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Cardioprotective effects of intracoronary administration of 4-chlorodiazepam in small and large animal models of ischemia-reperfusion

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ABSTRACT

Background: The Translocator Protein (TSPO) of the mitochondrial membrane has been recognized as a potential therapeutic target for mitigation of myocardial ischemia-reperfusion injury. Administration of 4-chlorodiazepam (4-CLD), a TSPO ligand, has been shown to confer acute cardioprotective effects in small animals; however, long-term studies and studies in clinically-relevant large animal models are lacking. In the present study we investigated a potential cardioprotective effect of intracoronary administration of 4-CLD in small and large animal models of ischemia-reperfusion.

Methods: Acute myocardial infarction was induced in 38 Wistar rats and 29 farm pigs by ligation of the left anterior descending coronary artery, followed by reperfusion. Animals were randomized to undergo intracoronary infusion of 2 μM 4-CLD or vehicle just prior (pigs) or immediately after (rats) reperfusion. Infarcted rats were euthanized either after 1 h of reperfusion (for histological assessment of the "no reflow" area) or after 60 days (for serial evaluation of cardiac function and structure by echocardiography and assessment of infarct size). Infarcted pigs were euthanized after 2 h of reperfusion for histological assessment of infarct size and "no reflow" area.

Results: In infarcted rats, intracoronary infusion of 4-CLD resulted in acute reduction of the "no reflow" area and conferred durable long-term structural and functional benefits (reduction in infarct size, attenuation of adverse remodeling, improvement in global systolic function). In infarcted pigs, intracoronary infusion of 4-CLD was well-tolerated from a hemodynamic standpoint and resulted in acute reduction in infarct size, reduction in "no reflow" area and more rapid resolution of ST-segment elevation.

Conclusions: In a rat model of myocardial infarction, intracoronary administration of 4-CLD attenuated the "no reflow" phenomenon and produced long-term structural and functional benefits. In a porcine model of myocardial infarction intracoronary administration of 4-CLD did not raise safety concerns and conferred acute cardioprotective effects.

1. Introduction

Prolonged myocardial ischemia results in irreversible cardiac injury and cell death. While timely reperfusion of ischemic myocardium remains the optimal treatment [1,2], restoration of blood flow in the ischemic region may paradoxically aggravate myocardial injury, a phenomenon termed "reperfusion injury" [3,4]. An important component of ischemia-reperfusion injury is endothelial cell damage, [5] resulting in microvascular obstruction and development of "no reflow" areas post-reperfusion. The "no reflow" phenomenon (i.e. development of microvascular obstruction despite of restoration of epicardial blood flow) is frequently observed in the clinical setting [6], and is associated with progression of left ventricular remodeling, development of congestive heart failure and premature death [7–10].

While the pathogenesis of myocardial reperfusion injury is complex, mitochondrial dysfunction appears to play a pivotal role [11]. Recently, the outer membrane mitochondrial peripheral benzodiazepine receptor (PBR), also known as translocator protein (TSPO), has been recognized as an important mediator of ischemia-reperfusion injury in cardiac

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myocytes [12]. Among the mechanisms through which PBRs have been shown to mediate myocardial ischemia-reperfusion injury is the regulation of the opening of mitochondrial permeability transition pore (a key step in development of reperfusion injury); this pivotal role of PBRs renders them an attractive target for therapeutic interventions [11–13].

4-Chlorodiazepam (4-CLD) is a benzodiazepine derivative of diazepam. Unlike most benzodiazepine derivatives, it lacks affinity for $GABA_A$ receptors and does not exert typical benzodiazepine effects [14]. 4-CLD is a potent ligand of TSPO, considered to exert both agonist and antagonist effects [15,16]. Studies in isolated hearts and acute small animal experiments suggest that 4-CLD confers acute cardioprotective effects [17–22]. However, long-term studies and studies in clinically-relevant large animal models are lacking. In the present study we investigated a potential cardioprotective effect of intracoronary administration of 4-CLD in small and large animal models of ischemia-reperfusion.

2. Methods

2.1. Ethical statement

All animals used in this study received humane care in compliance with the guidelines for care and use of laboratory animals of the US National Institutes of Health. The experimental protocol was approved by the Directorate of Agricultural & Veterinary Policy of the Region of Attica (protocol no. 2635/18-04-2012). Male Wistar rats, weighing 300 to 350 g, were used in the small animal study. Male farm pigs, weighing 30 to 40 kg, were used in the large animal study.

2.2. Small animal model

2.2.1. Surgical preparation and experimental protocol

Anesthesia was induced with 4% isoflurane inhalation and maintained with 2% inhalation. The heart was exposed through a left lateral thoracotomy at the fourth intercostal space, unfractionated heparin (100 IU/kg) was administered intraventricularly for prevention of thrombogenesis and myocardial infarction was induced by temporary ligation (45 min) of the mid portion of the left anterior descending coronary artery (LAD) using a silk 5.0 mm suture. 10 min prior to reperfusion animals were randomly assigned to two groups: 1) control group, n = 16; or 2) 4-CLD group, n = 16. Randomization of the animals was performed in permuted blocks of 4 experiments with the use of lot picks from an opaque box. The randomization process was performed by the laboratory technician, who was also responsible for preparing the solution or injection. After 45 min of ischemia the LAD ligation was removed, the inferior vena cava and the aorta were temporarily occluded (15 s) using a 2.0 mm silk suture [23], and animals underwent intraventricular injection (using a 28 G needle) of either 200 μL PBS (control group) or 2 μM of 4-CLD suspended in 200 μL of PBS (4-CLD group).

The animals underwent a 60-min reperfusion period. 10 min prior to completion of the 60-min reperfusion period, animals were randomized (1:1, in permuted blocks of 4 experiments with the use of lot picks from an opaque box) to the acute or the chronic experimental protocol (described below):

1. *Acute protocol*: After completion of 60 min of reperfusion, the inferior vena cava was electively catheterized with a 28 G venous catheter and 1% Thioflavin S (500 μL) was infused. Thioflavin S is a fluorescent dye, which stains endothelium receiving optimal blood flow; hypoperfused or non-perfused areas (i.e. areas of "no-reflow") are not stained and appear as non-fluorescent regions. Thioflavin S staining is an established method for assessing the extent of "no-reflow" [5,24–26]. 30 sec. later, the LAD was re-occluded (at the same site as during ischemia) and Gentian violet (500 μL) was infused for visualization of the area at risk. Gentian violet stains the non-ischemic zone blue; the region not perfused by Gentian violet (i.e., the region that does not stain blue) represents the ischemic area (area at risk). Immediately after the infusion of dyes animals were euthanized via decapitation and the heart was explanted. The LV, including the septum, was separated from the remainder of the heart and cut into 2-mm-thick sections perpendicular to the apex-base axis. The acute protocol is depicted schematically in Fig. 1A.
2. *Chronic protocol*: After completion of 60 min of reperfusion, the thoracic cavity was closed and animals were allowed to recover. Analgesia with subcutaneous administration of meloxicam (1 mg/kg) was provided to all animals. The experimental animals were followed over a 60-day period and underwent transthoracic echocardiography at 2 and 60 days post-MI. The animals were anesthetized prior to the echocardiographic study with 4% isoflurane inhalation and anesthesia was maintained throughout the study with 2% inhalation. Following completion of transthoracic echocardiography at day 60 post-MI animals were euthanized, while still under general anesthesia, via decapitation. The LV, including the septum, was separated from the remainder of the heart and cut into 2-mm-thick sections perpendicular to the apex-base axis. The chronic protocol is depicted schematically in Fig. 1B.

2.2.2. Echocardiography

Transthoracic echocardiography (Vivid Q GE Healthcare, Buckinghamshire, United Kingdom) was performed in 8 rats in each group to assess cardiac structure and function at 2 and 60 days post-MI. We chose to perform the baseline assessment of cardiac function at 2 days post-MI (rather than immediately post-reperfusion) in order to avoid any confounding effects of myocardial stunning occurring early post-reperfusion. During image acquisition heart rate was monitored and the animal's body temperature was maintained constantly at 37 ± 0.5 °C. Two dimensional long axis images were used for end-diastolic and end-systolic volume measurements and ejection fraction was calculated as the difference between end-diastolic and end-systolic volumes normalized to end-diastolic volume.

2.2.3. Dyes and morphometric measurements

In the acute protocol, the left ventricle area at risk was identified by the absence of Gentian violet dye staining. Afterwards, sections were examined in a dark room under ultraviolet light (365 nm wave length) and the area of "no-reflow" was traced as the non-fluorescent area (not stained by thioflavin) within the area at risk. All slices were weighed. For each experiment, the area at risk (AR) was calculated as a percentage of the mass of the left ventricle. The area of "no reflow" was calculated as a percentage of the AR. In the

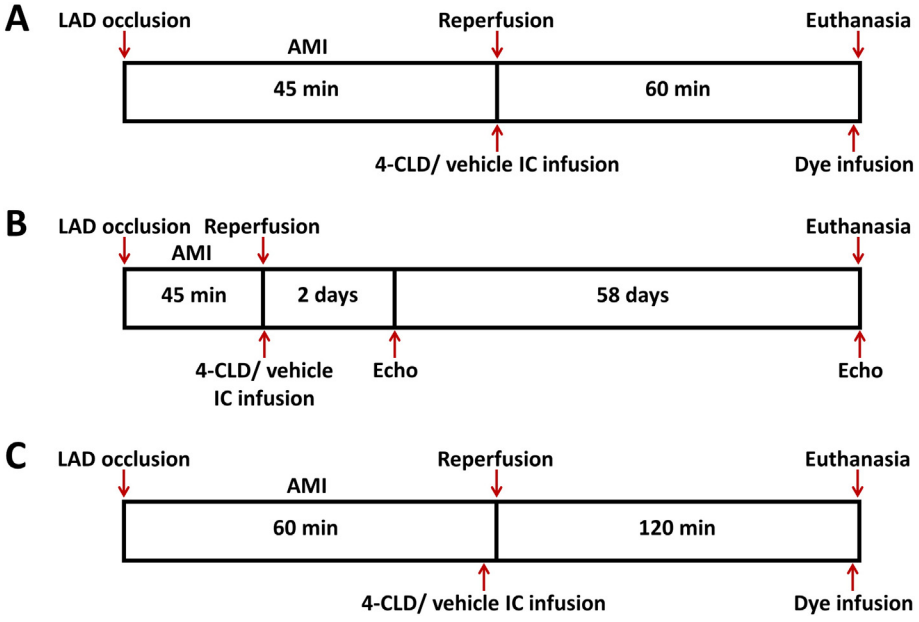

Fig. 1. Study protocol. Schematic depiction of the acute rat study protocol (A), the chronic rat study protocol (B) and the pig study protocol (C). (AMI: acute myocardial infarction; LAD: left anterior descending artery; and IC: intracoronary).