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1 **Identification of a mechanism promoting mitochondrial sterol accumulation**
2 **during myocardial ischemia-reperfusion: role of TSPO and STAR.**

3
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23
24
25
26 **List of abbreviations**

27 CYP: cytochrome P450; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; SOD2:
28 superoxide dismutase 2 cytochrome; COX-IV; cytochrome C oxidase; TSPO: translocator
29 protein; STAR: steroidogenic acute regulatory protein; mPTP: mitochondrial permeability
30 transition pore; VDAC: voltage-dependent anion channel; ROS: reactive oxygen species; AUC:
31 area under the curve; AOC: area over curve.

36 **Abstract**

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

60

61 **Keywords:** myocardial ischemia-reperfusion; mitochondria; translocator protein;
62 steroidogenic acute regulatory protein.

63

64

65 **Introduction**

66

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

73 Hypercholesterolemia is considered as a principal risk factor for coronary artery diseases. High
74 circulating levels of cholesterol contribute to the formation of atheroma plaques and thus to the
75 induction of ischemic pathologies. Thereby, patients with high hypercholesterolemia have an
76 elevated risk of ischemic events [60] and lipid-lowering therapies are associated with delayed
77 cardiovascular events and prolonged survival [43,54]. This elevated risk is attributed to the
78 development of atherosclerosis as a result of hypercholesterolemia. However, during the last
79 decades, several experimental and clinic observations have shown that hypercholesterolemia
80 per se could also exerts direct negative effects on the myocardium, inducing alterations of the
81 structure and the function of the cardiomyocytes [30,64]. It may interfere with cardioprotective
82 mechanisms [1]. The molecular mechanisms by which hypercholesterolemia directly alters
83 cardiac cells are ill known. Some data indicate that hypercholesterolemia induces oxidative
84 stress [36,46], apoptosis [47] and favors opening of the mitochondrial permeability transition
85 pore (mPTP) [36,41] which is a key event contributing to myocardial reperfusion injury [25].
86 The mitochondrial translocator protein (18 kDa; TSPO) is a high affinity cholesterol binding
87 protein primarily located in the outer mitochondrial membrane and is expressed in a wide
88 variety of species and ubiquitously distributed with a predominantly expression in steroid-
89 synthesizing tissues [39]. TSPO is also abundant in kidney and heart. In steroidogenic tissues,
90 TSPO seems to be part of a dynamic complex including outer mitochondrial membrane
91 proteins such as the voltage-dependent anion channel (VDAC) and cytosolic proteins such as
92 the steroidogenic acute regulatory protein (STAR) which is the principal mean pathway of
93 cholesterol transport from lipid droplets to mitochondria [37,50]. STAR is synthesized as a 37-
94 kDa protein, targeted to mitochondria, through its mitochondrial signal, where it is processed
95 by proteases in the outer mitochondrial membrane to a mature 30-kDa form [2,3]. The role of
96 TSPO in mPTP opening was largely discussed but its participation in mPTP formation was
97 dismissed using a cardiac mouse *Tspo* gene deleted model [59]. However, a body of evidence
98 suggests that TSPO plays a critical role in regulating physiological cardiac function and that
99 TSPO ligands may represent interesting drugs to protect the heart during ischemia-reperfusion
100 [39]. Indeed, several TSPO ligands such as SSR180575, 4'-chlorodiazepam or TRO40303
101 demonstrated cardioprotective effects by reducing infarct size in different animal models of
102 myocardial infarction [32,45,58]. This effect was associated with a protection of mitochondrial
103 function and a limitation of the increase in mitochondrial membrane permeability.
104 We previously demonstrated that the reperfusion of an ischemic myocardium causes a large
105 increase in mitochondrial cholesterol content and a subsequent strong generation of auto-
106 oxidized oxysterols resulting from the oxidation of cholesterol by reactive oxygen species
107 (ROS). Oxysterols, whether formed endogenously or brought in certain foods, have major
108 cytotoxic properties [65]. This mitochondrial accumulation of sterols at reperfusion was
109 dramatically enhanced in hyper-cholesterolemic animals [41]. Interestingly, TSPO ligands

110 inhibited cholesterol and concomitant auto-oxidized oxysterol accumulation, limited the
111 increase in mitochondrial membrane permeability and protected mitochondrial function both in
112 normo- and hyper-cholesterolemic animals [41,45,51]. Although some genetic experiments
113 questioned the involvement of TSPO in the mitochondrial transport of cholesterol [5,62], the
114 inhibition of cholesterol and oxysterol accumulation could represent a promising approach to
115 limit mitochondrial injury during myocardial ischemia-reperfusion and, thus, could participate
116 to the cardioprotective effect of TSPO ligands.

117
118 Here, to verify this hypothesis, we examined whether the inhibition of cholesterol synthesis led
119 to the inhibition of mitochondrial cholesterol and oxysterol accumulation and concomitantly
120 protected mitochondrial functions during myocardial ischemia-reperfusion. Then, we
121 investigated the mechanism by which cholesterol accumulates into mitochondria during
122 myocardial reperfusion by determining whether this accumulation results from an increase in
123 cholesterol influx and/or a decrease in cholesterol conversion. To do that: (1) we investigated
124 the potential role of the proteins that convert cholesterol inside mitochondria, *i.e.*, the
125 cytochromes P450 (CYP) 11A1 and 27A1; (2) we studied the role of the main proteins that
126 have been described to participate in the transport of cholesterol into mitochondria of
127 steroidogenic organs, *i.e.*, TSPO and STAR. For this purpose, we used the TSPO ligand 4'-
128 chlorodiazepam and TSPO global knock-out rats (*Tspo*^{-/-}).

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

133

134 **Methods**

135

136 **Animals**

137

138 Male Wistar and Sprague Dawley rats (\approx 300g) were used in this study. All animals were
139 maintained under optimal environmental conditions (temperature 22-25°C and a constant cycle
140 of 12-h light/dark) in the animal house of the faculty of Medicine of the University Paris-Est
141 (approval number E94028028).

142 Male Wistar rats were purchased from Janvier (Le Genest-St-Isle, France). A couple of
143 heterozygous global *Tspo* knock-out Sprague Dawley rats (*Tspo*^{+/-}) has been kindly provided
144 by Pr Papadopoulos (University of Southern California, USA). These Sprague Dawley rats
145 carried out a 89bp deletion in the *Tspo* gene resulting in the absence of protein expression
146 [48]. These animals were crossed to generate of wild-type (*Tspo*^{+/+}) and homozygous *Tspo*

147 deleted (*Tspo*^{-/-}) rats. *Tspo*^{+/+} and *Tspo*^{-/-} rats are either littermates or generated from
148 littermates to avoid generating high percentage of pups not needed from heterozygous
149 breeding for ethical considerations. In addition, the animals were used after height generations
150 of back-crossing. All rats used in this study were PCR genotyped using specific primers (Fig.
151 1a) and no TSPO immunoreactive protein was detected in myocardium (Fig. 1b) and cardiac
152 mitochondria (Fig. 1c) of *Tspo*^{-/-} rats. Cardiac morphology was similar between both genotypes
153 and *Tspo*^{-/-} rats did not display alterations in left ventricular function. This absence of cardiac
154 phenotype variation persisted during aging (Online Fig. S1 and Online Fig. S2). *Tspo* deletion
155 did not alter rat reproduction and morphology (Online Fig. S3a and S3b) although *Tspo*^{-/-} rats
156 tend to have a lower body weight (Online Fig. S3c). In the same way, we did not observe major
157 differences concerning circulating steroidogenic hormones (Online Fig. S3d), basal glucose
158 and lipid blood levels between both genotypes during aging (Online Fig. S4a) and both
159 genotypes respond identically when subjected to metabolic tests, *i.e.*, insulin and glucose
160 tolerance tests (Online Fig. S4b and S4c).

161

162 **In vivo coronary artery occlusion-reperfusion**

163

164 Rats were subjected to 30 min of ischemia followed by 15 min of reperfusion. Rats were
165 anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) given intraperitoneally. The
166 depth of anesthesia was monitored using the tail pinching response and the pedal reflex. Once
167 anesthetized, rats were placed on a warming pad to maintain body temperature at 37°C
168 (Homeothermic Blanket Control Unit, Harvard Apparatus, Les Ulis, France). Then, the animals
169 were intubated using an endotracheal tube and artificially ventilated with a rodent ventilator
170 model VentElite (respiratory rate, 63/min; respiratory volume, 2.20 mL; Harvard Apparatus,
171 Les Ulis, France) with a mixture of carbogen (95% O₂, 5% CO₂).

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

179 4'-Chlorodiazepam (10 mg/kg) or its vehicle (dimethylsulfoxide) were administered
180 intravenously through the jugular vein. This dose of 4'-chlorodiazepam was selected because
181 it was shown to be cardioprotective in a previous study [45]. In the ischemia-reperfused group,
182 4'-chlorodiazepam were injected 10 min before reperfusion during a 5 min infusion. For the
183 sham group, the drugs were administered 25 min before heart excision.

184 Pravastatin (10mg/kg) was given by gavage three days before surgery. We chose this dose
185 and a short duration of treatment because the aim was to limit cholesterol metabolism to
186 decrease cellular cholesterol available for mitochondria and not to induce
187 hypocholesterolemia. The molecule was dissolved in the drinking water at a concentration of
188 10mg/mL and administered orally at 10mg/kg per day. Animals underwent surgical procedures
189 24 hours after the last administration.

190 At the end of 15 minutes of reperfusion, the heart was excised and the left ventricular area at
191 risk of ischemia-reperfused rats or the left ventricle for sham rats were used to prepare
192 mitochondria and cytosols. To delineate the area at risk, both the position of the suture used
193 for the coronary artery occlusion and the change in coloration of the myocardium at the area
194 at risk level during the occlusion were used. This allowed avoiding any contamination of the
195 area at risk by a dye that could impair mitochondrial functions. The weight of the area at risk
196 varied from 190-220 mg according to the experiments. The same area of the myocardium was
197 excised from sham animals (myocardium not subjected to ischemia-reperfusion) to analyse
198 mitochondrial functions.

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

208

209 **Isolation of cardiac mitochondria and cytosols**

210

211 Rats were euthanized by intraperitoneal injection of pentobarbital (150 mg/kg). Rat left
212 ventricles were removed and immediately immersed in ice cold 0.9% NaCl, scissor minced
213 and homogenized using a Polytron homogenizer in a cold buffer (4 °C, pH 7.4) containing:
214 mannitol (220 mM), sucrose (70 mM), HEPES (10 mM) and EGTA (2 mM). The samples were
215 further homogenized for 10 consecutive times using a Potter homogenizer at 1500 rev/min.
216 The homogenates were then centrifuged at 1000 g for 5 min at 4°C to remove tissue debris
217 and nuclei. The supernatants were centrifuged for 10 min at 10,000 g. The final pellets
218 containing mitochondria were resuspended in the homogenization buffer with only 0.01mM of
219 EGTA and were used immediately (evaluation of mitochondrial function) or frozen at – 80°C
220 (western blot experiments and sterol determination). The supernatants were centrifuged at

221 100,000g for 60 min at 4°C. The pellets were discarded and the supernatants, corresponding
222 to the cytosols, were frozen at – 80°C after determination of the protein concentrations
223 (Pierce™ BCA Protein Assay Kit (23225, Thermofischer Scientific, Illkirch, France).

224 Testis and liver mitochondria were prepared using the same protocol without the Polytron
225 homogenization step.

226

227 **Evaluation of mitochondrial oxygen consumption and mitochondrial permeability** 228 **transition pore opening**

229

230 Oxygen consumption was measured with a Clark-type electrode (Hansatech Instruments Ltd,
231 Norfolk, UK). Mitochondria (0.4 mg /mL) were incubated in a respiration buffer (100 mM KCl,
232 50 mM sucrose, 10 mM HEPES and 5 mM KH₂PO₄, pH 7.4 at 30°C). Respiration was initiated
233 by addition of 2.5 mM pyruvate/malate. After 1 min, ATP synthesis was induced by addition of
234 1 mM ADP (ADP-stimulation respiration rate). ADP uptake was then inhibited by adding 1 μM
235 carboxyatractyloside (substrate-dependent respiration rate). The respiratory control ratio
236 (ADP-stimulation / substrate-dependent respiration rate) was then calculated.

237 mPTP opening was assessed by monitoring mitochondrial calcium retention capacity. Rat
238 cardiac mitochondria were loaded with increasing concentrations of calcium until the load
239 reached a threshold at which mitochondria underwent a fast process of calcium release, which
240 was due to mPTP opening. Mitochondria (1 mg/mL), energized with 5 mM pyruvate/malate,
241 were incubated in the respiration buffer supplemented with 1 mM Calcium Green-5N
242 fluorescent probe (C3737, Invitrogen, Eugene, OR, USA). The reaction was started by addition
243 of successive 10 μM Ca²⁺ pulses. The concentration of calcium in the extramitochondrial
244 medium was monitored by means of a Jasco FP-6300 spectrofluorimeter (Jasco, Bouguenais,
245 France) at excitation and emission wavelengths of 506 and 532 nm, respectively. The calcium
246 signal was calibrated by addition to the medium of known calcium amounts.

247

248 **Assessment of mitochondrial respiratory complex activities**

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

251

252 **Measurement of superoxide anion production in myocardial mitochondria**

253

254 Superoxide anion generation was assessed by measuring the rate of hydrogen peroxide
255 production. This was determined fluorometrically by the oxidation of Amplex Red to fluorescent
256 resorufin, coupled to the enzymatic reduction of hydrogen peroxide by horseradish peroxidase.
257 Essentially, the superoxide generated in mitochondria is converted endogenously to hydrogen

258 peroxide and then measured by the assay. Briefly, Amplex Red (10 μ M) and horseradish
259 peroxidase (1 U/mL) were added to isolated mitochondria (0.2 mg/mL protein) in respiration
260 buffer maintained at 30°C. The reaction was initiated by addition of the respiratory substrates
261 (pyruvate/malate or succinate, 5 mM) in the presence or absence of 1 μ M rotenone (inhibitor
262 of complex I) and 1 μ M antimycin A (inhibitor complex III). The subsequent increase in
263 fluorescence was monitored over time using a fluorescence spectrometer (Perkin-Elmer SA
264 LS 50B, excitation wavelength=563 nm; emission wavelength=587 nm).

265

266 **Measurement of 4-hydroxynonenal**

267

268 4-hydroxynonenal was measured using the Elabioscience competitive ELISA kit (Euromedex,
269 Souffelweyersheim, France) according to the manufacturer's instruction. Briefly, the samples
270 (0.2 mg protein) were added into a microplate pre-coated with 4-hydroxynonenal. After
271 incubation with 4-hydroxynonenal antibody and with labeled horse radish peroxidase,
272 substrate and stop solutions were added. The absorbance was measured at a wavelength of
273 450nm using Multiskan Sky (Thermo Scientific) plate reader, and the concentration of 4-
274 hydroxynonenal was determined by comparing the optical density of the sample to the
275 standard curve.

276

277 **Isolation of primary adult rat cardiomyocytes**

278

279 Left ventricular cardiomyocytes were isolated from *Tspo*^{+/+} and *Tspo*^{-/-} rats by an enzymatic
280 technique. The rat was euthanized by intraperitoneal injection of pentobarbital (150 mg/kg) and
281 the heart was excised. The heart was retrogradely perfused for 15 min at 37°C with a stock
282 perfusion buffer bubbled with 95%O₂/5%CO₂ containing 133 mM NaCl, 4.7 mM KCl, 0.6 mM
283 KH₂PO₄, 0.6 mM Na₂HPO₄, 1.2 mM MgSO₄, 12 mM NaHCO₃, 10 mM KHCO₃, 10 mM HEPES,
284 30 mM taurine, 0.032 mM phenol red, 5.5 mM glucose, 10 mM 2,3butanedionemonoxime pH
285 7.4 to wash out blood. After 2 min of perfusion, liberase (Blendzyme 10 mg/100 mL, Roche
286 Applied Science, Mannheim, Germany), trypsin EDTA (14 mg/100 mL) and 12.5 μ M Ca²⁺ were
287 added to the buffer and the heart was perfused for approximately 13-15 min. The heart was
288 placed into a beaker in the same buffer containing 10% bovine serum albumin pH 7.4 at 37°C
289 to stop the digestion. Left ventricles were then cut into small fragments and cells isolated by
290 stirring the tissue and successive aspirations of the fragments through a 10 mL pipette. After
291 10 min the supernatant was removed and the remaining tissue fragments were re-exposed to
292 10 mL of the same buffer. Then, the cells were suspended in the same buffer and Ca²⁺ was
293 gradually added from 12.5 μ M to 1 mM into an incubator at 37°C. Finally, the cardiomyocytes
294 were suspended in culture medium M199 supplemented with 0.1% insulin-transferine-

295 selenium. The cells were seeded on 35 mm Petri dishes (150000/dish) pre-coated with 10
296 $\mu\text{g}/\text{mL}$ sterilized laminin and incubated for 90 min before being used.

297

298 **Evaluation of mitochondrial reactive oxygen species (ROS) production in living** 299 **cardiomyocytes**

300

301 Assessment of mitochondrial superoxide production in cardiomyocytes was performed using
302 MitoSOX™ Red fluorescent probe which targets mitochondria and is oxidized by superoxide
303 anion. Cells were washed with a Tyrode's buffer (in mM: NaCl 130; KCl 5; HEPES 10; MgCl_2
304 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
305 then washed two times with the Tyrode solution.

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

309 Cardiomyocytes were imaged with the Olympus IX-81 motorized inverted microscope
310 equipped with a mercury lamp as a source of light for epifluorescence illumination and with a
311 cooled camera (Hamamatsu ORCA-ER). MitoSOX™ Red fluorescence were excited at 520-
312 550 nm and recorded at 580 nm. Images were acquired every 10 min from 0 to 60 min and
313 analyzed using a digital epifluorescence imaging software (XCellence, Olympus, Rungis,
314 France). Fluorescence was integrated over a region of interest ($\approx 80 \mu\text{m}^2$) for each
315 cardiomyocyte and a fluorescence background corresponding to an area without cells was
316 subtracted. The global response was analyzed by averaging the fluorescence changes
317 obtained from all the cardiomyocytes contained in a single field (20-30 cells).

318

319 **Blood sample analysis**

320

321 Blood was collected from 2, 6, and 12 months *Tspo*^{+/+} and *Tspo*^{-/-} rats. Rats were anesthetized
322 with isoflurane (0.2 L/min O₂, 2.5% isoflurane) and blood were collected from the jugular vein.
323 Serum lipid profile (total cholesterol, glycemia, HDL, free fatty acid, triglycerides) was
324 performed with an automaton (Olympus AU 400) by Bichat Hospital's Biochemistry platform.
325 Serum steroid hormones were measured with GC/MS by the mass spectrometry platform.
326 Serum oxysterols measurements were carried with GC/MS.

327

328 **Evaluation of CYP11A1 activity**

329

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

333

334 **Metabolic tests**

335

336 Metabolic tests were carried out according to Pitasi *et al.* [53]. Insuline tolerance tests were
337 performed in 2, 6 and 12 months *Tspo*^{+/+} and *Tspo*^{-/-} rats. One week later, glucose tolerance
338 tests were performed in the same rats. After 6 hours of fasting, rats received an intraperitoneal
339 injection of insulin (1 UI/kg, 3% BSA in saline solution) or D-glucose (1 g/kg in saline).
340 Glycaemia (fasting, 15, 30, 60, 90, 120 min after the injection) were measured with a hand-
341 held glucometer (Accu-Chek Performa, Roche, France) through blood collected from tail vein.
342 The area under the curve (AUC) were calculated using Graphpad Prism. The AUC values for
343 the glucose tolerance tests were normalized as follows: $AUC = totalAUC - (fasting\ glycaemia$
344 $* 120)$. The area over curve (AOC) for the insulin tolerance tests were calculated as follows:
345 $AOC = (fasting\ glycaemia * 120) - totalAUC$.

346

347 **Western Blot analysis**

348

349 After denaturing proteins at 90°C for 5 minutes, a total of 40µg of protein samples were loaded
350 onto 8-16% Mini-PROTEAN® TGX™ Precast Gel (4561104, Bio-Rad) and separated by SDS-
351 PAGE electrophoresis and then transferred to PVDF membranes. Next, membranes were
352 blocked with a 5% skim milk solution for 1h30 at room temperature. After, they were probed
353 with antibodies against CYP11A1 (1/250, sc18043, Santa Cruz Biotechnologies), CYP27A1
354 (1/250, sc14835, Santa Cruz Biotechnologies), TSPO (1/1000, ab154878, Abcam), STAR 37-
355 kDa (1/1000, MBS3223061, Cliniscience), STAR 30-kDa (1/1000, ab58013, Abcam), VDAC
356 (1/1000, 4866, Cell Signaling), superoxide dismutase 2 (SOD2; 1/1000, ab13533, Abcam),
357 cytochrome C oxidase (COX-IV; 1/2000, 4844, Cell Signaling), β-actin (1/2000, 4970, Cell
358 Signaling) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1/1000, 5174, Cell
359 Signaling). Membranes were then incubated with horseradish peroxidase conjugated
360 secondary antibodies, anti-mouse (1/5000, 7076, Cell Signaling), and anti-rabbit (1/5000,
361 7074, Cell Signaling). Finally, blots were revealed with ECL Western Blotting detection
362 reagents (Amersham, GERPN2209, Sigma-Aldrich). The signal intensities for specific bands
363 on the Western Blot were quantified using Image J software (1.30v - National Institute of
364 Health).

365

366 **Immunohistochemistry**

367

368 Formalin-fixed rat left ventricles were embedded in paraffin and cross-sections (5 µm
369 thickness) were performed using a rotary microtome. Briefly, after rehydration, antigen retrieval
370 was performed with heated citrate buffer. After endogenous peroxidase antigen blocking (H₂O₂
371 3% 15 min) followed by antigen blocking (1h, 30% goat serum), sections were incubated with
372 primary antibodies (TSPO, goat ab154878 1:180 - overnight at 4 °C). The following day
373 sections were washed and incubated with a horseradish peroxidase-conjugated secondary
374 antibody (Abcam; ab97051 1:200) for 30 minutes at room temperature. The sections were
375 washed again and incubated with 3,3'-diaminobenzidine (DAB; Vector Laboratories) under
376 visual observation until the signal appears. The reaction was stopped by immersing slides in
377 water. Images were acquired using an inverted optical microscope (Axioplan 2, Zeiss) at x400
378 magnification.

379

380 **Analysis of mRNA expression**

381

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

392

393 **Determination of cholesterol and oxysterol levels**

394

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

398

399 **Evaluation of cardiac hypertrophy**

400

401 To assess cardiac hypertrophy, formalin-fixed rat left ventricles were embedded in paraffin and
402 cross-sections (5 µm thickness) were performed using a rotary microtome. sections were
403 rehydrated and incubated for 45 minutes at room temperature with 10 µg/mL Alexa488-

404 conjugated wheat germ agglutinin (WGA; Invitrogen). Slides were then mounted using glycerol
405 and containing 4',6-diamidino-2-phenylindole (DAPI). Images were acquired using an inverted
406 fluorescent microscope at x200 magnification (AxioImager M2, Zeiss) and analyzed with the
407 Image J software. Cardiac hypertrophy was also assessed by measuring the ratio between the
408 weight of the left ventricle and the body weight of the animals.

409

410 **Echocardiography**

411

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

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

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

429

430

431 **Statistical analysis**

432

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

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

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

449

450 **Results**

451

452 **The inhibition of cholesterol synthesis reduces mitochondrial cholesterol accumulation** 453 **and protects mitochondria against myocardial ischemia-reperfusion injury.**

454

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

461 [Fig. 2a](#) shows that the reperfusion of an ischemic myocardium produced an increase in
462 mitochondrial cholesterol concentration. A similar result was obtained in the corresponding
463 cytosols ([Fig. 2b](#)) indicating that reperfusion promotes a global cellular accumulation of
464 cholesterol that can result from a cellular stimulation of synthesis and/or an efflux of cholesterol
465 from the blood compartment. This was associated with a significant increase in oxysterol
466 contents in both mitochondria and cytosols ([Fig. 2d and 2e](#)).

467 In sham animals, pravastatin did not modify cytosolic ([Fig. 2b](#)) but slightly decreased
468 mitochondrial cholesterol ([Fig. 2a](#)). We did not observe any effect of pravastatin on the
469 concentrations of oxysterols measured in cardiac mitochondria isolated from sham rats ([Fig](#)
470 [2d](#)). In cardiac cytosols pravastatin treatment did not modify the concentrations of 7 α -
471 hydroxycholesterol and 7-ketocholesterol but, intriguingly, increased the concentration of 7 β -
472 hydroxycholesterol ([Fig. 2e](#)).

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

475 This was associated with strong inhibition of oxysterol production in the mitochondria whereas
476 no decrease could be observed in the cytosols (Fig. 2d and 2e). It should be noted that
477 pravastatin treatment did not lower serum cholesterol whatever the group of rats (Fig. 2c).
478 We next examined the concomitant effect of pravastatin treatment on mitochondrial function.
479 Along with the reduction in mitochondrial cholesterol and oxysterols accumulation, pravastatin
480 significantly improved oxidative phosphorylation as demonstrated by the increase in both ADP-
481 stimulated respiration (state 3) and the respiratory control ratio (+48.4% versus ischemia-
482 reperfusion). Pravastatin also decreased the sensitivity of mitochondria to mPTP opening as
483 demonstrated by their increased capacity to retain calcium (+52.4% versus ischemia-
484 reperfusion) (Table 1).

485

486 *3.2. Cardiac mitochondrial cholesterol accumulation is not mediated by the inhibition of* 487 *cholesterol catabolism during ischemia-reperfusion*

488

489 Two CYP are responsible for the catabolism of cholesterol in mitochondria: CYP11A1 and
490 CYP27A1. They are located to the matrix side of the inner mitochondrial membrane and
491 convert cholesterol to pregnenolone and 27-hydroxycholesterol, respectively. In heart
492 mitochondria, the presence and/or the role of these proteins remains a source of questions [7].
493 As illustrated in Fig. 3a, Western Blot experiments confirmed the presence of CYP11A1 in
494 cardiac mitochondria, which is at the limit of detection when comparing the content observed
495 in the testis on the same gel (Fig. 3b). Original western blots of all the figures are shown in the
496 supplementary file. To go further, we next analyzed the activity of the enzyme in isolated
497 mitochondria using the fluorescent probe cholesterol-resorufin. We synthesised a CYP11A1
498 fluorescent probe in which a resorufin molecule was conjugated to the side chain of cholesterol
499 (see supplementary file). CYP11A1 cuts the cholesterol side chain and releases the
500 fluorescent resorufin, which can be measured at 590 nm. In accordance with the expression
501 of CYP11A1, the activity of the enzyme in the heart was very low compared to that observed
502 in the testis (Fig. 3c). This is corroborated by the fact that we were unable to detect
503 pregnenolone using isotope dilution mass spectrometry in isolated heart mitochondria (results
504 not shown). Western blot experiments allowed also to reveal the presence of CYP27A1 in rat
505 isolated cardiac mitochondria (Fig. 3a) which, as CYP11A1 compared to testis, was very low
506 compared to liver (Fig. 3b). This had not been established before and this was supported by
507 the presence of 27-hydroxycholesterol inside cardiac mitochondria (0.0442 ± 0.0073 $\mu\text{g}/\text{mg}$
508 protein; $n=15$ different preparations). Then, we examined the effect of ischemia-reperfusion on
509 both cytochromes. Fig. 3a shows that protein and mRNA expressions of both cytochromes
510 were not affected after ischemia-reperfusion nor was the activity of CYP11A1 (Fig. 3c). In the
511 same way, the mitochondrial concentration of 27-hydroxycholesterol significantly increased

512 after ischemia-reperfusion (from 0.0442 ± 0.0073 (n=15) to 0.071 ± 0.004 (n=10) $\mu\text{g}/\text{mg}$ protein)
513 ruling out an inhibition of CYP27A1 activity. These results tend to indicate that the
514 accumulation of mitochondrial cholesterol was not a consequence of a cholesterol metabolism
515 deficiency during ischemia-reperfusion.

516

517 *3.3. Cardiac cholesterol accumulation at reperfusion is related to an increase in mitochondrial*
518 *STAR but not in TSPO levels.*

519

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

534

535 *3.4. The effect of pravastatin on mitochondrial sterol accumulation is associated with the*
536 *inhibition of mitochondrial STAR processing.*

537

538 As pravastatin limited the accumulation of cholesterol and oxysterols in the mitochondria at
539 reperfusion (Fig. 2a and 2d), we wondered whether the limitation of mitochondrial STAR
540 processing could be involved in this effect. Thus, according to the protocol previously
541 described, we examined the effect of the administration of pravastatin on the induction of the
542 mitochondrial STAR processing. Fig. 4a confirmed the mitochondrial accumulation of STAR
543 30-kDa at reperfusion. It was totally inhibited by pravastatin administration (Fig. 4a).

544

545 *3.5. The TSPO ligand 4'-chlorodiazepam inhibits STAR mitochondrial processing.*

546

547 We previously demonstrated that TSPO ligands inhibited oxysterol formation by reducing the
548 accumulation of cholesterol in the mitochondrial matrix at reperfusion [51]. We reasoned that

549 this effect could be caused by the limitation of STAR mitochondrial membrane processing.
550 Thus, we examined the effect of the administration of 4'-chlorodiazepam on mitochondrial
551 STAR accumulation at reperfusion. When administrated before reperfusion, 4'-
552 chlorodiazepam greatly limited STAR mitochondrial levels (Fig. 4b). As previous data have
553 suggested that the effect of TSPO ligands could be independent from TSPO [26,63], we
554 wanted to ascertain the involvement of TSPO in the effect of 4'-chlorodiazepam. To do that,
555 we used *Tspo*^{-/-} rats.

556

557 *3.6. Tspo deletion reduces mitochondrial sterols accumulation without altering mitochondrial*
558 *function in rats subjected to ischemia-reperfusion.*

559

560 Fig. 5 shows the impact of *Tspo* deletion on mitochondrial sterol content. In sham animals,
561 *Tspo* deletion did not alter the level of mitochondrial cholesterol (Fig. 5a) but strongly
562 decreased the level of mitochondrial oxysterols (Fig. 5b-g). These oxysterols are known to be
563 formed by auto-oxidation, except 25-hydroxycholesterol which has also be found to result from
564 enzymatic conversion of cholesterol. This decrease in oxysterol content was not observed in
565 cardiac cytosols (Table 2) indicating that it could result from a limitation of mitochondrial ROS
566 production in *Tspo*^{-/-} mitochondria. To ascertain this hypothesis, we compared the basal level
567 of mitochondrial superoxide generation in cardiomyocytes isolated from *Tspo*^{+/+} and *Tspo*^{-/-} rats
568 and we measured lipid peroxidation in cardiac mitochondria isolated from *Tspo*^{+/+} and *Tspo*^{-/-}
569 rats. Fig. 6a and 6b provide direct evidence of this hypothesis. *Tspo* deletion limits
570 mitochondrial superoxide anion production over time in paced cardiomyocytes, as
571 demonstrated by the lower MitoSOX fluorescence in *Tspo*^{-/-} cardiomyocytes (Fig. 6a), and
572 decreases the concentration of the lipid peroxidation product 4-hydroxynonenal in
573 mitochondria isolated from *Tspo*^{-/-} rats (Fig. 6b). This decrease was not observed in the
574 corresponding cytosols (Fig. 6b). Mitochondrial superoxide anions can be produced by the
575 electron transport chain through electron leak and complexes I and III are recognized as the
576 major sites of production [67]. Therefore, we analyzed the rate of superoxide production by
577 measuring the rate of hydrogen peroxide production in cardiac mitochondria isolated from
578 *Tspo*^{+/+} and *Tspo*^{-/-} rats. We found that it was similar whatever the substrate used to induce
579 respiration, excluding a participation of the mitochondrial transport chain in this effect (Fig. 6c).
580 This in accordance with the activity of mitochondrial transport chain complexes as no difference
581 was found between *Tspo*^{+/+} and *Tspo*^{-/-} rats when the activity of each complex was measured
582 separately (Online Fig. S5). In the same way, the expression of superoxide dismutase 2, the
583 main mitochondrial antioxidant enzyme, was not altered in deleted animals, ruling out a role of
584 this enzyme in the decrease in superoxide anions production (Fig. 6d). We also analyzed the
585 expression of VDAC because the interaction between TSPO and VDAC was shown to

586 modulate oxidative stress [20,38]. Our results show that *Tspo* deletion did not alter the
587 expression of VDAC (Fig. 6d).

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

598 Therefore, rats of both genotypes were subjected to cardiac ischemia-reperfusion and
599 mitochondrial sterols and function were assessed. Ischemia-reperfusion induced an increase
600 in mitochondrial cholesterol and oxysterols in *Tspo*^{+/+} animals (Fig. 5) as previously described
601 [41,42,51]. It should be noted that these results were obtained in Sprague Dawleys animals
602 (the strain in which *Tspo*^{-/-} rats were generated) and confirmed all our previous results using
603 Wistar animals.

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

609

610 *3.7. Deletion of Tspo abolishes the effect of 4'-chlorodiazepam on mitochondrial STAR*
611 *processing and cholesterol accumulation without effect on STAR 37-kDa.*

612

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

621

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

629 As it was shown that *Tspo* depletion induced an alteration of the level of STAR 37-kDa in
630 Leydig tumor cells [27], we wondered whether global deletion of *Tspo* would alter the STAR
631 37-kDa cytosolic form. Cardiac ischemia-reperfusion in *Tspo*^{+/+} Sprague Dawley rats confirmed
632 the decrease in the level of STAR 37-kDa in cardiac cytosols (Fig. 8e) and this decrease was
633 not altered by 4'-chlorodiazepam (Fig. 8f). Similar results were observed in *Tspo*^{-/-} rats, *i.e.*,
634 the decrease in cytosolic STAR 37-kDa level observed after ischemia-reperfusion was
635 preserved and the administration of 4'-chlorodiazepam was without effect (Fig. 8g and 8h).

636

637 4. Discussion

638

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

651 An important step of this study was to rule out the possibility of a defect in mitochondrial
652 cholesterol catabolism that could explain mitochondrial cholesterol accumulation. We clearly
653 established in the rat heart the presence of the cytochromes, which are responsible for
654 cholesterol catabolism in mitochondria, *i.e.*, CYP11A1 and CYP27A1. To the best of our
655 knowledge, only one study described the presence of CYP11A1 in the heart (human heart)
656 [68] and that of CYP27A1 was unknown [7]. CYP11A1 constitutes an essential step for the
657 formation of steroid hormones and its presence suggests a possible *de novo* cardiac

658 steroidogenesis although we cannot yet detect pregnenolone. CYP27A1 is considered as a
659 major mechanism for cholesterol efflux from peripheral tissues, as emphasized by Babiker *et*
660 *al.* [4] showing that patients with CYP27A1 gene mutations do not secrete 27-oxygenated
661 products of cholesterol in peripheral macrophages. The role of these cytochromes in
662 cardiovascular physiology and physiopathology is largely unexplored. We demonstrate that
663 they are very poorly expressed in the rat heart compared to reference organs but, most
664 importantly, that their expression and activity are not modified after reperfusion. These results
665 provide evidence that the increased influx of cholesterol and not an alteration of its
666 mitochondrial metabolism is responsible for its mitochondrial accumulation during myocardial
667 ischemia-reperfusion.

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

685 During ischemia-reperfusion, pravastatin limited the cytosolic concentration of cholesterol but
686 not of oxysterols. This might be the consequence of an insufficient decrease in cytosolic
687 cholesterol, which did not reach the threshold needed to significantly reduce oxysterol content.
688 In contrast, pravastatin strongly inhibited cholesterol and oxysterol accumulation in the
689 mitochondria, and restored the capacity of mitochondria to synthesize ATP and to resist to
690 mPTP opening. These are key factors involved in cell protection and they may strongly
691 contribute to the cardioprotective effect of pravastatin. These novel results show that
692 decreasing mitochondrial cholesterol by inhibiting cholesterol metabolism protects
693 mitochondria from ischemia-reperfusion injury. However, it should be kept in mind that short

694 pravastatin treatment can initiate other mechanisms concomitantly to its sterol-lowering action
695 that can also contribute to its protective effect [6].

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

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

717 Our results also indicate that a role of TSPO in the heart may be a control of mitochondrial
718 ROS production. Indeed, we demonstrated that TSPO deletion attenuates mitochondrial
719 superoxide production in primary cardiomyocytes and lipoperoxidation in isolated
720 mitochondria. This is associated with a reduction of cardiac mitochondrial oxysterols, which
721 can be the consequence of a reduction of available ROS in mitochondria leading to a decrease
722 in cholesterol oxidation. This is in accordance with studies suggesting a relationship between
723 TSPO, ROS levels and oxidative stress [8,39]. This is in line with recent data, which report
724 significantly low ROS levels in the epithelial human breast cancer cell line MDA when TSPO
725 is down-regulated [14] and an accumulation of ROS to limit the efficiency of mitochondrial
726 autophagy when TSPO expression is enhanced [21]. So far, we did not determine the cause
727 of this decrease in mitochondrial superoxide anion generation but we have excluded an
728 alteration of the respiratory chain and of the expression of SOD2. The interaction between

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

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

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

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

761 More generally, this work shows that cholesterol, by means of a TSPO/STAR interaction,
762 participates in the changes in lipid cardiac profile and, thus, in the lipotoxicity contributing to
763 cardiac ischemia-reperfusion injury [61]. Other mitochondrial mechanisms such as the

764 increase in carnitine palmitoyl transferase 1A [35] have been recently shown to contribute to
765 this lipotoxicity. Further studies are required to explore a possible interaction between these
766 mechanisms since regulating mitochondrial metabolism is a promising strategy to limit the
767 extent of myocardial ischemia-reperfusion injury.

768 **Limitations and future perspective of the study**

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

783 **Conclusions**

784 In conclusion, the present study highlights STAR translocation to the mitochondria and its
785 coupling with TSPO to import cholesterol and to promote oxysterol formation during ischemia-
786 reperfusion. This interaction regulates mitochondrial homeostasis and play a key role during
787 mitochondrial injury.

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797

798 **Author contribution statement**

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

804

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809

810 **Availability of data and materials**

811 Availability of data and material Data are available from the corresponding author on
812 reasonable request.

813

814 **Declarations**

815 **Conflict of interests**

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

817

818 **Ethics approval**

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

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1072

1073 **Figure Legends**

1074 **Fig. 1.** *Tspo* deletion in Spague Dawley rats. (a): Agarose gel image of the typical PCR
1075 products used for genotyping screening of rat with *Tspo* locus modification (*Tspo*^{+/+}, 362 bp;
1076 *Tspo*^{+/-}, two bands 362 and 273 bp; *Tspo*^{-/-}, 273 bp) and primers used for genotyping. (b):
1077 Representative immunohistochemical images of TSPO in myocardium showing the lack of
1078 TSPO in *Tspo*^{-/-} rats of 2-month-old. Magnification: 400x, scale-bar: 50 µm. (c): Immunoblot
1079 analysis of cardiac mitochondria extracted from *Tspo*^{+/+} and *Tspo*^{-/-} rats. Voltage-dependent
1080 anion channel (VDAC) was used as a loading control. Each value represents the mean ± SEM
1081 of 3 independent mitochondrial preparations. Statistical analysis was done using an unpaired
1082 two-tailed t-test

1083
1084 **Fig. 2.** Effects of pravastatin (P) treatment on the level of cholesterol and oxysterols in cardiac
1085 mitochondria (a, d), cytosols (b, e) and plasma (c) of rats subjected or not to cardiac ischemia-
1086 reperfusion (I/R). 7α-hydroxycholesterol (7αOHC); 7β-hydroxycholesterol (7βOHC); 7-
1087 ketocholesterol (7KC); cholesterol-5α,6α-epoxide (αEpoxiC); cholesterol-5β,6β-epoxide
1088 (βEpoxiC). Each value is the mean of 9-15 independent experiments. All the figures were
1089 analyzed by a two-way ANOVA followed by a Tukey's multi-comparison test

1090

1091 **Fig. 3.** Effect of ischemia-reperfusion (I/R) on cardiac CYP11A, CYP27A1, TSPO and STAR
1092 expression. (a): I/R does not alter protein level mitochondrial protein levels and mRNA left
1093 ventricle expression of CYP11A1 and CYP27A. COX-IV was used as a loading control. (b):

1094 Expression of CYP11A1 and CYP27A1 in cardiac mitochondria compared to their respective
1095 control organs (testis and liver), respectively (n=3, independent mitochondrial preparations).
1096 (c): Representative experiment measuring CYP11A1 enzymatic activity in testis, sham and I/R
1097 cardiac mitochondria (left). Quantification of CYP11A1 enzymatic activity (right). Each value is
1098 the mean \pm SEM of 4-6 independent mitochondrial preparations. Statistical analysis: one way
1099 ANOVA followed by a Tukey's multi-comparison test. (d): I/R does not alter mitochondrial level
1100 of TSPO. (e): I/R decreases cytosolic level of STAR 37-kDa. (f): I/R increases mitochondria
1101 level of STAR 30-kDa. (g): I/R does not alter *Tspo* but decreases *Star* mRNA expression in
1102 the myocardium. Each value is the mean \pm SEM of at least 3 independent preparations (c-g).
1103 COX-IV (d and e) and actin (f) were used as a loading control. Statistical analyses were done
1104 using an unpaired two-tailed t-test (c-f) or a two-way ANOVA followed by a Sidak's multi-
1105 comparison test (g). ns: non-significant

1106

1107 **Fig. 4.** Effect of pravastatin and 4'-chlorodiazepam treatment on mitochondrial STAR level
1108 after cardiac ischemia-reperfusion (I/R). (a): Effect of pravastatin (P). Statistical analysis was
1109 done by a two-way ANOVA analysis followed by a Tukey's multi-comparison test. (b): Effect
1110 of 4'-chlorodiazepam (CDZ). Statistical analysis was done by a one-way ANOVA analysis
1111 followed by a Tukey's multi-comparison test. COX-IV was used as a loading control. Each
1112 value is the mean \pm SEM of at least 3 mitochondrial independent preparations

1113

1114 **Fig. 5.** Effects of *Tspo* deletion on cholesterol (a) and oxysterols (b-g) in cardiac mitochondria
1115 in rats subjected or not to cardiac ischemia-reperfusion (I/R). Each value is the mean \pm SEM
1116 of 6-7 independent experiments. All the panels were analyzed by a two-way ANOVA followed
1117 by a Tukey's multi-comparison test. White bars: *Tspo*^{+/+} rats. Grey bars: *Tspo*^{-/-} rats. ns: non-
1118 significant

1119

1120 **Fig. 6.** Effects of *Tspo* deletion on mitochondrial oxidative stress. (a): *Left:* time-dependent
1121 mitochondrial superoxide production in cardiomyocytes isolated from *Tspo*^{+/+} and *Tspo*^{-/-} rats.
1122 Each point is the mean of 9-10 independent cardiomyocyte preparations, each preparation
1123 including 20-30 cells. Statistical comparison was done by two-way ANOVA analysis. *Right:*
1124 representative images obtained at t=0 and 60 min. Scale bar: 200 μ m. (b): Lipid peroxidation
1125 as indicated by 4-hydroxynonenal levels in cardiac mitochondria (mito) and cytosols (cyto)
1126 isolated from *Tspo*^{+/+} and *Tspo*^{-/-} rats. Each value is the mean \pm SEM of 6 independent
1127 preparations. Statistical analysis was done using an unpaired two-tailed t-test. (c): Superoxide
1128 anion generation was assessed in isolated myocardial mitochondria by measuring the rate of
1129 hydrogen peroxide production. Superoxide anion production was induced by pyruvate/malate

1130 (P/M; 5/5 mM) or succinate (Succ;5 mM) in the presence or absence of 1 μ M rotenone (Rot)
1131 and 1 μ M antimycin A (AA) and was determined fluorometrically by the oxidation of Amplex
1132 red to resorufin. Each value is the mean \pm SEM of 5-6 independent mitochondrial preparations.
1133 **(d)**: Western blot analysis and quantification of SOD2 and VDAC in *Tspo*^{+/+} and *Tspo*^{-/-} cardiac
1134 mitochondria. Each value is the mean \pm SEM of 4-5 independent mitochondrial preparations.
1135 COX-IV was used as a loading control. A.U.: arbitrary units

1136
1137 **Fig. 7.** Effect of *Tspo* deletion on mitochondrial function in rats subjected or not to cardiac
1138 ischemia-reperfusion (I/R) and on infarct size. Mitochondrial yield **(a)**, oxygen consumption
1139 **(b,c)** and calcium retention capacity **(d)** in cardiac mitochondria isolated from *Tspo*^{+/+} and *Tspo*^{-/-}
1140 rats subjected to I/R and treated or not with 4'-chlorodiazepam (CDZ). Each value is the mean
1141 \pm SEM of at least 6 independent experiments. All the figures were analyzed by a two-way
1142 ANOVA followed by a Tukey's multi-comparison test. **(e)**: Infarct size (expressed as
1143 percentage of the area at risk) measured in *Tspo*^{+/+} and *Tspo*^{-/-} rats subjected to 30 min of
1144 coronary artery occlusion and 24 h of reperfusion. The areas at risk (insert) were similar in
1145 both groups.

1146
1147 **Fig. 8.** Deletion of *Tspo* inhibits the effect of 4'-chlorodiazepam (CDZ) on mitochondrial STAR
1148 and cholesterol accumulation after ischemia-reperfusion (I/R) but does not affect cytosolic
1149 STAR level. **(a)** and **(b)**: effect of CDZ on mitochondrial STAR and cholesterol accumulation in
1150 *Tspo*^{+/+} rats. **(c)** and **(d)**: effect of CDZ on mitochondrial STAR and cholesterol accumulation
1151 in *Tspo*^{-/-} rats. **(e)**: effect of I/R on cytosolic STAR 37-kDa level in *Tspo*^{+/+} rats. **(f)**: effect of CDZ
1152 treatment on cytosolic STAR 37-kDa level after I/R in *Tspo*^{+/+} rats. **(g)**: effect of I/R on cytosolic
1153 STAR 37-kDa level in *Tspo*^{-/-} rats. **(h)**: effect of CDZ treatment on cytosolic STAR 37-kDa level
1154 after I/R in *Tspo*^{-/-} rats. COX-IV and GAPDH was used as a loading control. Each value is the
1155 mean \pm SEM of 3-5, 3-6 and 6-7 independent preparations for mitochondrial western blot,
1156 cytosolic western blot and cholesterol dosage, respectively. Statistical analyses were done by
1157 an unpaired two-tailed t-test **(a, c, e-h)** and a two-way ANOVA analysis followed by a Tukey's
1158 multi-comparison test **(b and d)**. ns: non-significant

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.

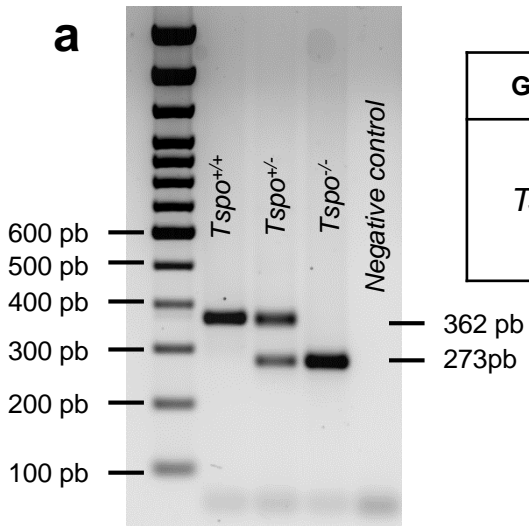
Each value is the mean ± SEM of 7-10 mitochondrial independent preparations. Data were analyzed by a one-way ANOVA followed by a Tukey's multi-comparison test. *p=0.0348, †p=0.0419 and ‡p=0.0017 vs respective I/R.

[§]p< 0.0001 vs respective Sham.

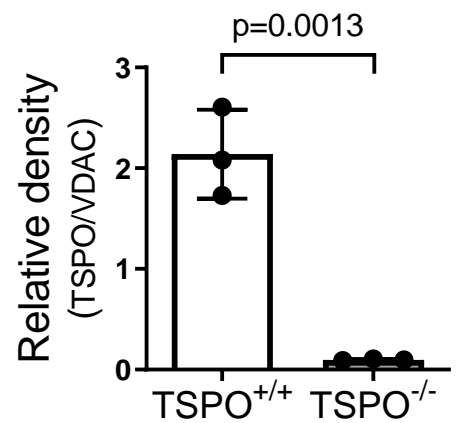
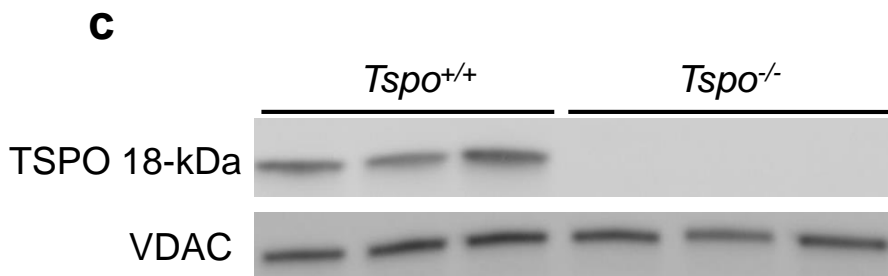
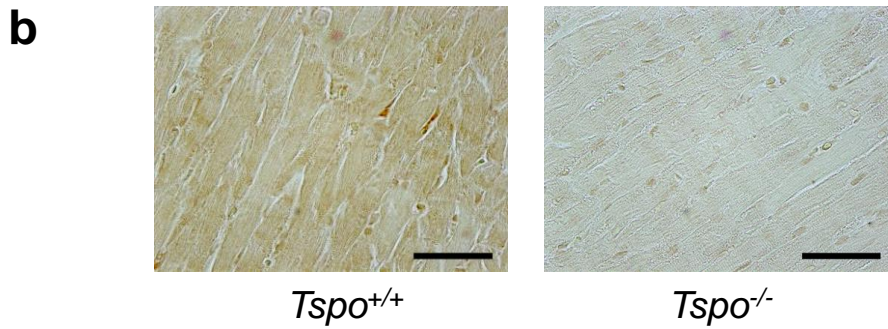
Table 2 Cardiac cytosolic concentrations of sterols in *Tspo*^{+/+} and *Tspo*^{-/-} rats

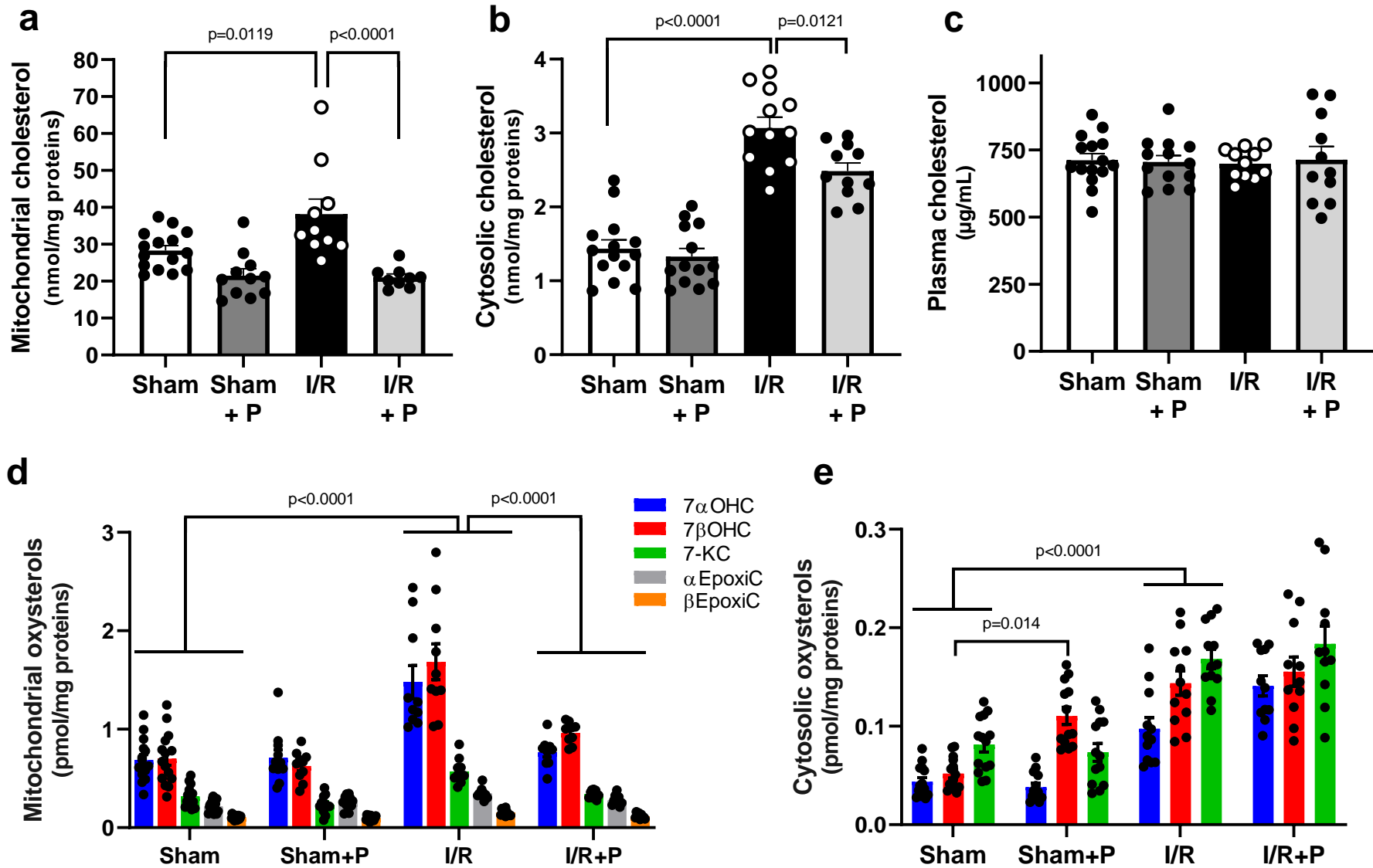
Sterols	<i>Tspo</i>^{+/+}	<i>Tspo</i>^{-/-}
Cholesterol (nmol/mg prot)	2.69 ± 0.08	2.60 ± 0.11
7 α -hydroxycholesterol (pmol/mg prot)	1.28 ± 0.05	1.30 ± 0.04
7 β -hydroxycholesterol (pmol/mg prot)	1.29 ± 0.04	1.31 ± 0.01
7-ketocholesterol (pmol/mg prot)	6.30 ± 0.19	6.31 ± 0.29
cholesterol-5 α ,6 α -epoxide (pmol/mg prot)	1.96 ± 0.15	4.76 ± 0.60 [‡]
cholesterol-5 β ,6 β -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 [*]

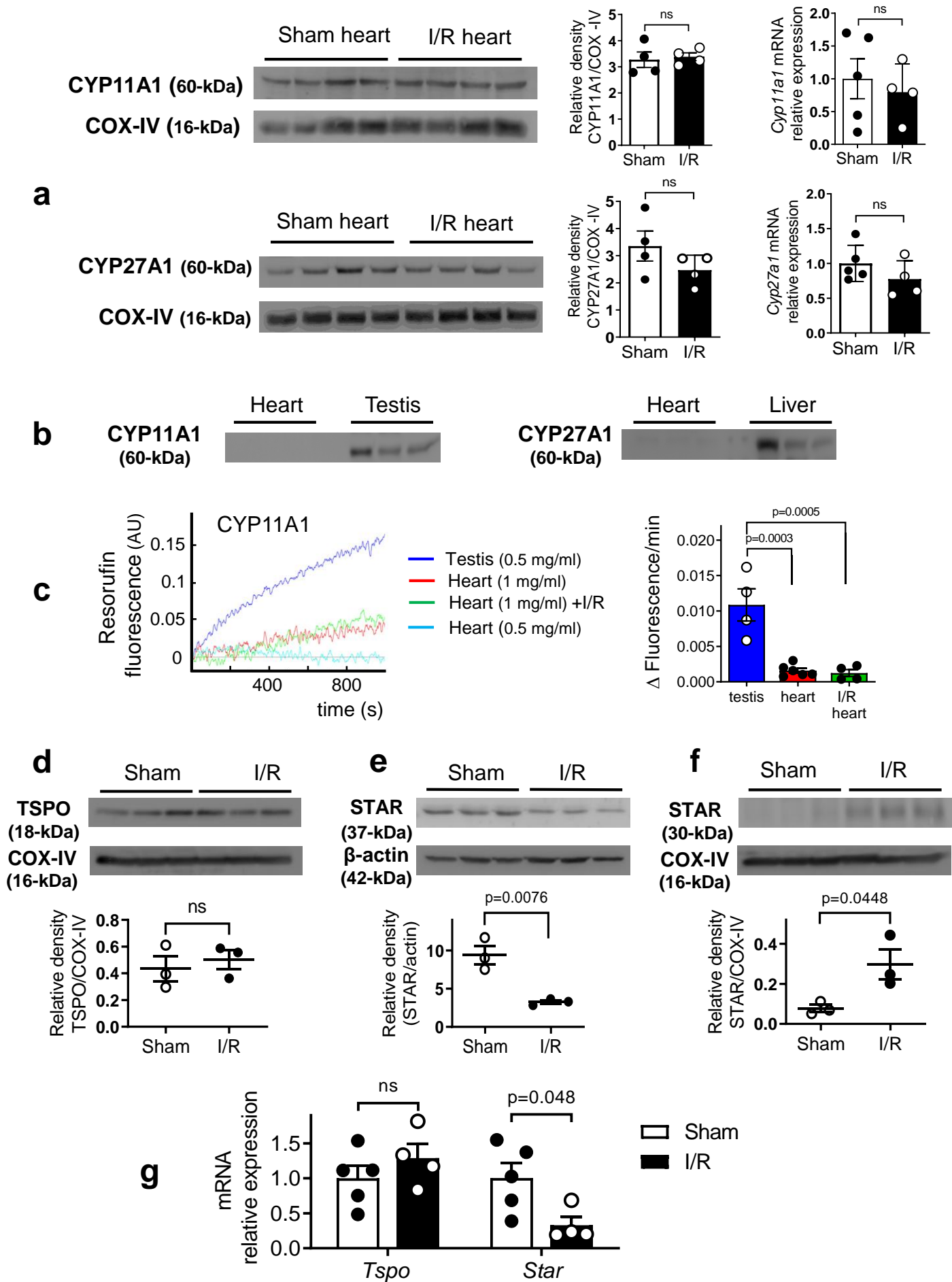
Each value is the mean \pm SEM of 6 independent preparations. Data were analyzed by an unpaired two-tailed t-test. *p=0.042, †p=0.0025 and ‡p=0.001 vs respective *Tspo*^{+/+}

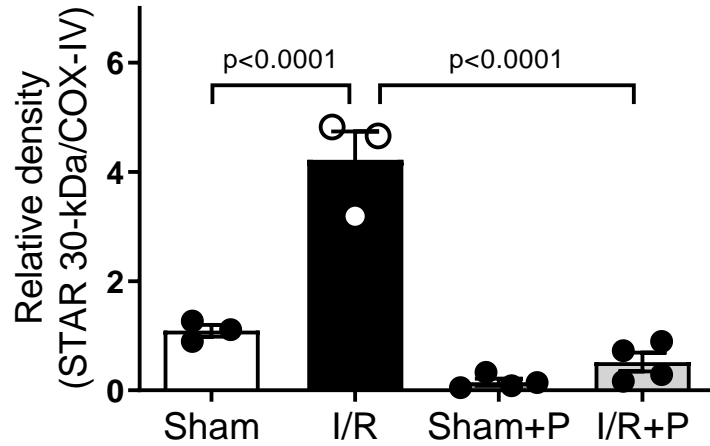
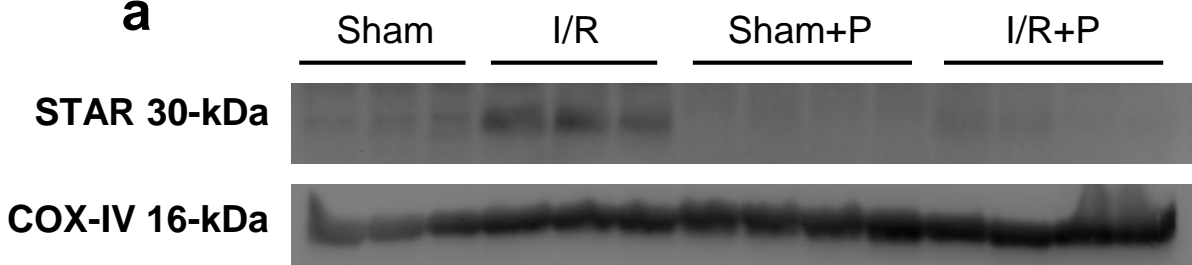
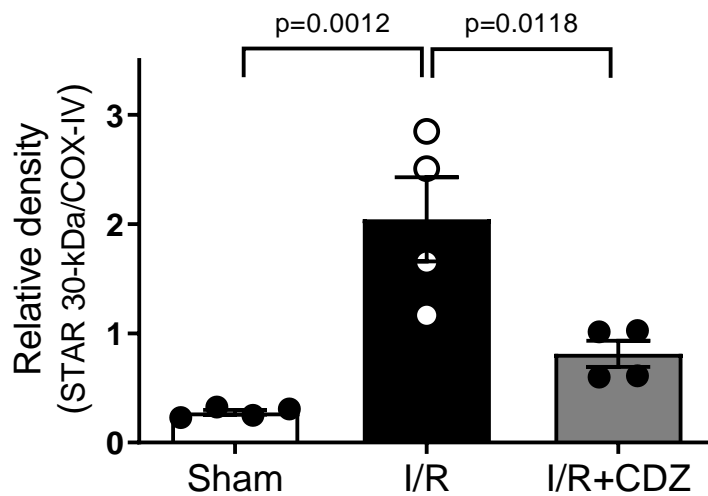
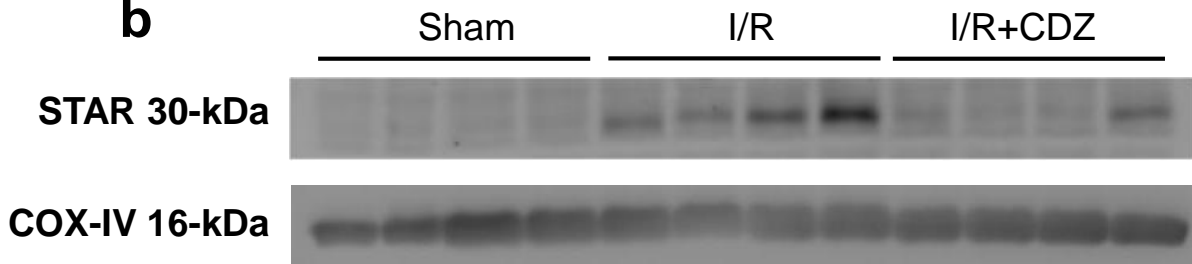


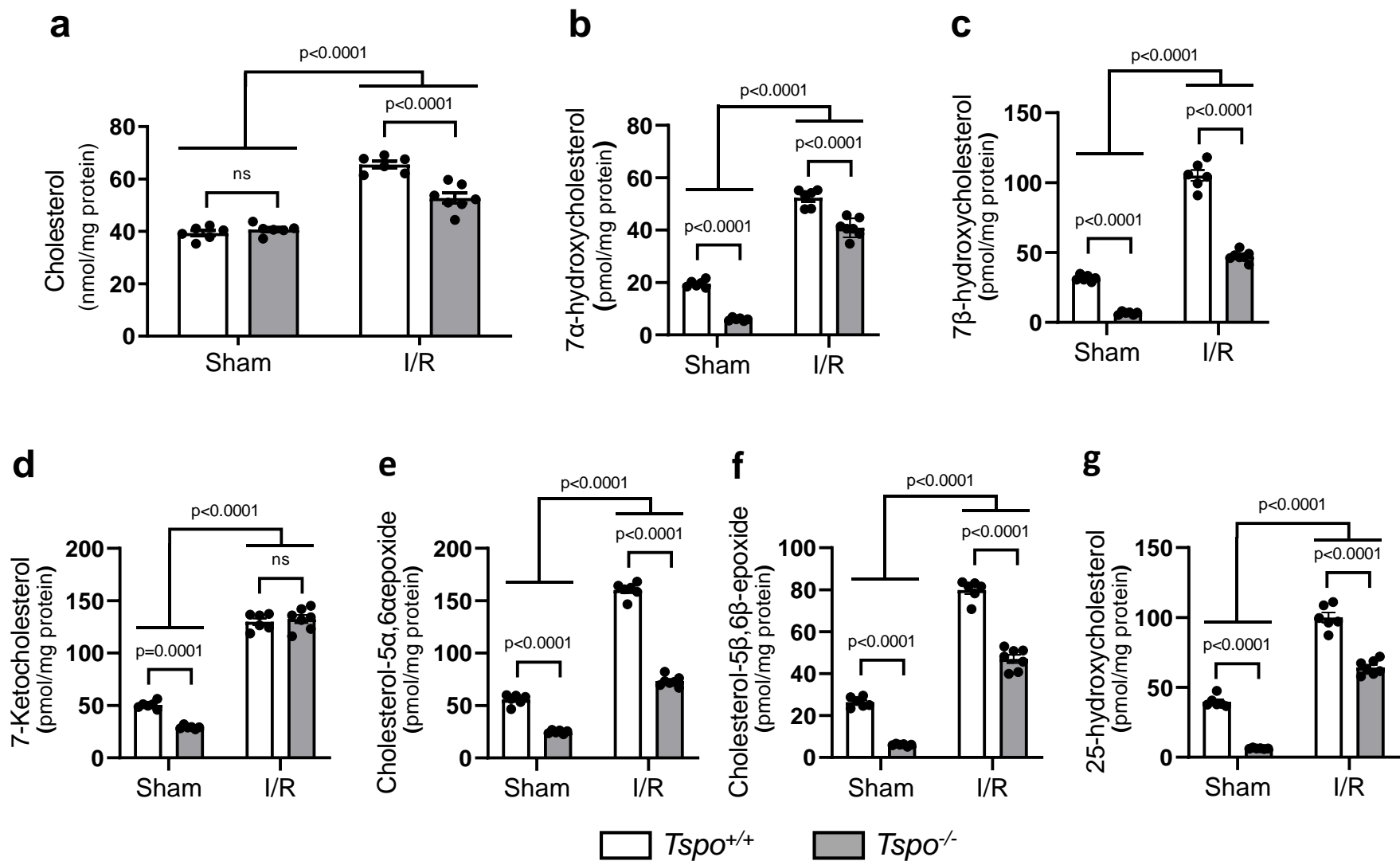
Gene	Primer name (reference)	Sequence (5' > 3')	Molecular weight	Melting temperature
<i>Tspo</i>	R5CKOZFN-R (6430489)	ACT-CCT-AAA-GGG-GTT-GCA-GG	6182,1 g/mol	62°C
	R5CKOZFN-F (6430488)	AGA-GCA-TAC-TCT-TGC-CGT-CG	6093,0 g/mol	62°C

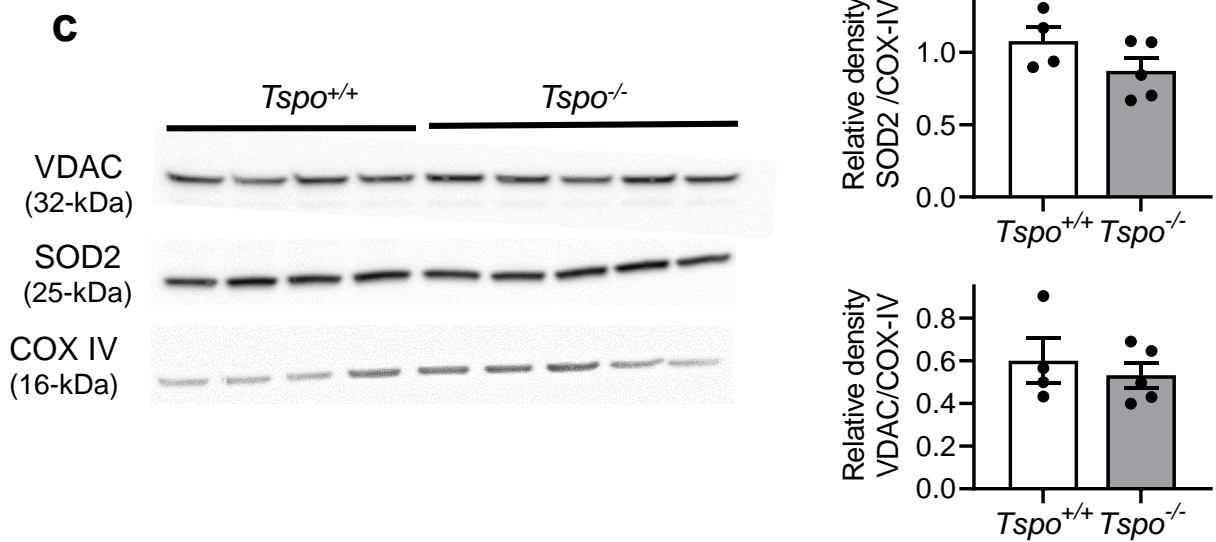
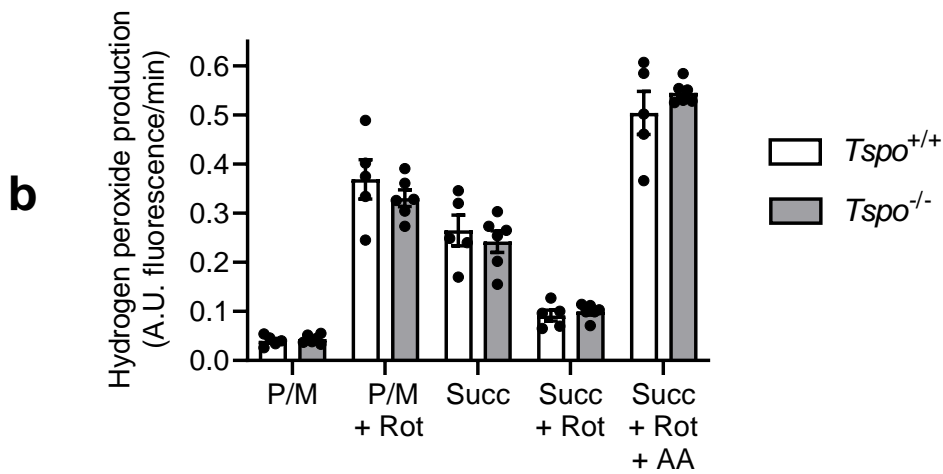
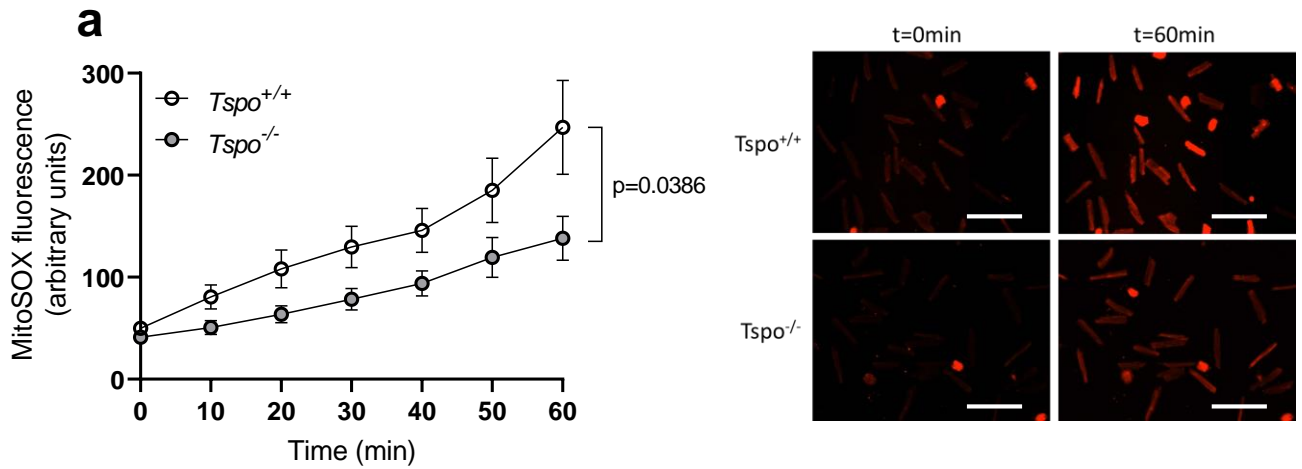


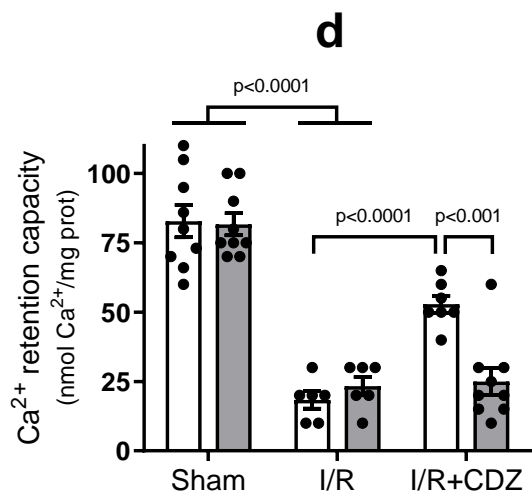
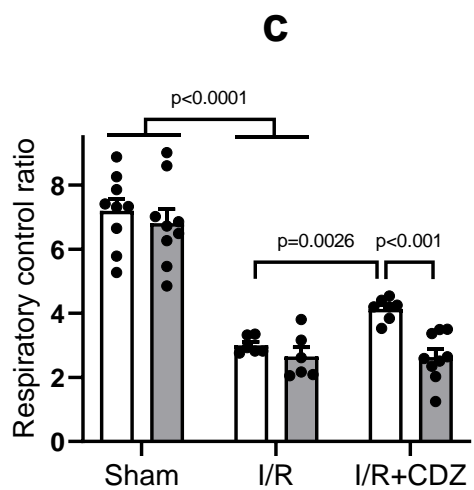
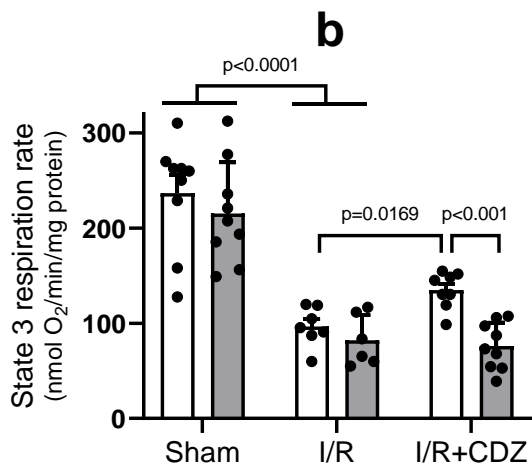
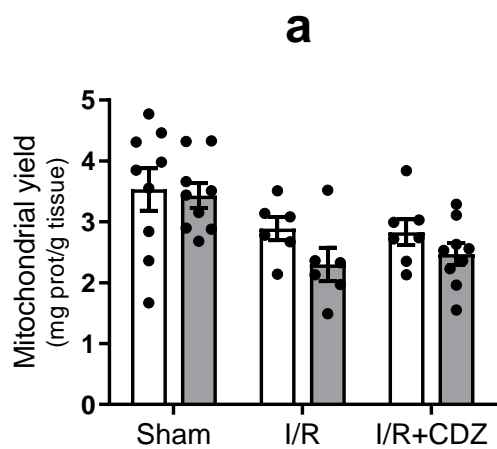




a**b**

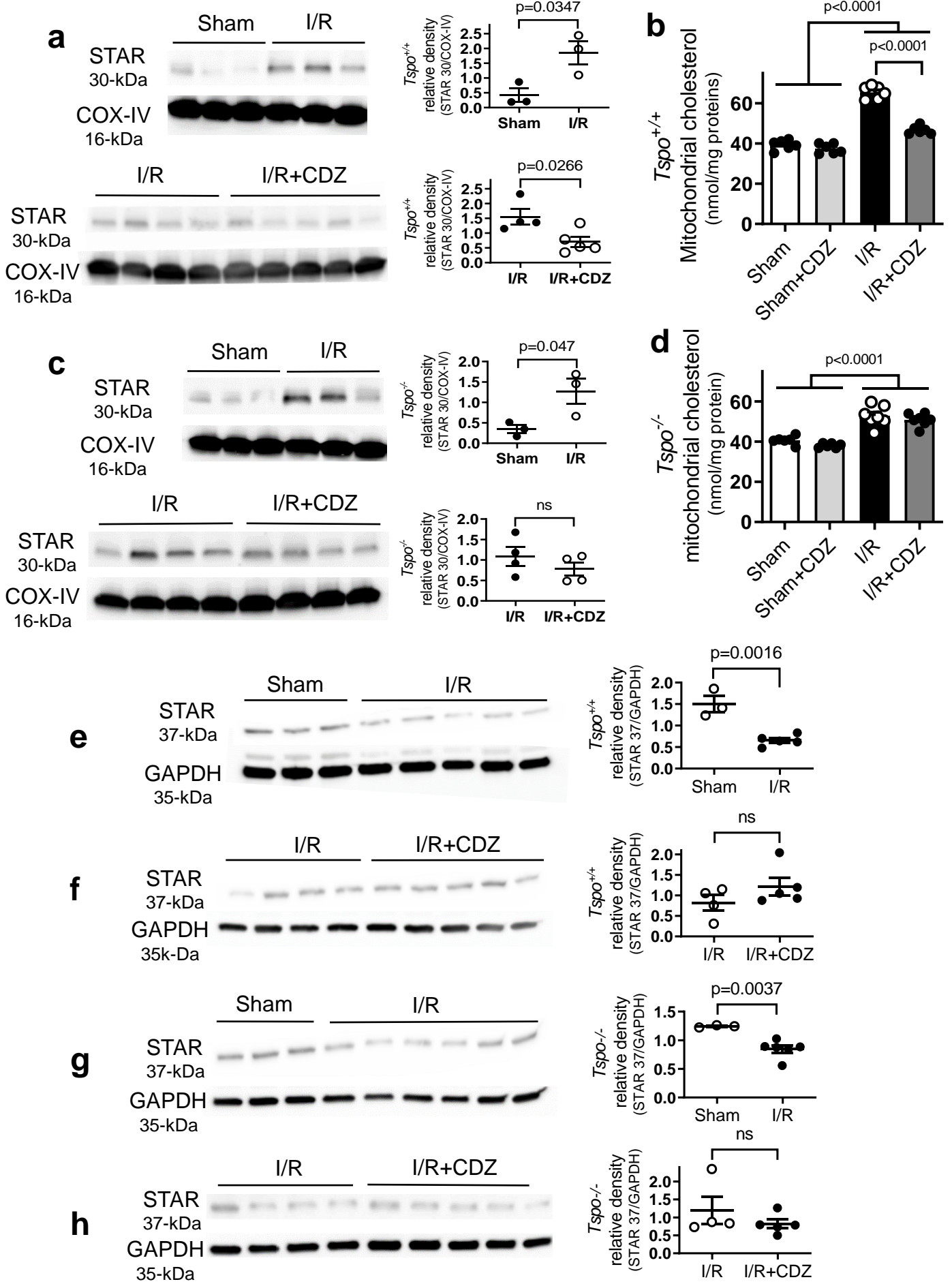






□ *Tspo*^{+/+}

■ *Tspo*^{-/-}



Journal: Basic Research in Cardiology

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

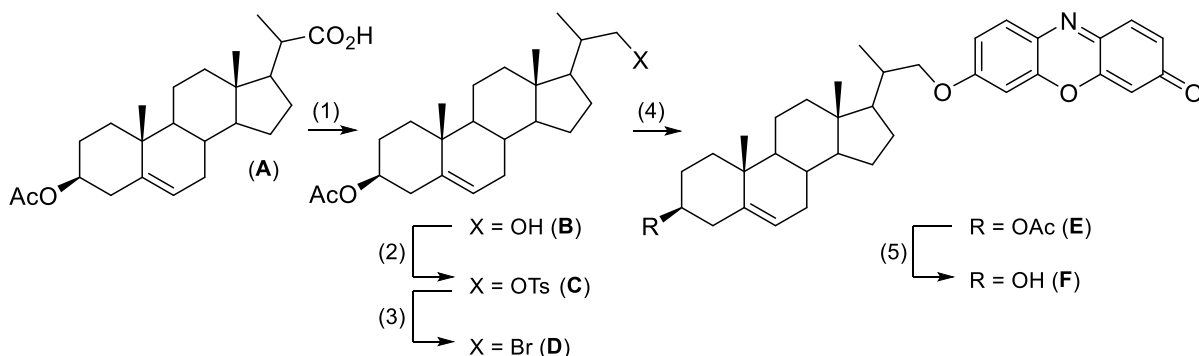
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SUPPLEMENTARY METHODS

Synthesis of the cholesterol-resorufin probe

Cholesterol-resorufin probe (**F**) was prepared from 3 β -acetoxy-22,23-bisnor-5-cholenic acid (**A**) according to a modified version of the procedure initially described (Simpson et al., 1991). This synthesis includes five steps as follows (following figure):



- (1) (a) oxalyl chloride, RT, tetrahydrofuran; (b) LiAlH(OtBu)₃, -60 °C, tetrahydrofuran, quantitative.
(2) tosyl chloride, triethylamine, 4-(dimethylamino)pyridine, 50 °C, dichloromethane, 50%.
(3) LiBr, 60 °C, *N,N*-dimethylformamide, quantitative.
(4) resorufin, K₂CO₃, 50 °C, *N,N*-dimethylformamide, 65%.
(5) LiAlH₄, 0 °C, tetrahydrofuran, 45%.

(1) Preparation of alcohol (**B**): to a stirred solution of acid (**A**) (1 g, 2.57 mmol) in anhydrous dichloromethane, was added oxalyl chloride (1.5 equiv, 325 microL) dropwise at 0 °C. After 2 h at room temperature, the reaction mixture was concentrated under reduced pressure and the resulting solid dissolved in anhydrous tetrahydrofuran under argon. To this solution, lithium tri-*t*-butoxy aluminum hydride (LiAlH(OtBu)₃ (2.7 equivalent, prepared from lithium aluminium hydride (LiAlH₄, 624 mg, 7 mmol) and tert-butanol (1.54 g, 20.8 mmol) in tetrahydrofuran) was added dropwise and under stirring at -60 °C. The mixture was kept at -60 °C for 2.5 h. After dilution with diethyl ether, an aqueous solution of concentrated NaOH was added dropwise to precipitate the aluminum salts. After filtration over a celite pad, the organic phase was washed with HCl (1N), with brine and dried over MgSO₄. The NMR analysis confirmed the complete conversion of the acid (**A**) into the alcohol (**B**), which was used without further purification. Alcohol (**B**) was obtained as a white powder (950 mg, quantitative).

(2) Preparation of tosylate (**C**): to a stirred solution of (**B**) (893 mg, 2.38 mmol) in anhydrous dichloromethane were successively added 4-toluenesulfonyl chloride (2 equiv, 910 mg), triethylamine (4.2 equiv, 1.39 mL) and 4-(dimethylamino)pyridine (5%, 14 mg). The mixture was refluxed for 15 h, then successively diluted with

dichloromethane, washed with HCl (1 N), dried over MgSO₄ and concentrated under reduced pressure. After purification by chromatography on silica gel (cyclohexane/ethyl acetate, 90:10), the tosylate (**C**) was obtained as a white powder (629 mg, 50%).

(3) Preparation of brominated compound (D): to a stirred solution of (**C**) (629 mg, 1.19 mmol) in anhydrous *N,N*-dimethylformamide (3.2 mL) was added LiBr (3 equiv, 310 mg), and the resulting mixture was heated at 60 °C for 15 h. After dilution with H₂O, the aqueous phase was extracted with cyclohexane. Brominated compound (**D**) was obtained as a white powder (515 mg, quantitative).

(4) Preparation of ether (E): to a stirred solution of (**D**) (160 mg, 0.36 mmol) in anhydrous *N,N*-dimethylformamide (3 mL), were successively added resorufin (1.3 equiv, 101 mg) and K₂CO₃ (2 equivalents, 100 mg). The resulting mixture was heated at 50 °C for 6 days under Ar. After dilution with H₂O, the aqueous phase was extracted with dichloromethane. The combined organic layers were washed with K₂CO₃ and dried over MgSO₄. After purification by chromatography on silica gel (cyclohexane/ethyl acetate, 70:30), the ether (**E**) was obtained as an orange solid (135 mg, 65%).

(5) Preparation of cholesterol-resorufin probe (F): to a stirred solution of (**E**) (101 mg, 0.18 mmol) in anhydrous tetrahydrofuran at 0 °C, was added LiAlH₄ (1.5 equiv, 10 mg). The mixture was let to react for 15 min at 0 °C and hydrolyzed with HCl (1N). The mixture was extracted with dichloromethane and the organic layers were dried over MgSO₄ and concentrated under reduced pressure. After purification by chromatography on silica gel (cyclohexane/ethyl acetate/triethylamine, 80:20:1%), the probe (**F**) was obtained as a red solid (42 mg, 45%).

Evaluation of CYP11A1 activity

CYP11A1 activity was measured in rat ventricular and testicular mitochondrial fractions as the ketokonazole inhibitable resorufine release induced by the enzyme according to a modified procedure described previously ([Rone et al., 2012](#)). Mitochondria were broken by 3 successive freeze-thaw cycles and were incubated at two concentrations 0.5 and 1 mg/ml in a buffer including 250 mM sucrose, 10 mM phosphate buffer, 15 mM triethanolamine-HCl, 20 mM KCl, 5 mM MgCl₂, 5 μM trilostane (pH=7 at 30°C). The reaction was then initiated by the successive addition of 5 μM cholesterol-resorufin and 500 μM NADPH. CYP11A1 activity was monitored over time by monitoring the

release of resorufin, which was followed by measuring the increase in fluorescence using a fluorescence spectrometer (Jasco FP-6300, excitation wavelength 530 nm; emission wavelength 595 nm). Ketokonazole (20 M) was added to evaluate the release of resorufin specifically related to CYP11A1 activity.

Assessment of mitochondrial respiratory complex activities

Mitochondrial respiratory chain enzymatic activities were measured as previously reported but with some modifications (Zini et al., 2007; Lo Iacono et al., 2011). Briefly, mitochondrial complex I activity (NADH decylubiquinone oxidoreductase) was measured at 37°C by monitoring the decrease in absorbance resulting from the oxidation of NADH at 340nm. The incubation medium contained 25 mM KH₂PO₄, 5 mM MgCl₂, 100 µM NADH, 250 µM KCN, 1 mg/ml bovine serum albumin and 0.04 mg/ml of freeze-thawed heart mitochondria. The reaction was started by the addition of 100 µM decylubiquinone.

Mitochondrial complex II activity (succinate ubiquinone reductase) was measured by monitoring the absorbance changes of 2,6-dichloroindophenol at 600 nm. The assay mixture contained 10 mM KH₂PO₄, 2 mM EDTA, 2 µM rotenone, 6 mM succinate, 250 µM KCN, 1 mg/ml bovine serum albumin and 0.02 mg/ml of freeze-thawed heart mitochondria. After a preincubation period of 5 min at 37°C, and addition of 80 µM 2,6-dichloroindophenol, the reaction was initiated by the addition of 100 µM decylubiquinone.

Ubiquinol cytochrome c reductase activity (complex III) was measured at 37°C as the rate of cytochrome c reduction at 550 nm. The reaction mixture contained 10 mM KH₂PO₄, 2 mM EDTA, 2 µM rotenone, 250 µM KCN, 1 mg/ml bovine serum albumin, 40 µM oxidized cytochrome c, 0.01 mg/ml of freeze-thawed heart mitochondria. The reaction was started by the addition of 100 µM decylubiquinol.

Mitochondrial complex IV activity (cytochrome c oxidase) was performed at 550 nm following the decrease in absorbance resulting from the oxidation of reduced cytochrome c. The reaction mixture contained 10 mM KH₂PO₄, 2 mM EDTA, 2 mM MgCl₂, 33 µM oxidized cytochrome c and 0.01 mg/ml of freeze-thawed heart mitochondria. The reaction was started by the addition of 1 mM of lauryl maltoside. Complex activities were quantified by measuring the initial slopes of the absorbance curves.

Determination of cholesterol and oxysterol levels

Sterol and oxysterol measurements were performed on cytosolic and mitochondrial extracts. To a screw-capped vial sealed with a Teflon septum, mitochondrial or cytosolic samples were added together with 50 ng of D7-7 α -hydroxycholesterol, D7-7 β -hydroxycholesterol, D7-7ketocholesterol, D6-cholesterol-5 α ,6 α -epoxide, D6-cholesterol-5 β ,6 β -epoxide and D6-27-hydroxycholesterol as internal standards, 50 μ l of butylated hydroxytoluene (5 g/L) and 50 μ l of K3-EDTA (10 g/L) to prevent auto oxidation. Each vial was flushed with argon for 20 min to remove air. Alkaline hydrolysis was allowed to proceed at room temperature (22°C) with magnetic stirring for 30 minutes in the presence of ethanolic 1M potassium hydroxide solution. After hydrolysis, the sterols were extracted twice with 5 ml cyclohexane and oxysterols were eluted on SPE cartridge by isopropanol:hexane 30:70 v/v. The organic solvents were evaporated under a gentle stream of argon and converted into trimethylsilyl ethers with BSTFA. Analysis was performed by gas chromatography – isotope dilution mass spectrometry (GC-MS) with a B-XLB column (30 m \times 0.25 mm i.d. \times 0.25 μ m film thick-ness, J&W Scientific Alltech, Folsom, CA, USA.) in a HP 6890 Network GC system (Agilent Technologies, USA) connected with a direct capillary inlet system to a quadruple mass selective detector HP5975B inert MSD (Agilent Technologies, USA). GC system was equipped with a HP 7687 series autosamplers and HP 7683 series injectors (Agilent Technologies, USA). The oven temperature program was as follows: initial temperature of 180 °C was held for 1 min, followed by a linear ramp of 20 °C/min to 270 °C, and then a linear ramp of 5 °C/min to 290 °C, which was held for 10 min. Helium was used as carrier gas at a flow rate of 1 mL/min and 1 μ L of sample was injected in splitless mode. Injection was carried at 250 °C with a flow rate of 20 ml/min. Transfer line temperature was 290 °C. Filament temperature was set at 150 °C and quadrupole temperature at 220 °C according with the manufacturer indication. Mass spectrometric data were acquired in selected ion monitoring mode (OTMSi-ethers) at m/z = 463 (M+-90) for 7 β -hydroxycholesterol-d7, m/z = 456 (M+-90) for 7 β -hydroxycholesterol, m/z = 479 (M+-90) for 7-ketocholesterol-d7, m/z = 472 (M+-90) for 7-ketocholesterol, m/z = 462 (M+-90) for 27-hydroxycholesterol-d6 and m/z = 456 (M+-90) for 27-hydroxycholesterol, m/z = 481 for 5 α ,6 α -epoxycholestanol-d7, m/z = 474 for 5 α ,6 α -epoxycholestanol, m/z = 481 for 5 β ,6 β -epoxycholestanol-d7, m/z = 474 for 5 β ,6 β -epoxycholestanol. Peak integration was performed manually, and oxysterols were quantified from selected-ion monitoring analysis against internal standards using standard curves for the listed sterols (Civra et al., 2019).

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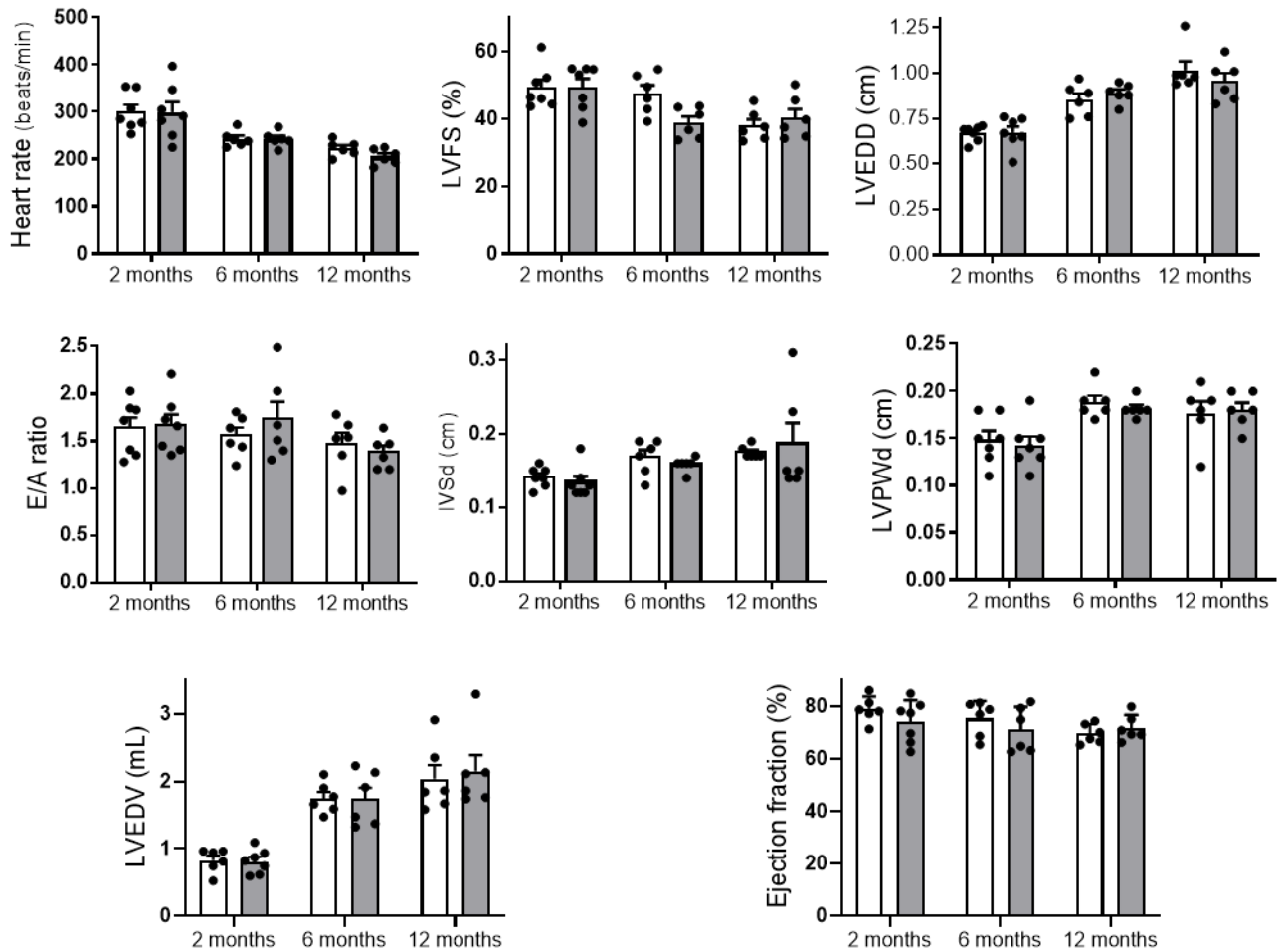
Rone MB, Midzak AS, Issop L, Rammouz G, Jagannathan S, Fan J, Ye X, Blonder J, Veenstra T, Papadopoulos V (2012) Identification of a dynamic mitochondrial protein complex driving cholesterol import, trafficking, and metabolism to steroid hormones. *Mol Endocrinol* 26:1868-1882 doi: 10.1210/me.2012-1159

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SUPPLEMENTARY FIGURES

Fig. S1

A



B

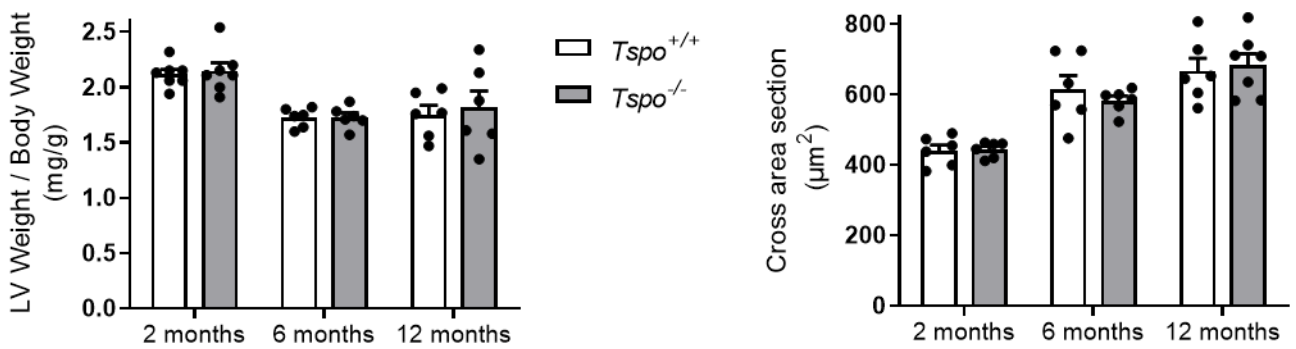


Fig. S1 TSPO deletion does not alter rat cardiac phenotype during aging.

Cardiac parameters were evaluated in 2-, 6- and 12-month-old rats.

A: Echocardiographic parameters: Left ventricular fractional shortening (LVFS), mitral E wave/A wave ratio (E/A ratio), left ventricular end-diastolic diameter (LVEDD), interventricular septum thickness in diastole (IVSd), Left Ventricular Posterior Wall thicknesses in Diastole (LVPWd), left ventricular end-diastolic volume (LVEDV). Each value is the mean \pm SEM of at least 6 animals.

B: Indexes of ventricular hypertrophy in $Tspo^{+/+}$ and $Tspo^{-/-}$ rats.

Left: ventricular (LV) weight to body weight ratio. Each value is the mean \pm SEM of at least 6 animals.

Right: cross sections of left ventricular were stained with FITC-conjugated WGA and cardiomyocyte surface in each section was quantified using Image J. Each value is the mean \pm SEM of at least 6 animals, i.e., 1080 cells (6 ventricles, 6 sections by ventricle and 30 cells by section).

In all panels, statistical comparison between genotypes were done using a two-way ANOVA. No difference between genotypes was observed whatever the parameter.

Fig. S2

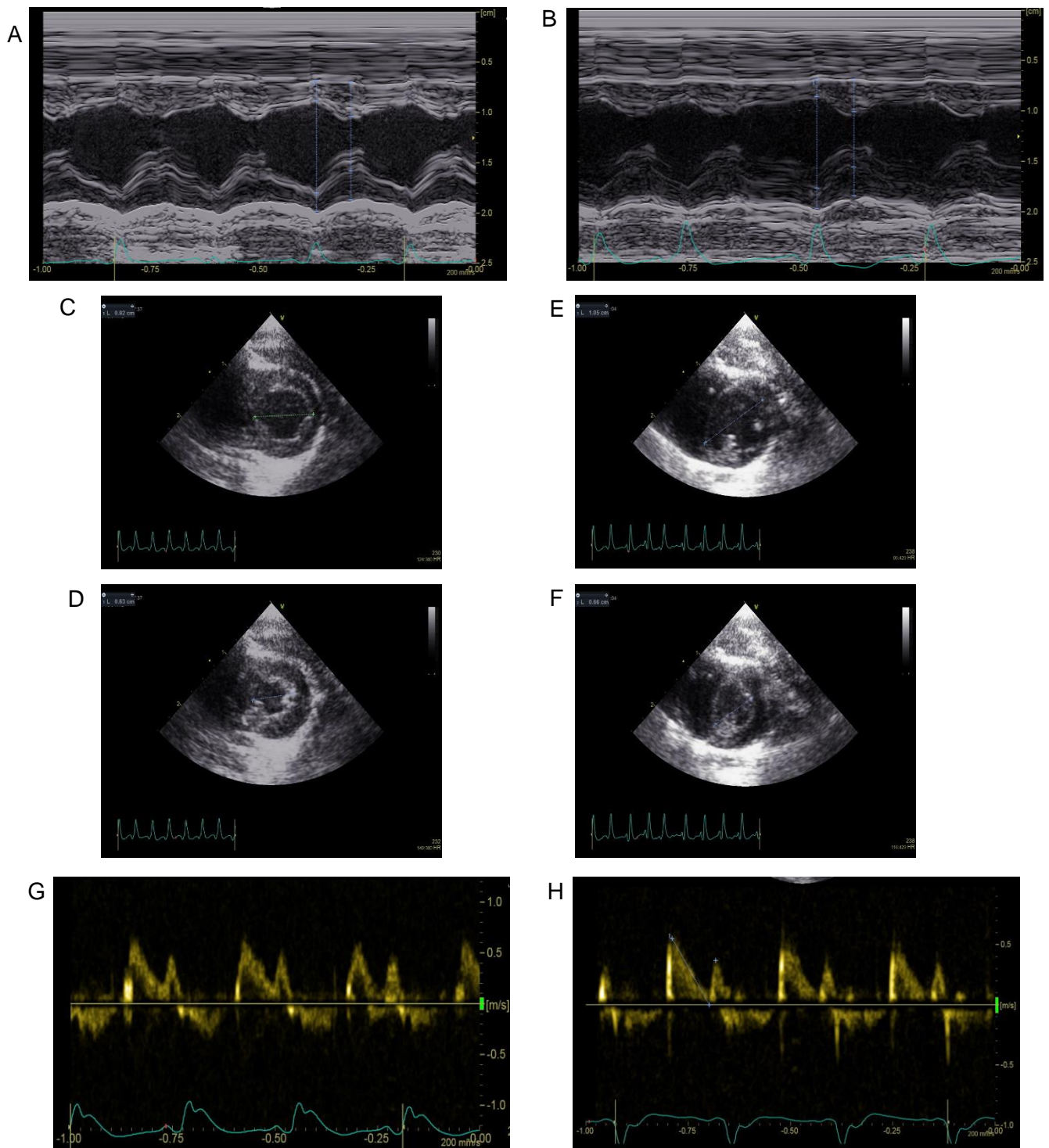


Fig. S2 Typical examples of analysis of LV dimensional parameters and LVFS from M-mode images obtained in one 6-month-old *Tspo^{+/+}* (A) and one 6-month-old *Tspo^{-/-}* rat (B).

Measurement of LV internal diameter in the end-diastole and end-systole from the cine loop of the parasternal short-axis view at the level of papillary muscles for the calculation of EF using Teicholz formula in one 6-month-old *Tspo^{+/+}* rat (C, D) and one 6-month-old *Tspo^{-/-}* rat (E, F), and measurement of E and A velocities and their ratio using transmitral flow tracing obtained by pulse wave *Doppler* in one 12-month-old *Tspo^{+/+}* rat (G) and one 12-month-old *Tspo^{-/-}* rat (H).

Fig. S3

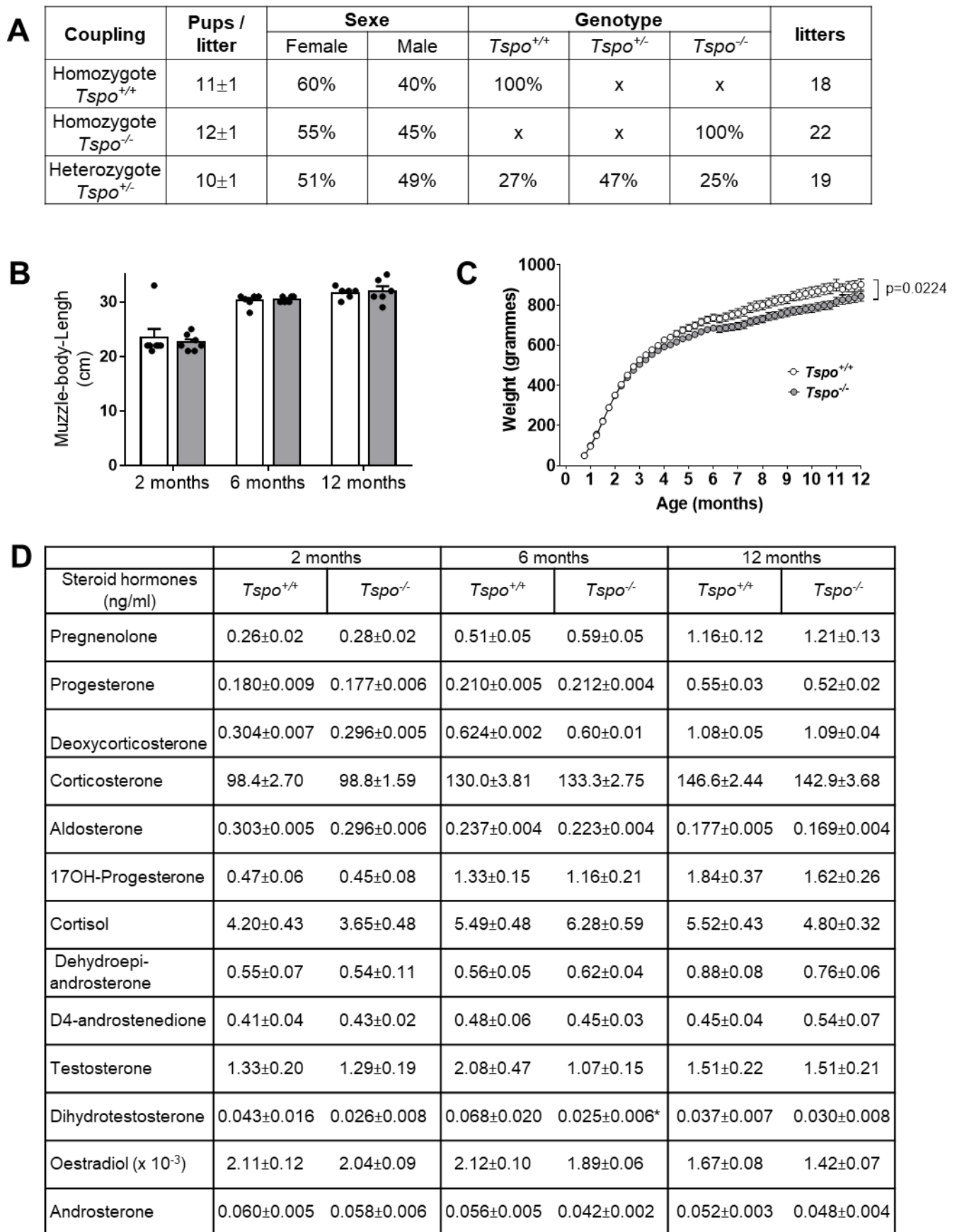


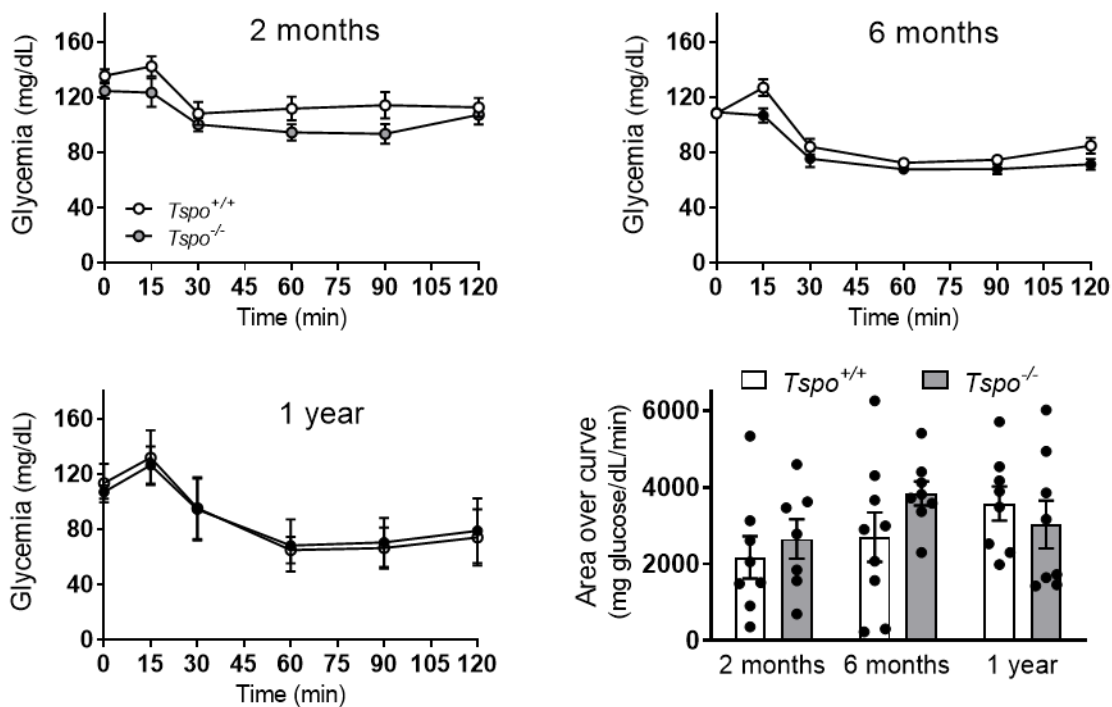
Fig. S3 Reproduction parameters (**A**) and evolution of body lengths (**B**), body weights (**C**) and circulating steroidogenic hormones (**D**) in *Tspo*^{+/+} and *Tspo*^{-/-} rats during aging. **B**: each value is the mean ± SEM of 6-7 animals. **D**: values are means ± SEM, n=8 (*Tspo*^{+/+}) and n= 10 rats (*Tspo*^{-/-}). Statistical comparison between genotypes in panel B and D were done by a two-way ANOVA analysis followed by a Sidak multi-comparison test. *p=0.0237 vs respective *Tspo*^{+/+}. Statistical analysis in panel **C** was done using a mixed effect analysis.

Fig. S4

A

Age months	Glycemia mg/dL		Cholesterol mmol/L		HDL mmol/L		Triglycerides mmol/L		Free Fatty Acid mmol/L	
	<i>Tspo</i> ^{+/+}	<i>Tspo</i> ^{-/-}	<i>Tspo</i> ^{+/+}	<i>Tspo</i> ^{-/-}	<i>Tspo</i> ^{+/+}	<i>Tspo</i> ^{-/-}	<i>Tspo</i> ^{+/+}	<i>Tspo</i> ^{-/-}	<i>Tspo</i> ^{+/+}	<i>Tspo</i> ^{-/-}
2	127 ± 4	119 ± 3	2.49 ± 0.08	2.52 ± 0.11	1.44 ± 0.06	1.36 ± 0.06	2.43 ± 0.24	2.13 ± 0.12	1.00 ± 0.06	0.93 ± 0.04
6	107 ± 3	99 ± 1	3.08 ± 0.15	3.22 ± 0.12	1.63 ± 0.09	1.72 ± 0.07	2.53 ± 0.23	2.64 ± 0.23	0.99 ± 0.06	1.00 ± 0.05
12	110 ± 2	105 ± 3	3.46 ± 0.16	3.44 ± 0.13	1.85 ± 0.09	1.92 ± 0.06	2.11 ± 0.21	1.81 ± 0.10	1.06 ± 0.05	1.00 ± 0.03

B



C

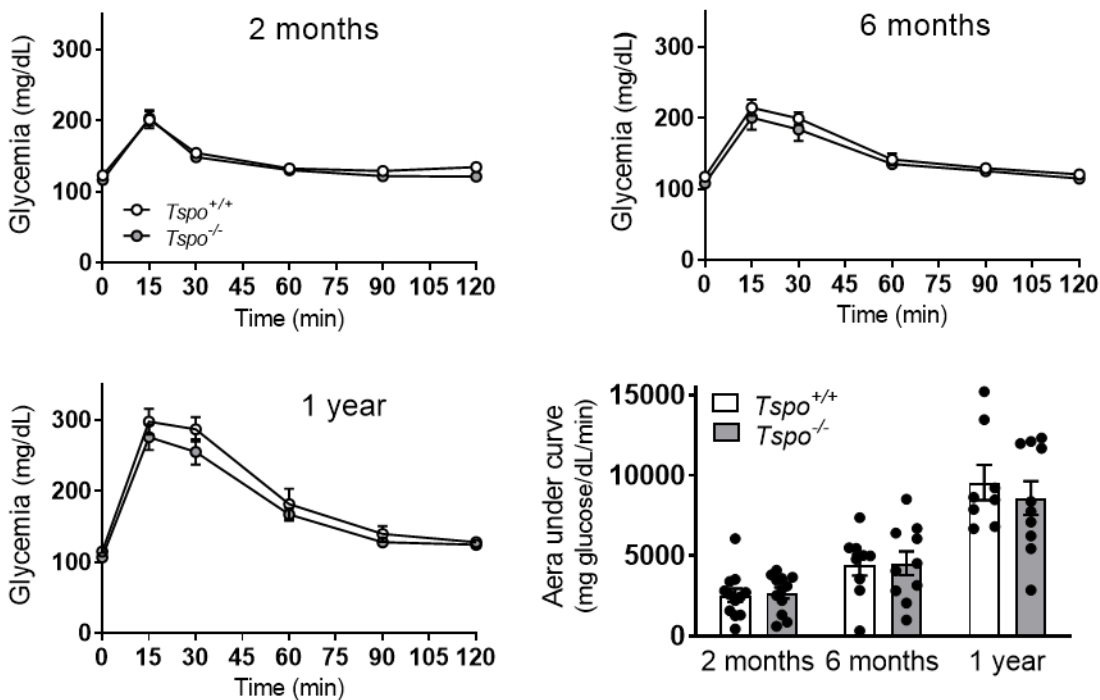


Fig. S4 Glycemia, lipid blood levels and effect of metabolic stresses in $Tspo^{+/+}$ and $Tspo^{-/-}$ rats during aging.

A: glycemia and lipid blood levels in 2-, 6- and 12-month-old rats. Each value is the mean \pm SEM, n=7-10 animals.

B: Insulin tolerance tests performed in 2-, 6- and 12-month $Tspo^{+/+}$ and $Tspo^{-/-}$ rats. At t=0, fasting rats received 1 UI/kg insulin. Glycemia was measured for 2 hours and the areas over the curve (AOC) were calculated (bar graph). Each value is the mean \pm SEM, n=7-9 animals.

C: Glucose tolerance tests performed in 2-, 6- and 12-month $Tspo^{+/+}$ and $Tspo^{-/-}$ rats. At t=0, fasting rats received 1 g/kg D-glucose. Glycemia was measured for 2 hours and the areas under the curve (AUC) were calculated (bar graph).

Each value is the mean \pm SEM, n=8-12 animals. Statistical comparison between genotypes (**A** and bar graphs of **B** and **C**) were done by a two-way ANOVA analysis. No difference between genotypes was observed whatever the parameter.

Fig. S5

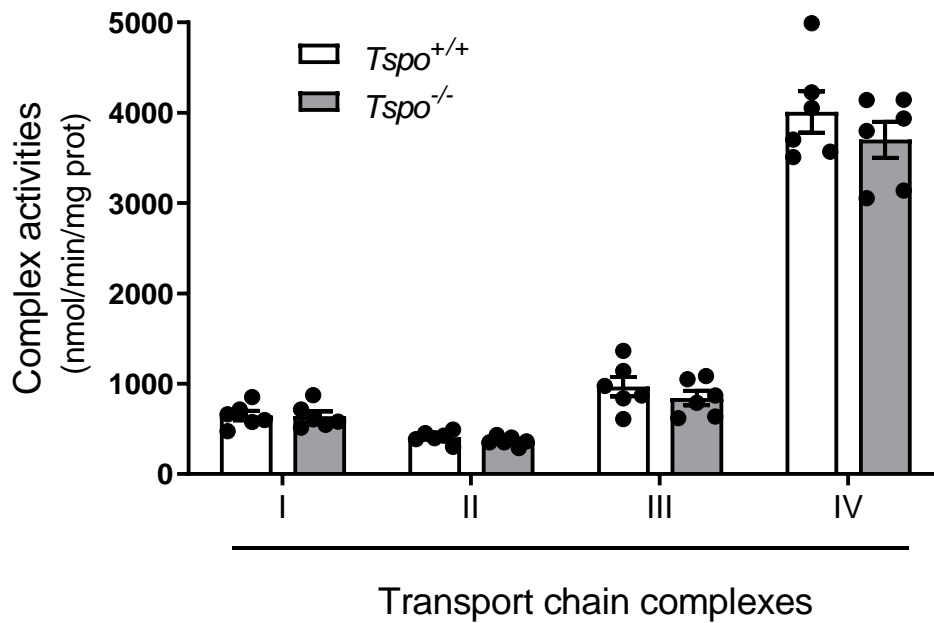


Fig. S5 Activities of transport chain complexes in $Tspo^{+/+}$ and $Tspo^{-/-}$ rats. Enzymatic activities of each respiratory chain complex (nmol/min/mg protein) were measured in mitochondrial fractions prepared from hearts of $Tspo^{+/+}$ and $Tspo^{-/-}$ rats. Each value is the mean \pm SEM of 6 independent preparations (6 animals). Statistical comparison between genotypes was done by using an unpaired two-tailed t-test. No difference between genotypes was observed whatever the complex.