25-hydroxycholesterol activates the Integrated Stress Response to reprogram transcription and translation in macrophages

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Keywords: Stress response, Translation, Lipids, Macrophages, Oxidative stress, Amino acid, 25-hydroxycholesterol, GCN2, MCMV, eIF2-alpha
Background: Interferons and viral infections stimulate the production of 25-hydroxycholesterol.

Results: 25-hydroxycholesterol significantly alters cholesterol ester and sphingolipid levels and activates the integrated stress response.

Conclusion: 25-hydroxycholesterol activates the GCN2/eIF2α/ATF4 integrated stress response likely by causing cysteine depletion and/or by generating oxidative stress.

Significance: Altering important membrane lipids and activating the integrated stress response may contribute to the anti-viral activity of 25-hydroxycholesterol

Abstract
25-hydroxycholesterol (25OHC) is an enzymatically-derived oxidation product of cholesterol that modulates lipid metabolism and immunity. 25OHC is synthesized in response to interferons and exerts broad anti-viral activity by as yet poorly characterized mechanisms. To gain further insights into the basis for anti-viral activity, we evaluated time-dependent responses of the macrophage lipidome and transcriptome to 25OHC treatment. In addition to altering specific aspects of cholesterol and sphingolipid metabolism, we found that 25OHC activates integrated stress response (ISR) genes and reprograms protein translation. Effects of 25OHC on ISR gene expression were independent of liver X receptors (LXRs) and sterol response element binding proteins (SREBPs) and instead primarily resulted from activation of the GCN2/eIF2α/ATF4 branch of the ISR pathway. These studies reveal that 25OHC activates the integrated stress response, which may contribute to its anti-viral activity.

Introduction
Macrophages and related cell types play key roles in both innate and adaptive immunity. Tissue-resident macrophages function as sentinels that detect invading pathogens through pattern recognition receptors (PRRs), which activate intrinsic anti-microbial activities and induce the elaboration of cytokines and chemokines that amplify the initial inflammatory response, recruit additional immune cells, and commence acquired immunity (1-3). The interferon (IFN) family of cytokines is a key component of innate immunity and a first line of defense against viral infection. Type-I IFNs trigger a signaling cascade resulting in amplification of hundreds of interferon-stimulated genes (ISGs), many of which have unclear roles in viral immunity (4).

Cholesterol 25-hydroxylase (Ch25h) is a recently identified ISG that encodes an ER-associated glycoprotein that catalyzes the production of 25OHC from cholesterol (5). Ch25h is highly induced in macrophages following
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stimulation with the TLR4 ligand Kdo2 lipid A contemporaneously with secondary response genes that are dependent on type I interferon production (6,7). 25OHC regulates cholesterol metabolism, acting as a suppressor of cholesterol biosynthesis through inhibition of SREBP proteolytic processing (8), and as a ligand for liver X receptors (LXRs), enabling LXR-mediated induction of genes that encode proteins mediating cholesterol export (9). CH25H and its product 25OHC play roles in regulating both innate and adaptive immunity. Mice lacking Ch25h exhibit abnormal immunoglobulin A production by B lymphocytes (6) and dysfunctional B cell migration within follicles (10). Recent studies demonstrated a broad and potent antiviral activity of CH25H and 25OHC (11,12) and suggested that 25OHC impedes viral infection at multiple stages.

The integrated stress response (ISR) is a shared stress response pathway that can be initiated by the four eIF2α kinases: Heme-regulated inhibitor (HRI), Protein kinase R (PKR), PKR-like ER kinase (PERK), and general control non-derepressible 2 (GCN2). Each kinase is activated by various sources of cellular stress and phosphorylates eIF2α (13,14). Phosphorylation of eIF2α rapidly suppresses initiation of protein translation but paradoxically there is increased production of the transcription factor ATF4 causing induction of certain stress-related genes (15,16). Together, suppression of protein translation and induction of stress response genes function to either alleviate cellular stress and restore homeostasis or trigger apoptosis if the stress cannot be corrected (17,18). Additionally, the phosphorylation of eIF2α by PKR and other eIF2α kinases is an important anti-viral mechanism that suppresses infection by inhibiting protein translation and stimulating apoptosis (4,19-21).

To gain further insights into the anti-viral activities of 25OHC, we performed lipidomic and transcriptomic analysis of 25OHC-treated bone marrow-derived macrophages (BMDMs). We found that 25OHC treatment significantly altered specific subsets of lipids and activated a robust integrated stress response that reprogrammed macrophage transcription and translation.

Experimental Procedures:
Materials
Tissue culture plastic was purchased from Fisher Scientific. Tissue culture medium and other tissue culture reagents were obtained from Mediatech Inc. 25OHC and Tunicamycin were purchased from Sigma-Aldrich, and EC from Biomol, and 27OHC from Research Plus, Inc., and Kdo2 from Avanti Polar Lipids. Supplemental amino acids, Glutathione and N-Acetyl Cysteine were purchased from Sigma-Aldrich.

Mice and Primary Cells
C57BL/6 thioglycollate-elicited macrophages and
Bone marrow-derived macrophages were obtained from WT and CH25H KO (6), LXR DKO (22,23), INSIG2 KO (24), and GCN2 KO (25) mice and cultured as described previously (26). For oxysterol experiments, BDMDs or thioglycollate elicited macrophages were plated in RPMI-1640 supplemented with 10% lipid reduced FBS (HyClone) for 18 h before treatment. For RNAi experiments in BMDMs, cells were transfected with control or smartpool siRNAs (40 nM, Dharmacon) directed against ATF4, HRI, PKR, PERK, GCN2, Insig-1 by using lipofectamine 2000 (Invitrogen). Cells were used for experiments after 48 h incubation, and target gene knockdown was validated by Q-PCR. MCMV infections of primary BMDMs used strain MW97.01 at the indicated multiplicities of infection.

**Lipid measurements**

Liquid chromatography/mass spectrometry measurements were performed to measure lipid species following detailed protocols available online:

http://www.lipidmaps.org/protocols/index.html

**Immunoblot analysis**

The BMDMs were lysed in a buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1% TritonX-100, 100 mM NaF, 100 mM Sodium pyrophosphate, 17.5mM β-glycerophosphate, and 1x complete protease inhibitor cocktail (Roche) and 1x PhosSTOP Phosphatase inhibitor cocktail (Roche). Each sample (20 mg) was separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were incubated with anti-eIF2a antibody, anti-phospho-eIF2a antibody, anti-CHOP antibody (Cell Signaling).

**RNA analysis**

Total RNA was purified and analyzed by microarray and quantitative PCR as previously described (27). cDNA was synthesized from 1 mg total RNA and used for quantitative PCR with gene-specific primers. Mouse 36b4 mRNA was used as an invariant control. PCR primers used were as follows (5’ to 3’): 36b4, GCT CGA CAT CAC AGA GCA GG and CCG AGG CAA CAG CAG TTG GGT AC; Ch25h, CCA TCT TTA CCT TTC ACG TGA TTA AC and CAG CCA AAG GCC ACA AGT CT; Ddit3/CHOP, GCA GCG ACA GAG CCA GAA TAA and TGT GGT GTT GTA TGA AGA TGC A; Chac1, GGC TTC GTT CGT GGC TAT AGC and CAG CCC TCA CGG TCT TCA AG; Trib3, CTT GCG CGA CCT CAA GCT and ATC ACG CAG GCA TCT TCC A; Atf4, CTC GGA ATG GCC GGC TAT and GTC CCG GAA AAG GCA TCC T; Asns, GCC ATG ACA GAA GAT GGG TTT C and AAG GGA GTG GTG GAG TGT TTT AAG

**Global protein translation**

After the indicated cell incubations, the medium
was switched to RPMI-1640 without methionine/cysteine. After 60 min, the cells were incubated with EasyTag EXPRESS \(^{35}\)S Protein Labeling Mix (PerkinElmer) at 20 mCi ml\(^{-1}\) for 20 min. The cells were then lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, and 1x complete protease inhibitor cocktail. Proteins in the lysate were precipitated using trichloroacetic acid and radioactivity of the precipitated proteins was measured by a scintillation counter.

Translating Ribosome Affinity Purification: Construct design and isolation protocol

The Csf1r-EGFP-L10a transgenic construct was produced by first subcloning EGFP-L10a fusion protein construct from S296.EGFP-L10a into p3XFLAG-CMV-7.1 (Sigma-Aldrich) at NotI and XbaI sites. The fragment containing 3XFLAG-EGFP-L10a was then subcloned into ApaI and NotI sites of p7.2cfms-egfp thereby replacing the egfp previously contained in the destination plasmid. The cfms (Csf1r) promoter-3XFLAG-EGFP-L10a transgenic construct was then linearized by digestion with MluI and Sall and utilized for production of transgenic mice in the C57Bl/6 strain. The cell specific expression of the Csf1r-EGFP-L10a construct was analyzed by flow cytometry. Mouse blood was extracted and anticoagulated with EDTA before blocking with 0.5ug anti-CD16/32 Fc Ab (eBioscience) for 30m at 4C. Cells were stained for 30 min on ice in staining buffer containing DPBS + 5% FBS + 5mM EDTA using CD115-APC Ab (eBioscience), CD11b-PE (Biolegend) Ab, and Gr1-PECy7 Ab (eBioscience). RBCs were lysed using whole blood lysis reagent kit (Beckman Coulter). Samples were processed and analyzed using a BD LRSII flow cytometer (Becton Dickinson, San Jose, CA) and FlowJo software. BMDMs (1x10^7) from Csf1r-EGFP-L10a transgenic mice were treated with DMSO, 25OHC, or Tunicamycin for indicated times and total RNA or translating RNA was isolated by RNeasy (Qiagen) directly or following TRAP as previously described (28) except that cell scraping in ice cold lysis buffer containing NP40 0.5% was used in lieu of homogenization. Ribosomal RNA was removed from total RNA or TRAP isolated RNA by polyA selection as previously described or using Ribo-Zero rRNA removal kit (Epicentre). Ribosomal depleted mRNA was prepared for sequencing as previous described (29). RNA libraries underwent Illumina sequencing at the UCSD Biogem Core. 2 (Tunicamycin) or 3 (DMSO and 25OHC) independent biological replicates were performed for each treatment.

Gene Ontology and Motif analysis

Gene Ontology (GO) enrichments for groups of regulated genes were performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (30,31). De novo motif
discovery was carried out using Homer software UCSD(26). The parameters used were mouse (promoter set), -start -500 –end 100 (promoter region search from -500 base pairs (bp) from TSS to +100 bp from TSS), -len 8,10,12 (motif length bp). Sequences were searched in both forward and reverse complement orientations.

Virus experiments
Tissue culture-derived MCMV strain K181 was prepared in BALB/c mouse embryonic cells and titered on NIH 3T3 cells (ATCC CRL 1658) as previously described (32,33). BMDMs were infected with MCMV at MOIs ranging from 0.1 to 10 PFU/cell. Virus was adsorbed for 6 h, the inoculum was removed, and the cells were fed with fresh medium. RNA analysis and global protein translation analysis were done as described above. Infectious MCMV was quantified by plaque assay on NIH 3T3 monolayers. Dilutions of the culture medium from the infected BMDMs were adsorbed to the cells in 0.25 ml of DMEM–5% CS with occasional agitation. After a 5 h adsorption period, an overlay of 1 ml of DMEM–5% CS and 0.4% agarose was added. Plaques were fixed 5 days later, stained with crystal violet, and counted. Each titer was calculated as the mean titer (PFU/ml) of triplicate infections and a single plaque assay for each infection.

Statistical analysis
Statistical analyses were performed with two-way ANOVA test for lipid analysis, with ANOVA and Bonferroni Multiple Comparison Test for MCMV plaque assay, and with Student’s t test for other experiments.

Results
25OHC alters cholesterol ester and sphingolipid formation
To investigate the effects of 25OHC on macrophages, BMDMs cultured in standard 10% serum-containing media or 10% lipid-deficient serum-containing media plus compactin were treated with 5 µM 25OHC for times ranging from 30min to 24h. Lipid and gene expression changes were evaluated by mass spectrometry and microarray analysis, respectively. Culture in lipid-deficient serum (LDS) plus compactin depletes cellular cholesterol, maximally increasing SREBP activity and inhibiting LXR activity as compared to culture under standard conditions. We quantified 569 lipid species in three independent experiments (Raw lipid data are available at: http://www.lipidmaps.org/data/results/primarymacrophage/25OHC/index.html). Two-way ANOVA analysis of 25OHC treated BMDMs identified significant changes in cholesterol ester (CE) and sphingolipids levels (Figure 1A). CE(18:1), CE(16:0) and CE(18:0) were quantitatively the most abundant species to accumulate, and exhibited 3 to 7-fold increases over baseline levels
25-hydroxycholesterol causes an integrated stress response (Figure 1B). Additionally, 25OHC increased ceramide and glucosylerceramide levels while decreasing sphingomyelins, primarily at late time points of 12 and 24 hours (Figure 1A). The majority of 25OHC dependent changes in CE and sphingolipid levels occurred in BMDMs cultured in both standard 10% serum-containing media and 10% LDS-containing media (Figure 1A).

**25OHC suppresses SREBPs, activates LXRs, and induces stress response genes**

Parallel analysis of the macrophage transcriptome demonstrated the expected changes in SREBP and LXR target genes in response to both alterations in media lipid content and treatment with 25OHC (Figure 1C, Supplemental Dataset S1). Culture in LDS caused induction of Hmger mRNA levels (Figure 1D) while 25OHC suppressed Hmger mRNA levels and induced Abca1 (Figure 1C, 1D). Larger magnitude differences in SREBP and LXR (Hmger and Abca1) regulated genes were notable in BMDMs grown in LDS + compactin, as these conditions maximally activate SREBPs and repress LXRs prior to treatment with 25OHC (Figure 1C). In light of the robust effects of 25OHC on SREBP and LXR target genes, the effects of 25OHC on the lipidome are more selective than would be expected, suggesting roles of post transcriptional mechanisms in maintenance of lipid homeostasis.

LXRs are the only known targets of 25OHC that directly induce gene expression, however, the majority of 25OHC stimulated genes identified in the transcriptional analysis are not established LXR targets. In fact, the upregulated set of genes was most significantly enriched for functional annotations related to ER Golgi transport and response to ER stress (Figure 2A). Aft4, Chop/Ddit3, Chac1, Trib3, and asparagine synthetase (Asns) are up-regulated during cellular responses to stress and were among the most highly induced genes by 25OHC (Figure. 1C, Supplemental Dataset S1) (13,34). Q-PCR assays in BMDMs confirmed that stress response genes were induced by 25OHC in a concentration and time-dependent manner (Figure 2B, 2C).

**MCMV infection stimulates 25OHC production and induces stress response gene transcription**

Ch25h is an interferon-stimulated gene and two recent independent publications demonstrated that CH25H and its product 25OHC have broad anti-viral activity (11,12). To determine if the concentrations of 25OHC produced during viral infections were capable of inducing ISR genes we infected BMDMs with MCMV. Consistent with previous findings, we independently found that 25OHC suppressed MCMV infection at various multiplicty of infection (MOI) (Figure 2D). Infection with MCMV strongly activated Ch25h dependent 25OHC production (Figure 2E). There were no increases in the production of other measurable oxysterols, exemplified by 27OHC.
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and 24,25-EC (Figure 2F, 2G). MCMV infection had no significant effect on total cholesterol (free + esterified), but had divergent effects on desmosterol and 7-dehydrocholesterol in both WT and CH25H KO BMDMs. (data not shown). As desmosterol and 7-dehydrocholesterol are the last intermediates of the Bloch and Kandutsh-Russel pathways of cholesterol biosynthesis, respectively, these results suggest complex effects of MCMV infection on late stages of cholesterol biosynthesis.

BMDMs infected with MCMV showed significant induction of Chop at 6, 24, and 48 hours (Figure 2H). Increases in Chop mRNA were partially dependent on endogenous synthesis of 25OHC as WT BMDMs had significantly more Chop mRNA when compared to CH25H KO BMDMs (Figure 2I). The decrease in Chop induction in CH25H KO macrophages was rescued by exogenous treatment with 25OHC (Figure 2I). MCMV, like human cytomegalovirus (HCMV), stimulates multiple stress response pathways during infection that likely account for the 25OHC-independent elevations in Chop mRNA (35,36). Thus, macrophage production of 25OHC during MCMV infection contributes to, but is not solely responsible for, stress gene activation during MCMV infection of macrophages.

25OHC induction of stress response genes is independent of LXRs and SREBPs

We next investigated potential roles of LXR agonists in mediating induction of stress response genes by 25OHC. Treatment of macrophages with the synthetic LXR agonist GW3965 did not stimulate stress response gene expression (Figure 3A). Additionally, 25OHC induced stress response genes in LXR DKO macrophages (Figure 3B). Furthermore, increases in stress gene transcription were significantly greater in response to 25OHC than 27-hydroxycholesterol (27OHC) or 24,25-epoxycholesterol (EC), which are also LXR agonists (Figure 3C). These results indicate that 25OHC activates stress response genes independently of LXRs.

SREBP processing and resulting transcriptional activity is regulated by the INSIGs and SCAP proteins (37). SCAP is required for transport of SREBPs from the endoplasmic reticulum (ER) to Golgi for proteolytic processing. INSIGs are directly bound by oxysterols, causing them to interact with SCAP and sequester SREBPs in the ER (8). Knockdown of Scap using siRNAs did not alter 25OHC induction of stress response genes (Figures 3D, 7A), indicating that this effect is independent of SREBP processing. We next investigated possible SREBP-independent roles of Insigs by performing loss of function experiments. BMDMs from INSIG2 KO mice showed similar stress response gene induction by 25OHC compared to WT BMDMs (Figures 3E, 7B). We detected a small but significant reduction of 25OHC-dependent stress response gene induction in both WT and INSIG2 KO BMDMs in which
Insig1 was knocked down (Figures 3F, 7B). The effect of knocking down INSIG1 is similar irrespective of the presence of INSIG2 even though binding of 25OHC to INSIG2 is sufficient to block SREBP processing. This suggests that INSIG1 partially contributes to 25OHC dependent stress response gene induction independent of its effects on SREBP processing.

Combined actions of 25OHC on LXR and SREBP can reduce cellular cholesterol levels by simultaneously suppressing cholesterol synthesis and promoting cholesterol efflux. To determine if cholesterol depletion was responsible for induction of stress response genes we treated macrophages with 25OHC in the presence and absence of exogenous supplementary cholesterol. The addition of exogenous cholesterol did not significantly inhibit the induction of stress response genes in 25OHC treated macrophages (Figure 3G).

25OHC activates the GCN2/eIF2α/ATF4 branch of the Integrated Stress Response

ATF4 and eIF2α integrate signals from four eIF2α kinases (HRI, PKR, PERK, and GCN2), which each respond to diverse stress signals, to activate a shared stress response pathway called the ISR. During the ISR, eIF2α is phosphorylated on serine 51 resulting in suppression of de novo protein synthesis and induction of ATF4 dependent stress response genes that together function to alleviate cell stress (13).

BMDMs treated with 25OHC significantly increased levels of eIF2α phosphorylation and CHOP protein levels (Figure 4A). Elevations in phosphorylated eIF2α occur in a time dependent manner with small increases in phosphorylation as early as 8 hours and maximal phosphorylation occurring after 16-24 hours (Figure 4B). Additionally, 25OHC was the only oxysterol that consistently increased eIF2α phosphorylation (Figure 4C). Phosphorylation of eIF2α inhibits GTP recycling in the ternary complex, eIF2-GTP-tRNA^Met, thereby suppressing initiation of protein translation. Relatively small increases in eIF2α phosphorylation can significantly inhibit protein translation as its target eIF2β is present in cells at relatively low concentrations (38). BMDMs treated with 25OHC showed depressed levels of active protein translation comparable with the well-known ER stress inducer, tunicamycin (Figure 4D). The kinetics of de novo protein synthesis inhibition in response to 25OHC occurred maximally at later time points consistent with the time dependent phosphorylation of eIF2α (Figure 4E).

Phosphorylation of eIF2α during the ISR paradoxically increases ATF4 protein levels due to preferential ATF4 translation with or without increases in Atf4 transcription leading to increased transcriptional activity at ATF4 target genes (16,39). Targeted knockdown of Atf4 significantly reduced induction of stress response genes by 25OHC demonstrating that full transcriptional
activation of these genes by 25OHC is ATF4-dependent (Figures 4F, 7C).

Four eIF2α kinases (HRI, PKR, PERK, and GCN2) recognize diverse forms of cell stress and trigger the ISR by phosphorylating eIF2α and activating ATF4 signaling (13). Knockdown of the eIF2α kinases in BMDMs suggested that GCN2 was primarily responsible for 25OHC dependent activation of stress related genes (Figures 4G, 7D). Consistently, BMDMs from GCN2KO mice showed significantly decreased transcription of stress related genes and eIF2α phosphorylation following 25OHC treatment (Figure 4H, 4I). The response to 25OHC in GCN2KO macrophages was not abolished, however, suggesting redundancy in ISR activation pathways that can be seen with overlapping stress stimuli (14).

25OHC reprograms transcription and translation

To further investigate effects of 25OHC on translation, we generated transgenic mice expressing EGFP-ribosomal protein L10a under the transcriptional control of Csf1r regulatory elements (Figure 5A). As previously described for the Csf1r-EGFP mouse (40) the expression pattern of the EGFP-L10a fusion protein in the Csf1r-EGFP-L10a mouse is restricted to the myeloid lineage as demonstrated by detection of GFP only in CD11b+ and CD115+ cells (Figure 5B, 5C). We detected GFP expression in both mononuclear cells (CD115+) and granulocytes (CD115low Gr1+) (Figure 5D, 5E). The EGFP-L10a system allows efficient isolation of polysomes containing mRNA undergoing active translation using a method termed translating ribosome affinity purification (TRAP) (28). Using TRAP we isolated sufficient quantities of mRNA undergoing active translation from BMDMs to perform RNA-Seq analysis. In parallel, RNA-Seq analysis was performed on total RNA. Relative changes in normalized tag counts for each RefSeq transcript identified by both TRAP and total RNA-Seq are shown in Figure 6A, plotted without (left scale bar) or with (right scale bar) an estimated correction (40%) for the global effect of 25OHC on translation. Genes shown to be induced by ATF4 and CHOP during the ER stress response demonstrated significantly increased transcription and translation in response to 25OHC treatment (Figure 6B) (41). The transcriptional and translational effects of 25OHC on ATF4 and CHOP targets were similar but less robust than effects seen following treatment with Tunicamycin, which is known as a potent inducer of ER stress (Figure 6B). 25OHC significantly increased the total mRNA of 455 genes and translating mRNA of 66 genes, with 46 genes significantly up-regulated both transcriptionally and translationally (Figure 6C). As this analysis does not correct for global effects on translation, transcripts undergoing decreased translation are under-represented and those undergoing increased translation are over-represented. GO analysis of
genes translationally up-regulated >1.5 fold were enriched for functional annotations related to response to ER stress, amino acid activation, and protein transport (Figure 6E). Multiple ISR genes, including Trib3, Ddit3/Chop, Asns, Atf4, and Ppp1r15a/Gadd34, were among the top 100 transcriptionally and top 66 translationally up-regulated genes (Dataset S2 and S3). Analysis of total and TRAP isolated mRNA revealed transcriptional suppression of 670 genes and decreased translation of 205 genes in 25OHC treated BMDMs, with 109 genes down-regulated both transcriptionally and translationally (Figure 6D). Thus, even without correction for global translation, 25OHC down-regulates 205 genes compared to up-regulation of only 66 genes at the translational level, which is consistent with a predominantly suppressive effect on protein translation. GO analysis of translationally suppressed genes >1.5 fold showed enrichment for functional annotations related to the immune and inflammatory responses (Figure 6E). Ldlr and Dhc24 genes were among the top 10 transcriptionally down-regulated genes consistent with the known suppression of SREBP processing by 25OHC (Dataset S4) (42).

Based on our experiments GCN2 is the primary stress response kinase responsible for activating the ISR in macrophages following 25OHC treatment. GCN2 primarily recognizes the stress of amino acid limitation but can be activated by UV irradiation, proteasome inhibition, and some viral infections, although the mechanism by which these stimuli activate GCN2 are less clear (43-45). GO analysis of 25OHC-induced genes identified enrichments in tRNA aminoacylation/amino acid activation. Additionally, we found significant induction of Atf4, Asns, Cebp, Ddit3, Slc38a2, Scl7a1, and Trib3, which are all genes known to be up-regulated in response to amino acid deprivation that carry an amino acid response element (AARE) bound by ATF4 (46). These findings are consistent with the idea that 25OHC activates the amino acid deprivation stress response via activation of GCN2. To test if amino acid limitation was responsible for 25OHC-dependent GCN2 activation we supplemented BMDMs with amino acids, including cysteine whose insufficiency has previously been shown to trigger GCN2 to activate the ISR (47). BMDMs supplemented with L-cysteine during treatment with 25OHC showed a significant decrease in transcription of stress related genes whereas supplementation with L-asparagine or L-alanine had no effect (Figure 6F). Our transcriptional analysis of 25OHC treated macrophages showed significant upregulation of Asns, Cars, Cebp, Cebp, Gadd45a, and Trib3, all of which were previously shown to be induced genes in response to cysteine deprivation in HepG2/C3a cells (46).

ATF4 induces genes important for amino acid import, glutathione biosynthesis, and resistance to oxidative stress. We performed de
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 novo motif analysis of promoters of genes significantly induced by 25OHC and identified the Nrf2 (NFE2L2) motif as the most highly enriched motif (Figure 6G). Nrf2 is a major regulator of the antioxidant response that is activated under conditions of oxidative stress, dimerizes with ATF4 and other bZIP transcription factors, and binds to antioxidant-responsive element sites in the promoters of target genes (48,49).

In addition to serving as an essential sulfur containing amino acid, cysteine is also a precursor for the biosynthesis of glutathione (44). The capacity of cysteine to act as a precursor for glutathione, the importance of the ISR in alleviating oxidative stress, and our motif analysis identifying Nrf2, a master regulator activated by oxidative stress, together led us to test whether treatment with anti-oxidants could reduce ISR gene transcription. BMDMs treated with either glutathione or N-acetyl cysteine (NAC) showed an almost complete loss of ISR gene induction in response to 25OHC (Figure 6H).

Discussion

The lipidomic and transcriptomic analysis of 25OHC-treated macrophages identified significant alterations in select subsets of lipid species including cholesterol esters and sphingolipids and the unexpected activation of the integrated stress response pathway. Activation of the ISR was specific to 25OHC, compared to other oxysterols, and this effect was independent of the known effects of 25OHC on SREBP processing and LXR activation. We independently corroborated recent publications demonstrating the anti-viral activity of 25OHC and showed that endogenously produced 25OHC contributes to activation of ISR genes during MCMV infection. Furthermore, 25OHC activates the ISR primarily by triggering the eIF2α kinase GCN2. This activation can be suppressed by cysteine supplementation and addition of antioxidants suggesting that 25OHC stimulates the ISR by altering amino acid metabolism and/or increasing oxidative stress in macrophages.

The observation of dramatic increases in cholesterol ester formation is consistent with previous studies indicating that 25OHC causes recycling of membrane cholesterol to the ER (50,51). The basis for the increase in ceramides and decrease in sphingomyelins is not yet clear. Sphingomyelin biosynthesis involves CERT-dependent transport of ceramide from the ER to Golgi, which might be disrupted by the ISR or perhaps by 25OHC itself since the transport protein has been shown to be inhibited by isoprenoids (liminoids) (52). In addition, under some conditions, cholesterol depletion can suppress de novo sphingomyelin production (53). Sphingomyelin might also decrease due to turnover in ceramide, which is elevated by ROS (54). This effect apparently depends on the cell type because an opposite effect (decreased ceramides and increased sphingomyelins) has been
reported for CHO cells (55). Further studies will be required to determine the mechanism for these changes.

In addition to its classical functions of suppressing SREBP processing and activating LXRs, 25OHC activated the GCN2/eIF2α/ATF4 branch of the ISR causing global transcriptional and translational reprogramming in the macrophage. The precise trigger by which 25OHC activates GCN2 remain to be established, but appears to involve increases in oxidative stress and/or depletion of specific amino acids including cysteine. Activation of the ISR by GCN2 is an important protective mechanism against both oxidative stress and amino acid limitation (44). The amino acid cysteine occupies an interesting position at the interface of amino acid metabolism and oxidative stress as it serves as both an amino acid required for efficient protein synthesis and as a precursor for thiol-containing peptides and proteins, like glutathione, involved in redox reactions. GCN2 is activated by uncharged tRNAs present during amino acid deficiency, however the mechanism by which GCN2 is activated by other stimuli such as UV radiation or oxidative stress is unclear (25,56). Cysteine is extremely unstable and the majority of intracellular cysteine is incorporated into glutathione, which serves as the major storage site of intracellular cysteine (57,58). One potential mechanism by which GCN2 could recognize increasing oxidative stress would be to recognize a deficiency in intracellular cysteine that develops as levels of glutathione are depleted. Our analysis provides initial evidence suggesting that oxidative stress and/or cysteine limitation is important for 25OHC mediated activation of GCN2. However, additional studies will be needed to determine the exact stress signal to which GCN2 is responding.

Although two recent independent studies demonstrated the broad anti-viral activity of 25OHC, the mechanism by which 25OHC suppresses viral infection is still unclear (11,12,59). We demonstrate that 25OHC significantly alters important plasma membrane lipid species and activates the ISR both of which may contribute to the anti-viral activity of 25OHC.

Experiments described by Liu et al. suggest that inhibition of viral entry is a major mechanism by which 25OHC suppresses viral infection (12). Our lipidomic analysis demonstrated large increases in cholesterol esters consistent with significant recycling of membrane cholesterol to the ER and decreases in sphingomyelin. Cholesterol and sphingomyelin are the major components of plasma membrane microdomains also referred to as “lipid rafts”. As many viruses rely on membrane microdomains for entry, alterations in plasma membrane cholesterol or sphingomyelins disrupt membrane microdomains and interfere with viral infectivity (60-62). As 25OHC significantly modifies both plasma membrane cholesterol and sphingomyelin it likely modifies membrane microdomains and
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this may account for the 25OHC-dependent inhibition of viral entry previously described.

Increasing evidence suggests that substantial crosstalk exists between innate immune signaling and stress response pathways (63). Viruses rely on host metabolic functions, including protein synthesis for survival and propagation (64). Activation of the ISR during viral infection is an important innate immune mechanism that can inhibit some but not all viral infections by suppressing protein translation (4,64).

Consistent with this idea, activation of GCN2 is an important protective immune response to both intracellular bacterial and viral infections (43,65-67). Our studies demonstrate that 25OHC treatment of macrophages activates GCN2 causing suppression of protein synthesis and that endogenous production of 25OHC by CH25H during MCMV infection contributes to ISR gene induction in macrophages. Activation of GCN2 by 25OHC may contribute to its anti-viral properties against certain viruses.

References

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Acknowledgements

We thank Dr. Joseph Goldstein (UT Southwestern Medical Center) for critical comments regarding 25OHC, Dr. David Hume (Roslin Institute) for providing the p7.2cfms-egfp plasmid, and Nathaniel Heintz (The Rockefeller University) for the S296.EGFP-L10A plasmid. Microarray analysis, RNA-Seq, and ribosome-protected RNA sequencing were carried out by BIOGEM at University of California, San Diego. Transgenic mouse generation was performed at the Transgenic and Gene Targeting Core at UCSD. These studies were primarily supported by NIH grant GM U54 069338 to the LIPID MAPS Consortium. Additional support was provided by the Clayton Foundation for Research (D.W.R.), and the American Heart Association (N.S.).

Figure legends

Figure 1 Lipidome and transcriptome analysis in 25OHC-treated BMDMs. (A) Heat map of (A) cholesteryl ester (CE) and sphingolipid changes. (B) Cellular content of the indicated CE in figure 1A. (C) Transcriptional profiling in BMDMs treated with 5mM of 25OHC for 0.5, 1, 2, 4, 8, 12, 24h. (D) Indicated genes expression changes from transcriptomic analysis in figure 1C. For figures 1A and 1C, log2 based transformation on ratio fold change values are used where red shading indicates up-regulation and green indicates down-regulation. In 1B and 1D data are plotted as mean values ± SEM. For each group, n=3.

Figure 2 25OHC induces stress related genes (A) Gene Ontology (GO) terms significantly enriched in up-regulated genes in BMDMs treated with 25OHC (vs DMSO solvent) for 24h. Benjamini-corrected –log p-values are shown. (B) Expression of Trib3 and Chac1 mRNA in BMDMs treated with 25OHC at increasing doses for 24h or (C) treated with fixed 25OHC concentration (5 µM) measured over increasing time. (D) BMDMs were infected with MCMV (MOI=0.1, 1, 5, 10) +/- 25OHC (5 µM) for 72h and yield of cell-free virus was determined by plaque assay. Data are plotted as mean values ± SD. For each group, n=3. *, p<0.05 or **, p<0.01 compared to control. Cellular content of (E) 25OHC, (F) 27OHC, or (G) 24,25 EC in WT or CH25H KO BMDMs infected with MCMV (MOI=3) for 24h. (H) Expression of Chop mRNA in BMDMs infected with MCMV (MOI=3) for indicated time. (I) Expression of Chop mRNA in WT or CH25H KO BMDMs infected with MCMV in the presence or absence of 25OHC for 24h at indicated MOI. Unless otherwise indicated data are plotted as mean values
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± SEM. For each group, n=3. *, p<0.05 or **, p<0.01.

Figure 3 25OHC induces stress related genes independently of LXRs and SREBPs (A)
Expression of Chop mRNA in WT or (B) LXR deficient BMDMs treated with 25OHC (5µM) or the LXR specific agonist GW3965 (1µM) for 24h. (C) Expression of Trib3 and Chac1 mRNA in BMDMs treated with (5µM) of the indicated oxysterols for 24h. (D) Expression of Chop mRNA in BMDMs +/- Scap knockdown treated with 25OHC (5µM) for 24h. Expression of Trib3 mRNA in WT and Insig2 KO BMDMs treated with 25OHC (5µM) for 24 h (E) without or (F) with concurrent knockdown of Insig1. (G) Expression of Trib3 mRNA in BMDMs treated with 5µM of 25OHC +/- 10mg/ml of cholesterol for 24h. For experiments in (E and F) each group represents samples from two separate experiment, n=6. *p<0.01 siCTL compared to siInsig1 treatment. For all other experiments data are plotted as mean values ± SEM. For each group, n=3 and *p<0.05 and ** p<0.01 compared to control.

Figure 4 25OHC activates the GCN2/eIF2α/ATF4 branch of the Integrated Stress Response (A, B, C) Protein levels of eIF2α, phosphorylated eIF2α (p-eIF2α), and CHOP in BMDMs treated with 25OHC (5µM), DMSO, or indicated oxysterols (5µM) for 24 hours, or for indicated times. (D, E) Incorporation of pulse-labeled 35S-methionine-cysteine into newly translated proteins in BMDMs treated with Tunicamycin (TN) (2.5 mg/ml) for 6h or 25OHC (5µM) and DMSO for 24 hours, or for indicated times. (F) Expression of Trib3 in BMDMs with knockdown of Atf4 treated with 25OHC (5µM) or DMSO for 24 h. (G) Expression of Chac1 mRNA in BMDMs with knockdown of eIF2α kinases Eif2ak4/GCN2, Eif2ak1/HRI, Eif2ak2/PKR, and Eif2ak3/PERK treated with 25OHC (5µM) or DMSO for 24 h. (H) Expression of Chac1 mRNA in WT or GCN2 KO BMDMs treated with 25OHC (5µM) or DMSO for 24h. (I) Protein levels of eIF2α and phosphorylated eIF2α (p-eIF2α) in WT and GCN2 KO BMDMs treated with 25OHC (5µM) or DMSO for 24 h. Data are plotted as mean values ± SEM. For each group, n=3. *, p<0.05 **, p<0.01 ***, p<0.001 compared to control.

Figure 5 Csf1r-EGFP-L10a mice express the fusion protein in monocytes and granulocytes (A) Cartoon depiction of Csf1r-EGFP-L10a transgenic construct containing the Csf1r promoter including intron, 3 FLAG repeats and EGFP-L10a fusion protein. Mouse blood was analyzed for the coexpression of GFP with (B) CD11b or (C) CD115. (D) Coexpression of Gr1 and CD115 with (E) subpopulation analysis of GFP expression in CD115+ monocytes or CD115low Gr1+ granulocytes or CD115’Gr1’ cells.

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Figure 6 25OHC reprograms macrophage transcription and translation (A) Ratio of transcriptional (total mRNA) and translational (TRAP mRNA) change in BMDMs treated with or without 25OHC (5µM) for 24h. Black and red dots correspond to Refseq ATF4 and CHOP target transcripts (41), respectively. Data plotted without (left scale bar) or with (right scale bar, blue shading) an estimated correction (40%) for the global suppressive effect of 25OHC on translation. (B) RNA-Seq-determined transcriptional and translational expression levels comparing all genes (Grey) with AT4 and CHOP target genes (Red) in BMDMs treated with 25OHC or Tunicamycin. P-values are from one-tailed t-tests comparing all genes to AT4 and Chop target genes. Venn diagram of transcriptionally and translationally (C) up-regulated and (D) down-regulated genes (>1.5x) and their overlap following 25OHC treatment of BMDMs. (E) GO terms associated with genes up-regulated or down-regulated (>1.5x) at the translational level (F) Expression of Trib3 mRNA in WT BMDMs treated with 25OHC (5µM) or DMSO +/- (1mM) L-cysteine, L-asparagine, or L-alanine for 24h. (G) Promoter motifs identified by de novo motif analysis of the promoters (interval from -500 to +100 bp from the TSS) of genes transcriptionally upregulated >1.5x by 25OHC. (H) Expression of Trib3 mRNA in WT BMDMs treated with 25OHC (5µM) or DMSO +/- Glutathione (15mM) or N-acetylcysteine (15mM) for 24h. Unless otherwise indicated data are plotted as mean values ± SEM. For each group, n=3. *, p<0.05 **, p<0.01 compared to control.

Figure 7 Knockdown efficiency of siRNA experiments (A) Expression of Scap mRNA in BMDMs in which Scap has been knocked down using Scap specific siRNAs treated with 5µM of 25OHC or DMSO for 24 h. (B) Expression of Insig1 mRNA in WT and Insig2 KO BMDMs in which Insig1 has been knocked down using Insig1 specific siRNAs treated with 5µM of 25OHC or DMSO for 24 h. (C) Expression of Atf4 mRNA in BMDMs in which Atf4 has been knocked down using Atf4 specific siRNAs treated with 5µM of 25OHC or DMSO for 24 h. (D) Expression of eIF2α kinase mRNA in BMDMs in which eIF2α kinases have been individually knocked down with specific siRNA to Eif2ak4/GCN2, Eif2ak1/HRI, Eif2ak2/PKR, and Eif2ak3/PERK and treated with 5µM of 25OHC or DMSO for 24 h as determined by Q-PCR and normalized to 36B4 expression. For experiments in (B) each group represents samples from two separate experiment, n=6. *p<0.01 siCTL compared to siInsig1 treatment. Data are plotted as mean values ± SEM. For all other experiments data are plotted as mean values ± SEM. For each group, n=3. *p<0.01 compared to siCTL treatment.
Figure 3

A) WT Chop

B) LXR DKO Chop

C) Trib3

D) WT Insig2KO

E) siCTL siScap

F) siCTL siInsig1

G) Mock Cholesterol Rx

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**Fig. 4**

**A**

DMSO 25OHC

- p-eIF2α
- CHOP
- eIF2α

**B**

25OHC (hrs)

- 1 2 4 8 12 16 24

- p-eIF2α
- eIF2α

**C**

DMSO 25OHC 27OHC EC 24OHC 22OHC

- p-eIF2α
- eIF2α

**D**

Incorporation rate of

- 3S Met & Cys

- DMSO
- 25OHC
- TN

**E**

Global Protein Translation

- Relative Incorporation

- S35

- 2h 4h 8h 12h 16h 24h

- DMSO
- 25OHC

**F**

Trib3

- Relative mRNA

- siCTL
- siAtf4

**G**

Chac1

- Relative mRNA

- DMSO
- 25OHC

- siCTL
- siAtf4
- siGCN2
- siHRI
- siPKR
- siPERK

**H**

Chac1

- Relative mRNA

- DMSO
- 25OHC

- WT
- GCN2KO

25OHC

- p-eIF2α
- eIF2α

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Figure 6: Analysis of gene expression and functional annotations.

A) Scatter plot showing Log2 translational change (25OH/DMSO) versus Log2 transcriptional change (25OH/DMSO) for all genes.

B) Box plots comparing transcriptional and translational changes induced by 25OH and tunicamycin.

C) Venn diagram for 25OH stimulated and suppressed genes, showing the overlap between total mRNA and translating mRNA.

D) Venn diagram for 25OH stimulated and suppressed genes, showing the overlap between total mRNA and translating mRNA.

E) Table of enriched functional annotations, indicating upregulated and downregulated pathways.

F) Bar graph showing relative mRNA levels for Trib3 in DMSO and 25OH conditions.

G) Heat map depicting the motif enrichment analysis, with best match Nrf2(bZIP) and P-value 1e-14.

H) Bar graph showing relative mRNA levels for Trib3 in DMSO and 25OH conditions, with statistical significance indicated by **.
25-hydroxycholesterol activates the Integrated Stress Response to reprogram transcription and translation in macrophages

J. Biol. Chem. published online November 4, 2013

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