The cardioprotective compound cloxyquin uncouples mitochondria and induces autophagy

Zhang J, Nadtochiy SM, Urciuoli WR, Brookes PS. The cardioprotective compound cloxyquin uncouples mitochondria and induces autophagy. Am J Physiol Heart Circ Physiol 310: H29–H38, 2016. First published October 30, 2015; doi:10.1152/ajpheart.00926.2014.—Mitochondrial quality control mechanisms have been implicated in protection against cardiac ischemia-reperfusion (IR) injury. Previously, cloxyquin (5-chloroquinolin-8-ol) was identified via phenotypic screening as a cardioprotective compound. Herein, cloxyquin was identified as a mitochondrial uncoupler in both isolated heart mitochondria and adult cardiomyocytes. Additionally, cardiomyocytes isolated from transgenic mice expressing green fluorescent protein-tagged microtubule-associated protein light chain 3 showed increased autophagosome formation with cloxyquin treatment. The autophagy inhibitor chloroquine abolished cloxyquin-induced cardioprotection in both cellular and perfused heart (Langendorff) models of IR injury. Finally, in an in vivo murine left anterior descending coronary artery occlusion model of IR injury, cloxyquin significantly reduced infarct size from 31.4 ± 3.4% to 16.1 ± 2.2%. In conclusion, the cardioprotective compound cloxyquin simultaneously uncouples mitochondria and induces autophagy. Importantly, autophagy appears to be required for cloxyquin-induced cardioprotection.

8-hydroxyquinoline; uncoupling; mitophagy; ischemia; heart

NEW & NOTEWORTHY

The cardioprotective compound cloxyquin uncoupled mitochondria and induced autophagy. Cardioprotection was blocked by an autophagy inhibitor, and the cardioprotective effects of cloxyquin were confirmed in vivo. Together these findings demonstrate a link between mitochondrial uncoupling and autophagy in the heart and suggest that cloxyquin is a useful tool to study this.

CORONARY HEART DISEASE (CHD) is a major source of mortality and morbidity in the United States, with myocardial infarction accounting for about a third of CHD deaths (10). Despite many investigational drugs being found to limit infarct size in animal models of cardiac ischemia-reperfusion (IR) injury (e.g., cyclosporine A, diazoxide, acadesine), not one of these has translated to a clinical therapeutic approved for reduction of infarct size in humans. Mitochondria, the major source of ATP for the cell as well as a significant source of reactive oxygen species (ROS), are particularly vulnerable in IR injury and therefore represent potential targets for intervention (45).

At reperfusion, a large burst of ROS coupled with an influx of Ca2+ from the cytosol triggers cell death via the opening of the mitochondrial permeability transition pore (6, 14, 40). Mild mitochondrial uncoupling has been shown to be protective in cardiac IR injury (4, 5, 15, 32, 43) although the downstream mechanisms are unclear and may include lowering of ROS at reperfusion (2, 9), prevention of mitochondrial Ca2+ overload at reperfusion (6), or generation of small amounts of “signaling ROS” (5). However, it is also known that the loss of mitochondrial membrane potential (e.g., via uncoupling) targets mitochondria for autophagic degradation (i.e., mitophagy) via a PTEN-induced putative kinase protein 1 (PINK1) and Parkin-dependent pathway (8, 37). Thus, together, uncoupling and autophagy may facilitate the recycling of cellular resources and removal of dysfunctional organelles.

Although the exact role of autophagy in cardiac IR injury remains to be established, enhanced autophagy appears to be protective during ischemia but detrimental during reperfusion (18, 19, 31). Whereas the ability of large-scale uncoupling (high-dose FCCP) to induce autophagy is well known (37), mild uncoupling has received less attention. Furthermore, a link between mild uncoupling and autophagy within the context of cardiac IR injury or cardioprotection has not been explored experimentally.

Previously, we developed a cell-based model for IR injury, which we used to screen a 2,000-molecule library (12). Cloxyquin (5-chloroquinolin-8-ol, Fig. 1A) was one of the compounds identified as a hit and was subsequently demonstrated to be protective in a rat Langendorff-perfused heart model of IR injury, where it reduced infarct size as well as improved functional recovery after reperfusion.

Cloxyquin has been shown to exhibit antibacterial, antifungal, antiprotozoal, and antimycobacterial properties (16) but has not been investigated for its effects on CHD. Its close structural relative, clioquinol (5-chloro-7-iodoquinolin-8-ol, aka “chinoform,” Fig. 1A), was used many decades ago for treatment of protozoal infections but was discontinued after it was shown to cause subacute myeloptic neuropathy (17). Clioquinol has been reported to uncouple oxidative phosphorylation, decrease mitochondrial membrane potential, and induce autophagy (3, 7, 20, 38). Thus we hypothesized that the structural similarities between cloxyquin and clioquinol may endow the former with similar properties.

Here we demonstrate that, similar to clioquinol, cloxyquin uncouples mitochondria and induces autophagy. We then show that cloxyquin-induced cardioprotection is blocked by the autophagy inhibitor chloroquine in vitro and perfused mouse heart models of IR injury. Finally, we show that cloxyquin
AUTOPHAGY IN CLOXYQUIN-INDUCED CARDIOPROTECTION

Fig. 1. Structure of cloxyquin and autophagy schematic. A: molecular structures of cloxyquin (5-chloroquinolin-8-ol) and cloiquinol (5-chloro-7-iodoquinolin-8-ol). B: brief overview of autophagy showing the targets of relevant inhibitors. 3MA: 3-methyladenine, CQ: chloroquine, BafA1: bafilomycin A1.

induced cardioprotection translates to an in vivo model of cardiac IR injury.

MATERIALS AND METHODS

Animals and reagents. Animal and experimental procedures complied with the National Institute of Health Guide for Care and Use of Laboratory Animals and were approved by the University of Rochester Committee on Animal Resources. Male 2–4–mo-old mice on a C57BL/6j background were housed in a pathogen-free vivarium with 12-h:12-h light/dark cycles and food and water ad libitum. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Mitochondria isolation and oxygen consumption. Hearts from four mice were removed and immersed in 10 ml of ice-cold isolation medium (IM), consisting of the following (in mM): 300 sucrose, 20 Tris·HCl, 2 EGTA, and 0.1% (wt/vol) fat-free BSA, pH 7.35 at 4°C. Tissue was chopped, washed, and homogenized in 10 ml IM using a Tekmar Tissumizer (IKA Instruments, Wilmington, NC). The homogenate was centrifuged at 500 g for 5 min, the pellet was discarded, and the supernatant was centrifuged at 8,000 g for 5 min. The pellet was resuspended in 1.5 ml IM and centrifuged again at 8,000 g for 5 min. The top layer of the pellet (broken mitochondria and microsomes) was discarded, and the remaining pellet was resuspended in 75 μl IM. Protein content was determined using the Folin-Phenol method (29) against a standard curve constructed using bovine serum albumin. This protocol yielded ~4 mg of mitochondrial protein.

Respiration buffer consisted of the following (in mM): 120 KCl, 25 sucrose, 5 MgCl$_2$, 5 KH$_2$PO$_4$, 1 EGTA, and 10 HEPES, pH 7.3 at 37°C. For each experiment, 0.5 ml respiration buffer, 0.1% (wt/vol) fat-free BSA, and 0.3 mg protein/ml mitochondria were added to a water-jacketed (37°C) and magnetically stirred chamber. The chamber was sealed with a plug containing a capillary through which substrates and drugs were added using Hamilton syringes. The O$_2$ concentration was measured using a Clark electrode (YSI, Yellow Springs, OH). After baseline measurements, 10 mM glutamate, 5 mM malate, 100 μM ADP, 1 μg/ml oligomycin, and DMSO (0.1% final) or cloxyquin (500 nM or 50 μM) were added sequentially. Oxygen consumption rates were obtained from the slope after each addition. Daily averages (i.e., $n = 1$) were calculated from the average of 2–3 traces per mitochondrial preparation.

Adult mouse cardiomyocyte isolation. Following tribromoethanol (Avertin) anesthesia, the heart was excised and perfused for 3 min (flow rate 4 ml/min) with perfusion buffer (PB), consisting of (in mM) the following: 120.0 NaCl, 15.0 KCl, 0.6 Na$_2$HPO$_4$, 0.6 KH$_2$PO$_4$, 1.2 MgSO$_4$, 1.0 HEPES, 4.6 NaHCO$_3$, 30.0 taurine, 5.5 glucose, and 10.0 butadione monoxide, pH 7.4 at 37°C. The heart was then perfused for 10 min with digestion buffer (DB), consisting of PB plus 12.5 μM CaCl$_2$, 0.025% (wt/vol) trypsin, 6.525 U collagenase A (Roche, Indianapolis, IN), and 15.375 U collagenase D (Roche). Ventricular tissue was teased apart, resuspended in Stop Buffer (SB), consisting of PB plus 12.5 μM CaCl$_2$ and 10% (vol/vol) FBS, and filtered through a 200-μm mesh. Cells were allowed to settle by gravity for 10 min and then resuspended in 10 ml of SB. [Ca$^{2+}$] was increased stepwise to 1.8 mM in a shaking water bath. The final pellet was resuspended with MEM (Life Technologies, Grand Island, NY) containing penicillin/streptomycin and 2.5% (vol/vol) FBS (for use in cellular metabolism and simulated IR studies) or DMEM containing (in mM) 5.5 glucose 0.1 and pyruvate (for use in fluorescent imaging studies). This protocol yielded about 4 × 10$^5$ cells per heart with about 80% viability.

Cellular metabolism using Seahorse XF96 analyzer. Seahorse XF-96 V3-PS plates (Seahorse Bioscience, Billerica, MA) were coated with laminin (Bioscience, San Jose, CA). Cardiomyocytes were plated at a density of 1,500 cells per well and allowed to equilibrate for 1 h at 37°C in 5% CO$_2$. Shortly before measurements, MEM was replaced with bicarbonate-free DMEM containing (in mM) 4.0 glutamine, 5.0 glucose, and 0.1 pyruvate, pH 7.4 at 37°C. Wells were evenly distributed into five experimental groups: vehicle (0.2% DMSO) and four doses of cloxyquin (10, 50, 100, and 500 nM). After 2 min for equilibration, the cells were subjected to 20 cycles of 1-min mixing and 2-min measurements. After four baseline measurements, 1 μg/ml oligomycin was injected. Vehicle and cloxyquin were injected 7 min later. Individual well oxygen consumption rate (OCR) values were normalized to a range in which the baseline OCR was defined as 100% and the oligomycin-inhibited respiration was 0% (a sample trace of this normalization is shown in Fig. 2B). Daily averages (i.e., $n = 1$) were calculated from the average of 18–20 wells per cardiomyocyte isolation.

Fluorescent imaging. Transgenic mice expressing green fluorescent protein (GFP)-tagged microtubule-associated protein light chain 3 (LC3) (33) were a kind gift of John Lemasters (Medical University of South Carolina). Cardiomyocytes isolated from these mice were plated onto laminin-coated glass-bottom plates (Greiner Bio-One, Monroe, NC) at a density of 2.5 × 10$^4$ cells/ml. After 1 h of equilibration at 37°C in 5% CO$_2$, cells were treated with vehicle (0.2% DMSO) or cloxyquin (10, 50, 100, 500 nM) and optionally with 3-methyladenine (10 mM), chloroquine (5 μM), or bafilomycin A1 (100 nM) (see Fig. 3B for treatment strategy). Cells were examined using a confocal microscope (Nikon Instruments, Melville, NY) using the ×40 oil objective, under appropriate illumination. Cells were excited using a 488-nm laser, and fluorescence emission was detected at 515 nm. Puncta count and cell area were analyzed using ImageJ 1.48v software (NIH). Daily averages (i.e., $n = 1$) were calculated from the average of 5–10 cells per cardiomyocyte isolation.

Simulated IR using modified Seahorse XF24 analyzer. Cardiomyocytes were plated onto laminin-coated Seahorse XF V7-PE PET plates (Seahorse Bioscience) at a density of 5,000 cells per well and allowed to equilibrate for 1 h at 37°C in 5% CO$_2$. MEM was then replaced with bicarbonate-free DMEM with (in mM) 4.0 glutamine, 5.0 glucose, and 0.1 pyruvate, pH 7.4 at 37°C. Wells were evenly distributed into experimental groups: 0.2% DMSO and 1 nM cloxyquin, with or without 5 μM chloroquine. The Seahorse XF24 analyzer was modified for gas flow in the cartridge headspace to provide an in vitro model of IR injury as previously described (12). Drugs were injected 30 min before the onset of simulated IR. Cells were subjected to 1 h of ischemia followed by 1 h of reperfusion. Lactate dehydrogenase (LDH) release into the supernatant was measured using a kit (Roche) according to the manufacturer’s protocol and expressed as a percentage of total LDH content in both the supernatant and the Triton X-100 lysed cell pellet. Daily averages (i.e., $n = 1$) were calculated from the average of 5–7 wells per cardiomyocyte isolation. The autophagy
**RESULTS**

_Cloxyquin is a mitochondrial uncoupler._ Because clioninol, a close structural relative of cloxyquin (Fig. 1A), is a mitochondrial uncoupler (20), we hypothesized that cloxyquin would have similar effects on isolated mitochondria. The respiratory control ratio for mitochondria used in these studies would have similar effects on isolated mitochondria. The mitochondrial uncoupler (20), we hypothesized that cloxyquin 3-methyladenine (3MA) could not be used with this system because the 3MA stock solution could not be maintained at a high enough temperature in the cartridge and rapidly precipitated before the time of drug delivery.

**Ex vivo IR** The Langendorff-perfused heart model of IR injury was performed as previously described (47). Following tribromoethanol (Avertin) anesthesia, the heart was excised and perfused with Krebs-Henseleit buffer (KH) consisting of the following (in mM): 118.0 NaCl, 4.7 KCl, 1.2 MgSO4, 25.0 NaHCO3, 1.2 KH2PO4, 11.0 glucose, and 2.5 CaCl2, with 95% O2-5% CO2 at 37°C. A balloon connected to a pressure transducer was inserted into the left ventricle, and cardiac function was recorded for the duration of the protocol. Following equilibration, cloxyquin (1 nM) or vehicle (KH with <0.01% DMSO) with or without chloroquine (5 μM) was infused for 20 min. The heart then underwent global no-flow ischemia for 25 min followed by reperfusion for 60 min. Infarct was assessed following staining with triphenyltetrazolium chloride. The autophagy inhibitor 3MA could not be used with this system because the 3MA stock solution could not be maintained at a high enough temperature in the syringe pump and rapidly precipitated during the infusion period.

**Western blot analysis.** Cloxyquin (1 mg/kg in sodium phosphate buffer, pH 7.4 at 37°C with 3.6% DMSO, sterile filtered) was administered via tail-vein injection. After 20 min, the heart was excised under anesthesia and homogenized in 2 ml SDS-PAGE loading buffer. Samples were resolved on 12% SDS-PAGE gels and transferred to nitrocellulose. The membrane was cut at 25 kDa. The top and bottom halves were probed with primary antibodies against actin and LC3, respectively, at 1:1,000 dilutions. A horseradish peroxidase-linked secondary antibody (GE Biosciences, Pittsburgh, PA) was used at 1:2,500 dilution with enhanced chemiluminescence detection.

**Statistical analysis.** Student’s t-test and ANOVA were applied where appropriate. ANOVA was followed through by post hoc t-tests.
0.1% fat-free BSA in the respiration buffer to chelate free fatty acids that could uncouple mitochondria. Following cloxyquin, further addition of fat-free BSA to 0.5% returned OCR back to oligomycin-treated levels (data not shown), suggesting that BSA can indeed inhibit the uncoupling effects of cloxyquin.

We next investigated the effects of cloxyquin on respiration in adult mouse cardiomyocytes using the Seahorse XF96 analyzer. While normalizing the OCR data to baseline = 100%, oligomycin-inhibited = 0% (see schematic in Fig. 2B), we found cloxyquin to increase OCR in the presence of oligomycin (Fig. 2C). Notably, uncoupling was observed at much lower concentrations than in isolated mitochondria, likely because cardiomyocytes were incubated without fat-free BSA or serum.

**Autophagy is induced by cloxyquin in adult cardiomyocytes.** Loss of mitochondrial membrane potential can trigger mito-
To investigate whether cloxyquin modulates autophagy, cardiomyocytes were isolated from adult transgenic mice expressing GFP-tagged LC3 (33). In this model, autophagosomes appear on confocal microscopy as fluorescent green puncta. Cardiomyocytes were subjected to the protocol shown in Fig. 3B, in which cloxyquin or vehicle were administered, with optional prior administration of 3MA, or postadministration of chloroquine or bafilomycin A1. Cloxyquin at 500 nM significantly increased puncta formation (Fig. 3, A and B), which was abolished by pretreatment with 3MA, an inhibitor of autophagy induction.

However, contrary to expectations, subsequent addition of chloroquine or bafilomycin A1 (inhibitors of downstream autophagic flux) only slightly increased (and not significantly) puncta formation (Fig. 3B). Two explanations for this could be 1) that cloxyquin acts as an inhibitor of autophagosome degradation or 2) that the maximum capacity of the system to tolerate a given abundance of autophagosomes had been reached with cloxyquin alone. Although, in theory autophagosome abundance should continue to rise when lysosomal disposal is blocked, other limitations in cardiomyocytes (e.g., availability of membrane lipid, or ATP) may cause the system to reach a plateau. Therefore, to specifically examine autophagosome disposal and to distinguish between these two possibilities, a different type of autophagic flux assay was performed.

Cardiomyocytes were treated with cloxyquin, chloroquine, or vehicle and allowed to equilibrate. 3MA was then administered to inhibit new autophagosome formation, allowing only for autophagosome degradation (see treatment schematic in Fig. 3D). With vehicle treatment (DMSO), posttreatment with 3MA resulted in 58 ± 10% fewer puncta 30 min later, suggesting that at baseline a little more than half of the autophagosomes in a cardiomyocyte are degraded within 30 min. As expected, addition of 3MA after chloroquine treatment did not lead to fewer puncta (i.e., disposal of autophagosomes that had already been formed), indicating the expected inhibition of autophagosome disposal by chloroquine (Fig. 3, C and D). However, when 3MA was added after cloxyquin, similar to the DMSO vehicle condition, 60 ± 3% fewer puncta were observed (Fig. 3, C and D), demonstrating that autophagosome degradation was not inhibited by cloxyquin.

Blocking autophagy blocks cloxyquin-induced cardioprotection in vitro and ex vivo. To investigate the requirement for autophagy in cloxyquin-induced cardioprotection, cardiomyocytes were subjected to simulated IR injury using the modified Seahorse XF24 analyzer (12). Cloxyquin at 1 nM, administered before the onset of ischemia, significantly reduced LDH release compared with DMSO (Fig. 4A). Interestingly, moderate

**Fig. 4.** Autophagy is required for cloxyquin-induced cardioprotection. A: post-ischemia-reperfusion (IR) injury lactate dehydrogenase (LDH) release in adult mouse cardiomyocytes subjected to simulated IR injury. Cloxyquin (1 nM) reduces LDH release; this protective effect is abolished by coadministration with 5 μM CQ. Data are presented as means ± SE, n = 4, *P < 0.05 vs. DMSO. B: top: representative slices of hearts subjected to a perfused heart model of IR injury. Live tissue is pink, and infarcted tissue is pale (red and green in the pseudo-colored images, respectively). Bottom: quantification of infarct size as percentage of total heart area. C: functional recovery of perfused hearts in B, as measured by the rate pressure product. Data are presented as means ± SE, n = 6–8 mice per group, *P < 0.05.
concentrations (~50 nM) of cloxyquin were ineffective at preventing IR injury, and higher concentrations (>100 nM) exacerbated the effects of IR injury (data not shown). Although the concentrations of cloxyquin required to demonstrate induction of autophagy in cells (100–500 nM) or to detect uncoupling in cells (500 nM) were several orders of magnitude greater than that required to protect against simulated IR injury (1 nM), this is likely attributable to the poor sensitivity of the GFP-LC3 system to detect low levels of autophagy and the poor sensitivity of the Seahorse system to detect uncoupling. Importantly, protection against IR injury induced by 1 nM cloxyquin was completely abolished by the coadministration of chloroquine (1 nM) to mouse hearts before ischemia, thus demonstrating a requirement for autophagy in cloxyquin-induced protection at the cellular level.

Using a Langendorff-perfused mouse heart model, we next sought to investigate whether autophagy was required for cloxyquin-induced cardioprotection ex vivo. In agreement with our previous findings in perfused rat heart (12), 20-min infusion of cloxyquin (1 nM) to mouse hearts before ischemia decreased post-IR infarct size (Fig. 4B) and improved functional recovery (Fig. 4C). Importantly, and consistent with the cardiomyocyte data above, chloroquine abolished the cardioprotective effects of cloxyquin pretreatment (Fig. 4, B and C). This reversal was not due to a toxic effect of chloroquine because administration of chloroquine alone did not exacerbate IR injury.

Cloxyquin modulates autophagy and is cardioprotective in vivo. We next investigated whether the effects of cloxyquin could be recapitulated in vivo. The formation of autophagosomes requires the lipidation of LC3, converting LC3-I to LC3-II. To examine the modulation of autophagy in vivo, homogenates of whole hearts isolated from mice that had been treated with cloxyquin (1 mg/kg) for 20 min were Western blotted for LC3, and indeed cloxyquin increased the LC3-II/LC3-I ratio (Fig. 5A).

Finally, we sought to test whether cloxyquin could elicit cardioprotection in an in vivo model of IR injury, left anterior descending coronary artery occlusion. Indeed, cloxyquin treatment (1 mg/kg) 20 min before occlusion significantly decreased infarct size from 31.4 ± 3.4% to 16.1 ± 2.2% of the area at risk (Fig. 5, B and C). The area at risk was not significantly different between experimental groups (Fig. 5D). Unfortunately, the ability of chloroquine to block cloxyquin-induced autophagy or cardioprotection could not be determined in vivo. Although separately cloxyquin (1 mg/kg iv) or chloroquine (10 mg/kg ip) were nonfatal treatments, mice administered with the combination of these agents did not survive (n = 5). Furthermore, attempts to inhibit autophagy in vivo using intraperitoneal injection of 1.8 mg/kg bafilomycin A1 did not elicit expected changes in LC3-II/LC3-I ratio at 20 min or 1 h (data not shown). Overall, our data indicate that cloxyquin induces mitochondrial uncoupling, autophagy, and cardioprotection, in multiple experimental systems.
DISCUSSION

In the present study, cloxyquin uncoupled oxidative phosphorylation in isolated mitochondria and in adult cardiomyocytes, induced autophagy in cardiomyocytes and in vivo, and elicited protection against IR injury in cardiomyocytes, in ex vivo perfused hearts and in vivo. A summary of the optimal concentrations at which these effects were observed is shown in Table 1. Although these numbers are largely agreeable, some mismatches are noted. This includes the higher concentration required to uncouple isolated mitochondria (likely attributable to the presence of BSA), the induction of protection in myocytes at a lower level than required to uncouple or induce autophagy (likely attributable to different sensitivities of the experimental systems), and the relatively high concentration required to protect and induce autophagy in vivo (vs. ex vivo, likely attributable to serum albumin and other factors that limit bioavailability). Nevertheless, there were common observations at common concentrations across systems, and furthermore, the protection induced by cloxyquin was blocked by an inhibitor of autophagy in all systems examined. Therefore, these data together suggest that the phenomena of uncoupling, autophagy, and cardioprotection all induced by cloxyquin may be mechanistically linked.

In pursuing the mechanism of cloxyquin-induced cardioprotection, we showed that cloxyquin is a mitochondrial uncoupler. Cloxyquin and its close relative clioquinol are both 8-hydroxyquinolines, a class of metal chelators. The effects of clioquinol on the mitochondrial membrane have been linked to its ability to chelate zinc (3), but here cloxyquin was shown to uncouple isolated mitochondria in zinc-free buffers. Although it is possible that endogenous zinc was sufficient to form cloxyquin-zinc chelates, the molecular structure of cloxyquin contains a hydroxyl moiety in series with multiple double bonds, making it reasonable to hypothesize that it may be a protonophore. It is also possible that cloxyquin may affect other mitochondrial functions by acting at the level of the respiratory chain, by stimulating activity of uncoupling proteins, or by acting on mitochondrial inner membrane ion channels. Alternatively, it is possible that cloxyquin may stimulate mitochondrial ROS generation, which could also induce either autophagy alone or could stimulate uncoupling. In this regard, it is notable that the related compound clioquinol has been shown to stimulate ROS generation although this appears to be via chelation of metals from nonmitochondrial Cu/Zn-SOD (25) and not via an effect on mitochondria.

The uncoupling effect of cloxyquin was inhibited by BSA, which is consistent with previous findings that serum albumin diminished the ability of clioquinol to uncouple rat liver mitochondria (13). Serum was therefore omitted from our cellular experiments because of this effect, as well as the fact that serum is known to be protective in cardiac IR injury because of its content of insulin and other important signaling molecules (21, 22, 49). Unsurprisingly, the removal of serum drastically increased the potency of cloxyquin, i.e., it uncoupled cells at a much lower concentration than seen in BSA-containing mitochondrial experiments.

This absence of serum also affected levels of autophagy. Here, no serum was used in the incubation of cardiomyocytes; this effectively mimicked a starved metabolic state (26), and indeed baseline levels of autophagy were quite high. Unexpectedly, although cloxyquin increased autophagosome formation above this baseline, neither chloroquine nor bafilomycin A1 further increased the puncta count. One potential explanation could be because autophagy in the presence of cloxyquin, and ramped up by the absence of serum, was already at a very high level (the so-called “autophagic ceiling”), leaving a very small window for detection of further increases upon addition of chloroquine or bafilomycin A1. This ceiling may be due to a limited availability of membrane lipid, energy, or other factors governing autophagosomal maximal abundance. An alternative explanation is that cloxyquin may simply inhibit autophagosome degradation. However, we found that autophagosomal degradation was not impaired by cloxyquin (Fig. 3D), thus favoring the autophagic ceiling theory. This observation suggests that cloxyquin is likely an inducer of autophagy, which would also be consistent with its mitochondrial uncoupling effect.

Given the limitations of this in vitro system, an in vivo autophagic flux assay would have been an excellent alternative to study the effects of cloxyquin on autophagy. However, although separate administration of cloxyquin or chloroquine was nonfatal, and cloxyquin resulted in the expected increase in LC3-II/LC3-I ratio (i.e., cloxyquin increased autophagosome abundance), simultaneous administration of cloxyquin and chloroquine was fatal to mice. Unfortunately, this also meant that chloroquine could not be used to test whether blocking autophagy could block cloxyquin-induced cardioprotection in vivo. Furthermore, attempts to inhibit autophagy in vivo using bafilomycin A1 did not elicit expected changes in LC3-II/LC3-I ratio.

The lack of effect of chloroquine on baseline IR injury (Fig. 4) suggests that there may be no role for baseline levels of autophagy in protecting the heart from IR injury. In contrast, the data showing an effect of chloroquine on cloxyquin-induced protection suggest that it is the extra autophagy in-

Table 1. Optimal concentrations of cloxyquin observed to impact biological phenomena in different experimental model systems

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<th>Mitochondrial uncoupling</th>
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Superscripts denote differences, as follows (see also RESULTS for full descriptions): ^a Isolated mitochondrial experiments contain BSA, which likely binds to hydrophobic cloxyquin, necessitating a higher concentration. ^b The concentration of cloxyquin that protects cardiomyocytes from ischemia-reperfusion (IR) injury likely does still induce autophagy and uncoupling but at levels that are below the limit of detection for these measurement systems. ^c Assuming a volume of 25 ml for a 25-g mouse, 1 mg/kg cloxyquin (MW 179.6) would equate to 5.5 µM with whole body distribution. As seen in the comparison between mitochondria and cardiomyocytes, presence of serum, albumin, and other factors (e.g., metabolism by liver, distribution to adipose tissue) likely limits bioavailability of cloxyquin in vivo, necessitating a higher concentration than ex vivo. X denotes that the parameter was not measured (or not measurable) in the particular biological system. *The combined delivery of cloxyquin and chloroquine (CQ) in animals was fatally toxic in vivo.
duced by cloxyquin treatment that serves a protective role in IR injury, so blocking this with chloroquine exacerbates the injury (or, put differently, removes the protection). Overall, our data suggest that autophagy is similar to a number of other important biological phenomena, with a baseline level serving a particular function and a stimulated/induced level serving a different function. We do not view the lack of effect of chloroquine on baseline IR injury as mechanistically inconsistent with a blocking effect of chloroquine on cloxyquin-induced protection from IR injury because these phenomena are likely to operate via distinct mechanisms.

By identifying cloxyquin as a cardioprotective compound that both uncoupled mitochondria and induced autophagy, this study demonstrates a potential link between mitochondrial uncoupling and autophagy within the context of cardioprotection. In this regard, mitochondrial uncoupling is known to induce autophagy of mitochondria via classical PINK1/Parkin-mediated signaling (37). In addition, low levels of uncoupling are cardioprotective (4). The mechanisms linking uncoupling to autophagy remain unclear but could include activation of metabolic signaling pathways such as AMPK or mammalian target of rapamycin, which are known to regulate autophagy (11, 27, 30, 34).

Although it has long been established that mitochondrial dysfunction is a key player in the pathology of IR injury, mechanisms to target this damage from a therapeutic perspective have been limited. In this regard, mitochondria with damaged electron transport chain proteins are known to generate more ROS (28, 41), which would be particularly detrimental at reperfusion. Fission, selective fusion, and mitochondrial autophagy (mitophagy) can selectively remove dysfunctional mitochondria (44) and can thereby limit ROS-mediated damage. As such, a mild global decrease in mitochondrial membrane potential could selectively remove a fragile population of mitochondria via autophagy, allowing the remaining more robust population to endure the oxidative stress of IR and then generate less ROS at reperfusion. This highlights that mitochondrial quality control may play a crucial role in the outcome of IR injury.

Similar quality control mechanisms may exist in other cardioprotective models, such as ischemic preconditioning (IPC) (35). IPC also both induces and requires mitochondrial autophagy (18, 19). Therefore, it may be interesting to determine whether other important preconditioning signals and phenomena (e.g., Akt activation, GSK-3β, K$_{\text{ATP}}$ channel opening) are also activated by cloxyquin. Given that volatile anesthetics are also known to be cardioprotective, potentially by acting at the mitochondrial level (1), it may also be interesting to explore whether mitochondrial uncoupling and autophagy are induced in anesthetic preconditioning.

Although mitochondrial uncoupling has been shown to induce cardioprotection in numerous model systems (4, 5, 15, 32, 43), autophagy may be only one mechanism linking these phenomena. Alternative mechanisms may include inhibition of mitochondrial Ca$^{2+}$ accumulation and overload, effects on mitochondrial ROS generation, or the recruitment of cytosolic signaling pathways that respond to uncoupling (e.g., the mitochondrial unfolded protein response, UPR$_{\text{mit}}$).

Finally, the present study reaffirms the utility of our previously established cell-based high-throughput screening model of IR injury (12), which initially identified cloxyquin as a protective molecule. With this study, cloxyquin has now been confirmed to be protective in vitro, ex vivo, and in vivo models of cardiac IR injury. These results illustrate the potential of cell-based screening to identify protective agents for use in IR injury and the ability to repurpose existing drugs to target CHD. In this regard, a number of “mild uncoupling agents” have also recently been identified (39, 42), and it may be interesting to determine whether these compounds can elicit cardioprotection and autophagy in a manner similar to cloxyquin.

**Limitations.** An in vivo autophagic flux assay using chloroquine or bafilomycin A1 could not be performed. In the case of chloroquine, there was toxicity when used in combination with cloxyquin, and mice died within 20 min of injection. Furthermore, we have learned from speaking with other investigators in the field that the combination of an autophagy stimulator and chloroquine is often lethal in animal models. As for bafilomycin A1, its pharmacokinetics make it unsuitable for analyzing the effects of cloxyquin on autophagy in vivo. In our hands, LC3-II/I ratio was unaltered following up to 1 h of bafilomycin A1 treatment at various doses. Most protocols that use bafilomycin A1 to inhibit autophagic flux in vivo require daily treatment via feeding or repeat intraperitoneal injections (23, 24, 46, 48). These models of chronic autophagic flux inhibition are unsuitable for analyzing the effects of 20 min of cloxyquin treatment. As such, there does not appear to be a ready solution to this problem. It is also important to note that no specific markers of mitophagy were used in this study, and, despite evidence that mitochondrial uncouplers are inducers of mitophagy (8), the conclusions of this study only point at general autophagy. It has been demonstrated that hearts lacking Parkin cannot be preconditioned via IPC (18). Therefore, it would be interesting to investigate whether PINK1 and Parkin are mediators of cloxyquin-induced cardioprotection.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: J.Z., S.M.N., and P.S.B. conception and design of research; J.Z. and W.R.U. performed experiments; J.Z. analyzed data; I.Z., S.M.N., and P.S.B. interpreted results of experiments; J.Z. prepared figures; J.Z. drafted manuscript; J.Z., S.M.N., and P.S.B. edited and revised manuscript; J.Z., S.M.N., and P.S.B. approved final version of manuscript.

**REFERENCES**

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