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Autophagosome formation is required for cardioprotection by chloramphenicol

Zoltán Giricz[1,*], **Zoltán V. Varga**[1], **Gábor Koncsos**[1], **Csilla Terézia Nagy**[1], **Anikó Görbe**[1], **Robert M. Mentzer Jr.**[2], **Roberta A. Gottlieb**[2], and **Péter Ferdinandy**[1,3]

[1]Cardiometabolic Research Group, Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest, Hungary

[2]Heart Institute, Cedars-Sinai Medical Center, CA. USA

[3]Pharmahungary Group, Szeged, Hungary

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), 300μM 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.

[*]Corresponding author: Zoltán Giricz, Cardiometabolic Research Group, Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest, Hungary, Nagyvárad tér 4. H-1089, Tel.: +36 1 2104416; fax: +36 1 2104412, giricz.zoltan@med.semmelweis-univ.hu.

Declaration of Conflicting Interests

The authors declare that there is no conflict of interest.

Keywords

(3-5) autophagy; cardioprotection; chloramphenicol

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 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 200nM 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×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 hours, 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 300μM CAP for 1 hour. Then cell cultures were washed with ice cold PBS and lysed for 5 minutes in 500μL homogenization buffer (1x 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 seconds on ice. The homogenate was centrifuged at 10,000×g, 4°C for 10 minutes; 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 6mL 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 hours cells were treated with 300μM CAP or vehicle (CON). Cells were incubated for 1 h, then scraped in 200μL RIPA lysis buffer and sonicated (Ultrasonic Processor UP200H, Hielscher, USA) for

3×15 sec at 50% power. Homogenates were centrifuged for 10 min at 10,000×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 (250-300g) were anesthetized with i.p. pentobarbital (30 mg/kg) and anticoagulated with i.v. heparin (100U/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 300μM CAP. To inhibit CAP-induced autophagosome formation a group of hearts were perfused with KH containing 300μM CAP and 200nM 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 300μM CAP and 10μM CQ (CAP+CQ) throughout the protocol to inhibit lysosomal degradation of CAP-induced autophagosomes. Further groups of hearts were perfused with 10μM CQ or 200nM 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 2mm-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 500μL RIPA buffer with Tissuelyser LT (Quiagen, Germany) at 4°C. Lysates were centrifuged at 10,000×g at 4°C for 15 min. Supernatants were aliquoted and stored at −80°C. Protein concentration was assessed by BCA kit, then samples containing 40μg protein were prepared in Laemmli-buffer and electrophoresed on 4-12% polyacrylamide gels (Thermo Fisher Scientific, USA), then transferred to polyvinylidene difluoride membranes. Even loading was assessed with Ponceau staining. Membranes were blocked with 5% non-fat milk or 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.05% Tween 20 (TBST) for 2 h. Antibodies against LC3 A/B, p-Akt, Akt, p-Erk1/2, Erk1/2 (Cell Signaling, USA)

were diluted 1:1000 in 5% milk or 5% BSA in TBST and added to membranes and incubated for overnight at 4°C. After washing in TBST for 3×5 min, secondary antibody conjugated to horseradish peroxidase diluted 1:2000 in 5% milk or 5% BSA in TBST was added for 1 hour at room temperature. Signals were detected by enhanced chemiluminescence kit (Thermo Fisher Scientific, USA) and quantified with ImageQuant software (Bio-Rad, USA).

2.8. Statistical analysis

Differences between treatments were evaluated by One-way ANOVA using Fisher's LSD as post-hoc test or non-parametric Kruskal-Wallis test (coronary flow data, 14-20 min timepoint) by Prism 5 (GraphPad, GraphPad Software, USA). Data are presented as means ± SEM.

3. Results

3.1. Pilot study: CAP induces autophagy in *ex vivo* perfused hearts but not in NRCMs and H9c2 cells

In a pilot study we aimed to compare the effect of CAP on autophagy in neonatal cardiomyocytes, in H9c2 cells and in *ex vivo*-treated hearts. Therefore, we used the most common autophagy marker LC3 to measure changes in autophagy. In isolated rat neonatal cardiomyocytes and in H9c2 cells there was no significant difference in LC3-II to I ratio between corresponding CON and CAP groups (Fig. 1A, 1B). Since CAP failed to induce autophagy in NRCMs and H9c2 cells, in our further experiments we used the isolated rat heart model. In the left atrium of hearts perfused with CAP, the ratio of LC3-II to LC3-I was significantly increased after 35 minutes compared to CON samples indicating that CAP induced cardiac autophagy (Fig. 1C). The discrepancy might be attributed to the difference in metabolic status of cultured cells and the *ex vivo* perfused hearts. Since insulin, fatty acids and other factors are present in cell culture medium due to the use of foetal calf serum, and since these factors are not included in the heart perfusion buffer, signaling pathways related to autophagy might be differentially modulated by CAP. Furthermore, the presence of cell types other than cardiomyocytes (endothelial cells, fibroblasts) in the heart might also contribute to the cardioprotective effects of CAP (see for a recent review: [20]).

3.2. Main study: TAT-HA-Atg5^{K130R} blocks CAP-induced cardioprotection in isolated hearts

In order to assess whether the sequestration and/or degradation phases of autophagy are necessary for the cardioprotective effect of CAP, we used *ex vivo* Langendorff heart perfusion experiments. CAP treatment significantly reduced infarct size and CK release as compared to CON hearts (Fig. 3A, 3B). Pretreatment with TAT-HA-Atg5^{K130R} abolished the infarct size limiting effect of CAP, while pretreatment with CQ did not interfere with CAP-induced cardioprotection.

We also measured LC3 expression in isolated heart samples and the western blot results showed that CAP increased LC3-II/I ratio. Meanwhile, administration of TAT-HA-Atg5^{K130R} but not CQ reduced the increase of LC3-II due to CAP administration (Fig. 3C).

Since cardioprotection, as assessed by infarct size, and autophagosome formation, as assessed by LC3-II to LC3-I ratio, was lower in CAP+K130R group than in CAP group, these results indicate that CAP-induced cardioprotection requires the process of autophagosome formation but not autophagosomal clearance.

To characterize cardioprotective signaling mechanisms modulated by CAP, we assessed activation of proteins involved in the RISK/SAFE pathway. We found that the phosphorylation of Erk1/2 but not of Akt increased significantly due to CAP treatment (Fig. 4).

Coronary flow was significantly lower at the beginning of reperfusion (4-5 min) in CAP, CQ and CAP+CQ groups compared to CON group. Prior to ischemia (14-20 min) the coronary flow was lower in CQ and CAP+CQ. After the initiation of reperfusion (65-79 min) the coronary flow was decreased in K130R and CQ groups. At the end of the reperfusion time (169-185 min) the coronary flow was lower in CAP+CQ group compared to CON (Table 1).

4. Discussion

Here we demonstrate that pretreatment with an inhibitor of autophagosome formation (TAT-HA-Atg5^{K130R} functional null-mutant protein) abolished the infarct size limiting effect of CAP, while pretreatment with an inhibitor of autophagosome-lysosome fusion (CQ blocker of autophagosomal clearance) did not interfere with CAP-induced cardioprotection. Furthermore, CAP administration induced autophagy as assessed by an increased LC3-II/LC3-I ratio, which was inhibited by the administration of TAT-HA-Atg5^{K130R}, but not of CQ. These results support that inhibition of autophagosome formation abolishes cardioprotection induced by CAP. Furthermore, this is the first demonstration that autophagosome formation but not autophagosomal clearance is required for cardioprotection afforded by the induction of autophagy (Fig. 5).

In our present study, CAP significantly increased the LC3-II/I ratio in *ex vivo* hearts which indicates the induction of autophagy. Furthermore, CAP treatment reduced infarct size and creatine kinase activity in isolated rat hearts. These results are in agreement with those of Granville *et al.* [10] who showed that CAP reduced infarct size in an open chest rabbit model of regional ischemia. Moreover, similar results were obtained in a previous study with a more water soluble derivative of CAP, chloramphenicol succinate (CAPS), in a swine model of myocardial ischemia-reperfusion injury by Sala-Mercado *et al.* [12]. They found that CAPS, administered as a pre-treatment or before reperfusion, rendered the porcine heart resistant to ischemia-reperfusion injury, however, they did not investigate which stage of autophagy is necessary for cardioprotection. Interestingly, in our present *ex vivo* rat heart experiment, CAPS did not reduce infarct size (data not shown). Since CAPS is a prodrug activated by esterases in the blood [21] which are not present in isolated heart perfusion, our data suggests that *in vivo* metabolism of CAPS into CAP plays a significant role in its cardioprotective effect. In 2014 Shiomi *et al.* [22] demonstrated that in isolated guinea pig hearts sevoflurane reduced infarct size after 30 min, but not 45 min of cardiac ischemia. In this experiment, CAP pretreatment extended the time of ischemia after which sevoflurane

preconditioning reduced infarct size. These results well demonstrate that CAP or its derivatives are able to induce or facilitate cardioprotection.

We further investigated whether CAP exerts cardioprotection via autophagy. Therefore, in the present study we determined whether autophagy is required for CAP-induced cardioprotection and which stage of autophagy is essential for the cardioprotective effect of pharmacologically induced autophagy. We used TAT-HA-Atg5^{K130R} to inhibit autophagosome formation and CQ to investigate the role of lysosomal degradation. Atg5 is not known to participate in other pathways besides autophagy, and therefore TAT-HA-Atg5^{K130R} is the most specific inhibitor available. In the present study we found that pretreatment with TAT-HA-Atg5^{K130R} abolished the infarct size limiting effect of CAP. Similarly, Shiomi *et al.* found that a non-specific autophagy inhibitor, 3-methyladenine, abolished the CAP-induced restoration of sevoflurane preconditioning after prolonged ischemic insult [22]. These results demonstrate that the sequestration phase of autophagy is essential for CAP-induced cardioprotection against acute ischemia/reperfusion injury.

The role of autophagosomal clearance in cardioprotection has been investigated in several models. Guo *et al.* [23] showed that one hour after cardiac ischemia LC3-II/I ratio was significantly elevated, and that ischemic postconditioning limited the elevation in the LC3-II/I ratio evidencing that cardioprotective interventions might inhibit cardiac autophagy. However, the same study indicated that inhibition of autophagosomal clearance by CQ abolished cardioprotection by ischemic postconditioning, which suggests that inhibition of cardiac autophagy might be controversial in ischemic conditioning. Similarly, Wang *et al.* investigated the mechanisms underlying resveratrol-mediated cardioprotection, and found that chronic inhibition of autophagic clearance with bafilomycin A1 abrogated resveratrol-mediated cardiac protection in diabetic mice [24]. On the contrary, in our present study, acute treatment with CQ did not abolish CAP-induced cardioprotection in isolated healthy hearts. These findings suggest that although *in vivo* inhibition of autophagic clearance interferes with several types of cardioprotective stimuli, autophagic clearance is not likely to be necessary for cardioprotection triggered by pharmacologically induced autophagy in an *ex vivo* setting, the basis of which is unclear.

5. Conclusion

Here we have shown for the first time in the literature that the sequestration, but not the clearance phase of autophagy is necessary for the cardioprotective effect of CAP. Therefore, therapeutic tools developed on the basis of inducing autophagy sequestration might lead to novel therapeutic options against acute myocardial ischemia/reperfusion injury.

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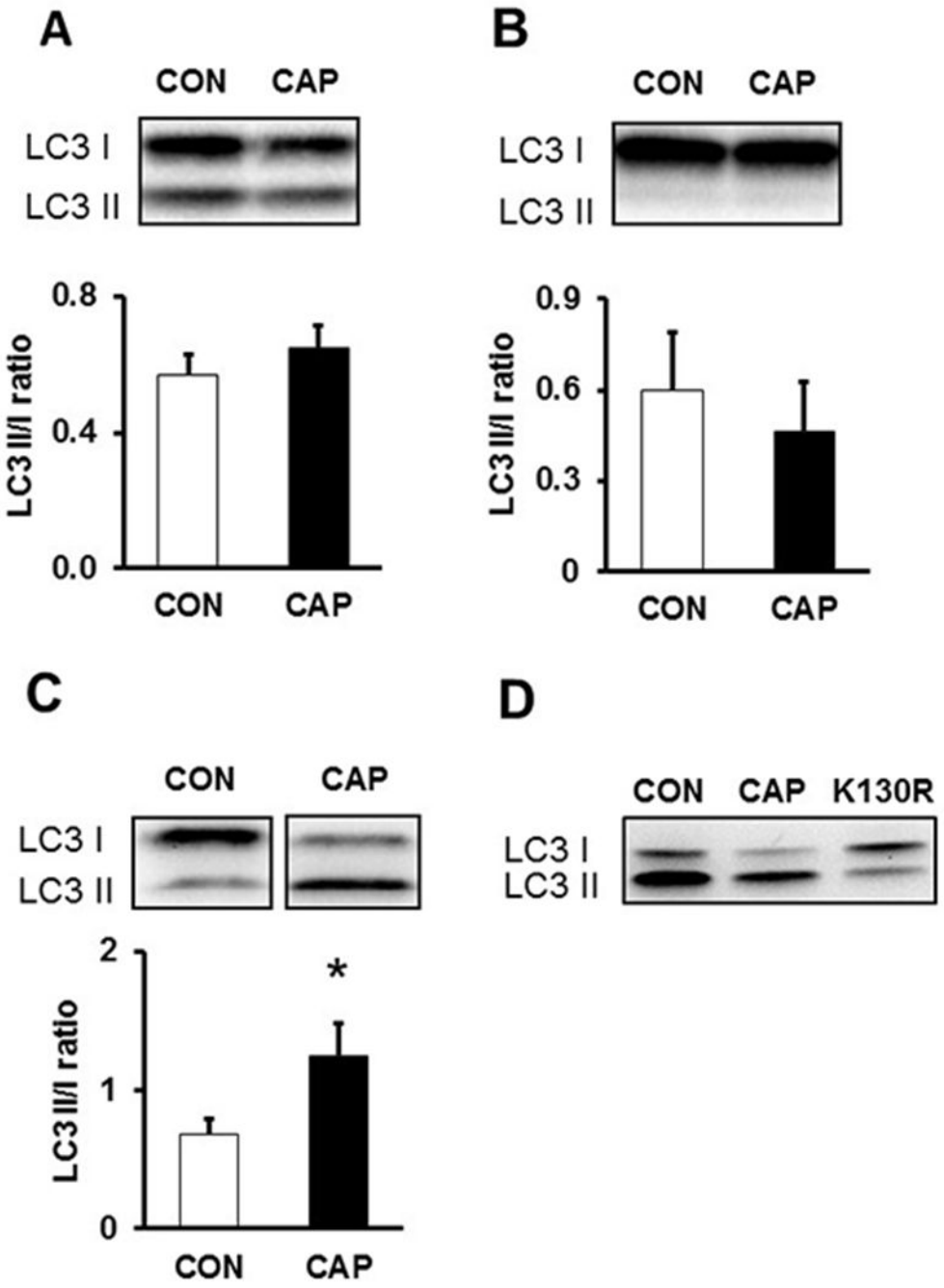

Fig. 1.

Pilot study: The effect of CAP on autophagy-related protein LC3 levels in **(A)** NRCMs **(B)** in H9c2 cells and **(C)** *ex vivo* heart lysates *p <0.05 vs. CON n=3-8 **(D)** Pilot study: effect of CAP and TAT-HA-Atg5^{K130R} on autophagy-related protein LC3 levels in whole heart. n=2 Data are presented as means ± SEM. Chloramphenicol - CAP, Control - CON, Microtubule-associated protein 1A/1B-light chain 3 - LC3, Neonatal rat cardiac myocytes – NRCMs

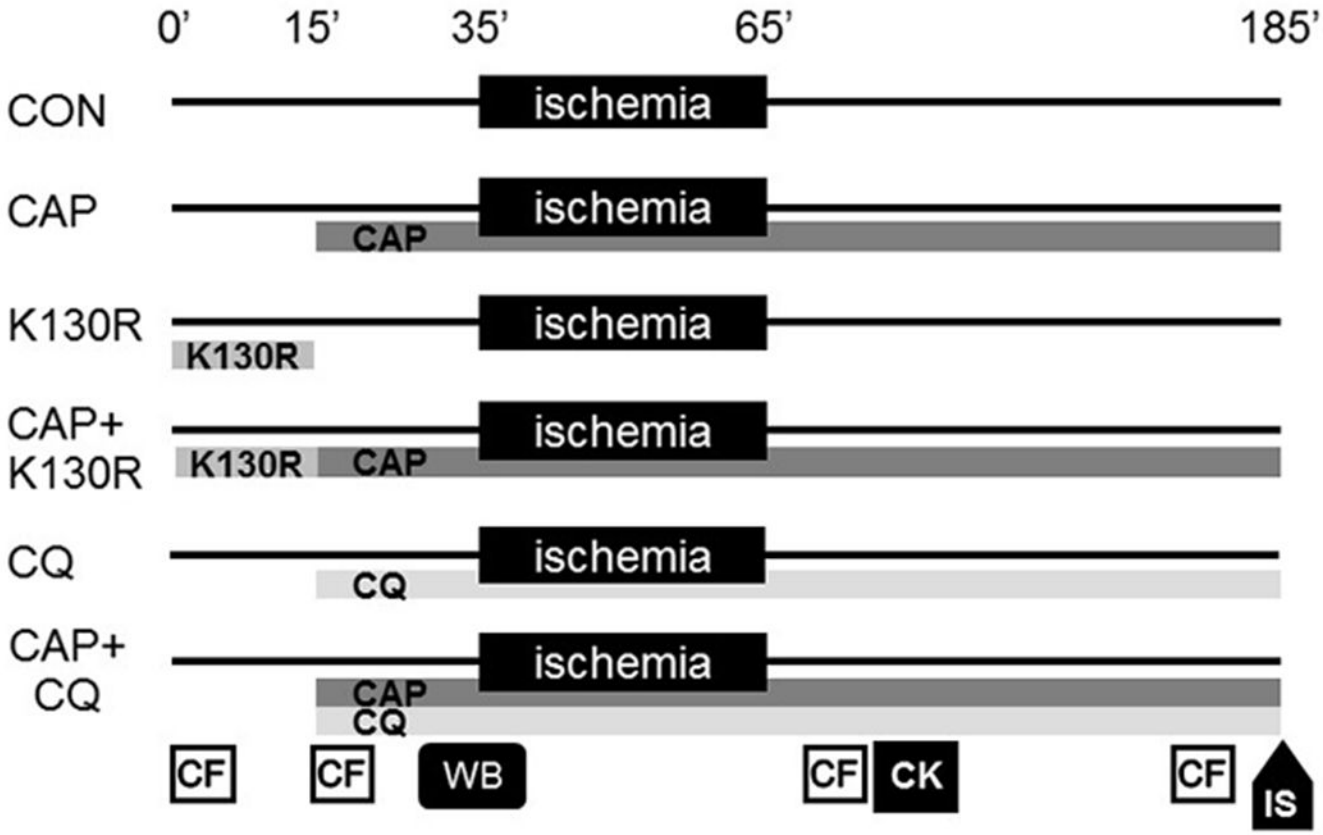

Fig. 2.

Experimental protocol. Chloramphenicol - CAP, Control - CON, TAT-HA-Atg5^{K130R} - K130R, CK - Creatine kinase, CF – Coronary flow measurement, WB – Western blot, IS – Infarct size.

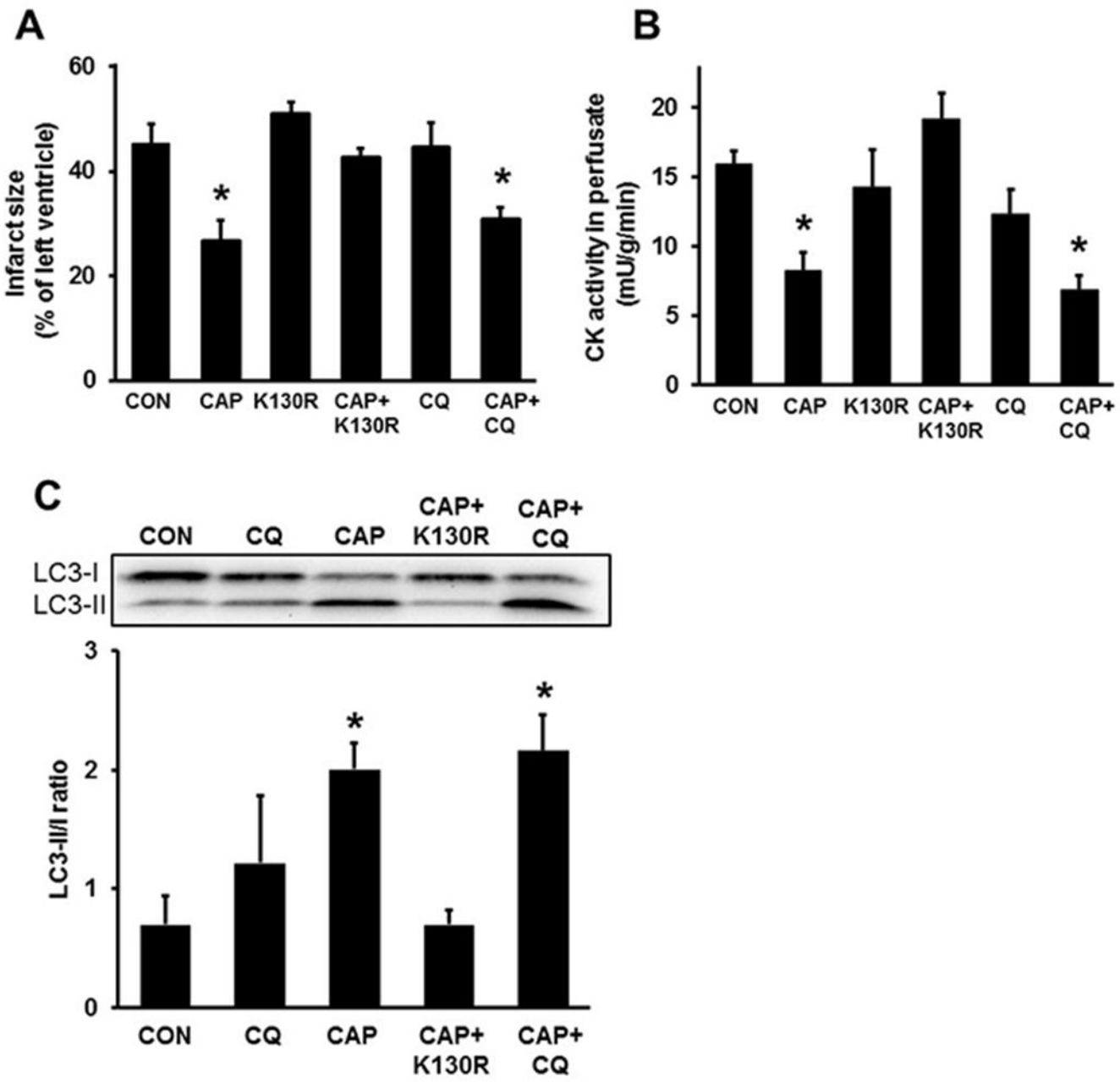

Fig. 3.

(A) Inhibition of autophagosome formation by TAT-HA-Atg5^{K130R} abolishes CAP-induced cardioprotection (**B)** Inhibition of autophagosome formation (K130R) abolishes the CAP induced CK activity reduction in perfused rat hearts. n=3-8 **(C)** The effect of CAP on autophagy-related protein LC3 levels in isolated hearts n=3 *p <0.05 vs. CON Data are presented as means ± SEM. Chloramphenicol - CAP, Chloroquine – CQ, Control - CON, TAT-HA-Atg5^{K130R} - K130R, Creatine kinase – CK

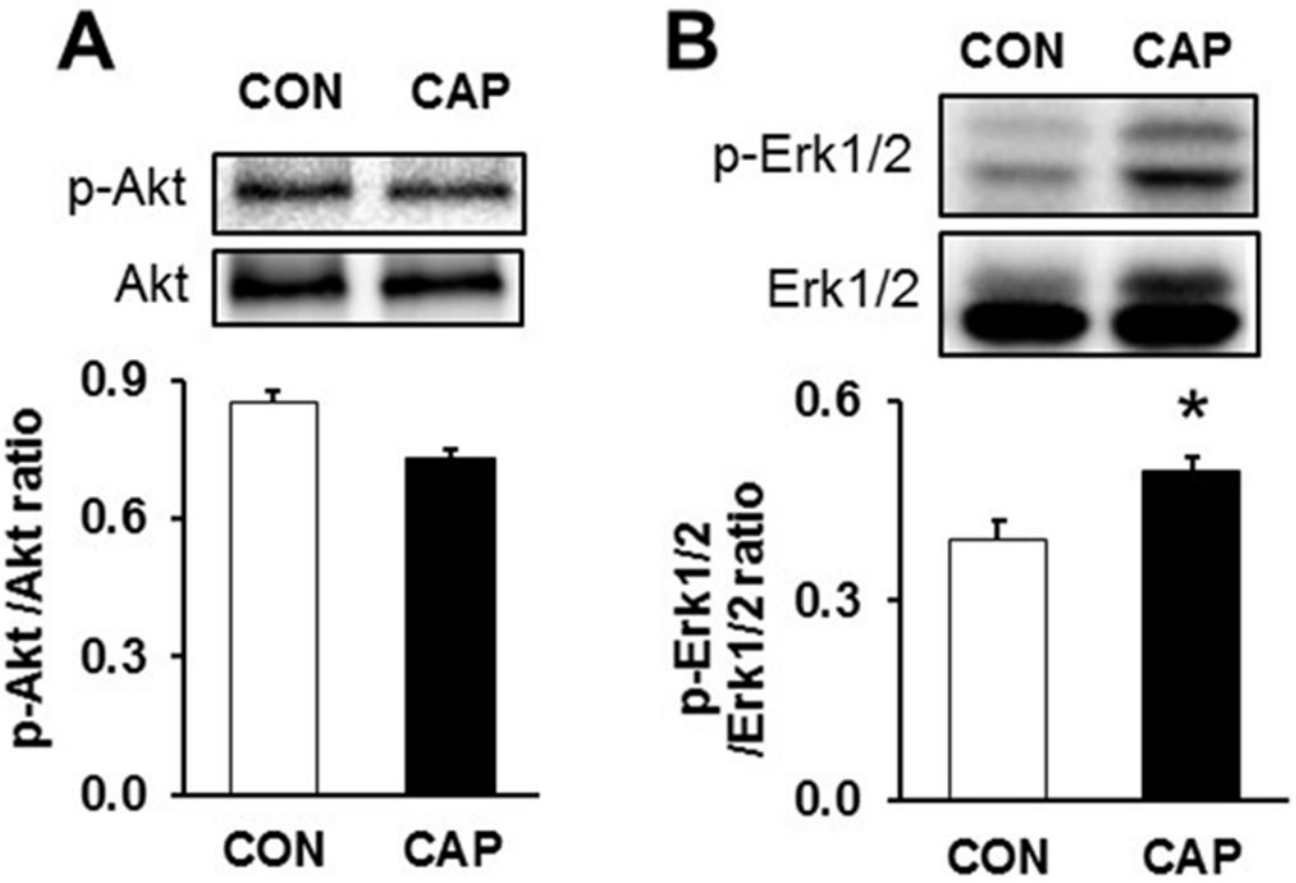

Fig. 4.

Assessment of cardioprotective pathways from *ex vivo* whole heart lysates. (A) Ratio of p-Akt to Akt signals (B) ratio of p-Erk1/2 to Erk1/2. n=5. Chloramphenicol - CAP, Control - CON

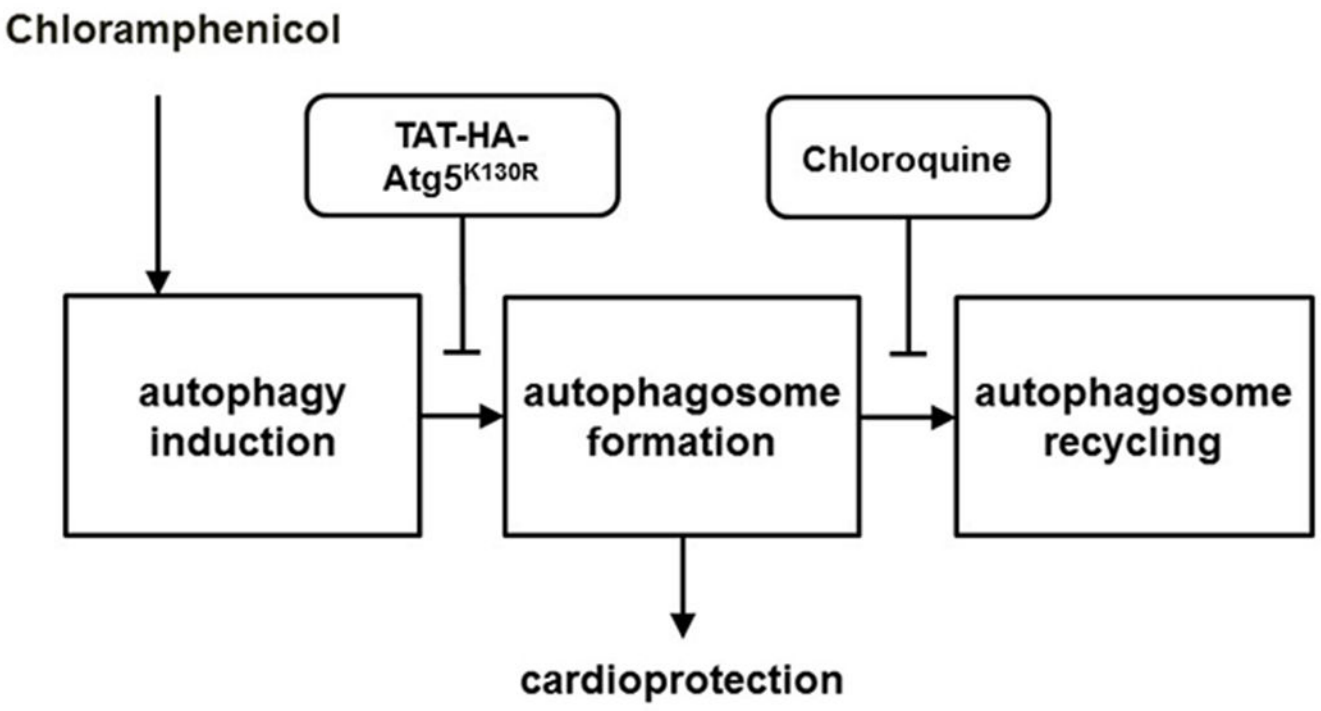

Fig. 5.

Chloramphenicol induced cardioprotection requires the process of autophagosome formation, but not autophagosome recycling.

Table 1 –

Measurement of coronary flow in *ex vivo* perfused hearts *p <0.05, vs. CON n=3-8 Data are presented as means ± SEM. Chloramphenicol - CAP, Chloroquine – CQ, Control - CON, TAT-HA-Atg5^{K130R} - K130R

Groups	Perfusion time (min)			
	4-5	14-20	65-79	169-185
CON	14±0.6	14±0.9	8.3±0.7	6.7±0.6
CAP	12±0.5*	10±1.1	9.8±0.7	6.6±0.4
K130R	14.3±0.9	9.5±2.5	5.7±0.9*	5.7±0.7
CAP+K130R	14.9±0.6	10.6±0.3	7.8±0.4	5.3±0.2
CQ	11±0.3*	9.2±0.4*	5±1.1*	5.7±2.2
CAP+CQ	10.7±1.0*	8.5±0.7*	7.8±0.7	4±0.4*