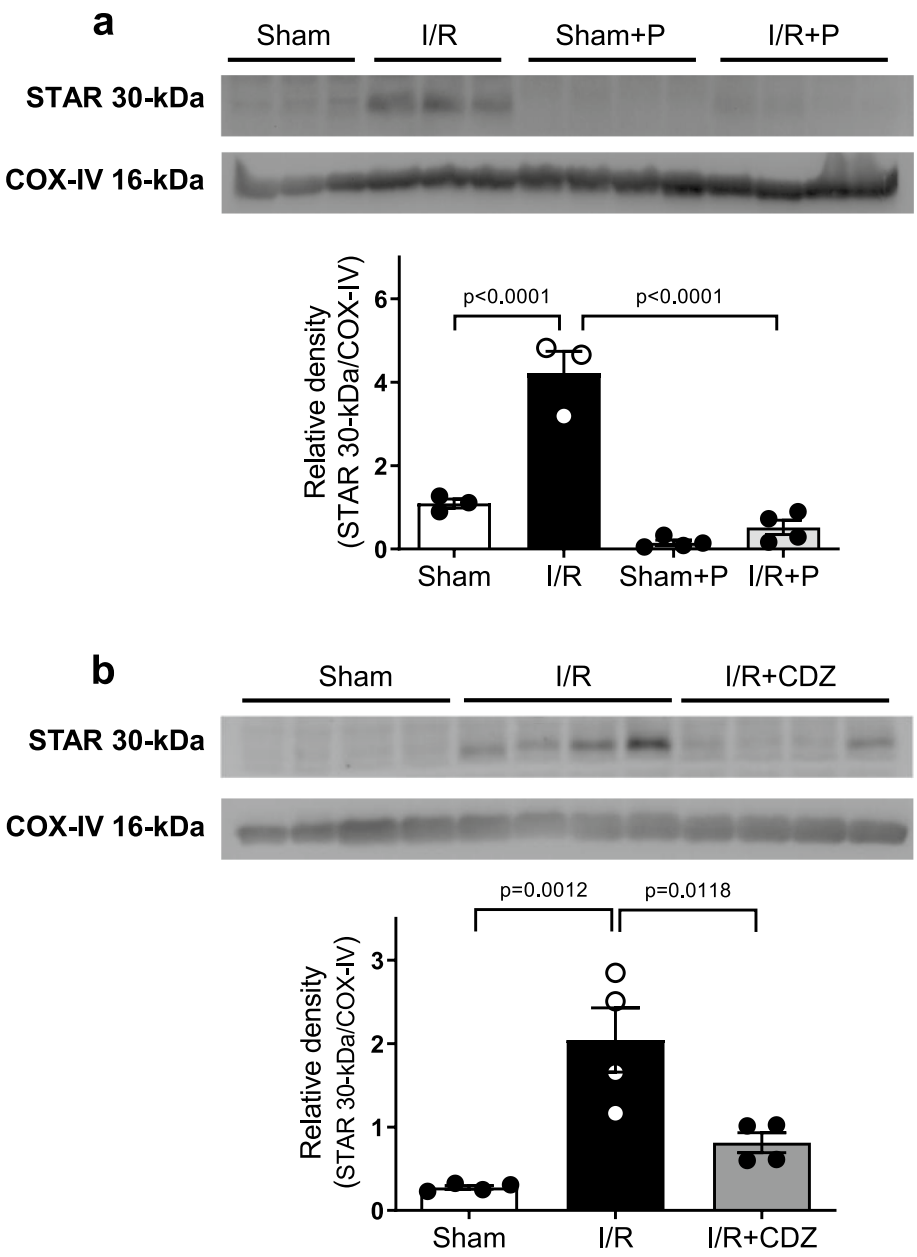


Fig. 4 Effect of pravastatin and 4'-chlorodiazepam treatment on mitochondrial STAR level after cardiac ischemia–reperfusion (I/R). **a** Effect of pravastatin (P). Statistical analysis was done by a two-way ANOVA analysis followed by a Tukey's multi-comparison test. **b** Effect of 4'-chlorodiazepam (CDZ). Statistical analysis was done by a one-way ANOVA analysis followed by a Tukey's multi-comparison test. COX-IV was used as a loading control. Each value is the mean ± SEM of at least 3 mitochondrial independent preparations

paced cardiomyocytes, as demonstrated by the lower MitoSOX fluorescence in *Tspo*$^{-/-}$ cardiomyocytes (Fig. 6a), and decreases the concentration of the lipid peroxidation product 4-hydroxynonenal in mitochondria isolated from *Tspo*$^{-/-}$ rats (Fig. 6b). This decrease was not observed in the corresponding cytosols (Fig. 6b). Mitochondrial superoxide anions can be produced by the electron transport chain through electron leak and complexes I and III are recognized as the major sites of production [67]. Therefore, we analyzed the rate of superoxide production by measuring the rate of hydrogen peroxide production in cardiac mitochondria isolated from *Tspo*$^{+/+}$ and *Tspo*$^{-/-}$ rats. We found that it was similar whatever the substrate used to induce respiration, excluding a participation of the mitochondrial transport chain in this effect (Fig. 6c). This is in accordance with the activity of mitochondrial transport chain complexes as no difference was found between *Tspo*$^{+/+}$ and *Tspo*$^{-/-}$ rats when the activity of each complex was measured separately (Online Fig. S5). In the same way, the expression of superoxide dismutase 2, the main mitochondrial antioxidant enzyme, was not altered in deleted animals, ruling out a role of this enzyme in the decrease in superoxide anions production (Fig. 6d). We also analyzed the expression of VDAC because the interaction between TSPO and VDAC was shown to modulate oxidative stress [20, 38]. Our results show that *Tspo* deletion did not alter the expression of VDAC (Fig. 6d).

We did not observe any consequences of these sterol alterations in sham animals on cardiac mitochondrial

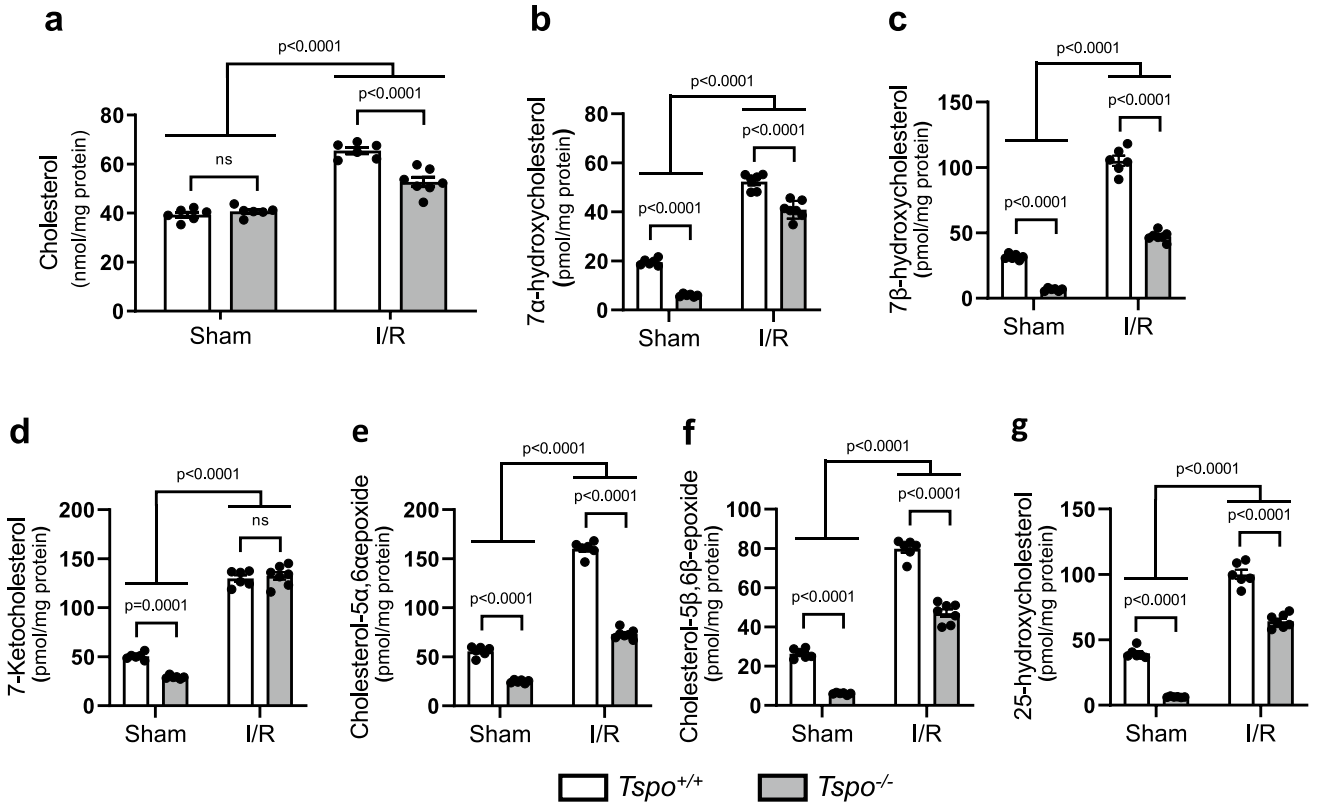


Fig. 5 Effects of Tspo deletion on cholesterol (**a**) and oxysterols (**b**–**g**) in cardiac mitochondria in rats subjected or not to cardiac ischemia–reperfusion (I/R). Each value is the mean ± SEM of 6–7 independent experiments. All the panels were analyzed by a two-way ANOVA followed by a Tukey's multi-comparison test. White bars: *Tspo*$^{+/+}$ rats. Gray bars: *Tspo*$^{-/-}$ rats. ns: non-significant

Table 2 Cardiac cytosolic concentrations of sterols in *Tspo*$^{+/+}$ and *Tspo*$^{-/-}$ rats

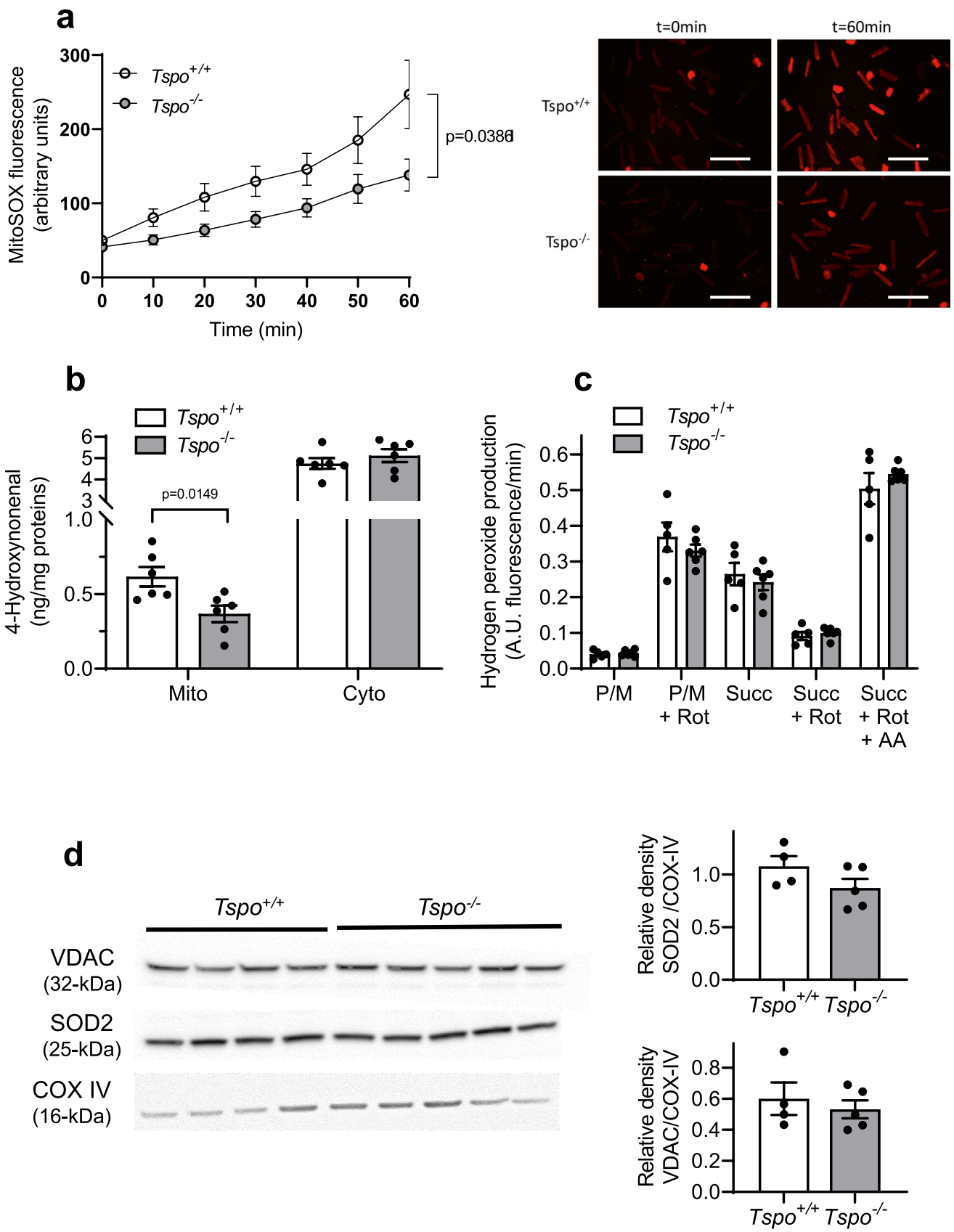
Sterols	*Tspo*$^{+/+}$	*Tspo*$^{-/-}$
Cholesterol (nmol/mg prot)	2.69 ± 0.08	2.60 ± 0.11
7α-hydroxycholesterol (pmol/mg prot)	1.28 ± 0.05	1.30 ± 0.04
7β-hydroxycholesterol (pmol/mg prot)	1.29 ± 0.04	1.31 ± 0.01
7-ketocholesterol (pmol/mg prot)	6.30 ± 0.19	6.31 ± 0.29
cholesterol-5α,6α-epoxide (pmol/mg prot)	1.96 ± 0.15	4.76 ± 0.60‡
cholesterol-5β,6β-epoxide (pmol/mg prot)	0.97 ± 0.04	0.77 ± 0.03†
25-hydroxycholesterol (pmol/mg prot)	3.58 ± 0.14	4.11 ± 0.18*

Each value is the mean ± SEM of 6 independent preparations. Data were analyzed by an unpaired two-tailed *t* test. *$p = 0.042$, $^{†}p = 0.0025$ and $^{‡}p = 0.001$ vs respective *Tspo*$^{+/+}$

function (Fig. 7). When mitochondria were extracted from *Tspo*$^{+/+}$ and *Tspo*$^{-/-}$ rats, the yield of extraction, determined by measuring the concentration of proteins in the mitochondrial pellet per g of cardiac tissue, was identical, reflecting a similar number of mitochondria in hearts (Fig. 7a). Similarly, *Tspo* deletion did not affect mitochondrial respiration or oxidative phosphorylation as illustrated by the similar V3 (Fig. 7b) and respiratory coefficient rate (Fig. 7c) values and did not alter the capacity of mitochondria to retain calcium, a marker of the mitochondrial membrane impermeability (Fig. 7d). *Tspo* deletion being without consequence on basal cardiac mitochondrial function, we assumed that the effect of *Tspo* deletion could appear following a cardiac stress.

Therefore, rats of both genotypes were subjected to cardiac ischemia–reperfusion and mitochondrial sterols and function were assessed. Ischemia–reperfusion induced an increase in mitochondrial cholesterol and oxysterols in *Tspo*$^{+/+}$ animals (Fig. 5) as previously described [41, 42, 51]. It should be noted that these results were obtained in Sprague–Dawley animals (the strain in which *Tspo*$^{-/-}$ rats were generated) and confirmed all our previous results using Wistar animals.

Tspo deletion did not prevent but blunted cholesterol and oxysterol accumulation following ischemia–reperfusion (Fig. 5). However, this decrease in sterols had no consequences on mitochondrial parameters, which remained similar between *Tspo*$^{+/+}$ and *Tspo*$^{-/-}$ rats (Fig. 7a–d). As a result, no difference in infarct size was observed in rats subjected to 30 min ischemia followed by 24-h reperfusion (Fig. 7e).


a
MitoSOX fluorescence (arbitrary units)
Tspo+/+
Tspo-/-
p=0.0386
Time (min)
t=0min
t=60min
Tspo+/+
Tspo-/-
b
4-Hydroxynonenal (ng/mg proteins)
p=0.0149
Mito
Cyto
c
Hydrogen peroxide production (A.U. fluorescence/min)
P/M
P/M + Rot
Succ
Succ + Rot
Succ + Rot + AA
d
VDAC (32-kDa)
SOD2 (25-kDa)
COX IV (16-kDa)
Relative density SOD2 /COX-IV
Relative density VDAC/COX-IV

◂**Fig. 6** Effects of *Tspo* deletion on mitochondrial oxidative stress. **a** *Left:* time-dependent mitochondrial superoxide production in cardiomyocytes isolated from $Tspo^{+/+}$ and $Tspo^{-/-}$ rats. Each point is the mean of 9–10 independent cardiomyocyte preparations, each preparation including 20–30 cells. Statistical comparison was done by two-way ANOVA analysis. *Right:* representative images obtained at t = 0 and 60 min. Scale bar: 200 µm. **b** Lipid peroxidation as indicated by 4-hydroxynonenal levels in cardiac mitochondria (mito) and cytosols (cyto) isolated from $Tspo^{+/+}$ and $Tspo^{-/-}$ rats. Each value is the mean ± SEM of 6 independent preparations. Statistical analysis was done using an unpaired two-tailed t test. **c** Superoxide anion generation was assessed in isolated myocardial mitochondria by measuring the rate of hydrogen peroxide production. Superoxide anion production was induced by pyruvate/malate (P/M; 5/5 mM) or succinate (Succ;5 mM) in the presence or absence of 1 µM rotenone (Rot) and 1 µM antimycin A (AA) and was determined fluorometrically by the oxidation of Amplex red to resorufin. Each value is the mean ± SEM of 5–6 independent mitochondrial preparations. **d** Western blot analysis and quantification of SOD2 and VDAC in $Tspo^{+/+}$ and $Tspo^{-/-}$ cardiac mitochondria. Each value is the mean ± SEM of 4–5 independent mitochondrial preparations. COX-IV was used as a loading control. *A.U.* arbitrary units

Deletion of Tspo abolishes the effect of 4′-chlorodiazepam on mitochondrial STAR processing and cholesterol accumulation without effect on STAR 37-kDa

$Tspo^{-/-}$ rats being generated from a different strain (Sprague–Dawley animals) than that used in all our previous experiments, we assessed the effect of 4'-chlorodiazepam on mitochondrial STAR in Sprague–Dawley $Tspo^{+/+}$ rats subjected to ischemia–reperfusion before analyzing its effect in $Tspo^{-/-}$ rats. Ischemia–reperfusion induced an increase in mitochondrial STAR 30-kDa level, which was inhibited by 4'-chlorodiazepam administration (Fig. 8a). Similarly, 4'-chlorodiazepam prevented mitochondrial cholesterol accumulation (Fig. 8b) confirming the results obtained with Wistar rats. These data legitimized the use of Sprague–Dawley $Tspo^{-/-}$ rats.

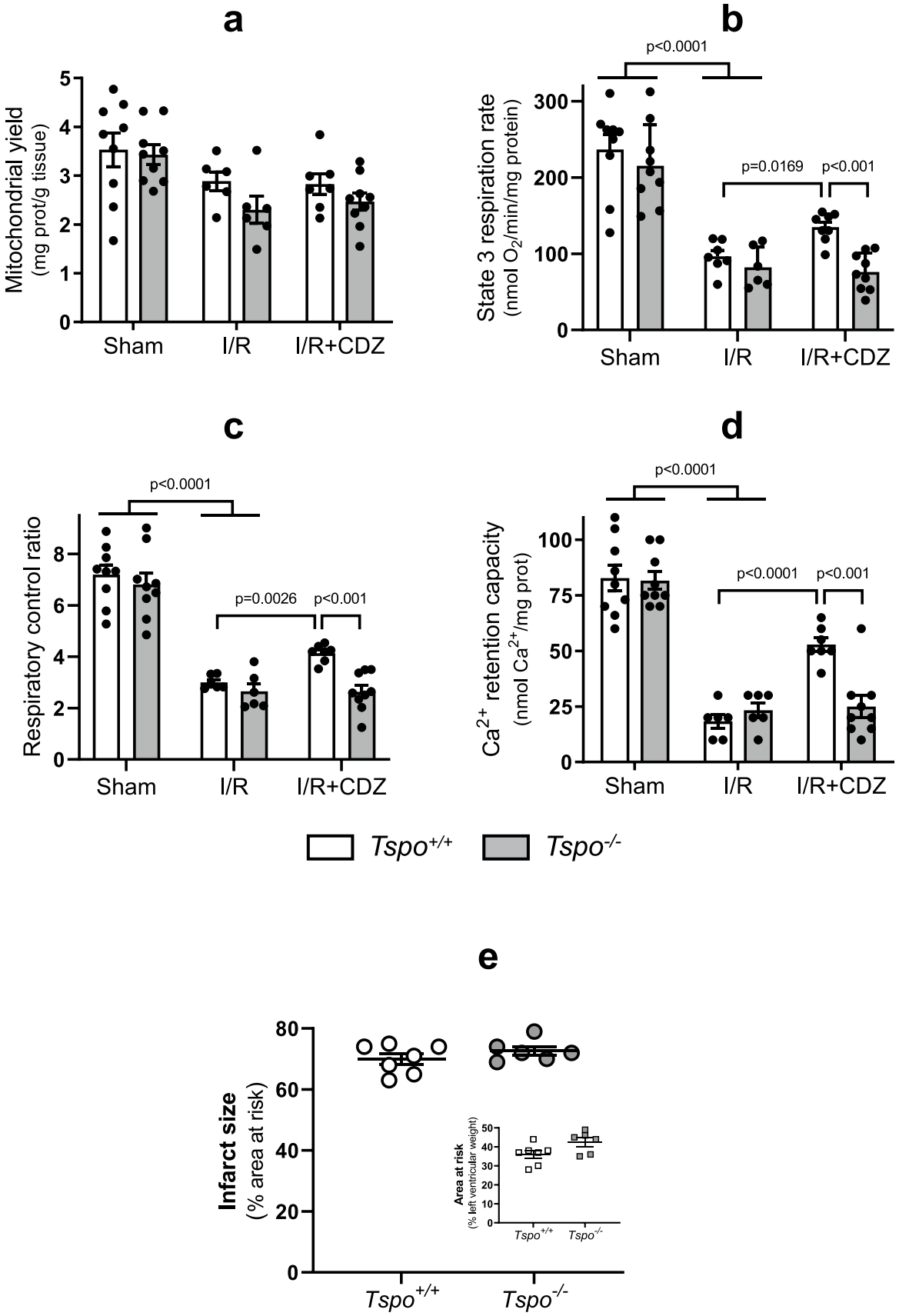
Figure 8c shows that the mitochondrial enhancement of STAR 30-kDa caused by ischemia–reperfusion persisted in $Tspo^{-/-}$ rats, demonstrating a TSPO-independent mitochondrial processing of STAR. Nevertheless, *Tspo* deletion abolished the effect of 4'-chlorodiazepam, the administration of the drug being unable to prevent cholesterol accumulation (Fig. 8d), to inhibit STAR 30-kDa enhancement in mitochondria (Fig. 8c), and to improve mitochondrial functional parameters (Fig. 7) after ischemia–reperfusion. This confirms the role of TSPO in the mitochondrial protecting effect of 4'-chlorodiazepam.

As it was shown that *Tspo* depletion induced an alteration of the level of STAR 37-kDa in Leydig tumor cells [27], we wondered whether global deletion of *Tspo* would alter the STAR 37-kDa cytosolic form. Cardiac ischemia–reperfusion in $Tspo^{+/+}$ Sprague–Dawley rats confirmed the decrease in the level of STAR 37-kDa in cardiac cytosols (Fig. 8e) and this decrease was not altered by 4'-chlorodiazepam (Fig. 8f). Similar results were observed in $Tspo^{-/-}$ rats, *i.e.*, the decrease in cytosolic STAR 37-kDa level observed after ischemia–reperfusion was preserved and the administration of 4'-chlorodiazepam was without effect (Fig. 8g and h).

Discussion

Early reperfusion of an ischemic myocardium induces an accumulation of mitochondrial cholesterol and oxysterols, which is potentiated by hypercholesterolemia [41, 51]. In this setting, administration of TSPO ligands prevented mitochondrial sterol accumulation, protected mitochondrial function, and reduced infarct size [45, 51]. This protective effect is preserved during hypercholesterolemia [41]. This makes controlling mitochondrial cholesterol concentration an interesting strategy to protect mitochondria during myocardial ischemia–reperfusion and particularly in hypercholesterolemic conditions that are known to amplify cellular injury and to impede cardioprotective mechanisms [1]. In the present study, we provide evidence that inhibiting cholesterol synthesis protects mitochondrial function during myocardial ischemia–reperfusion independently from any blood level lowering effect. We also provide novel findings that extend the understanding on the mechanisms responsible for mitochondrial cholesterol entry and accumulation during myocardial ischemia–reperfusion.

An important step of this study was to rule out the possibility of a defect in mitochondrial cholesterol catabolism that could explain mitochondrial cholesterol accumulation. We clearly established in the rat heart the presence of the cytochromes, which are responsible for cholesterol catabolism in mitochondria, *i.e.,* CYP11A1 and CYP27A1. To the best of our knowledge, only one study described the presence of CYP11A1 in the heart (human heart) [68] and that of CYP27A1 was unknown [7]. CYP11A1 constitutes an essential step for the formation of steroid hormones and its presence suggests a possible de novo cardiac steroidogenesis, although we cannot yet detect pregnenolone. CYP27A1 is considered as a major mechanism for cholesterol efflux from peripheral tissues, as emphasized by Babiker et al. [4], showing that patients with CYP27A1 gene mutations do not secrete 27-oxygenated products of cholesterol in peripheral macrophages. The role of these cytochromes in cardiovascular physiology and physiopathology is largely unexplored. We demonstrate that they are very poorly expressed in the rat heart compared to reference organs, but, most importantly, that their expression and activity are not modified after reperfusion. These results provide evidence that the increased influx of cholesterol and not an alteration of its mitochondrial metabolism is responsible for its mitochondrial accumulation during myocardial ischemia–reperfusion.


a
Mitochondrial yield
(mg prot/g tissue)
Sham
I/R
I/R+CDZ
b
p<0.0001
State 3 respiration rate
(nmol O2/min/mg protein)
p=0.0169
p<0.001
c
p<0.0001
Respiratory control ratio
p=0.0026
p<0.001
d
p<0.0001
Ca2+ retention capacity
(nmol Ca2+/mg prot)
p<0.0001
p<0.001
Tspo+/+
Tspo-/-
e
Infarct size
(% area at risk)
Area at risk
(% left ventricular weight)

◂**Fig. 7** Effect of *Tspo* deletion on mitochondrial function in rats subjected or not to cardiac ischemia–reperfusion (I/R) and on infarct size. Mitochondrial yield (**a**), oxygen consumption (**b**, **c**), and calcium retention capacity (**d**) in cardiac mitochondria isolated from $Tspo^{+/+}$ and $Tspo^{-/-}$ rats subjected to I/R and treated or not with 4'-chlorodiazepam (CDZ). Each value is the mean ± SEM of at least 6 independent experiments. All the figures were analyzed by a two-way ANOVA followed by a Tukey's multi-comparison test. **e** Infarct size (expressed as percentage of the area at risk) measured in $Tspo^{+/+}$ and $Tspo^{-/-}$ rats subjected to 30 min of coronary artery occlusion and 24 h of reperfusion. The areas at risk (insert) were similar in both groups

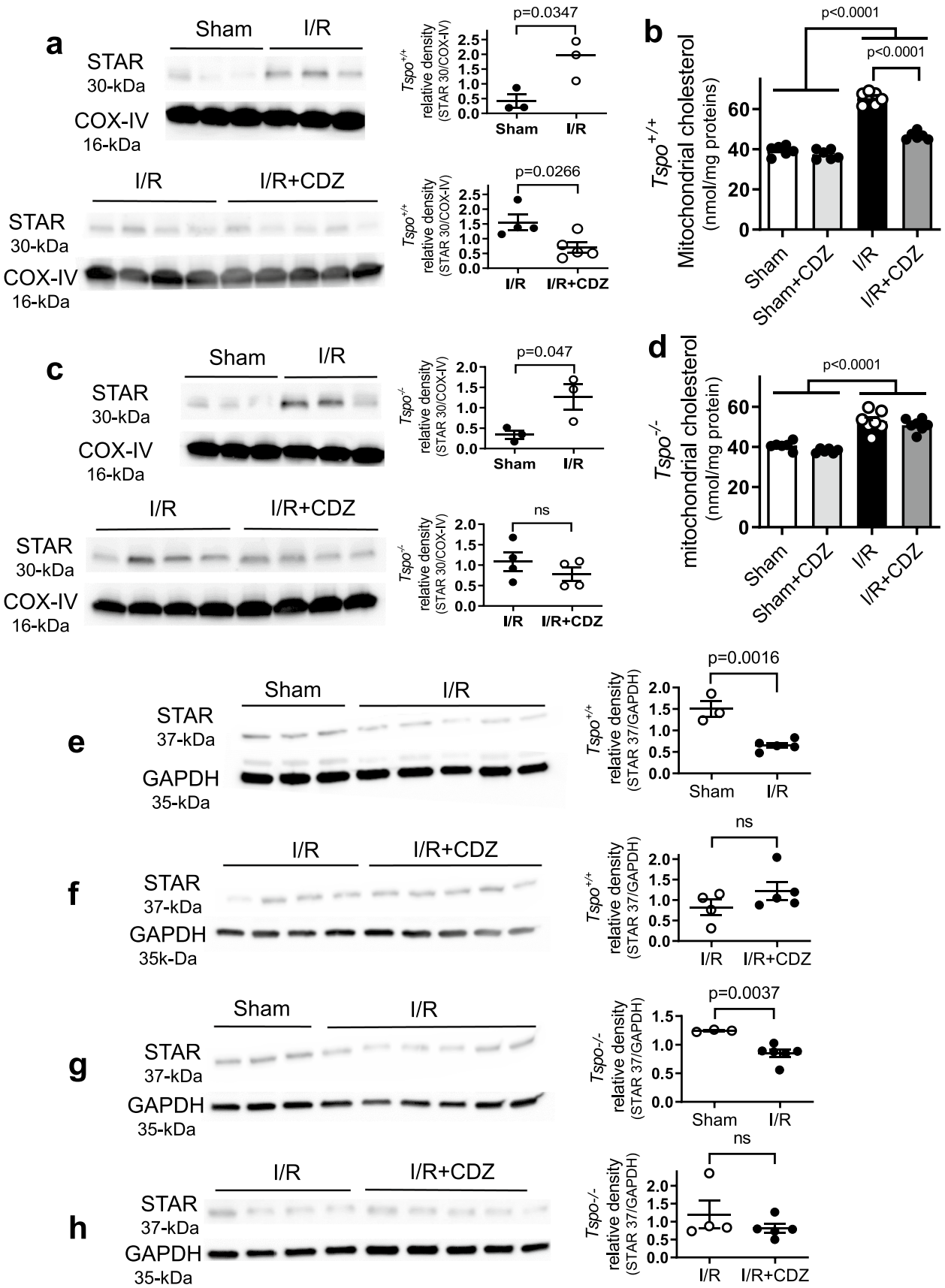
To investigate the mechanisms explaining mitochondrial accumulation of cholesterol, we reduced cholesterol synthesis using pravastatin that inhibits 3-hydroxy-3-methyglutaryl-CoA reductase and thus acts upstream of mitochondria. We chose this statin, because it is freely soluble in water and thus easy to use in animal models. As previously observed [18, 33], pravastatin treatment did not lower serum cholesterol in our experimental conditions. This is in accordance with studies indicating that pravastatin only exerts a hypocholesterolemic effect in dyslipidemic animal models [12, 15, 52]. Here, pravastatin did not alter the concentration of sterols in cardiac cytosols isolated from sham rats, which is in line with this hypothesis. The brevity of the pravastatin treatment (3 days) can also participate to this lack of effect. We observed, however, an increase in 7β-hydroxycholesterol in the cytosols of sham animals. This increase was unexpected but was associated with the inversion of the ratio 7-ketocholesterol/7β-hydroxycholesterol. A balance exists between the circulating and the tissue levels of 7-ketocholesterol and 7β-hydroxycholesterol in vivo, and this balance is regulated by the 11β-hydroxysteroid dehydrogenase type 1 [23]. The reduction of 7-ketocholesterol in 7β-hydroxycholesterol is stereospecific and this enzyme is present in the rat heart [66]. We can assume that pravastatin treatment favors the formation of 7β-hydroxycholesterol, but this needs to be confirmed.

During ischemia–reperfusion, pravastatin limited the cytosolic concentration of cholesterol but not of oxysterols. This might be the consequence of an insufficient decrease in cytosolic cholesterol, which did not reach the threshold needed to significantly reduce oxysterol content. In contrast, pravastatin strongly inhibited cholesterol and oxysterol accumulation in the mitochondria, and restored the capacity of mitochondria to synthetize ATP and to resist to mPTP opening. These are key factors involved in cell protection and they may strongly contribute to the cardioprotective effect of pravastatin. These novel results show that decreasing mitochondrial cholesterol by inhibiting cholesterol metabolism protects mitochondria from ischemia–reperfusion injury. However, it should be kept in mind that short pravastatin treatment can initiate other mechanisms concomitantly to its sterol-lowering action that can also contribute to its protective effect [6].

Importantly, we also identified a mechanism involved in the accumulation of cholesterol and oxysterols into mitochondria during myocardial ischemia–reperfusion. We demonstrated a mitochondrial targeting and processing of STAR 37-kDa leading to a mitochondrial STAR 30-kDa mature protein. The mitochondrial accumulation of STAR 30-kDa and sterols during reperfusion are blocked by both the inhibition of cholesterol synthesis by pravastatin and the TSPO ligand 4'-chlorodiazepam. In addition, 4'-chlorodiazepam was ineffective to inhibit ischemia–reperfusion induced STAR import and cholesterol accumulation and to improve mitochondrial functional parameters in $Tspo^{-/-}$ rats. All these findings clearly demonstrate that a functional interaction exists between STAR and TSPO in cardiac cells as observed in Leydig cells [27]. They also validate that 4'chlorodiazepam acts through its binding to TSPO.

It should also be stressed that TSPO is not mandatory for mitochondrial STAR import and cholesterol accumulation, as both processes were not abolished in $Tspo^{-/-}$ rats in basal conditions. This is consistent with data obtained on steroidogenic organs where TSPO appears non-essential for low-flux steroidogenesis [16]. This hypothesis is supported by the data showing no variation of cardiac phenotypes between $Tspo^{+/+}$ and $Tspo^{-/-}$ rats. It was suggested that adaptive mechanisms could take place to compensate for the lack of TSPO [16, 48]. This raises questions concerning the role of TSPO in the heart in vivo, since it must bind a ligand to exert an action. Moreover, in the heart, TSPO appears as a spectator unable to control STAR processing, but its absence limits cholesterol movements during reperfusion. This suggests that the interaction between STAR and TSPO is necessary for an efficient transport of mitochondrial cholesterol particularly when its strong activation is needed.

Our results also indicate that a role of TSPO in the heart may be a control of mitochondrial ROS production. Indeed, we demonstrated that TSPO deletion attenuates mitochondrial superoxide production in primary cardiomyocytes and lipoperoxidation in isolated mitochondria. This is associated with a reduction of cardiac mitochondrial oxysterols, which can be the consequence of a reduction of available ROS in mitochondria leading to a decrease in cholesterol oxidation. This is in accordance with studies suggesting a relationship between TSPO, ROS levels, and oxidative stress [8, 39]. This is in line with recent data, which report significantly low ROS levels in the epithelial human breast cancer cell line MDA when TSPO is down-regulated [14] and an accumulation of ROS to limit the efficiency of mitochondrial autophagy when TSPO expression is enhanced [21]. So far, we did not determine the cause of this decrease in mitochondrial superoxide anion generation, but we have excluded an alteration of the respiratory chain and of the


a
Sham
I/R
STAR
30-kDa
COX-IV
16-kDa
p=0.0347
Tspo+/+
relative density
(STAR 30/COX-IV)
Sham
I/R
I/R
I/R+CDZ
p=0.0266
b
p<0.0001
p<0.0001
Tspo+/+
Mitochondrial cholesterol
(nmol/mg proteins)
Sham
Sham+CDZ
I/R
I/R+CDZ
c
p=0.047
Tspo-/-
ns
d
p<0.0001
Tspo-/-
mitochondrial cholesterol
(nmol/mg protein)
e
STAR
37-kDa
GAPDH
35-kDa
p=0.0016
Tspo+/+
relative density
(STAR 37/GAPDH)
f
35k-Da
ns
g
p=0.0037
Tspo-/-
h

◂**Fig. 8** Deletion of *Tspo* inhibits the effect of 4'-chlorodiazepam (CDZ) on mitochondrial STAR and cholesterol accumulation after ischemia–reperfusion (I/R) but does not affect cytosolic STAR level. **a** and **b** Effect of CDZ on mitochondrial STAR and cholesterol accumulation in $Tspo^{+/+}$ rats. **c** and **d** Effect of CDZ on mitochondrial STAR and cholesterol accumulation in $Tspo^{-/-}$ rats. **e** Effect of I/R on cytosolic STAR 37-kDa level in $Tspo^{+/+}$ rats. **f** Effect of CDZ treatment on cytosolic STAR 37-kDa level after I/R in $Tspo^{+/+}$ rats. **g** Effect of I/R on cytosolic STAR 37-kDa level in $Tspo^{-/-}$ rats. **h** Effect of CDZ treatment on cytosolic STAR 37-kDa level after I/R in $Tspo^{-/-}$ rats. COX-IV and GAPDH was used as a loading control. Each value is the mean ± SEM of 3–5, 3–6 and 6–7 independent preparations for mitochondrial western blot, cytosolic western blot and cholesterol dosage, respectively. Statistical analyses were done by an unpaired two-tailed t test (**a**, **c**, **e**–**h**) and a two-way ANOVA analysis followed by a Tukey's multi-comparison test (**b** and **d**). *ns* non-significant

expression of SOD2. The interaction between TSPO and VDAC, which was shown to modulate ROS production [20, 38] and is absent in deleted animals, could be involved in this effect, but this hypothesis needs additional experiments to be confirmed. Moreover, the effect of 4'-chlorodiazepam treatment on mitochondrial ROS generation in wild-type and knock-out animals is lacking and the control of ROS production by TSPO deserves further investigations in the future.

We also observed that myocardial ischemia–reperfusion at its acute phase does not induce changes in TSPO expression. This is in line with data indicating that modulation of TSPO expression probably requires chronic processes and is most of the time observed during chronic pathologies such as neurodegenerative, neuroinflammatory, and neuropsychiatric diseases [10, 44], cerebral ischemia [56], or diabetic cardiomyopathy [22].

Our study highlights the role of STAR as a key protein during the reperfusion of an ischemic myocardium. STAR probably answers to the metabolic needs of the cells but acts as a deleterious agent stimulating cholesterol accumulation and generation of oxysterols, which are harmful for mitochondria during reperfusion. In contrast, Anuka et al. [2] demonstrate a cardioprotective role of STAR in cardiac fibroblasts in post-infarction conditions caused by an expression of the protein showing antiapoptotic activity, thus unrelated to the traditional role of the protein in steroidogenesis as recently described [19]. This apparent discrepancy could be explained by different physiopathological setting as Anuka et al. [2] used a permanent occlusion mouse model with investigations performed more lately during reperfusion (3 days). We cannot exclude the possibility for STAR to possess two properties in stress events, a short-term cholesterol transport property and a long-term antiapoptotic effect.

Another point to be studied is the characterization of the cellular signaling pathway leading to the accumulation of mitochondrial cholesterol during cardiac ischemia–reperfusion. In steroid organs and brain, the cyclic AMP-dependent protein kinase (PKA) is a primary modulator of StAR and TSPO activity and facilitates mitochondrial cholesterol transport [11, 57]. In the heart, PKA has been involved in both the harmful effect of ischemia–reperfusion and the protection effect conferred by preconditioning. Moreover, specific isoforms of phosphodiesterases, cAMP-degrading enzymes regulating the cellular level of cAMP and thus PKA activity, are targets to protect the heart against ischemia–reperfusion injury [13], and modulate mitochondrial function [34]. It will be interesting to know if phosphodiesterases/PKA signaling pathways participate in the control of mitochondrial cholesterol accumulation during cardiac ischemia–reperfusion.

More generally, this work shows that cholesterol, by means of a TSPO/STAR interaction, participates in the changes in lipid cardiac profile and, thus, in the lipotoxicity contributing to cardiac ischemia–reperfusion injury [61]. Other mitochondrial mechanisms such as the increase in carnitine palmitoyltransferase 1A [35] have been recently shown to contribute to this lipotoxicity. Further studies are required to explore a possible interaction between these mechanisms, since regulating mitochondrial metabolism is a promising strategy to limit the extent of myocardial ischemia–reperfusion injury.

Limitations and future perspective of the study

This study has several limitations: (1) the use of only male rats is not consistent with contemporary standards on sex as a biological variable and sex is an important variable to take into account. We used male rats, because cardiovascular studies are generally performed with male, especially because female hormones have cardioprotective properties [17] but also because all our previous studies were performed with male animals; (2) some experiments were performed with a rather low number of samples which might limit the statistical analysis power; (3) the present study focuses on subsarcolemmal mitochondria. Future studies will be aimed at investigating whether interfibrillar mitochondria, which differ in their functional responses and protein content, behave in the same way with respect to TSPO and Star during ischemia–reperfusion; (4) future works are needed to visualize the increase in mitochondrial cholesterol uptake and, thus, to confirm the causal role of mitochondrial sterol accumulation in ischemia–reperfusion injury. This can be done in cardiomyocytes subjected to hypoxia-reoxygenation when a suitable cholesterol probe will be available to monitor mitochondrial cholesterol uptake.

Conclusions

In conclusion, the present study highlights STAR translocation to the mitochondria and its coupling with TSPO to import cholesterol and to promote oxysterol formation

during ischemia–reperfusion. This interaction regulates mitochondrial homeostasis and play a key role during mitochondrial injury.

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Author contribution VL, MR, SP, BG, and DM contributed to the experimental design; JB, SL, JM, JBS, NE, FG, LAB, CT, FV, CC, and DM conducted the experiments; JB, SL, JM, SP, and DM performed data analysis; SP, BG and DM wrote or contributed to the writing of the manuscript with input from all co-authors. All authors have read and approved the content, and agreed to submit for consideration for publication in the journal.

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Data availability Data are available from the corresponding author on reasonable request.

Declarations

Conflict of interests On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethics approval All animal procedures used in this study were conformed to the Directives of the European Parliament (2010/63/EU-848 EEC). The experimental protocols were reviewed and approved (APAFIS13504#-201820130912402v3 and APAFIS#23908–2020012712028279 v4) by the local Ethic Committee Cometh (Afssa/ENVA/UPEC, N° 16).

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