The endoplasmic reticulum (ER) regulates cellular protein and lipid biosynthesis. ER dysfunction leads to protein misfolding and the unfolded protein response (UPR), which limits protein synthesis to prevent cytotoxicity. Chronic ER stress in skeletal muscle is a unifying mechanism linking lipotoxicity to metabolic disease. Unidentified signals from cells undergoing ER stress propagate paracrine and systemic UPR activation. Here, we induce ER stress and lipotoxicity in myotubes. We observe ER stress-inducing lipid cell non-autonomous signal(s). Lipidomics identifies that palmitate-induced cell stress induces long-chain ceramide 40:1 and 42:1 secretion. Ceramide synthesis through the ceramide synthase 2 de novo pathway is regulated by UPR kinase Perk. Inactivation of CerS2 in mice reduces systemic and muscle ceramide signals and muscle UPR activation. The ceramides are packaged into extracellular vesicles, secreted and induce UPR activation in naïve myotubes through dihydropyrophosphate accumulation. This study furthers our understanding of ER stress by identifying UPR-inducing cell non-autonomous signals.
The endoplasmic reticulum (ER) is a cellular organelle with a key role in both protein synthesis and folding, and lipid biosynthesis. Disruption to ER function results in organellar stress and the accumulation of misfolded proteins. Unchecked accretion of unfolded proteins can result in cell death. ER stress has been implicated in a wide range of pathologies including aging, certain cancers and metabolic diseases including dyslipidaemia, obesity and type 2 diabetes (T2D). Metabolic diseases including obesity and T2D are characterised by elevated plasma concentrations of saturated fatty acids, particularly palmitate. These lipid species are thought to induce metabolic dysfunction in insulin-sensitive tissues such as liver, adipose tissue and skeletal muscle through effects termed lipotoxicity. Skeletal muscle is a key regulator of systemic metabolic homeostasis.

Recently, ER stress has emerged as a potential unifying mechanism linking lipotoxicity to metabolic dysfunction in metabolic disease. Lipotoxicity-induced ER stress in skeletal muscle causes metabolic dysfunction and contributes to the development of metabolic disease. However, the mechanisms linking palmitate with the induction of ER stress remain unclear. The cell has adaptive responses to maintain protein homeostasis and survival during ER stress. ER stress triggers the unfolded protein response (UPR), a protective signalling cascade. The UPR is composed of three arms, mediated by the kinases protein kinase R-like endoplasmic reticulum kinase (Perk; encoded byEIF2AK3) and inositol-requiring enzyme 1 (Ire1; encoded by ERMN), and the transmembrane transcription factor, activating transcription factor 6 (Atf6). Signalling through these proteins increases cellular protein chaperones and disulphide isomerases, activates protein degradation pathways, and inhibits protein translation. These protective responses reduce protein load on the ER and improve protein folding. The intracellular mechanisms of ER stress and regulation of the UPR are well understood. However, recent studies suggest that UPR signalling can be propagated in both a paracrine manner and systemically by cell non-autonomous signals. The nature of these extracellular paracrine and endocrine signals remains unknown.

In this study, we hypothesise that cell non-autonomous signalling may be important in the communication of lipotoxicity-induced ER stress in skeletal muscle. We demonstrate the presence of lipotoxicity-induced long-chain ceramides secreted in response to lipotoxicity, which function as a paracrine signal to activate the UPR in myotubes. We describe the mechanism through which the long-chain ceramide signal is linked to UPR activation, synthesised, transported extracellularly and initiates UPR activation. This study identifies the nature of a cell non-autonomous signal capable of propagating ER stress. We develop our understanding of the control of UPR signalling beyond a cell-autonomous state, with clear implications in the development and propagation of metabolic disease.

**Results**

**Myotubes secrete a non-autonomous ER stress-inducing lipid signal in response to chronic lipotoxicity.** To confirm UPR induction in response to chronic lipotoxicity in muscle cells, murine C2C12 myotubes were exposed to physiological concentrations of palmitate for the final 6 days of an 8 day differentiation protocol. Cells were treated with either 100 or 200 µM palmitate conjugated to fatty-acid-free bovine serum albumin (BSA), or BSA vehicle control. Concentrations of 100 µM and 200 µM palmitate reflect the physiological palmitate plasma concentration range in mice (100–300 µM) and chronic treatment (6 days) models continual palmitate exposure in vivo. Palmitate-treatment increased expression of the UPR genes activating transcription factor 3 (Atf3), activating transcription factor 4 (Atf4), Heat Shock Protein Family A (Hsps70) Member 5 (Hspa5) and ER Degradation Enhancing Alpha-Mannosidase Like Protein 1 (Edem1) (Supplementary Fig. 1a).

To assess a role for cell non-autonomous secreted signals during lipotoxicity-induced cell stress, C2C12 myotubes were exposed to 200 µM palmitate or the BSA vehicle during differentiation to induce ER stress. Culture media was then changed to serum-free and palmitate-free media for 24 h. Compounds accumulating in the media would therefore originate from the myotubes. Lipidomic profiling confirmed that the conditioned media was exogenous palmitate-free (Supplementary Fig. 1b). This conditioned media was transferred to naïve differentiated C2C12 myotubes (Fig. 1a). Conditioned media from palmitate-treated C2C12 myotubes increased expression of UPR genes Atf4, Hspa5 and Edem1 (Fig. 1b). To determine the relevance of this phenotype in a human model, primary human skeletal muscle cells (HSkMCs) were exposed to 50 or 100 µM palmitate, or the BSA vehicle during a 6 day differentiation. Concentrations of 50 µM and 100 µM palmitate reflect the physiological palmitate plasma concentration range in humans (50–150 µM) and chronic treatment (6 days) models continual palmitate exposure in vivo. Palmitate-treatment increased the expression of UPR genes ATF3, ATF4, HSPA5 and EDEM1 (Supplementary Fig. 1c). Conditioned serum- and palmitate-free media collected from HSkMCs treated with 100 µM palmitate or BSA was transferred to naïve HSkMCs. Conditioned media from palmitate-treated HSkMCs increased UPR gene expression in human myocytes (Fig. 1c). These data suggest the presence of cell non-autonomous UPR-inducing signal secreted by both murine and human myocytes in response to chronic lipotoxicity.

Next we sought to determine the nature of the UPR-inducing secreted signal. Conditioned media was boiled to denature proteins prior to transfer to naïve myotubes. Boiled conditioned media from palmitate-treated myotubes retained UPR-inducing properties, suggesting that there are non-protein secreted signal(s) (Fig. 1d). The UPR-inducing signal was preserved in boiled media conditioned from palmitate-treated cells with no evidence of altered UPR-inducing activity in boiled control media. However, boiling denaturation has the potential to destabilise small molecules. Therefore, to further characterise the physicochemical nature of the UPR-inducing signal, molecular weight cut-off dialysis of 1 kDa was used to dialyse conditioned media from control and palmitate-treated myocytes. Dialysate (containing the dialysed molecules of 1 kDa and below) was dried down under N₂, reconstituted in serum-free media and transferred to naïve myocytes. The UPR-inducing signal in conditioned media from palmitate-treated myocytes was observed in the dialysate reconstituted media (Supplementary Fig. 1d). These data suggest the signal(s) is a small molecule of 1 kDa or less.

We hypothesised that the UPR-inducing signal may be a bioactive lipid. The lipid-containing organic component and metabolite containing aqueous component of the conditioned media from both control and palmitate-treated myocytes was extracted using protein-denaturing organic-aqueous solvent partition. The lipids, isolated from the organic fractions of conditioned media from both control and palmitate-treated myocytes, were reconstituted in serum-free media and transferred on to naïve differentiated myotubes. The organic lipid fraction of media from palmitate-treated myotubes increased the expression of UPR target genes compared to the organic lipid fraction of media from control myotubes (Fig. 1e). Next, we examined whether aqueous soluble metabolites may represent additional UPR-inducing signals. The metabolites isolated from the aqueous
fractions of conditioned media from both control and palmitate-treated myocytes were reconstituted in serum-free media and transferred to naïve myocytes. The aqueous metabolite fraction of media from palmitate-treated myotubes also increased the expression of UPR target genes compared to the aqueous fraction of media from control myotubes (Supplementary Fig. 1e). These data suggest that both a bioactive lipid(s) and metabolite(s) may function as cell non-autonomous UPR-inducing secreted signals.

Lipotoxicity-induced UPR-activating long-chain ceramide secretion from myotubes. To identify the cell non-autonomous UPR-inducing lipid species we performed lipidomic profiling of serum and palmitate-free conditioned media from palmitate-treated myotubes. Multivariate analysis of the media lipidomic data identified lipids species discriminating 100 μM and 200 μM treated myocyte media from control (Supplementary Fig. 2). Palmitate increased media concentrations of diacylglycerides (DG), lysophosphatidylcholines (LPC) and ceramides (Fig. 2a; Fig. 1 Myotubes secrete a cell non-autonomous unfolded protein response (UPR) -inducing lipid signal in response to lipotoxicity. a C2C12 myotubes were treated with 200 μM palmitate or bovine serum albumin (BSA) vehicle, serum-free media was conditioned on these cells (24 h) and transferred to naïve myocytes. b Activating transcription factor 3 (Atf3), activating transcription factor 4 (Atf4), Heat Shock Protein Family A (Hspa5) and ER Degradation Enhancing Alpha-Mannosidase Like Protein 1 (Edem1) UPR gene expression in C2C12 myotubes receiving conditioned media from myotubes treated with 200 μM palmitate (grey) or BSA (red) (n = 4; two-tailed Student’s t test; Atf4 P = 0.00009, Hspa5 P = 0.005, Edem1 P = 0.000024). c UPR gene expression in human skeletal muscle cells (HSkMCs) receiving conditioned media from HSkMCs treated with 100 μM palmitate (grey) or BSA (red) (n = 3, two-tailed Student’s t test; ATF3 P = 0.005, ATF4 P = 0.0017, Hspa5 P = 0.0088). d UPR gene expression in myotubes receiving boiled media conditioned on C2C12 myotubes treated with 200 μM palmitate or BSA (control = red; control boiled = grey; 200 μM palmitate = blue; 200 μM palmitate boiled = green; n = 3; one-way ANOVA; control media vs 200 μM palmitate media, Atf3 P = 0.017, Atf4 P = 0.005; control vs 200 μM palmitate boiled, Atf3 P = 0.03, Atf4 P = 0.0065; control boiled vs 200 μM palmitate, Atf3 P = 0.016, Atf4 P = 0.005; control boiled vs 200 μM palmitate boiled, Atf3 P = 0.027, Atf4 P = 0.007). e UPR gene expression in myotubes receiving reconstituted lipid extract isolated from media conditioned on C2C12 myotubes treated with 200 μM palmitate (grey) or BSA (red) (n = 4; two-tailed Student’s t test: Atf3 P = 0.05, Atf4 P = 0.0026, Hspa5 P = 0.0097, Edem1 P = 0.0048). *P ≤ 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Data are expressed as mean ± SEM with individual data points. Source data are provided as a Source Data file.
Supplementary Fig 3a). Specific species within these lipid classes - LPC 16:0, LPC 18:0, DG 32:0, ceramide 40:1 (N-(docosanoyl)-ceramide; Ceramide d18:1/22:0) and ceramide 42:1 (N-(tetra-cosanoyl)-ceramide; Ceramide d18:1/24:0) - were selected as candidate signals based on their increased concentration in conditioned media following palmitate treatment (post lipid class numbering represents carbon acyl-chain length and unsaturation). DG 32:0 (0–10 µM) (Fig. 2b), LPC 16:0 (0–100 µM) (Fig. 2c) and LPC 18:0 (0–50 µM) (Fig. 2d), at a dose response of physiological concentrations found in plasma\textsuperscript{17,22}, had no significant effect on UPR gene expression. However, treatment of myotubes with ceramides 40:1 (Fig. 2e) and 42:1 (Fig. 2f), at a dose response in the physiological range found in plasma\textsuperscript{22–24}, increased expression of \textit{Atf4} and \textit{Hspa5}. Combined treatment of myotubes with both ceramides 40:1 and 42:1 increased expression of \textit{Atf4}, \textit{Hspa5} and \textit{Edem1}, (increased expression of \textit{Atf3} trended to significance, \(p = 0.06\)) (Fig. 2g). However, treatment of myocytes with ceramide 34:1 had a limited effect on UPR gene expression, suggesting specificity within lipid class (Supplementary Fig. 3b). Ceramides 40:1 and 42:1 (and ceramide 34:1) were also increased in concentration in media collected from HSkMCs treated with palmitate (Fig. 2h; Supplementary Fig. 3c). Treatment of HSkMCs with ceramides 40:1 and 42:1, was consistent with effects on murine myotubes with increased expression of
ATF3, ATF4 and EDEM1 (Fig. 2i). We confirmed induction of the UPR at the protein level using immunoblotting for the C/EBP Homologous Protein (CHOP), X-box binding protein 1 (XBP-1s) and Glyceroldehyde-3-phosphate dehydrogenase (GAPDH) expression control from HSkMCs treated with vehicle control (red) and a combination of both ceramides (green) (n = 4; one-way ANOVA; control vs 10 M Cer 40:1, 10 M Cer 42:1, CHOP P = 0.014). j Western blot for C/EBP Homologous Protein (CHOP), X-box binding protein 1 active splice variant (XBP-1s) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression control from HSkMCs treated with 10 M ceramides 40:1 and 42:1 consistent with increased secretion in people with T2D (Fig. 3d, Supplementary Fig 6b).  

**Long-chain ceramides are enriched in murine and human skeletal muscle during metabolic disease.** We then explored whether the long-chain ceramide 40:1 and ceramide 42:1 species were increased in the skeletal muscle of murine models and human patients with obesity and insulin resistance, metabolic diseases characterised by dyslipidaemia and lipotoxicity. Mice were fed either a western diet (WD) or a low-fat control diet (LFD) for 12 weeks. Western diet increased body weight and fat mass in the mice, while lean mass was unaffected (Supplementary Fig. 4a, b). Blood glucose concentrations were elevated in mice fed a western diet following an insulin tolerance test (ITT, Supplementary Fig. 4e, f) at week 10 of the study. Therefore, mice fed a western diet exhibited increased glucose intolerance and insulin resistance. The long-chain ceramide signals were increased in the skeletal muscle (Fig. 3a, Supplementary Fig. 5a) and blood plasma (Fig. 3b, Supplementary Fig. 5b) of western diet-fed mice. Muscle and plasma concentrations of ceramide 40:1 and 42:1 were significantly positively correlated in the mice (Supplementary Fig 5c, d).  

Next, we examined the association of skeletal muscle long-chain ceramides 40:1 and 42:1 with metabolic disease in human volunteers. Skeletal muscle biopsies of the pectoralis major were taken from age- and sex-matched patients with and without T2D (Supplementary Table 1). Lipidomic analysis of the biopsies demonstrated greater concentrations of ceramides 40:1 and 42:1 in the muscle from patients with T2D (Fig. 3c, Supplementary Fig 5e). Lipidomic analysis of the blood plasma from a random subset of patients identified increased concentrations of ceramides 40:1 and 42:1 in people with T2D (Fig. 3d, Supplementary Fig. 5f). Muscle and plasma concentrations of ceramide 40:1 and 42:1 were significantly positively correlated in the patients (Supplementary Fig 5g, h). These data highlight that increases in skeletal muscle and blood plasma long-chain ceramides are associated with metabolic disease in mice and humans.

**Palmitate-induced lipotoxicity increases ceramide synthesis by the ceramide synthase 2 de novo pathway through the UPR kinase Perk.** Ceramides are synthesised either via the salvage pathway or the de novo pathway, which begins with the condensation of palmityl-CoA with serine (Fig. 4a). To confirm that increases in ceramide secretion are coupled to increased intracellular synthesis, lipid extracts from C2C12 myotubes treated with 100 or 200 µM palmitate, or the BSA vehicle, were analysed using lipidomics. Palmitate increased intra-myocyte concentration of ceramide 40:1 and 42:1 consistent with increased secretion of these species (Fig. 4b, Supplementary Fig 6a). Similar increases in intra-myocyte ceramide 40:1 and 42:1 concentration following palmitate treatment were observed in primary HSkMCs (Fig. 4c, Supplementary Fig 6b).  

We hypothesised that exogenous palmitate would increase flux through the de novo ceramide synthesis pathway through conversion to palmityl-CoA. C2C12 myotubes, with and without palmitate treatment, were exposed to either myriocin (Fig. 4d), which inhibits serine palmitoyl transferase (SPT), or fumonisin B1 (FB1) (Fig. 4e), an inhibitor of multiple ceramide synthases (CerS). Inhibition of the de novo pathway at both SPT and CerS greatly abrogated palmitate-induced increases in the intracellular concentrations of ceramide 40:1 and ceramide 42:1, suggesting that palmitate-induced ceramide 40:1 and ceramide 42:1 synthesis is via the de novo pathway. There are six CerS isoforms, which exhibit a preference to produce ceramides of differing acyl-chain...
Our results suggest palmitate stimulates increases in long-chain ceramide 40:1 and ceramide 42:1 production and secretion. Long-chain ceramides, including ceramide 40:1 and 42:1, are predominantly synthesised by CerS2. However, CerS4 overlaps with CerS2 in its specificity for the production of long-chain ceramides. CerS4 production of long-chain ceramides is unaffected by FB1, suggesting that the mechanism for palmitate-induced ceramide 40:1 and 42:1 production is independent of CerS4. The expression of CerS4 was decreased in response to palmitate treatment in C2C12 myocytes (Supplementary Fig. 6c). In contrast palmitate-treatment of C2C12 myotubes and HSkMCs induced CerS2 expression (Fig. 4f, g). To confirm long-chain ceramides 40:1 and 42:1 are generated through CERS2 in response to palmitate treatment in myocytes, we decreased CERS2 expression in HSkMCs by 70% using siRNA (Fig. 4h). Knockdown of CERS2 expression decreased intracellular (Fig. 4i) and extracellular media (Fig. 4j) ceramide 40:1 and 42:1 concentrations and inhibited the palmitate-induced increase in the myocyte media concentration of the lipids. These data suggest increased ceramide 40:1 and ceramide 42:1 concentrations in response to palmitate are generated through the de novo pathway. We then transferred conditioned serum- and palmitate-free media collected from HSkMCs treated with 100 μM palmitate or BSA control, with and without siRNA-mediated CERS2 knockdown, to naive HSkMCs. The induction of UPR gene expression induced by media conditioned on palmitate-treated HSkMCs was inhibited by CERS2 knockdown (Fig. 4k) in agreement with the effect of CERS2 knockdown on ceramide 40:1 and ceramide 42:1 media concentrations. These data confirm that the CERS2 generated long-chain ceramides are cell non-autonomous UPR-inducing lipid signals.

Next, we hypothesised that long-chain ceramide synthesis in response to lipotoxicity was directly regulated by specific UPR pathways. Intracellular ceramide 40:1 and 42:1 concentrations were analysed in C2C12 myotubes co-treated with 200 μM palmitate or the BSA vehicle, and either 10 μM 4µ8C, which inhibits Ire1 activity (Fig. 5a) or 10 µM AMG PERK 44, an inhibitor of the UPR kinase Perk (Fig. 5b). While inhibition of Ire1 had no effect on ceramide concentrations, inhibition of Perk abrogated palmitate-induced increases in ceramides 40:1 and 42:1. To confirm PERK contributed to the palmitate-mediated secretion of ceramide 40:1 and 42:1 from myocytes, we decreased PERK expression in HSkMCs by greater than 85% using siRNA (Fig. 5c). Knockdown of PERK decreased intracellular (Fig. 5d) and the extracellular media (Fig. 5e) ceramide 40:1 and 42:1 concentrations and inhibited the palmitate-induced increase in the myocyte media concentration of the lipids. These data identify a role for the UPR, and specifically Perk, in regulating sphingolipid synthesis.

In vivo transgenic inactivation of Cers2 decreases the plasma and muscle long-chain ceramide signals and muscle UPR activation. Cers2 was implicated in the biosynthesis of the UPR-inducing ceramide signals in myocytes in vitro. We next explored whether transgenic catalytic inactivation of Cers2 in the Cers2 H/A mouse (two consecutive histidine for alanine substitutions in the Cers2 catalytic centre) affected synthesis of the ceramides. Lipidomics was used to analyse the concentration of ceramide species in the skeletal muscle (Fig. 6a, Supplementary Fig. 7a) and plasma (Fig. 6b, Supplementary Fig. 7b) of mice heterozygous for Cers2 H/A (Het Cers2 H/A) and mice homozygous for Cers2 H/A (Homo
CerS2 H/A) compared to wild type littermate controls. Tissues of the Homo CerS2 H/A mouse exhibit a compensatory increase in ceramide 34:1 concentration (Supplementary Fig. 7a, b)\(^29\). To limit compensatory effects and generate a more physiological model we also used the Het CerS2 H/A mouse which is not characterised by compensatory increases in shorter chain ceramides (Supplementary Fig. 7a, b). The concentrations of ceramide 40:1 and 42:1 were depleted in plasma and muscle of Het CerS2 H/A, and Homo CerS2 H/A mice in a gene-dosage responsive manner. We then assessed whether heterozygous and homozygous catalytic inactivation of CerS2 and subsequent decreases in the long-chain ceramides altered skeletal muscle expression of UPR genes. The expression of both Atf4 and Hspa5 was significantly decreased in the skeletal muscle of Het CerS2 H/A, and Homo CerS2 H/A mice (Fig. 6c).

These data indicate that CerS2 regulates the systemic and muscle concentration of the long-chain ceramide signals and disruption in biosynthesis of the ceramides decreases muscle UPR activation in vivo.

Extracellular vesicles mediate secretion and transport of the UPR-inducing long-chain ceramide signal. Ceramides are hydrophobic. If ceramides are to function as a paracrine or...
Fig. 4 Long-chain ceramides are synthesised via the Cers2 de novo pathway. a The de novo synthesis pathway and salvage pathway of ceramide synthesis with de novo synthesis pathway inhibitors (red). Intracellular ceramides (Cer) in b C2C12 myotubes (control, 200 µM palmitate n = 4; 100 µM palmitate n = 3; one-way ANOVA; control vs 200 µM palmitate, Cer 40:1 P < 0.0001; Cer 42:1 P = 0.0026) (control = red, 100 µM palmitate = grey, 200 µM palmitate = blue) and c human skeletal muscle cells (HSKMCs) (control = red, 50 µM palmitate = grey, 100 µM palmitate = blue) (n = 4; one-way ANOVA; control vs 100 µM palmitate, Cer 40:1 P = 0.043; control vs 200 µM palmitate, Cer 40:1 P = 0.0003, Cer 42:1 P = 0.05; 100 µM palmitate vs 200 µM palmitate, Cer 40:1 P = 0.014) treated with palmitate. Long-chain ceramides in C2C12 myotubes co-treated with either d 10 µM myricitrin (serine palmitoyl transferase inhibitor) (control = red, 10 µM myricitrin = grey, 200 µM palmitate = blue, 200 µM palmitate + 10 µM myricitrin = green) (n = 3; one-way ANOVA; control vs 100 µM myricitrin, Cer 40:1 P < 0.0001, Cer 42:1 P < 0.0001; control vs 200 µM palmitate, Cer 40:1 P < 0.0001, Cer 42:1 P = 0.014; control vs 200 µM palmitate + 10 µM myricitrin, Cer 40:1 P < 0.0001, Cer 42:1 P < 0.0001; 200 µM palmitate vs 100 µM myricitrin, Cer 40:1 P < 0.0001, Cer 42:1 P = 0.0001, Cer 42:1 P = 0.016; control vs Cer 42:1 siRNA P = 0.014; control vs CERS2 siRNA + 100 µM palmitate P = 0.011; 100 µM palmitate vs CERS2 siRNA P = < 0.0001; 100 µM palmitate vs CERS2 siRNA + 100 µM palmitate P = 0.001). j CER expression in control HSKMCs (red) and HSKMCs treated with 100 µM palmitate (grey), scrambled control siRNA (con siRNA; white), siRNA against CERS2 (CERS2 siRNA; blue) or CERS2 siRNA and 100 µM palmitate (green) (n = 3; one-way ANOVA; control vs 100 µM palmitate P = 0.0016; control vs CERS2 siRNA P = 0.014; control vs CERS2 siRNA + 100 µM palmitate P = 0.011; 100 µM palmitate vs CERS2 siRNA P = < 0.0001; 100 µM palmitate vs CERS2 siRNA + 100 µM palmitate P = 0.001). k UPR gene expression in HSKMCs receiving conditioned media on control HSKMCs (red) and HSKMCs treated with 100 µM palmitate (grey), con siRNA (white), CERS2 siRNA (blue) or CERS2 siRNA and 100 µM palmitate (green) (n = 4; one-way ANOVA; control vs 100 µM palmitate, Cer 40:1 P = 0.0001, Cer 42:1 P = 0.001; control vs CERS2 siRNA Cer 40:1 P = 0.0001, Cer 42:1 P = 0.0001; 100 µM palmitate vs CERS2 siRNA Cer 40:1 P = 0.0001, Cer 42:1 P = 0.0001; 100 µM palmitate vs CERS2 siRNA + 100 µM palmitate Cer 40:1 P = 0.0001, Cer 42:1 P = 0.0001; CERS2 siRNA vs CERS2 siRNA + 100 µM palmitate Cer 40:1 P = 0.023). We then explored whether small EVs or large EVs released from palmitate-treated myocytes contained the UPR-inducing signal. Small EVs (Fig. 7d) and large EVs (Fig. 7e) isolated from palmitate-treated myotubes increased UPR gene expression in naïve myotubes following their reconstitution in serum-free media. EV-depleted conditioned media, however, did not induce UPR gene expression in naïve myotubes (Fig. 7f). Lipidomic analysis of small EVs (Fig. 7g) and large EVs (Fig. 7h) showed that palmitate-treated myotubes produced EVs enriched in ceramides 40:1 and 42:1. Reciprocally, LC-MS analysis of EV-depleted media demonstrated a ~92% reduction in long-chain ceramides 40:1 and 42:1. This indicates ceramides are imported into the myotubes. Lipidomic analysis also demonstrated an increase in the intracellular concentration of the cognate dihydroceramides 40:0 and 42:0 (Fig. 8b). Dihydroceramides are ceramide precursors. Therefore, exogenous ceramides may be imported and recycled within the muscular signal, propagating the induction of the UPR, then there must be an appropriate physiological mechanism to overcome limitations to solubility and mediate extracellular transport. Due to their physicochemical nature this is unlikely to be via canonical membrane transporters. Extracellular vesicles (EVs) have emerged as important vehicles for signalling mediators30. There is developing recognition that bioactive lipid signals may be partitioned to their physicochemical nature this is unlikely to be via canonical systemic signalling pathways. Given the importance of EVs in physiological and pathological processes, the role of EVs in the propagation of the UPR is an area of active research. In this study, we demonstrate that EVs can transport ceramides and ceramide biosynthetic enzymes. The long-chain ceramide signals extracellularly. Conditioned media from control and 200 µM palmitate-treated myocytes were conditioned media on control HSKMCs (red) and HSKMCs treated with 100 µM palmitate (grey), con siRNA (white), CERS2 siRNA (blue) or CERS2 siRNA and 100 µM palmitate (green) (n = 4; one-way ANOVA; control vs 100 µM palmitate, Cer 40:1 P = 0.0001, Cer 42:1 P = 0.0005; control vs CERS2 siRNA + 100 µM palmitate, Cer 40:1 P = 0.019, Cer 42:1 P = 0.011; 100 µM palmitate vs CERS2 siRNA Cer 40:1 P < 0.0001, Cer 42:1 P < 0.0001; 100 µM palmitate vs CERS2 siRNA + 100 µM palmitate Cer 40:1 P < 0.0001, Cer 42:1 P < 0.0001; Cer 42:1 siRNA vs CERS2 siRNA + 100 µM palmitate Cer 40:1 P = 0.024).
Fig. 5 Palmitate-induced long-chain ceramide synthesis requires Perk. a Ceramides (Cer) in C2C12 myotubes treated with palmitate or Bovine Serum Albumin (BSA), in combination with 10 µM 4p8c, an inhibitor of Ire1 (Ire1i) (control = red, Ire1i = grey, palmitate = blue, palmitate + Ire1i = green) (n = 3; one-way ANOVA; control vs 200 µM palmitate Cer 40:1 P < 0.0001, Cer 42:1 P = 0.0062; control vs 200 µM palmitate + 10 µM Ire1i Cer 40:1 P < 0.0001; 10 µM Ire1i vs 200 µM palmitate Cer 40:1 P < 0.0001, Cer 42:1 P = 0.013; 10 µM Ire1i vs 200 µM palmitate + 10 µM Ire1i Cer 40:1 P < 0.0001). b Ceramides in C2C12 myotubes treated with palmitate or BSA, in combination with 10 µM AMG PERK 44, an inhibitor of PERK (Perki) (control = red, Perki = grey, palmitate = blue, palmitate + Perki = green) (n = 3; one-way ANOVA; control vs 200 µM palmitate Cer 40:1 P < 0.0001, Cer 42:1 P = 0.022; control vs 200 µM palmitate + 10 µM Perki Cer 40:1 P = 0.0003; 10 µM Perki vs 200 µM palmitate Cer 40:1 P < 0.0001, Cer 42:1 P = 0.023; 10 µM Perki vs 200 µM palmitate + 10 µM Perki Cer 40:1 P = 0.0002; 200 µM palmitate vs 200 µM palmitate + 10 µM Perki Cer 40:1 P = 0.0017; Cer 42:1 P = 0.028). c PERK expression in control HSKMCs (red) and HSKMCs treated with 100 µM palmitate (grey), scrambled control siRNA (con siRNA; white), siRNA against PERK (PERK siRNA; blue) or PERK siRNA and 100 µM palmitate (green) (n = 3; one-way ANOVA; control vs 100 µM palmitate P = 0.05; control vs PERK siRNA P = 0.016; control vs PERK siRNA + 100 µM palmitate P = 0.016; 100 µM palmitate vs PERK siRNA P = 0.0004; 100 µM palmitate vs PERK siRNA + 100 µM palmitate P = 0.0004). d Cer 40:1 and Cer 42:1 normalised concentration in control HSKMCs (red) and HSKMCs treated with 100 µM palmitate (grey), con siRNA (white), PERK siRNA (blue) or PERK siRNA and 100 µM palmitate (green) (n = 4; one-way ANOVA; control vs 100 µM palmitate, Cer 40:1 P < 0.0001, Cer 42:1 P = 0.015; control vs PERK siRNA Cer 40:1 P < 0.05; Cer 42:1 P = 0.05; 100 µM palmitate vs PERK siRNA Cer 40:1 P < 0.0001, Cer 42:1 P = 0.0002; 100 µM palmitate vs PERK siRNA + 100 µM palmitate Cer 40:1 P < 0.0001, Cer 42:1 P = 0.0015; PERK siRNA vs PERK siRNA + 100 µM palmitate Cer 40:1 P = 0.062); e Cer 40:1 and Cer 42:1 normalised concentration in media conditioned on control HSKMCs (red) and HSKMCs treated with 100 µM palmitate (grey), con siRNA (white), PERK siRNA (blue) or PERK siRNA and 100 µM palmitate (green) (n = 3; one-way ANOVA; control vs 100 µM palmitate, Cer 40:1 P = 0.0487; Cer 42:1 P = 0.0007; control vs PERK siRNA Cer 40:1 P = 0.0009; Cer 42:1 P < 0.0001; control vs PERK siRNA + 100 µM palmitate, Cer 40:1 P = 0.0006; Cer 42:1 P < 0.0001; 100 µM palmitate vs PERK siRNA Cer 40:1 P < 0.0001, Cer 42:1 P < 0.0001; 100 µM palmitate vs PERK siRNA + 100 µM palmitate Cer 40:1 P < 0.0001, Cer 42:1 P < 0.0001). * P ≤ 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001. Data are expressed as mean ± SEM with individual data points. Source data are provided as a Source Data file.

cell. Similar effects on intracellular ceramide 40:1 and 42:1, and dihydroceramide 40:0 and 42:0 were seen in long-chain ceramide-treated primary HSKMCs (Fig. 8c, d).

Dihydroceramides drive activation of the UPR35. We assessed the role of dihydroceramides in lipotoxicity-induced ER stress. Palmitate-treated myotubes were co-incubated with 5 µM fenretinide, an inhibitor of dihydroceramide desaturase 1 (Des1), the enzyme catalysing the conversion of dihydroceramides to ceramides. Co-treatment with palmitate and fenretinide decreased ceramide 42:1 concentration without effect on ceramide 40:1 concentration (Supplementary Fig. 10a). However, the intracellular concentrations of dihydroceramides were greatly increased by palmitate and fenretinide co-treatment (Supplementary Fig. 10b). Concomitant palmitate treatment and Des1 inhibition synergistically enhanced expression of UPR genes (Supplementary Fig. 10c). To confirm a role for dihydroceramides in lipotoxicity-induced UPR activation we used siRNA to decrease DEGS1 expression (the gene encoding Des1) by > 50% in the translational primary HSKMC model (Fig. 8e). DEGS1 knockdown in HSKMCs decreased the intracellular concentration of ceramides 40:1 and 42:1 (Fig. 8f). Concomitant palmitate treatment of human primary myotubes with DEGS1 knockdown increased the intracellular concentrations of dihydroceramides 40:0 and 42:0 (Fig. 8g). Palmitate treatment in combination with DEGS1 knockdown synergistically enhanced UPR gene expression in human myotubes (Fig. 8h). These data implicate dihydroceramides in the cell non-autonomous ceramide-induced activation of UPR signalling in lipotoxicity.

Discussion

ER stress has emerged as a potentially unifying mechanism underpinning the development of lipotoxicity and metabolic...
dysfunction in metabolic disease. Cell non-autonomous signalling represents a mode of regulation in the control of UPR activation\(^{16}\). The identity of these UPR-inducing signals remained unknown. This study identifies the nature of a cell non-autonomous signal that propagates the activation of the UPR and describes such a signal in the context of lipotoxicity (Supplementary Fig. 11). We show that palmitate-mediated activation of the Perk-arm of the UPR stimulates an increase in the de novo synthesis, via CerS2, of long-chain ceramides (ceramide 40:1 and 42:1). These ceramides are packaged into extracellular vesicles, secreted, and propagate activation of the UPR, potentially via the accumulation of dihydroceramides in naïve myotubes. The ER is the key cellular site of de novo ceramide biosynthesis\(^{36}\). Using long-chain ceramides, a product of this pathway, as signals of ER stress to induce cell non-autonomous UPR activation appears advantageous. However, our work does not preclude the presence of other metabolite factors, protein signals, or bioactive lipids that may contribute to cell non-autonomous UPR activation. Indeed, our data suggest the presence of an, as yet unidentified, aqueous soluble metabolite factor(s) secreted from palmitate-treated myocytes which also activate UPR gene expression. These findings have consequences for our understanding of both the mechanisms of lipotoxicity and the regulation of ER stress and UPR activation.

Ceramides have previously been implicated in both the development of metabolic disease and the regulation of ER stress. Blood plasma and tissue ceramide and dihydroceramide concentrations are associated with a range of cardiometabolic abnormalities. A recent study by our group showed that palmitate-mediated activation of the Perk-arm of the UPR stimulates an increase in the de novo synthesis of long-chain ceramides, which are then secreted and propagate activation of the UPR. These ceramides are transported in extracellular vesicles and may act as signals of ER stress.

**Fig. 6** Transgenic CerS2 inactivation decreases plasma and muscle long-chain ceramides and muscle UPR gene expression. a Ceramides (Cer) 40:1 and 42:1 in the soleus muscle of transgenic mice heterozygous (Het CerS2 H/A; grey) and homozygous (Homo CerS2 H/A; blue) for catalytically inactivated ceramide synthase 2 compared to wildtype (WT; red) (WT, Het CerS2 H/A \(n = 6\); Homo CerS2 H/A \(n = 3\); one-way ANOVA; WT vs Het CerS2 H/A, Cer 40:1 \(P = 0.03\), Cer 42:1 \(P = 0.03\); WT vs Homo CerS2 H/A, Cer 40:1 \(P = 0.0001\), Cer 42:1 \(P = 0.0005\)). b Cer 40:1 and Cer 42:1 in the plasma of Het CerS2 H/A (grey) and Homo CerS2 H/A (blue) mice compared to WT (red) (WT, Het CerS2 H/A \(n = 6\); Homo CerS2 H/A \(n = 3\); one-way ANOVA; WT vs Het CerS2 H/A, Cer 42:1 \(P = 0.0018\); WT vs Homo CerS2 H/A, Cer 40:1 \(P = 0.0006\), Cer 42:1 \(P < 0.0001\)). c Activating transcription factor 4 (Atf4), Heat Shock Protein Family A (Hsp70) Member 5 (Hspa5) and ER Degradation Enhancing Alpha-Mannosidase Like Protein 1 (Edem1) soleus muscle UPR gene expression in Het CerS2 H/A (grey) and Homo CerS2 H/A (blue) mice compared to WT (red) (WT, Het CerS2 H/A \(n = 6\); Homo CerS2 H/A \(n = 3\); one-way ANOVA; WT vs Het CerS2 H/A, Atf4 \(P < 0.0001\), Hspa5 \(P = 0.019\); WT vs Homo CerS2 H/A, Atf4 \(P < 0.0001\), Hspa5 \(P = 0.019\) \(* P \leq 0.05\), \(**P < 0.01\), \(***P < 0.001\), \(****P < 0.0001\). Data are expressed as mean ± SEM with individual data points. Source data are provided as a Source Data file.
diseases\textsuperscript{37–41}. Here, we observe an increase in the concentrations of long-chain ceramides 40:1 and 42:1 in the blood plasma and muscle of western diet-induced obese mice and humans with T2D. Confirming that ceramides 40:1 and 42:1 are enriched in muscle by metabolic disease in mouse and humans. We do not preclude that circulating concentrations of long-chain ceramides in this setting may be contributed to by additional tissues besides skeletal muscle. Ceramides have been identified as key mechanistic contributors to the lipotoxicity-induced development of adipose tissue and hepatic insulin resistance\textsuperscript{42}. Inhibition of ceramide synthesis ameliorates palmitate-induced ER stress in pancreatic \(\beta\)-cells\textsuperscript{43}. Similarly, palmitate-induced the production of long-chain ceramides, and siRNA knockdown of CerS 5 and 6 prevented myristate-induced ER stress, in intestinal epithelial cells\textsuperscript{44}. A short-chain ceramide analogue, infused into the hypothalamus of rats increases hypothalamic ER stress, and reduces the thermogenic capacity of brown adipose tissue, thus, highlighting that ceramide-induced ER stress in one tissue can have a metabolic impact on distal tissues\textsuperscript{45}. Evidence is emerging that skeletal muscle ceramide metabolism has a key role in the
regulation of systemic physiology. Skeletal muscle ceramides are elevated in both patients with, and animal models of, obesity and diabetes and negatively correlate with insulin sensitivity. In addition, muscle-specific disruption of ceramide synthesis by CerS1 improves systemic insulin sensitivity in diet-induced obese mice. Here we demonstrate that lipotoxicity-induced ER stress can stimulate biosynthesis and secretion of ceramides which regulate UPR activation in a cell non-autonomous manner in skeletal muscle.

In the present study, we focus on skeletal muscle as a key regulator of systemic metabolic homoeostasis, where lipotoxicity-induced ER stress causes metabolic dysfunction and contributes to the development of metabolic disease. This led to our hypothesis that cell non-autonomous signalling may be important in the communication of lipotoxicity-induced ER stress in skeletal muscle. Our work in skeletal muscle may have relevance to the mechanisms of lipotoxicity-induced ER stress in other tissues such as adipose tissue, liver, pancreas and the hypothalamus, where future work may identify that cell non-autonomous ceramide signalling contributes to dysfunction in metabolic disease in these tissues.

Ceramides are highly hydrophobic molecules and therefore unlikely to be secreted via canonical membrane transporters. Our data suggests that EVs represent a vehicle for long-chain ceramide extracellular transport in ER stress. This is consistent with a role for ceramides in small EV biogenesis in which inhibition of neutral sphingomyelinase 2 (nSMase2) reduces small EV secretion. The transport of ceramides by EVs in propagating cell non-autonomous UPR activation further highlights the important role of extracellular vesicles in skeletal muscle endocrine and paracrine signalling. This study describes the signalling of bioactive ceramide lipids delivered through EVs.

We highlight the importance of acyl-chain length in ceramide signalling. Six mammalian CerS isoforms have been identified (CerS1-6). Each CerS enzyme produces ceramides with a unique, but over-lapping, acyl-chain distribution. Long-chain ceramides, ceramide 40:1 and ceramide 42:1 are produced by CerS2 and were identified as the UPR-inducing signals. This is consistent with studies which emphasise the importance of long-chain, rather than C16 and other shorter chain, ceramides in muscle metabolic disease. For instance, despite ceramide 36:1...
being the predominately ceramide species in muscle, we do not identify this ceramide species in the media of skeletal myocytes in response to palmitate treatment, suggesting it is not a key signal in the palmitate-induced cell non-autonomous activation of the UPR in muscle. The importance of different chain length ceramides in metabolic disease may alter depending on the tissue type and context. Through the use of heterozygous transgenic catalytic inactivation of CerS2, a model which represents a more physiological condition than total loss of function, preventing compensatory increases in shorter chain ceramides, we are able to directly manipulate the concentration of the long-chain ceramide signals in vivo and show that this modulates the expression of UPR genes in muscle.

Lipidomic profiling of ceramide-treated myotubes showed increases in cognate dihydroceramides. Inhibition of Des1, or knockdown of DEGS1, increased the dihydroceramide/ceramide ratio, and exacerbated palmitate-induced ER stress. Although a role for dihydroceramide accumulation in ER stress has been reported in several cancers including gastric carcinoma and glioblastoma, here we present data suggesting the conversion of ceramide to dihydroceramide. We present evidence of an emerging role for dihydroceramides in the induction of ER stress.

This study may have repercussions beyond the understanding of ER stress. Mitochondria also contain a discreet UPR initiated when mitochondrial integrity and function are impaired. This mitochondrial UPR may also be regulated by paracrine signals when mitochondrial integrity and function are impaired. This may have consequences across pathophysiological activation of ER-stress and cell-cell signalling. In addition, by defining the mechanisms of ER-stress activated long-chain ceramide production, packaging, secretion, and UPR activation we highlight potential targets for therapeutic intervention in lipotoxicity-associated metabolic diseases.

**Methods**

**C2C12 myoblast culture and differentiation.** C2C12 myoblasts (91031101, Sigma Aldrich) were grown to confluence in DMEM media (D6429, Sigma Aldrich) containing 10% foetal bovine serum (FBS, Sigma Aldrich) and 1% penicillin-streptomycin (F/S, Sigma Aldrich). Approximately 100,000 cells were seeded per well in 12-well collagen I-coated plates (Corning Biocoat, 734-0295). Myoblasts were then differentiated to myotubes from time of plating in differentiation media, which constituted DME containing 10% horse serum (HS, Sigma Aldrich) and
1% FBS. Cells were differentiated for 8 days. After 2 days of differentiation, cells were treated with either 200 µM palmitate, or the BSA vehicle control, for 6 days or ceramide 40:1 or 42:1 for the final 10 h. Media was changed every day during differentiation. Furthermore, cells were co-treated with the following pharmacological inhibitors: 10 µM AMG PERK 44 (Tocris Bioscience), 10 µM 4q8c (Tocris Bioscience), 10 µM Fumonisin B1 (Tocris Bioscience), 5 µM Ferrentinide (Tocris Bioscience), 10 µM Myriocin (Sigma Aldrich).

**Primary human skeletal muscle cell culture and differentiation.** HSKMCs were purchased from Cell Applications Inc (S150a-05a). They were grown to confluence in Skeletal Muscle Growth Medium (Cell Applications) in collagen I-coated 12-well plates (approximately 100,000 cells were seeded per well). Once confluent, HSKMCs were differentiated for 6 days using Skeletal Muscle Differentiation Media (Cell Applications, 151D-250). During differentiation cells were treated with either fatty acid-free BSA (200 µg/ml palmitate, or the BSA vehicle control, to support a cholinergic model of ER stress, or with ceramide 40:1 or 42:1 for the final 10 h of differentiation. Media was changed every 2 days during differentiation.

Informed consent was obtained from donors. The cells were approved and compiled with ethics according to:
- Collection, generation, research purpose and sale: Cell Applications, Inc. 5820 Oberlin Dr. Suite 101, San Diego, CA 92121,
- Use in compliance with Human Tissue Act (UK) by Leeds Institute of Cardiovascular and Metabolic Medicine, University of Leeds, Leeds, LS2 9JT UK.

**Conjugation of palmitate to BSA.** The conjugation of palmitate to BSA was carried out as previously described. PBS (75 ml) was heated to 37 °C. Fatty-acid-free BSA (200 µg/ml palmitate, or the BSA vehicle control, to support a cholinergic model of ER stress, or with ceramide 40:1 or 42:1 for the final 10 h of differentiation. Media was changed every 2 days during differentiation. Informed consent was obtained from donors. The cells were approved and compiled with ethics according to:

- Preparation of ceramides. Ceramide 40:1 (N-docosanoyl)-ceramide; Ceramide d18:1/22:0 (860501) and ceramide 42:1 (N-tetracosanoyl)-ceramide; Ceramide d18:1/24:0 (860524) were purchased from Avanti Polar Lipids Inc. Preparation of ceramides followed standard protocols in the literature. Ethanol and dodecanol were mixed at a ratio of 98:2, followed by vortexing and pre-warming to 37 °C. Meanwhile, ceramide 40:1 and ceramide 42:1 (Avanti Polar Lipids Inc) were dissolved in chloroform-methanol 1:1. The required volume was then dried down under N2 gas. The pre-warmed ethanol-dodecanol mixture was added to the dried ceramide 40:1 and ceramide 42:1 such that the final stock concentration was 2.5 mM. This mixture was thoroughly vortexed and incubated at 37 °C for a further 20 min followed by further vortexing. The stock solution was then diluted to the required concentration using ethanol/dodecanol. The solution used at 0°C or 4°C was diluted in cell culture medium (37 °C) to the required treatment concentration followed by vortex mixing. Ethanol/dodecanol was included as a vehicle control at the same concentration as the treatment.

**Preparation of lysophosphocholines and diacylglycerols.** LPC 16:0 (855675), LPC 18:0 (855775), DG 32:0 (800816) (Avanti Polar Lipids) were dissolved in 50% ethanol in sterile water at a stock concentration of 50 mM. LPC and DG stocks were made up fresh for each media change during cell culture experiments. The stock solution of LPC 16:0, LPC 18:0 and DG 32:0 were diluted in cell medium (37 °C) to the required treatment concentration followed by vortex mixing. Ethanol/water was included as a vehicle control at the same concentration as the treatment.

**Conditioned media collection and processing.** Following treatment with either palmitate or the BSA vehicle for 6 days, culture media was removed and cells washed with PBS. Serum-free media, free of any agonists, was then added for 24 h. This conditioned media was collected and frozen at −80 °C. For boiling experiments, conditioned media was incubated at 100°C in a water bath for 5 min. For molecular weight cut off dialysed experiments, conditioned media was dialysed for 24 h using molecular weight cut off dialysis of 1000 Da and deionised water as dislocation, and subsequently dissected. Tissue was flash frozen in liquid nitrogen and stored at −80 °C. All animal studies complied with national and local ethical regulations for animal research. Animal studies were regulated under the Animals (Scientific Procedures) Act 1986 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body. All procedures were carried out in accordance with U.K. Home Office protocols by a UK Home Office Personal License Holder.

**Intraperitoneal glucose tolerance tests.** Intraperitoneal glucose tolerance tests were performed as previously described. Mice were fasted for 8 h with access to water prior to baseline glucose measurement. Administration of glucose (Sigma-Aldrich) was performed by intraperitoneal injection (glucose 1.5 mg/g of body weight; glucose solution 150 mg/ml). Blood was obtained from the tail vein immediately prior to glucose injection and then at 10, 20, 30, 60, 90 and 120 min post injection. Glucose levels were measured using a Bayer Contour Glucose Meter (Bayer Healthcare).

**Human skeletal muscle biopsies.** 75 eligible and consecutive patients undergoing routine pacemaker therapy at Leeds General Infirmary volunteered to participate in the study following written informed consent. Eligible patients were grouped into two age and sex-matched cohorts with the presence of diabetes (n = 22) or absence of diabetes (n = 53) of T2DM. The T2DM cohort had a previous diagnosis of T2DM (>3 months) and/or was receiving treatment for diabetes. In such cases, T2DM was defined as a documented history of diabetes, fasting plasma glucose ≥ 7.0 mmol/L, plasma glucose ≥ 11.1 mmol/l.2 h after the OGTT or a glycated haemoglobin (HbA1c) ≥ 6.5% (≥48 mmol/L). Patient demographic data is given in Supplementary Table 1.

Human skeletal muscle (pectoralis major) biopsies were obtained from eligible participants during their pacemaker operation. Pacemaker procedures were otherwise entirely routinely carried out: lidocaine was initially injected to anaesthetise the area and a small incision was made under the left clavicle. During creation of the pre-pectoral pocket for the generator above the pectoralis major a portion of the muscle (approximately 100 mg) was sampled. This sample was immediately placed into a 1.5 mL Eppendorf tube and snap frozen in liquid nitrogen. The study was approved by the Leeds West Research Ethics Committee (11/YH/0921) and Leeds Teaching Hospitals R&D Committee (CD11/10015) and conforms to the Declaration of Helsinki.

**RNA handling and reverse transcription quantitative polymerase chain reaction.** RNA was extracted and purified in situ, using the RNeasy mini kit (Qiagen), following the manufacturer’s instructions. cDNA was synthesised by a two-step process with the RT’s: First Strand Kit (Qiagen), following the manufacturer’s instructions. cDNA was re-analysed using a Step One Plus Real Time Cycler (Applied Biosystems), quantified using the ΔΔ-CT method with expression normalised to the housekeeping gene.
For murine samples, R818 (PPM72041A) was used as the housekeeping gene, while ACTB (PPH00073G) was used for analysis of primary HSKMCs. The induction of ER stress by lipopolysaccharide (LPS) and thapsigargin was confirmed throughout this study using primers for Ali1 (PPM40669C), Atf4 (PPM40670E), Hspa5 (PPM33586B), Edem1 (PPM26188B), CerS2 (PPM32482A) and CerS4 (PPM28335A) for murine samples, and ATF3 (PPH00408C), ATF7 (PPH00216A), HSPA5 (PPM416021C), EIF2AK3 (PERK) (PPK) (PPH10874A), CERS2 (PPH16029F) and DEGO1 (PPH16415F) for human samples. Data were collected and analysed using StepOne™ Software (version 2.1 Applied Biosystems). Details of primers are given in Supplementary Table 2.

Metabolite extraction. Metabolites from cell pellets, media (1 mL), tissue (approximately 25 mg) and plasma (20 µL) were extracted using a modified Bligh and Dyer method as previously described. Methanol-chloroform (2:1) 600 µL was added to the samples and cells were homogenised in a TissueLyser II (Qiagen) (2 min, 25 Hz) and the samples were sonicated for 15 min. Chloroform-water (1:1) was then added (200 µL of each). Samples were centrifuged (16,100 g, 20 min) and the aqueous and organic phases were separated. For each sample, 250 µL of a deuterated internal standard mixture was added to organic fraction samples. Peaks were then selected from a mass standard mix with a retention time ( RT) of 25–35 min. Peaks had to be present in a minimum of 25% of the samples. Peaks were annotated by accurate mass comparison to the LipidMaps database (https://www.lipidmaps.org/) and confirmed by data-directed fragmentation and comparison with commercial standards as described above. Intensity was normalised to an appropriate internal standard, and cell number (or dry protein weight) for animal tissue.

Extracellular vesicle isolation and quantification. Conditioned media was centrifuged first at 10,000 g for 5 min and the pellet discarded. This was followed by a second spin at 10,000 g for 20 min to pellet large EVs. The supernatant was then spun at 110,000 g for 75 min. The supernatant was discarded and the pellet resuspended in a small volume of PBS prior to a final ultracentrifugation at 110,000 g for 75 min at 4°C. The super- natant was discarded and the pellet resuspended in a small volume of PBS. EV particles were quantified using a NTA3.0 system (Malvern). Six movies of 60 seconds were acquired per sample at room temperature, using a camera level of 15. The processing threshold was set to 6. Data was collected, and particle number and size distribution of each sample was analysed, using NTA3.0 software (NanoSight, Malvern Panalytical).

Preparation of EV protein lysate for protein quantification and SDS-PAGE electrophoresis. After isolation, an aliquot of the EV sample was lysed using RIPA buffer (containing protease (c)Complete ULTRA, 0.5-892.791.001 Roche, supplied by Merck Dorset, UK) and phosphatase inhibitors (PhosSTOP, 490 684 5001 Roche, supplied by Merck Dorset, UK) on ice for 30 min. The samples were centrifuged at 13,000 g for 15 min and the clarified supernatant used for protein quantification using a bicinchoninic acid protein assay kit (ThermoFisher Scientific, 23227) as per the manufacturer’s instructions.

SDS-PAGE and western blot analysis. Samples (containing 25 µg protein for EV analysis, 30 µg for UPF markers) were resolved by electrophoresis through a 4–12% Bis-Tris gradient gel (Invitrogen, Paisley, UK). The resolved proteins were transferred to nitrocellulose membrane (Bio-rad, Hertfordshire UK) and blocked by incubation for 1 h with TBS containing 0.1% (v/v) Tween 20 at room temperature. Incubation with primary antibody (TRF (1:1000), GE MAB (1:1000, 1D4) and –Invitrogen, Paisley, UK), CPT1a (1:1000, EPR21843-72-F), SOD (1:1000, 716G), XBP-1s (1:1000, E9V33; CHOP (1:1000, L63F7) (All Cell Signalling Technologies, London UK) and peroxidase-conjugated secondary antibodies (sheep anti-mouse (1:3000, 7134V, GE Healthcare, Amersham, UK) for GAPDH and TRF and goat anti-rabbit (1:3000, G21234, Invitrogen, Paisley, UK) for all other antibodies) were performed in the same relevant solution as the blocking step. Bound peroxidase conjugates were visualised using an enhanced chemiluminescence detection system (WBKLS0500 Millipore, supplied by Merck Dorset, UK). Images were recorded using Syngene Gbox gel doc system and Genesys software version 1.83 (Syngene, Cambridge UK) for image collection and densitometry analysis. Uncropped and unprocessed blot images are presented in the Source Data file.

Cellular confocal microscopy imaging of ER swelling. HSKMCs were plated onto collagen-coated coverslips at a density of 4 x 10^4 cells/mL. Once 90% confluent, the growth media was changed to differentiation media and the cells were allowed to differentiate for 5 days. On day 5 of differentiation, CellLight ER-RFP BacMam 2.0 (25000 particles/mL, Invi- tron, Paisley, UK) was added to the cells at a multiplicity of infection of 30. After 18 h of treatment (day 6 of differentiation), 10 µM ceramide 40:1, 10 µM ceramide 42:1 or 10 µM of both ceramide 40:1 and 42:1, or vehicle only (982 Ethanol: Dodecane) was added to the cells. The coverslips were fixed in 4% paraformaldehyde for 10 mins at room temperature after 10 h of exposure, washed and mounted onto slides using VectaMount-G containing DAPI (1000-20, Southern Biotech, Birmingham, Alabama).

HSKMCs were then imaged using a Zeiss LSM 880 confocal microscope in the Cy3 (excitation 555 nm) and DAPI channels (excitation 358 nm, emission 460 nm) to visualise ER and nuclei respectively. Images were obtained using a 40x objective lens.

The amount of ER-specific staining was recorded using the ZEN software (Version 2.60, ZEISS). 25 fields of view were imaged from 3–4 slides taken from 3 biological replicates. Each view had a threshold applied to establish the ER-specific staining and a mean fluorescence intensity recorded. This was then corrected to the number of nuclei present in that field.

Statistical analysis. Multivariate data analysis was performed using SIMCA-P+ 13.0 (Umetrics AB, Umeå, Sweden) as previously described. LC-MS data sets were mean-centred and Pareto-scaled prior to analysis. Data sets were analysed using principal components analysis and partial least squares-discriminant analys- is. Lipids responsible for clustering or regression trends within the pattern recognition models were identified by interrogating the corresponding loadings plots. The variable importance in projections/coefficients plots were deemed to have changed globally if they contributed to separation in the models with a confidence limit of 95%.

Univariate statistics was used to assess the significance of changes in individual metabolites. Sample sizes were calculated using power calculations. Samples were randomly assigned to experimental groups. Group variance was analysed with an F test. Statistical significance was assessed using one-way ANOVA (Tukey’s, Holm–Sidak’s and Dunnett’s post hoc test) or two-tailed Student’s t test, as...
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Author contributions

B.D.M. designed and carried out the majority of experiments, interpreted the results and wrote the paper with input from all co-authors. D.F.A. performed LC-MS analysis on CerS2 H/A mice and EV isolations. L.H. performed CerS2 H/A mouse experiments. H.N.D. designed and performed siRNA knockdown experiments in human myocytes. N.T.W. designed and performed protein assays for UPR protein analysis and extracellular vesicle characterization. S.A.M. provided experimental support in mass spectrometry experiments. A.D.V.M. and A.W. performed gene expression analysis for CerS2 mouse studies. T.S.B. contributed to the design of protein quantitation experiments. F.W.B.S. and M.V. provided tissue from murine western diet studies. K.W. provided human skeletal muscle biopsies. R.B led CerS2 H/A mouse research. J.L.G. supported experiment design and interpretation and secured funding. L.D.R. led the study and experiment design, interpretation and manuscript writing and secured funding. All authors commented on and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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