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Differences in the profile of protection afforded by TRO40303 and mild hypothermia in models of cardiac ischemia/reperfusion injury

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ABSTRACT

The mode of protection against cardiac reperfusion injury by mild hypothermia and TRO40303 was investigated in various experimental models and compared to MitoQ *in vitro*.

In isolated cardiomyocytes subjected to hypoxia/reoxygenation, TRO40303, MitoQ and mild hypothermia delayed mPTP opening, inhibited generation of mitochondrial superoxide anions at reoxygenation and improved cell survival. Mild hypothermia, but not MitoQ and TRO40303, provided protection in a metabolic starvation model in H9c2 cells and preserved respiratory function in isolated rat heart mitochondria submitted to anoxia/reoxygenation. In the Langendorff-perfused rat heart, only mild hypothermia provided protection of hemodynamic function and reduced infarct size following ischemia/reperfusion. In biopsies from the left ventricle of pigs subjected to *in vivo* occlusion/reperfusion, TRO40303 specifically preserved respiratory functions in the peri-infarct zone whereas mild hypothermia preserved both the ischemic core area and the peri-infarct zones. Additionally in this pig model, only hypothermia reduced infarct size.

We conclude that mild hypothermia provided protection in all models by reducing the detrimental effects of ischemia, and when initiated before occlusion, reduced subsequent reperfusion damage leading to a smaller infarct. By contrast, although TRO40303 provided similar protection to MitoQ *in vitro* and offered specific protection against some aspects of reperfusion injury *in vivo*, this was insufficient to reduce infarct size.

1. Introduction

The treatment of acute myocardial infarction (AMI) involves restoring blood flow to the ischemic myocardium as soon as possible using thrombolytics or percutaneous coronary intervention (PCI). The sudden re-supply of oxygen and nutrients saves a large part of the heart leading to a limited infarct extension. However, in the peri-infarct zone, it can exert a deleterious effect on cardiomyocytes localized in the ischemic area at risk, where cells may die due to oxidative stress, calcium overload, opening of the mitochondrial permeability transition pore (mPTP) and further induction of apoptosis or necrosis depending on the ATP level at that stage of the injury. This process is known as reperfusion injury (Garcia-Dorado et al., 2009; Yellon and Hausenloy, 2007).

Abbreviations: AMI, acute myocardial infarction; ANOVA, Analysis of Variance; AAR, area at risk; CF, constant flow; DMSO, dimethyl sulfoxide; LDH, Lactate dehydrogenase; MAS, Mitochondrial Assay Solution; mPT, mitochondrial permeability transition; mPTP, mitochondrial permeability transition pore; OCR, oxygen consumption rate; PCI, percutaneous coronary intervention; RCR, respiratory control ratio; TSPO, translocator protein; TTC, triphenyltetrazolium chloride

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In both preclinical models and small clinical trials, several new approaches have been proposed to reduce reperfusion injury (Atar et al., 2009; Gotberg et al., 2010; Piot et al., 2008; Staat et al., 2005). However, these techniques have not yet been fully validated and are not used in general practice.

TRO40303 is a newly discovered cytoprotective compound that reduces mitochondrial permeability transition (mPT) and decreases infarct size in rodent models of reperfusion injury (Le Lamer et al., 2014; Schaller et al., 2010). As part of a European consortium, TRO40303 was used as a tool to further study reperfusion injury both on the clinic and in preclinical models in order to gain knowledge on translational aspects in this pathology.

TRO40303 had been evaluated in a phase I trial (Le Lamer et al., 2014) and was tested in a phase II clinical trial in patients undergoing primary PCI following an acute ST-elevated myocardial infarction (STEMI) (The MITOCARE Study Group, 2012). The results showed that TRO40303 was not able to reduce infarct size (Atar et al., 2014).

In the present part of the work, the effects of TRO40303 were compared with two controls: hypothermia, that has been shown to be a potent cardioprotective technique in animal models of ischemia/reperfusion injury (Chien et al., 1994; Duncker et al., 1996; Schwartz et al., 1997; Tissier et al., 2009; van den Doel et al., 1998); and the mitochondria targeted anti-oxidant, MitoQ (Neuzil et al., 2007), because reoxygenation is associated with an increase in reactive oxygen species (Di Lisa and Bernardi, 2006; Halestrap, 2006) on which TRO40303 might act (Le Lamer et al., 2014; Schaller et al., 2010).

The effects of TRO40303, mild hypothermia and MitoQ on ischemia/reoxygenation induced reactive oxygen species production, mPT, and subsequent cardiac cell death were studied in several *in vitro* model systems. These included isolated rat cardiomyocytes subjected to *in vitro* hypoxia/reoxygenation, H9c2 cells subjected to metabolic starvation and isolated cardiac mitochondria submitted to anoxia/reoxygenation. Additional models explored the effects of some of these treatments. The Langendorff isolated rat heart was used to evaluate hemodynamic functional recovery and infarct size following ischemia/reperfusion. The pig model was used to evaluate infarct size and mitochondrial respiratory function in heart tissue biopsies following *in vivo* ischemia/reperfusion.

2. Materials and methods

2.1. Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless specified. TRO40303 (3,5-seco-4-nor-cholestan-5-one oxime-3-ol) (Schaller et al., 2010) was synthesized by Synkem (Dijon, France) and MitoQ (10-(4,5-dimethoxy-2-methyl-3,6-dioxocyclohexa-1,4-dien-1-yl) decyl-triphenylphosphanium;methanesulfonate) was synthesized by Idealp (Lyon, France). For the *in vitro* and *ex vivo* tests, the compounds were dissolved in dimethyl sulfoxide (DMSO) and for *in vivo* administration in pig, it was prepared as a 16.3 mg/ml solution in liposomes (Northern Lipids Inc, Burnaby, Canada).

2.2. Animals

All animal procedures used were in strict accordance with the Directive 2010/63/EU of the European Parliament. Trophos, INSERM/Institut Mondor de Recherche Biomédicale, University of Bristol and Lund University, had valid licenses and ethical approval for animal experimentation (agreements B13-055-15, C13-055-06, C94-028-245) delivered by the French government, the Bristol ethics committee (UB/09/012), and the local Swedish ethics committee (M179-07). Additionally, within the MITOCARE consortium, a Safety and Ethics monitoring Committee composed of an independent data protection specialist and an ethics specialist was set-up in order to ensure ethics of the project. Hearts excised from male Wistar rats were used for isolation of cardiomyocytes (Janvier, Le Genest Saint Isle, France, 250–280 g) and for the *ex vivo* rat heart perfusion models (Harlan UK Ltd, 225–300 g). Hearts excised from male Sprague–Dawley rats were used for isolation of cardiac mitochondria (Janvier, Le Genest Saint Isle, France, 250–280 g). Healthy domestic male and female pigs from a local farm weighing 40–50 kg were used for the *in vivo* ischemia/reperfusion protocol in pigs. Animals were maintained in the local animal house under conventional conditions, in a room with controlled temperature (21–25 °C), a reverse 12 h light/dark cycle for rats, and with food and water available *ad libitum*.

2.3. Isolation of adult rat cardiomyocytes

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

2.4. Model of hypoxia/reoxygenation in adult rat cardiomyocytes

The cardiomyocytes were placed into a thermo-stated (37 °C) chamber (Warner Instruments Inc, Connecticut) which was mounted on the stage of a IX81 Olympus microscope (Olympus, Center Valley, Pennsylvania) and were perfused with a Tyrode's solution (in mM: NaCl 130; KCl 5; HEPES 10; $MgCl_2$ 1; $CaCl_2$ 1.8, pH 7.4 at 37 °C) at a rate of 0.5 ml/min. The chamber was connected to a gas bottle diffusing a constant stream of O_2 (21%), N_2 (74%) and CO_2 (5%) maintaining an O_2 concentration of 21%. Oxygen in the perfusate was measured in the chamber using a fibre optic sensor system (Ocean Optics Inc., Florida). Cardiomyocytes were paced to beat by field stimulation (5 ms, 0.5 Hz).

To simulate ischemia, the perfusion was stopped and cardiomyocytes exposed for 2 h to a hypoxic medium maintaining an O_2 concentration of 2–3%. This medium was the Tyrode's solution (bubbled with 100% N_2) supplemented with 20 mM 2-deoxyglucose and subjected to a constant stream of N_2 (100%). At the end of the ischemic period, reoxygenation was induced by restoring rapidly the Tyrode's flow and 21% O_2 in the chamber.

TRO40303 (3 μM) was added to the cardiomyocytes 15 min before reoxygenation and this concentration was maintained in the reoxygenation medium during the first 10 min of reoxygenation. MitoQ (0.2 μM) was added to the cardiomyocytes 30 min before the induction of the hypoxia. In both cases, doses were based on previous experience with TRO40303 (Schaller et al., 2010) or preliminary