Mitigation of autophagy ameliorates hepatocellular damage following ischemia-reperfusion injury in murine steatotic liver

Nitika A. Gupta,1,2* Vasantha L. Kolachala,1,6 Rong Jiang,1 Carlos Abramowsky,1,3 Asha Shenoi,1,2 Astrid Kosters,1 Haritha Pavuluri,1 Frank Anania,4 and Allan D. Kirk1,2,5
1Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia; 2Transplant Services, Children’s Healthcare of Atlanta, Atlanta, Georgia; 3Department of Pathology, Emory University School of Medicine, Atlanta, Georgia; 4Department of Medicine, Emory University School of Medicine, Atlanta, Georgia; and 5Department of Surgery, Emory University School of Medicine, Atlanta, Georgia

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Ischemia-reperfusion injury (IRI) is a common clinical consequence of hepatic surgery, cardiogenic shock, and liver transplantation. A steatotic liver is particularly vulnerable to IRI, responding with extensive hepatocellular injury. Autophagy, a lysosomal pathway balancing cell survival and cell death, is engaged in IRI, although its role in IRI of a steatotic liver is unclear. The role of autophagy was investigated in high-fat diet (HFD)-fed mice exposed to IRI in vivo and in steatotic hepatocytes exposed to hypoxic IRI (HII) in vitro. Two inhibitors of autophagy, 3-methyladenine and bafilomycin A1, protected the steatotic hepatocytes from HII. Exendin 4 (Ex4), a glucagon-like peptide 1 analog, also led to suppression of autophagy, as evidenced by decreased autophagy-associated proteins [microtubule-associated protein 1A/1B-light chain 3 (LC3) II, p62, high-mobility group protein B1, beclin-1, and autophagy-related protein 7], reduced hepatocellular damage, and improved mitochondrial structure and function in HFD-fed mice exposed to IRI. Decreased autophagy was further demonstrated by reversal of a punctate pattern of LC3 and decreased autophagic flux after IRI in HFD-fed mice. Under the same conditions, the effects of Ex4 were reversed by the competitive antagonist exendin 9-39. The present study suggests that, in IRI of a steatotic liver, autophagy ameliorates hepatocellular injury, and preserves mitochondrial integrity. These data suggest that therapies targeting autophagy, by Ex4 treatment in particular, may ameliorate the effects of IRI in highly prevalent steatotic livers.

ISCHEMIA-REPERFUSION INJURY (IRI) is a common mechanism of clinical injury that, in the presence of hepatic steatosis, results in poor outcomes in shock/heart failure and after hepatic resection, hepatobiliary surgery, hepatopathy, and liver transplantation (5, 10, 36, 51). At a mechanistic level, the complexity of IRI on the background of steatosis arises mostly as a result of ischemia-induced hypoxia, reperfusion-induced oxidative damage, and steatosis-induced damage, each of which contributes a different type of injury (24, 25). A network of interactions among altered sinusoidal spaces, reduced microcirculatory blood flow, mitochondrial damage, and recruited immune cells further exacerbate this complexity (50). Hepatic IRI has thus been an area of increasingly intense investigation over the past decade, with a growing emphasis on mechanisms of IRI-mediated cell death in the presence of hepatic steatosis.

Studies have shown that, in addition to the more commonly described mechanisms of necrosis and apoptosis, autophagy is involved in cell death pathways (49). Autophagy plays a critical role in removing protein aggregates and damaged or excess organelles to maintain intracellular homeostasis (34). The ultimate effect of the lysosomal degradation in autophagy remains a matter of controversy, with various reports alluding to it as a prosurvival pathway (54, 55), intimating its role in programmed cell death (7, 31), or suggesting a dual functionality of prosurvival and prodeath (1, 9, 39). Hence, the effect of autophagy is likely contextual, with environmental factors and cellular stressors contributing to autophagy’s influence on cell survival (12, 35). However, the relative significance of autophagy and its related lysosomal pathways in cell death is incompletely defined, particularly in the settings of IRI and steatosis (8, 14, 53).

Glucagon-like peptide 1 (GLP1) hormone is released from the L cells of the small intestine and has been shown to have a multitude of effects on different organs. We have shown that exendin 4 (Ex4), a GLP1 analog, has a role in modulating hepatic steatosis (21) and plays a protective role in hepatic IRI by alleviating apoptosis and necrosis (20). In this study we sought to investigate two critical missing links. 1) Does autophagy play a role in the IRI of a steatotic liver? 2) Does Ex4 modulate autophagy, thereby decreasing the hepatocellular dysfunction in a fatty liver ischemia-reperfusion insult? We demonstrate that 3-methyladenine (MA) and bafilomycin A1 (BafA1), known inhibitors of autophagy, and Ex4 protect steatotic hepatocytes from ischemia-reperfusion-induced cell death. In addition, we show that Ex4 decreases the levels of several proteins associated with autophagy. Given the substantial increase in the incidence of hepatic steatosis in the general population and the frequency of varying degrees of hepatic IRI (e.g., systemic hypotension, liver surgery, and transplantation), these observations are of utmost clinical significance.

MATERIALS AND METHODS

Experimental animals. The Institutional Animal Care and Use Committee of Emory University approved all procedures, which were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, published by the US Public Health Service. Four-week-old male C57BL/6 mice were
obtained from Jackson Research Laboratories (Bar Harbor, ME). Over a period of 12 wk, half the mice were fed regular chow and the other half received a high (60%)-fat diet (HFD; Research Diets, New Brunswick, NJ). Mice were maintained on a 12:12-h dark-light cycle and allowed free access to food and water. Body weights were measured at regular intervals.

**Hepatic IRI.** After 12 wk of feeding, mice were subjected to hepatic IRI, as described in our previous publication (20). The abdomen was exposed, and a clamp was applied across the hepatic artery, portal vein, and bile duct; partial ischemia was induced for 20 min followed by 24 h of reperfusion (selected after 6 h to 3 days of reperfusion). Ex4 (20 μg/kg iv; Sigma Aldrich, St. Louis, MO) was administered 2 h prior to and immediately after surgery. Sham surgeries, in which the abdomen was opened and closed without clamping, were performed in anesthetized animals.

**Hepatocyte culture and in vitro hypoxic IRI.** Earlier studies employed human hepatoma (HuH7) cells as a substitute for primary hepatocytes to study in vitro hypoxic IRI (HIRI) (17). Accordingly, in the present study we also used HuH7 cells. Cells were cultured using DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (HyClone, Logan, UT). Steatosis was induced by addition of free fatty acids (FFA): palmmitic and oleic acids (400 nM each) with 10% fatty acid-free albumin (Sigma Aldrich). Steatotic and control HuH7 cells were placed in a hypoxia chamber for 30 min in serum-free medium with or without Ex4 (20 nM) and then reperfused (medium with 10% serum) with or without Ex4 for 2 h, as described elsewhere (20). A competitive antagonist of GLP1 receptor (GLP-1R), exendin 9-39 (Ex9-39, 1 μM), was added 30 min before Ex4 treatment.

**Hepatocellular damage.** Hepatocellular damage was assessed in vivo by measurement of serum alanine aminotransferase (ALT) levels using Infinity ALT/GTP reagent (Thermo Fisher, Scientific, Waltham, MA) and in vitro by the lactate dehydrogenase (LDH) cytotoxicity assay (Clontech, Mountain View, CA), which determines LDH release into the culture medium from dead or membrane-damaged cells. Cell culture media from steatotic HuH7 cells exposed to HIRI and treated with Ex4 were used, along with appropriate controls. Percent cytotoxicity was measured according to the manufacturer’s protocol using a Synergy 2 plate reader (BioTek, Winooski, VT) at 490 nm and expressed as international units per liter. Percent cytotoxicity was calculated using the following formula: cytotoxicity (%) = [(sample absorbance – low control absorbance) (high control absorbance – low control absorbance)] × 100, where low control represents the level of spontaneous LDH release from untreated cells and high control represents the maximum LDH activity that can be released from the 100% dead cells in response to Triton X-100.

**Autophagy inhibitors.** To determine whether autophagy indeed had a role in HIRI, we pretreated steatotic HuH7 and control cells for 30 min with 2.5 mM MA or 50 nM BafA1 (Sigma Aldrich), two known inhibitors of autophagy, and exposed the cells to HIRI, as described above. Percent cytotoxicity was measured using the LDH assay, as described above.

**Electron microscopy.** After IRI, liver tissues were fixed in glutaraldehyde. Electron microscopy was performed by the Emory Electron Microscopy Core facility to assess the damage to liver mitochondria. Sections were viewed on a transmission electron microscope (model H-7500, Hitachi High Technologies America, Pleasanton, CA). Mitochondrial damage score was based on membrane and cristae morphology and integrity, as described previously (11) and shown in Table 1.

**Mitochondrial enzyme activity analysis.** Mitochondrial enzyme activity was evaluated using the 2,3-bis-(2-methyl-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) Cell Viability Kit (Cell Signaling Technology, Danvers, MA) according to the manufacturer’s protocol. The XTT assay is based on cleavage of the tetrazolium salt XTT, which occurs in the presence of viable mitochondrial enzymes. Cultured cells were rinsed and treated with XTT for 12 h, and the formazan dye that formed was quantified using a Synergy 2 plate reader at 450 nm. Percent enzyme activity was calculated according to the manufacturer’s instructions.

**Western blotting.** Liver tissues and HuH7 cells were processed and subjected to Western blotting. Equal amounts of protein were separated on SDS-polyacrylamide gels and probed with specific antibodies against high-mobility group protein 1 (HMGB1), microtubule-associated protein 1A/1B-light chain 3 (LC3) II, p62, beclin-1, and autophagy-related protein 7 (ATG7) (Cell Signaling Technology). Relative band densities were analyzed using Image Lab software (version 3.0, Bio-Rad, Hercules, CA).

In vitro hepatocyte transfection with pSELECT green fluorescent protein-LC3 vector and confocal microscopy. Steatotic HuH7 cells were transfected with the pSELECT green fluorescent protein (GFP)-LC3 vector (a gift from Dr. Arianne Thiess, Department of Internal Medicine, Baylor Research Institute, Baylor University Medical Center, Dallas, TX) or the empty vector (27). After 48 h of transfection, cells were exposed to HIRI and treated with Ex4. Cells were then fixed in 4% paraformaldehyde, counterstained with the nuclear stain 4’,6-diamidino-2-phenylindole, and analyzed using a confocal laser scanning microscope (model FV1000, Olympus America, Center Valley, PA). Six random fields (per slide) were captured using ×60 and ×100 objectives, and the number of cells showing punctate GFP-LC3 staining was determined.

**Measurement of autophagy flux.** Autophagy flux was assessed in HIRI-exposed and non-HIRI-exposed steatotic HuH7 cells. A difference in LC3 II levels in the presence and absence of lysosomal degradation (BafA1, 10 nM) represents autophagic flux (30); BafA1 prevents maturation of autophagic vacuoles by inhibiting fusion of autophagosomes to lysosomes. To assess the autophagic flux, lean and steatotic cells were pretreated with or without BafA1 for 2 h and then exposed to HIRI and treated with Ex4 and Ex9-39. Western blots were performed as described above. LC3 II band intensities were analyzed using Image Lab software (version 3.0). LC3 II intensities were normalized to band intensities of β-actin, as in previous studies (40, 57). Autophagy flux (AF) was evaluated by the following equations according to the instructions of the VIVA Detect Autophlux Kit instruction manual (VIVA Bioscience, UK): UT AF = (UT + BafA1) – (UT – BafA1), MT AF = (MT + BafA1) – (MT – BafA1), and ∆AF = (MT AF) – (UT AF), where UT is untreated and MT is modulator (Ex4/Ex9-39)-treated.

**Statistical analysis.** Statistical analyses were performed using Student’s t-test (Prism 5.0, Graphpad, San Diego, CA). P < 0.05 was regarded as significant.

**RESULTS**

**Autophagy inhibitors MA, BafA1, and Ex4 decrease IRI-induced hepatocellular cytotoxicity.** To determine if autophagy plays a role in IRI of a steatotic liver, we utilized steatotic HuH7 cells exposed to HIRI, as described in MATERIALS AND METHODS. The autophagy pathway was blocked by pretreatment of cells with MA or BafA1, known inhibitors of autophagy. As
expected, LDH levels at baseline were significantly higher in steatotic than lean cells [13.6 ± 2.0 vs. 2.0 ± 0.8 (P < 0.008); Fig. 1A]. Pretreatment with MA mitigated cytotoxicity induced by HIRI [lean/HIRI+/MA−: 11.4 ± 0.9 vs. lean/HIRI+/MA+: 1.0 ± 0.3 (P < 0.001) and FFA/HIRI+/MA−: 59.4 ± 2.8 vs. FFA/HIRI+/MA+: 11.32 ± 1.3 (P < 0.0001); Fig. 1A]. Similarly, treatment with BafA1 (50 nM) also mitigated cytotoxicity induced by HIRI [lean/HIRI+/BafA1−: 30.6 ± 2.1 vs. lean/HIRI+/BafA1+: 16.4 ± 6.6 (P < 0.08) and FFA/HIRI+/BafA1−: 66.5 ± 3.5 vs. FFA/HIRI+/BafA1+: 45.6 ± 3.3 (P < 0.02); Fig. 1B]. These results imply that autophagy is a critical pathway in cellular toxicity of HIRI and warrant further investigation. Treatment with Ex4 had a similar effect, with decreases in cellular cytotoxicity in the steatotic HuH7 cells [lean/HIRI+/Ex4−: 22.5 ± 2.3 vs. lean/HIRI+/Ex4+: 15.1 ± 0.8 (P < 0.01) and FFA/HIRI+/Ex4+: 54.8 ± 3.1 vs. FFA/HIRI+/Ex4+: 40.9 ± 1.3 (P < 0.006); Fig. 1C], confirming the protective effect of Ex4 in our in vitro system (20). The concentrations of Ex4, BafA1, and MA were not normalized for comparison but were selected arbitrarily on the basis of findings in the literature. BafA1 and MA were used to determine whether autophagy is involved in the IRI-exposed steatotic hepatocytes, making it an important area for further study. MA inhibits autophagy by targeting phosphatidylinositol 3-kinase signaling, whereas BafA1 inhibits autophagy by inhibiting the fusion of autophagosomes to lysosomes. Even though MA and BafA1 act at different sites in the autophagic process, the observation that both inhibitors protected steatotic hepatocytes subjected to hypoxic reperfusion injury is significant.
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A

![Graph showing ALT (UI/L) for different groups: Lean IRI-/Ex4-, Lean IRI+/Ex4-, HFD IRI-/Ex4-, HFD IRI+/Ex4-.]

B

![Micrographs of Mitochondria, Cristae, and Membrane for Lean IRI-/Ex4-, Lean IRI+/Ex4-, HFD IRI-/Ex4-, HFD IRI+/Ex4-.]

C

D

E

F

G

H

![Bar graph showing Mitochondrial Damage score for Lean and HFD: IRI-/Ex4-, IRI+/Ex4-, IRI+/Ex4+, IRI+/Ex4+ with error bars.]

I

![Bar graph showing Percent Mitochondrial Enzyme activity for Lean and FFA: IRI-/Ex4-, IRI+/Ex4-, IRI+/Ex4+, IRI+/Ex4+ with error bars.]

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Ex4 treatment improved IRI/HIRI-induced hepatocellular injury and mitochondrial damage. Having determined the protective effect of Ex4 in cell death and the importance of autophagy in the cytotoxicity after IRI, we investigated the mitochondrial integrity and mitochondrial enzyme activity that are known to be altered in autophagy. Results presented in Fig. 2A clearly indicate that hepatocellular injury, monitored as serum ALT levels, was increased in steatotic livers compared with the corresponding lean mice and that exposure to IRI further exacerbated the injury in steatotic livers compared with livers from lean mice. Ex4 conferred almost complete protection against IRI in lean, as well as HFD-fed mice, [lean/IRI−/Ex4−: 46.8 ± 7.5 vs. lean/IRI+/Ex4−: 370 ± 33.7 (P < 0.0001); lean/IRI+/Ex4+: 95.5 ± 23.8 (P < 0.001); HFD/IRI−/Ex4−: 124.7 ± 16.9 vs. HFD/IRI+/Ex4−: 700.7 ± 43.5 (P < 0.0002); HFD/IRI+/Ex4+: 700.7 ± 43.5 vs. HFD/IRI+/Ex4+: 148.5 ± 39.1 (P < 0.0002), where all values are expressed as IU/L]. Mitochondrial damage, as shown by electron microscopic images of liver tissues, mirrored serum ALT levels. Lean control mice, with intact liver mitochondrial architecture with well-defined membranes and normal cristae, were given baseline scores of 0 (Fig. 2, B and H). In contrast, after exposure to IRI, the HFD-fed mice showed pronounced mitochondrial damage, as evidenced by a discontinuous membrane, particulate cristae, and mitochondrial ghosts; mitochondrial damage scores of these HFD-fed mice were significantly higher than those of the lean mice: 3.5 ± 0.28 vs. 1.5 ± 0.2 (P < 0.001; Fig. 2, C, F, and H). Treatment with Ex4 offered significant mitochondrial protection, with the mitochondrial score improving to 1.2 ± 0.37 (P < 0.002) in HFD-fed mice and 0.33 ± 0.3 (P < 0.04) in lean mice (Fig. 2, D, G, and H).

We also assessed mitochondrial enzyme activity using the XTT assay. Lean and steatotic HuH7 cells exposed to HIRI demonstrated decreased activity (Fig. 2H). Ex4 improved the mitochondrial enzyme activity [FFA/HIRI+/Ex4+: 66.8 ± 3.6 vs. FFA/HIRI+/Ex4−: 55.7 ± 1.2 (P < 0.008) and lean/HIRI+/Ex4+: 73.5 ± 1.6 vs. lean/HIRI+/Ex4−: 62.0 ± 0.6 (P < 0.0007)]. Thus, in the setting of IRI, Ex4 appeared to confer substantial mitochondrial protection (structural and functional) in lean and HFD-fed mice.

Ex4 mitigates the autophagy marker LC3 II in livers of IRI-exposed lean and HFD-fed mice. After establishing the protective effect of Ex4 after IRI in vivo and in vitro, we assessed biochemical markers for autophagy. For in vivo studies we assessed the autophagy marker LC3 II (48). IRI-exposed lean and HFD-fed mice demonstrated significantly higher levels of LC3 II than controls [lean/IRI−: 0.31 ± 0.07 vs. lean/IRI+/Ex4−: 0.86 ± 0.03 (P < 0.04) and HFD/IRI+: 2.4 ± 0.1 vs. HFD/IRI+/Ex4+: 0.92 ± 0.21 vs. HFD/IRI+/Ex4−: 2.4 ± 0.1 (P < 0.002); Fig. 3D]. Lean mice demonstrated a similar trend but to a lesser extent. These results demonstrate that Ex4 can mitigate IRI-induced autophagy as monitored by the levels of LC3 II.

Ex4 mitigates autophagy in steatotic HuH7 cells exposed to HIRI. In the next series of experiments, a detailed study was undertaken to determine the effect of Ex4 on autophagy in lean and steatotic HuH7 cells exposed to normoxia (HIRI−) as well as hypoxia (HIRI+). Autophagy was assessed by evaluating the autophagy markers LC3 II, p62, HMG1, beclin-1, and ATG7. As shown in Fig. 4, A and B, in normoxia-exposed steatotic hepatocytes, Ex4 increased the levels of autophagy markers; for example, LC3 II and p62 were statistically significantly increased. Although the increase in other markers was not statistically significant, the positive trend is clear (Fig. 4B). In contrast, in HIRI-exposed steatotic cells, Ex4 clearly and significantly decreased all the autophagy markers (Fig. 4, C and D). Lean hepatocytes failed to show statistically significant changes in the autophagy markers with exposure to normoxia or HIRI and with or without Ex4.

Ex4 treatment increases autophagy as evidenced by an enhanced punctate staining of GFP-LC3 in steatotic cells exposed to normoxia. To verify our observation that Ex4 exhibits dual mechanisms of accentuating and attenuating
Fig. 4. A–D: Ex4 mitigates autophagy in steatotic HuH7 cells during HIRI. Lean and steatotic hepatocytes were treated with and without Ex4 during normoxia (HIRI−) and hypoxia (HIRI+). Total cell lysates were subjected to electrophoresis followed by Western blotting with anti-LC3 I/II, p62, high-mobility group protein 1 (HMGB1), beclin-1, autophagy-related protein 7 (ATG7), and β-actin antibodies. A and C: HIRI− and HIRI+. Lanes 1 and 3, lean and FFA with no treatment; lanes 2 and 4, lean and FFA with Ex4 treatment. B and D: relative band density. Experiments were repeated 3 times in triplicate. Values are means ± SD. Ex4 treatment resulted in a statistically significant reduction of autophagy markers in steatotic cells exposed to HIRI. In steatotic cells that were not exposed to HIRI, Ex4 treatment resulted in an increase, rather than a decrease, in autophagy markers. Increase in LC3 II and p62 proteins was statistically significant. While the other proteins did not reach statistical significance, the increasing trend appears clear. *P < 0.05.
autophagy with exposure to normoxia or HIRI, respectively, we performed additional studies. Lean and steatotic HuH7 cells were transfected with pSELECT GFP-LC3 vector. In steatotic cells exposed to normoxia, GFP-LC3 showed a diffuse pattern (Fig. 5C). Upon treatment with Ex4 the diffuse pattern was transformed into a distinct punctate pattern, indicating increased autophagosome formation (Fig. 5D). The number of cells with puncta was quantified [FFA/Ex4−: 2.6 ± 0.42 vs. FFA/Ex4+: 7.46 ± 0.78 (P < 0.0009); Fig. 5E], and the number of puncta per cell was also quantified [FFA/Ex4−: 5.33 ± 0.8 vs. FFA/Ex4+: 12.67 ± 4.2 (P < 0.04); Fig. 5F]. Ex4 also led to a mild increase in autophagy in lean cells, but the effect was less robust than the increase in the steatotic cells and was not statistically significant (Fig. 5, A, B, E, and F). These data confirm that Ex4 increases autophagy during normoxia.

Ex4 treatment mitigates punctate staining of GFP-LC3 in lean and steatotic hepatocytes exposed to HIRI, indicating attenuation of autophagy. After verifying that Ex4 increases autophagosome formation in normoxia-exposed steatotic cells, we exposed GFP-LC3-transfected cells to HIRI. The steatotic HuH7 cells demonstrated an increase in punctate GFP-LC3 staining (Fig. 5F) compared with non-HIRI-exposed cells (Fig. 5C), indicating increased autophagosome formation. Quantitative data presented in Fig. 5, E and F (non-HIRI-exposed steatotic hepatocytes) and in Fig. 5, K and L (HIRI-exposed steatotic hepatocytes) indicate increased autophagosome formation in steatotic hepatocytes exposed to HIRI. Treatment with Ex4 led to a remarkable change, i.e., replacement of the punctate pattern with a prominent diffuse pattern of GFP-LC3 (Fig. 5J), implying attenuation of autophagy. The number of cells with puncta was quantified [FFA/Ex4+: 6.7 ± 0.6 vs. FFA/Ex4−: 3.1 ± 0.6 (P < 0.0009); Fig. 5K]. Lean HuH7 cells demonstrated a similar trend (Fig. 5, G and H). The number of cells with puncta was quantified [lean/Ex4−: 4.0 ± 0.6 vs. lean/Ex4+: 1.9 ± 0.3 (P < 0.008); Fig. 5K]. The number of puncta per cell was also quantified [FFA/Ex4−: 37.07 ± 5.0 vs. FFA/Ex4+: 21.85 ± 2.4 (P < 0.004); Fig. 5L]. Lean HuH7 cells demonstrated a similar trend [lean/Ex4−: 25.09 ± 4.4 vs. lean/Ex4+: 13.40 ± 2.6 (P < 0.03)]. These results corroborate our earlier data on the effect of Ex4 in reducing autophagy under HIRI conditions.

Ex4 increases autophagic flux in steatotic hepatocytes exposed to normoxia. Thus far, the results from monitoring autophagy biomarkers and from the GFP-LC3 transfection studies are consistent with the dual action of Ex4 in increasing or decreasing autophagy, depending on the presence or absence of HIRI. Next, we evaluated autophagic flux, which has been deemed the gold standard for precise and consistent evaluation of autophagosome formation. We used BafA1, an autophagy inhibitor, which inhibits fusion of autophagosomes to lysosomes and, thus, can detect the levels of LC3 II before degradation. After BafA1 (10 nM) treatment, autophagosome flux (monitored as changes in LC3 II levels) increased considerably in normoxia-exposed steatotic cells after treatment with Ex4, indicating accentuation of autophagy and confirming our above-mentioned findings [FFA/Ex4−: 0.15 ± 0.1 vs. FFA/Ex4+: 0.32 ± 0.03 (P < 0.03); Fig. 6, A and B]. Although the increase in lean cells did not reach statistical significance, the increasing trend is clear (Fig. 6B).

Ex4 decreases autophagic flux in steatotic hepatocytes exposed to HIRI. Next, we evaluated autophagic flux in steatotic HuH7 cells exposed to HIRI. After treatment with Ex4, a significant attenuation was observed in autophagic flux in the steatotic cells [FFA/Ex4−: 0.49 ± 0.05 vs. FFA/Ex4+: 0.2 ± 0.4 (P < 0.01); Fig. 6, C and D], in contrast to the increase observed in normoxia-exposed cells, thus confirming the dual effect of Ex4 on autophagy. The lean cells showed a mild increase in flux; however, the increase was not statistically significant.

To rule out the possible influence of cytotoxicity or viability induced by BafA1 on the flux data, we studied the effect of BafA1 on autophagy, as well as lean, hepatocytes exposed to normoxia and HIRI. BafA1 at 10 nM, a concentration at which BafA1 inhibited autophagy flux, is not cytotoxic, nor did it confer cell survival advantage. These results clearly rule out any complications that might arise by cytotoxicity or survival advantage on flux data (Fig. 6E).

The GLP-1R antagonist Ex9-39 reverses the effects of Ex4 on autophagy. Because Ex4 is an agonist of GLP-1R, we chose to determine whether Ex4 exerts its effects on autophagy via GLP-1R. We utilized Ex9-39, a competitive inhibitor, which competes with Ex4 for GLP-1R. The results presented in Fig. 7 indicate a significant increase in LC3 II levels in steatotic hepatocytes exposed to HIRI (lane 4) compared with non-HIRI-exposed cells (lane 2): 0.78 ± 0.14 vs. 0.31 ± 0.05 (P < 0.01). These effects were significantly attenuated on treatment with Ex4 in steatotic cells exposed to HIRI: 0.78 ± 0.14 in lane 4 vs. 0.39 ± 0.04 in lane 8 (P < 0.02). This attenuation was significantly reversed upon treatment with Ex9-39, a GLP-1R antagonist, under similar conditions: 0.39 ± 0.04 in lane 8 vs. 0.96 ± 0.2 in lane 12 (P < 0.01). In contrast, there was a
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Fig. 6. A and B: Ex4 increases autophagic flux in steatotic hepatocytes exposed to normoxia (HIRI –). Autophagic flux was assessed using BafA1 (10 nM). A: Western blot for LC3 I/II and β-actin (loading control). Lanes 1 and 5, Ex4/BafA1–; lanes 2 and 6, Ex4+/BafA1–; lanes 3 and 7, Ex4–/BafA1+; lanes 4 and 8, Ex4+/BafA1+. Lanes 1–4 are from lean cells, and lanes 5–8 are from steatotic (FFA) cells. B: graphical representation of autophagic flux, which was calculated using equation in MATERIALS AND METHODS. Values are means ± SD. FFA/Ex4 vs. FFA/Ex4+/BafA1 (P < 0.03). C and D: Ex4 decreases autophagic flux in steatotic hepatocytes exposed to HIRI. C: Western blot for LC3 I/II and β-actin. Lanes 1 and 5, Ex4–/BafA1–; lanes 2 and 6, Ex4+/BafA1–; lanes 3 and 7, Ex4–/BafA1+; lanes 4 and 8, Ex4+/BafA1+. Lanes 1–4 are from lean cells, and lanes 5–8 are from steatotic (FFA) cells. Autophagic flux was assessed as described in MATERIALS AND METHODS. D: graphical representation of autophagic flux. There was a significant decrease in autophagic flux in the Ex4-treated FFA cells: FFA/Ex4– vs. FFA/Ex4+/BafA1 (P < 0.01). E: BafA1 at 10 nM, the concentration at which BafA1 inhibits autophagy flux, is not cytotoxic, nor does it confer survival advantage to lean and steatotic hepatocytes exposed to normoxia, as well as HIRI. Lean and steatotic HuH7 cells were treated with BafA1 (10 nM) during normoxia and HIRI, and LDH activity was measured to calculate percent cytotoxicity. Values are means ± SD of triplicate plates.

*P < 0.05.

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significant increase in LC3 II levels upon treatment with Ex4 in non-HIRI-exposed steatotic hepatocytes: 0.31 ± 0.05 in lane 2 vs. 1.0 ± 0.15 in lane 6 (P < 0.0002). These effects were reversed upon treatment with Ex9-39 under similar conditions, although the reversal was not statistically significant: 1.0 ± 0.15 in lane 6 vs. 0.48 ± 0.13 in lane 10 (P < 0.07). These changes were not seen in normoxia- or hypoxia-exposed lean HuH7 cells: lane 5 vs. lane 9 and lane 7 vs. lane 11. These results are consistent with a possible role of GLP-1R in autophagy.

**DISCUSSION**

Autophagy is considered a fundamental component of several liver diseases (43, 46, 58), including fatty liver disease (2, 44), with an overall protective effect on lipid metabolism (16, 45). Few reports have alluded to autophagy in IRI of the heart and liver (37, 47, 52), and our study is the first to demonstrate that autophagy can be detrimental in the presence of hypoxia and steatosis. In addition, Ex4, a GLP-1R agonist, can decrease autophagy in IRI of a steatotic liver and increase autophagy in normoxia, with both scenarios resulting in cell protection. It should be interesting to explore the dual effect of Ex4. Nevertheless, this offers an exciting therapeutic target for a clinical condition of surmounting significance. Because the ultimate effect of Ex4 is cell survival, the autophagic balance between prosurvival and prodeath in conditions of IRI is pivotal.

We clearly show that IRI (and HIRI in the in vitro model) leads to an increase in autophagy, which is associated with cell death, as evidenced by increased serum ALT and mitochondrial damage in vivo and LDH in vitro, and that MA and BafA1, inhibitors of autophagy, confer protection to steatotic hepatocytes exposed to HIRI. The upregulation of autophagy is demonstrated by autophagic flux, biomarker upregulation, and the punctate pattern of GFP-LC3 II protein, monitored as puncta per cell, as well as number of punctate-positive cells per field. Autophagic flux is considered the gold standard for evaluation of autophagy, as treatment with BafA1 prevents degradation of autophagosomes, permitting accurate quantification. In our studies, the autophagic flux was increased in steatotic cells exposed to HIRI, with a corresponding increase in cell death. Several autophagy markers such as LC3 II, ATG7, beclin-1, HMGBl, and p62, also showed increased levels. The observation that increased levels of p62 were associated with increased autophagy deserves a comment. Since p62 is degraded during autophagy, it is argued that decreased levels of p62 can be observed when autophagy is
induced, and when autophagy is inhibited p62 accumulates. It is not surprising, therefore, that most of the reports, especially the nutrition/starvation-related studies, allude to an increase in p62 levels in response to a decrease in autophagy. Our model, although highly relevant clinically, is complicated at a molecular level: an environment of excess fat (steatosis) superimposed with hypoxia and nutrient deprivation (ischemia) followed by oxidative injury (reperfusion) makes comparison with autophagy in nutrition models difficult. This model is an interplay of several different signaling pathways. A probable explanation for our observation would be that Nrf2, which is decreased p62 levels, which in turn can result in decreased autophagy. Our model, although a similar trend was seen, differences were not statistically significant. *P < 0.05.

Microscopically, an increase in the punctate pattern of GFP-LC3-transfected proteins signified autophagosome formation (3, 27). Increased autophagy resulting in cell death has been reported in myocardial IRI (38, 47), whereas increased autophagy after hepatic IRI alludes to a protective role (18, 52). Hence, there is evidence for a dual role of autophagy, with mitochondrial integrity being an important determinant of cell survival or death.

Mitochondria respond to the stress of hypoxia, reactive oxygen species, and loss of growth factors by regulating cell death, as demonstrated in steatotic liver IRI (19). In our study, increased autophagy was associated with disruption of mitochondrial membranes and cristae in the liver of HFD-fed mice exposed to IRI, with decreased mitochondrial function confirmed in our in vitro model. Ex4 reversed the structural and functional damage. It remains to be determined, however, whether this mitochondrial damage is due to mitophagy (33) or other mechanisms, such as signaling pathways, lipid, or receptor dynamics. Additionally, although it is our contention that increased autophagy in the steatotic liver after IRI is a cell death mechanism because of the association with concurrent increased hepatocellular injury and mitochondrial dysfunction, we acknowledge that there are other facets to autophagy (29, 35), and its exact role remains elusive.

While the role of autophagy in promoting cell survival or cell death depends greatly on the surrounding environment, we have shown that Ex4, a GLP-1R agonist, is capable of modulating the level of autophagy under hypoxic conditions in steatotic cells, thus influencing the fate of the cell. GLP-1R agonists have been shown to be protective in IRI of the heart (4, 6), which corroborates our finding of a protective role against necrotic and apoptotic cell death in IRI of the steatotic liver (20). In normoxia, our results are consistent with a previous report (44) that shows the protective effect of Ex4 by increasing autophagy.

The novel finding in our study is that Ex4 decreases hepatocellular damage while decreasing autophagy, demonstrated in vivo and in vitro. Furthermore, this is mechanistically proven by blocking the GLP1 pathway with Ex9-39, a competitive antagonist of Ex4, leading to a reversal of the protective effects. Although the presence of GLP-1R in liver is...
controversial (23, 56, 59), we previously demonstrated its presence and function (15, 20, 44), and we confirmed its presence by real-time PCR in the present study in mouse liver tissue (data not shown). We acknowledge that several signaling pathways are likely to be involved in this phenomenon.

The controversy over the role of autophagy in cell survival or cell death is ongoing, with many unanswered questions (9, 13, 28, 32). In the present study we show that autophagy is a critical player in the cell death pathway of a steatotic liver exposed to IRI. It is our premise that the GLP-1R agonist Ex4, a relatively safe and commercially available agent, has the potential for mitigating this hepatocellular injury, although the exact underlying mechanisms remain under investigation. In addition, since different signaling pathways regulate the autophagic process (22), it should be of considerable significance and an exciting opportunity to design strategies to target one or more of these signaling pathways and, thus, mitigate hepatocellular injury.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

N.A.G. and V.L.K. are responsible for conception and design of the research; N.A.G., V.L.K., C.A., A.K., and A.D.K. interpreted the results of the experiments; N.A.G. and V.L.K. drafted the manuscript; N.A.G., V.L.K., A.K., C.A., F.A.A., and A.D.K. edited and revised the manuscript; N.A.G. and V.L.K. approved the final version of the manuscript; V.L.K., R.J., A.S., A.K., and H.P. performed the experiments; N.A.G., V.L.K. and C.A. analyzed the data; V.L.K. prepared the figures.

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