Gastrointestinal, Hepatobiliary, and Pancreatic Pathology

The Glucagon-Like Peptide-1 Receptor Agonist Exendin 4 Has a Protective Role in Ischemic Injury of Lean and Steatotic Liver by Inhibiting Cell Death and Stimulating Lipolysis

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Nonalcoholic fatty liver disease is an increasingly prevalent spectrum of conditions characterized by excess fat deposition within hepatocytes. Affected hepatocytes are known to be highly susceptible to ischemic insults, responding to injury with increased cell death, and commensurate liver dysfunction. Numerous clinical circumstances lead to hepatic ischemia. Mechanistically, specific means of reducing hepatic vulnerability to ischemia are of increasing clinical importance. In this study, we demonstrate that the glucagon-like peptide-1 receptor agonist Exendin 4 (Ex4) protects hepatocytes from ischemia reperfusion injury by mitigating necrosis and apoptosis. Importantly, this effect is more pronounced in steatotic livers, with significantly reducing cell death and facilitating the initiation of lipolysis. Ex4 treatment leads to increased lipid droplet fission, and phosphorylation of perilipin and hormone sensitive lipase – all hallmarks of lipolysis. Importantly, the protective effects of Ex4 are seen after a short course of perioperative treatment, potentially making this clinically relevant. Thus, we conclude that Ex4 has a role in protecting lean and fatty livers from ischemic injury. The rapidity of the effect and the clinical availability of Ex4 make this an attractive new therapeutic approach for treating fatty livers at the time of an ischemic insult. (*Am J Pathol* 2012, 181:1693–1701; <http://dx.doi.org/10.1016/j.ajpath.2012.07.015>)

The incidence of obesity and fatty liver disease is increasing worldwide. Non alcoholic fatty liver disease (NAFLD) includes a spectrum of liver abnormalities ranging from simple steatosis with preserved synthetic function to end-stage liver disease requiring transplantation.^{1,2} The cause of hepatic dysfunction related to steatosis remains incompletely defined.³ However, it is known that a steatotic liver has increased susceptibility to ischemic insults, such as those induced during liver resections and liver surgery,^{4–6} heart failure,⁷ and shock.⁸ In addition, steatotic livers are known to weather the ischemic insult of transplantation poorly,⁹ resulting in increased rates of primary nonfunction and initial graft dysfunction.^{10,11} As such, fatty livers are routinely turned down for transplantation and this impacts transplant wait list morbidity and mortality.¹² Thus, liver steatosis contributes to the public health burden and methods to mollify the adverse effects of liver steatosis are relevant across a large spectrum of hepatic diseases.

The inability of a steatotic liver to withstand ischemic insult is directly related to increased post ischemic cell death, which can occur through necrosis and apoptosis. The fundamental connection between intracellular fat and poor hepatic cell survival¹³ is incompletely understood. However, it has been suggested that methods that decrease intracellular fat reverse this susceptibility and the use of glucagon-like peptide-1 (GLP-1) analogues is one such approach. GLP-1 is secreted from the L cells of the small intestine and its cognate receptor (GLP-1R) is present in several organs, such as the pancreas, brain, heart, kidney, and liver. Although it is well known for its incretin action,¹⁴ it also has pleiotropic effects.^{15–19} In the liver we have shown that GLP-1 or its homologue Exendin 4 (Ex4) acts directly on steatotic hepatocytes to decrease their lipid con-

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tent.^{20,21} In addition, a cytoprotective action of Ex4 with improvement in cell survival has also been reported.²² Thus, we hypothesize that anti-steatotic effects of Ex4 in hepatocytes and cytoprotective effects in other organs make it a rational target for investigation in steatotic livers undergoing ischemia reperfusion injury (IRI), a common clinical scenario in people with NAFLD. In this study, we explore the role of Ex4 in protecting against necrosis and apoptosis, the two forms of cell death encountered in hepatic IRI, and we provide evidence to show that Ex4 stimulates lipolysis with a short course of treatment. To our knowledge, this is the first study showing a direct and rapid action of Ex4 in acutely reversing the vulnerability of a steatotic liver to ischemic insults, supporting the investigation of Ex4 as a potential therapeutic agent for treatment of people with NAFLD undergoing ischemic injury and at the time of procurement of a fatty liver for transplantation.

Materials and Methods

Experimental Animals

The Institutional Animal Care and Use Committee (IACUC) of Emory University approved all procedures performed on animals and all experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the United States Public Health Service.

C57BL/6 male mice were obtained from Jackson Research Laboratories (Bar Harbor, ME) at 4 weeks of age and were maintained on a 12-hour dark-light cycle and allowed free access to food and water under conditions of controlled temperatures ($25 \pm 2^\circ\text{C}$). Animals were divided into two groups. Half the animals were fed a regular diet and the other half received a high fat diet (60% fat; Research Diets, Inc, NJ) *ad libitum* for 12 weeks. Body weights were monitored during this period.

Hepatic IRI

After 12 weeks of feeding, mice on a lean and a high fat diet were subdivided into control and IRI groups with and without Ex4 ($n = 8$ per group). Sham controls were also included in the study. Ex4 (20 $\mu\text{g/kg}$; Sigma-Aldrich, St. Louis, MO) was given 2 hours before surgery and 2 hours after surgery through the tail vein. Hepatic IRI was performed under general anesthesia, induced, and maintained by ketamine/xylazine. A vertical incision was made through the skin and peritoneum, exposing the porta hepatis. A small clamp was applied to the portal vein and hepatic artery, and ischemia was induced for 20 minutes. The clamp was then removed and blood flow was restored (reperfusion). After closing the abdomen, the mice were placed in a recovery cage and allowed free access to food and water. Sham surgery animals underwent a laparotomy and closure without hepatic vascular clamping. All animals were sacrificed 24 hours after closure. Serum samples were collected for serum alanine aminotransferase (ALT) measurement and liver tissues were frozen or fixed in buffered formalin. Serum ALT was used as a measure of hepatocellular damage and was determined by using Infinity ALT reagent (Thermo Scientific, Middletown, VA).

Histological Evaluation

Paraffin sections of liver were stained with H&E. Six random images were taken from each slide and necrotic areas were quantified using Metamorph software (version 7.5.6) (Molecular Devices, Sunnyvale, CA). Percent area of necrosis was calculated out of the total area. Steatosis was assessed in frozen liver sections using Oil Red O (ORO) stain, as represented by the red color.

In Vitro Ischemia-Hypoxia Model of Steatotic Hepatocytes

HuH7 cells were cultured using Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS, Hyclone, Logan, UT). After overnight serum starvation, the cells were treated with free fatty acids: palmitic and oleic acid (400 nmol/L), along with 10% essential free fatty acids free bovine serum albumin (BSA, Sigma Aldrich) for 10 hours. Steatosis was assessed using ORO stain. The steatotic hepatocytes were kept in a hypoxia chamber (1% O_2 , 9% CO_2 , and 95% N_2) for 30 minutes, followed by a 2-hour reperfusion by adding serum containing medium and placing the cells in the regular incubator at normal atmospheric conditions (20% O_2 , 5% CO_2). Ex4 was added 30 minutes before and after hypoxia, and the monolayers were tested for apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining.

TUNEL Staining

TUNEL staining was performed according to the manufacturer's instructions (Roche Applied Sciences, Indianapolis, IN) both in the liver tissue from mice undergoing IRI and in *in vitro* steatotic hepatocytes with appropriate control groups. Nuclei were stained with DAPI. Images were scanned and processed as immunofluorescent samples (described as follows).

Immunofluorescence and Confocal Microscopy

HuH7 cells were plated on chamber slides, and on reaching confluence they were treated with palmitic and oleic acid for 10 hours followed by treatment with Ex4 (20 nmol/L) for 10 minutes (controls were treated with Ex4 free media), washed, permeabilized, and fixed in 4% paraformaldehyde. These cells were further blocked in 2% bovine serum albumin and incubated with phospho-perilipin mouse monoclonal antibody (Ser 497, Vala Sciences, San Diego, CA) and rabbit phospho-hormone-sensitive lipase (p-HSL Ser563, Cell Signaling Technology, Inc., Danvers, MA) overnight at 4°C . They were then incubated with Alexa fluor 594 secondary antibody (Cell Signaling Technology, Inc) for 60 minutes, at 37°C . Nuclei were stained with DAPI. Samples were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and scanned using an inverted laser confocal microscope (Olympus Fluoview FV1000, IX81; Olympus America Inc; Center Valley, PA). Each image was acquired three-dimensionally (x, y, and z), sequentially with appro-

priate lasers at 800×800 pixels, 12 bits with the speed of $4 \mu\text{s}/\text{pixel}$). Six random fields were captured using $\times 40$ objective into z stacks (1 micron each) for each sample. All of the independent experiments were acquired similarly with same settings. Image processing and analysis were performed using FV10-ASW Version 2.1 (Olympus America Inc., Center Valley, PA). Briefly, the following arithmetic numbers were obtained from independent experiments and fields (3 z-stacks from each field) of all samples to perform statistical analysis: intensity, area, overlay, graphics, co-localization, and file conversions. These experiments were repeated three times.

Adipocyte Culture

3T3-L1 cells were grown on chamber slides and differentiated into adipocytes by placing in a differentiation medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum supplemented with dexamethasone ($1 \mu\text{mol/L}$), isobutylmethylxanthine ($500 \mu\text{mol/L}$), and insulin ($10 \mu\text{g/mL}$). The differentiated adipocytes were then treated with Ex4 and forskolin (positive control) for 10 minutes. Immunofluorescence was performed with phospho-perilipin and phospho-HSL antibodies, as previously

described. The cells were stained with a fluorescent lipid staining kit (Vala Sciences, San Diego, CA) for 1 hour at 37°C followed by nuclear staining with DAPI.

Tissue Triglyceride Quantification

Liver tissue triglycerides were measured using a triglyceride assay according to the manufacturer's instructions (Biovision, CA) and were expressed as nmol/mg liver tissue.

Electron Microscopy

Liver tissues were fixed in glutaraldehyde and electron microscopy was performed by the Electron Microscopy Core facility, Emory University.

Statistical Analysis

Statistical analysis was performed by Student's *t*-test (Graph Pad Prism 4.0, San Diego, CA). A probability value of $P < 0.05$ was regarded as significant. One-way analysis of variance was used where more than two groups were compared.

Figure 1. Exendin 4 (Ex4) mitigates necrosis after ischemia reperfusion injury. **A:** Body weights of mice fed a high fat diet and regular chow were monitored for 12 weeks. **B:** Oil Red O staining showing fat globules (as depicted by a red color) in the livers of mice fed HFD (left) and regular chow (right). **C–H:** Light microscopic images of H&E staining showing percent necrosis in liver tissue specimens. Representative image of lean control mice (**C**), lean mice subjected to hepatic ischemia reperfusion injury (IRI) without Ex4 (**D**) and with Ex4 ($20 \mu\text{g}/\text{kg}$) (**E**), control mice fed HFD diet (**F**), HFD fed mice subjected to hepatic IRI without Ex4 (**G**) and with Ex4 (**H**). **Arrows** indicate the areas of necrosis. **Insets** represent higher magnification of lipid droplets. **I:** Graphical representation of percent necrosis calculated out of total area per field using Metamorph software ($n = 8$ per group; $P < 0.0001$). Open bars represent lean and HFD controls; gray bars represent lean and HFD IRI+; and black bars represent lean and HFD IRI+/Ex4+. **J:** Serum alanine aminotransferase levels in lean and HFD mice subjected to IRI with and without Ex4 treatment. Open bars represent lean and HFD controls; gray bars represent lean and HFD IRI+; and black bars represent lean and HFD IRI+/Ex4+. Data are mean $\pm$ SD; $n = 8$ per group; lean IRI+ versus HFD IRI+; * $P < 0.05$; † $P < 0.01$; HFD IRI versus HFD IRI+Ex4+; ‡ $P < 0.008$.

Results

Ex4 Mitigates Necrosis after IRI

To investigate the effect of Ex4 in IRI of lean and steatotic livers, we used an established *in vivo* model of hepatic steatosis. Body weights were monitored at regular intervals and mice were fed a high fat diet, which showed a significant increase in weight relative to regular chow fed controls (high fat diet 37.1 ± 1.0 grams versus lean 25.2 ± 0.5 grams; $P < 0.0001$) (Figure 1A). The presence of hepatic steatosis was verified by ORO staining (Figure 1B, top panel, red staining). After IRI, an extensive area of necrosis was evident in the steatotic livers as compared to livers of lean mice ($31.0\% \pm 3.0$ versus $5.2\% \pm 0.1$) (Figure 1, G versus D). When treated with Ex4, before and after IRI, a protective effect was seen leading to significantly decreased necrosis in steatotic mice ($3.2\% \pm 0.9$; $P < 0.0001$) (Figure 1H). In addition, lean mice treated with Ex4 also demonstrated minimal to no necrosis (Figure 1E).

Hepatocellular damage was also assessed by measuring serum ALT levels. As expected, the baseline serum ALT was numerically, but not statistically significantly higher in steatotic mice as compared to the lean mice. However, after IRI and 24 hours of reperfusion, serum ALT levels were markedly increased in the steatotic mice as compared to the lean mice (700 ± 43.5 IU/L versus 370 ± 33.7 IU/L; $P < 0.01$) (Figure 1J), representing more extensive hepatocellular affliction in the steatotic mice. On treatment with Ex4, a protective effect was seen with significant reduction of serum ALT levels in both steatotic and lean mice undergoing IRI. A fourfold decrease in serum ALT was demonstrated in the Ex4 treated steatotic mice undergoing IRI (180 ± 37.16 IU/L versus 700 ± 43.5 IU/L; $P < 0.008$) (Figure 1J). Improvement in serum ALT was also evident in Ex4-treated lean mice undergoing IRI

(95.76 ± 24.05 versus 370 ± 33.72 IU/L; $P < 0.001$), but the effect was twofold. These data show that a short course of Ex4 treatment has a protective effect on the liver undergoing IRI, as evidenced by significantly less necrosis and reduced elevations in post ischemic serum ALT, and this effect is particularly evident in the steatotic liver.

Ex4 Decreases Apoptosis Induced by IRI

One of the mechanisms by which hepatocyte death occurs during IRI is apoptosis.²³ To determine whether Ex4 had an anti-apoptotic effect, H&E stained liver sections were assessed by a pathologist blinded to the treatment groups. Apoptotic bodies were identified by the presence of pyknotic nuclei and various stages of karyorrhexis and karyolysis. The total number of apoptotic bodies were counted in each field and normalized to the live cells. We observed that on treatment with Ex4, there was a significantly lower number of apoptotic bodies in the steatotic liver as compared to the nontreated steatotic mice undergoing IRI (6.9 ± 3.06 versus 25.3 ± 4.3 ; $P < 0.03$) (Figure 2, F versus E). Although after IRI, the apoptotic bodies were not significantly increased in the lean liver (as compared to the steatotic liver), Ex4 treatment lead to lower apoptosis as compared to nontreated lean IRI (1.5 ± 0.6 versus 6.0 ± 1.4 ; $P < 0.003$) (Figure 2, C versus B). To further verify these findings, we performed a TUNEL assay on frozen liver tissues. Treatment with Ex4 led to a fourfold reduction in TUNEL-positive cells in liver sections of steatotic mice undergoing IRI (23.67 ± 2.9 versus 9.0 ± 0.7 ; $P < 0.0008$) (Figure 2, M versus L). This result, along with direct histological evidence shows that Ex4 protects the steatotic liver from cell death not only by necrosis as previously shown, but also from

Figure 2. Exendin 4 (Ex4) decreases apoptosis induced by ischemia reperfusion injury (IRI). High fat diet (HFD)-fed mice were subjected to hepatic IRI with or without Ex4 treatment. **A–F:** Light microscopic images of H&E stain showing apoptotic bodies in lean and HFD mice with or without Ex4 treatment. The number of apoptotic bodies was counted relative to live cells in four random areas per slide, eight slides per group. Representative images of lean control (A), lean IRI+ (B), lean IRI+/Ex4+ (C), HFD control (D), HFD IRI+ (E), HFD IRI+ Ex4+ (F) are shown. Arrows indicate apoptotic bodies. **Inset** shows higher magnification of apoptotic bodies. **G:** Graphical representation of the number of apoptotic bodies with or without Ex4 treatment ($n = 8$ per group; one-way analysis of variance; $*P < 0.01$). *In vivo* terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining showing TUNEL-positive cells (green fluorescence) in lean and HFD mice controls (H and K) subjected to IRI (I and L), and lean and HFD IRI+/Ex4+ (J and M), graphical representation of TUNEL-positive cells (N). Open bars lean and HFD controls, gray bars lean and HFD IRI+, and black bars represent lean and HFD IRI+/Ex4+, respectively. Data are mean $\pm$ SD; $n = 8$; $*P < 0.001$.

Figure 3. Exendin 4 (Ex) decreases apoptosis induced by ischemia reperfusion injury in hepatocytes *in vitro*. HuH7 cells were plated on chamber slides and made steatotic by addition of free fatty acids. Cells were then subjected to hypoxia (Hy+) and reperfusion with or without Ex4 treatment, before and after hypoxia. Shown here are representative confocal images of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive stain [green fluorescence (A–F)]. Lean and steatotic hepatocytes exposed to hypoxia and reperfusion is shown in (B and E), respectively. Lean and steatotic hepatocytes exposed to hypoxia and treated with Ex4 are shown in (C and F), respectively. Arrows represent TUNEL positive cells. Graphical representation of TUNEL-positive cells is shown in (G). These data are representative of three independent experiments done in triplicate (* $P < 0.001$).

apoptosis, resulting in overall improvement in resilience of the liver to IRI, in particular in the steatotic liver.

Ex4 Decreases Apoptosis Induced by IRI in Hepatocytes in Vitro

To verify that the effect of Ex4 on steatosis was indeed a direct action, we performed TUNEL assays in HuH7 cells that were made steatotic *in vitro* and exposed to a hypoxia chamber. As shown in Figures 3, C and F, there was a significantly lower number of TUNEL-positive cells in lean and steatotic hepatocytes exposed to Ex4 as compared to nontreated lean and steatotic cells (5.8 ± 0.29 versus 26.6 ± 2.2 in lean cells; $P < 0.001$ and 13.53 ± 0.94 versus 34.52 ± 5.3 in steatotic cells; $P < 0.001$) (Figure 3G). This mitigation of apoptosis by Ex4 in hepatocytes undergoing ischemia-hypoxia-reperfusion *in vitro* provides support to the *in vivo* protective effect of Ex4 seen in our previous experiment in decreasing cell death in hepatocytes undergoing IRI. Although the reduction in apoptosis is seen in both lean and steatotic cells, it is several-fold higher in the

steatotic hepatocytes. The effectiveness of Ex4 in normal and steatotic livers undergoing IRI broadens the potential of its clinical applicability.

Ex4 Decreases Fat Content and Causes Lipid Droplet Fission

To assess the effect of Ex4 on lipid droplets, we examined liver histology by H&E staining. In steatotic mice undergoing IRI without Ex4, there was an increase in coalescence of fat droplets or lipid droplet “fusion” leading to macrovesicular steatosis (Figures 4, B and E), a striking difference from the predominantly microvesicular

Figure 4. Exendin 4 (Ex4) decreases fat content and causes lipid droplet fission in ischemia reperfusion injury (IRI) of a steatotic liver. Steatotic mice fed a high fat diet (HFD) were sacrificed 24 hours after IRI, liver tissues were fixed in formalin, paraffin sections were processed for H&E, and frozen sections for Oil Red O staining, respectively. Representative image of fat droplets by H&E stain in HFD control mice (A), HFD IRI+ (B) and HFD IRI+/Ex4+ (C). Panel below represents Oil Red O stain in HFD control mice (D), HFD IRI+ (E), and HFD IRI+/Ex4+ (F). Electron microscopic image of liver tissues of steatotic control mice (G and J) and steatotic mice subjected to IRI without Ex4 (H and K) and with Ex4 treatment (I and J). Lipid droplet fusion and fission are indicated by short arrows. Representative image of steatotic mice subjected to IRI showing fusion of lipid droplet (H) and fission of lipid droplets after Ex4 treatment (I) are shown. Higher magnifications of the previously described images (K and L, respectively) are also shown. Graphical representation of triglyceride levels in steatotic mice (M). Open bars represent mice fed HFD control, gray bar represent HFD IRI without Ex4, and the black bar represents HFD IRI+/Ex4+. These data are representative of $n = 8$ per group; * $P < 0.009$.

Figure 5. Exendin 4 (Ex4) decreases fat content in steatotic HuH7 cells undergoing ischemia-reperfusion *in vitro*. Representative images for Oil Red O (ORO) staining of control, non-steatotic HuH7 cells (A), HuH7 free fatty acids (FFA) + (B), HuH7 FFA+/hypoxia (Hy+) (C), and HuH7 FFA+ Hy+/Ex4+ (D). Graphical representation of quantification of ORO stain by image pro software. Open bars are lean and FFA+ controls, gray bar is FFA+/Hy+, and black bar represents FFA+Hy+ Ex4+; **P* < 0.006. Shown is a representative figure from three independent experiments done in triplicate.

fat before IRI (Figures 4, A and D). In the Ex4 treated mice, this effect was ameliorated with a significant reduction in large fat droplets (Figures 4, C and F). To study these lipid droplets at a subcellular level, we performed

electron microscopy. In the Ex4-treated mice undergoing IRI, electron microscopic images showed that the vast majority of lipid droplets in a state of “fission” in which the otherwise smooth contour of lipid droplets had become irregular, showing multiple pseudopodia-like protrusions (Figures 4, I and L). These results clearly demonstrated a direct effect on the lipid droplet, leading to fission, a known precursor of lipolysis.²⁴ In addition, total liver triglycerides were significantly reduced in the Ex4-treated steatotic mice undergoing IRI (49.51 ± 4.85 versus 95.75 ± 15.32 nmol/mg of liver tissue; *P* < 0.009). The baseline triglyceride level of the control steatotic mice (without IRI and Ex4) was similar to that of the steatotic mice undergoing IRI without Ex4 (53.06 ± 8.7 nmol/mg) (Figure 4M). The reduction in liver triglyceride content by Ex4 provides further evidence for the lipolytic effects of Ex4.

Ex4 Decreases Fat Content in Steatotic HuH7 Cells Undergoing Ischemia-Reperfusion in Vitro

To confirm that the lipolytic action was indeed a direct effect of Ex4, we performed *in vitro* studies and exposed steatotic HuH7 cells to ischemia and hypoxia followed by reperfusion, with and without Ex4 treatment. Ex4-treated cells showed substantially less steatosis than the non-treated steatotic HuH7 cells. ORO staining confirmed the decrease steatosis in Ex4-treated steatotic HuH7 cells (Figure 5) (*P* < 0.006). This provides evidence for the

Figure 6. Exendin 4 (Ex4) upregulates phosphorylation of perilipin and hormone sensitive lipase; and initiates lipolysis in adipocytes and steatotic HuH7 cells. **A–F:** Confocal immunofluorescence images of adipocytes representing lipolysis. Adipocytes were grown and differentiated and treated with or without Ex4 and subjected to immunofluorescence with phospho-perilipin and phospho-hormone sensitive lipases (HSL) antibodies. Green fluorescence represents fat staining, blue represents nuclear staining, and red represents phospho-perilipin (A–C), and phospho-HSL (D–F). Untreated adipocytes are shown in A and D, adipocytes treated with Ex4 and stained for phospho-perilipin (B) and stained for phospho-HSL (E). Positive control for lipolysis, forskolin (FSK) is shown in C and F. Graphical representation of co-localization intensity of fat and phospho-perilipin (G) fat and phospho-HSL (H) are shown. Open bar represents control, gray bar represents Ex4 treatment, black bar represents FSK-treated cells. Similar immunofluorescence staining for phospho-HSL was performed in HuH7 cells. Nuclear stain (blue), fat stain (green), and phospho-HSL (red) (arrow) and merged images of HuH7 control (I), HuH7 treated with Ex4 (J), and graphical representation of mean staining intensity of phospho-HSL (K). Open bar represents untreated cells and black bar represents Ex4-treated HuH7 cells. This is representative from three independent experiments done in triplicate; **P* < 0.04.

direct action of Ex4 on hypoxic hepatocytes causing a reduction in hepatic steatosis.

Ex4 Upregulates Phosphorylation of Perilipin and Hormone-Sensitive Lipase and Initiates Lipolysis

To investigate the underlying mechanism of the effects of Ex4 on the lipid droplet, we investigated proteins that have a crucial role in lipid droplet stabilization by using adipocytes as our experimental model. Perilipin is one such protein that is bound to the lipid droplet preventing its degradation from hormone sensitive lipases (HSL). When perilipin is phosphorylated, access of HSL to the lipid droplet is facilitated, leading to lipolysis. To investigate this action, adipocytes and steatotic HuH7 cells (Ex4-treated and nontreated controls) were probed with phospho-perilipin (Ser 497) and phospho-HSL (Ser 563) antibodies and examined by confocal microscopy. As shown in Figure 6, A–C, perilipin was phosphorylated in the Ex4 treated adipocytes as compared to nontreated controls, an effect that was similar to that seen with forskolin, a positive control for lipolysis.²⁵ In addition, HSL, which in a nonstimulated state, has a cytoplasmic distribution that becomes phosphorylated and aligned along the lipid droplet surface, indicating initiation of the lipolytic process (Figures 6, D–F). In adipocytes, Ex4 treatment leads to a significant increase in co-localization of lipid droplet with phospho-perilipin and phospho-HSL, as quantified by confocal microscopy (Figure 6, G and H). This was also evident in the steatotic HuH7 cells (Figure 6, I–K). This provides evidence for the direct effect of Ex4 through phosphorylation of HSL enzymes and perilipin, in the process of lipolysis.

Discussion

With the rising incidence of obesity, there is an increase in the number of people with fatty liver disease. A fatty liver is vulnerable to ischemic insults which are encountered during shock, heart failure, liver resection, and transplantation, and manifest during the ensuing reperfusion.^{4,10} Thus, IRI results in extensive damage to the fatty liver, increasing morbidity in this burgeoning population and further escalating obesity-related health care costs. The effects of Ex4 are pleotropic and have been described in several organs, including the pancreas,^{26,27} hypothalamus,²⁸ and heart,^{29–31} and we have recently demonstrated its role in the reduction of hepatic steatosis.²⁰ In the present study, we show that Ex4 protects the liver from cell death induced during IRI, an effect that is significantly more pronounced in the fatty liver. We also show that Ex4 initiates lipolysis through perilipin and HSL, and we postulate that this is likely the reason for increased protection seen from cell death after IRI in the fatty liver. These effects make clinical applicability of Ex4 in patients with fatty livers at the time of liver surgery or organ procurement for transplantation of an exciting therapeutic option worth additional investigation.

The exact mechanism for the inability of a steatotic liver to withstand ischemic insults is unclear, but it is known that there is extensive hepatic damage caused by activation of cell death pathways.³² Several studies have reported the presence of necrosis or apoptosis in the liver after IRI,^{32–34} with some reporting predominance of one over other,³⁵ or the presence of both.²³ In our study, we demonstrate that both necrosis and apoptosis are present in the liver subjected to IRI, resulting in worse damage in the steatotic liver than in a lean liver. The treatment of lean and steatotic mice with Ex4-protected livers from necrosis and apoptosis is compared to nontreated mice in the respective groups. Reports have shown the attenuation of apoptosis and necrosis by Ex4 in other organs^{22,36,37}; however, to our knowledge this is the first study showing this protective effect in the IRI of the liver, particularly the steatotic liver. In addition, this is also the first study to demonstrate that the protective effect of Ex4 is observed after a short course of treatment, making this an agent with potential for use in the peri-ischemic period, or at the time of organ procurement. These data offer a view of steatosis as a dynamic state susceptible to acute modification, and in doing so provide a basis for intervention *in vivo* or perhaps *ex vivo* in the case of organ procurement.

The lipid droplet has been recognized as an actual cell organelle instead of an inert substance deposited within the cell.³⁸ One of the processes by which growth of lipid droplets has been reported to occur is “fusion or coalescence.”³⁹ In our study, we observed that lipid droplets in

Figure 7. Schematic representation of GLP-1R signaling. We have previously shown that activation of GLP-1R by its agonist Exendin 4 (Ex4) increases intracellular cyclic adenosine monophosphate (cAMP) levels. Here, we propose that the activation GLP-1R by Ex4 mediates phosphorylation of perilipin and hormone sensitive lipases (HSL) leading to initiation of lipolysis and increased cell survival in steatotic hepatocytes.

the hepatocytes undergo fusion after being subjected to IRI, resulting in an increase in the size of the lipid droplet or macrovesicular steatosis, which is associated with increased hepatocellular damage and poor clinical outcomes in animal and human studies.^{3,40} In addition, lipid droplets have also been reported to undergo “fission,” eventually leading to breakdown or lipolysis.⁴¹ In our study, using electron microscopy, we demonstrate that in the steatotic liver of Ex4-treated mice undergoing IRI, the vast majority of the lipid droplets are in a state of fission within a few hours of treatment. The otherwise smooth contour of the droplet (Figures 4, H and K) becomes irregular, showing multiple pseudopodia-like protrusions (Figures 4, I and L), eventually leading to smaller droplets. This decrease in macrovesicular fat seen after Ex4 treatment may be due to breakdown of the large lipid droplets into small lipid droplets. Fission occurs under maximum stimulation of lipolysis, as shown by Guo et al,⁴² in turn offering a mechanistic advantage in providing a larger surface area for lipases.

Several proteins have also been detected on lipid droplets, which include the PAT domain proteins, particularly perilipin.⁴³ When lipolysis is initiated, it leads to phosphorylation of perilipin and HSL,⁴⁴ both of which are directly involved with activation of lipolysis.⁴⁵ There is evidence from adipose tissues that lipolysis is a highly regulated process, and Zechner et al⁴⁶ have shown that it can be induced by catecholamines, which then bind to the G protein coupled receptors leading to an increase in intracellular cyclic-adenosine monophosphate (cAMP) via activation of adenylate cyclase and protein kinase A. Subsequent phosphorylation of perilipin and HSL^{47,48} triggers release of co-activators of adipose triglyceride lipase, which then enter the lipid droplet. The access of lipase to the lipid droplet core is regulated by phosphorylation of perilipin, both of which play a major role in regulation of lipolysis.^{41,49} In our study, phosphorylation of perilipin and HSL is increased as an early event in Ex4-treated adipocytes and steatotic HuH7 cells, alluding to the fact that Ex4 initiates lipolysis by a direct action through perilipin and HSL. Other studies have also speculated that a lipase allows access of the neutral fat core to its catalytic site on the lipid monolayer.⁵⁰ Perilipin is downstream of cAMP,⁴⁷ and we have shown in our previous study that GLP-1R acts through activation of cAMP.²¹ In this study, we provide evidence for direct activation of perilipin and hormone-sensitive lipase by Ex4 through the cAMP pathway. This is schematically represented in Figure 7. Although we acknowledge that additional mechanistic studies are needed, to our knowledge this is the first study highlighting a direct, rapid, and clinically relevant action of Ex4 in causing lipid droplet fission, stimulating lipolysis. Future studies will be directed toward testing these observations in a GLP-1 KO mouse model.

In conclusion, we show that Ex4, a clinically available GLP-1R agonist, can protect the liver against IRI by decreasing necrosis and apoptosis. This protective effect is also evident in the steatotic liver, where significant reduction of the hepatocellular damage is inflicted by IRI. We also provide evidence to show that Ex4 has a direct

action on the lipid droplet, initiating lipid fission, and lipolysis. This study presents a potentially new therapeutic role for Ex4, a drug already approved by the Food and Drug Administration for treatment of type 2 diabetes, substantially increasing the translational potential for this approach. Enthusiasm for targeting GLP-1R is increased by the rapidity with which Ex4 exerts its action, as this suggests that therapy at the time of injury, during surgery, and before liver procurement could make meaningful improvements in subsequent hepatic function. Furthermore, studies to understand the exact mechanisms behind these observations are required, but the current evidence supports clinical investigation of Ex4 in the setting of liver injury, particularly that of a steatotic liver.

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References

- Day CP: Non-alcoholic fatty liver disease: a massive problem. *Clinical Med* 2011, 11:176–178
- Hanouneh IA, Zein NN: Metabolic syndrome and liver transplantation. *Minerva Gastroenterol Dietol* 2010, 56:297–304
- Selzner M, Clavien PA: Fatty liver in liver transplantation and surgery. *Semin Liver Dis* 2001, 21:105–113
- Gomez D, Malik HZ, Bonney GK, Wong V, Toogood GJ, Lodge JP, Prasad KR: Steatosis predicts postoperative morbidity following hepatic resection for colorectal metastasis. *Br J Surg* 2007, 94:1395–1402
- Veteläinen R, van Vliet A, Gouma DJ, van Gulik TM: Steatosis as a risk factor in liver surgery. *Ann Surg* 2007, 245:20–30
- Chavin KD, Fiorini RN, Shafizadeh S, Cheng G, Wan C, Evans Z, Rodwell D, Polito C, Haines JK, Baillie GM, Schmidt MG: Fatty acid synthase blockade protects steatotic livers from warm ischemia reperfusion injury and transplantation. *Am J Transplant* 2004, 4:1440–1447
- Marcos A, Ham JM, Fisher RA, Olzinski AT: Posner: Single-center analysis of the first 40 adult-to-adult living donor liver transplants using the right lobe. *Liver Transpl* 2000, 6:296–301
- Matheson PJ, Hurt RT, Franklin GA, McClain CJ, Garrison RN: Obesity-induced hepatic hypoperfusion primes for hepatic dysfunction after resuscitated hemorrhagic shock. *Surgery* 2009, 146:739–748
- Selzner M, Clavien PA: Failure of regeneration of the steatotic rat liver: disruption at two different levels in the regeneration pathway. *Hepatology* 2000, 31:35–42
- Clavien PA, Selzner M: Hepatic steatosis and transplantation. *Liver Transpl* 2002, 8:980
- Sharkey FE, Lytvak I, Prihoda TJ, Speeg KV, Washburn WK, Half GA: High-grade microsteatosis and delay in hepatic function after orthotopic liver transplantation. *Hu Pathol* 2011, 42:1337–1342
- Cheng Y, Chen C, Lai CY, Chen T, Huang T, Lee T, Lin C, Lord R, Chen Y, Eng H, Pan TL, Lee TH, Wang YH, Iwashita Y, Kitano, Goto S: Assessment of donor fatty livers for liver transplantation. *Transplantation* 2001, 71:1206–1207
- Sorrentino P, Terracciano L, D'Angelo S, Ferbo U, Bracigliano A, Tarantino L, Perrella A, Perrella O, De Chiara G, Panico L, De Stefano N, Lepore M, Mariolina, Vecchione R: Oxidative stress and steatosis are cofactors of liver injury in primary biliary cirrhosis. *J Gastroenterol* 2010, 45:1053–1062

14. Thorens B, Waeber G: Glucagon-like peptide-I and the control of insulin secretion in the normal state and in NIDDM. *Diabetes* 1993, 42:1219–1225
15. Acitores A, Gonzalez N, Sancho V, Valverde I, Villanueva-Penacarrillo ML: Cell signalling of glucagon-like peptide-1 action in rat skeletal muscle. *J Endocrinol* 2004, 180:389–398
16. Drucker DJ: Biological actions and therapeutic potential of the glucagon-like peptides. *Gastroenterology* 2002, 122:531–544
17. Ban K, Noyan-Ashraf MH, Hoefler J, Bolz SS, Drucker DJ, Husain M: Cardioprotective and vasodilatory actions of glucagon-like peptide 1 receptor are mediated through both glucagon-like peptide 1 receptor-dependent and -independent pathways. *Circulation* 2008, 117:2340–2350
18. Baggio LL, Holland D, Wither J, Drucker DJ: Lymphocytic infiltration and immune activation in metallothionein promoter-exendin-4 (MT-Exendin) transgenic mice. *Diabetes* 2006, 55:1562–1570
19. Alvarez E, Roncero I, Chowen JA, Thorens B, Blazquez E: Expression of the glucagon-like peptide-1 receptor gene in rat brain. *J Neurochem* 1996, 66:920–927
20. Gupta NA, Mells J, Dunham RM, Grakoui A, Handy J, Saxena NK, Anania FA: Glucagon-like peptide-1 receptor is present on human hepatocytes and has a direct role in decreasing hepatic steatosis in vitro by modulating elements of the insulin signaling pathway. *Hepatology* 2010, 51:1584–1592
21. Ding X, Saxena NK, Lin S, Gupta NA, Anania FA: Exendin-4, a glucagon-like protein-1 (GLP-1) receptor agonist, reverses hepatic steatosis in ob/ob mice. *Hepatology* 2006, 43:173–181
22. Li L, El-Kholy W, Rhodes CJ, Brubaker PL: Glucagon-like peptide-1 protects beta cells from cytokine-induced apoptosis and necrosis: role of protein kinase B. *Diabetologia* 2005, 48:1339–1349
23. Cursio R, Colosetti P, Saint-Paul MC, Pagnotta S, Gounon P, Iannelli A, Auberger P, Gugenheim J: Induction of different types of cell death after normothermic liver ischemia-reperfusion. *Transplant Proc* 2010, 42:3977–3980
24. Marcinkiewicz A, Gauthier D, Garcia A, Brasaemle DL: The phosphorylation of serine 492 of perilipin directs lipid droplet fragmentation and dispersion. *J Biol Chem* 2006, 281:11901–11909
25. McDonough PM, Ingemannson RS, Loy PA, Koon ED, Whittaker R, Laris CA, Hilton JM, Nicoll JB, Buehrer BM, Price JH: Quantification of hormone sensitive lipase phosphorylation and colocalization with lipid droplets in murine 3T3L1 and human subcutaneous adipocytes via automated digital microscopy and high-content analysis. *Assay Drug Dev Technol* 2011, 9:262–280
26. Ali S, Lamont BJ, Charron MJ, Drucker DJ: Dual elimination of the glucagon and GLP-1 receptors in mice reveals plasticity in the incretin axis. *J Clin Invest* 2011, 121:1917–1929
27. Lamont BJ, Li Y, Kwan E, Brown TJ, Gaisano H, Drucker DJ: Pancreatic GLP-1 receptor activation is sufficient for incretin control of glucose metabolism in mice. *J Clin Invest* 2012, 122:388–402
28. Cabou C, Vachoux C, Campistron G, Drucker DJ, Burcelin R: Brain GLP-1 signaling regulates femoral artery blood flow and insulin sensitivity through hypothalamic PKC-delta. *Diabetes* 2011, 60:2245–2256
29. Axelsen L, Keung W, Pedersen H, Meier E, Riber D, Kjolbye A, Petersen J, Proctor S, Holstein-Rathlou NH, Lopaschuk G: Glucagon and a glucagon-GLP-1 dual-agonist increases cardiac performance with different metabolic effects in insulin-resistant hearts. *Br J Clin Pharmacol* 2012, 165:2736–2748
30. Chaykovska L, von Websky K, Rahnenfuhrer J, Alter M, Heiden S, Fuchs H, Runge F, Klein T, Hofer B: Effects of DPP-4 inhibitors on the heart in a rat model of uremic cardiomyopathy. *PloS One* 2011, 6:e27861
31. Hlebowicz J, Lindstedt S, Bjorgell O, Dencker M: The effect of endogenously released glucose, insulin, glucagon-like peptide 1, ghrelin on cardiac output, heart rate, stroke volume, and blood pressure. *Cardiovasc Ultrasound* 2011, 9:43
32. Suzuki T, Yoshidome H, Kimura F, Shimizu H, Ohtsuka M, Takeuchi D, Kato A, Furukawa K, Yoshitomi H, Iida A, Dochi T, Miyazaki M: Hepatocyte apoptosis is enhanced after ischemia/reperfusion in the steatotic liver. *J Clin Biochem Nutr* 2011, 48:142–148
33. Savateev AV, Savateeva-Liubimova TN: [Apoptosis—universal mechanisms of cell death and survival in ischemia and reperfusion: ways to pharmacological control]. *Eksp Klin Farmakol* 2010, 73:44–49
34. Xu WH, Ye QF, Xia SS: Apoptosis and proliferation of intrahepatic bile duct after ischemia-reperfusion injury. *Hepatobiliary Pancreat Dis Int* 2004, 3:428–432
35. Jaeschke H, Gujral JS, Bajt ML: Apoptosis and necrosis in liver disease. *Liver Int* 2004, 24:85–89
36. Marziani M, Alpini G, Saccomanno S, Candelaresi C, Venter J, Rychlicki C, Fava G, Francis H, Trozzi L, Benedetti A: Exendin-4, a glucagon-like peptide 1 receptor agonist, protects cholangiocytes from apoptosis. *Gut* 2009, 58:990–997
37. Bose AK, Mocanu MM, Carr RD, Yellon DM: Myocardial ischaemia-reperfusion injury is attenuated by intact glucagon like peptide-1 (GLP-1) in the in vitro rat heart and may involve the p70s6K pathway. *Cardiovasc Drugs Ther* 2007, 21:253–256
38. Martin S, Parton RG: Lipid droplets: a unified view of a dynamic organelle. *Nat Rev Mol Cell Biol* 2006, 7:373–378
39. Krahmer N, Guo Y, Farese RV Jr, Walther TC: SnapShot: lipid droplets. *Cell* 2009, 139:1024–1024
40. Selzner N, Selzner M, Jochum W, Amann-Vesti B, Graf R, Clavien PA: Mouse livers with macrosteatosis are more susceptible to normothermic ischemic injury than those with microsteatosis. *J Hepatol* 2006, 44:694–701
41. Marcinkiewicz A, Gauthier D, Garcia A, Brasaemle DL: The phosphorylation of serine 492 of perilipin directs lipid droplet fragmentation and dispersion. *J Biol Chem* 2006, 281:11901–11909
42. Guo Y, Walther TC, Rao M, Stuurman N, Goshima G, Terayama K, Wong JS, Vale RD, Walter P, Farese RV: Functional genomic screen reveals genes involved in lipid-droplet formation and utilization. *Nature* 2008, 453:657–661
43. Tansey JT, Sztalryd C, Hlavin EM, Kimmel AR, Londos C: The central role of perilipin in lipid metabolism and adipocyte lipolysis. *IUBMB Life* 2004, 56:379–385
44. Londos C, Sztalryd C, Tansey JT, Kimmel AR: Role of PAT proteins in lipid metabolism. *Biochimie* 2005, 87:45–49
45. Sztalryd C, Xu G, Dorward H, Tansey JT, Contreras JA, Kimmel AR, Londos C: Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. *J Cell Biol* 2003, 161:1093–1103
46. Zechner R, Kienesberger PC, Haemmerle G, Zimmermann R, Lass A: Adipose triglyceride lipase and the lipolytic catabolism of cellular fat stores. *J Lipid Res* 2009, 50:3–21
47. Brasaemle DL, Subramanian V, Garcia A, Marcinkiewicz A, Rothenberg A: Perilipin A and the control of triacylglycerol metabolism. *Mol Cell Biochem* 2009, 326:15–21
48. Shen WJ, Patel S, Miyoshi H, Greenberg AS, Kraemer FB: Functional interaction of hormone-sensitive lipase and perilipin in lipolysis. *J Lipid Res* 2009, 50:2306–2313
49. Londos C, Sztalryd C, Tansey JT, Kimmel AR: Role of PAT proteins in lipid metabolism. *Biochimie* 2005, 87:45–49
50. Farese RV, Jr., Walther TC: Lipid droplets finally get a little R-E-S-P-E-C-T. *Cell* 2009, 139:855–860