Mitohormesis reprogrammes macrophage metabolism to enforce tolerance

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Macrophages generate mitochondrial reactive oxygen species and mitochondrial reactive electrophilic species as antimicrobials during Toll-like receptor (TLR)-dependent inflammatory responses. Whether mitochondrial stress caused by these molecules impacts macrophage function is unknown. Here, we demonstrate that both pharmacologically driven and lipopolysaccharide (LPS)-driven mitochondrial stress in macrophages triggers a stress response called mitohormesis. LPS-driven mitohormetic stress adaptations occur as macrophages transition from an LPS-responsive to LPS-tolerant state wherein stimulus-induced pro-inflammatory gene transcription is impaired, suggesting tolerance is a product of mitohormesis. Indeed, like LPS, hydroxyoestrogen-triggered mitohormesis suppresses mitochondrial oxidative metabolism and acetyl-CoA production needed for histone acetylation and pro-inflammatory gene transcription, and is sufficient to enforce an LPS-tolerant state. Thus, mitochondrial reactive oxygen species and mitochondrial reactive electrophilic species are TLR-dependent signalling molecules that trigger mitohormesis as a negative feedback mechanism to restrain inflammation via tolerance. Moreover, bypassing TLR signalling and pharmacologically triggering mitohormesis represents a new anti-inflammatory strategy that co-opts this stress response to impair epigenetic support of pro-inflammatory gene transcription by mitochondria.

Macrophage inflammatory responses are important for host defence but, if not tightly controlled, can be detrimental to the host in acute and chronic inflammatory diseases. Macrophage tolerance is a form of trained immunity that evolved to protect the host from overproduction of inflammatory mediators; however, this immunoparalysed state impairs the ability of macrophages to clear pathogens, tumours and perform tissue homeostatic functions. Thus, understanding how the balance between responsiveness versus tolerance is regulated has far-reaching implications in health and disease.

Mitochondria are critical for macrophage-mediated immunity. On encountering a pathogen, TLR ligands such as LPS increase macropage production of mitochondrial reactive oxygen species (mtROS) for bactericidal purposes. Moreover, TLR-driven expression of Acod1 (encoding IRG1) results in tricarboxylic acid (TCA) cycle remodelling and production of itaconate, a mitochondrial reactive electrophilic species (mtRES) with antimicrobial properties. Rapid production of mtROS and mtRES following TLR engagement coincides with oxidative damage, glutathione (GST) depletion and activation of cytoprotective genes (for example, Nfe2l2 and Aft4), suggesting acute oxidative and electrophilic mitochondrial stress is caused by local production of these reactive molecules. Whether this stress impacts macrophage function long term is unknown.

Mitochondrial integrity is closely monitored by quality-control systems. These include nuclear-encoded transcription factors that respond to mitochondrial stress (for example, increased mtROS and impaired mitochondrial proteostasis) and alter gene expression via retrograde mitochondrial–nuclear signalling. Most thoroughly studied in model organisms such as Caenorhabditis elegans and Saccharomyces cerevisiae, stress-induced activation of these transcription factors can promote persistent cytoprotective and mitochondrial protective adaptations and stress resistance in a process known as mitohormesis, which influences organismal metabolism, health and longevity. Whether mitohormesis influences immunity is unknown.

Here we demonstrate that both pharmacological and TLR-driven mitochondrial stress triggers mitohormesis in macrophages. Mitohormetic adaptions, including enhanced mitochondrial proteostasis, mitochondrial oxidative stress resistance (OSR) and the suppression of oxidative metabolism, leave macrophages in an immunoparalysed, LPS-tolerant state. Furthermore, by pharmacologically triggering mitohormesis with reactive, lipophilic small molecules that mimic the actions of endogenously generated mtROS and mtRES, we show the transition to an LPS-tolerant state can occur independently of TLR signalling and other mechanisms previously proposed to enforce tolerance. Thus, mtROS and mtRES trigger mitohormesis as a negative feedback mechanism to restrain macrophage inflammation via tolerance, and this process can be exploited therapeutically to counteract acute and chronic inflammation.
Fig. 1 | Hydroxyoestrogens are anti-inflammatory in macrophages in vitro. a, BMDMs pretreated with ethanol vehicle control or 1 μM oestrogens for 1 h, followed by 6 h of LPS stimulation (100 ng ml$^{-1}$) and Nos2 qPCR. EtOH, ethanol. b, GO analysis of 253 genes repressed by 1 h of hydroxyoestrogen pretreatment (1 μM) in 6-h LPS-stimulated BMDMs identified by RNA-seq. c, RNA-seq hierarchical clustering dendrogram, with heat map highlighting genes with reduced relative expression (log$_2$-transformed reads per kilobase of transcript per million mapped reads (RPKM) values centred on the mean of each gene) in hydroxyoestrogen-pretreated, LPS-stimulated BMDMs. d, BMDMs pretreated with ethanol or indicated concentrations of 4-OHE1 for 1 h, followed by 6 h of LPS stimulation and Il1b qPCR. Percentages indicate induction relative to maximum (100%) in ‘ethanol + LPS’ control BMDMs. e, BMDMs pretreated with ethanol, E2 or 4-OHE1 (both 5 μM) for 1 h, followed by 6 h of LPS stimulation before pro-IL-1β western blot. f, BMDMs pretreated with ethanol, E2 or 4-OHE1 (both 5 μM) for 1 h, followed by 6 h of LPS stimulation (with 2.5 μM Nigericin added in the last hour) before ELISA for secreted IL-1β. g, THP-1 cells pretreated with ethanol, E2 or 4-OHE1 (both 5 μM) for 1 h, followed by 6 h of LPS stimulation (alone, or with 2.5 μM Nigericin added in the last 1 h) before IL-1β ELISA. h, BMDMs from ER$^\alpha$ fl/fl Lyz2-Cre mice pretreated with ethanol or 1 μM of oestrogens for 1 h, followed by 6 h of LPS stimulation and Il1b qPCR. For bar graphs, each data point is an independent biological replicate. For n = 2, data are represented as the mean; for n = 3, mean ± s.e.m. P values are from an unpaired, two-sided Student’s t-test (planned comparisons). All qPCR and THP-1 ELISA data are representative of at least two independent experiments. BMDM western blot (e; n = 2 independent biological replicates per condition) and ELISA (f; n = 3 independent biological replicates per condition) were each performed once. NT, not treated.

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Results

Hydroxyoestrogens are anti-inflammatory in vitro. Biological sex affects immune responses, and oestrogens have conflicting immunomodulatory effects on macrophages\textsuperscript{4,15}, which control macrophage inflammatory responses. While most studies focus on 17β-oestradiol (E2), the most abundant oestrogen, metabolism can alter the structure and immunomodulatory properties of sterols\textsuperscript{16}. We screened a panel of 14 endogenous oestrogens (Supplementary Table 1) to identify metabolites with anti-inflammatory activity in macrophages that might have therapeutic utility. Bone marrow-derived macrophages (BMDMs) were pretreated with oestrogen metabolites for 1 h, followed by a 6-h LPS stimulation and Nos2 real-time quantitative PCR (qPCR). This revealed the hydroxyoestrogens 2-hydroxyoestrone (2-OHE1), 4-hydroxyoestrone (4-OHE1) and 2-hydroxyoestradiol (2-OHE2) repressed Nos2 induction, while other oestrogens including E2 and 16-epiestriol lacked this activity (Fig. 1a). RNA sequencing (RNA-seq) identified 253 genes repressed by hydroxyoestrogen pretreatment in LPS-stimulated BMDMs (Supplementary Table 2), and Gene Ontology (GO) analysis revealed enrichment for categories including ‘inflammatory response’ (Fig. 1b). Hierarchical clustering revealed a broad set of pro-inflammatory cytokines and chemokines repressed by hydroxyoestrogens (Fig. 1c and Extended Data Fig. 1a), making them suitable for mechanistic studies. In addition, 4-OHE1 repressed Il1b in THP-1 and U937 human monocytic cell lines (Extended Data Fig. 1c). In agreement with these transcriptional effects, 4-OHE1, but not E2, repressed LPS-induced pro-interleukin (IL)-1β protein levels in BMDMs (Fig. 1e) and RAW macrophages (Extended Data Fig. 1d), and inhibited IL-1β release from BMDMs and THP-1 cells (Fig. 1g).

Hydroxyoestrogen 4-OHE1, but not E2, repressed Il1b induction by multiple TLR agonists (Extended Data Fig. 1e), suggesting that a common pathway downstream of TLRs is targeted. To test if these effects were dependent on ERα, the primary ER in macrophages\textsuperscript{17}, we repeated these experiments using BMDMs from Erα\textsuperscript{-/-} Lys2-Cre mice. Surprisingly, the anti-inflammatory activity of the hydroxyoestrogens was intact (Fig. 1b). Moreover, co-treatment with the high-affinity ERα antagonist ICI 182780 had no effect on the ability of hydroxyoestrogens to repress Il1b (Extended Data Fig. 1f). Together, these results demonstrate hydroxyoestrogens are ERα-independent repressors of macrophage pro-inflammatory gene transcription in vitro.

Hydroxyoestrogens are anti-inflammatory in vivo. To test if hydroxyoestrogens have anti-inflammatory activity in vivo during acute inflammation, we intraperitoneally (i.p.) injected mice with ethanol, E2 or 4-OHE1, followed by LPS. Hydroxyoestrogen 4-OHE1, but not E2, significantly repressed the LPS-induced increase in serum IL-1β, and splenocyte pro-inflammatory gene expression (Fig. 2a,b). Thus, 4-OHE1 can antagonize acute LPS-induced inflammation in vivo.

To test if hydroxyoestrogens have anti-inflammatory effects in vivo in a chronic inflammatory setting, we examined effects on gene expression in visceral white adipose tissue (vWAT) macrophages during the early stages of high-fat diet (HFD)-induced inflammation and metabolic dysfunction. Mice were placed on a HFD, and ethanol, E2 or 4-OHE1 was administered subcutaneously (s.c.) every 6 d. After 30 d, vWAT macrophages were profiled by RNA-seq (Fig. 2c and Extended Data Fig. 2a). While both E2 and 4-OHE1 reduced adiposity and vWAT macrophage cellularity (Extended Data Fig. 2b,c), vWAT macrophages from 4-OHE1-treated mice displayed a distinct gene expression signature compared to those of macrophages from ethanol-treated mice and E2-treated mice (Fig. 2c). Hydroxyoestrogen 4-OHE1, compared to E2, repressed expression of a distinct set of genes (Fig. 2d and Supplementary Table 3). GO analysis of genes uniquely repressed by 4-OHE1 revealed enrichment for categories including ‘inflammatory response’, while genes uniquely repressed by E2 showed no enrichment for inflammatory processes (Fig. 2c). Many genes repressed in macrophages in vitro were repressed by 4-OHE1, but not E2, in vWAT macrophages (Extended Data Fig. 2d).

To test if the anti-inflammatory effects of 4-OHE1 coincided with improved metabolic control, we subjected mice fed normal chow (NC) or HFD and s.c. injected with ethanol or 4-OHE1 for 30 d to a glucose tolerance test. We found that 4-OHE1 significantly enhanced glucose disposal in HFD-fed mice compared with that in HFD and even NC controls (Fig. 2f and Extended Data Fig. 2e). This occurred despite reduced glucose-stimulated insulin secretion (GSIS) in 4-OHE1-treated HFD-fed mice (Fig. 2g), raising the possibility of enhanced insulin sensitivity and glucose uptake in tissue(s) due to reduced inflammation. Thus, 4-OHE1 both repressed inflammation and improved metabolic control during HFD-induced metabolic dysfunction in vivo. Interestingly, challenging these mice with LPS 48 h after their last injection revealed that this extended, intermittent dosing of 4-OHE1 via the s.c. route provided protection against systemic LPS-induced inflammation (Fig. 2h and Extended Data Fig. 2f). Thus, a 4-OHE1 dosing schedule with more relevance to human drug administration can protect mice from inflammatory insults at a dosage with potential for clinical translation.

Fig. 2 | Hydroxyoestrogens are anti-inflammatory in vivo. a, Experimental set-up (top) and serum IL-1β levels (bottom) in 8-week-old male C57BL/6 mice injected i.p. with ethanol vehicle control or oestrogens (10 mg kg\textsuperscript{-1}) before i.p. LPS injection (2 mg kg\textsuperscript{-1}). n = 2, 4, 5 and 4 mice for the ethanol, ethanol + LPS, 4-OHE1 + LPS and E2 + LPS groups, respectively. b, qPCR for pro-inflammatory gene expression in splenocytes isolated from mice in a. c, Experimental set-up (top) and hierarchical clustering of vWAT macrophage RNA-seq data (bottom) from 8-week-old male C57BL/6 mice fed a HFD and injected s.c. every 6 d with ethanol, E2 or 4-OHE1 (10 mg kg\textsuperscript{-1}). Relative expression heat map displays the log\textsubscript{2}-transformed RPKM values centred on the mean of each gene. n = 5 mice per treatment group. d, Venn diagram displaying the overlap between genes significantly repressed by E2 or 4-OHE1 in vWAT macrophages from HFD-fed mice. e, GO analysis of genes uniquely repressed by E2 (left) or 4-OHE1 (right) in vWAT macrophages from HFD-fed mice. ECM, extracellular matrix. f, Glucose tolerance test (GTT) in 12-week-old male C57BL/6 mice after 30 d of NC or HFD feeding and s.c. injection with either ethanol or 4-OHE1 (10 mg kg\textsuperscript{-1}). n = 10, 10 and 11 for NC + ethanol, HFD + ethanol and HFD + 4-OHE1 groups, respectively. P values from NC + ethanol versus HFD + ethanol comparisons (top), and HFD + ethanol versus HFD + 4-OHE1 comparisons (bottom). g, Serum insulin ELISA measuring GSIS in fasted mice from f at 15 min after glucose injection. Number of mice reduced from f due to random sample collection errors (n = 9, 8 for NC + ethanol; n = 9,10 for HFD + ethanol; and n = 9, 9 for HFD + 4-OHE1 groups for each respective time point). h, Serum IL-1β levels in a subset of mice from f, 4.5 h after i.p. LPS injection (3 mg kg\textsuperscript{-1}). n = 3, 5, 6 and 6 mice per condition, respectively. For bar graphs, all data are represented as the mean ± s.e.m. P values are from unpaired, two-sided Student’s t-tests (planned comparisons). Acute inflammation model was performed once. HFD chronic inflammation model was performed once for transcriptional profiling and once for metabolic studies.
Hydroxyoestrogens do not require NRF2 to repress inflammation. Given their ERα-independent anti-inflammatory effects, we considered other mechanisms by which hydroxyoestrogens might act, given what is known about their natural production and metabolism (Fig. 3a). Hydroxylation of oestrone (E1) or E2 by CYP1 family cytochrome P450 monooxygenases creates a catechol moiety, giving hydroxyoestrogens their ‘catechol oestrogens’ nickname18. Present in a variety of approved drugs and natural compounds, this catechol moiety can cause cellular stress in two ways. First, it can be oxidized to its quinone form, and redox cycling between these forms (which involves a semiquinone free-radical intermediate) can produce reactive oxygen species (ROS). Second, because the quinone form possesses α,β-unsaturated carbonyls and is highly electrophilic, it can be attacked by nucleophiles, such as reactive...
cysteines in proteins, forming covalent adducts. Accordingly, cells detoxify hydroxyoestrogens via catechol methylation by COMT (which reduces redox cycling) and GST conjugation of the quinone. Given that hydroxyoestrogens, but not their precursors or methylated metabolites, repressed LPS-induced pro-inflammatory gene transcription (Fig. 3b and Extended Data Fig. 1b), we hypothesized their anti-inflammatory effects depend on their ability to cause oxidative and electrophilic stress.

The Keap1–Nrf2 system regulates cytoprotection in response to oxidative and electrophilic stress. Our LPS-treated BMDM RNA-seq dataset identified 341 genes significantly upregulated in hydroxyoestrogen-pretreated cells versus control pretreatments (Supplementary Table 2). GO analysis revealed enrichment of categories including ‘response to oxidative stress’ and ‘GSH metabolism’ (Extended Data Fig. 3a), and the Nrf2 binding motif was enriched in the gene promoters (Fig. 3c).

Expression of Nrf2 targets Hmox1, Nqo1, ROS detoxification and GST biosynthesis genes were significantly upregulated in hydroxyoestrogen-pretreated, LPS-stimulated BMDMs (Fig. 3d). In non-LPS-stimulated macrophages, treatment with 4-OHE1 alone, but not E2, was sufficient to rapidly increase intracellular oxidative stress that is sensitive to N-acetyl cysteine (NAC), which generates hydrosulfides with both direct antioxidant activity, and the ability to protect protein thiols from oxidation by electrophiles (Fig. 3e). This oxidative stress coincides with Nrf2 stabilization, and NAC-sensitive upregulation of Hmox1 expression by 4-OHE1, but not E2 (Fig. 3f,g). Given Nrf2 has been described as a negative regulator of LPS-induced inflammation, we tested if Nrf2 was required for the anti-inflammatory activity of 4-OHE1. Impaired Hmox1 induction confirmed lack of Nrf2 function in BMDMs from Njε2l2−/− mice (Nrf2 knockout (KO) BMDMs; Extended Data Fig. 3b). However, 4-OHE1 still repressed LPS-induced Il1b in these macrophages (Fig. 3b), demonstrating Nrf2 is dispensable for 4-OHE1’s repression of pro-inflammatory gene transcription.

Hydroxyoestrogens cause mitochondrial stress. A recent report demonstrated that E2 is highly abundant in mitochondrial membranes, and that E2 from distal and exogenous sources localizes to mitochondria in target cells and tissues. Given Nrf2 monitors mitochondrial integrity, we hypothesized Nrf2 activation by hydroxyoestrogens might be indicative of mitochondrial stress caused by these lipophilic electrophiles as they localize to mitochondria, producing ROS and covalently modifying mitochondrial proteins. In support of this hypothesis, Hmox1 induction by 4-OHE1 was sensitive to the mitochondrial-matrix-targeted antioxidant MitoQ (Extended Data Fig. 4a). Deeper mining of the GO analysis of the 341 genes upregulated in hydroxyoestrogen-pretreated, LPS-stimulated BMDMs revealed three additional transcriptional signatures indicative of mitochondrial stress (Fig. 4a–c and Extended Data Fig. 4d–j). The heat shock factor 1 (HSF1) signature includes putative HSF1 target genes, suggesting HSF1 activation in response to mitochondrial stress. The ATF4/mitochondrial damage signature includes Atf4, which coordinates cytoprotection in response to mitochondrial stress in mammalian cells, and the ATF4 target Gdf15, encoding a mitokine indicative of mitochondrial dysfunction. Finally, the glycolysis/plasminogen activator pathway signature includes enzymes in these pathways known to be upregulated in response to mitochondrial stress in other systems.

We performed steroid extraction and liquid chromatography–mass spectrometry (LC–MS) to determine if 4-OHE1 was enriched in mitochondrial fractions relative to whole cells (Extended Data Fig. 4e,f). However, we were unable to detect free 4-OHE1 in macropahes treated for 1 h (Extended Data Fig. 4g), suggesting 4-OHE1 is rapidly covalently conjugated by GST and/or proteins (Fig. 3a). To identify covalent 4-OHE1 protein targets acting through reactive cysteines, we performed competitive isotope tandem orthogonal proteolysis–enabled activity-based protein profiling (isoTOP-ABPP) in 4-OHE1-treated BMDMs (Fig. 4d). This revealed 127 cysteines on 118 proteins targeted by 4-OHE1 (Fig. 4e). Cross-referencing this target list with MitoCarta 2.0 revealed 18 targets that were mitochondrial proteins (Fig. 4f). A chi-square test comparing the observed frequency of mitochondrial targets with the expected frequency of mitochondrial proteins confirmed significant enrichment of mitochondrial proteins in the target list (Extended Data Fig. 4h). Together, these transcriptional and proteomic studies suggest hydroxyoestrogens cause mitochondrial stress via local ROS production and covalent targeting of mitochondrial proteins.

**Hydroxyoestrogens impair mitochondrial acetyl-CoA production.** We next considered how oxidative and electrophilic mitochondrial stress caused by hydroxyoestrogens might exert anti-inflammatory effects. Mitochondria utilize oxidized glucose for acetyl-CoA production, which in turn is used to acetylate histones, a process crucial for Il1b upregulation in LPS-stimulated macrophages. Pharmacological inhibition of glucose utilization in LPS-stimulated macrophages has selective repressive effects on Il1b, but not cytokines such as those encoded by Il6 and Tnf, suggesting the glucose-to-acetyl-CoA axis is more important for transcription of the former gene. Hydroxyoestrogen 4-OHE1 had stronger repressive effects on Il1b than Il6 and Tnf in RAW macrophages (Fig. 5a) and BMDMs (Extended Data Fig. 5a), suggesting 4-OHE1 interferes with this axis.

To test this hypothesis, we performed metabolomics to identify metabolites whose levels were acutely affected by 4-OHE1. BMDMs were treated with ethanol or 4-OHE1 for 2 h, followed by 30 min of [13C6]glucose labelling and metabolite extraction (Fig. 5b). Of the 136 metabolites measured, just 7 were significantly changed by 4-OHE1 (Fig. 5c). Free-GSH levels were reduced (Fig. 5c), sup-
porting the notion that 4-OHE1 is rapidly conjugated by GSH after treatment (Fig. 3a). In line with our hypothesis, the metabolite with the most statistically significant change was acetyl-CoA, as levels in 4-OHE1-treated BMDMs decreased by 45% (Fig. 5c,d). Interestingly, coenzyme A (CoA) levels were also significantly reduced (41%; Fig. 5c,e). As 80–90% of intracellular CoA
is intra-mitochondrial\(^3\), this suggests mitochondrial stress caused by 4-OHE1 disrupts CoA homeostasis, and in turn acetyl-CoA production. Of the TCA cycle metabolites quantified, total levels of citrate and aconitate (immediately downstream of acetyl-CoA) showed the largest decreases in abundance (Extended Data Fig. 5b). \(^{13}\)C\(_6\)glucose tracing revealed increased labelling of TCA cycle intermediates in 4-OHE1-treated BMDMs (Extended Data Fig. 5c).

**Fig. 4 | Hydroxyoestrogens cause mitochondrial stress.** a-c, Relative expression (log\(_2\)-transformed RPKM values centred on the mean of each gene) of genes indicative of mitochondrial stress in 1-h oestrogen-pretreated, 6-h LPS-stimulated BMDM RNA-seq dataset. d, IsoTOP-ABBP strategy to identify covalent targets of 4-OHE1 acting through reactive cysteines. BMDMs were treated with ethanol or 1\(\mu\)M 4-OHE1 for 1h (n = 3 independent biological replicates per condition). e,f, All targets (left) and mitochondrial targets (right) of 4-OHE1 identified by isoTOP-ABBP. Cysteine-containing peptides above the dashed line have a light/heavy ratio > 2.0, indicating at least a 50% reduction in cysteine-reactive probe targeting of these cysteines in 4-OHE1-treated BMDMs relative to control BMDMs. In total, 18 of 20 mitochondrial targets are in MitoCarta 2.0. FKBP4 and GCLC mitochondrial localization prediction is from UniProt. PPP, pentose phosphate pathway.
metabolites (and amino acids derived from these metabolites), except citrate and aconitate, after 4-OHE1 treatment (Extended Data Fig. 5c), suggesting alternative entry routes of glucose-derived carbon into the TCA cycle in response to 4-OHE1-induced mitochondrial stress and disruption of CoA homeostasis. Together, these data demonstrate mitochondrial stress caused by 4-OHE1 impairs mitochondrial acetyl-CoA production.

Because acetyl-CoA is required for histone acetylation and pro-inflammatory gene transcription, we performed chromatin immunoprecipitation followed by sequencing (ChIP–seq) to take an unbiased, genome-wide look at how LPS-induced transcription factor binding and histone acetylation were affected by hydroxyoestrogens. For the nuclear factor kappa B (NFkB) subunit p65, we identified 6,149 peaks induced by 30 min of LPS stimulation (Fig. 5f). Pretreatment with the hydroxyoestrogen 2-OHE2 had minimal effects on NFkB binding, as only 13% of LPS-induced p65 binding peaks were significantly reduced in read density. For histone acetylation, we identified 10,999 regions of H3K27ac, a mark of active promoters and enhancers, induced by 30 min of LPS stimulation (Fig. 5f). In contrast with NFkB binding, pretreatment with the hydroxyoestrogen 2-OHE1 strongly impaired H2K27ac deposition, as nearly two-thirds (65%) of LPS-induced H3K27ac regions were significantly reduced in read density (Fig. 5f and Extended Data Fig. 5d). Thus, while hydroxyoestrogens largely leave TLR4 signalling and transcription factor nuclear localization/dNA binding intact, they strongly impair histone acetylation required for pro-inflammatory gene transcription, further supporting the hypothesis that hydroxyoestrogens impair mitochondrial acetyl-CoA production (Fig. 5g).

Exogenous CoA can rescue histone acetylation and gene expression defects in cells with impaired CoA homeostasis19,30. Indeed, exogenous CoA and acetyl-CoA fully rescued LPS-induced \(Ll1b\) in 4-OHE1-pretreated macrophages at early time points (Fig. 5h), and partially at 6h (Extended Data Fig. 5e). Acetate supplementation failed to rescue \(Ll1b\) (Extended Data Fig. 5f), suggesting necrolyytic acetyl-CoA generation by ASCC2 does not contribute to histone acetylation in this context. These rescue experiments further support a model whereby impairment of mitochondrial acetyl-CoA production by hydroxyoestrogens underlies their anti-inflammatory activity.

Finally, we wondered if this mechanism might extend to other anti-inflammatory electrophiles. Diethyl maleate (DEM) and celastrol are electrophiles that repress anti-inflammatory electrophiles. Diethyl maleate (DEM) and celastrol, 100 \(\mu\)M DEM and 10 \(2,2',\) data are represented as the mean; red bars) for 3 h before 1 h of electrophile pretreatment, 1.5 h of LPS stimulation and \(Ll1b\) qPCR. I, RAW macrophages cultured in the absence of acetyl-CoA (200 \(\mu\)M); red bars) for 2 h before 1 h of electrophile pretreatment, 1.5 h of LPS stimulation and \(Ll1b\) qPCR. Concentrations: 250nM celastrol, 100 \(\mu\)M DEM and 10 \(\mu\)M FCCP. For bar graphs, each data point is an independent biological replicate. For \(n = 2\), data are represented as the mean; for \(n = 3\), data are the mean ± s.e.m. All qPCR data are representative of at least two independent experiments. Metabolomics and ChIP–seq studies were performed once.

**Hydroxyoestrogen-driven mitochondrial stress triggers mitohormesis.** We next wondered how the mitochondrial stress caused by hydroxyoestrogens might influence macrophage function beyond these acute anti-inflammatory effects. Mitochondrial stress can trigger apoptosis; however, we observed no changes in oxygen consumption (Extended Data Fig. 6a), mtMP (Extended Data Fig. 6b) or cell viability during acute (<1 h) hydroxyoestrogen treatment when stress is first detectable (Fig. 3e–g). Alternatively, mild doses of mitochondrial stress can trigger persistent stress adaptations that provide cytoprotection and mitochondrial protection during subsequent stress exposure in a process known as mitohormesis37. Two hallmarks of mitohormesis are mitochondrial biogenesis and increased mitochondrial chaperone activity. To test if acute hydroxyoestrogen-driven mitochondrial stress triggered these adaptations, we expressed a mitochondrial-matrix-targeted, oxidation-resistant green fluorescent protein in RAW macrophages, creating the RAW matrix-oxGFP reporter cell line. Treatment with 4-OHE1, but not E1 or 4-MeOE1, induced a transient decrease at 1h, followed by a steady increase in RAW matrix-oxGFP fluorescence at 8h and 24h as measured by flow cytometry (Fig. 6a). This dose-dependent effect occurred with no change in matrix-oxGFP transcription from the integrated reporter construct (Extended Data Fig. 6c,d). Quantification of the mitochondrial DNA/genomic DNA (mtDNA/gDNA) ratio ruled out increased mitochondrial DNA replication (Extended Data Fig. 6e). As matrix-oxGFP is a mitochondrial chaperone client that must be folded after mitochondrial import38, this suggests RAW matrix-oxGFP cells report mitochondrial chaperone activity. Increased matrix oxGFP fluorescence was sensitive to NAC, MitoQ and inhibition of HSF1 transcriptional activity with KRIIB11, suggesting HSF1-dependent chaperone expression is required for this stress adaptation37 (Extended Data Fig. 6f,g). Increased mitochondrial chaperone activity occurred concurrently with increased mitochondrial volume and/or surface area, as 4-OHE1, but not E1 or 4-MeOE1, increased MitoTracker Green signal in RAW macrophages and BMDMs (Extended Data Fig. 6h,i). THP-1 and U937 matrix-oxGFP reporter cells responded similarly to 4-OHE1, demonstrating this stress adaptation is conserved in human cells (Extended Data Fig. 6j). Thus, acute hydroxyoestrogen-driven mitochondrial stress triggers an adaptive increase in mitochondrial chaperone activity to protect mitochondrial proteostasis.

Another hallmark of mitohormesis is mitochondrial OSR19 in which mild oxidative stress triggers redox adaptations that both...
lower steady-state levels of mtROS and provide defence against subsequent oxidative stress insults. Like all cells, macrophages produce mtROS from the electron transport chain (ETC), and LPS enhances mtROS production for bactericidal purposes. Thus, we tested if acute hydroxyoestrogen-driven mitochondrial stress influenced basal and LPS-induced mtROS levels using the mitochondrial-targeted H2O2 sensor MitoPY1. Macrophages treated with 4-OHE1, but not E1 or 4-MeOE1, showed reduced basal MitoPY1 fluorescence (Fig. 6b).
LPS enhanced mtROS levels as expected (Fig. 6b). However, in macrophages pretreated with hydroxyoestrogens, but not with their precursor or methylated metabolites, there was a significant decrease in LPS-induced mtROS (Fig. 6b and Extended Data Fig. 6k). To corroborate this effect, we expressed redox sensitive-GFPs (roGFPs) in the cytosol (cyto-roGFP), mitochondrial inner membrane space (IMS-roGFP) and mitochondrial matrix (matrix-roGFP) to quantify subcellular redox status in RAW macrophages (Fig. 6c). H$_2$O$_2$ and dithiothreitol treatment confirmed responsiveness of the roGFPs to altered redox states (Extended Data Fig. 6l). LPS triggered oxidation of IMS-roGFP, but not matrix-roGFP or cyto-roGFP (Fig. 6c), demonstrating roGFPs can monitor compartment-specific redox changes in macrophages, and supporting the hypothesis that LPS-induced mtROS is primarily produced by complex III into the IMS$^{39,40}$. Corroborating the MitoP71 data, pretreatment with hydroxyoestrogens, but not their precursor or methylated metabolites, significantly reduced IMS-roGFP oxidation by LPS (Fig. 6d). Together, these results demonstrate acute hydroxyoestrogen-driven mitochondrial stress triggers mitohormetic mitochondrial OSR adaptations in macrophages, reducing basal and LPS-driven mtROS production.

We also tested whether mitohormetic OSR adaptations could protect macrophages against subsequent oxidative stress insults. For murine macrophages, our strategy is outlined in Fig. 6e. Following overnight vehicle or 4-OHE1 treatment, macrophage viability assessment by flow cytometry revealed most cells fell into a live forward scatter/side scatter (FS/SS) gate and excluded DAPI (Fig. 6e). We then treated these macrophages with menadione, a redox cycling quinone that produces toxic levels of mitochondrial superoxide$^{41}$. While an apoptotic cell population (indicated by FS/SS shift and DAPI uptake) appeared in our control cultures, this population did not appear in 4-OHE1-pretreated macrophages (Fig. 6e). Menadione resistance was specifically conferred by 4-OHE1, and not E1 or 4-MeOE1 (Fig. 6f), was sensitive to NAC but not MitoQ pretreatment (Extended Data Fig. 6m), and occurred in BMDMs (Extended Data Fig. 6n). THP-1 and U937 cells (which we found to be menadione resistant) treated with 4-OHE1 acquired resistance to increased intracellular oxidative stress caused by exogenous H$_2$O$_2$, demonstrating this OSR adaptation is conserved in human cells (Extended Data Fig. 6o). Thus, mitohormesis triggered by acute hydroxyoestrogen-driven mitochondrial stress confers macrophages with increased mitochondrial OSR and lasting ‘vaccine-like protection$^{13}$ against subsequent oxidative stress insults.

**Lipopolysaccharide-driven mitochondrial stress also triggers mitohormesis.** Having characterized the adaptations to hydroxyoestrogen-driven mitochondrial stress, we next wondered what this pharmacologically induced mitohormesis could teach us about how macrophages respond to acute LPS-driven mitochondrial stress. Oxidative damage, GST depletion and upregulation of cyto-protective genes occurs rapidly (1–6 h) following LPS treatment$^{14–16}$, likely in response to a combination of increased mitochondrial oxygen consumption and mtROS production$^{17,27,28}$, and increased mitochondrial production of electrophilic itaconate. However, whether LPS-driven oxidative and electrophilic mitochondrial stress triggers mitohormesis is unknown.

To test the similarity between the mitochondrial stress caused by 4-OHE1 and LPS at the transcriptional level, we performed RNA-seq on BMDMs treated for 6 h and 24 h with either 4-OHE1 or LPS. This revealed an extremely high degree of overlap for both activated and repressed genes at each time point (Fig. 7a and Supplementary Table 7). Chi-squared tests confirmed such overlap would be extremely unlikely by chance (Supplementary Table 8), suggesting 4-OHE1 closely mimics physiological oxidative and electrophilic mitochondrial stress induced by LPS. GO analysis of genes upregulated by both 4-OHE1 and LPS revealed enrichment for categories including ‘regulation of cellular response to stress’, ‘protein folding’ and ‘detoxification of ROS’ (Fig. 7b and Extended Data Fig. 7a). Examination of genes in these categories revealed upregulation of HSF1-regulated chaperones that control mitochondrial protein folding$^{29}$, and enzymes of the peroxiredoxin–thioredoxin system that scavenge mitochondrial H$_2$O$_2$, by both 4-OHE1 and LPS (Fig. 7c and Extended Data Fig. 7b). Co-treatment with 4-OHE1 and LPS further enhanced upregulation of many of these genes (Fig. 7d).

Given the highly similar transcriptional response to mitochondrial stress caused by 4-OHE1 and LPS, we tested if LPS treatment triggered mitohormesis. With regards to mitochondrial chaperone activity, treatment of RAW matrix-oxGFP reporter cells with LPS drove a progressive increase in fluorescence in a manner identical to that with 4-OHE1 treatment (Fig. 7e), and like stress-induced gene expression, 4-OHE1/LPS co-treatment enhanced this effect. This suggests that the increased chaperone expression in response to both 4-OHE1 and LPS contributes to increased mitochondrial chaperone activity. Indeed, HSF1 transcriptional inhibition blunted increased matrix-oxGFP fluorescence in response to both 4-OHE1 and LPS (Extended Data Fig. 7c). Furthermore, like 4-OHE1, LPS enhanced MitoTracker Green signal in macrophages without a significant increase in mtDNA content (Extended Data Fig. 7d,e), suggesting enhanced mitochondrial volume and/or surface area for dye uptake. Finally, LPS enhanced matrix-oxGFP fluorescence in THP-1 and U937 reporter cells, demonstrating conservation of this response across species (Extended Data Fig. 7f).

To test if acute LPS-driven mitochondrial stress resulted in adaptive OSR, we repeated our menadione resistance experiments.
Overnight treatment with either 4-OHE1 or LPS increased BMDM viability relative to that of controls, with 4-OHE1/LPS co-treatment enhancing this effect (Fig. 7f). Menadione decreased viability in control cells; however, both 4-OHE1-treated and LPS-treated macrophages were resistant to menadione-induced toxicity, with 4-OHE1/LPS co-treatment enhancing resistance (Fig. 7f). This suggests the increased expression of mtROS scavenging enzymes in response to both 4-OHE1 and LPS contributes to adaptive OSR. Induction of mtROS scavengers by 4-OHE1/LPS was impaired in NRF2 KO BMDMs, suggesting NRF2 controls this programme (Extended Data Fig. 7g). LPS treatment afforded THP-1 cells resistance against oxidative stress, showing conservation of this response in human cells (Extended Data Fig. 7h). Together, these data demonstrate that 4-OHE1-driven mitochondrial stress closely mimics physiological, LPS-driven mitochondrial stress, and that both trigger classic mitohormetic adaptations in macrophages.

Mitohormetic metabolic reprogramming enforces LPS tolerance. Following primary LPS exposure, macrophages transition to an LPS-tolerant state where pro-inflammatory gene induction is refractory to upregulation by secondary LPS treatment (Fig. 8a). Many mechanistic explanations for LPS tolerance have been...
proposed, including upregulation of negative regulators of TLR4 signalling, and production of anti-inflammatory cytokines. However, recent evidence suggests that suppression of mitochondrial oxidative metabolism following LPS exposure limits acetyl-CoA production required for pro-inflammatory gene transcription. ETC inhibition by LPS-induced nitric oxide has been proposed to drive this suppression; however, Nos2-deficient macrophages still show suppression of mitochondrial oxidative metabolism following LPS treatment. Metabolic reprogramming often acts in concert with mitochondrial stress, with aerobic glycolysis providing a damaged mitochondrial network an opportunity to recover from stress while simultaneously augmenting ATP and NADPH production for energy and antioxidant defence, respectively. Thus, we wondered if the mitohormesis we characterized in LPS-treated macrophages, which includes increased mitochondrial chaperone activity and OSR, also includes metabolic reprogramming that enforces tolerance via suppression of mitochondrial oxidative metabolism.

If this hypothesis is true, then mitochondrial stress alone, separately from other TLR4-dependent events, should be sufficient to trigger metabolic reprogramming to an LPS-tolerant state. Given 4-OHE1-driven mitochondrial stress closely mimics LPS-driven mitochondrial stress, as we hypothesized that mitochondrial stress is a key signal that triggers the transition from an LPS-responsive to LPS-tolerant state via mitohormesis.
Fig. 8 | Mitohormesis in macrophages involves metabolic reprogramming that enforces an LPS-tolerant state. a, Schematic describing how mitochondrial oxidative metabolism (blue line) supports pro-inflammatory gene expression (orange line) after LPS treatment, but is suppressed as macrophages transition to an LPS-tolerant state where pro-inflammatory genes are refractory to upregulation by secondary LPS exposure. Mitohormetic adaptations (red line) occur in parallel with this process, but whether suppression of mitochondrial oxidative metabolism is a coincident mitohormetic adaptation is unknown. b, Seahorse energy map plotting basal OCR versus basal ECAR in RAW macrophages treated overnight (18–24 h) with ethanol, 4-OHE1 (5 μM), LPS, or both 4-OHE1/LPS. c, Seahorse mitochondrial stress test in RAW macrophages treated overnight (18–24 h) with ethanol, 4-OHE1 (5 μM), LPS, or both 4-OHE1/LPS, after which the treatments were washed out and cells allowed to recover (1–2 h) before secondary LPS stimulation for 6 h. d, iIlb qPCR in RAW macrophages treated overnight (18–24 h) with ethanol, 4-OHE1 (5 μM), LPS, or both 4-OHE1/LPS. e, Seahorse energy map plotting basal OCR versus basal ECAR in RAW macrophages treated overnight (18–24 h) with ethanol, 4-OHE1 (5 μM), LPS, or both 4-OHE1/LPS. f, RNA-seq 24 h. g, Model depicting how oxidative and electrophilic stress in LPS-stimulated macrophages drives mitohormesis and tolerance, and how hydroxyoestrogens co-opt this stress response. h, RAW macrophages pretreated for 1h with vehicle or MitoQ (1 μM), then treated overnight (18–24 h) with primary LPS, after which the treatments were washed out and cells allowed to recover (1–2 h) before 6 h secondary LPS stimulation and iIlb qPCR. DMSO, dimethylsulfoxide. For qPCR, each data point is an independent biological replicate (for n = 2, data are represented as the mean), and data are representative of two independent experiments. Seahorse data are representative of two independent experiments with n = 5 independent biological replicates per condition (represented as the mean ± s.e.m.). Energy map represents the average of three consecutive basal OCR and ECAR measurements. NO, nitric oxide.
treated with vehicle control, 4-OHE1, LPS, or both 4-OHE1/LPS for a tolerizing duration before treatments were washed out and cells subjected to Seahorse respirometry. Plotting extracellular acidification rate (ECAR, a proxy for glycolysis) versus oxygen consumption rate (OCR, a proxy for mitochondrial oxidative metabolism) showed that while control macrophages are relatively aerobic, both 4-OHE1 and LPS treatment shifted the macrophages away from mitochondrial oxidative metabolism and towards aerobic glycolysis, with 4-OHE1/LPS co-treatment enhancing this metabolic shift (Fig. 8b). Mitochondrial stress testing revealed both 4-OHE1 and LPS significantly reduced basal and maximal OCRs, with 4-OHE1/LPS co-treatment causing an even stronger reduction (Fig. 8c and Extended Data Fig. 8a,b). Thus, 4-OHE1-driven mitochondrial stress is sufficient to trigger metabolic reprogramming similar to that observed in LPS-treated macrophages.

To test if macrophages metabolically reprogrammed by 4-OHE1 were LPS tolerant, cells were treated with vehicle, 4-OHE1, LPS or both OHE1 and LPS, for a tolerizing duration (18–24h) before treatments were washed out and cells allowed to recover before secondary LPS stimulation and Il1b qPCR (Fig. 8d and Extended Data Fig. 8c). While naïve macrophages responded robustly, cells treated with primary LPS showed classic tolerance and impaired Il1b upregulation during restimulation. Cells treated with 4-OHE1 also displayed impaired Il1b induction, demonstrating that 4-OHE1-induced metabolic reprogramming coincides with transition to an LPS-tolerant state. In agreement with 4-OHE1/LPS co-treatment driving a stronger metabolic shift, co-treated macrophages displayed a more severely impaired secondary LPS response. Cells tolerized with LPS, 4-OHE1 or both 4-OHE1/LPS displayed impaired upregulation of mtROS in response to secondary LPS compared to that in naïve macrophages (Extended Data Fig. 8d), likely due to reduced OCR, and the OSR programme upregulated during mitohormesis. As mtROS regulates inflammatory activation, we confirmed that 4-OHE1, but not E2, induced tolerance that impaired IL-1β secretion (Extended Data Fig. 8e). The hydroxyoestrogen 4-OHE1 also induced tolerance in THP-1 cells, demonstrating this effect is conserved (Extended Data Fig. 8f). CoA supplementation during the washout and recovery period boosted secondary LPS responsiveness in tolerized macrophages, suggesting impaired CoA-acetyl-CoA homeostasis in the tolerized state (Fig. 8e). Together, these data demonstrate that, in addition to increased mitochondrial chaperone activity and OSR, 4-OHE1-induced mitohormesis involves metabolic reprogramming to an LPS-tolerant state essentially identical to that induced by LPS. Our RNA-seq data revealed that, unlike LPS, 4-OHE1-induced LPS tolerance occurs in the absence of transcriptional upregulation of negative regulators of TLR4 signalling (Tnfaip3), anti-inflammatory cytokines (Il10) and without Nos2 induction (Fig. 8f). Thus, mitohormetic metabolic reprogramming to an LPS-tolerant state can be uncoupled from TLR4 signalling, and mitochondrial stress is a sufficient signal to trigger this reprogramming in the absence of TLR4-dependent events that have been proposed to enforce tolerance.

We propose a model whereby mitochondrial stress induced by mtROS and mtRES triggers mitohormesis and, in turn, a state of ‘mitohormetic tolerance’ (Fig. 8g). One prediction of this model is that reducing mtROS/mtRES during primary LPS exposure would impair the oxidative stress signalling that leads to tolerance, and enhance secondary LPS responsiveness. mtMP has been reported to increase following LPS treatment, inducing reverse electron transport to drive mtROS production33; however, ratiometric mtMP measurements with JC-9 revealed no increase after LPS treatment (Extended Data Fig. 8g), suggesting mtROS is a product of increased mitochondrial ETC flux and oxygen consumption that occurs 0.5–2h after LPS treatment33. Scavenging superoxide with MitoQ during primary LPS exposure resulted in a partial rescue of secondary LPS responsiveness (Fig. 8h). This suggests that mtROS is partially responsible for mitohormetic tolerance and restraining macrophage inflammatory responsiveness, a model that we propose warrants further investigation.

**Discussion**

Whether intrinsically generated oxidative and electrophilic mitochondrial stress affects macrophage function is unknown. Moreover, the mechanisms controlling macrophage tolerance are unclear. We identified hydroxyoestrogens as potent anti-inflammatories, and through study of their pharmacological effects, we demonstrate that both hydroxyoestrogen-driven and LPS-driven mitochondrial stress trigger a set of stress adaptations known as mitohormesis. One of these adaptations, the suppression of mitochondrial oxidative metabolism, suppresses the macrophage response to secondary LPS, revealing mitohormesis as a stress response that enforces LPS tolerance to prevent excessive inflammation. Thus, in addition to their antimicrobial roles, mtROS/mtRES are signalling molecules that trigger negative feedback to restrain inflammation via mitohormesis, a process that can be pharmacologically targeted (Fig. 8g).

While we provide evidence that HSF1 and NRF2 regulate specific mitohormetic adaptations (increased mitochondrial chaperone activity and OSR, respectively), the identity of the transcription factor(s) coordinating suppression of mitochondrial oxidative metabolism remains unclear. ATF4 is an attractive candidate, as its *C. elegans* orthologue ATFS-1 simultaneously suppresses ETC genes and upregulates glycolytic genes in response to mitochondrial stress34. For LPS-induced mitohormesis, the relative contributions of mtROS, itaconate and other mtRES (for example, 4-hydroxynonenal) in triggering mitohormesis and enforcing tolerance will require further investigation. While we provide evidence suggesting mtROS is partially required for mitohormetic tolerance, macrophages with reduced IRG1 expression are resistant to LPS-induced suppression of mitochondrial oxidative metabolism and tolerance35, suggesting multiple reactive molecules contribute to triggering mitohormesis. Overall, we speculate that metabolic reprogramming involves transcriptional changes, along with post-transcriptional alterations in mitochondrial composition by the ubiquitin–proteasome system, which can tune mitochondrial composition and function in response to oxidative and metabolic stress36. Importantly, whether ROS/RES trigger mitohormetic tolerance in clinical situations of immunosuppression where restoring myeloid inflammatory responses would be beneficial will be important to investigate. Transcriptional profiling of myeloid cells from sepsis and cancer patients has revealed overlapping signatures of reduced pro-inflammatory gene expression37 and increased aerobic glycolysis38, and ROS/RES production has been linked to myeloid cell metabolic alterations and immunosuppression39. We propose myeloid cells performing high levels of aerobic glycolysis as a consequence of undergoing mitohormesis may represent ‘exhausted’ cells unable to convert glucose-derived carbon into acetyl-CoA to epigenetically activate inflammatory responses. Further work with preclinical models and clinical samples is needed to test the relevance of mitohormesis to in vivo immunosuppression. Better defining the transcriptomic, proteomic, metabolic and functional adaptations associated with macrophage mitohormesis can help determine whether this stress response occurs in vivo, and whether it can be used to stratify outcomes, as a diagnostic to inform clinical decisions, or as a therapeutic target.

From an anti-inflammatory therapeutic perspective, these findings demonstrate that, beyond acute depletion of acetyl-CoA, inducing mitohormetic tolerance represents a mechanism by which the reactive, lipophilic hydroxyoestrogens achieve lasting immunosuppression. We propose triggering mitohormesis in macrophages by causing oxidative and electrophilic mitochondrial
stress with lipophilic electrophiles represents a new therapeutic strategy to combat inflammation that works by co-opting the physiological response to mitochondrial stress that occurs naturally after TLR engagement and during transition to a tolerant state. In other words, by promoting eustrress\(^{(10)}\) (that is, moderate mitochondrial stress), a benefit is achieved (that is, reduced inflammation). This concept of mitochondria-targeted, pro-oxidant therapy to repress inflammation via triggering mitohormetic metabolic reprogramming directly opposes the concept of using mitochondria-targeted antioxidants as anti-inflammatory drugs\(^{(13,19,40)}\).

In situations where mitochondrially targeted antioxidants show anti-inflammatory effects, it will be important to delineate whether this activity is due to quenching of redox signals, or secondary effects of mitochondrial stress adaptations triggered by the small molecule\(^{(42)}\). More broadly, given the failures of antioxidants in clinical trials, perhaps in inflammation and other pathologies (for example, ageing), transiently increasing oxidative eustrress to trigger the body’s own stress defences (as occurs during physical activity and caloric restriction) represents a more tractable therapeutic approach than trying to mimic endogenous defence systems that have evolved over millions of years with loosely targeted antioxidants.

**Methods**

**Animals.** All experiments were approved by the UC Berkeley Animal Care and Use Committee to ensure ethical use of animals (AUP-2017-02-9539-1), and performed under the supervision of the UC Berkeley Office of Laboratory and Animal Care. N/2KO (C07009) and C57BL/6J (000664) mice were purchased from Jackson Labs, and C57BL/6N (027) mice were from Charles River. Elox flox mice and Lyz2-Cre mice were generous gifts from K. Korach (National Institutes of Health (NIH)) and G. Barton (UC Berkeley), respectively. Animals were housed under standard conditions (20–23 °C, 50% humidity, 12h/12h light/dark cycle).

**Cell culture.** BMDMs were prepared from 6- to 12-week-old female C57BL/6N or C57BL/6J mice by plating bone marrow in DMEM (Corning 10–013-CV) supplemented with 10% FBS (HyClone SH30071.03), penicillin–streptomycin, and 0.45% Tween-20. Lysate was supplemented with Proteinase K (NEB) and incubated for 1 h at 56 °C. Proteinase K was inactivated and 5 μl of lysate was used in qPCR reactions for mitochondrial and genomic DNA ampiclons (see Supplementary Table 6 for primers).

**RNA-seq.** See Gene Expression Omnibus ( GEO) under accession code GSE169731 for detailed experimental design and sample information. Libraries were prepared from total RNA using an mRNA HyperPrep kit (KAPA/Roche) and unique dual index adaptors (IDT), qPCR quantified and pooled for sequencing (Illumina HiSeq 2500 or HiSeq 4000). Reads were aligned using STAR and counted with HOMER. EdgeR and DESeq were used for differential expression analysis. Hierarchical clustering was performed using Cluster and visualized with Java TreeView. Heat maps were produced using Java TreeView and GraphPad Prism 8. GO analysis was performed with Metascape. Promoter motif finding was performed using HOMER.

**Western blotting.** Cells/mitochondrial fractions were lysed in RIPA buffer with protease inhibitor cocktail (Roche) for 20 min on ice, followed by centrifugation at 10,000g for 10 min at 4 °C. Supernatant was quantified with DC Protein Assay (BioRad); 15–30 μg of protein was mixed with 5× NuPAGE loading dye and 2× NuPAGE reducing reagent (Life Technologies), and samples were heated for 10 min at 70 °C. Samples were separated on NuPAGE 4–12% Bis-Tris gels (Life Technologies) and transferred to PVDF membranes (GE Healthcare), which were blocked (SuperBlock, Thermo Fisher) for 1 h. Membranes were probed with primary antibodies overnight at 4 °C, followed by 0.1% TBS-T washes and fluorophore-conjugated secondary antibody probed for 1 h. After addition with HRP-conjugated secondary antibodies, membranes were washed with TBS-T and exposed to ECL (Thermo Fisher) for 1 min. Bands were visualized using Licor Odyssey imaging system. Primary antibodies and dilutions used were as follows: tubulin (CP06, Cal Biochem; 1:1000), pro-IL-1β (AF-401-NA, R&D Systems; 1:500), RNF2 (MABE1799, EMD Millipore; 1:500), vinculin (sc-73614, Santa Cruz; 1:2000), VDAC (ab154856, Abcam; 1:2000). Secondary antibodies were (1:10,000 dilution for all) Alexa Fluor 680 conjugates from Invitrogen, and IRDye 800CW conjugates from Rockland. All antibodies were diluted in 1% PBS-T with 5% BSA.

**IL-1β ELISA.** Detection of murine and human IL-1β in serum and cell supernatants was performed using ELISA kits according to the manufacturer’s instructions (Invitrogen, nos. 88-703-12 and 88-7261-22, respectively). For supernatants, BMDMs (7.5×10⁵ cells) and THP-1 cells (1.5×10⁵ cells) were plated in 24-well plates, and 200 μl of medium was collected for ELISA.

**Acute in vivo inflammation.** C57BL/6J male mice aged 8 weeks old were injected i.p. with ethanol or oestrogens (10 mg kg⁻¹), followed by i.p. injection of PBS or LPS (2 mg kg⁻¹) 1 h later. After 3 h, submandibular bleeding was performed for measurement of serum IL-1β. At 4 h, mice were euthanized and splenocytes isolated by crushing spleen through a 40-μm filter, red blood cell lysis and cell pellet lysis in TRIzol.

**Chronic in vivo inflammation.** For RNA-seq studies, 8-week-old C57BL/6N male mice were placed on a HFD (Research Diets, ID14294; 60 kcal% fat; Fisher, NC0004611) with s.c. ethanol or oestrogen injections (10 mg kg⁻¹) every 6 d in the rear flank. After 30 d, cWAT was isolated and stromal vascular fraction prepared by digesting cWAT in DMEM with 0.1% collagenase (Sigma C6885) and 5% BSA for 1 h at room temperature with gentle shaking. The sample was passed through a 70-μm filter, and red blood cells were lysed, and the stromal vascular fraction was stained on ice in FACS buffer (PBS with 10 mM HEPES and 5% BSA). After Fc block (BD Biosciences), cells were labelled with anti-CD45-PerCP-Cy5.5 (clone 30-F11, BioLegend), anti-F4/80-PE (clone BM8, eBioscience) and anti-CD11b-APC (clone M1/70, eBioscience; all 1:100 dilutions). Cells were resuspended in FACS buffer with DAPI (Thermo Fisher D1300, stock: 1×10⁻⁴ M) and F4/80/CD11b macrophages sorted directly into TRIzol-LS (Invitrogen). Total live cell counts were also determined after pulsing a haemocytometer and trypan blue.

For metabolic studies using 8-week-old male C57BL/6J mice, after 30 d of NC or HFD feeding and injections, mice were fasted overnight with water. The next morning, blood samples were collected from tail tips to measure fasting blood glucose (Accu-Chek glucometer, Aviva Plus test strips). Mice were then i.p. injected with 2% glucose solution (2 g glucose per kg bodyweight), and blood glucose measurements were recorded over time. For GSIS, this process was repeated for only the 0-min and 15-min time points, with larger blood samples collected from the lateral tail vein for serum insulin ELISA (Crystal Chem 90880). At 48 h after final s.c. injections, mice were i.p. injected with PBS or LPS (3 mg kg⁻¹), and cardiac puncture was performed at 4.5 h to collect blood for serum IL-1β ELISA. Splenocytes were isolated as described above.

**Mitochondrial fractions for steroid extraction and LC-MS.** RAW macrophages (+15–30 million total) were treated with ethanol or 5 μM 4-OHE1 for 1 h before PBS wash and collection. For whole-cell steroid extraction, 1×10⁶ macrophages were pelleted (800 g for 10 min at 4 °C), flash frozen and stored at −80 °C. From remaining cells, mitochondrial fractions were isolated using a previously described method with modification\(^{(4)}\). Briefly, cells were resuspended in 2 ml cell isolation buffer (IBc) in a 15 ml conical tube and disrupted with sonication (Bioruptor, ...
Diagenode. Mitochondrial fractions were isolated by centrifugal filtration, flash frozen and stored at −80°C. Fractions were checked for mitochondrial protein enrichment versus whole-cell lysates by western blot.

For steroid extraction, whole-cell and mitochondrial fraction pellets were thawed and resuspended in 1 ml of acetoneitrile (to confirm the method could extract 4-OHE1, we also performed extraction from 20 μl of cell culture medium to which 2 μl of 0.5 mM 4-OHE1 was added). Samples were stored for 30 min at −20°C, then centrifuged for 5 min at 12,000g at 4°C. Supernatant was transferred to a glass V-bottom 96-well plate that was dried and evaporated under a nitrogen stream. Residue was resuspended in 2 ml of 0.2 M sodium acetate buffer (pH 5.0) and mixed with 10 ml of hexane. The mixture was centrifuged for 2 min at 1,200g, and the upper hexane layer transferred to a glass tube and evaporated under nitrogen with gentle heating at 25–30°C in a water bath (N-EVAP 112, Organomation Associates). The residue was reconstituted in 50 μl of methanol.

Samples were subjected to a flash liquid chromatography system (LC-1200 series, Agilent Technologies) equipped with a reversed-phase analytical column (length of 150 mm, inner diameter of 1.0 mm, particle size of 5 μm; Viva C18, Restek). The LC system was connected in line with an ETTQ-Orbitrap-XL mass spectrometer equipped with an electrospray ionization (ESI) source and operated in the positive ion mode (Thermo Fisher Scientific). Mass spectrometry data acquisition and processing were performed using Xcalibur software (version 2.0.7, Thermo Fisher Scientific). Injection volumes were between 2 μl and 5 μl for all samples. The LC–MS is located in the QB3/Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

iPGO-APBB. iPGO-APBB was performed as previously described. Briefly, proteomes (prepared from BMDMs treated with ethanol or 1 μM 4-OHE1 for 1 h) were labelled with iRay (100 μM) for 1 h at room temperature, and subsequently treated with 100 μM isotopically light (control) or heavy (treated) TEV-biotin, and click chemistry was performed. Proteins were precipitated, washed and resublimated, and insoluble components were precipitated. Soluble proteome was diluted 100-fold in labeling buffer and was bound to avidin-agarose beads at room temperature overnight at 4°C. Bead-bound proteins were enriched, then resuspended, alkylated with iodoacetamide, then washed and resuspended with sequencing-grade trypsin overnight. Non-bead-bound tryptic peptides were washed away, and the TEV-biotin tag was digested overnight in TEV buffer containing an Ac-TEV protease at 29°C. Liberated peptides were diluted in water, acidified and stored at −80°C. Analysis was performed using multidimensional protein identification technology (MudPIT) with an Orbitrap Q Exactive Plus mass spectrometer (Thermo Fisher). Data were extracted in the form of MS1 and MS2 files using Raw Extractor 1.9.9.2 (Scirrps Research Institute) and searched against the UniProt mouse database using ProLuCID search methodology in IP2 v3 (Integrated Proteomics Applications). ProLuCID data were filtered through DTSelect to achieve a peptide false-positive rate below 1%, and cysteine residues were searched with a static modification for carboxyamidomethylation (+57.02146 Da) and up to two differential modifications for the light or heavy TEV tags (+464.26181 Da and +470.29977 Da, respectively). Mitochondrial target enrichment analysis was performed by comparing the iPGO-APBB target list to MitoCarta (v2.0). Metabolomics. BMDMs were cultured in DMEM (Corning 17–207–CV) supplemented with One Shot dialyzed FBS (Thermo Fisher), 1 mM sodium pyruvate, 4 mM l-glutamine and 25 mM glucose (all from Gibco). Approximately 5 × 10^7 cells were treated with ethanol or 5 μM 4-OHE1. After 2 h, cells were washed with unsupplemented medium, and provided DMEM as described above except with 25 mM [13C6]glucose (Cambridge Isotope Laboratories). Cells were harvested for the last 20 min of staining. Cells were placed on ice, washed with PBS and scraped into PBS + DAPI. For roGFP macrophages, emission after excitation with 450-nm (violet) and 488-nm (blue) lasers was collected using a 505-nm longpass:525/50 bandpass filter combination coupled to each respective dye for flow cytometry. Data were analysed with FlowJo v.10.5.2 (Tree Star).

Pro-II-1β intracellular staining for flow cytometry. Cells were stained using a FIX & PERM Kit (Thermo Fisher) according to the manufacturer's instructions with anti-pro-II-1β (AF-401-NA, R&D Systems) and anti-goat Alexa Fluor 647 (Invitrogen) antibodies.

Matrix-oxGFP and roGFP RAW macrophages. For matrix-oxGFP macrophages, cells were transduced with lentivirus encoding N-terminal fusions of oxGFP to COX4L mitochondrial matrix targeting sequence and selected with 10 μg/mL puromycin (Thermo Fisher). For roGFP macrophages, cells were transduced with lentiviral constructs encoding N-terminal fusions of roGFP to COX4L targeting sequence (matrix-roGFP), LACTB targeting sequence (IMS-roGFP) or nuclear export sequence (cyto-roGFP), and selected with 10 μg/mL puromycin and 500 μM H2O2 (Fisher, H325) added in culture medium for staining at 37 °C. Cells were treated with ethanol or 5 μM 4-OHE1 was added. Samples were stored for 30 min at −80°C, then centrifuged at 25–30°C in a water bath (N-EVAP AP 112, Organomation Associates). The residue of 150 mm, inner diameter of 1.0 mm, particle size of 5 μm (Zymo Research, D4007) was washed with wash buffer II (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 0.5% NaDOC) three times, wash buffer III (10 mM HEPES pH 8.0, 250 mM NaCl, 2 mM EDTA, 1% NP-40 and 0.5% NaDOC) three times, and TE buffer with 50 mM NaCl two times (all supplemented with protease inhibitors, all washes on ice). Beads were then resuspended in 200 μl elution buffer (50 mM Tris–HCl (pH 8.0), 10 mM EDTA and 1% SDS) for 30 min at 37 °C to elute immunoprecipitated complexes. Eluent was collected, 10 μl of 5 M NaCl added, and DNA precipitation cross-links reversed by heating at 65°C. Sequencing libraries were then prepared using a new tube and evaporated overnight at room temperature using a SpeedVac. DNA was blunted, A-tailed and ligated to NEXTFlex adaptors (Bioro). Libraries were PCR amplified, quantified, pooled and sequenced (Illumina HiSeq 2500). Reads were aligned using STAR, and HOMER findPeaks was used to call peaks/regions relative to inputs. HOMER getDifferentialPeaks was used to identify peaks/regions with significantly altered read density, and HOMER annotatePeaks.pl was used to make read density histograms. Data are available at the GEO under accession GSE169731.

Flow cytometry and cell sorting. All analysis was performed using LSR II, LSR Fortessa or LSR Fortessa X20 analyzers (BD). All sorting was performed on Influx or Aria Fusion sorters (BD). Data were collected using BD FACSDiva software and analysed with Flowjo v.10.5.2 (Tree Star).

ChIP-seq. Approximately 30 million BMDMs were treated with ethanol or 1 μM hydroxyoestrogen for 1 h, followed by PBS or LPS (100 ng ml−1) stimulation for 30 min. Cells were washed with PBS and fixed with 0.67 ml ml−1 DSB (Thermo Fisher) in PBS for 30 min at room temperature with shaking, followed by addition of paraformaldehyde (Electron Microscopy Sciences) to 1% and an additional 15 min of fixation. Fixation was quenched with 125 mM glycine and shaking 10 min, after which cells were scraped and washed with PBS. Cell pellet was resuspended in 1.5 ml ChIP RIPPA buffer (20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 0.1% SDS and 1% Triton X-100) with protease inhibitors (Roche) and sonicated at 4°C for 60 min (Bioruptor sonicator, continuous cycles of 10 on/40 off, ‘medium’ setting). Samples were spun for 20 min at maximum speed at 4°C to remove insoluble material, and supernatant containing soluble sheared chromatin was transferred to new tube, saving 1% volume for input library preparation. Immunoprecipitation was performed overnight at 4°C with 2 μg of primary antibody (pAb; Santa Cruz, sc-372; HK272:ac; Abcam, ab3729), or species-appropriate IgG control antibody (GenScript), conjugated to Protein A Dynabeads (Invitrogen). The next day, beads were captured with magnet and washed with wash buffer II (20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 0.5% NaDOC) three times, wash buffer III (10 mM Tris–HCl (pH 8.0), 250 mM NaCl, 2 mM EDTA, 1% NP-40 and 0.5% NaDOC) three times, and TE buffer with 50 mM NaCl two times (all supplemented with protease inhibitors, all washes on ice). Beads were then resuspended in 200 μl elution buffer (50 mM Tris–HCl (pH 8.0), 10 mM EDTA and 1% SDS) for 30 min at 37 °C to elute immunoprecipitated complexes. Eluent was collected, 10 μl of 5 M NaCl added, and DNA precipitation cross-links reversed by heating at 65°C. Sequencing libraries were then prepared with RNase A (Thermo Fisher) for 1 h at 37°C. Proteinase K (NEB) for 1 h at 50°C, and DNA was recovered with Zymo ChIP DNA Clean and Concentrator kit.

Sequenceing libraries were prepared using an in-house protocol. Briefly, DNA was blunted, A-tailed and ligated to NEXTFlex adaptors (Bioro). Libraries were PCR amplified, quantified, pooled and sequenced (Illumina HiSeq 2500). Reads were aligned using STAR, and HOMER findPeaks was used to call peaks/regions relative to inputs. HOMER getDifferentialPeaks was used to identify peaks/regions with significantly altered read density, and HOMER annotatePeaks.pl was used to make read density histograms. Data are available at the GEO under accession GSE169731.
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Author contributions

G.A.T. and K.S. conceptualized the project. G.A.T., K.M.T., A.T.I., J.t.H., D.K.N., A.S. and K.S. procured funding and resources. G.A.T., K.M.T., B.F., A.T.I. and J.t.H. designed experimental methodology. G.A.T., K.M.T., B.F., J.M.W., J.W., S.Z., R.I.K., S.K.L., A.T.I. and J.t.H. performed experiments and analysed data. G.A.T. curated data, wrote the original manuscript, edited the manuscript with input from all authors and prepared the revised manuscript.

Competing interests

G.A.T. and K.S. are co-inventors on provisional patent application no. 63/091,217 filed by UC Berkeley describing methods of use of hydroxyoestrogen for treatment of inflammatory and metabolic disease. G.A.T. is co-founder of a start-up focused on therapeutic applications of hydroxyoestrogen derivatives. D.K.N. is a shareholder and adviser for Frontier Medicines. The other authors declare no competing interests.

Additional information

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Extended Data Fig. 1  | Hydroxyestrogens are anti-inflammatory in macrophages in vitro.  

**a.** BMDMs pretreated for 1 h with vehicle or 1 μM hydroxyestrogens before 6 h LPS stimulation and qPCR to validate targets identified in RNA-seq data.  

**b.** RAW macrophages pretreated for 1 h with vehicle or 5 μM estrogens before 6 h LPS stimulation and Il1b qPCR.  

**c.** THP-1s and U937s pretreated for 1 h with vehicle control or 10 μM estrogens before 6 h LPS stimulation and qPCR for indicated transcripts.  

**d.** RAW macrophages pretreated with ethanol or 5 μM 4-OHE1 for 1 h, followed by 6 h LPS stimulation before pro-IL-1β measurement by western blot (left, n = 2 biological replicates), or by intracellular staining and flow cytometry (right, representative data from 1 independent biological replicate for each condition shown).  

**e.** RAW macrophages pretreated with EtOH or 2.5 μM estrogens for 1 h, followed by 3 h stimulation with LPS (TLR4), Pam3CSK4 (PAM, TLR2), polyinosinic-polycytidylic acid (pIC, TLR3), or CpG oligodeoxynucleotides (CpG, TLR9), and Il1b qPCR. Percentages indicate induction relative to max (100%) in the ‘EtOH + TLR ligand’ control BMDMs for each ligand.  

**f.** BMDMs pretreated with EtOH or 1 μM hydroxyestrogens in the absence (left) or presence (right) of 10 μM ICI 182780 for 1 h, followed by 6 h LPS stimulation and Il1b qPCR. Each data point is an independent biological replicate. For n = 2, data represented as mean; for n = 3, mean ± SEM. P values from unpaired, two-sided Student’s T Test versus ‘EtOH + LPS’ sample (planned comparison). qPCR and flow cytometry representative of 2 independent experiments. Western blot was performed once.
Extended Data Fig. 2 | Hydroxyestrogens are anti-inflammatory in vivo. a. Representative gating strategy for identifying forward scatter/side scatter (FS/SS) live gate+, DAPI−, CD45+, F4/80+CD11b+ visceral white adipose tissue (vWAT) macrophages for sorting and flow cytometry analysis. b. vWAT mass in mice after 30 days HFD feeding and EtOH control or estrogen injections. n = 5 mice per group. c. vWAT macrophage cellularity in mice after 30 days HFD feeding and EtOH control or estrogen injections. n = 5 mice per group. d. Relative expression* of select pro-inflammatory genes in vWAT macrophages from HFD-fed mice injected with EtOH, 4-OHE1, or E2 (*log2-transformed RPKM centered on the mean of each gene). n = 5 mice per group. e. Blood glucose levels at 30min and 1h post-glucose injection in mice from Fig. 2f. n = 10, 10, and 11 mice per condition. f. qPCR for Nos2 expression in splenocytes isolated from mice in Fig. 2h. n = 3, 5, 5, and 5 mice per condition. For bar graphs, each data point is an independent biological replicate, and data is represented as mean ± SEM. All P values from unpaired, two-sided Student’s T Test (planned comparisons). HFD chronic inflammation model was performed once for transcriptional profiling (a–d), and a second time for metabolic studies (e,f).
Extended Data Fig. 3 | Hydroxyestrogens activate NRF2, but NRF2 is dispensable for their anti-inflammatory effects. a. GO analysis of 341 genes significantly upregulated in hydroxy estrogen-pretreated, LPS-stimulated BMDMs relative to control pretreatments. Red highlights GO categories involved in oxidative stress resistance (OSR) and detoxification of reactive oxygen species (ROS). b. WT and Nrf2 KO BMDMs were pretreated 1h with EtOH or indicated concentrations of 4-OHE1 before 6h LPS stimulation and qPCR for the NRF2 target gene Hmox1. Data represented as mean ± SEM. n = 3 independent biological replicates per condition. P values from unpaired, two-sided Student’s T Test against corresponding WT sample (planned comparisons). Data representative of 2 independent experiments.
Extended Data Fig. 4 | Hydroxyestrogens cause mitochondrial stress. a. RAW macrophages treated with 5 μM E2, or 5 μM 4-OHE1 (with or without 30 min MitoQ pretreatment, 1 μM) for 1 h before Hmox1 qPCR. n = 3 independent biological replicates per condition. Data represented as mean ± SEM. P values from unpaired, two-sided Student’s T test (planned comparisons). Data representative of 2 independent experiments. b–d. GO analysis of 341 genes significantly upregulated in hydroxyestrogen-pretreated, LPS-stimulated BMDMs relative to control pretreatments. Red highlights GO categories indicative of HSF1 and ATF4 activity, and upregulation of glycolysis/pentose phosphate pathway (PPP) genes. e. Experimental setup for steroid extraction and liquid chromatography/mass spectrometry (LC-MS) to measure 4-OHE1 extracted from cell culture media (top, control), or from whole cell and mitochondrial fractions prepared from RAW macrophages treated with 5 μM 4-OHE1 for 1 h (n = 2 independent biological replicates per condition). f. Uncropped western blot confirming enrichment of mitochondrial marker VDAC, and depletion of cytoplasmic marker vinculin, in mitochondrial fractions versus whole cell lysates prepared from RAW macrophages. Blot is representative of two independent fractionations for LC-MS. g. top – Extracted ion chromatograms for 4-OHE1 (m/z = 287.1642). Peaks at retention time (RT, minutes) = 13.14 and RT = 13.23 for the 4-OHE1 standard and media + 4-OHE1 sample (denoted by red arrows) confirm our ability to extract and detect 4-OHE1. Lack of a defined, quantifiable chromatographic peak at RT = 13.1–13.2 for whole cell or mitochondrial extracts prepared from RAW macrophages indicates lack detectable, free 4-OHE1 in these samples. Data is representative of two independent fractionations and LC-MS runs with n = 2 independent biological replicates per condition. bottom – Mass spectrum (positive ion mode) measured for 1 mg/mL 4-OHE1 standard (injection volume = 2 μL) showing detail for the [M+H]+ ion of 4-OHE1 at m/z = 287.1642. Mass range displayed: m/z = 287.1624–287.1660. h. Chi-square test comparing the observed frequency of mitochondrial targets (18) in our isoTOP-ABPP target list (118 total targets) versus the expected frequency of mitochondrial targets from MitoCarta 2.0.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Hydroxyestrogens impair mitochondrial acetyl-CoA production and histone acetylation required for LPS-induced proinflammatory gene transcription. **a.** BMDMs pretreated 1h with EtOH or 4-OHE1 before 6h LPS stimulation and qPCR. The average percent induction at each 4-OHE1 concentration relative to control for each gene was calculated (n = 3 independent biological replicates) and plotted. **b.** Abundance (area under the curve, AUC) of TCA cycle metabolites in EtOH versus 4-OHE1-treated BMDMs. **c.** Fractional contribution (FC) of 13C6 glucose-derived carbons to TCA cycle metabolites and amino acids in EtOH versus 4-OHE1-treated BMDMs (significant labeling in **bold**). **d.** UCSC genome browser H3K27ac ChIP-seq tracks displaying regions of decreased read density (arrows) in the Il1b, C6d9, Ifitm3, and Nfkbid loci. Read density was locally scaled, and black bar added to provide common reference point across tracks. **e.** RAW macrophages cultured in CoA (500μM, top) or acetyl-CoA (Ac-CoA, 500μM, bottom) (red bars) for 3h prior to 1h EtOH or 5μM 4-OHE1 pretreatment, 6h LPS stimulation, and Il1b qPCR. **f.** RAW macrophages cultured in sodium acetate (5mM, purple bar) or CoA (500μM, red bar) for 15 minutes prior to 1h EtOH or 5μM 4-OHE1 pretreatment, 1h LPS stimulation, and Il1b qPCR. **g.** Data from reference24. Acetyl-CoA levels in HeLa cells treated with vehicle or 10μM FCCP for 24h. n = 7 independent biological replicates per condition. **h.** RAW macrophages pretreated with vehicle or itaconate (7.5mM) for 1h before 3h LPS stimulation and Il1b qPCR. **i.** RAW macrophages cultured in acetyl-CoA (Ac-CoA, 200μM, bottom) for 2h prior to pretreatment with vehicle or itaconate for 1h before 3h LPS stimulation and Il1b qPCR. Each data point is an independent biological replicate. For n = 2, data represented as mean; for n = 3 or more, data represented as mean ± SEM. All P values from unpaired, two-sided Student’s T Test (planned comparisons). qPCR data representative of at least 2 independent experiments. Metabolomics and ChIP-seq studies were performed once.
Extended Data Fig. 6 | Hydroxyestrogen-driven mitochondrial stress triggers mitohormesis. a. Oxygen consumption in RAW macrophages after treatment with vehicle or 4-OHE1. 5 repeated measurements per condition were taken over 45min and averaged (n = 4 independent biological replicates). b. Mitochondrial membrane potential in RAW macrophages treated 20min with vehicle, oligomycin, FCCP, or 4-OHE1 (5μM). c. RAW matrix-oxGFP macrophages treated 8h with vehicle or 4-OHE1 and fluorescence quantified by flow cytometry. d. qPCR for matrix-oxGFP transcript in parental macrophages and RAW matrix-oxGFP macrophages treated 8h with vehicle or estrogens (5μM). e. Mitochondrial DNA content in RAW macrophages treated with vehicle or 5μM 4-OHE1. f. RAW matrix-oxGFP macrophages pretreated 1h with NAC (5mM) or MitoQ (1μM) prior to 8h vehicle or 5μM 4-OHE1 treatment. g. RAW matrix-oxGFP macrophages pretreated 1h with KRIIB11 (10, 7.5, 5μM) before 8h treatment with 5μM 4-OHE1. h. RAW macrophages and BMDMs treated 8h with vehicle or estrogens (5μM) before MitoTracker Green staining and flow cytometry. i. THP-1 and U937 matrix-oxGFP cells treated 24h with vehicle or estrogens (5μM). j. BMDMs treated 1h with vehicle or estrogens (5μM) before 6h LPS stimulation, MitoPY1 staining, and flow cytometry. k. BMDMs pretreated 1h with vehicle or estrogens (5μM) before 6h LPS stimulation, MitoPY1 staining, and flow cytometry. l. RAW macrophages pretreated 1h with NAC (5mM) or MitoQ (1μM) prior to vehicle or 5μM 4-OHE1 treatment (18h). Cells were then treated 2.5h with vehicle or 50μM menadione before viability assessment by flow cytometry. o. THP-1 and U937s treated 7h with vehicle or estrogens (5μM) before H2O2 challenge, CM-H2DCFDA staining, and flow cytometry. Each data point is an independent biological replicate. For n = 2, data represented as mean; for n = 3 or more, data represented as mean ± SEM. All P values from unpaired, two-sided Student’s T Test (planned comparisons). All data representative of at least 2 independent experiments.
Extended Data Fig. 7 | LPS-driven mitochondrial stress triggers mitohormesis. a, GO analysis of 1622 genes upregulated by both 4-OHE1 and LPS at 6h in BMDMs. b, Heatmap showing relative expression* of select genes upregulated by both 4-OHE1 and LPS at 6h in BMDMs. (*DESeq2 counts centered on the mean of each gene). c, RAW matrix-oxGFP macrophages pretreated with vehicle or 10 μM KRB11 for 1h before treatment with 4-OHE1 (5 μM, left) or LPS (right) for 8h. Matrix-oxGFP fluorescence was quantified by flow cytometry. d, MitoTracker Green signal in RAW macrophages and BMDMs measured by flow cytometry after 24h LPS simulation. e, Mitochondrial DNA/genomic DNA (mtDNA/gDNA) ratio in RAW macrophages treated with PBS vehicle control or LPS for indicated times. f, THP-1 and U937 matrix-oxGFP reporter cells treated 24h with LPS before matrix-oxGFP fluorescence was quantified by flow cytometry. g, WT (left) and Nrf2 KO (right) BMDMs were treated with EtOH vehicle control, or 4-OHE1/LPS (5 μM/100ng/mL) for 7h before Prdx6 qPCR. h, THP-1 cells were left untreated, or stimulated with LPS for 7h, before H₂O₂ treatment (500 μM, 5min) and intracellular oxidative stress measurement via CM-H₂DCFDA staining and flow cytometry. Each data point is an independent biological replicate. For n = 2, data represented as mean; for n = 3, mean ± SEM. P values from unpaired, two-sided Student’s T Test (planned comparisons). RNA-seq study was performed once. Flow cytometry and qPCR data representative of 2 independent experiments.
Extended Data Fig. 8 | Mitohormesis in macrophages involves metabolic reprogramming that enforces an LPS-tolerant state. a. Basal and maximal OCR from RAW macrophage mitochondrial stress test. Data is average of 3 repeated measurements prior to oligomycin injection, or following FCCP injection, respectively. b. Mitochondrial stress test in BMDMs treated 20h with EtOH, 4-OHE1 (5 μM), LPS, or both. n = 5 independent biological replicates per condition. c. Il1b qPCR in BMDMs treated overnight (18–24h) with EtOH, 4-OHE1 (5 μM), LPS, or both, before treatment wash out, recovery (1–2h), and 6h secondary LPS stimulation. Data is plotted as secondary LPS fold-induction versus cells with the same primary treatment but no secondary LPS. d. RAW macrophages treated overnight with EtOH, 4-OHE1 (5 μM), LPS, or both, before wash out, recovery, and 6h secondary LPS stimulation for MitoPY1 staining and flow cytometry. Data plotted as percent change in MitoPY1 fluorescence versus no secondary LPS control for each primary treatment. P values are from comparison of treatment vs. naïve control. e. BMDMs treated overnight with EtOH or estrogens (5 μM) before wash out, recovery, and 6h secondary LPS stimulation (100ng/mL, 2.5 μM nigericin added the last hour) for IL-1β ELISA. f. Il1b qPCR in THP-1s treated overnight with EtOH, LPS, or 4-OHE1 (5 μM) before wash out, recovery, and 6h secondary LPS stimulation. Data plotted as LPS fold-induction for cells with the same primary treatment but no secondary LPS. g. Mitochondrial membrane potential (mtMP) in RAW macrophages treated with LPS and stained with JC-9 for flow cytometry. Except for b, each data point is an independent biological replicate. For n = 2, data represented as mean; for n = 3 or greater, mean ± SEM. P values are from unpaired, two-sided Student’s T Test (planned comparisons). BMDM Seahorse and ELISA were performed once. All other data representative of 2 independent experiments.
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| Sample size | No statistical methods were used to predetermine sample size. Sample size was chosen based on previous experience with the experiments performed, and the effect size/level of biological variability inherent to each type of experiment in published work. |
|-------------|------------------------------------------------------------------------------------------------------------------|
|             | For in vivo measurement of proinflammatory cytokine production and gene expression, we chose to use a smaller sample size than previous studies, such as Tanahill et al. and Rodriguez et al., because our in vitro effect sizes were larger, and to minimize the number of animals used in the study. |
|             | [https://doi.org/10.1038/nature11986](https://doi.org/10.1038/nature11986)                                           |
|             | [https://doi.org/10.1016/j.cmet.2019.01.014](https://doi.org/10.1016/j.cmet.2019.01.014)                              |
|             | For in vivo metabolic measurements, a larger sample size was chosen because although metabolic dysfunction can be detected within 3-4 weeks of high-fat diet feeding, the effect sizes are small at this timepoint compared to later timepoints as shown by He et al. |
|             | [https://doi.org/10.1016/j.cdtm.2020.06.003](https://doi.org/10.1016/j.cdtm.2020.06.003)                              |
|             | For in vitro macrophage measurements of gene expression, cytokine secretion, metabolites, and various parameters measured via dyes/reporters and flow cytometry, Cameron et al. and Langston et al. provide guidance for sample size selection. |
|             | [https://doi.org/10.1038/s41390-019-0336-y](https://doi.org/10.1038/s41390-019-0336-y)                                |
|             | [https://doi.org/10.1016/j.apsb.2019.04.053-7](https://doi.org/10.1016/j.apsb.2019.04.053-7)                         |
| Data exclusions | No data was excluded during analysis. |
| Replication | 2-3 independent experiments at minimum were performed for all data presented, which display representative results. Exceptions are: RNA-seq studies (not independently replicated due to cost, but gene expression changes/signatures were always followed-up by qPCR and/or functional studies), metabolomics & ChIP-seq studies (not independently replicated due to cost, and because the results are corroborated by each other, and by the rescue of pro-inflammatory gene expression by CoA/acetyl-CoA supplementation), in vivo acute LPS study (reduction in IL-1β was corroborated in chronically 4-OHE1-dosed animals injected with LPS), in vivo metabolic study (improvements in metabolic parameters were in line with the reduction in adipose tissue macrophage proinflammatory gene expression observed using the same high-fat diet model for RNA-seq studies), and ELISAs where indicated (they confirmed transcriptional and protein level effects observed across multiple cell lines and via other methods, such as western blotting and intracellular staining/flow cytometry). |
| Randomization | For in vivo experiments, mice were randomized and grouped such that mean bodyweight between treatment groups was similar. For in vitro experiments, cells were always plated from a common culture the day before the experiment. |
| Blinding | Investigators were not able to be blinded during data collection or analysis because a single individual was responsible for the experimental set-up, data collection, and data analysis. |

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### Reporting for specific materials, systems, and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

#### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | Antibodies            |
| ☑   | Eukaryotic cell lines |
| ☑   | Palaeontology and archaeology |
| ☑   | Animals and other organisms |
| ☑   | Human research participants |
| ☑   | Clinical data         |
| ☑   | Dual use research of concern |

#### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChIP-seq              |
| ☑   | Flow cytometry        |
| ☑   | MRI-based neuroimaging |
### Antibodies

| Antibodies used | Description |
|-----------------|-------------|
| pro-IL-1beta [AF-401-NA, R&D Systems], 1:50 for intracellular staining, 1:500 for western blot |
| donkey anti-goat Alexa Fluor 647 [A-21447, Invitrogen], 1:500 for intracellular staining |
| Tubulin [clone DM1A, CP06-100UG, Cal Biochem], 1:1000 for western blot |
| NRF2 [clone 103, MABE1799, EMD Millipore], 1:500 for western blot |
| Vinculin [clone 7F9, sc-73614, Santa Cruz], 1:20000 for western blot |
| VDAC [ab154856, Abcam], 1:2000 for western blot |
| Western blot secondary antibodies [all used at 1:10,000]: AlexaFluor 680 conjugates from Invitrogen, and IRDye 800DCW conjugates from Rockland. |
| p65 [sc-372, Santa Cruz], 2ug for ChIP-seq |
| I3K27ac [ab4729, AbCam], 2ug for ChIP-seq |
| CD11b APC-eFluor 780 conjugate [clone M1/70, 47-0112-82, eBioscience/ThermoFisher], 1:100 for flow cytometry |
| F4/80 PE conjugate [clone BM8, 12-4801-82, eBioscience/ThermoFisher], 1:100 for flow cytometry |
| CD45 PerCP5.5 conjugate [clone 30-F11, 103132, BioLegend], 1:100 for flow cytometry |

### Validation

| Validation | Description |
|------------|-------------|
| pro-IL-1beta [AF-401-NA, R&D Systems]: confirmed by manufacturer to detect pro-IL-1beta in LPS-stimulated, but not control, RAW264.7 cells by western blot. |
| https://www.rndsystems.com/products/mouse-il-1beta-il-1f2-antibody_af-401-na |
| Tubulin [clone DM1A, CP06-100UG, Cal Biochem]: confirmed to detect alpha-tubulin structures via IF. |
| https://doi.org/10.1016/j.mex.2018.02.003 |
| NRF2 [clone 103, MABE1799, EMD Millipore]: Certificate of Analysis states and product website displays that the antibody is validated for western blot using Krap1 KO MEFs vs. Nrfl2 KO MEFs. |
| https://www.emdmillipore.com/US/en/product/Anti-Nrf2-Antibody-clone-103-MM_NF-MABE1799?ReferrerURL=https%3A%2F%2Fwww.google.com%2F |
| Vinculin [clone 7F9, sc-73614, Santa Cruz]: antibody raised against human vinculin detects band of the correct molecular weight across 6 independent mouse and human cell lines. |
| https://www.scbt.com/p/vinculin-antibody-7F9 |
| VDAC [ab154856, Abcam]: product webpage displays western blot showing loss of VDAC band from VDAC KO vs WT HAP1 and HFK23T cells. |
| https://www.abcam.com/vdacr--porin-antibody-epr10852b-mitochondrial-loading-control-ab154856.html |

### Eukaryotic cell lines

| Policy information about: cell lines |
|-------------------------------------|
| Cell line source(s) | RAW 264.7, HEK293T, THP-1, and U937 cells were from ATCC via the UC Berkeley Cell Culture Facility. |
| Authentication | Cells were not authenticated. |
| Mycoplasma contamination | Cell lines were not tested for mycoplasma contamination. |
| Commonly misidentified lines (See Eukaryotic Cell Line Register) | None were used in this study. |

### Animals and other organisms

| Policy information about: studies involving animals, ARRIVE guidelines recommended for reporting animal research |
|-----------------------------------------------------|
| Laboratory animals | Male CS7BL/6 mice at 8 weeks of age were used for in vivo studies. Female CS7BL/6 mice at 6-12 weeks of age were used for preparation of BMOMs for in vitro studies - this includes NRF2 KO mice purchased from Jackson Labs [Stock No. 017009]. |
| Wild animals | No wild animals were used in this study. |
| Field-collected samples | No field-collected samples were used in this study. |
| Ethics oversight | All experiments were approved by the UC Berkeley Animal Care and Use Committee (ACUC) to ensure ethical use of animals (AUP-2017-02-9539-1), and performed under the supervision of the UC Berkeley Office of Laboratory and Animal Care [OLAC]. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.
ChIP-seq

Data deposition

- Confirm that both raw and final processed data have been deposited in a public database such as GEO.
- Confirm that you have deposited or provided access to graph files [e.g. BED files] for the called peaks.

Data access links

- Link to GEO submission: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE169731
- ChIP-seq data is a subseries: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE169729

Files in database submission

- Raw Fastq files and processed BedGraph files are available at the GEO.

Genome browser submission

- [UCSC](http://genome.ucsc.edu/) provides access to UCSC Genome Browser session with ChIP-seq data loaded in BedGraph format: http://genome.ucsc.edu/s/gimmlin/Timblin_Saijo_mm9_BMDM_p65_H3K27ac_Chipseq_tracks

Sample key (all BedGraph tracks are normalized to total read number & input samples):
- Dtag0 - p65, ETOH
- Etag0 - p65, ETOH + LPS
- Ftag0 - p65, 2-OHE2 + LPS
- Jtag0 - H3K27ac, ETOH
- Ktag0 - H3K27ac, ETOH + LPS
- Ltag0 - H3K27ac, 2-OHE1 + LPS

Methodology

Replicates

For each experimental condition, 3 independent biological replicates were pooled for chromatin immunoprecipitation step.

Sequencing depth

100bp single-end reads were generated on HiSeq2500. Mapping percentage to mouse genome build mm9 was generally around 60-80%, if not better. p65 ChIP experiment has approximately 40-50 million uniquely-mapped reads per experimental sample, while H3K27ac ChIP experiment has approximately 10 million uniquely-mapped reads per experimental sample.

Antibodies

- p65 (sc-372, Santa Cruz), 2ug for ChIP-seq
- H3K27ac (ab4729, AbCam), 2ug for ChIP-seq

Peak calling parameters

Mapping was performed using STAR, setting parameters to allow for continuous read mapping (--alignMaxIntronMax 1 --alignEndType EndToEnd). HOMER was used for peak finding and analysis. After using "makeTagDirectory" to create tag directories for input and experimental samples, "findPeaks" was used to find significant peaks (p65) or regions (H3K27ac) for each experimental sample relative to appropriate input control. "getDifferentialPeaks" was then used to identify peaks/regions enriched in one sample versus another, such as the 6,149 p65 peaks and 10,999 H3K27ac regions enriched in ETOH + LPS-treated BMDM samples versus ETOH control samples described in Figure S5. Using those LPS-induced peaks/regions, "getDifferentialPeaks" was used again to identify what percentage of those LPS-induced peaks/regions has significantly more read coverage in the ETOH + LPS samples versus the hydroxyestrogen + LPS samples (13% and 65%, respectively). "annotatePeaks" was used to generate the histograms displaying read density for all samples/experimental conditions across these 6,149 p65 peaks and 10,999 H3K27ac regions as shown in Figure S5.

Data quality

IgG control ChIPs yielded insufficient amounts of recovered chromatin for library preparation and sequencing, meaning the DNA enriched was specific to our antibodies. Following library sequencing, mean quality score of the raw sequencing reads was generally >38.0. Mapping percentage to the mm9 reference genome was 60-80% or better. For both ChIPs, peaks/regions appeared in LPS-treated samples versus controls at promoters/enhancers of genes known to be upregulated by LPS. Motif enrichment analysis of peaks/regions in LPS-treated samples revealed highly significant enrichments (P value<1e-100 or smaller) for NFkB motifS, other pro-inflammatory transcription factor motifs (e.g. AP-1), and macrophage lineage determining transcription factor motifs (e.g. PU.1), meaning bona fide regulatory regions are being identified.

Software

Data collection software information will be obtained from our sequencing facility and noted here & in the manuscript methods prior to publication. Analysis software information is noted above and in the methods section of the manuscript.

Flow Cytometry

Plots

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage [with statistics] is provided.
Methodology

Sample preparation
For adipose tissue macrophages, stromal vascular fraction (SVF) was then prepared from visceral white adipose tissue. Tissue was minced with scissors and incubated in DMEM with 0.1% collagenase (Sigma C6885) and 5% BSA for 1 hour at room temperature with gentle shaking. Digested tissue was filtered through a 70um filter, RBCs lysed, and SVF stained for analysis and cell sorting on ice in FACs buffer (PBS with 10mM HEPES and 5% BSA) using standard techniques.

For monocyte/macrophages cell lines, cells were stained as described, then placed on ice & harvested in cold PBS + viability dye, with adherent cells lifted by scraping.

Instrument
All sample analysis was done on LSR II, LSR Fortessa, or LSR Fortessa X20 analyzers [BD]. All cell sorting was performed on Influx or Aria Fusion sorters [BD].

Software
Data was collected using BD FACSDiva software v6.2 and analyzed using FlowJo 10.5.2 (Tree Star).

Cell population abundance
Post-sort analysis was not performed because cells were sorted directly into Trizol LS lysis buffer to prevent gene expression changes during the post-sort period. Gene expression profiles could be compared with existing adipose tissue macrophage profiles to confirm unbiased clustering of samples versus other tissue-resident macrophages.

Gating strategy
For adipose tissue macrophage sorting, broad FS/SS gates (avoiding small debris) were drawn. DAPI-negative, CD45+ cells were selected based on clear distinction between positive and negative populations. Cells expressing the highest levels of CD11b and F4/80 were then gated for sorting as done in published protocols.

For monocyte/macrophage cell lines, a similar strategy was used for live gate and live/dead exclusion. Most all experiments present data as a mean fluorescence intensity (MFI) of a dye, fluorescent protein, or genetic probe. Controls are included to confirm the various dyes/proteins/probes are faithfully reporting their intended target measure (e.g. hydrogen peroxide treatment as a positive control for MitoPy1 detection of ROS, FCCP to dissipate membrane potential measured by TMRE and JC-9, etc.).

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.