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A TSPO ligand prevents mitochondrial sterol accumulation and dysfunction during myocardial ischemia-reperfusion in hypercholesterolemic rats

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ABSTRACT

A major cause of cell death during myocardial ischemia-reperfusion is mitochondrial dysfunction. We previously showed that the reperfusion of an ischemic myocardium was associated with an accumulation of cholesterol into mitochondria and a concomitant strong generation of auto-oxidized oxysterols. The inhibition of mitochondrial accumulation of cholesterol abolished the formation of oxysterols and prevented mitochondrial injury at reperfusion. The aim of this study was to investigate the impact of hypercholesterolemia on sterol and oxysterol accumulation in rat cardiac cytosols and mitochondria and to analyse the effect of the translocator protein ligand 4′-chlorodiazepam on this accumulation and mitochondrial function. Hypercholesterolemic ZDF fa/fa rats or normocholesterolemic lean rats were submitted to 30 min of coronary artery occlusion followed by 15 min reperfusion where cardiac cytosols and mitochondria were isolated. Hypercholesterolemia increased the cellular cardiac concentrations of cholesterol, cholesterol precursors and oxysterols both in cytosol and mitochondria in non-ischemic conditions. It also amplified the accumulation of all these compounds in cardiac cells and the alteration of mitochondrial function with ischemia-reperfusion. Administration of 4′-chlorodiazepam to ZDF fa/fa rats had no effect on the enhancement of sterols and oxysterols observed in the cytosols but inhibited cholesterol transfer to the mitochondria. It also alleviated the mitochondrial accumulation of all the investigated sterols and oxysterols. This was associated with a restoration of oxidative phosphorylation and a prevention of mitochondrial transition pore opening.

The inhibition of cholesterol accumulation with TSPO ligands represents an interesting strategy to protect the mitochondria during ischemia-reperfusion in hypercholesterolemic conditions.

1. Introduction

A prominent mechanism in cardiac ischemia-reperfusion injury is the onset of the mitochondrial permeability transition. This is caused by the formation of a high conductance pore called the mitochondrial permeability transition pore (mPTP) in the mitochondrial inner membrane under the synergistic effect of high calcium concentration, adenine nucleotide depletion and oxidative stress [1], conditions prevailing during the reperfusion of an ischemic myocardium. The detailed molecular identity of the mPTP remains controversial. Recent studies identified the F0F1 ATPase as a possible major component of the pore [1]. A number of genetic ablation experiments excluded other proteins such as the voltage-dependent anion channel from the composition of the pore. This is also the case for the mitochondrial translocator protein (TSPO) [2] which is a high affinity cholesterol binding protein primarily located in the outer mitochondrial membrane. A body of

Abbreviations: TSPO, translocator protein; ZDF, Zucker diabetic fatty; mPTP, mitochondrial permeability transition pore.

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evidence suggests however the ability of TSPO ligands to regulate physiological cardiac function and to protect the heart during ischemia-reperfusion [3]. Indeed, TSPO ligands such as SSR180575, 4′-chlorodiazepam or TRO40303 were able to protect mitochondrial function, to limit the increase in mitochondrial membrane permeability and to reduce experimental infarct size but the protecting mechanism of these ligands remains to be established [3]. TSPO has long been considered as the transporter of cholesterol to the inner mitochondrial membrane but this property was recently challenged [4,5]. In steroidogenic tissues, it seems more likely that TSPO is a part of a dynamic complex including cytosolic proteins such as the steroidogenic acute regulatory protein and outer mitochondrial membrane proteins including voltage-dependent anion channel [6].

We recently showed that the reperfusion of an ischemic myocardium was associated with an accumulation of cholesterol into mitochondria and a concomitant strong generation of auto-oxidized oxysterols resulting from the oxidation of cholesterol by reactive oxygen species [7]. Oxysterols have been involved in the development of atherosclerosis and genesis of neurodegenerative diseases [8,9] and to cause cell death in various cell lines by increasing oxidative stress [10,11]. In the heart, the TSPO ligands 4′-chlorodiazepam and TRO40403 reduced the mitochondrial accumulation of cholesterol and abolished the formation of oxysterols at reperfusion [7]. This effect may be particularly important in the presence of factors of co-morbidities such like dyslipidemia and hypercholesterolemia which are risk factors for arterial stenosis, thrombosis and myocardial ischemia. Indeed, hypercholesterolemia has been shown to amplify myocardial injury in animal models [12–14] and to impede the ability of cardioprotective strategy to protect the myocardium against infarction [15–18]. Therefore, in addition to its role in the development of atherosclerosis, hypercholesterolemia appears to have direct deleterious effects on cardiac cells [19,20] but the mechanisms involved in are still poorly known.

In the present study, we used Zucker diabetic fatty (ZDF) fa/fa rats to investigate the impact of hypercholesterolemia on the concentrations of sterols and oxysterols in the cytosols and mitochondria of cardiac cells. We also analysed their cardiac cellular accumulation when rats were subjected to myocardial ischemia-reperfusion. In addition, we examined whether the TSPO ligand 4′-chlorodiazepam remained efficient to inhibit the mitochondrial accumulation of cholesterol and oxysterols and to protect mitochondria against ischemia-reperfusion injury in these hypercholesterolemic conditions.

2. Material and methods

2.1. Animals

All animal procedures used in this study were conformed to the Directives of the European Parliament (2010/63/EU-848 EEC). The experimental protocols were reviewed and approved by the Animal Ethics Committee Afssa/ENVA/Universite Paris Est-Créteil (approval number: 08/03/11-05). The Zucker diabetic fatty (ZDF) fa/fa rat was used as an animal model of hypercholesterolemia and was compared to ZDF lean (+/?) rat. Thirteen week old male animals were supplied by Charles River laboratories (L'arbresle, France). They were housed in an air-conditioned room with a 12 h light–dark cycle and received standard rodent chow (ZDF lean), a Purina 5008 diet (ZDF fa/fa) and drinking water ad libitum.

2.2. Isolation of mitochondria and cytosols

The left ventricle from sham rats (noninfarcted) or from the area at risk of rats that underwent ischemia-reperfusion were minced and homogenized with an Ultra-Turrax T25 (set at position 5; IKA-Werke GmbH & Co. KG, Staufen, Germany) for 5 s in a buffer containing 300 mM sucrose, 10 mM HEPES and 2 mM EGTA, pH 7.4 at 4 °C. This was followed by repeated passes in a Potter-Elvehjem glass homogenizer (motor-driven Teflon pestle set at 1500 rpm) in a final volume of 15 ml. Cardiac homogenates were centrifuged at 1000*g* for 5 min at 4 °C, and the supernatants were centrifuged at 10,000*g* for 10 min at 4 °C. The pellets, containing mitochondria, were resuspended in the MSH buffer (220 mM mannitol, 70 mM sucrose, 10 mM HEPES and 10 μM EGTA, pH 7.4 at 4 °C) to obtain a protein concentration of about 15 mg protein/ml. Isolated mitochondria were kept on ice until use. The supernatants were centrifuged at 100,000*g* for 60 min at 4 °C. The pellets were discarded and the supernatants, corresponding to the cytosols, were frozen at −80 °C after determination of the protein concentration. Protein concentrations were determined by the Advanced Protein Assay Reagent (57697, Sigma-Aldrich, St. Louis, MO, USA).

2.3. Mitochondrial oxygen consumption

Mitochondrial oxygen consumption was measured at 30 °C with a Clark-type electrode (Hansatech Instruments Ltd, Norfolk, UK). Mitochondria (0.4 mg protein/ml) were incubated in a respiration buffer containing 100 mM KCl, 50 mM sucrose, 10 mM HEPES and 5 mM KH2PO4, pH 7.4 at 30 °C. Respiration was initiated by addition of 2.5 mM pyruvate/2.5 mM malate (state 2 respiration rate). After 1 min, ATP synthesis was induced by addition of 1 mM ADP (state 3 respiration rate). Upon depletion of ADP, the rate of state 4 respiration was measured, and the respiratory control ratio (state 3/state 4) was evaluated.

2.4. Evaluation of mitochondrial permeability transition pore (mPTP) opening

mPTP opening was assessed by monitoring mitochondrial calcium retention capacity. Mitochondria were loaded with increasing concentrations of calcium until the load reached a threshold at which mitochondria underwent a fast process of calcium release, which was due to mPTP opening as previously described [21]. Cardiac mitochondria (1 mg/ml), energized with 5 mM pyruvate/malate, were incubated in the respiration buffer supplemented with 1 μM Calcium Green-5N fluorescent probe (C3737, Invitrogen, Eugene, OR, USA). The concentration of calcium in the extramitochondrial medium was monitored by means of a Jasco FP-6300 spectrofluorimeter (Jasco, Bouguenais, France) at excitation and emission wavelengths of 506 and 532 nm, respectively. The calcium signal was calibrated by addition to the medium of known calcium amounts.

2.5. Determination of mitochondrial sterol and oxysterol levels by isotope dilution-mass spectrometry analysis

Sterol and oxysterol measurements were performed on mitochondria extracts. To a screw-capped vial sealed with a Teflon septum, 150 μl of mitochondria were added together with 2.5 μg of D6-cholesterol (D-3373, CDN Isotopes Inc., Pointe-Claire, Canada), 20 ng of D7-7α-hydroxycholesterol (D-4064, CDN Isotopes Inc.), D7-7β-hydroxycholesterol (D-4123, CDN Isotopes Inc.), D7-7ketocholesterol (700046P, Avanti Polar Lipids, Alabaster, AL 35007-9105, USA), D6-cholesterol-5α,6α-epoxide (700047P, Avanti Polar Lipids), D6-cholesterol-5β,6β-epoxide (700014P, Avanti Polar Lipids), and D6-27-hydroxycholesterol (700059P, Avanti Polar, Lipids) as internal standards, 50 μl of butylated hydroxytoluene (5 g/l, Sigma-Aldrich, St. Louis, MO, USA) and 50 μl of K3-EDTA (10 g/l, Sigma-Aldrich) to prevent auto oxidation. Each vial was flushed with argon for 20 min to remove air. Alkaline