Profound Cardioprotection With Chloramphenicol Succinate in the Swine Model of Myocardial Ischemia-Reperfusion Injury

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Background—Emerging evidence suggests that “adaptive” induction of autophagy (the cellular process responsible for the degradation and recycling of proteins and organelles) may confer a cardioprotective phenotype and represent a novel strategy to limit ischemia-reperfusion injury. Our aim was to test this paradigm in a clinically relevant, large animal model of acute myocardial infarction.

Methods and Results—Anesthetized pigs underwent 45 minutes of coronary artery occlusion and 3 hours of reperfusion. In the first component of the study, pigs received chloramphenicol succinate (CAPS) (an agent that purportedly upregulates autophagy; 20 mg/kg) or saline at 10 minutes before ischemia. Infarct size was delineated by tetrazolium staining and expressed as a % of the at-risk myocardium. In separate animals, myocardial samples were harvested at baseline and 10 minutes following CAPS treatment and assayed (by immunoblotting) for 2 proteins involved in autophagosome formation: Beclin-1 and microtubule-associated protein light chain 3-II. To investigate whether the efficacy of CAPS was maintained with “delayed” treatment, additional pigs received CAPS (20 mg/kg) at 30 minutes after occlusion. Expression of Beclin-1 and microtubule-associated protein light chain 3-II, as well as infarct size, were assessed at end-reperfusion. CAPS was cardioprotective: infarct size was 25% and 41%, respectively, in the CAPS-pretreated and CAPS-delayed treatment groups versus 56% in saline controls (P=0.01 and P=0.05 versus control). Moreover, administration of CAPS was associated with increased expression of both proteins.

Conclusion—Our results demonstrate attenuation of ischemia-reperfusion injury with CAPS and are consistent with the concept that induction of autophagy may provide a novel strategy to confer cardioprotection.

Key Words: myocardial infarction ■ ischemia ■ reperfusion ■ autophagy

Autophagy is the cellular process responsible for the degradation and removal of damaged organelles and protein aggregates. Although the role of autophagy in cardiac pathophysiology is complex and poorly understood, emerging data suggests that an “adaptive” induction of autophagy may confer a cardioprotective phenotype. This concept raises the intriguing possibility that pharmacological upregulation of autophagy may represent a novel therapeutic strategy to protect the heart against ischemia-reperfusion injury.

Three discrete pieces of evidence have identified chloramphenicol, a cytochrome P450 monooxygenase inhibitor and bacteriostatic antimicrobial, as a potential candidate agent to test this paradigm. First, P450 monooxygenase inhibitors, including chloramphenicol, have been reported to limit infarct size in small animal (rat and rabbit) models of ischemia-reperfusion. Second, chloramphenicol has been shown to induce an autophagic response in osteosarcoma cells. Moreover, recent preliminary data from our laboratory revealed a similar response in rat myocardium following in vivo administration of the succinated form of the agent: a rapid upregulation in molecular markers of autophagosome formation. Our aim in the current study was to extend these observations and interrogate the paradigm of adaptive autophagy in a clinically relevant, in vivo large animal model of myocardial ischemia-reperfusion injury. Specifically, we sought to establish: (1) whether preischemic administration of chloramphenicol succinate (CAPS) reduces infarct size in the anesthetized porcine model of coronary artery occlusion-reperfusion, (2) whether cardioprotection can be achieved if...
administration of CAPS is “delayed” (ie, given before reperfusion, rather than as a pretreatment), and (3) whether administration of CAPS was associated with induction of autophagy.

Methods
This study was approved by the Institutional Animal Care and Use Committee of Wayne State University and was performed in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute of Laboratory Animals Resources (National Institutes of Health Publication Vol. 25 No. 28, revised 1996).

Surgical Preparation
Twenty-one domestic swine (10 male, 11 female; 26±2 kg [mean±SD]) were premedicated with ketamine (33 mg/kg IM), and an IV catheter was positioned in an ear vein. All pigs were then anesthetized with sodium pentobarbital (20 to 40 mg/kg IV), intubated, and ventilated with room air. Core body temperature was monitored with a rectal temperature probe and maintained between 37 to 38°C throughout the experiment. Fluid-filled catheters were positioned in the right carotid artery for continuous monitoring and measurement of systemic hemodynamic parameters (heart rate and arterial pressure) and in the right external jugular vein for administration of fluids and supplemental anesthesia. The ECG was continuously monitored throughout each experiment.

The heart was exposed via a median sternotomy and suspended in a pericardial cradle. A fluid-filled catheter was inserted into the left atrial appendage, and a segment of the left anterior descending coronary artery was isolated, usually midway along its course.

CAPS: Pretreatment

Infarct Size
For the first 12 pigs enrolled into the study, our aim was to establish whether prophylactic administration of CAPS was cardioprotective. After stabilization, baseline values of body temperature, heart rate, and arterial pressures were tabulated. Each pig was then assigned to receive CAPS (20 mg/kg dissolved in 12 mL PBS, n=6) or a matched volume of PBS (n=6). CAPS (rather than chloramphenicol) was used, because the succinated form is soluble in aqueous media and, thus, more suitable for in vivo administration. Treatment was administered in a blinded manner and given as a slow bolus (over 2 to 3 minutes) into the left atrial appendage.

Ten minutes after treatment, pigs underwent 45 minutes of coronary occlusion followed by 3 hours of reperfusion (achieved by placement/removal of an atraumatic vascular clamp on the isolated left anterior descending coronary artery segment). To minimize the possible confounding effects of occlusive thrombosis at the site of coronary artery occlusion, a segment of the left anterior descending coronary artery was isolated, usually midway along its course.

Atrial fibrillation was induced by applying low-energy DC countershocks directly to the heart (energy of 25 J; maximum of 4 attempts). At the conclusion of the 3-hour reperfusion period, the left anterior descending coronary artery was ligated, the ascending aorta was occluded, and the pigs were euthanized, and LV tissue blocks were rapidly excised. All tissue samples were immediately snap frozen and stored at −80°C until processed.

Our aim was to assess the expression of 2 pivotal proteins involved in autophagosome formation: Beclin-1 and microtubule-associated protein light chain (LC) 3-II.1–5 Frozen myocardial samples (50 to 100 mg) were minced and homogenized in an extraction buffer containing 50 mmol/L Tris-HCl (pH 7.5)/5 mmol/L EDTA/10 mmol/L EGTA/10 mmol/L benzamidine/0.3% β-mercaptoethanol/1% Complete Mini EDTA-Free Protease Inhibitor Cocktail (Roche, Mannheim, Germany). Homogenates were centrifuged at 1000 × g for 10 minutes, the supernatant was decanted, and protein concentration was determined by Bradford assay (Thermo Scientific). Proteins (20 μg per lane) were separated by SDS-PAGE, transferred onto nitrocellulose membranes (Bio-Rad), and probed with primary antibodies for Beclin-1 and the B-isofrom of LC3-II (Cell Signaling Technology and Novus Bio). Immunoblotting for GAPDH (Sigma-Aldrich) was conducted to control for protein loading. After washing, the blots were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc) and developed with the Pierce ECL chemiluminescence kit (Thermo Scientific). Blots were scanned and quantified using image analysis software (SigmaScan Pro, SPSS, Inc).

CAPS: Delayed Treatment

Infarct Size
The final 7 pigs enrolled in the study underwent 45 minutes of left anterior descending coronary artery occlusion and 3 hours of reperfusion as described previously. Our objective in these supplemental experiments was to determine whether cardioprotection could be achieved if CAPS is given before reperfusion, rather than as a pretreatment. Accordingly, animals received CAPS (20 mg/kg dissolved in 12 mL PBS, n=6) or PBS alone (n=1) at 30 minutes after occlusion. AR and AN were quantified as described above, and hemodynamics were assessed throughout each experiment.

Molecular Markers of Autophagy
LV samples were obtained at 3 hours after reperfusion (3.25 hours after administering CAPS or PBS), before delineation of infarct size. Tissue from remote, normally perfused myocardium (ie, not contounded by the presence of necrosis or the effect of ischemia-reperfusion per se on autophagy)11 was processed and probed for expression of Beclin-1, LC3-II, and as a loading control, GAPDH.

Statistics
AR/LV and AN/LV were compared among the control, CAPS-pretreated, and CAPS-delayed treatment groups by ANOVA followed by the Newman–Keuls post hoc test. Comparisons of heart rate and mean arterial pressure among groups were made using generalized estimating equations. The decision to use parametric (rather than nonparametric) statistics and present the data as mean±SEM (rather than as medians, first, and third quartiles) was made after applying the Kolmogorov–Smirnov normality test and obtaining probability values of >0.10. Incidence of VF among groups was compared using an extension of Fisher’s exact test. Expression of Beclin-1 and LC3-II following CAPS treatment,
corrected for GAPDH expression and normalized to either baseline values (for CAPS-pretreatment) or control values (for CAPS-delayed treatment), is reported, but because of the limited number of samples obtained, no conclusions regarding statistical significance are made.

Results

Incidence of Ventricular Fibrillation
Of the 19 pigs that underwent coronary artery occlusion-reperfusion, 8 developed VF during ischemia and/or at the time of reflow: 4/7 in the control group, 3/6 pretreated with CAPS, and 1/6 that received CAPS-delayed treatment ($P=0.38$ [not significant]). There were no premature deaths: all animals were successfully resuscitated and completed the protocol.

Hemodynamics
There were no significant differences in heart rate ($P=0.33$) or mean arterial pressure ($P=0.65$) among control, CAPS-pretreated, and CAPS-delayed treatment groups (Figure 1).

Infarct Size
AR was comparable in control, CAPS-pretreated, and CAPS-delayed treatment groups, averaging 23±2, 21±1, and 23±1% of the total LV weight, respectively. Mean infarct size in the control cohort was 56±5% of the risk region. In contrast, AN/AR was significantly reduced with CAPS ($P=0.009$ by ANOVA): AN averaged 25±5 and 41±4% of the myocardium at risk in the CAPS-pretreated and CAPS-delayed treatment cohorts, respectively ($P<0.01$ and $P<0.05$ versus control) (Figure 2).

Molecular Markers of Autophagy
In pigs pretreated with CAPS, myocardial expression of Beclin-1 and LC3-II was 2.4- and 6.2-fold higher at 10 minutes following treatment versus baseline (Figure 3). Delayed administration of CAPS was associated with in-

Figure 1. Heart rate and mean arterial pressure (mean±SEM), measured at baseline, immediately before occlusion (Pre), during coronary occlusion and throughout reperfusion.

Figure 2. Reduction of infarct size with CAPS. Top, Infarct size, expressed as a % of the myocardium at risk (mean±SEM) for control, CAPS-pretreated, and CAPS-delayed treatment cohorts. Bottom, Images of heart slices obtained from 1 control, 1 CAPS-pretreated pig, and 1 pig that received CAPS-delayed treatment. Heart slices were incubated in triphenyltetrazolium chloride; using this method, viable myocardium stains red, whereas necrotic tissue (denoted by arrows) is unstained and thus appears pale.
creased expression of LC3-II (5.1-fold greater than control), but not Beclin-1, at 3 hours following relief of ischemia (Figure 4).

Discussion
We report that CAPS, administered as a pretreatment or before reperfusion, rendered the porcine heart resistant to ischemia-reperfusion injury. Moreover, our results suggest that the infarct-sparing effect of CAPS is associated with increased myocardial expression of molecular markers of autophagy.

Autophagy: Beneficial Versus Detrimental?
Autophagy is the highly conserved cellular process whereby subcellular components, including damaged organelles and protein aggregates, are sequestered in autophagosomes for delivery to lysosomes and subsequent degradation by lysosomal enzymes.1–4 There is compelling evidence that constitutive autophagy is crucial for the maintenance of cardiomyocyte structure and function under basal conditions.1,16 In addition, it is well documented that acute and chronic stressors, including ischemia-reperfusion and ischemia per se, hypoxia, starvation, hypertrophy, and heart failure, induce an upregulation in autophagy.1–5,8,16,17 However, the role of autophagy in the setting of stress (protective versus detrimental) is complex, poorly understood, and in all likelihood dependent on the severity and duration of the insult.

Recent attention has focused on the concept that preischemic induction of autophagy may confer a cardioprotective phenotype and render the heart resistant to lethal ischemia-reperfusion injury, possibly via the removal of damaged and dysfunctional mitochondria, disposal of misfolded and aggregated proteins, and subsequent recycling of amino acids and fatty acids to provide a compensatory source of energy production during the sustained ischemic insult.2–4,7,11 In support of this concept, induction of autophagy has been implicated to play a role in the protection achieved with ischemic preconditioning and administration of the adenosine A1 receptor agonist and classic “preconditioning-mimetic” 2-chloro-N(6)-cyclopentyl-adenosine (CCPA).6,7,9 However,
in apparent contrast, other studies have reported an attenuation (rather than exacerbation) of ischemia-reperfusion injury in hearts from mice in which autophagy is downregulated via knock-down of Beclin-1.18,19 Our current results, demonstrating reduction of infarct size and increased expression of Beclin-1 and LC3-II with CAPS pretreatment, are consistent with the proposed association between preischemic induction of autophagy and cardioprotection. Moreover, we extend this concept and show that prophylactic administration of CAPS is not a prerequisite for protection; infarct size reduction was maintained (although lesser in magnitude) when CAPS was given during, rather than before, sustained ischemia. Addition of chloramphenicol at the time of reperfusion has been reported to limit infarct size in isolated buffer-perfused hearts.13 However, our study provides the first evidence of infarct size reduction with delayed CAPS treatment in an in vivo, large animal model.

Reduction of Infarct Size with CAPS
Our aim was to investigate the paradigm of adaptive autophagy by establishing whether a pharmacological agent capable of initiating an autophagic response would limit myocardial infarct size. In this regard, our results provide qualitative evidence that CAPS triggers an increase in myocardial expression of Beclin-1 and LC3-II. Furthermore, data obtained with delayed administration of CAPS imply a persistent increase in markers of autophagy (specifically, LC3-II) at 3.25 hours after treatment.

Beclin-1 and LC3-II are crucial proteins required for autophagosome formation.2,3,20,21 Moreover, although the mechanisms of autophagy are highly integrated with phosphatidylinositol 3-kinase/Akt signaling2,3 and recent data have identified the involvement of protein kinase C,22 the 2 proteins are not, based on present knowledge, components of conventional, cardioprotective signaling pathways.23 Rather, Beclin-1 and LC3-II are considered specific markers of autophagy and autophagosome formation. Thus, the increase in expression of Beclin-1 and LC3-II seen with administration of CAPS imply induction of autophagy by this agent.

It appears implausible that the increased expression of autophagic markers seen at 10 minutes after CAPS treatment can reflect de novo protein synthesis. A brisk increase in markers of autophagy, which can reflect de novo protein synthesis. A brisk increase in autophagic markers seen at 10 minutes after CAPS treatment, are consistent with the proposed association between preischemic induction of autophagy and cardioprotection. Moreover, we extend this concept and show that prophylactic administration of CAPS is not a prerequisite for protection; infarct size reduction was maintained (although lesser in magnitude) when CAPS was given during, rather than before, sustained ischemia. Addition of chloramphenicol at the time of reperfusion has been reported to limit infarct size in isolated buffer-perfused hearts.13 However, our study provides the first evidence of infarct size reduction with delayed CAPS treatment in an in vivo, large animal model.

Indeed, we cannot exclude the possibility that other mechanisms, including purported protective effects of succinate,24 may contribute to the infarct-sparing effect of CAPS. Efforts to obtain definitive proof that upregulation of autophagy is necessary and sufficient for CAPS-induced cardioprotection are confounded by the lack of selectivity of current pharmacological tools: ie, Wortmannin and 3-methyladenine inhibit the initiation of autophagy but also inhibit the classic, phosphatidylinositol 3-kinase/Akt “survival” pathway.2,3 Moreover, despite the clinical relevance afforded by the use of the in vivo porcine model, application of selective molecular tools, such as suppression of autophagy by point mutation of the essential autophagy gene atg5,7,22 is not feasible in this preparation.

Although our data imply an upregulation of Beclin-1 and LC3-II with CAPS, expression of autophagic markers was assessed in a small number of pigs (thereby precluding statistical comparisons) and at limited time points following drug administration. Indeed, it could be argued that in the CAPS-delayed treatment group, a more meaningful approach may have been to obtain tissue samples during the early minutes after reflow rather than at end-reperfusion. Given the propensity of the pig to develop reperfusion-induced VF, we elected not to undermine the successful completion of these experiments and assessment of our primary end point (infarct size) by obtaining biopsies at this vulnerable time point. In addition, we did not quantify autophagosome abundance or autophagic flux (ie, the dynamic formation and clearance of autophagosomes) in CAPS-treated hearts versus controls.2,3,21

In addition to the resolution of these mechanistic issues, future studies are required to establish whether alterations in the dose, timing, or regimen of CAPS treatment (bolus + infusion rather than bolus alone) might yield even greater cardioprotective efficacy and whether addition of CAPS to cardioplegic solution confers benefit in the setting of cardiopulmonary bypass surgery. Nonetheless, our results demonstrate attenuation of ischemia-reperfusion injury with CAPS and are consistent with the concept that induction of autophagy may provide a novel therapeutic strategy to confer cardioprotection.

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Disclosures
R.A.G. is Founder and CEO of Radical Therapeutix, Inc, and R.M.M., Jr, is on the Scientific Advisory Board of Radical Therapeutix, Inc.

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