NADPH and glutathione redox link TCA cycle activity to endoplasmic reticulum stress

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Abstract

Endoplasmic reticulum (ER) stress is associated with dysregulated metabolism, but little is known about how the ER responds to metabolic activity. Here, working primarily in mouse hepatocytes, we show that decreasing the availability of substrate for the TCA cycle diminished NADPH production and attenuated ER stress in a manner that depended on glutathione oxidation. ER stress was also alleviated by impairing either TCA-dependent NADPH production or Glutathione Reductase. Conversely, stimulating TCA activity favored NADPH production, glutathione reduction, and ER stress. Validating these findings, we show that deletion of the mitochondrial pyruvate carrier, which is known to decrease TCA cycle activity and protect the liver from diet-induced injury, also diminished NADPH, elevated glutathione oxidation, and alleviated ER stress. These results provide independent genetic evidence that mitochondrial oxidative metabolism is linked to ER homeostasis. Our results demonstrate a novel pathway of communication between mitochondria and the ER, through relay of redox metabolites.

Introduction

As the gateway to the secretory pathway, the ER must properly synthesize and fold nascent secretory and membrane proteins. Disruption of this process, known as ER stress, is associated with many disease states, including obesity and its comorbidities (Mohan, R, Brown, Ayyappan, & G, 2019). Thus, it is important to understand how ER homeostasis is perturbed, particularly by metabolic disruption. The ER and the mitochondrial network, although not connected to each other by secretory pathway traffic, are intertwined both physically and functionally. The ER makes close physical contacts with mitochondria to facilitate the exchange of metabolites (Raffaello, Mammucari, Gherardi, & Rizzuto, 2016; Vance, 2014; Yoboue, Sitia, & Simmen, 2018). The ER also communicates with mitochondria via signaling from the unfolded protein response (UPR), which is activated by ER stress and signals through the three ER-resident stress sensors IRE1, PERK, and ATF6 (Walter & Ron, 2011). The UPR regulates mitochondrial activity at several levels, including enhancing mitochondrial protein quality control, augmenting ER-mitochondrial interactions and calcium signaling, and contributing to mitochondrial depolarization and initiation of apoptosis (Y. Fan & Simmen, 2019; Gutierrez & Simmen, 2018; Rainbolt, Saunders, & Wiseman, 2014). While the pathways through which ER stress and the UPR regulate mitochondrial function are becoming clearer, the converse—how mitochondrial function impacts ER homeostasis—is less understood.

In the liver, the UPR suppresses a large number of genes involved in metabolic processes (Rutkowski et al., 2008). One of the processes suppressed is fatty acid β -oxidation (DeZwaan-McCabe et al., 2017), which takes place in mitochondria. The role of the UPR is to restore ER function during stress, suggesting that suppressing β -oxidation in the mitochondria is functionally beneficial to achieving ER homeostasis. However, it is not clear how metabolic activity within the mitochondria might be conveyed to the ER.

The impact of metabolic activity on ER homeostasis is most evident from the association of ER stress with obesity—particularly in highly metabolically active tissues such as liver, pancreas, and adipose (Cnop, Foufelle, & Velloso, 2012). Lipotoxicity (i.e., damage caused by the inappropriate accumulation of lipids in non-adipose tissue), inflammation, and oxidative stress have all been shown to contribute to obesity-associated ER stress (Fu, Watkins, & Hotamisligil, 2012; Salvado, Palomer, Barroso, & Vazquez-Carrera, 2015). Yet, independent of diet content, feeding after a fast is sufficient to elicit ER stress in the liver (Gomez & Rutkowski, 2016; Oyadomari, Harding, Zhang, Oyadomari, & Ron, 2008; Pfaffenbach et al., 2010) and to alter the extent of physical contacts between the ER and mitochondria (Theurey et al., 2016). These findings suggest that ER homeostasis is acutely and intrinsically connected with metabolism even apart from the problems brought on by obesity. However, the biochemical pathways linking catabolism to ER function are not known.

The tricarboxylic acid (TCA) cycle is the central hub of metabolism, participating in both catabolism and anabolism. Acetyl-CoA enters the cycle after either oxidative breakdown of lipids within the mitochondria or after conversion of pyruvate, generated from glycolysis in the cytosol, by the mitochondrial pyruvate dehydrogenase complex. Canonically, TCA activity yields NADH and FADH₂ for the electron transport chain. However, the TCA cycle also provides precursors for biosynthetic pathways (Owen, Kalhan, & Hanson, 2002). In addition, the cycle can produce NADPH in addition to NADH, due to the activity of isozymes that reside either in the mitochondria (isocitrate dehydrogenase 2/IDH2) or cytosol (IDH1 or malic enzyme/ME1) (Rydstrom, 2006). Therefore, activity of the TCA cycle is likely to influence cellular processes by mechanisms beyond just the production of ATP from the electron transport chain.

NADPH is used as a cofactor by glutathione reductase (GR) to reduce oxidized glutathione (GSSG→2GSH), and likewise by thioredoxin reductase to reduce oxidized thioredoxin. Both of these molecules contribute to defense against oxidative stress (Sies, Berndt, & Jones, 2017), and both have connections to ER protein biogenesis. Thioredoxin has

1 been shown to be a source of electrons for reduction and isomerization of disulfide bonds of ER
2 client proteins (Poet et al., 2017). The oxidized form of glutathione (GSSG), which predominates
3 in the ER lumen compared to the cytosol, was formerly thought to reoxidize protein disulfide
4 isomerase (PDI) to promote ER disulfide bond formation. However, since the discovery of
5 alternative pathways for PDI reoxidation (Frandsen & Kaiser, 1999; Tu, Ho-Schleyer, Travers, &
6 Weissman, 2000; Zito, Melo, et al., 2010), the role of glutathione in the ER is now much less
7 clear (Delaunay-Moisan, Ponsero, & Toledano, 2017; Tsunoda et al., 2014). Whether elevated
8 GSSG might be beneficial to ER function under some cellular conditions but detrimental in
9 others is also unknown.

10 Despite the centrality of the TCA cycle to cellular function and hints that its activity might
11 be tied to ER stress (Mogilenko et al., 2019; Xin et al., 2018), its involvement in ER homeostasis
12 has not been investigated. Here, we used primary hepatocytes and other metabolically active
13 cell types to investigate the relationship between metabolic activity and ER stress. We show that
14 TCA cycle activity links lipid and carbohydrate catabolism to ER homeostasis through
15 production of NADPH and redox regulation of glutathione. Our findings delineate a novel
16 mechanism of communication from mitochondria to the ER, reveal an unexpected protective
17 role for GSSG, and provide a plausible pathway by which ER homeostasis is linked to metabolic
18 activity.

Results

Inhibition of β -oxidation alleviates ER stress in metabolically active cell types

We have previously shown *in vivo* and in hepatoma cells *in vitro* that inhibiting β -oxidation either pharmacologically or genetically diminishes ER stress signaling (Tyra, Spitz, & Rutkowski, 2012). In order to identify the pathway by which β -oxidation and ER homeostasis are linked, we first asked whether etomoxir, which blocks β -oxidation by inhibiting the CPT1-dependent transport of fatty acyl-CoAs into the mitochondria for oxidation (Weis, Cowan, Brown, Foster, & McGarry, 1994; Yao et al., 2018), could diminish ER stress signaling in primary hepatocytes *in vitro* as it does in the liver *in vivo*. Treatment of primary hepatocytes with the ER stressor tunicamycin (TM) upregulated UPR-responsive mRNAs, whereas etomoxir cotreatment suppressed this effect (Figure 1A). Dampened ER stress signaling was also evident from diminishment of the splicing of the IRE1 α nuclease target *Xbp1* (Figure 1B) and of the upregulation of the stress-regulated factor CHOP (Figure 1C). Because these events are differentially regulated by the three limbs of the UPR, our data suggest that etomoxir diminishes signaling from all three UPR pathways. This result is consistent with our previous findings, in which ER stress signaling in hepatoma cells was diminished by etomoxir, by inhibition of β -oxidation with a separate agent, and by knockdown of CPT1 (Tyra et al., 2012).

We next determined if the apparent protective effects of etomoxir were limited to one cell type or to one ER stressor. Using the selection of UPR-regulated mRNAs in Figure 1A as a broad indicator of ER stress signaling, we found that etomoxir also diminished UPR activation in C2C12 myoblasts (Figure 1D), and immortalized (data not shown) and primary (Figure 1E) brown adipocytes. Hepatocytes, myocytes, and brown adipocytes are characterized by particularly high lipid metabolic activity (Frayn, Arner, & Yki-Jarvinen, 2006). In contrast, etomoxir had no significant effect on the expression of UPR target genes in primary mouse

embryonic fibroblasts (Figure S1). In primary hepatocytes subjected to ER stress by palmitate loading, etomoxir likewise diminished ER stress signaling (Figure 1G). (As expected, the unsaturated fatty acid oleate did not cause ER stress.) Palmitate is thought to elicit ER not by disrupting protein folding *per se* but by altering ER membrane fluidity, activating the UPR stress sensors through their transmembrane domains (Volmer, van der Ploeg, & Ron, 2013). Thus, etomoxir diminishes ER stress induced by stressors that act by divergent mechanisms.

The effects of etomoxir on ER stress signaling could be due to improvement of ER homeostasis in some way, or to simple inhibition of UPR signaling. We did not observe any robust differences in the efficiency with which ER client proteins were secreted in hepatocytes treated with etomoxir compared to those that were not treated (data not shown). However, as a separate assessment of ER homeostasis, we examined ER ultrastructure in hepatocytes treated with TM, with or without etomoxir. Consistent with previous reports (Finnie, 2001; Rutkowski et al., 2006), TM elicited marked ER dilation accompanied by a loss of lamellar structure and overall disorganization, although some areas of grossly normal ER were present and were predominantly juxtaposed near mitochondria. In contrast, these disruptions were largely prevented by cotreatment with etomoxir (Figure 2A) as confirmed by two independent, blinded scorers (Figure 2B). Although etomoxir diminished ER stress signaling, it did not block it entirely. Higher doses of TM elicited UPR activation in etomoxir-treated cells to an extent comparable to that observed with lower doses in non-etomoxir-treated cells (Figure 2C). Therefore, the UPR in etomoxir-treated cells remained competent for signaling. Together, these results suggest that inhibiting β -oxidation alleviates ER stress.

To provide a direct test of whether etomoxir could alleviate ER stress caused by the accumulation of misfolded protein, we examined its ability to reverse ER stress associated with overexpression of the null Hong Kong (NHK) protein. NHK is encoded by a nonsense mutation of α 1-antitrypsin that results in a truncated product which is retained in the ER and undergoes

ER-associated degradation, resulting in ER stress in diverse cell types (Nagasawa, Higashi, Hosokawa, Kaufman, & Nagata, 2007; Ordonez et al., 2013; Sifers, Brashears-Macatee, Kidd, Muensch, & Woo, 1988; J. Wu et al., 2007). We transduced primary hepatocytes with a recombinant adenovirus expressing NHK under doxycycline (Dox)-inducible control (Ad-TetOn-NHK). As a control, cells were transduced with adenovirus constitutively overexpressing GFP instead (Ad-GFP). No ER stress was observed in Ad-TetOn-NHK-transduced cells in the absence of Dox (not shown). As expected, Dox treatment elicited ER stress in cells transduced with Ad-TetOn-NHK, but not in cells expressing Ad-GFP. The stress induced by NHK was markedly diminished by etomoxir (Figure 3A). Overexpression of NHK disrupted ER structure, resulting in the appearance of structurally amorphous ER puncta that were diminished by etomoxir treatment (Figure 3B). This finding was again substantiated by blinded analysis of EM images (Figure 3C). In principle, etomoxir treatment could alleviate NHK-dependent ER stress by reducing protein synthesis. However, we found no evidence that global translation rates were altered by etomoxir (Figure S2). Consistent with this observation, we found that, 4 hours after NHK induction, steady-state expression of NHK did not differ in cells treated or not with etomoxir (Figure 3D). However, at later time points, we observed diminished steady state expression of NHK in etomoxir-treated cells, and this distinction was lost when degradation of NHK was blocked by the proteasomal inhibitor MG-132 (Figure 3D). While the mechanisms by which etomoxir enhances ERAD remain under investigation, these results provide a separate line of evidence that inhibiting β -oxidation improves ER homeostasis, perhaps by affecting misfolded protein clearance.

Inhibiting β -oxidation protects ER function through glutathione redox

We and others have previously shown that etomoxir raises the cellular ratio of oxidized (GSSG) to reduced (GSH) glutathione (Merrill et al., 2002; Pike, Smift, Croteau, Ferrick, & Wu, 2010;

Tyra et al., 2012). Given the association of GSSG with the oxidative protein folding environment in the ER, we examined whether etomoxir could make the ER more oxidizing to proteins in the lumen. This was done by monitoring the oxidation of albumin, an endogenous hepatocyte ER client protein that forms 17 disulfide bonds (Peters & Davidson, 1982). We tested the resistance of albumin to reduction by treating cells with DTT, lysing them, and then modifying free sulfhydryls with PEG-maleimide, which retards migration upon SDS-PAGE (Winther & Thorpe, 2014). We found that treatment with DTT led to almost complete reduction of albumin in otherwise untreated cells, whereas etomoxir caused a significant increase in the preponderance of albumin that remained oxidized (Figure 4A). These results suggest that glutathione redox might mediate the protective effects of etomoxir.

To first determine if glutathione redox plays a role in the protective effects of etomoxir, we used 2-AAPA to inhibit glutathione reductase (Seefeldt et al., 2009; Zhao et al., 2009), an enzyme with both mitochondrial and cytosolic activity that couples NADPH oxidation to GSSG reduction. We predicted that this treatment would phenocopy the protective effects of etomoxir. As expected, treatment with 2-AAPA diminished cellular GSH levels and increased GSSG, thus significantly elevating the GSSG/GSH ratio, similar to the effects of etomoxir (Figure 4B). Also similarly to etomoxir, 2-AAPA treatment alleviated ER stress, as determined by attenuated *Xbp1* splicing (Figure 4C) and diminished upregulation of UPR target genes (Figure 4D). Therefore, inhibiting glutathione reduction phenocopies the protective effects of etomoxir.

We next tested the converse prediction: that promoting glutathione reduction would cause ER stress. We treated hepatocytes with auranofin, which inhibits redox-active selenoproteins, including thioredoxin reductases and, at the dose used here, glutathione peroxidases (Chaudiere & Tappel, 1984; Roberts & Shaw, 1998; Scarbrough et al., 2012). In the liver, glutathione peroxidase 1 is two orders of magnitude more abundant than any thioredoxin reductase (Lai, Kolippakkam, & Beretta, 2008). Therefore, presumably due to its effects on glutathione peroxidase, auranofin treatment elevated cellular GSH levels and

diminished GSSG, leading to a decrease in the GSSG/GSH ratio (Figure 5A). The relative resistance of albumin to reduction by DTT upon treatment with auranofin suggested that the ER environment was more reducing, whereas treatment with 2-AAPA made it more oxidizing (Figure 5B). Auranofin treatment alone was sufficient to induce a modest level of ER stress, seen in upregulation of UPR target genes in the absence of any other ER stress stimulus (Figure 5C). More importantly, auranofin completely blocked the ability of etomoxir to alleviate ER stress (Figure 5D). This epistatic relationship suggests that the protective effect of etomoxir requires glutathione oxidation.

TCA cycle activity links oxidative metabolism to ER homeostasis

Although glutathione reductase uses NADPH to reduce glutathione, β -oxidation yields NADH and FADH₂ but not NADPH. However, β -oxidation yields acetyl-CoA, which enters the TCA cycle by condensation with oxaloacetate for further oxidation. We therefore speculated that etomoxir might diminish production of NADPH from the TCA cycle, thereby resulting in elevated GSSG. In this model, it is not β -oxidation *per se* that is linked to ER homeostasis, but rather TCA cycle activity and the resultant production of NADPH. This model first predicted that etomoxir would diminish levels of both acetyl-CoA and NADPH—predictions which we then confirmed (Figure 6A, B). The model also predicted that impairing TCA-dependent NADPH production would elevate the GSSG/GSH ratio and alleviate ER stress, similarly to etomoxir treatment. Three TCA cycle isozymes generate NADPH: mitochondrial isocitrate dehydrogenase 2 (IDH2) and cytosolic IDH1 and malic enzyme (ME1). (The NADPH-producing mitochondrial ME3 is not expressed in the mouse liver). Using siRNAs, we specifically knocked down mRNA expression of each of these (Figure 6C). Knockdown of each gene diminished GSH levels, and knockdown of *Idh2* and *Me1* also increased GSSG levels, thus elevating the

GSSG/GSH ratio (Figure 6D). Further, ER stress was diminished by knockdown of each of the three genes (Figure 6E).

The Pyruvate Dehydrogenase complex oxidizes pyruvate produced by glycolysis to acetyl-CoA, and is a major control point of TCA cycle activity for carbohydrate metabolism (Gray, Tompkins, & Taylor, 2014). Pyruvate Dehydrogenase Kinases (PDKs) phosphorylate and inactivate this enzyme complex. To determine the effects of stimulating TCA cycle activity, we treated hepatocytes with dichloroacetate, which stimulates substrate entry into the TCA cycle by inhibiting PDKs (Constantin-Teodosiu, Simpson, & Greenhaff, 1999; C. Y. Wu et al., 2018). Dichloroacetate treatment substantially elevated the NADPH/NADP⁺ ratio (Figure 7A). In addition, dichloroacetate treatment alone caused ER stress to an extent that was nearly as robust as the bona fide ER stressor TM, as seen by upregulation of UPR target genes (Figure 7B) and splicing of *Xbp1* (Figure 7C). Therefore, these data provide direct evidence that mitochondrial oxidative catabolic activity causes ER stress.

Ablation of the Mitochondrial Pyruvate Carrier alleviates ER stress

Finally, we wished to test whether ER stress could be alleviated by non-pharmacological manipulation of TCA cycle activity. The Mitochondrial Pyruvate Carrier (MPC) is composed of two subunits, MPC1 and MPC2, and mediates mitochondrial import of pyruvate (Bricker et al., 2012; Herzig et al., 2012). Loss of either subunit eliminates MPC activity, and liver-specific ablation of the MPC diminishes liver damage and inflammation in mice on obesogenic diets (Gray et al., 2015; McCommis et al., 2015; McCommis et al., 2017). In the absence of MPC activity, import of pyruvate into the mitochondria is greatly diminished, meaning that less acetyl-CoA can be produced by pyruvate dehydrogenase and therefore that TCA cycle flux is diminished (Gray et al., 2015; Rauckhorst et al., 2017). Therefore, ablating the MPC should mimic the effects of etomoxir treatment since both ultimately result in diminished acetyl-CoA availability for oxidation in the TCA cycle. To test this prediction, we first examined the effects of

1 deleting MPC1 by CRISPR in C2C12 myocytes. We found that loss of MPC1 diminished the
2 NADPH/NADP⁺ ratio and elevated the GSSG/GSH ratio in these cells (Figure 8A, B).
3 Diminished UPR activation at both the protein (Figure 8C) and mRNA (Figure 8D) levels
4 indicated that MPC1-deficient cells were also remarkably resistant to ER stress induced by TM
5 or by the ER calcium-disrupting agent thapsigargin (TG). These findings were confirmed in
6 primary hepatocytes taken from mice lacking MPC2 in the liver (*Mpc2*^{LKO}), when compared to
7 cells from mice with an intact allele (*Mpc2*^{fl/fl}). In cells lacking MPC2, both the upregulation of
8 UPR target genes (Figure 8E) and *Xbp1* mRNA splicing (Figure 8F, G) were attenuated. These
9 findings provide direct genetic evidence that ER homeostasis is responsive to the availability of
10 TCA cycle substrates.
11

Discussion

Our results elucidate a pathway by which the ER senses metabolic activity (Figure 9). We propose that NADPH and GSSG convey TCA cycle status: decreasing the availability of acetyl-CoA—either from lipid or carbohydrate catabolism—dampens NADPH production and disfavors glutathione reduction, with the increased GSSG ultimately rendering the ER lumen more oxidizing and protecting ER homeostasis. Conversely, enhancing acetyl-CoA availability ultimately favors GSH over GSSG and causes ER stress. That these relationships were seen in hepatocytes, myocytes, and brown adipocytes, but were conspicuously absent in fibroblasts, hints that oxidative metabolism might represent an intrinsic challenge to ER functionality and might explain why feeding itself appears to be an ER stressor. Although there are many potential reasons that it might benefit the cell to tie ER protein processing to TCA cycle status—for example amino acid availability, ATP supply, lipid availability, etc.—the one we currently favor is the balancing of the cellular redox budget. Both the oxidation of nutrients in the mitochondria and the oxidation of nascent proteins in the ER generate toxic reactive oxygen species (ROS): in the mitochondria by electron leakage during oxidative phosphorylation (Murphy, 2009), and in the ER by catalysis of disulfide bond formation by ER oxidoreductase (ERO1) (Higa & Chevet, 2012). That inhibiting β -oxidation renders the ER more oxidizing suggests that the cell might be able to safely oxidize nutrients or nascent proteins, but not both simultaneously. As with the elucidation of any new pathway, our findings raise many further questions about how the pathway is initiated and regulated, and how it ultimately impacts cellular and organismal physiology.

That increased TCA activity elicits ER stress does not necessarily portend cellular dysfunction. It is possible that ER stress induced by enhanced metabolic activity might “prime” the ER by eliciting prophylactic ER stress. Brief exposures to stress are known to precondition cells against subsequent stresses (Rutkowski & Kaufman, 2007). Metabolic fluxes are, at least

outside the context of overnutrition and obesity, transient. Insulin action on the liver during feeding is known to promote both oxidative catabolism through PDH dephosphorylation (Moule & Denton, 1997; Wieland, Patzelt, & Loffler, 1972) and protein biogenesis through mTOR activation (Howell & Manning, 2011). UPR activation induced by enhanced TCA flux could enhance the functionality of the organelle in anticipation of the increase in protein synthesis—including synthesis of ER client proteins—that follows in the post-absorptive state.

While our data suggest that production of NADPH by the TCA cycle is a key event in our proposed pathway, it is not yet clear how NADPH production corresponds to actual activity of the cycle—given that metabolites can enter and exit the cycle at multiple points depending on nutritional conditions—nor how the cellular compartmentalization of NADPH production contributes to the transmission of the NADPH status to the ER. The NADPH/NADP⁺ ratio is regulated by nutritional state, exercise, diet, and circadian rhythms (Bradshaw, 2019; Ying, 2008). Only a portion of this NADPH is generated by the TCA cycle; other contributors include the pentose phosphate pathway of glucose metabolism and one-carbon folate metabolism, both of which are cytosolic. The fractional contribution of these pathways to total NADPH pools varies by cell type (J. Fan et al., 2014). In addition, we found here that inhibition of either the mitochondrial (IDH2) or cytosolic (IDH1 and ME1) NADPH-producing enzymes diminishes ER stress, and we have found previously that inhibition of the pentose phosphate pathway does likewise (Tyra et al., 2012). These findings imply that inhibiting NADPH production from any source protects ER function. How, then, are the cytosolic sources of NADPH linked to glutathione redox, since glutathione reductase contains a mitochondrial targeting signal? (Cytosolic glutathione reductase activity has been reported, possibly arising from translation initiation at a downstream start codon or inefficient mitochondrial targeting; (Kelner & Montoya, 2000)) Nicotinamide nucleotides are not thought to be competent for *trans*-mitochondrial transport (Lewis et al., 2014). One possibility is that diminishment of NADPH also inhibits thioredoxin reductase in the cytosol, and that cytosolic glutathione becomes more oxidized

because it must compensate in the reduction of reactive oxygen species for the shortage of reduced thioredoxin. An alternate possibility is that cytosolic NADPH manipulation ultimately affects matrix NADPH status through metabolite shuttles, which are used to convey reducing equivalents across the mitochondrial membranes by coupling mitochondrial redox reactions with their cytosolic counterparts (for example, using the transport of citrate to couple IDH2 and IDH1) (Taylor, 2017). A next step will be to determine how TCA activity affects compartmentalization of NADPH arising from various sources.

Our results are surprising also in their implication that oxidized glutathione has a beneficial role in ER function. It is well-established that reducing agents elicit ER stress and activate the UPR, which speaks to the importance of the ER oxidative environment in the protein folding process. However, reducing equivalents are needed as well in order to activate ERO1 (Kim, Sideris, Sevier, & Kaiser, 2012) and to reduce disulfide isomerases so that they can catalyze reduction of improper disulfide bonds (Schwaller, Wilkinson, & Gilbert, 2003). Disruption of this capacity impairs the secretory pathway transport of model proteins with non-sequential disulfide bonds that must undergo such isomerization to avoid being trapped in non-native conformations (Poet et al., 2017). How oxidation and reduction are balanced within the ER lumen is not well-understood, and that understanding is confounded by the observations that neither ablation of both ERO1 isoforms (Zito, Chin, Blais, Harding, & Ron, 2010) nor apparent depletion of total ER glutathione (Tsunoda et al., 2014) appreciably impacts ER protein oxidation capacity or stress sensitivity except for specialized substrates. It could be that ER oxidative capacity and the relative need for oxidized glutathione varies by cell type.

An important question for future work is how elevated GSSG in the mitochondria and/or cytosol is transmitted to the ER. Does a change in the total cellular GSSG/GSH ratio ultimately change that ratio in the ER, or does GSSG exert its effects on the ER environment indirectly? To date, no mechanism for import of GSSG into the ER has been described. One possibility is that diminishing the cytosolic level of GSH suppresses its import into the ER, thereby elevating

the ER GSSG/GSH ratio as well. However, given that the ratio of GSH to GSSG in the cytosol is approximately 100:1, it seems unlikely that cytosolic GSH is limiting in this way. Alternatively, perhaps sites of ER-mitochondrial contact facilitate spatially restricted exchange of glutathione as they do for other metabolites such as calcium and reactive oxygen species (Joseph, Booth, Young, & Hajnoczky, 2019). Ultimately, determining how elevated GSSG outside the ER leads to a more oxidizing ER lumen will require compartment-specific monitoring and manipulation of the glutathione redox state.

Our results also raise the question of what aspect(s) of ER functional capacity are altered by TCA cycle activity and glutathione redox. Activation of the UPR generally serves as a readout for ER stress, but it is not clear what diminished ER stress implies in terms of an actual accumulation of unfolded proteins, particularly because some stressors, such as palmitate loading, activate the UPR apparently independent of the protein folding process (Volmer et al., 2013). There are relatively few approaches for disentangling truly dysfunctional protein processing from the criteria by which the UPR perceives ER stress, which are still not well understood. For example, one might expect a dysfunctional ER to secrete proteins more slowly; on the other hand, perhaps enhanced ER retention time and chaperone association of client proteins facilitates proper protein folding under stressful conditions. In any case, we have yet to find any robust differences in nascent ER protein trafficking, processing, or aggregation in cells in which TCA activity is manipulated other than apparently enhanced clearance of NHK (Figure 4D). It remains to be determined whether this effect extends to other substrates and cell types. One intriguing—but speculative—possibility is that GSSG might paradoxically protect the ER by exacerbating protein misfolding, if doing so renders misfolded client proteins more readily recognized and either refolded or cleared rather than futilely engaged with the ER quality control machinery. Whatever the case, we infer that inhibiting TCA activity actually protects ER homeostasis in part because of its effects on ER ultrastructure. In principle, a hyperoxidizing ER might simply blunt the UPR rather than improve ER function, since activation of at least the UPR

sensors ATF6 and IRE1 can be inhibited by oxidation (Nadanaka, Okada, Yoshida, & Mori, 2007; J. M. Wang et al., 2018). However, there is no evidence that enforcing reduction of these sensors is sufficient for their activation, meaning there would then be no reason to expect DCA to cause ER stress on its own. Moving forward, the redox status and activity of BiP will be of particular interest, given its roles in protein folding, ERAD, protein translocation, and UPR signaling (Pobre, Poet, & Hendershot, 2019), and the fact that oxidative modifications can alter the nature of its associations with nascent proteins (J. Wang, Pareja, Kaiser, & Sevier, 2014) and with the translocation channel (Ponsero et al., 2017). Perhaps manipulations of TCA activity will provide a new tool for understanding the relationships between ER oxidation, ER stress, and UPR activation.

Although our findings extend to other cell types beyond hepatocytes, they seem particularly relevant to liver disease. Obesity is the leading cause of non-alcoholic fatty liver disease (NAFLD) which, along with its downstream consequences—steatohepatitis, cirrhosis, and liver cancer—is the most common liver disease in the world (Araujo, Rosso, Bedogni, Tiribelli, & Bellentani, 2018). ER stress and dysregulation of the UPR are associated with NAFLD/NASH in humans (Gonzalez-Rodriguez et al., 2014; Lake et al., 2014; Lebeaupin et al., 2015; Lebeaupin et al., 2018), and in mice on NASH-promoting diets (Charlton et al., 2011; Rahman et al., 2007). ER stress might promote NASH by aggravating diet-induced steatosis (Lee, Scapa, Cohen, & Glimcher, 2008; Oyadomari et al., 2008; Rutkowski et al., 2008), and ER stress has also been shown to directly activate inflammatory signaling cascades (Lebeaupin et al., 2018; Özcan et al., 2004; Willy, Young, Stevens, Masuoka, & Wek, 2015) and to promote hepatocyte cell death (Iracheta-Vellve et al., 2016; Olivares & Henkel, 2015). NAFLD is associated with increased TCA flux in the liver (Rauckhorst et al., 2017; Satapati et al., 2012; Sunny, Parks, Browning, & Burgess, 2011), raising the question of whether NAFLD progression is accelerated by ER stress arising from this increased flux, and whether diminished hepatic TCA flux in mice lacking MPC activity protects the liver at least in

part by alleviating or preventing ER stress. On one hand, oxidative stress is recognized to contribute to NAFLD progression, and glutathione is known to protect against NAFLD progression (Liu, Baker, Baker, & Zhu, 2015), although most of those studies have examined glutathione synthesis rather than glutathione redox. Conversely, our observation of elevated GSSG in cells lacking MPC1 (Figure 8B) suggests that, at a minimum, GSSG is not incompatible with diminished sensitivity to NAFLD. Whether TCA-dependent NADPH production contributes to NAFLD is not known. IDH1 and IDH2 function *in vivo* has mostly been examined in the context of transforming mutations in gliomas that change IDH activity. Both IDH1 and IDH2 are widely expressed, and to our knowledge no liver-specific deletion of either has been created. Thus, the role of the axis identified here in NAFLD has not been directly tested.

In conclusion, we have identified a novel NADPH- and glutathione-dependent pathway through which TCA cycle activity impacts ER homeostasis. We expect that this pathway will be relevant to the physiology and pathophysiology of liver, muscle, adipose, and other highly metabolically active cell types.

Materials and Methods

Cell culture and drug treatments

Primary hepatocytes were isolated from mice of both sexes between 6-12 weeks of age. Mice were anesthetized with isoflurane for the duration of the isolation. The liver was perfused through the portal vein with freshly prepared Perfusion Medium followed by digestion with Liver Digest Medium. Media formulas were as follows: Liver Perfusion Medium: HBSS, no calcium, no magnesium, no phenol red (Life Technologies, Carlsbad, CA), 0.5 mM EDTA, 0.5 mM EGTA, 25 mM HEPES, and penicillin-streptomycin (10,000 U/mL); Liver Digest Medium: HBSS, calcium, magnesium, no phenol red, 25 mM HEPES, penicillin-streptomycin (10,000 U/mL), 3.6 mg Trypsin Inhibitor (Sigma, St. Louis, MO), 25 mg Collagenase Type IV (210 U/mg) (Worthington Biochemical Corp., Lakewood, NJ). Flow rates were 4 ml/min for 5 min for perfusion, and 8 min for digestion. The liver was quickly excised, placed in cold Wash Medium (DMEM, 10 mM HEPES, 5% FBS, 100 µg/mL penicillin-streptomycin), dispersed by tearing Glisson's capsule, and filtered through a sterile 70 µm cell strainer. Hepatocyte suspensions were centrifuged at 500 rpm for 3 min and resuspended in 30 mL of Wash Medium with 35% Percoll. Cells were centrifuged for 5 min at 1000 rpm, followed by resuspension in Wash Medium for a final wash with centrifugation for 3 min at 500 rpm. Viable hepatocytes were resuspended in Hepatocyte Medium (William's E, 5% FBS, 10 nM insulin, 100 nM dexamethasone, 100 nM triiodothyronine, and 100 µg/mL penicillin-streptomycin), or, for *Mpc^{fl/fl}* and liver-specific knockout (*Mpc2^{LKO}*) hepatocytes, in high glucose DMEM, 10% FBS, penicillin-streptomycin, and 0.5 µg/ml amphotericin B, and plated on collagen-coated tissue culture plates. Media was changed 4 h after plating to remove any non-adherent cells. MPC2-deficient hepatocytes were isolated from *Mpc2^{fl/fl}* animals bred into the Albumin-CRE line. MPC1-deficient C2C12 cells were generated by CRISPR and cultured as described (Oonthonpan, Rauckhorst, Gray, Boutron, & Taylor,

2019). gRNA sequences were 5'-GCGCTCCTACCGGTGCCCCGA-3' and 5'-GCCAACGGCACGGCCATGGC-3'.

Primary (pBAT) and immortalized (iBAT) brown adipocytes were isolated and cultured as described (Markan et al., 2014). Primary mouse embryonic fibroblasts were isolated and cultured as described (Scheuner et al., 2001). Drug treatments used the times and concentrations indicated in the figure legends. TM, auranofin, and DCA were from Millipore Sigma; etomoxir, 2-AAPA, and MG-132 from Cayman Chemical; stocks of each of these were stored at -20°C in DMSO. PA and OA (Millipore Sigma) were diluted stepwise in DMSO to 200 mM, and then to 100 mM in 10% fatty acid-free BSA (Millipore Sigma), followed by incubation at 40°C for 90 min with gentle agitation. Doxycycline (Millipore Sigma) stocks were stored at -20°C in water.

Adenovirus experiments

A cDNA encoding the NHK allele of α 1-antitrypsin was cloned into an adenoviral shuttle vector downstream of a TRE-Tight promoter, and the shuttle was recombined with a backbone expressing rtTA under the RSV promoter. Virus was purified by the University of Iowa Viral Vector Core. Primary hepatocytes were infected with Ad-TetOn-NHK or Ad-CMV-eGFP at a multiplicity of infection of 1:1. Infection began when media was changed 4 h after cells were plated. Cells were incubated with adenovirus for at least 12 h prior to addition of doxycycline or other treatments. NHK expression was induced with 500 ng/mL doxycycline in fresh media at the time of treatment.

dsiRNA knockdown experiments

Primary hepatocytes were cultured overnight prior to transfection. Hepatocytes were transfected with 2.75 μ M dsiRNA (Integrated DNA Technologies, Coralville, IA) in nuclease-free duplex

buffer using the Viromer® Blue transfection kit (Origene) following the manufacturer's protocol. A non-targeting dsRNA was used as a control and two dsRNAs for each gene of interest (*Idh1*, *Idh2*, and *Me1*) were used. Hepatocytes were transfected with dsRNA for 24 h before experimental treatments. Targeting sequences were as follows: *Idh1*: GUACAACCAGGAUAAGUCAAUUGAA, GUUGAAGAAUUCAAGUUGAAACAAA; *Idh2*: AUUUUAUUAUUGCUCUGGAAUCACATG, AUCUUUGACAAGCACUAUAAGACTG; *Me1*: GCCAUUGUUCAAAAGAUAAAACCAA, ACCUUUCUAUCAGAUUAUAAAAUAT; non-targeting control: CGUUAUAUCGCGUAUAAUACGCGUAT

Biochemical Assays

Levels of total (GSx), oxidized (GSSG), and reduced (GSH) glutathione were measured using a Glutathione Fluorometric Assay Kit (Biovision, Milpitas, CA) following the manufacturer's protocol. NADPH levels were measured using an NADP/NADPH Colorimetric Quantification Kit, and acetyl-CoA levels using the PicoProbe™ Acetyl-CoA Fluorometric Assay Kit (Biovision) following the manufacturer's protocols, with the addition of 6N perchloric acid to precipitate proteins.

RNA and Protein Analyses

Protein lysates were processed for immunoblot as described (Rutkowski et al. 2006). Primary antibodies were: CHOP (Santa Cruz sc-7351 or Proteintech 15204-1-AP), BiP (BD Biosciences 610978), PeIF2 α (Invitrogen 44-728G), MPC1 (Cell Signaling Technology 14462), calnexin (loading control; Enzo ADI-SPA-865), actin (loading control; MP Biomedicals 691001), α 1-antitrypsin (Dako A0012). The oxidative state of the ER was measured by incorporation of PEG-maleimide (mm(PEG)₂₄) (ThermoFisher) as described (Tyra et al., 2012). Samples were run on Tris-tricine or Tris-HCl SDS-PAGE gels and transferred to 0.45 μ m Immobilon-P Polyvinylidene

Fluoride (PVDF) (Millipore) for Western blotting using ECL Prime substrate (GE Healthcare). qRT-PCR, including primer validation by standard curve and melt curve analysis, was as described (Rutkowski et al., 2006). Briefly, RNA was isolated following the standard Trizol protocol and RNA concentrations were obtained using the Qubit RNA Broad Range kit. Concentrations were normalized, and cDNA was synthesized using 400 ng RNA with PrimeScript RT Master Mix (Takara). PCR reactions were performed using TB Green Premix Ex Taq (Takara) in a CFX96 cycler (Bio-Rad). Oligonucleotide sequences are listed in Table 1. Two loading controls were included in each experiment (*Btf3* and *Ppia*). Conventional RT-PCR was performed to assess splicing of *Xbp1* as described (Tyra et al., 2012).

Table 1: Oligonucleotide sequences

Gene	Sequences
<i>Btf3</i> (loading)	Fwd: CCAGTTACAAGAAAGGCTGCT Rev: CTTCAACAGCTTGTCCGCT
<i>Ppia</i> (loading)	Fwd: AGCACTGGAGAGAAAGGATT Rev: ATTATGGCGTGTAAGTCACCA
<i>Bip</i>	Fwd: CATGGTTCTCACTAAAATGAAAGG Rev: GCTGGTACAGTAACAACCTG
<i>Chop</i>	Fwd: CTGCCTTTACCTTGGAGAC Rev: CGTTTCCTGGGGATGAGATA
<i>Derl3</i>	Fwd: TGGGATTTCGGCTTCTTTTC Rev: GAACCCTCCTCCAGCAT
<i>Edem</i>	Fwd: CGATCTGGCGCATGTAGATG Rev: AAGTCTAGGAGCTCAGAGTCATTAA
<i>Dnajc3</i>	Fwd: TCCTGGTGGACCTGCAGTACG Rev: CTGCGAGTAATTTCTTCCCC
<i>Xbp1</i>	Fwd: TTGTGGTTGAGAACCAGG Rev: TCCATGGGAAGATGTTCTGG

Transmission Electron Microscopy

Primary hepatocytes were cultured on collagen-coated glass coverslips and fixed in 2.5% glutaraldehyde prior to imaging by the University of Iowa Central Microscopy Research Facility. TEM images were scored blindly and binned into categories based on ER morphology: Normal (<25% dilated), Intermediate (25-75% dilated), or Severe (>75% dilated).

Statistical Analyses

Continuous variables were reported as the mean $\pm$ standard deviation and were analyzed using the two-tailed Student's t-test with Benjamini-Hochberg post-hoc correction for multiple comparisons. For qRT-PCR, significance was calculated prior to transformation of C_t values out of the log phase. A post-correction alpha of 0.05 was used to determine statistical significance.

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Author Contributions

ERG and DTR conceived and designed the experiments. ERG, KSM, MM, and AQK-M performed the experiments. ERG, KSM, MJP, BNF, EBT, and DTR interpreted the data. ERG and DTR wrote the manuscript. All authors edited and approved the manuscript.

Competing Interests

BNF is a shareholder and member of the Scientific Advisory Board for Cirius Therapeutics. KSM received research support from Cirius Therapeutics between 2017-2019. EBT receives research grant funding from MPC-related research administered through the University of Iowa from Cirius Therapeutics and Poxel SA.

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Figure Legends

Figure 1: Inhibiting β -oxidation attenuates UPR activity in metabolically active cells and protects against different ER stressors. (A-C) Primary hepatocytes were treated with vehicle or 250 ng/mL tunicamycin (TM) in the presence or absence of 25 μ g/mL etomoxir (ET) for 8 h. Activation of the UPR was assessed by qRT-PCR of a sampling of UPR target genes (A), splicing of *Xbp1* by conventional RT-PCR (B), and CHOP protein expression by immunoblot (C). In (B) and (C), and elsewhere, each lane represents an independently treated well. Loading control for immunoblot was calnexin, which does not change in response to TM or ET. **(D, E)** Same as except using C2C12 myoblasts (D) or primary brown adipocytes (E). **(F)** mRNA expression of UPR markers was assessed by qRT-PCR in primary hepatocytes treated with 200 μ M palmitate (PA) or oleate (OA) and 25 μ g/mL ET for 8 h. For this and all subsequent figures, error bars represent means $\pm$ S.D.M. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ by two-tailed t-test and Benjamini-Hochberg adjustment for multiple comparisons. p-values between 0.05 and 0.1 are given as values. NS, $p > 0.1$.

Figure 2: Inhibiting β -oxidation improves ER ultrastructure. (A) Primary hepatocytes were treated with vehicle, 250 ng/mL TM, 25 μ g/mL ET, or TM and ET for 8 h and fixed in 2.5% glutaraldehyde. Fixed cells were imaged by transmission electron microscopy. Inset panels are indicated. Blue arrowheads represent areas of structurally normal ER, while orange arrowhead represent dysmorphic ER. **(B)** Images were scored blindly and binned based on the percentage of “dilated” ER in the image. Normal (blue): $< 25\%$, Moderate (gray): $25-75\%$, and Severe (orange): $> 75\%$. $n = 9-10$ cell images per group. **(C)** Primary hepatocytes were treated with 25 μ g/mL ET in the presence of 0.5, 2, or 10 μ g/mL TM for 8 h, and expression of *Bip* and *Chop* was detected by qRT-PCR.

Figure 3: Inhibiting β -oxidation attenuates ER stress induced by overexpression of a constitutively misfolded protein. (A) Primary hepatocytes were infected with Ad-CMV-eGFP (control) or Ad-TetOn-NHK at an MOI of 1:1. NHK expression was induced with 500 ng/mL doxycycline (Dox) in the presence or absence of 25 μ g/mL ET. mRNA expression of UPR markers was measured by qRT-PCR. (B) Primary hepatocytes infected with Ad-TetOn-NHK and treated with Dox and/or ET for 8 h were fixed in 2.5% glutaraldehyde for TEM. Orange arrowheads in insets denote dysmorphic ER. (C) Images were scored as described in Figure 2; n = 10 cell images per group. (D) Primary hepatocytes infected with Ad-TetOn-NHK were treated with Dox and ET as in (A) for 4 or 8 h in the presence or absence of 5 μ M MG-132. NHK expression was analyzed by immunoblot. Loading control was calnexin.

Figure 4: Inhibiting Glutathione Reductase attenuates ER stress. (A) Primary hepatocytes were treated with 25 μ g/mL ET for 4 h followed by addition of 10 mM DTT for 0, 30, or 60 min. Protein lysates were treated with 4 μ M mm(PEG)₂₄ prior to SDS-PAGE and immunoblotting to detect endogenous albumin. The percentage of oxidized albumin is given below each group, with statistical comparison between ET-treated and non-treated cells. (B) Primary hepatocytes were treated with 25 μ g/mL ET or 25 μ M 2-AAPA for 8 h. Levels of total (GSx_{tot}), reduced (GSH), and oxidized (GSSG) glutathione, and the ratio of GSSG to GSH were measured fluorometrically and expressed relative to untreated cells. (C) Splicing of *Xbp1* mRNA was measured by conventional RT-PCR, and (D) mRNA expression of UPR markers was measured by qRT-PCR in cells treated for 8 h with TM and/or 2-AAPA.

Figure 5: Glutathione oxidation is required for inhibition of β -oxidation to alleviate ER stress. (A) Levels of total, reduced, and oxidized glutathione were measured in primary

hepatocytes treated with 10 μ M auranofin (AuF) for 8 h. Glutathione was quantified as in Figure 4. **(B)** Hepatocytes were treated with 2-AAPA or AuF for 4 h prior to DTT and mm(PEG)₂₄ as in Figure 4. **(C)** mRNA expression of UPR markers after treatment with AuF for 8h was quantified. **(D)** Hepatocytes were treated with TM, ET, and AuF as indicated. mRNA expression of UPR markers was measured by qRT-PCR.

Figure 6: Diminishing TCA-dependent production of NADPH alleviates ER stress. (A)

Acetyl-CoA levels or **(B)** the NADPH/NADP⁺ ratio were measured in primary hepatocytes treated with ET for 8 h and is expressed relative to untreated cells. **(C)** *Idh1*, *Idh2*, and *Me1* were knocked down with dsRNA prior to treatment with TM for 8 h. A non-targeting dsRNA was used as a control. Relative mRNA expression of *Idh1*, *Idh2*, and *Me1* was measured by qRT-PCR. **(D)** Glutathione was quantified in cells with knocked down *Idh1*, *Idh2*, or *Me1* (first oligo pair from Figure 6C in each case). **(E)** Expression of UPR target genes was assessed in cells from (C, D).

Figure 7: Stimulating TCA substrate entry causes ER stress. (A-C) Primary hepatocytes were treated with 4 μ M dichloroacetate (DCA) or TM for 8 h. The NADPH/NADP⁺ ratio (A), UPR target gene expression (C), or *Xbp1* mRNA splicing (B) were analyzed as in previous figures.

Figure 8: Attenuation of TCA flux by deletion of the Mitochondrial Pyruvate Carrier

alleviates ER stress. (A, B) The NADPH/NADP⁺ ratio (A), and the GSSG/GSH ratio (B) measured as above in parental wild-type and *Mpc1*^{-/-} C2C12 myoblasts. **(C)** Expression of BiP, CHOP, phosphorylated eIF2 α , and MPC1 was analyzed by immunoblot in wild-type and *Mpc1*^{-/-} C2C12 myocytes treated with 100 ng/mL TM, 1 μ g/mL TM, or 100 nM thapsigargin (TG). Loading control was actin. **(D)** Relative mRNA expression of UPR markers from cells treated similarly to (C) was quantified by qRT-PCR. **(E)** Primary hepatocytes from *Mpc2*^{fl/fl} and *Mpc2*^{LKO}

mice were treated with 250 ng/mL TM, and gene expression was measured by qRT-PCR. The *Mpc2*^{-/-} untreated and w.t. TM-treated were reversed by cut/paste to keep ordering consistent with the rest of the data in the paper but the image was otherwise unmodified. **(F)** Splicing of *Xbp1* in TM-treated hepatocytes from *Mpc2*^{fl/fl} or *Mpc2*^{LKO} mice was assessed by conventional RT-PCR. (G) *Xbp1* splicing was quantified from 8 independent samples (4 from cells taken from a male mouse, 4 from cells taken from a female mouse).

Figure 9: Proposed model describing the relationship between TCA cycle activity and ER homeostasis. See Discussion for details.

Figure S1. ET treatment does not diminish ER stress in primary mouse embryonic fibroblasts. Primary mouse embryonic fibroblasts treated were treated with 25 μ g/ml ET and 250 ng/ml TM as indicated for 8h. mRNA expression was assessed by qRT-PCR as in Figure 1.

A **B** **C**

Figure S2. ET treatment does not affect protein synthesis. Primary hepatocytes treated were treated with 25 μ g/ml ET and 250 ng/ml TM as indicated in media with 10 percent the normal amount of methionine and cysteine, and EasyTag Express³⁵S (Perkin Elmer NEG772002MC) was added to a final concentration of 50 μ Ci/ml. After the indicated times, cell lysates were collected in 1% SDS 100 mM Tris, pH 8.8, denatured by heating, spotted onto gridded 3MM filter paper, and air dried. Filter was then precipitated by serial incubations in ice-cold 10% trichloroacetic acid (TCA) for 60 min., 5% TCA at room temperature for 5 min., 5% TCA preheated to 75° for 5 min., 5% TCA at room temperature for 5 min., and finally 2:1 ethanol:ether preheated to 37° for 5 min. Filter was then air dried, sliced into individual samples, and immersed in scintillation cocktail, and counted. n = 6 samples per condition. The modest effect of this dose of TM on protein synthesis is consistent with previous findings (Rutkowski et al., 2006)

A **B** **C** **D**

A NADPH/NADP⁺ **C** **B**