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Metabolic preconditioning of donor organs: Defatting fatty livers by normothermic perfusion ex vivo

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ABSTRACT

Fatty liver is a significant risk factor for liver transplantation, and accounts for nearly half of the livers rejected from the donor pool. We hypothesized that metabolic preconditioning via ex vivo perfusion of the liver graft can reduce fat content and increase post-transplant survival to an acceptable range. We describe a perfusate medium containing agents that promote the defatting of hepatocytes and explanted livers. Defatting agents were screened on cultured hepatocytes made fatty by pre-incubation with fatty acids. The most effective agents were then used on fatty livers. Fatty livers were isolated from obese Zucker rats and normothermically perfused with medium containing a combination of defatting agents. This combination decreased the intracellular lipid content of cultured hepatocytes by 35% over 24 h, and of perfused livers by 50% over 3 h. Metabolite analysis suggests that the defatting cocktail upregulated both lipid oxidation and export. Furthermore, gene expression analysis for several enzymes and transcription factors involved in fatty acid oxidation and triglyceride clearance were elevated. We conclude that a cocktail of defatting agents can be used to rapidly clear excess lipid storage in fatty livers, thus providing a new means to recondition donor livers deemed unacceptable or marginally acceptable for transplantation.

1. Introduction

Orthotopic liver transplantation (OLT) is a highly successful therapeutic modality for the treatment of end-stage liver disease. The main limitation of OLT is the scarcity of donor livers. Currently, the majority of donor livers are obtained from cadavers with irreversible loss of brain function who still have functioning circulation and respiration. Among such donors, the most common single predisposing significant risk factor for post-operative liver failure is increased fat content (also called steatosis) of the liver (Canelo et al., 1999; Strasberg et al., 1994). Steatosis accounts for nearly half of the livers rejected from the donor pool (Chan et al., 2004). Thus, salvaging these steatotic donor livers that would be otherwise discarded may significantly expand the donor pool and help close the gap between supply and demand in liver transplantation. While there are many reports focusing on protecting the fatty donor liver from transplantation-related injury (Belghiti et al., 1999; Nakamuta et al., 2005; Tanaka et al., 1990), so far none of these approaches have been successfully applied to clinical practice.

In the quest to better understand the pathology of obesity and diabetes, several compounds have been discovered that increase lipid export and/or oxidation, thus providing an opportunity to develop a metabolic preconditioning strategy specifically aimed at reducing the fat content of steatotic livers to a normal range prior to transplantation. For example, several lipophilic ligand-activated transcription factors, such as peroxisome proliferators-activated receptors (PPARs), have emerged as important regulators of lipid and glucose metabolism (Dressel et al., 2003; Gilde et al., 2003; Luquet et al., 2004; Nagasawa et al., 2006; Tamura et al., 2006). Among those, pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are known to activate xenobiotic pathways, but their role in lipid metabolism remains poorly

Abbreviations: OLT, Orthotopic liver transplantation; PPARs, Peroxisome proliferators-activated receptors; PXR, Pregnane X receptor (PXR); CAR, Constitutive Androstane Receptor; PBEF, pre-B cell colony-enhancing factor; TG, triglyceride; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; MEM, Minimum Essential Medium; OURs, Oxygen uptake rates; VLDL, Very low-density lipoprotein; PPAR α, peroxisome proliferator-activated receptor α; PPAR δ, peroxisome proliferator-activated receptor δ; SCO, Scoparone; FOR, forskolin; VIS, visfatin; ACO, acyl coenzyme A oxidase (ACO); PGC1α, peroxisomal proliferator-activated receptor coactivator; CPT1α, carnitine palmitoyltransferase I; apoB-100, apolipoprotein B-100; TGH, triacylglycerol hydrolase

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understood. Visfatin, also known as pre-B cell colony-enhancing factor (PBEF), which is secreted by visceral adipocytes, exerts insulin-mimetic effects and decreases plasma triglyceride levels (TG) in vivo (Fukuhara et al., 2005; Kloting and Kloting, 2005); insulin is known to increase TG secretion from perfused livers isolated from fed rats (Rennie et al., 2000; Zammit et al., 1999). Forskolin (FOR) increases intracellular cyclic AMP, which results in increased β-oxidation and ketogenesis (Pegorier et al., 1989).

Herein, we tested the effect of a combination of amino acids, visfatin, forskolin, nuclear receptor ligands (GW7647 for PPARα; GW501516 for PPARδ; hypericin for PXR; scoparone (SCO) for CAR) on lipid metabolism in cultured fatty hepatocytes, as well as in ex vivo perfused fatty livers. We found that a mixture containing these factors significantly upregulates pathways involved in fatty acid oxidation and triglyceride secretion, which results in a rapid reduction in lipid storage.

2. Material and Methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/l glucose, 10× DMEM, fetal bovine serum (FBS), penicillin and streptomycin, Minimum Essential Medium (MEM) vitamin solution, and phenol red were purchased from Life Technologies (Gaithersburg, MD). Insulin was purchased from Novo Nordisk A/S (Bagsuaerd, Denmark), glucagon from Eli Lilly and Co. (Indianapolis, IN), epidermal growth factor from Collaborative Biomedical Products (Becton Dickinson, Bedford, MA). Type I collagen suspension (1.2 mg/ml in 1 mM HCl) was prepared from Lewis rat tail tendons as described elsewhere (Dunn et al., 1991). Heparin-treated human plasma was purchased from Rockland Immunochemicals, Inc. (Gilbertsville, PA). Sodium monophosphate, sodium bicarbonate, glutamine, the PPARα agonist GW7647, and forskolin were purchased from Sigma Chemical (St. Louis, MO). Scoparone was purchased from Calbiochem (La Jolla, CA). Visfatin, hypericin, and GW501516 were purchased from Axxora Alexis (San Diego, CA).

2.2. Hepatocyte isolation and culture

Hepatocytes were isolated from adult female Lewis rats (Charles River Laboratories, Boston, MA) weighing 75–125 g, according to a two-step collagenase perfusion technique described by Seglen (1976) and modified by Dunn et al. (1991). Each isolation yielded $150–300 \times 10^6$ hepatocytes. Viability ranged from 90% to 98% as determined by trypan blue exclusion.

A collagen gelling solution was prepared by mixing 9 parts of the 1.2 mg/ml collagen suspension in 1 mM HCl with 1 part of 10× concentrated DMEM at 4 °C. Six-well culture plates were coated with 0.15 ml/well of this collagen gelling solution and the coated plates were incubated at 37 °C for 1 h. The hepatocytes were suspended in standard hepatocyte culture medium at 10^6 cells/ml and seeded at a density of 10^6 cells/well. The standard hepatocyte culture medium consisted of DMEM supplemented with 10% FBS, 14 ng/ml glucagon, 20 ng/ml epidermal growth factor, 7.5 μg/ml hydrocortisone, 200 μg/ml streptomycin (10,000 μg/ml)–penicillin (10,000 U/ml) solution, and 0.5 U/ml insulin (high insulin). DMEM contains 3.7 g/l $NaHCO_3$ (corresponding to 44.1 mM) and has a pH of 7.4 when equilibrated with 10% CO_2. After incubating the cells at 37 °C in a 90% air/10% CO_2 atmosphere for 24 h, the culture medium was removed and a second layer of collagen gelling solution (0.25 ml/well) was added to form a collagen sandwich. After gelling for 45 min at 37 °C, 1 ml of fresh standard hepatocyte culture medium was applied on top

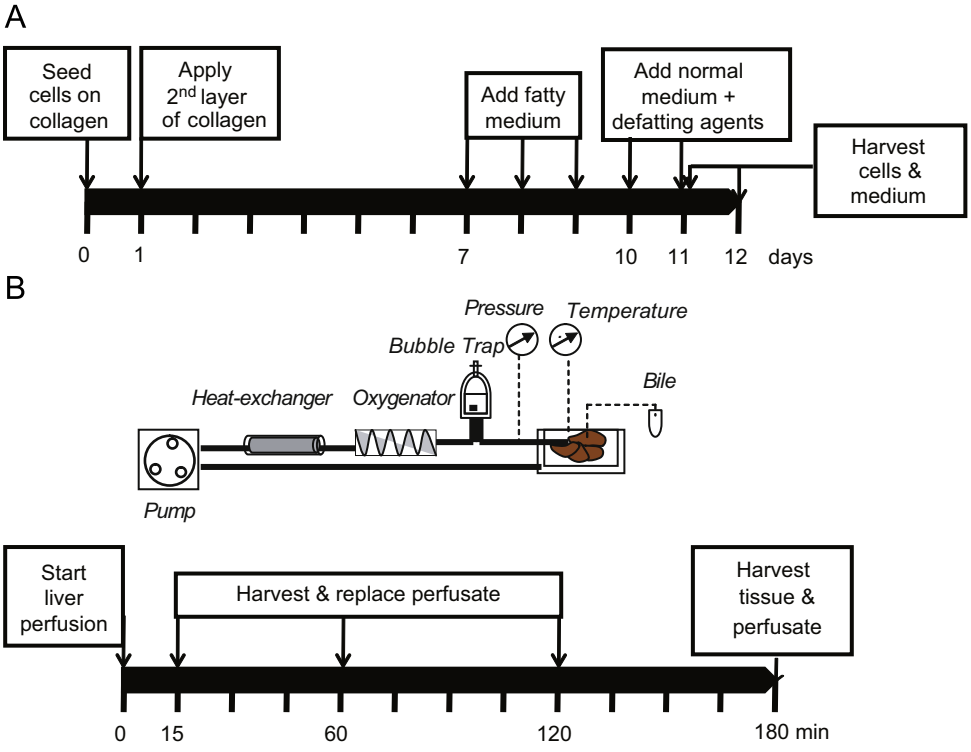

Fig. 1. (A) Experimental design of in vitro defatting experiments. (B) Schematic of isolated ex vivo fatty liver perfusion setup.

of the culture. Fresh medium (1 ml) was supplied to the cultures daily after removal of the supernatant. The hepatocytes were cultured in this fashion for at least 6 days prior to any use.

2.3. Induction of hepatocellular steatosis

The functionally stable 6-day-old sandwiched hepatocyte cultures in hepatocyte medium were switched to medium supplemented with 2 mM oleic acid, 2 mM linoleic acid, and 4% bovine serum albumin for 3 days (Fig. 1a), which caused the cells to become fatty due to increased intracellular triglyceride content (Stefanovich et al., 1996).

2.4. Preparation of defatting media

The medium/perfusate consisted of Minimum Eagles's Medium (MEM; Sigma Chemical, MA), supplemented with 3% wt/vol bovine serum albumin (Fraction V, Sigma Chemical Co.), 1.07 mM lactic acid, 0.11 mM pyruvic acid, and several amino acids so that the concentrations of individual amino acids in the medium/perfusate were approximately twice those in postabsorptive rat plasma (Arai et al., 2001). The pH of the medium/perfusate was adjusted to 7.4. The medium/perfusate was then supplemented with various defatting agents (10 μM forskolin; 1 μM GW7647; 10 μM hypericin; 10 μM scoparone; 0.4 ng/ml visfatin; 1 μM GW501516). This medium served as the basal medium for both in vitro defatting and ex vivo perfusions. In the vehicle controls, an equivalent amount of dimethylsulfoxide (DMSO) was added, which was $<0.1\%$ v/v. For ex vivo perfusions, perfusate was equilibrated with a humidified 95% O_2/5% CO_2 gas mixture.

2.5. Isolated liver perfusion

Homozygous (Obese) and heterozygous (Lean) Zucker rats (Charles River Laboratories, Boston, MA) aged 14–15 weeks were used. After being anesthesized with ketamine and xylazine (100 and 8 mg/kg, respectively), the animals were subjected to transverse laparotomy. The portal vein, the common bile duct, and the inferior vena cava were exposed by retracting the bowel to the left. The bile duct was transected after distal ligation, and a polyethylene tube (PE-10, BD-Clay Adams, Sparks, MD) was inserted into the lumen of the bile duct and secured with a circumferential 4-0 silk suture. 0.5 ml of saline containing 200 units of heparin was injected through the infrahepatic vena cava. A 14-gauge Teflon catheter (Deseret Medical, Inc, Sandy, UT) was inserted into the portal vein and secured with 4-0 silk. The liver was then immediately perfused with washing buffer (MEM) for 10 min to wash out the blood. Then, the liver was freed from the animal's body cavity, weighed, and transferred to the perfusion chamber. A parafilm cover was placed over the liver and chamber to prevent excessive evaporation. Fig. 1b shows the timeline of ex vivo perfusions.

The perfusion reservoir contained 40 ml of perfusate. Perfusate was pumped from the reservoir through a silicone tubing oxygenator exposed to a gas mixture of 95% O_2/5% CO_2, a heat exchanger, and an air-bubble trap before entering the liver. Outflow from the liver was returned to the perfusion chamber and recycled. After sampling at the end of each hour, 30 ml of perfusate was replaced with fresh perfusate. The total volume of perfusate in circulation was 50 ml for the first hour and 40 ml for the last 2 h. During the 3-hour perfusion experiment, flow rate into the liver was kept constant at 2.5 ml min^{-1} g $liver^{-1}$ and the temperature of the perfusate maintained between 36.5 and 37 °C. Bile was collected and gravimetrically measured for assessment of bile production.

2.6. Oxygen uptake rate measurement in cultured hepatocytes

Oxygen uptake rates (OURs) of cultured hepatocytes were measured using a device described by Foy et al. (1994). Briefly, the device consists of a polycarbonate disc machined to fit onto a 60-mm tissue culture dish to form an airtight sealed chamber containing the cells and the liquid medium on top of them. A magnetic stir bar provides uniform mixing of the medium within the chamber. After closing the chamber, oxygen tension within the chamber was measured using an oxygen-sensitive ruthenium-coated optical probe (Ocean Optics, Dunedin, FL). Since the chamber is closed during the oxygen tension measurement, the decrease in oxygen tension versus time can be directly related to the OUR of the cells. Prior to OUR measurement, a calibration was performed using a 21% oxygen tension standard consisting of PBS incubated in a 21% oxygen atmosphere at 37 °C for 2 h, and a 0% oxygen tension standard consisting of a freshly prepared 20% w/v solution of sodium hydrosulfite in pre-warmed de-ionized water at 37 °C. Oxygen sensor calibration was verified before each measurement. For the actual OUR measurement, the culture medium was replaced with fresh medium pre-equilibrated in 90% air/10% CO_2 at 37 °C. The drift of the electrode over the course of 12 h, which represents the maximum time in which the electrode was used for the experiment, was monitored and found to be less than 5%. The electrode drift due to protein deposition was found to be less than 5% as evidenced by the steadiness of the signal after re-calibration at 158 mm Hg, during the course of the experiment. The OUR was obtained from the slope of the linear portion of the oxygen uptake curve using the following equation, assuming Michaelis–Menten kinetics:

$$\frac{dP}{dt} = \frac{-V_m P}{K_{0.5} + P}\left(\frac{N_c}{kV}\right)$$

where P is the measured oxygen partial pressure (mmHg), t is time (s), $N_{c\ is}$ the number of cells in the device chamber, k is the solubility of oxygen in water at 37 °C (1.19 nmol/ml mmHg), and V is the liquid volume in the device chamber (11.5 ml).

To determine the fraction of total OUR due to mitochondrial oxygen consumption, a second OUR measurement was carried out after replacing the medium with fresh medium containing 1 mM potassium cyanide for 20 min. The cyanide-inhibitable fraction of the total OUR was assumed to represent mitochondrial uptake.

2.7. Oxygen uptake rate measurement of perfused livers

OURs were calculated by multiplying the perfusate flow rate by the outflow–inflow difference in the oxygen concentration as follows:

$$\mathrm{OUR} = V[C_{O2,\mathrm{inflow}} - CO_{2,\mathrm{outflow}}]/\text{liver weight}$$

where V is the perfusion flow rate (mL/min), C_{O2} is the oxygen concentration (nM) calculated as $C_{O2} = 0.0031\ PO_2$, where PO_2 is the oxygen partial pressure (mmHg) measured by a blood-gas analyzer (Bayer Diagnostics, Rapidlab 865, Germany), and 0.0031 (ml O_2/(mmHg dl)) is the solubility of oxygen in aqueous solutions at 37 °C.

2.8. Determination of mRNA levels of key metabolic enzymes

After incubation with the defatting or control medium for 24 h, hepatocyte cultures were washed with PBS, incubated with 0.1% collagenase for about 5 min, and the cells harvested by centrifugation. Cell pellets were flash frozen in liquid nitrogen and stored at −80 °C. RNA was isolated from the cell pellets using a NucleoSpin RNAII kit (catalog #740955.50, Clontech, Mountain View, CA).

Table 1
Primers used for RT-PCR.

Gene name	Forward primer sequence	Backward primer sequence
PGC1	GTGTCGCCTTCTTGCTCTTC	GTGTGCGGTGTCTGTAGTGG
ACC	CGATGACCCTTCAAAAGTGC	CTTCTCCACCCAGTCCTTCA
CPT1α	CAAGCGGGACCATAGAGAAG	GCAGCCTTTGACTACCAAGC
LPL	ACTCCTACTTCCGCTGGTCA	TGGCATTTCACAAACACTGC
ACO	TGGGAGCAGCCTCTACAATC	CGAATGCCACAGACACAGAC
ApoB	ACCGCACCTTCTTGATTCTG	GCAGCCAGTCTCTTCTCCAC
PPARα	GTCCGATTCTTCCACTGCTG	GCATCCCGTCTTTGTTCATC
PPARδ	TCATCCACGACATTGAGACG	GAAGAGGCTGCTGAAGTTGG
TGH	CACTGCTGCTCTGATTACAACAG	GCCTTCAGCGAGTGGATAGC
β-Actin	GAGGGAAATCGTGCGTGA	CCAAGAAGGAAGGCTGGAA

Reverse transcription was conducted using the ImProm-II™ Reverse Transcription System (catalog # A3802, Promega Corporation, Madison, WI). First strand cDNA was amplified with the gene-specific primers shown in Table 1 using the Promega PCR core system I (catalog # M7660). PCR products were separated and visualized on 2% agarose gels and gel images were scanned using a Bio-Rad Fluor-S MultiImage system.

2.9. Nile red staining protocol

Nile red staining was used to assess lipid accumulation in cultured hepatocytes. Immediately prior to the assay, cultures were rinsed with PBS, and 5 ml of PBS with 140 μl of AdipoRed was added per well. The content of each well was mixed carefully to prevent aggregate formation, and the tissue culture plates were incubated for 10–15 min. Fluorescence in each well was measured in an F_{max} fluorescence plate reader (Molecular Devices) using an excitation of 485 nm and an emission of 572 nm.

2.10. Lipid extraction and triglyceride assay

The lipid extraction method was adopted from a published protocol (Bligh and Dyer, 1959). Briefly, 1 ml of collagenase-treated cell culture sample was mixed with 3.75 ml $CHCl_3$:MeOH (1:2, v/v), 1.25 ml $CHCl_3$, and 1.25 ml dH_2O in this order, with vigorous vortexing after each reagent addition. The mixture was centrifuged at 3000 rpm for 5 min, and the bottom ~1 ml organic phase was collected, dried under nitrogen, and dissolved in 2-propanol. The resulting solution was assayed for triglyceride content using a commercial kit (Wako Diagnostics) following the manufacturer's instructions.

2.11. Histological evaluation

After liver perfusion, liver tissue samples were harvested from the left lateral lobe for histological analysis. The pieces of liver tissue were immersed immediately in 10% buffered formalin overnight and thereafter washed with 70% ethanol three times. Subsequently, the tissues were dehydrated in a graded series of ethanol and embedded in paraffin. Four-micrometer-thick sections were mounted on glass slides and stained with hematoxylin and eosin for light microscopy observation. All histological evaluations were performed by a blinded investigator.

2.12. VLDL measurement in perfusate

Very low-density lipoproteins (VLDL) secreted in the perfusate during ex vivo perfusion were measured using a Lipid Panel on a Piccolo portable blood analyzer (Abaxis, Inc., Sunnyvale, CA).

3. Results

3.1. Defatting of fatty hepatocytes in vitro

Hepatocytes were cultured in standard hepatocyte culture medium for one week to allow for stabilization of the metabolic parameters, and then switched to fatty acid (oleic and linoleic acid)-supplemented medium for 3 days, which causes extensive intracellular lipid droplet accumulation. Fatty hepatocytes were then exposed to various defatting agents that were selected based on their known stimulatory effect on lipid export and/or β-oxidation. The defatting agents tested were the peroxisome proliferator-activated receptor α (PPAR α) ligand GW7647 (GW7), the peroxisome proliferator-activated receptor δ (PPAR δ) ligand GW501516 (GW5), the pregnane X Receptor ligand hypericin (HPC), the constitutive androstane receptor ligand (SCO), the glucagon mimetic and cAMP activator forskolin, and the insulin-mimetic adipokine visfatin (VIS), to reduce intracellular lipid content by promoting export from cultured hepatocytes.

These agents were first tested individually for lipid reduction and were found to reduce TG accumulation, concomitantly with an increase in ketone body production, suggesting increased β-oxidation, as well as a trend towards increased TG secretion into the medium (Supplementary Fig. 1).

Next, we examined the effect of a cocktail of defatting agents for up to 48 h. Nile red lipid staining was used to assess intracellular lipid content (Fig. 2). Cells incubated in the vehicle control clearly showed more staining, indicating significantly higher remaining lipid content. Cells incubated with the cocktail had a lipid content that was 24% lower and 31% lower than the control after 24 and 48 h, respectively. Observation of the cells at higher magnification revealed that incubation with defatting agents led to smaller size lipid droplets and reduced lipid content (Fig. 2). Then, we measured the effect of the defatting cocktail on ketone body and TG secretion. As seen in Figs. 3A and B, the cocktail increased ketone body production – used as an index of β-oxidation – by 33% and 24% compared to the vehicle control after 24 and 48 h of incubation, respectively. Mitochondrial oxygen uptake rate, which is required for β-oxidation, increased 1.8-fold when fatty hepatocytes were switched to the defatting cocktail, but not at all when they were switched to fresh medium with vehicle but no defatting agents (Fig. 3C). Interestingly, total cellular oxygen uptake also increased in the vehicle control group; however, the difference between total cellular and mitochondrial rates likely represents non-mitochondrial processes. To evaluate the effect of the cocktail on TG secretion, the TG content was measured in cell culture supernatants. Fig. 3D shows that TG secretion by hepatocytes was 1.4-fold higher in the cocktail than using vehicle. Thus, as previously found with the individual defatting agents, the cocktail mediates defatting via both TG export and β-oxidation.

To further assess the lipid reduction mechanism, the effect of defatting agents on the gene expression of enzymes acyl coenzyme A oxidase (ACO), peroxisomal proliferator-activated receptor coactivator (PGC1α), carnitine palmitoyltransferase I (CPT1α) and transcription factors (PPARα, PPARδ) involved in lipid oxidation, and factors involved in lipid export (apolipoprotein B-100 [apoB-100] and triacylglycerol hydrolase [TGH]) was measured (Fig. 4). Notably, the expression levels of genes regulating oxidation (ACO, PGC1α, CPT1α, PPARα, and PPARδ) and secretion (ApoB-100, TGH) were significantly higher in the presence of the defatting cocktail compared to the control cultures at both 24 and 48 h. These results corroborate the metabolic data, suggesting that both pathways are active and responsible for lipid clearance.

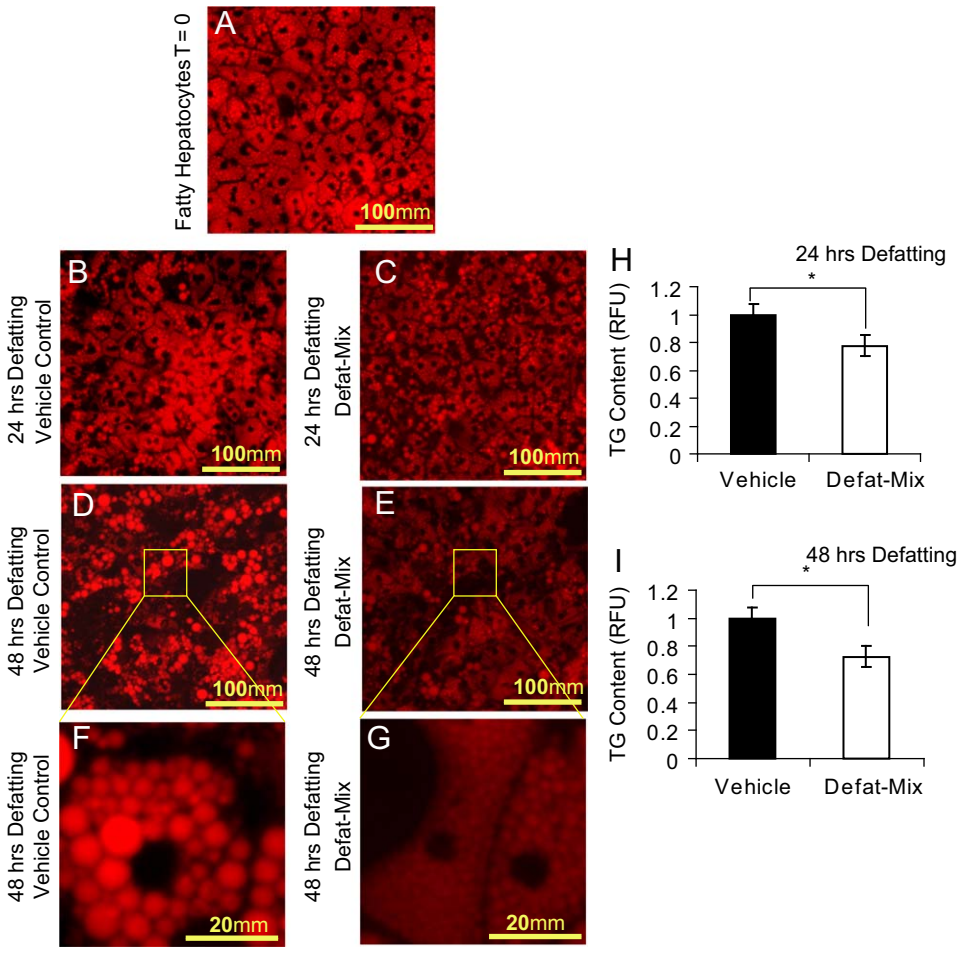

Fig. 2. Effect of cocktail of defatting agents on hepatocyte TG content 24 h after defatting. Primary cultured rat hepatocytes were made steatotic by incubation with fatty acid-supplemented medium for 3 days, and then switched to regular medium supplemented with a cocktail of defatting agents (1 μM GW7, 1 μM HYP, 10 μM SCO, 10 μM FOR, 1 ng/ml VIS, and 1 μM GW5). Vehicle controls represent similar cultures exposed to vehicle control (<0.1% DMSO). Intracellular lipid-specific Nile Red staining of hepatocyte cultures (A) at the start of defatting; (B)–(C) after 24 h of defatting; (E)–(G) after 48 h of defatting with the cocktail as compared to the vehicle controls. (H)–(I) Quantification of Nile Red staining in hepatocytes by image analysis. $^{*}p<0.05$ by one-tailed *t*-test compared to vehicle control.

3.2. *Ex vivo defatting of livers by normothermic perfusion*

Based on the results obtained with cultured fatty hepatocytes, we tested the effect of the defatting cocktail on normothermically perfused fatty livers isolated from Zucker rats. The perfusions were carried out for 3 h and the perfusate consisted of supplemented MEM as detailed in the Methods section. The perfusate was additionally supplemented with the defatting cocktail (combination of HPC, GW7, GW5, VIS, SCO, and FOR). Fig. 5A shows that the TG content decreased by 65% after only 3 h of perfusion. A 30% decrease was observed when control perfusate with no defatting agents was used. In addition, the use of the defatting cocktail led to greater bile production compared with control perfusate after 2 and 3 h of perfusion (Fig. 5B). Histologically, significant defatting effects were observed in the periportal but not in the perivenous region (Fig. 6), i.e., lipid vesicles in hepatocytes, especially those in zone 1 (periportal area), were diminished and the hepatocellular cytoplasmic volume was restored as evidenced by the increased area of eosinophilic staining in hepatocytes. In addition, the sinusoid diameter in this region appeared to be increased in the liver perfused with medium containing defatting agents compared to that of livers in the control group.

To determine the mechanism of action of the defatting cocktail, we monitored TG secretion, oxygen uptake, and ketone body production during the perfusions. As seen in Fig. 7A, the defatting cocktail increased secretion of TG by 40% compared to the control perfusate after 2 h of perfusion. Oxygen uptake of the livers perfused with the defatting cocktail was at least 70% higher than that of vehicle controls during the first hour of perfusion (Fig. 7B). To determine whether this change could be due to increased lipid oxidation, we measured total ketone body secretion as an index of β-oxidation during the perfusions. The data show that, consistent with our observations *in vitro*, ketone body secretion was over 2-fold higher with the defatting cocktail compared to the control medium (Fig. 7C), suggesting that β-oxidation is increased and could therefore account for at least part of the increased oxygen uptake with the defatting cocktail. Taken together, these results suggest that the defatting cocktail increased both β-oxidation and TG secretion.

4. Discussion

In this study, we report that a cocktail of agents that stimulate lipid export and oxidation can speed up the process of defatting fatty hepatocytes and perfused livers. Defatting agents were initially screened using cultured hepatocytes that were made fatty by pre-incubation in fatty acid-supplemented medium. Then, a combination of the most effective agents was tested on steatotic livers isolated from obese Zucker rats in a perfusion system. We found that a combination of peroxisome proliferator activated-

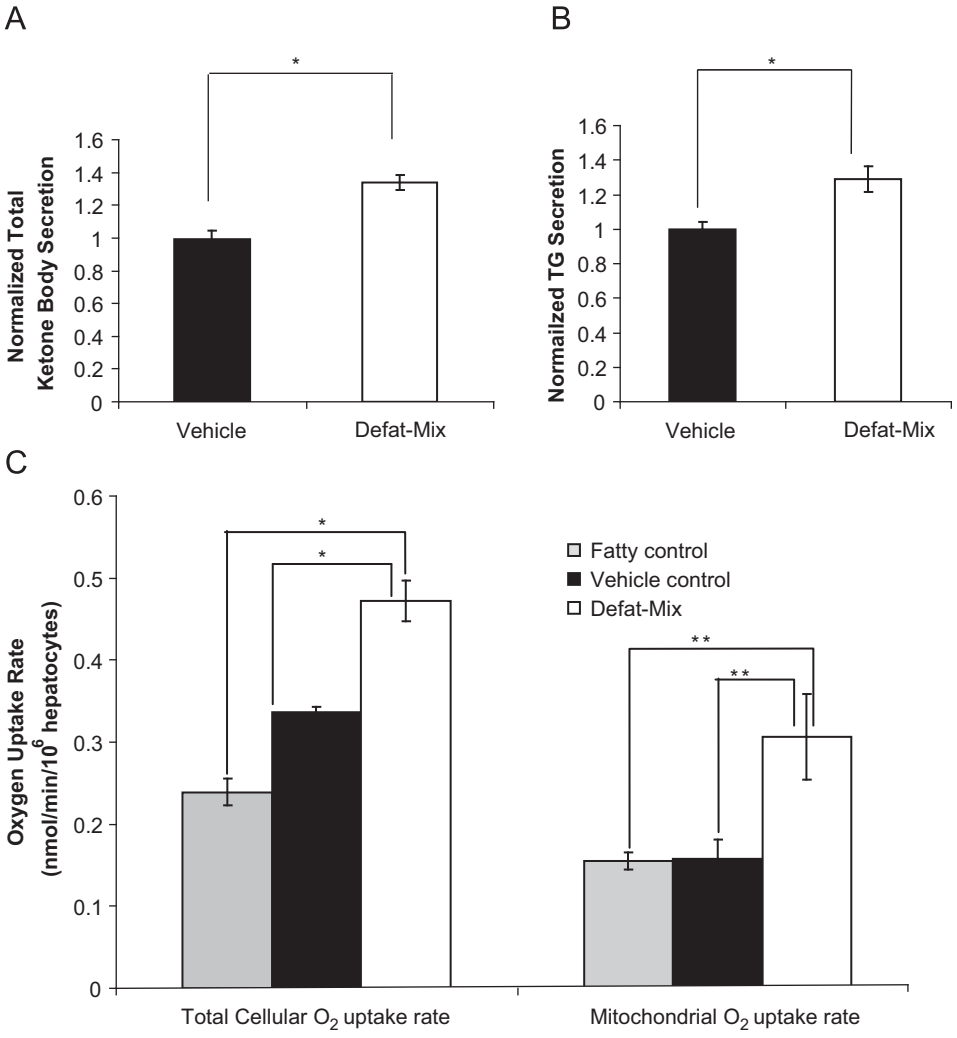

Fig. 3. Mechanism of defatting of steatotic hepatocytes. Steatotic hepatocytes were incubated in culture medium supplemented with the cocktail of defatting agents, or vehicle control ($<0.1\%$ DMSO). (A) Total ketone bodies (acetoacetate+β-hydroxybutyrate) secreted after 24 h of defatting. Data are normalized to the vehicle control. Data shown are the means $\pm$ SE of results obtained from two separate isolations and performed in 2–5 replicate culture dishes ($N = 4$–10). (B) TG secreted after 24 h of defatting. Data are normalized to vehicle controls. Data shown were obtained from two separate isolations and performed in duplicate cultures ($N = 4$), and expressed as means $\pm$ SE. White bars are significantly lower ($^*p<0.05$) than vehicle control. (C) Total and mitochondrial oxygen uptake rates measured after 24 h of defatting incubation. Data shown are the means $\pm$ SE of results obtained from two separate isolations and performed in 2–5 replicate culture dishes ($N \geq 4$).

receptor α ligand GW7647 (GW7), peroxisome proliferator-activated receptor δ ligand GW501516 (GW5), pregnane X Receptor ligand hypericin (HPC), the constitutive androstane receptor ligand (SCO), the glucagon mimetic and cAMP activator forskolin, and the insulin-mimetic adipokine visfatin was an effective defatting cocktail resulting in the decrease of intracellular lipid content by more than 50% during 3 h liver perfusions.

There are two major pathways for reducing the content of intracellular lipids, which are stored as TG: (a) secretion of TG through VLDL and (b) fatty acid oxidation. In both cases, the stored TG first undergoes lipolysis followed by either re-esterification and assembly with apolipoproteins (such as apolipoprotein B-100) into TG-rich VLDL (Lankester et al., 1998; Lehner et al., 1999), or complete hydrolysis into glycerol and fatty acids (Wiggins and Gibbons, 1992). Triacylglycerol hydrolase is responsible for TG hydrolysis in hepatocytes, and has been shown to play a major role in TG lipolysis and reestrification (Dolinsky et al., 2004; Lehner et al., 1999). Interestingly, TGH mRNA levels were significantly higher in hepatocytes cultured in medium with the defatting cocktail compared to the control unsupplemented culture medium, suggesting that the defatting cocktail upregulates the pathway that makes TG available for both export and oxidation.

To stimulate TG secretion, we used the insulin-mimetic visfatin (Fukuhara et al., 2005; Dominici et al., 2005; Haider et al., 2006; Kloting and Kloting, 2005; Kralisch et al., 2005; Wen et al., 2006; Yonezawa et al., 2006), in combination with amino acid supplements to support increased apolipoprotein synthesis. Insulin has previously been shown to stimulate triacylglycerol secretion in perfused rat livers (Rennie et al., 2000; Zammit et al., 1999); however, visfatin was used instead because it does not depend on the insulin signaling pathway, which is known to be inhibited by steatotis and high lipid levels, leading to insulin resistance. Apolipoprotein B-100 gene expression was increased when using the defatting cocktail. Interestingly, visfatin, even when used alone (unpublished observations), stimulated ketone body secretion and β-oxidation in fatty hepatocytes, suggesting that this agent also promotes β-oxidation of lipids. Thus, visfatin and insulin may exert a differential effect on lipid metabolism in hepatocytes.

The β-oxidation pathways are known to be regulated by several nuclear receptors. For example, PPARα has been shown to

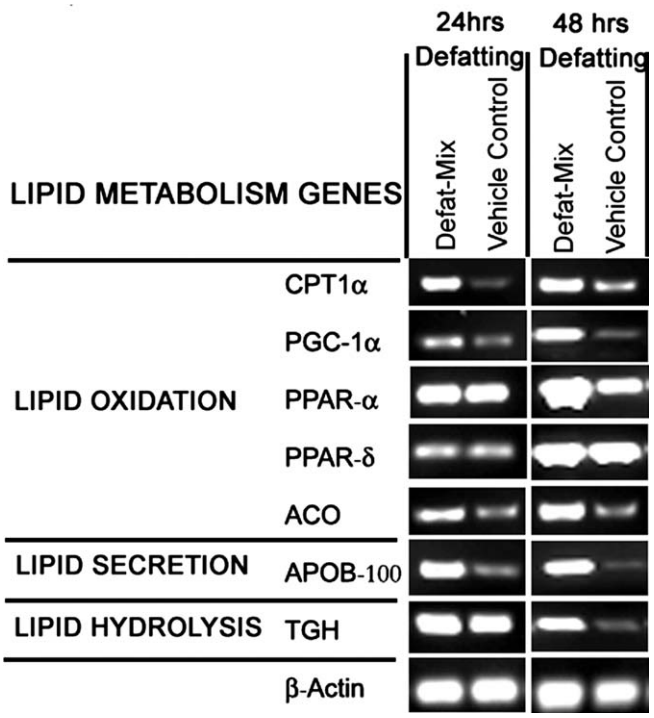

Fig. 4. Gene expression of lipid metabolic pathways involved during defatting. Steatotic hepatocytes were incubated in culture medium supplemented with the cocktail of defatting agents, or vehicle control (<0.1% DMSO). Fatty hepatocytes treated with defatting agents showed increased expression of genes involved in the pathways of lipid oxidation (ACO, PGC1α, CPT1α, PPARα, and PPARδ), lipid secretion (ApoB-100), and lipid hydrolysis (TGH), compared to the vehicle control. The increased expression was observed for defatting durations of both 24 and 48 h.

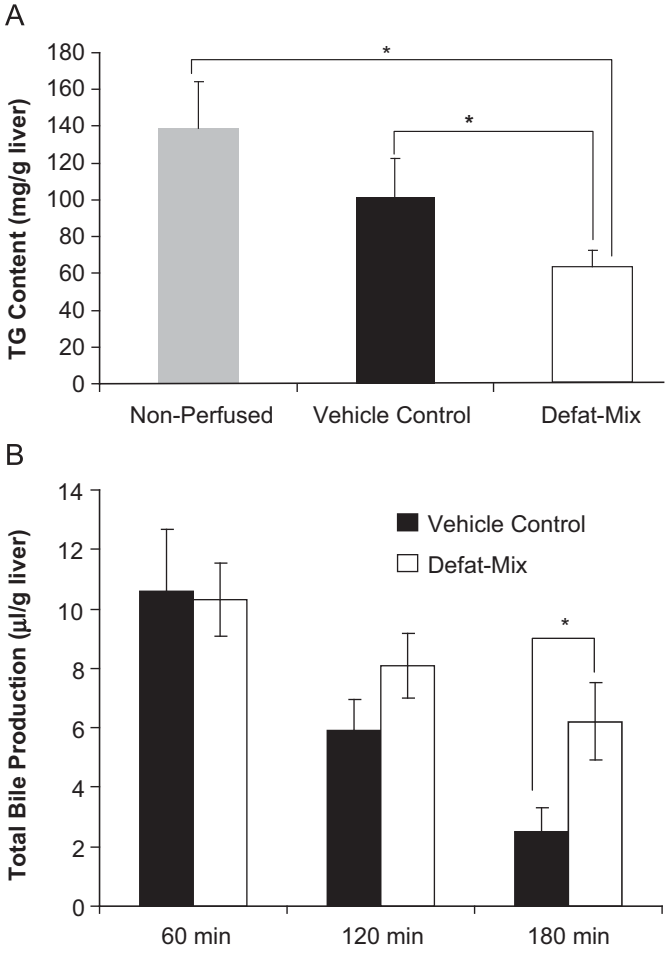

Fig. 5. Defatting of steatotic livers isolated from obese fa/fa Zucker rats. Isolated rat livers were perfused at 37 °C with perfusate containing the defatting agent cocktail ($N=7$) or perfusate with DMSO vehicle ($N=5$) for 3 h. (A) TG content remaining after perfusion. Data are normalized and compared to unperfused freshly isolated steatotic livers (138 mg TG/g liver). (B) Total bile production by perfused livers. *$p<0.05$ by one-tailed t-test compared to vehicle control.

increase fatty acid oxidation through increased expression of carnitine palmitoyltransferase I, a key player in the import of fatty acids into mitochondria (Le May et al., 2005), and acyl coenzyme A oxidase, an enzyme involved in peroxisomal β-oxidation (Tamura et al., 2006). PPARδ has also been implicated in fatty acid catabolism (Luquet et al., 2004) and has been shown to increase the levels of ACO, CPT-1, and peroxisomal ketothiolase (Nagasawa et al., 2006). Therefore, in order to develop a defatting regimen, we investigated the effects of PPARα and PPARδ agonists, as well as other nuclear receptors (PXR and CAR ligands), and the cyclic AMP activator forskolin, which has been previously shown to increase ketogenesis and fatty acid oxidation (Pegorier et al., 1989). Herein we observed that the defatting cocktail led to increased gene expression of PPARα and PPARδ, as well as several other enzymes and factors involved in β-oxidation, including ACO, CPT-Iα (Djouadi et al., 2005; Kitz Kramer et al., 2007; Le May et al., 2005; Tamura et al., 2006), and PGC1α (Meirhaeghe et al., 2003; Napal et al., 2005) in cultured hepatocytes.

It is noteworthy that the extent of reduction of lipid content that occurred after 24 h in cultured fatty hepatocytes took only 3 h in *ex vivo* liver perfusions. The reason for the faster kinetics in perfused livers may be attributed to greater availability of oxygen, as the perfusate was equilibrated with 95% v/v oxygen in the gas phase, while cultured hepatocytes were maintained in a normoxic incubator, and under static conditions, there is a significant decrease of oxygen concentration going from the air–liquid interface to the cell surface (Yarmush et al., 1992). Greater oxygen availability is expected to favor a higher rate of oxidation, as well as help maintain mitochondrial energy production, which is necessary for the processing and export of intracellular lipids in the form of LDL. It is interesting to note that PPAR α expression in steatotic hepatocytes increased significantly after 48 h exposure in defatting media. This increased expression may be attributed to existence of the positive feedback loop, i.e. some of the downstream target proteins might be responsible for increasing the expression of transcription factor PPAR α. The increased expression may not directly be a result of PPAR α receptor agonist since there is a delay in the increased expression of PPAR α as seen in the figure (marginal increase in expression at 24 h).

Fatty livers have been considered as "marginal donors" for liver transplantation because of the higher risk of graft primary non-function. Numerous studies have demonstrated that fatty livers are susceptible to transplantation-related injury such as ischemia-reperfusion injury (Selzner et al., 2006; Sun et al., 2001; Takahashi et al., 2000). Multiple injury mechanisms have previously been suggested to be responsible for this susceptibility, including compromised hepatic microcirculation (Teramoto et al., 1993, 1994), increased lipid peroxidation (Serkova et al., 2006), or decreased hepatocyte ATP levels (Chaudry et al., 1984; Fukumori et al., 1997). Based on these findings, many strategies have been developed to protect fatty liver from being damaged by these mechanisms. For instance, Serafin et al. (2002) reported that ischemic preconditioning increased fatty liver tolerance to ischemia-reperfusion injury by improving liver microcirculatory function. By overexpressing superoxide dismutase, an endogenous radical scavenger, Lehmann et al. (2003) showed decreased lipid peroxidation and organ injury, as well as increased survival in a

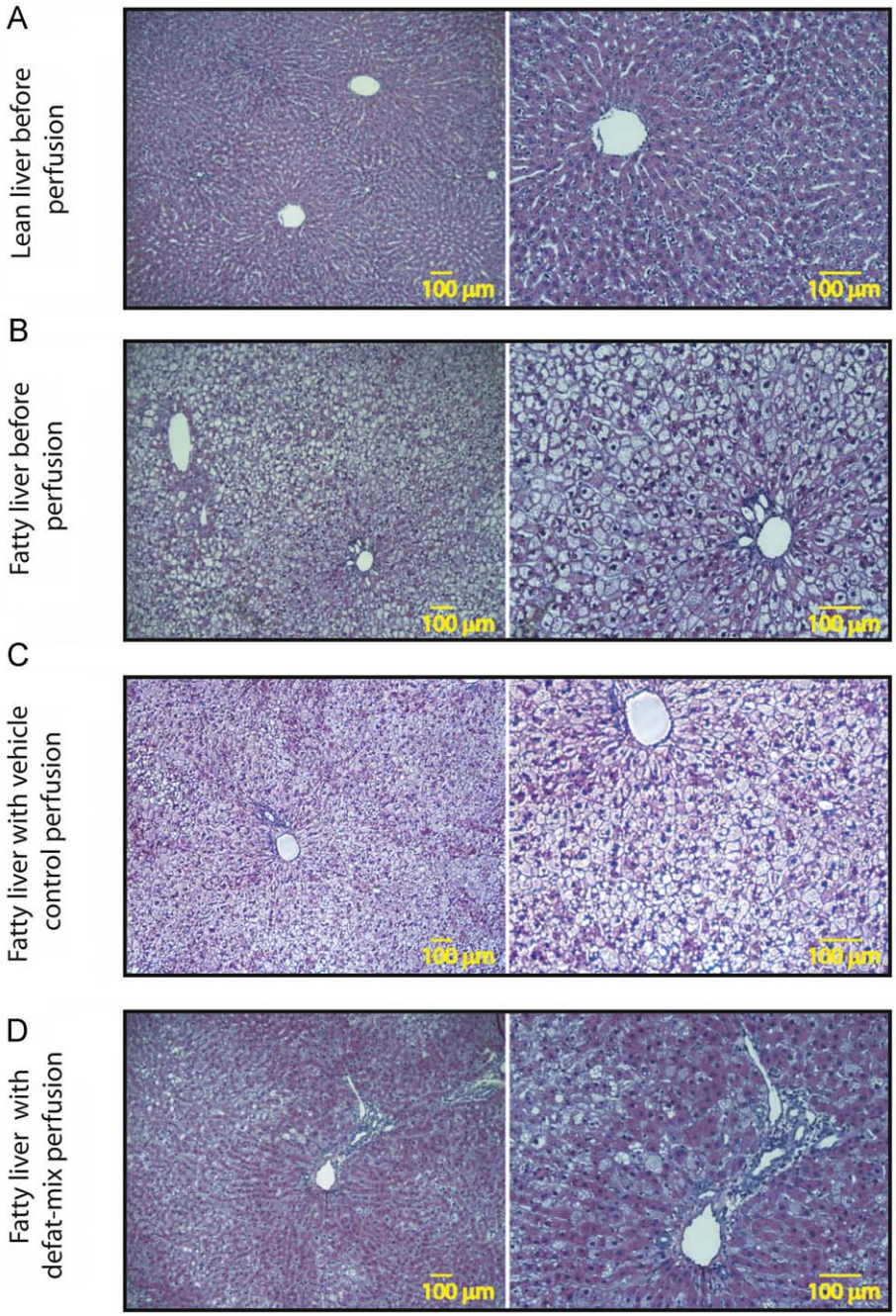

Fig. 6. Histological appearance of defatted steatotic livers. Livers (14–15 weeks of Zucker rats) were harvested before and after 3 h of perfusion, fixed and stained with hematoxylin and eosin. (A) Normal lean liver. (B) Fatty liver before perfusion. (C) Fatty liver after perfusion with the vehicle control. (D) Fatty liver after perfusion with the defatting cocktail. Higher magnification images are shown in right panel.

rat model of fatty liver transplantation. In the present study, rather than focusing on the individual consequences of steatosis, we pursued a different strategy, namely hepatocyte defatting. Because most of these aforementioned pathophysiologies stem from a fundamental pathological change—the excess accumulation of lipids in hepatocytes, reduction of lipid content in hepatocytes and restoration of normal liver microcirculation and histological architecture may be the most efficient way of minimizing these various steatosis-derived injuries.

One of the major challenges in this study was to achieve rapid defatting kinetics such that it is possible to recondition rejected liver grafts and release them back to the donor pool for transplantation within the typical timeframe of liver transplantation (6–12 h between harvest of donor and transplantation into recipient). By targeting multiple biochemical pathways of lipid catabolism and export in the liver using a defatting cocktail, we showed an effective method that can significantly reduce the triglyceride content in liver by 65% in only 3 h of normothermic perfusion. Since there is a strong correlation between increased degree of steatosis and graft primary non-function (Adam et al., 1991; D'Alessandro et al., 1991), rapid defatting via normothermic perfusion with a defatting cocktail may make it possible to salvage moderately (30–60%) or even severely (>60%) steatotic livers for transplantation. This 3 h defatting regimen could be implemented

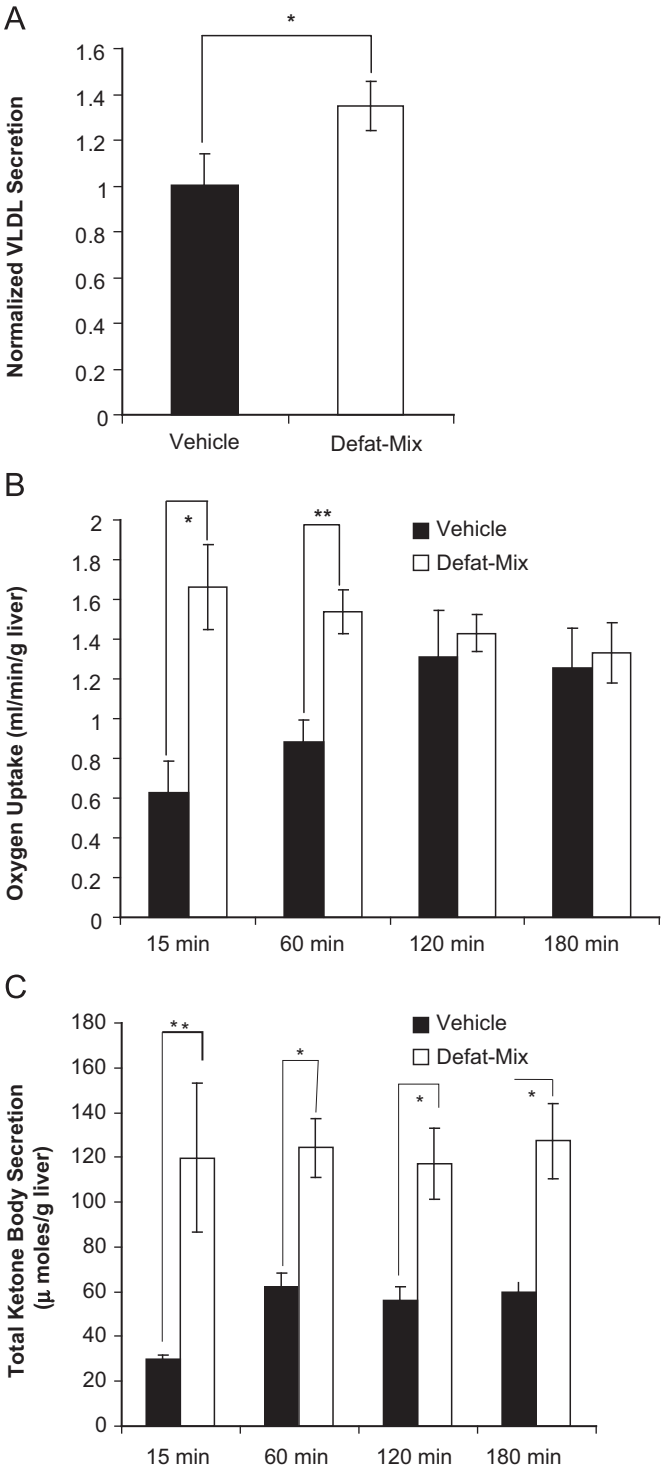

Fig. 7. Mechanism of defatting during perfusion of steatotic livers. (A) VLDL secreted into the perfusate after 2 h of perfusion with the defatting cocktail ($N=4$) as compared to vehicle controls ($N=3$). (B) Total oxygen uptake rate during fatty liver perfusion with defatting cocktail ($N=4$) as compared to vehicle control ($N=5$). (C) Total ketone body secretion during fatty liver perfusion with defatting cocktail ($N=3$) as compared to vehicle control ($N=3$). Data shown are expressed as means $\pm$ SE. $^{*}p<0.05$, $^{**}p<0.01$ by one tailed t-test compared to vehicle control.

as part of a normothermic extracorporeal liver perfusion preservation method, which has been shown to be a superior way to preserve donor livers that have suffered from warm ischemia (Butler et al., 2002; Chaudry et al., 1984; Lee et al., 2002; Tolboom et al., 2007).

In conclusion, we show for the first time that a cocktail of defatting agents can be used to significantly reduce the intracellular fat content of cultured hepatocytes and perfused livers within a few hours. Short-term ex vivo normothermic perfusion of severely steatotic donor livers using this defatting cocktail could set the stage for metabolic reconditioning of such livers, which would otherwise be rejected from the donor pool. Such an outcome would significantly alleviate the donor liver shortage and facilitate donor liver allocation.

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Appendix A. Supporting Information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2009.05.005.

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