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Autophagosome formation is required for cardioprotection by chloramphenicol

Zoltán Giricz[a,*], Zoltán V. Varga[a], Gábor Koncsos[a], Csilla Terézia Nagy[a], Anikó Görbe[a], Robert M. Mentzer Jr[b], Roberta A. Gottlieb[b], Péter Ferdinandy[a,c]

[a] Cardiovascular and Metabolic Research Group, Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest, Hungary
[b] Heart Institute, Cedars-Sinai Medical Center, CA, USA
[c] Pharmahungary Group, Szeged, Hungary

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ABSTRACT

Aims: Chloramphenicol (CAP), a broad spectrum antibiotic, was shown to protect the heart against ischemia/reperfusion (I/R) injury. CAP also induces autophagy, however, it is not known whether CAP-induced cardioprotection is mediated by autophagy. Therefore, here we aimed to assess whether activation of autophagy is required for the infarct size limiting effect of CAP and to identify which component of CAP-induced autophagy contributes to cardioprotection against I/R injury.
Main methods: Hearts of Sprague-Dawley rats were perfused in Langendorff mode with Krebs-Henseleit solution containing either vehicle (CON), 300 μM CAP (CAP), CAP and an inhibitor of autophagosome-lysosome fusion chloroquine (CAP + CQ), or an inhibitor of autophagosome formation, the functional null mutant TAT-HA-Atg5^{K130R} protein (CAP + K130R), and K130R or CQ alone, respectively. After 35 min of aerobic perfusion, hearts were subjected to 30 min global ischemia and 2 h reperfusion. Autophagy was determined by immunoblot against LC3 from left atrial tissue. Infarct size was measured by TTC staining, coronary flow was measured, and the release of creatine kinase (CK) was assessed from the coronary effluent.
Key findings: CAP treatment induced autophagy, increased phosphorylation of Erk1/2 in the myocardium and significantly reduced infarct size and CK release. Autophagy inhibitor TAT-HA-Atg5^{K130R} abolished cardioprotection by CAP, while in CAP + CQ hearts infarct size and CK release were reduced similarly to as seen in the CAP-treated group.
Conclusion: This is the first demonstration that autophagosome formation but not autophagosomal clearance is required for CAP-induced cardioprotection.
Significance: Inducing autophagy sequestration might yield novel therapeutic options against acute ischemia/reperfusion injury.

1. Introduction

Autophagy is an intracellular degradation process which eliminates dysfunctional organelles and long-lived proteins through lysosomal breakdown [1]. During starvation degradation of intracellular components promotes cell survival by maintaining cellular energy levels. Autophagy consists of sequential steps: the early stage, which includes initiation of the autophagosomal membrane and autophagosome formation, and the late stage, where autophagosome-lysosome fusion and lysosomal degradation happen [2,3]. Autophagy has a constitutive, low activity under normal conditions in most cells, including cardiac myocytes. A growing amount of evidence suggests that increased autophagy plays a role in cardioprotective interventions [4,5].

Previous studies reported that several therapeutic agents which are already in clinical use, for example hydrophobic statins [6], sevoflurane [7], sulfaphenazole [8] and certain antibiotics, such as chloramphenicol (CAP) [9], may also induce autophagy in addition to their primary effects. Since previous studies reported that CAP protects the heart against ischemia/reperfusion injury and upregulates autophagy markers [10–12], we hypothesized that CAP induces cardioprotection *via* induction of autophagy.

It has been shown that the induction of autophagy is required for cardioprotective mechanisms but no detailed investigation has been performed on which stage of autophagy is necessary for

* Corresponding author at: Cardiometabolic Research Group, Department of Pharmacology and Pharmacotherapy, Semmelweis University, Nagyvárad tér 4, Budapest H-1089, Hungary.
E-mail address: giricz.zoltan@med.semmelweis-univ.hu (Z. Giricz).

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cardioprotection. Since autophagy is a multi-step process, it can be inhibited at different steps. Previous studies have shown that inhibition of early-stage autophagy can be achieved by 3-methyladenine [13] or TAT-HA-Atg5^{K130R}, a dominant negative mutant fusion protein of a key mediator of autophagy, Atg5 [14–16]. The late phase of autophagy, lysosomal fusion and degradation, can be arrested by elevating lysosomal pH, *e.g.*, by the use of an antimalarial drug, chloroquine (CQ, see for review: [17]). Although a few studies utilized these substances to investigate the mechanism of cardioprotective stimuli, it is unknown which phase of CAP-induced autophagy is necessary for cardioprotection.

Therefore, in this study we aimed to investigate whether CAP-induced autophagy is necessary for cardioprotection. Furthermore, we assessed whether sequestration and/or degradation phases of autophagy are necessary for the cardioprotective effect of CAP.

2. Materials and methods

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and was approved by the animal ethics committee of the San Diego State University, San Diego, California and Semmelweis University, Budapest, Hungary. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless indicated otherwise.

2.1. Study design

To identify the most suitable model system, in a pilot study we examined the effect of CAP on autophagy in neonatal rat cardiomyocytes (NRCMs), in H9c2 cardiac myoblast cells and in isolated hearts. We found that CAP induced autophagy in isolated hearts but not in NRCMs or in H9c2 cells (see Fig. 1A–C). The efficacy of TAT-HA-Atg5^{K130R} was also assessed in a pilot experiment where we observed that in the left atrium LC3-II/I ratio was decreased after 15 min of administration of 200 nM TAT-HA-Atg5^{K130R} to isolated hearts as compared to vehicle controls (Fig. 1D).

Therefore, in the main series of experiments, we used an *ex vivo* model of acute cardiac ischemia/reperfusion injury to assess the effects of autophagy inhibitors (TAT-HA-Atg5^{K130R} and CQ) on CAP-induced cardioprotection. Since the availability of TAT-HA-Atg5^{K130R} was limited due to technical limitations in production and purification of the protein in quantities necessary for *ex vivo* heart perfusion experiments, we had to reduce the number of isolated hearts treated with TAT-HA-Atg5^{K130R}.

2.2. Isolation and treatment of NRCMs

Preparation of NRCM culture used in the pilot study was described before in details [18]. Briefly, neonatal rats were sacrificed by cervical dislocation and hearts were removed and placed into ice-cold phosphate buffered saline (PBS) solution. Ventricles were minced and resuspended in 0.25% trypsin (Thermo Fisher Scientific, USA) solution. Digested tissue was centrifuged at 400 $\times g$, for 15 min at 4 °C. Cell pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-glutamine, Antibiotic-Antimycotic solution and 10% fetal bovine serum (FBS, Thermo Fisher Scientific, USA). Cells were counted in a hemocytometer, and seeded into 75 cm^2 flasks at a density of 4×10^6 cell/flask. After 24 h, the growth medium was replaced with differentiation medium containing 1% FBS. Cardiomyocytes were kept under normoxic conditions (37 °C, in 95% air and 5% CO_2 gas mixture) for three days prior to treatment and cell collection.

On the 3rd day of culturing, cell medium was supplemented with vehicle (saline, 5% *v*/v) or 300 μM CAP for 1 h. Then cell cultures were washed with ice cold PBS and lysed for 5 min in 500 μL homogenization buffer (1 × Radio-Immunoprecipitation Assay Buffer, RIPA, supplemented with a protease and phosphatase inhibitor cocktail). Lysed cells were scraped, collected and sonicated with an ultrasonic homogenizer (Ultrasonic Processor UP200H, Hielscher, USA) for 10 s on ice. The homogenate was centrifuged at 10,000 $\times g$, 4 °C for 10 min; and the supernatant was collected and stored at − 80 °C. Protein concentration was assessed by Bicinchoninic Acid Protein Assay Kit (BCA Protein Assay kit, Pierce, USA).

2.3. Treatment of H9c2 cells

H9c2 cells were seeded in T25 flasks (10^6 cells/flask) in 6 mL DMEM supplemented with 10% FBS, Antibiotic-Antimycotic solution, glucose, MEM Non-Essential Amino Acid Solution and L-glutamine. Then incubated at 37 °C in 5% CO_2. After 24 h cells were treated with 300 μM CAP or vehicle (CON). Cells were incubated for 1 h, then scraped in 200 μL RIPA lysis buffer and sonicated (Ultrasonic Processor UP200H, Hielscher, USA) for 3 × 15 s at 50% power. Homogenates were centrifuged for 10 min at 10,000 $\times g$, 4 °C. Supernatants were aliquoted and stored at − 80 °C. Protein concentration was assessed with BCA kit.

2.4. Preparation of K130R

TAT-HA-Atg5^{K130R} protein was purified from BL21(DE3)pLysS *E. coli* bacteria transformed with pTAT-HA-Atg5^{K130R} plasmid as described elsewhere [8]. Briefly, crude cellular extract was purified on a Ni-NTA column (Thermo Fisher Scientific, USA) followed by desalting on a PD-10 column (GE Healthcare, United Kingdom) into PBS. Purified protein was used immediately after assessing its concentration by the BCA method.

2.5. Ex vivo heart perfusion

Sprague-Dawley rats (250–300 g) were anesthetized with i.p. pentobarbital (30 mg/kg) and anticoagulated with i.v. heparin (100 U/kg). Hearts were excised and perfused in Langendorff mode with Krebs-Henseleit solution (KH) for 15 min [19]. From the 15th min, a group of heart received KH containing 300 μM CAP. To inhibit CAP-induced autophagosome formation a group of hearts were perfused with KH containing 300 μM CAP and 200 nM cell-permeable recombinant TAT-HA-Atg5^{K130R} (CAP + K130R) for 15 min at the beginning of the protocol, then received CAP alone.

Another group of hearts received 300 μM CAP and 10 μM CQ (CAP + CQ) throughout the protocol to inhibit lysosomal degradation of CAP-induced autophagosomes. Further groups of hearts were perfused with 10 μM CQ or 200 nM TAT-HA-Atg5^{K130R} alone (CQ and K130R, respectively; see Fig. 2).

2.6. Measurement of infarct size, coronary flow and creatine kinase release

At the end of perfusion, hearts were sliced into 2 mm-thick slices, and right ventricles were removed. Slices were immersed in 1% triphenyltetrazolium-chloride for 20 min, then in 4% formalin for 24 h and scanned. Weight of slices was measured. Necrotic area was evaluated by planimetry (ImageJ). Data is expressed as a percentage of left ventricular mass. Coronary flow rate was measured throughout the protocol by timed collection of coronary effluent. Creatine kinase (CK) release was measured in the coronary effluent collected from 10 min to 20 min after the onset of the reperfusion by a colorimetric assay (Stanbio™ CK Liqui-UV™ Test, Stanbio Laboratory, USA).

2.7. Western blotting

After 35 min aerobic perfusion, left atria or whole hearts were snap frozen. Samples were lysed in 500 μL RIPA buffer with Tissuelyser LT (Quiagen, Germany) at 4 °C. Lysates were centrifuged at 10,000 $\times g$ at

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