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Elevated Sensitivity of Macrosteatotic Hepatocytes to Hypoxia-Reoxygenation Stress Is Reversed by a Novel Defatting Protocol

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Abstract

Macrosteatotic livers exhibit elevated intrahepatic triglyceride (TG) content in the form of large lipid droplets (LDs), reduced ATP, and elevated reactive oxygen species (ROS), contributing to their elevated sensitivity to ischemia-reperfusion injury during transplantation. Decreasing macrosteatosis in living donors through dieting has been shown to improve transplantation outcome. Accomplishing the same feat in deceased donor grafts would require ex-vivo exposure to potent defatting agents. Herein, we used a rat hepatocyte culture system exhibiting macrosteatotic LD morphology, elevated TG levels, and elevated sensitivity to hypoxia and reoxygenation (H/R), to test for such agents and ameliorate H/R sensitivity. Macrosteatotic hepatocyte preconditioning for 48h with a defatting cocktail, previously developed to promote TG catabolism, reduced the number of macrosteatotic LDs and intracellular TG levels by 82% and 27%, respectively, but did not ameliorate sensitivity to H/R. L-carnitine supplementation to this cocktail, together with hyperoxic exposure, yielded a similar reduction in macrosteatotic LD numbers, and to a 57% reduction in intrahepatic TG storage, likely by increasing the supply of acetyl-CoA to mitochondria, as indicated by a 70% increase in ketone body secretion. Furthermore, this treatment reduced ROS levels by 32%, increased ATP levels by 27%, nearing ATP levels of lean controls, and completely abolished H/R sensitivity as indicated by ~85% viability post H/R and return of cytosolic lactate dehydrogenase release down to levels seen in lean controls. Cultures maintained for 48h post H/R were ~83% viable and exhibited superior urea secretion and bile canaliculi transport compared to untreated macrosteatotic cultures. These findings show that the elevated sensitivity of macrosteatotic hepatocytes to H/R can be overcome by defatting agents, suggesting a possible route for the recovery of discarded macrosteatotic grafts.

Keywords

Hypoxia; macrosteatosis; hyperoxia; carnitine; ATP; ROS

Introduction

Orthotopic liver transplantation is the only therapeutic option for end-stage liver disease, but is limited by the scarcity of suitable grafts (1-4). Macrosteatotic livers contain excessive intrahepatic TG, which is stored in the form of large LDs, and exhibit elevated sensitivity to ischemia-reperfusion (I/R) stress associated with graft preservation injury. Such livers are known to exhibit increased rates of primary non-function post transplantation and are therefore excluded from the donor pool (1-5). The extent of preservation injury in both whole livers and hepatocyte cultures, as demonstrated by elevated hepatocellular death, is likely mediated by reduced baseline ATP levels and elevated ROS-related stress, and can be correlated to intrahepatic TG content and the number of macrosteatotic LDs (5-11). In addition, due to the lipid accumulation in their cytoplasm, macrosteatotic hepatocytes increase their size causing sinusoidal space narrowing at the organ level, which was shown to exacerbate I/R injury (6, 10). Reduction of liver intrahepatic macrosteatosis and TG content while in the donor's body by dieting for several days to weeks has been shown to ameliorate I/R sensitivity and enable successful liver transplantation in both humans and animal models (5, 6, 12). The donor shortage could be significantly alleviated by implementing methods to recondition macrosteatotic livers explanted from deceased donors. Such methods may require ex-vivo exposure of macrosteatotic grafts to agents that decrease LD size and the removal of stored TG, which would in turn reduce hepatocyte sensitivity to I/R stress (6).

For efficient evaluation of such agents, we have recently described a macrosteatotic primary rat hepatocyte culture system, which exhibits clinically relevant features observed in human macrosteatotic livers (13). Using this system, macrosteatotic cultures were preconditioned for 48h with a defatting cocktail previously developed using a microsteatotic hepatocyte culture system. The cocktail contained steatosis reduction supplements (SRS) shown to promote various TG catabolism pathways including LD breakdown and FFA oxidation (14). This preconditioning method was shown to decrease the number of macrosteatotic LDs to levels similar to that in control lean cultures; however, the hepatocytes maintained a relatively high content of intrahepatic TG, possibly due to re-esterification of FFAs released during LD breakdown (13, 15). It is unclear whether the decrease in LD size alone, or more complete removal of TG, is required to ameliorate hepatocyte elevated sensitivity to H/R induced stress, which simulates I/R injury in a static culture system.

Herein, we hypothesized that L-carnitine, which is involved in the shuttling of FFAs from the cytosol into the mitochondria (16, 17), may promote FFA β -oxidation and decrease re-esterification to TG, thus leading to decreased TG storage. We also investigated the impact of the various defatting strategies on the sensitivity of hepatocytes to H/R induced stress in terms of viability and functional preservation using urea secretion and bile canalicular transport as biomarkers (13, 18). The data herein show that L-carnitine addition to the defatting cocktail, in combination with hyperoxia, led to the greatest decrease in TG content. These interventions also decreased oxidative stress markers and increased ATP stores, all of which likely contributed to improved survival of macrosteatotic hepatocytes following H/R induced stress.

Methods

Hepatocyte Isolation and Culture

Male lean Zucker rats, (Charles River, Wilmington, MA) (310±20g) were housed in a 12h light-dark cycle and temperature-controlled environment (25°C) with water and standard chow ad libitum. All experimental procedures followed National Research Council guidelines and were approved by the Rutgers University Animal Care and Facilities Committee. Hepatocytes were isolated using a two-step in-situ collagenase perfusion technique. Hepatocytes with viability of 92±3%, as determined by trypan-blue exclusion, were seeded in 6-well plates in a collagen sandwich configuration at a concentration of 10⁶ cells/well (5). Cultures were maintained in standard hepatocyte medium for 4 days with a fresh medium change every other day (13, 14) (Figure 1A).

Steatosis Induction

Five-day hepatocyte cultures were switched to steatosis-inducing medium. Standard hepatocyte medium was supplemented with 2,000µM oleic acid, 2,000µM linoleic acid and 4% (weight to volume) bovine serum albumin (Sigma-Aldrich) for 3 days. Medium was replaced with fresh steatosis-inducing medium for another 3 days of steatosis induction to induce macrosteatosis and the spent medium was collected as previously described (13) (Figure 1A, experimental days 5-11). Note that we opted for in-vitro steatosis induction of lean hepatocytes because attempts to isolate steatotic primary hepatocytes from obese Zucker rat livers led to hepatocyte viability <10%, primarily consisting of the lean fraction of the isolated cells (data not shown).

Steatosis Reversal

Following 6-day steatosis induction, the macrosteatotic cultures were incubated for 48h in a 90% air/10% CO₂ gas mixture at 37°C in a standard incubator with fresh hepatocyte medium supplemented with a combination of SRS or with SRS media supplemented with 0.8mM L-carnitine (3-hydroxy-4-N, N, N-trimethylaminobutyric acid, Sigma-Aldrich) (SRS + L-car) (Figure 1A). SRS media was previously shown to accelerate macrosteatosis reduction in-vitro by activating hepatocellular TG metabolism (13, 14), and its component concentrations are described in supplementary table 1. L-carnitine concentration was determined based on cytotoxicity titration experiments (data not shown) and is in agreement with concentrations used in in-vitro studies using steatotic human hepatocyte cell line, HepG2 (17), which were used in an ex-vivo perfusion experiments exploring L-carnitine effect on ketogenesis in perfused steatotic mouse livers (19). In parallel, macrosteatotic cultures were incubated in fresh SRS or SRS+L-car medium for 48h under 90% oxygen at atmospheric pressure (SRS+ 90% O₂ or SRS+ L-car+ 90% O₂) using a hyperoxia induction chamber consisting of a hermetically closed plastic box (8×12×10 inches) with drilled ports for gassing and an oxygen probe (5). The tissue culture plates were placed in the humidified chamber, the chamber closed tightly, and purged for 5 minutes with 90% O₂/10% CO₂ gas mixture and the ports were closed. The chamber was inserted to a 37°C incubator for 48h, where the oxygen level was maintained ~90% in the chamber. Then, spent medium from all cultures was collected (Figure 1A). The uptake rate of L-carnitine was determined based on the difference in its concentration in the defatting medium between the onset (t=0h) and end

(t=48h) of the defatting period as measured using a commercially available kit (Abcam, Cambridge, MA). L-carnitine uptake amounted to only ~20% of the initial supply in the medium, and therefore was available in excess (Supplementary Figure 1).

Exposure of Hepatocytes to Hypoxia-Reoxygenation

The tissue culture plates were placed in the chamber described above. The humidified chamber was purged for 5 minutes with 90% N₂/10% CO₂ gas mixture and the ports were closed. The chamber was inserted in a 37°C incubator for 8h, where the oxygen level was maintained ~0% in the chamber. This hypoxia induction duration was determined based on its ability to display significant macrosteatotic culture elevated sensitivity to H/R stress while keeping lean cultures unaffected (Supplementary figure 2A). After the prescribed hypoxia time, the plates were retrieved from the chamber, the culture medium collected for further analysis, and the cells fed with fresh hepatocyte medium pre-equilibrated at normoxic conditions. The plates were then placed back in a standard 37°C and 90% air/10% CO₂ atmosphere incubator for 6h of reoxygenation (Figure 3A). Then, the cultures were tested for viability using the ethidium homodimer-1 (EthD-1) assay (Supplementary figure 2) and the collected media during hypoxia and reoxygenation was tested for enzymatic release using a commercially available LDH release assay (Roche, Indianapolis, IN) (5, 13, 18) as described below. The media was changed again at 12, 24, 36 and 48h post hypoxia or normoxia induction and tested for urea secretion as a marker for liver specific detoxification ability (13, 18). At the end of the 48h, relevant cultures were assayed for viability (EthD-1 assay) as well as for bile canalicular transport as an indicator of their ability to metabolize and excrete compounds using the 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (DCFDA) assay as described elsewhere (13, 18). The same treatment to the cultures that underwent 8h of exposure to normoxic was used as control.

Hepatocyte Steatosis Assessment

Hepatocyte cultures were fixed in 4% paraformaldehyde, stained with the lipid-specific Nile red stain (Adipored™, Lonza, Walkersville, MD), counterstained with 1ug/ml nuclei-specific Hoechst-33342 stain (Invitrogen) and confocal fluorescence images were obtained with an Olympus IX-80 microscope (13). In addition, hepatocytes were scraped and sonicated in standard hepatocyte medium and using a lipase-based assay kit (Sigma-Aldrich), where the TG content is measured by quantification of liberated glycerol (5, 13).

Hepatocyte Viability Assessment

Cultures were washed with PBS, and dead cells quantified following incubation with 4.29ug/ml EthD-1, 5ug/ml Calcein-AM and 1ug/ml Hoechst-33342 (Invitrogen) to stain all cells, for 20 min at 37°C (13). Five 20X epifluorescence images were obtained in 5 separate wells/experimental condition. Cells containing EthD-1 labeled nuclei were counted as dead, while those with Hoechst-stained nuclei were counted as live. Dead cells were confirmed to be negative for Calcein-AM (Invitrogen) esterase dependent metabolic assay (Supplementary figure 2B). Percentage of viable cells was determined in each well and averaged for 5wells/condition (13). Hepatocyte injury was assessed by measuring LDH released in the culture medium using a commercial kit (Roche).

Hepatocyte Function Assessment

Urea nitrogen secretion was measured using a biochemical assay (Stanbio), ketone body secretion using a biochemical assay (Bioassay systems, Hayward, CA) and VLDL secretion using a lipase assay kit (Sigma-Aldrich). All assays used spent media samples following the manufacturer's recommendations. Concentrations were normalized to the time period between medium changes and to the number of viable hepatocytes to convert into specific secretion rates. Intracellular ATP content was measured in deproteinized cultures using a colorimetric assay (Biovision, Milpitas, CA). ROS stress was assessed by the ratio of glutathione oxidized to non-oxidized forms (GSSG/GSH) as well as the sum of the two in deproteinized cultures using a colorimetric assay (Biovision) (20). To control for a potential artefact due to the difference in culture duration in standard culture medium between lean cultures and those exposed to fattening medium, lean un-fatted cultures were monitored as an additional control for hepatocyte functional stability on each experimental day. In addition, in a separate experiment, the experimental days were "time-shifted" so that cultures were subjected to fattening/defatting after the same number of days of culture in standard medium as the lean controls. Results were similar in both study designs (data not shown), which we attribute to the stability of collagen-sandwiched hepatocytes (21).

Statistical Analysis

Results shown in text and figures are means $\pm$ 1 standard error. One way ANOVA followed by Fisher's Least Significant Difference post-hoc test was performed using KaleidaGraph (Synergy Software, Reading, PA). Values of $p < 0.05$ indicate statistical significance.

Results

The primary rat macrosteatotic hepatocyte system previously developed in our laboratory exhibits morphological characteristics of large LDs displacing the hepatocyte nucleus to the periphery and elevated intrahepatic TG, as seen in humans and animal models of macrosteatotic livers (Figure 1) (13). Preconditioning of these cultures for 48h with the SRS cocktail (steatosis reduction supplements, SRS) or L-carnitine supplemented SRS cocktail under normoxic (SRS or SRS+ L-car) or hyperoxic (SRS+ 90% O₂ or SRS+ L-car+ 90% O₂) conditions led to a similar reduction of ~82% in the number of macrosteatotic LDs to levels of control lean cultures (Figure 1B, C). As indicated in Figure 1D, the SRS cocktail reduced intrahepatic TG levels by only 27%, while the SRS+L-car and SRS+90% O₂ led to a ~41% and ~45% decrease, respectively. These differences in intrahepatic TG defatting did not however reach statistical significance compared to treatment with SRS alone. On the other hand, the combination of the two added to SRS, namely the combination SRS+L-Car +90% O₂, reduced intrahepatic TG levels by ~57%, which was statistically significantly different compared to the value obtained by SRS alone ($p < 0.05$). Therefore, both L-carnitine and elevated O₂ tension were required to promote significant TG removal.

To determine whether these treatments reduce intrahepatic TG levels by mitochondrial oxidation, total ketone body secretion (often used as surrogate marker of β -oxidation) was measured. SRS+L-car and SRS+90% O₂ led to a ~35% and ~20% increase in ketone body secretion rate, respectively, compared to the SRS cocktail alone. These rates were

statistically significant compared to that of macrosteatotic cultures (Figure 2A). However, the combination of the two treatments (SRS+L-Car+90%O₂), increased the ketone body secretion rate by ~70%, levels that were statistically significantly higher than those obtained by SRS alone ($p<0.05$). The intrahepatic ATP levels in macrosteatotic hepatocytes were reduced by 30% compared to control lean cultures (Figure 2B). While SRS treatment increased this value by 11% (under normoxia or hyperoxia), L-carnitine supplemented SRS increased it by 22% under normoxia, and by 27% under hyperoxia (Figure 2B). Importantly, only the combined SRS+L-car+90% O₂ treatment increased ATP levels to be statistically significantly higher than that for macrosteatotic cultures, also reaching values similar to lean controls (Figure 2B). The uptake rate of L-carnitine was similar under normoxic and hyperoxic conditions (Supplementary Figure 1). To assess whether the change in intracellular TG levels was affected by TG secretion rates, the latter were measured in the extracellular medium and found to be the same for all 4 treatments (Figure 2C), thus suggesting that altered TG secretion was not likely contributing to the observed changes in intrahepatic TG levels. A hallmark of macrosteatotic livers is their increased ROS stress as measured by the ratio of oxidized to reduced glutathione (GSSG/GSH) (20). While the total glutathione levels in all cultures were similar ($p=0.3$, Figure 2E), the GSSG/GSH in the macrosteatotic cultures was ~4.6 fold higher compared to control lean cultures. This ratio was reduced by ~33% ($p<0.05$) following 48h of preconditioning with any of the 4 treatments, although it remained significantly above that of lean control cultures (Figure 2F).

The viability of lean, microsteatotic, and macrosteatotic hepatocytes that underwent 8h of normoxia followed by 6h of reoxygenation remained >87% (Figure 3A, B, C). However, following 8h of hypoxia followed by 6h of reoxygenation, macrosteatotic cultures exhibited elevated sensitivity to the H/R induced stress as indicated by a reduction in viability down to ~41% compared to only ~78% in the microsteatotic group and ~90% in the lean control group (Figure 3A, B, C). Macrosteatotic H/R elevated sensitivity was also demonstrated by the ~17 fold increased LDH release after 8h of hypoxia and 6h of reoxygenation compared to the lean controls. Microsteatotic cultures also exhibited elevated sensitivity to H/R, although the LDH increase was only ~5.9 times that of the lean controls (Figure 3D).

Treatment of macrosteatotic cultures with the SRS cocktail alone for 48h prior to exposure to 8h of hypoxia and 6h of reoxygenation did not ameliorate H/R elevated sensitivity and even showed a trend (although not statistically significant) towards further reduced viability (~10%) and increased LDH release (~25%) compared to macrosteatotic cultures (Figure 3). SRS+L-car treatment led to a ~10% increase in viability and ~60% reduction in LDH release compared to macrosteatotic cultures, bringing the treated cultures up to ~51% viability post H/R (Figure 3C, D, $p<0.05$). SRS+90% O₂ treatment further increased viability to ~79% and reduced LDH release by ~80% compared to macrosteatotic cultures (Figure 3C, D, $p<0.05$). The combination of all treatments (SRS+L-car+90% O₂) further increased culture viability post H/R stress to reach ~85% viability overall, which was comparable to that of control lean cultures (Figure 3B, C). In the same experiment, LDH release post H/R stress was reduced by ~97% compared to macrosteatotic cultures ($p<0.05$), reaching a level comparable to control lean cultures undergoing the same H/R protocol (Figure 3D).

Since the L-carnitine supplemented SRS cocktail combined with hyperoxia abolished H/R elevated sensitivity and hepatocyte death at 6h post hypoxia induction, we extended the observation period to 48h post hypoxia in standard hepatocyte culture media under normoxia to determine whether viability was maintained and to assess functional parameters. Macrosteatotic, lean and SRS+ L-car+ 90%O₂ pretreated macrosteatotic cultures that underwent 8h of normoxia exhibited ~90% viability 48h post induction (Figure 4A, B), consistent with the data in Figure 3. The viability of macrosteatotic cultures 48h post 8h of hypoxia induction was further reduced to ~28%, whereas macrosteatotic cultures preconditioned with SRS+ L-car+ 90%O₂ maintained an elevated viability at about ~83%, which was similar to that of control lean cultures that underwent the same H/R protocol (Figure 4A, B). Urea secretion levels during 48h following 8 hours of normoxia or hypoxia was higher in the control lean hepatocytes compared with either macrosteatotic or macrosteatotic cultures preconditioned with SRS+ L-car+ 90%O₂ (p<0.001) (Figure 4C and supplementary Figure 3). However, the accumulated urea in macrosteatotic cultures preconditioned with SRS+ L-car+ 90%O₂ was 95% higher than in macrosteatotic cultures at 48h following 8h of hypoxia (p<0.001) (Figure 4C). The accumulation of the fluorescent DCFDA molecule in the bile canaliculi 48h post 8h of hypoxia induction was used as indicator of the hepatocyte cultures' ability to utilize this transport mechanism for excretion of bile and other metabolized compounds (13, 18). Macrosteatotic cultures exhibited compromised bile canalicular transport of DCFDA compared with control lean cultures. Preconditioning macrosteatotic cultures with SRS+ L-car+ 90%O₂ for 48h prior to H/R enabled full recovery of bile canalicular transport to levels comparable to those in control lean hepatocytes (Figure 4D).

Discussion

Herein, we show that macrovesicular steatotic hepatocyte cultures are significantly more sensitive to H/R injury than control lean and microvesicular steatotic hepatocytes. Furthermore, macrosteatotic hepatocytes exhibit lower levels of ATP and increased levels of ROS-related stress (as evidenced by the increased GSSG/GSH ratio) compared to control lean cultures. Therefore, this in-vitro system exhibits many clinically relevant features with human macrosteatotic livers in terms of LD content and morphology, as well as with respect to elevated sensitivity to preservation-related stress such as H/R (albeit requiring a longer ischemic period than that typically seen in actual whole livers). The combination of these features makes this system suitable to characterize the hepatocellular responses and biochemical pathways that differentiate macrosteatotic, microsteatotic, and lean hepatocytes, as well as the lipid lowering effects of individual “defatting” agents and combinations thereof. Thus, this system could also potentially be used as a screen for such agents that could be used to decrease hepatic macrosteatosis and H/R sensitivity in actual whole livers.

Preconditioning macrosteatotic hepatocytes for 48h using the SRS cocktail reduced macrosteatotic LD numbers while TG remained relatively high and intrahepatic ATP levels low, with an overall effect that it did not ameliorate sensitivity to H/R stress. Performing this treatment with L-carnitine supplementation (SRS+ L-car) or under elevated O₂ tension (SRS +90% O₂) did not lead to a statistically significant decrease in TG, but significantly improved hepatocyte resistance against H/R induced death. Using all three components, i.e.

the combination of L-carnitine supplementation to SRS under 90% O₂ tension (SRS+L-carnitine+90% O₂) led to the greatest decrease in TG content, which was statistically significant compared to that achieved by SRS alone, down to a level slightly lower than that in microsteatotic cultures. It is also the only treatment that exhibited ATP levels and hepatocyte viability post H/R stress that were similar to lean un-fatted control cultures. Taken together, these results suggest that improved viability post H/R may not be the simple result of defatting, but likely involves other metabolic effects of these defatting agents on hepatocytes, especially increasing ATP content. We did not attempt to separate the role of defatting from these other likely benefits of the defatting protocol, as it is generally very difficult to decouple metabolic pathways from each other. Interestingly, TG levels did not need to be decreased to the control lean baseline to recover the typical H/R responses of lean hepatocytes.

Hepatic ketone body production is a function of acetyl-CoA levels in the mitochondria, which are affected by several intrahepatic metabolic pathways, including FFA β -oxidation as well as glycolysis followed by oxidative decarboxylation by pyruvate dehydrogenase (22). Nevertheless, it is often used as a surrogate marker of β -oxidation in patients with steatotic livers (23). Furthermore, since L-carnitine putatively increases FFA supply into the mitochondria, an increase in ketone body secretion in this case likely reflects a change in β -oxidation, as previously reported in perfused livers from steatotic mice (19). Based on the increased ketone body production during defatting, it is plausible that the reduction of intracellular TG levels was achieved by directing cytoplasmic FFA liberated from the LDs towards β -oxidation before it underwent re-esterification to TG, as summarized in figure 2D (6, 15, 24). In addition, the fact that we observed similar TG secretion rates for all tested defatting treatments indicates that the different outcomes in terms of intrahepatic TG levels were likely due to other metabolic routes, of which catabolism of TG followed by FFA β -oxidation is likely the main one. More definite conclusions may be reached using FFA isotopic labeling, fractionating the different pools of intracellular FFA, and quantifying label appearance in CO₂, although capturing CO₂ evolved from cultures is prone to error (15, 25). Preconditioning methods, such as defatting of microsteatotic hepatocyte cultures (5) or perfusion of macrosteatotic livers with high oxygen tension (26) lead to reduced ROS stress, among others alterations, and lower levels of hepatic injury markers following preservation stress. Here we found that all 4 preconditioning methods led to similar reduction in ROS stress (GSSG/GSH), independent of the oxygen tension or the final TG levels. Therefore, reduction in GSSG/GSH was not a single cause for abolishing H/R elevated sensitivity of macrosteatotic cultures preconditioned with L-carnitine supplemented SRS cocktail under hyperoxia. However, it is likely that the combination of (1) ROS stress reduction; (2) increased ATP levels, possibly arising from the stimulated TG metabolism towards β -oxidation; and (3) reduced TG levels together with additional protective mechanisms inherent to high O₂ environments and to L-carnitine supplementation, improved hepatocyte resistance to H/R stress (14, 20, 26, 27). Other antioxidant mechanisms besides glutathione (e.g. thioredoxin, vitamins C and E) may also come into play, although since glutathione is one of the major anti-oxidant systems, the GSSG/GSH likely reflects the redox state of the liver cells.

The data herein indicate that L-carnitine supplementation to the SRS cocktail was critical for macrosteatotic hepatocyte defatting and improving resistance to H/R stress when using hyperoxic (90% O₂) conditions. The defatting effect of L-carnitine is consistent with studies showing that administration of L-carnitine as a nutritional supplement to dieting patients with nonalcoholic steatohepatitis or to rats with steatotic livers reduced hepatic steatosis and TG levels (28, 29). While a large body of literature describes the ability of L-carnitine supplementation to promote FFA oxidation (16, 17, 19, 30), L-carnitine may have contributed in other ways to the increase in hepatocyte resistance to H/R stress. For example, L-carnitine addition to a cold preservation solution for 24h was reported to increase ATP levels in steatotic rat livers (27). Furthermore, the use of hyperoxic conditions, either by oxygenation of isolated perfused livers, or by hyperbaric treatment of whole animals, effectively prevented rat liver graft preservation injury, including in steatotic livers (20, 26, 31). While the mechanism of action was not fully elucidated, it is noteworthy that increased ATP and reduced ROS levels were observed in several of these studies while oxygen consumption was elevated during hepatocyte defatting (14). L-carnitine has also been reported to have anti-oxidant protective effects mediated by peroxisome proliferator-activated receptor alpha in human hepatocytes (27, 32), as well as to inhibit ceramide generation and protect against cell death in cardiac myocytes, a mechanism which may also occur in hepatocytes (33).

A limitation of our hepatocyte culture model is that it does not reproduce the tissue scale abnormalities associated with macrosteatosis, such as sinusoid narrowing, which causes poor microcirculation. On the other hand, the narrowing is largely due to the increased size of the macrosteatotic hepatocytes; thus, hepatocyte defatting would likely address this issue, in addition to promoting intracellular ATP production and reducing ROS stress (5, 6, 13, 14). Serafin, et al. have previously explored the effect of reducing ROS levels in macrosteatotic obese Zucker rat livers using an experimental model of I/R (34). In that study, the administration of glutathione ester 5 minutes before reperfusion led to a ~30% reduction in hepatocyte death (as assessed by intrahepatic enzyme levels released into the circulation), albeit hepatic death was still ~60% above that in control lean livers. It is unlikely that any defatting took place in the short time span between glutathione administration and reperfusion. The defatting approach may be advantageous compared to methods that solely focus on increasing ATP levels or reducing ROS without addressing sinusoidal narrowing due to macrosteatosis (6). The in-vitro system described herein may facilitate the exploration of combination therapies that would promote defatting together with other agents that promote ATP generation, ROS removal, as well as stress preconditioning methods such as heat-shock (6, 35) or ischemic preconditioning (6, 7), all of which have individually shown some ability to reduce I/R related damage in steatotic livers.

We have previously shown that urea secretion is significantly reduced in macrosteatotic hepatocytes induced with oleic and linoleic acids and that defatting of these cultures improves urea secretion (13). Oleic acid has been reported to suppress urea cycle enzyme gene expression in livers of a juvenile visceral steatosis animal model, which was reversed by L-carnitine administration (36). Herein, we observed that macrosteatotic cultures treated with SRS+ L-car+ 90% O₂ exhibited ~2 fold increase in urea secretion 48h following hypoxia induction compared with macrosteatotic cultures. This significant difference may be

explained in part by a combination of higher viability of pretreated macrosteatotic cultures as well as functional improvement in these cultures. The bile canalicular impairment observed in macrosteatotic hepatocytes was consistent with impaired hepatobiliary transport in steatotic livers previously reported in several animal models (37, 38). We have also shown that defatting of macrosteatotic hepatocytes completely reverses the fat-induced impairment in bile canalicular excretion of DCFDA (13). Here, we also show that this recovery is maintained for at least 48h post H/R induced stress.

In summary, we describe a preconditioning method which is effective in salvaging macrosteatotic hepatocytes from in-vitro H/R induced death and promotes high viability and functionality for at least 48h post H/R stress. Scaling up this approach to actual livers will most likely need to be performed in an organ perfusion system. Recent ex-vivo perfusion studies using obese Zucker rat livers have indicated that normothermic but not subnormothermic perfusion reduced the defatting timescale from days to hours compared to static culture systems, as described herein (14, 39). This time scale would make the defatting approach clinically feasible; interestingly, there is a recent report of the successful transplantation of two livers that were normothermally perfused for 10h using an OrganOx® perfusion device (40, 41). While these perfused and transplanted livers were lean, studies involving normothermic perfusion of discarded macrosteatotic human livers with a perfusate containing a defatting cocktail such as the one developed here may be a promising avenue to recover macrosteatotic livers and alleviate the persistent shortage of suitable livers for transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

TG	Triglyceride
LDs	lipid droplets
ROS	reactive oxygen species
I/R	ischemia and reperfusion
H/R	hypoxia and reoxygenation
FFAs	free fatty acids
LDH	lactate dehydrogenase
SRS	steatosis reduction supplements
SRS+ L-car	SRS media supplemented with L-carnitine

SRS+ L-car+ 90% O₂	SRS+L-car medium under 90% oxygen at atmospheric pressure
EthD-1	ethidium homodimer-1
carboxy-DCFDA	5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate
GSSG/GSH	ratio of oxidized to reduced glutathione

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Figure 1. Hepatocyte morphology and lipid content during macrosteatosis reversal

Macrosteatotic hepatocyte cultures were preconditioned for 48h with SRS, SRS+L-car, SRS + 90% O₂ or SRS+L-car+ 90% O₂. (A) Experimental timeline. (B) Hepatocyte morphology post-FFA-supplementation (microsteatosis and macrosteatosis), and macrosteatotic cultures after 48h of preconditioning with 3 selected treatments. Bright-field (top) and fluorescent images of Nile red (showing LDs) and Hoechst (showing nuclei) stained hepatocytes (bottom). Bar= 50μm². (B and C) Macrosteatotic (>350 μm²) droplet number and

intracellular TG content for different treatments. Means $\pm$ S.E. N = 6. *p<0.001 vs. lean, +p<0.01 vs. macrosteatosis and \$p< 0.05 vs SRS. N.S.= not statistically significant.

Figure 2. Hepatocyte macrosteatosis reversal mechanism of action

Macrosteatotic cultures preconditioned for 48h with SRS, SRS+L-car, SRS+90% O₂ or SRS+L-car+90% O₂ were assayed for total ketone body secretion during treatment (A), intrahepatic ATP content post treatment (B) and TG secretion during treatment (C) to evaluate potential defatting mechanisms illustrated (D). In addition, the total intracellular glutathione (E, $p=0.3$) and the ratio between the oxidized (GSSG) to non-oxidized (GSH) forms of glutathione were determined as indicators of ROS stress in the cultures. Means $\pm$

S.E. N = 6. * $p < 0.05$ vs. lean, + $p < 0.05$ vs. macrosteatosis and \$ $p < 0.05$ vs SRS. N.S.= not statistically significant.

Figure 3. Hepatocyte sensitivity to in-vitro hypoxia and reoxygenation stress

Lean, microsteatotic and macrosteatotic cultures as well as macrosteatotic hepatocytes preconditioned with SRS, SRS+L-car, SRS+90% O₂ or SRS+L-car+ 90% O₂ were exposed to 8h of hypoxia or normoxia followed by 6h of reoxygenation under normoxic conditions as indicated in the flow diagram (A). (B) Bright-field (top) and EthD-1 (red)/ Calcein-AM (green)/ Hoechst (blue) stained epi-fluorescent images of the culture following 8h of hypoxia and 6h of reoxygenation. Bar=50μm. (C) EthD-1⁺ hepatocytes were considered dead, as confirmed by being Calcein-AM⁻, to quantify the cultures' % viability post 8h of

hypoxia (red bars) or normoxia (blue bars) induction followed by 6h of reoxygenation. (D) Intrahepatic LDH release during 8h of hypoxia and 6 hours of reoxygenation. Means $\pm$ S.E. N = 6. *p<0.05 vs. lean, +p<0.05 vs. macrosteatosis, \$p<0.03 vs. SRS and #p<0.05 vs. same condition following 8h of normoxia.

Figure 4. Hepatocyte viability and function 48h following in-vitro hypoxic induction

Lean, macrosteatotic and macrosteatotic cultures preconditioned with SRS+L-car+ 90% O₂ treatment were evaluated for viability and hepatocyte specific functions 48h post hypoxic induction. (A) Bright-field (top) and EthD-1 (red)/ Calcein-AM (green)/ Hoechst (blue) stained epi-fluorescent images of the culture 48h post 8h of hypoxia. Bar=50um. (B) EthD-1⁺ hepatocytes were scored dead, as confirmed by being Calcein-AM⁻ to quantify the percentage viability post 8h of hypoxia (red bars) or normoxia (blue bars) followed by 48h of reoxygenation. Means $\pm$ S.E. N = 6. *p<0.001 vs. lean or SRS+L-car+ 90% O₂, +p<0.001

vs. macrosteatosis and $\# < 0.05$ vs. same condition following 8h of normoxia. (C) Urea secretion for 48h following 8h of hypoxia stress. Means $\pm$ S.E. N = 6. * $p < 0.001$ vs. lean, + $p < 0.001$ vs. macrosteatosis. (D) Bright-field (top) and DCFDA (green) accumulation in the bile canaliculi epi-fluorescent images as an indicator of bile canalicular transport function, as demonstrated by the white arrows. Bar=50 μ m.