Lipid-induced NOX2 activation inhibits autophagic flux by impairing lysosomal enzyme activity

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Abstract Autophagy is a catabolic process involved in maintaining energy and organelle homeostasis. The relationship between obesity and the regulation of autophagy is cell type specific. Despite adverse consequences of obesity on cardiac structure and function, the contribution of altered cardiac autophagy in response to fatty acid overload is incompletely understood. Here, we report the suppression of autophagosome clearance and the activation of NADPH oxidase (Nox)2 in both high-fat-fed murine hearts and palmitate-treated H9C2 cardiomyocytes (CMs). Defective autophagosome clearance is secondary to superoxide-dependent impairment of lysosomal acidification and enzyme activity in palmitate-treated CMs. Inhibition of Nox2 prevented superoxide overproduction, restored lysosome acidification and enzyme activity, and reduced autophagosome accumulation in palmitate-treated CMs. Palmitate-induced Nox2 activation was dependent on the activation of classical protein kinase Cs (PKCs), specifically PKCβII. These findings reveal a novel mechanism linking lipotoxicity with a PKCβ-NOX2-mediated impairment in pH-dependent lysosomal enzyme activity that diminishes autophagic turnover in CMs.—Jaishy, B., Q. Zhang, H. S. Chung, C. Riehle, J. Soto, S. Jenkins, P. Abel, L. A. Cowart, J. E. Van Eyk, and E. D. Abel. Lipid-induced NOX2 activation inhibits autophagic flux by impairing lysosomal enzyme activity. J. Lipid Res. 2015. 56: 546–561.

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Obesity is an independent risk factor for CVD (1). A prominent factor in obesity-related cardiomyopathy is the ectopic deposition of toxic lipid intermediates in the heart that may have deleterious effects (“lipotoxicity”) on myocardial structure and function (2). Studies using mouse models of increased myocardial lipid availability showed that increasing lipid accumulation directly in cardiomyocytes (CMs) was sufficient to induce lipotoxic cardiomyopathy and premature cell death independently of systemic or neurohumoral effects of obesity (3–5). However, gaps remain in our mechanistic understanding of the interactions between lipid accumulation and cardiac dysfunction and the impact of lipotoxicity on cardiac autophagy has been relatively understudied.

Macroautophagy (hereafter “autophagy”) is a catabolic process wherein cytoplasmic materials are delivered to lysosomes in double-membrane structures called autophagosomes for degradation (6). Autophagy regulates lipid metabolism in multiple tissues (7–9). Impaired autophagy

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Abbreviations: Akt, protein kinase B; apo, apolipoprotein; BCAA, branched chain amino acid; CatL, cathepsin L; CM, cardiomyocyte; CMH, 1-hydroxy-3-methylxacyctophenone; CQ, chloroquine; DAG, diacylglycerol; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; ESR, electron spin resonance; FOX, forkhead box; GFP, green fluorescent protein; HFD, high fat diet; I/R, ischemia and reperfusion; IRE1α, endoplasmic reticulum to nucleus signaling 1; LAP, lysosomal acid phosphatase; LC3, light chain 3; LC3-II, light chain 3β II; 3MA, 3-methyladenine; mCLC3, mCherry-light chain 3; MR, Magic Red; mTOR, mechanistic target of rapamycin; mTORC1, mechanistic target of rapamycin complex 1; NCD, control chow; ND, nutrient-depleted; Nox, NADPH oxidase; ODM, Optiprep density gradient medium; PERK, eukaryotic translation initiation factor 2α kinase 3; PKC, protein kinase C; ROS, reactive oxygen species; Ulk1, unc-51-like protein autophagy activating kinase 1; vATPase, vacuolar ATPase; XBP1, X-box binding protein.

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leads to insulin resistance in the liver and is associated with reduced insulin secretion and cell survival in pancreatic β cells in animal models of obesity (10, 11). Conversely, inhibition of autophagy in adipose tissue improves insulin sensitivity and attenuates diet-induced obesity (8). Disruption of autophagy was observed in multiple cardiac pathologies including ischemic and hypertensive heart disease and heart failure (12). Altered cardiac autophagy has been reported in genetic and dietary models of obesity, suggesting that autophagy might be modulated by increased myocardial lipid uptake or utilization (13–15). However, the regulation of cardiac autophagy in response to lipid overload is incompletely understood.

Increased levels of reactive oxygen species (ROS) leading to oxidative stress represent an important consequence of lipotoxic cardiomyopathy. Chronic lipid supply to the heart promotes excessive ROS production (16), which damages various organelles and macromolecules by oxidative modifications. Given its role in clearing damaged organelles and macromolecules (17), it is plausible that autophagy is impacted by ROS in cardiac lipotoxicity. ROS overproduction was linked to impaired lysosomal function in certain neurological diseases and in cultured hepatocytes, which could impact autophagic turnover (18, 19). Whether ROS modulates cardiac autophagy in response to lipid overload, as in obesity and diabetes, is unclear. Here, we have explored the mechanistic link between lipid overload and cardiac autophagy in mouse models of diet-induced obesity and in cultured CMs exposed to FFAs. These experiments reveal a novel molecular mechanism by which lipid overload impairs autophagosome turnover via a ROS- and protein kinase C (PKC)-mediated impairment of lysosomal function.

MATERIALS AND METHODS

Animal experiments

This study protocol was approved by the Institutional Animal Care and Use Committee of the University of Utah and the Carver College of Medicine of the University of Iowa. FVB mice with cardiac-specific expression of an mCherry-light chain 3 (mCLC3) transgene were backcrossed to the C57BL6 background for 10 generations. mCLC3 mice or their littermate controls on the C57BL6 background were housed at 22°C with free access to water and food with a 12 h light/dark cycle. For the high fat feeding study, 8-week-old male mice were fed with high fat diet (HFD) (45% kcal fat, D12451; Research Diets, New Brunswick, NJ) or control chow (NCD) (4% kcal fat, 8656; Teklad, Harlan Laboratories, Madison, WI) for 12 weeks. For all studies, mice were fasted for 6 h (6:00 AM to 12:00 PM) before euthanization. Mice were injected with 0.01 unit of human recombinant insulin (Novolin R; Novo Nordisk, Princeton, NJ) via the inferior vena cava (ivc) as described previously (20). Hearts were collected 5 min after the injection. For mechanistic target of rapamycin (mTOR) activation, mice were injected ip with a branched chain amino acid (BCAA) solution [46 mM isoleucine, 91 mM leucine, 61 mM valine, and 35 mM arginine (pH 7.4)] at a daily dose of 100 μl/g body weight for 2 weeks prior to harvest. To inhibit autophagosome turnover and to determine autophagic flux, mice were injected with chloroquine (CQ) at 48 h (30 mg/kg body weight), 24 h (30 mg/kg), and 2 h (50 mg/kg) prior to harvest.

Cell culture and treatments

All reagents were purchased from Sigma (St. Louis, MO) unless stated otherwise. H9C2 (rat embryonic cardiomyoblasts, ATCC CRL-1446) and Ad-293 (Aglent) cells were maintained in DMEM (Invitrogen, Carlsbad, CA) with 25 mM glucose and 10% FBS (Thermo Scientific, Logan, UT). H9C2 cardiomyoblasts were differentiated to CMs in DMEM containing 5 mM glucose and 1% FBS for 5 days. All treatments were performed in nutrient-rich medium (DMEM with 5% glucose and 10% FBS) except for nutrient-depleted (ND) conditions when CMs were incubated with HBSS supplemented with 0.5 mM sodium-pyruvate. Cellular superoxide was scavenged by treating CMs with 10 mM tiron. Lysosomal acidification was inhibited with 80 μM CQ, denovo ceramide synthesis with 50 μM myriocin, and carnitine palmitoyl transferase 1 (CPT1) with 50 μm etomoxir. NADPH oxidase (Nos2) activity was inhibited with 10 mM 4′-hydroxy-3′-methoxyacetophenone (apocynin), total PKC activity with 2 μM GF109203X (Santa Cruz, Dallas, TX), and classical and novel PKC-isoform specific activities with 2 μM G66976 (EMD Millipore, San Diego, CA) and 10 μM rottlerin, respectively. Additionally, pan-PKCβ and PKCBp-specific activity were blocked with 10 μM PKCB pseudosubstrate (Tocris, Ellisville, MO) and 2 μM PKCBII peptide inhibitor I, respectively. For knockdown studies, H9C2 CMs were transfected with 80 nM scramble or targeted siRNAs for 48 h using Lipofectamine 2000 (Life Technologies, Eugene, OR). siRNAs for p47phox (HS302656) and PKCα (sc-45918) were from Invitrogen and Santa Cruz, respectively.

Immunoblotting

Total proteins were extracted from hearts and H9C2 CMs in a lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 mM Na3PO4, 100 mM NaF, and 1× Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL). Proteins were resolved on SDS-PAGE and analyzed by Western blotting using the LI-COR Odyssey imager (LI-COR), an infrared fluorescence-based detection system. The following primary antibodies (catalog number in parentheses) were used for immunoblotting. Rabbit antibodies for LC3 (L9189) and p62/SQSTM1 (P0067), and mouse antibody for α-tubulin (T8020) were from Sigma. Rabbit antibodies for Beclin-1 (3495), phospho-protein kinase B (Akt) S473 (4058), phosphoryl-AMPKα (sc-21175) (2535), phospho-mTOR (sc-25148) (2971), phospho-forkhead box (FOXO)1 (sc-28793) (9464), phospho-unc-51-like protein autophagy activating kinase 1 (ULK1) S511 (5869), phosphoULK1 S657 (6888), phospho-S6 (sc-2211), FOXO1 (2880), GAPDH (2118), phospho-eukaryotic translation initiation factor 2α kinase 3 (PERK) (3179), phospho-eukaryotic initiation factor 2α (eIF2α) (sc-3597), and mouse antibodies for pan AKT (2920), AMPKα (F6, 2793), mTOR (4517), S6 (2317), and Parkin (4211) were from Cell Signaling (Danvers, MA). Rabbit antibodies for ULK1 (sc-33182), phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) (sc-33796), endoplasmic reticulum (ER) to nucleus signaling 1α (IRE1α) (sc-20790), superoxide dismutase 2 (SOD2) (sc-30080), and PKCa (sc-208), mouse antibodies for eIF2α (sc-81261) and X-box binding protein (XBP)-1 (sc-7160) and goat antibodies for PERK (sc-9476) and p47phox (sc-7660) were from Santa Cruz (Dallas, TX). Rabbit antibodies for Lamp1 (ab349) and phospho-IRE1α (ab8187) were from Abcam (Cambridge, MA). Rabbit antibody for VDAC (PA1-954A) was from Thermo Scientific. Mouse antibody for p47phox (07-500) was from Millipore, and was used to detect p47phox protein in mouse tissues.
IRDye 800CW anti-mouse (LI-COR, Lincoln, NE) and Alexa Fluor anti-rabbit 680 (Invitrogen) were used as secondary antibodies. For the Western blot data from murine hearts, n represents the number of animals in each group. For cell culture studies, the data are representative of a minimum of two independent experiments with n representing the number of replicates per independent experiment.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from H9C2 CMs using TRIzol reagent (Invitrogen), purified with the RNeasy kit (Qiagen, Valencia, CA), and reverse transcribed. Quantitative real-time PCR was performed using SYBR Green I with 6-carboxyl-X-rhodamine (ROX) as an internal reference dye. The expression level was normalized to the levels of cyclophilin A transcript. The primer sequences used for quantitative real-time PCR are listed in supplementary Table 1.

Fluorescence microscopy

For mCLC3 imaging, murine hearts were embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA) and sliced in 5 μm transverse sections on a cryostat (Leica) at −20°C. Heart sections were fixed in 4% formaldehyde (Fisher Scientific, Fair Lawn, NJ) for 15 min and stained with Hoechst 33342 (Invitrogen) for 30 min at room temperature, as described previously (21). For mCherry-green fluorescent protein (GFP)-LC3 or Ad-GFP-LC3 imaging in cells, H9C2 CMs were fixed and stained identically as heart sections. Images were acquired with excitation/emission of 405/461 nm to detect Hoechst 33342, 488/520 nm to detect GFP, and 543/461 nm to detect mCherry fluorescence, respectively. For immunofluorescence, CMs were fixed in 4% paraformaldehyde (PFA) and permeabilized in PBS containing 5% goat serum, 5% horse serum, and 0.2% Triton X-100. After fixation, CMs were incubated with rabbit anti-Lamp1 (described above; 1:500 dilution), rabbit anti-translocase of outer mitochondrial membrane 20 (Tom20) (Santa Cruz, sc-11415, 1:250 dilution), or mouse anti-ceramide with rabbit anti-Lamp1 (described above; 1:500 dilution), rabbit anti-translocase of outer mitochondrial membrane 20 (Tom20) (Santa Cruz, sc-11415, 1:250 dilution), or mouse anti-ceramide (Enzo Life Sciences, ALX-804-196-T050; 1:250 dilution) antibody for 1 h and subsequently with Alexa Fluor 594 goat anti-rabbit (Invitrogen, R37117) or Alexa Fluor 488 goat anti-mouse (Invitrogen, A11001) secondary antibody (1:500 dilution) for 1 h at room temperature. Ceramide was detected with excitation/emission of 488/520 nm and Lamp1 was detected with excitation/emission of 543/618 nm by indirect fluorescence. For live cell imaging, CMs were grown on an eight chamber coverglass (Lab-Tek, Nunc, Rochester, NY) for 15 min and stained with Hoechst 33342 (Invitrogen) for 30 min at room temperature, as described previously (21). For mCherry-green fluorescent protein (GFP)-LC3 or Ad-GFP-LC3 imaging in cells, H9C2 CMs were fixed and stained identically as heart sections. Images were acquired with excitation/emission of 405/461 nm to detect Hoechst 33342, 488/520 nm to detect GFP, and 543/603 nm (using a 500-550 filter). Fluorescent intensity was quantified from each CM using ImageJ software (National Institutes of Health, Bethesda, MD). Colocalization analyses were performed using Coloc2 plugin in Fiji image processing software. A GFP-LC3 micrograph of a particular region was selected as the “region of interest” for a colocalization analysis of that region. For all quantification, n represents the number of CMs in each group from two to three independent experiments.

FFA-BSA complex solution

Solutions of FFA complexed with BSA were prepared essentially as previously described (22). Briefly, a 100 mM stock solution of FFA was prepared in 0.1 M NaOH at 70°C. The stock solution was mixed with a 20% (w/v) solution of FFA-free BSA at 50°C to prepare a 10 mM FFA-20% BSA complex solution. The solution was added to a desired concentration in DMEM with 5 mM glucose and 10% FBS and used immediately after filtration. For vehicle control, CMs were incubated with 1% FFA-free BSA solution.

Viral infections and transfections

Retrovirus harboring pBABE-Puro-mCherry-EGFP-LC3 and adenovirus harboring GFP-LC3 were generated and amplified in Ad-293 cells. Adenoviruses were purified by repeated freeze-thaw in a 10% glycerol solution in PBS followed by centrifugation at 5,000 rpm for 5 min. Viral supernatant was titered and used to infect CMs in DMEM supplemented with 5 mM glucose and 10% FBS (23). All subsequent treatments were performed 18 h post infection. For retroviral infection, viral supernatants derived from Ad-293 cells were filtered through a 0.45 μm-pore filter and added to H9C2 cell culture medium supplemented with 4 μg/ml polybren (Millipore). For siRNA-mediated gene silencing, H9C2 CMs (~70% confluent) were transfected with 80 nM of p47 phox (sc-45918) or scramble siRNA duplexes (Santa Cruz) for 48 h using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s protocol.

Subcellular fractionation

Murine hearts or H9C2 CMs were homogenized with a Dounce homogenizer in buffer A containing 20 mM HEPES (pH 7.4), 10 mM NaCl, 1 mM DTT, 50 mM NaF, 1 mM Na3VO4, and 1× Halt protease and phosphatase inhibitor (Thermo Scientific). Intact cells and nuclei were pelleted by centrifugation at 1,000 g for 10 min. Membrane fractions were obtained by three successive centrifugations of 100,000 rpm for 1 h. The pellet was resuspended in buffer A following each ultracentrifugation. For the isolation of crude mitochondrial fractions, H9C2 CMs were homogenized in buffer B containing 20 mM HEPES (pH 7.5) and 250 mM sucrose. The supernatant obtained following 1,000 g centrifugation was pelleted by centrifugation at 2,000 g for 30 min. The pellet was resuspended in buffer B following two washes in the same buffer and treated as the mitochondrial fraction.

Lysosomal fractions were prepared as described previously with minor modifications (24). Briefly, H9C2 CMs were resuspended in 220 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH (pH 7.4), and 1 mM EDTA (isotonic buffer) and homogenized with a Dounce homogenizer. Following centrifugation at 1,000 g for 15 min, the resulting supernatant was layered on
the top of a gradient solution containing (bottom to top) 35% (w/v) and 17% (w/v) Optiprep density gradient medium (ODM) (Sigma) and 6% (w/v) Percoll (GE Healthcare) in isotonic buffer and centrifuged at 50,000 g for 1 h at 4°C. The crude lysosomal fraction at the 6% Percoll-17% ODM interface was adjusted to 35% ODM and placed on the bottom of a second gradient solution containing 17 and 5% ODM and centrifuged at 50,000 g for 1 h at 4°C. The resultant 5–17% ODM interface containing lysosomes was resuspended in isotonic buffer and pelleted by centrifugation at 55,000 g for 1 h at 4°C.

Lysosomal enzyme activity

For lysosomal enzyme activities, live CMs were incubated with an enzymatic substrate for 1 h at 37°C and the product formation was assessed by fluorescence microscopy. For CatL activity, CMs were incubated with MR-CatL substrate (Immunocytometry Technologies, Bloomington, MN). For lysosomal acid phosphatase (LAP) activity, CMs were incubated with LysoLive Phosgreen substrate (Marker Gene Technologies, Eugene, OR). Images were acquired as described in the supplementary Methods section. The lysosomal fraction described earlier was used to measure β-galactosidase activity using the mammalian β-galactosidase assay kit (Thermo Scientific) and β-hexosaminidase activity as previously reported (25).

Superoxide measurement by electron spin resonance spectroscopy

CMs were washed and incubated for 30 min at 37°C with 1 M HEPES buffer (pH 7.4) containing 25 μM deferoxamine mesylate and 0.2 mM 1-hydroxy-3-methoxycarbonyl-2,5,5,6-tetramethylpiperidine HCl (CMH) (Enzo Life Sciences, Farmingdale, NY) to trap superoxide as a CMH-free radical adduct. The cell sample was homogenized and aspirated into a capillary tube for the detection of the adduct by electron spin resonance (ESR) spectroscopy. ESR spectra were recorded using a Bruker EMX micro EPR spectrometer with the following settings: center field, 3511 G; field sweep, 70 G; microwave frequency, 9.85 GHz; microwave power, 20 mW; modulation amplitude, 0.5 G; conversion time, 5.0 ms; time constant, 1.28 ms; resolution, 1,400 points; and receiver gain, 50 dB.

Measurement of apoptosis

Apoptosis was assessed in H9C2 CMs using FITC annexin V apoptosis detection kit with propidium iodide (BioLegend, San Diego, CA) following the supplier’s protocol. Briefly, following each treatment, CMs were washed and resuspended in a staining buffer, stained with FITC-annexin V and propidium iodide for 15 min at room temperature, and subsequently analyzed by flow cytometry using BD FACSCalibur using appropriate filters.

ATP measurement

Total ATP content in CMs was measured by a bioluminescence assay based on the luciferase/luciferin reaction using the ATP assay system kit (Thermo LabSystems, Milford, MA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5. All data are presented as mean ± SEM. Statistical significance (P < 0.05) was determined by unpaired Student’s t-test. Estimation of the minimal sample size for the variables measured was determined by power analysis with an initial setting of α = 0.05 and power = 0.8, using G*Power 3.1 software.

RESULTS

High fat feeding increases autophagosome abundance in the heart independent of changes in autophagy induction pathways

To study the effect of high fat feeding on cardiac autophagy, we fed C57BL6 male mice with either HFD providing 45 kcal% fat or NCD for 12 weeks and assessed markers of autophagy in the heart. HFD significantly increased (~40%) microtubule-associated protein 1 light chain 3 β II (LC3-II) levels compared with NCD controls (Fig. 1A). Accordingly, in cardiac-specific mCLC3 transgenic mice, HFD markedly increased mCherry-positive autophagosome formation in the heart (Fig. 1B). However, levels of Beclin1 and phosphorylation of proteins known to regulate autophagy induction, such as Akt, AMPK, and FOXO1, were unchanged by HFD (Fig. 1C). Although HFD mice developed obesity and generalized insulin resistance (supplementary Table 2), cardiac function was preserved (supplementary Table 3), and basal and insulin-stimulated Akt signaling were intact in the heart (Fig. 1D). These data suggest that HFD-induced autophagosome accumulation might be independent of changes in signaling pathways that initiate autophagy.

The mTOR complex 1 (mTORC1) regulates autophagy via insulin-dependent and -independent mechanisms (26, 27). Phosphorylation of mTOR was significantly lower (~34%) in HFD-fed hearts (Fig. 1E), as were the downstream targets Ulk1 (S757) and ribosomal protein S6 (S235/236), raising the possibility that diminished mTORC1 activity could contribute to the activation of autophagy following HFD. In parallel, levels of BCAA, which can activate mTOR, were significantly reduced in the heart and serum of HFD mice (Fig. 1F, G), although changes in other amino acids were less pronounced (supplementary Table 4). To determine whether HFD-induced autophagy is mTORC1 dependent, we stimulated mTOR signaling by injecting mice with a BCAA solution. Despite significant activation of mTORC1, as evidenced by increased S6 phosphorylation, BCAA injection did not suppress autophagy in HFD-fed hearts (Fig. 1H). Thus, HFD-induced autophagosome accumulation may be independent of changes in cardiac mTOR signaling.

Autophagosome accumulation could also be due to lysosomal dysfunction (28). To determine whether HFD impairs cardiac autophagosome turnover, we injected mice with the lysosomal acidification inhibitor CQ to block autophagosome turnover, and monitored autophagosome accumulation in the heart. In NCD-fed hearts, CQ significantly induced autophagosome accumulation relative to saline control, as measured by a 2-fold increase in LC3-II levels and a 3.8-fold accumulation of the autophagy substrate p62 (Fig. 1I). In HFD-fed hearts, baseline LC3-II and p62 levels were higher than in NCD controls. However, CQ caused no additional accumulation of these autophagosome markers over HFD controls, consistent with the hypothesis that HFD does not induce autophagic flux, but impairs autophagosome turnover.
Palmitate facilitates autophagosome accumulation in H9C2 CMs

To elucidate the molecular mechanisms linking lipid overload to autophagosome accumulation in CMs, we treated H9C2 CMs with the saturated fatty acid, palmitate. Of note, palmitate treatment caused no cell death during the first 4 h (supplementary Fig. 1A, B) and total cellular ATP content was unchanged (supplementary Fig. 1C). Consistent with in vivo observations, palmitate significantly induced LC3-II levels (~2.5-fold vs. vehicle-saline) without affecting Akt, AMPK, and mTOR signaling (Fig. 2A, supplementary Fig. 1D). Importantly, palmitate-induced autophagosome accumulation was reversed when CMs were returned to medium without palmitate supplementation (supplementary Fig. 1E). Thus palmitate temporally regulates autophagy without inducing cytotoxicity.

Treatment with CQ increased LC3-II levels in both vehicle- and palmitate-treated CMs. However, the fold increase in LC3-II accumulation was greater in vehicle-treated (3.4-fold) versus palmitate-treated (1.8-fold) CMs relative to their respective non-CQ controls. Importantly, palmitate treatment increased p62 accumulation, which was not
enhanced further by CQ treatment (Fig. 2A). Palmitate led to a 4-fold accumulation of GFP-LC3-positive autophagic puncta relative to vehicle controls (Fig. 2B). Upon CQ treatment, the number of puncta was increased by 12-fold in vehicle controls compared with 2.3-fold in palmitate-treated CMs. Of note, palmitate treatment increased the colocalization of GFP-LC3-positive autophagosomes with lysosomes stained with anti-Lamp1 antibody or Lysotracker Red, suggesting that autophagosome-lysosome fusion was not impaired by palmitate (supplementary Fig. 1F, G).

We further examined autophagosome turnover in H9C2 CMs stably expressing mCherry-GFP-tagged LC3.

![Image](image_url)

**Fig. 2.** Palmitate promotes autophagosome accumulation in H9C2 CMs by suppressing autophagic turnover. A: Immunoblot analysis of autophagy proteins [LC3 (left) and p62 (right)] in H9C2 CMs incubated with either vehicle (Veh) (1% BSA) or 500 µM palmitate (Palm) for 4 h and in the presence or absence of 80 µM CQ for the last 1 h (n = 3). B, C: H9C2 CMs expressing adenoviral GFP-LC3 (B) or stably expressing retroviral mCherry-GFP-LC3 (C) were incubated with vehicle or palmitate in the presence or absence of CQ as described in (A). B: Confocal images (top) showing the distribution of adenoviral GFP-LC3 (green). Graph (bottom) represents the average number of GFP-positive puncta/CMs ± SEM (n = 50 CMs). C: Confocal images (top) showing GFP (green) and mCherry (red) fluorescence of mCherry-GFP-LC3. DAPI (blue) stains the nuclei. Table (bottom) shows mean fluorescence intensity ± SEM of GFP and mCherry signals from images in (C) and the ratio of GFP to mCherry fluorescence in response to different treatments (n = 21 CMs). Scale bar, 10 µm. *P < 0.05; **P < 0.01; ***P < 0.001 versus vehicle + saline. D: LC3 immunoblot (top) from CMs incubated in ND medium for 0.5 h and treated with vehicle or palmitate in the presence or absence of 3MA and CQ for an additional 2 h in ND medium. CMs incubated with nutrient-rich (NR) or ND medium initially for 0.5 h and then for 2.5 h with vehicle (Veh) or palmitate (Palm) in NR medium for the final 2 h were used as baseline controls (n = 3). Immunoblot lane numbers, correspond with x axis label of densitometry. For all panels except (C), *P < 0.05; **P < 0.01; ***P < 0.001.
The GFP (green) fluorescence was quenched in the acidic lysosomal lumen, whereas mCherry (red) fluorescence was maintained. Vehicle-treated CMs predominantly contained mCherry-positive puncta, whereas palmitate-induced accumulation of both GFP- and mCherry-positive autophagic puncta was similar to that observed when autophagosome turnover was blocked by CQ (Fig. 2C). Moreover, both GFP and mCherry fluorescence were increased by palmitate treatment. The increased fluorescence in palmitate-treated CMs was not augmented further by CQ addition, which indicates that palmitate treatment impairs autophagosome clearance in CMs.

The CM studies described thus far were performed under nutrient replete conditions. Therefore, we performed studies under conditions of increased autophagic flux in ND CMs and assessed turnover of existing autophagosomes following incubation with vehicle or palmitate in the presence of 3-methyladenine (3MA), a class III phosphatidylinositol-4,5-bisphosphate 3-kinase inhibitor, that blocks neo-autophagosome formation, and CQ, that blocks the degradation of existing autophagosomes (Fig. 2D). Nutrient depletion increased autophagic flux in CMs, as evidenced by a 5-fold increase in LC3-II in the presence of CQ. Although palmitate increased basal autophagy in ND CMs, the increase in LC3-II following CQ treatment was blunted, which is consistent with impaired autophagosome turnover. Blockade of new autophagosome formation with 3MA reduced CQ-induced levels of LC3-II. In the presence of 3MA, LC3-II levels remained higher in palmitate-treated CMs and addition of CQ did not increase these levels above those observed in vehicle-treated cells. Taken together, these in vitro studies suggest that palmitate promotes autophagosome accumulation primarily by reducing autophagosome clearance under conditions of high or low autophagic flux. Thus, for the remainder of this study we focused on mechanisms regulating autophagic turnover in response to lipid overload.

**Palmitate-induced autophagosome accumulation is superoxide dependent**

We initially examined whether palmitate-induced autophagy is mediated by ceramides or ER stress (29–32). Palmitate induced a 2-fold accumulation of ceramides in CMs (Fig. 3A). However, inhibition of de novo ceramide biosynthesis with the serine-palmitoyl transferase 1 (SPT1) inhibitor, myriocin, had no effect on palmitate-induced autophagy (Fig. 3B). Analysis of proteins that indicate activation of the unfolded protein response and ER stress revealed that signaling through PERK, eIF2α, IRE1α, and XBP-1 were unchanged following 4 h of palmitate treatment (Fig. 3C). The unfolded protein response pathway only became activated after 8 h of treatment, as evidenced by increased IRE1α phosphorylation and increased XBP-1 splicing. These data suggest that palmitate-induced modulation of autophagy is independent of ceramide accumulation and precedes activation of ER stress. However, 4 h palmitate treatment also increased total diacylglycerol (DAG) content by ~3-fold, which has been implicated in the regulation of autophagy (supplementary Fig. 11).

Palmitate-induced ROS generation is known to mediate its lipotoxic effects. We observed a 3.8-fold increase in superoxide anion accumulation in palmitate-treated CMs (Fig. 4A). Treatment of these CMs with the generalized superoxide scavenger tiron significantly lowered LC3-II levels (~35%) and p62 accumulation (~32%) and partially restored autophagic flux (Fig. 4B, C). Tiron treatment also normalized palmitate-induced GFP-LC3-positive autophagosome accumulation (Fig. 4D). These data provide evidence for a superoxide-dependent mechanism by which palmitate impairs autophagosome turnover.

We next investigated whether superoxide-dependent regulation of autophagy is specific to palmitate, a saturated fatty acid, or represents a general effect of lipid overload. Treatment with oleate, the most abundant unsaturated fatty acid, had no effect on autophagy or superoxide production in H9C2 CMs (supplementary Fig. 3A, B). Importantly, when coincubated at equimolar concentration, oleate reversed palmitate-induced autophagosome accumulation to basal levels, but increased TG content in palmitate-treated CMs (supplementary Fig. 3C, D). These data suggest that a palmitate-specific ROS-induction pathway regulates autophagosome accumulation.

**Nox2-derived superoxide impairs lysosomal acidification and enzyme activity in response to palmitate**

Lysosomal pH is a key determinant of lysosomal enzyme activity. In vitro studies revealed impaired lysosomal acidification following cholesterol and fatty acid overload, although the underlying mechanisms are incompletely understood (33, 34). We stained lysosomes with LysoSensor yellow/blue to assess lysosomal acidification. Yellow fluorescence predominates at an acidic pH, whereas blue fluorescence intensifies as lysosomal pH rises. These changes in LysoSensor dual fluorescence were validated by treating live CMs with CQ as a positive control to increase lysosomal pH. CQ treatment significantly increased blue fluorescence (supplementary Fig. 4A) and led to an impairment in lysosomal enzyme activities, namely CatL activity, as measured by the cleavage of the MR-CatL substrate into a fluorescent product, and LAP activity, as measured by the generation of fluorescent byproducts (supplementary Fig. 4B, C). Similar to CQ, palmitate significantly increased LysoSensor blue staining (Fig. 5A) and inhibited lysosomal function as evidenced by blunted CatL activity (Fig. 5B). Lysosomal acidification and CatL activity were normalized by tiron treatment. Importantly, the biochemical activity of two lysosomal hydrolases, β-galactosidase and β-hexosaminidase, when examined in vitro, were unaffected by palmitate (Fig. 5C), as were the expression of lysosomal genes (Fig. 5D). Thus, palmitate impairs lysosomal acidification and pH-dependent enzyme activity in living cells independently of lysosome biogenesis, via mechanisms involving enhanced superoxide production.

To explore the mechanisms linking superoxide overproduction to lysosomal dysfunction, we performed proteomics to evaluate oxidative modifications of the vacuolar ATPases (vATPases) that regulate its activity. Palmitate
Lipid-induced lysosome dysfunction

autophagy is independent of mitochondrial superoxide and mitophagy induction.

Noxs, particularly Nox2 and Nox4, are another major source of superoxides in the heart (36). Total levels of Nox4 were unchanged (supplementary Fig. 6A, B), but total levels and plasma membrane localization of the Nox2 subunit p47phox were increased in HFD hearts (Fig. 6A) and in palmitate-exposed CMs (Fig. 8C). We evaluated whether inhibiting Nox would improve lysosomal function and autophagosome turnover in palmitate-treated CMs. Inhibition of Nox2 with apocynin improved CatL activity and significantly reduced LC3-II accumulation in palmitate-treated CMs (Fig. 6B, C). As additional evidence of Nox2 as the source of superoxides, siRNA-mediated depletion of its regulatory subunit, p47phox, completely abolished superoxide production in palmitate-treated CMs (Fig. 6D). Importantly, p47phox depletion restored treatment of CMs increased S-nitrosylation of a highly conserved cysteine residue (Cys-277) of the Atp6v1a1 subunit that could impair vATPase function to acidify lysosomes (Fig. 5E) (35).

As an important source of superoxides, we explored to determine whether mitochondrial ROS modulates palmitate-induced autophagy. Overexpression of the mitochondrial superoxide, SOD2, failed to attenuate palmitate-induced autophagy (supplementary Fig. 5A). Moreover, inhibition of CPT1 with etomoxir to limit mitochondrial long chain fatty acid uptake accentuated palmitate-induced autophagy, suggesting an extramitochondrial mode of action (supplementary Fig. 5B). Markers of mitophagy, PINK1 and Parkin, were unchanged by palmitate (supplementary Fig. 5C) and we did not observe any colocalization of Tom20-positive mitochondria with GFP-LC3-positive autophagosomes (supplementary Fig. 5D). Thus palmitate-induced autophagy is independent of mitochondrial superoxide and mitophagy induction.

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**PKCβ regulates Nox2 activity in palmitate-treated CMs**

We next explored molecular signals linking palmitate treatment to Nox2 activation by focusing on PKC isoforms. The rationale for examining these enzymes stems from observations that palmitate treatment significantly increases the levels of DAG (supplementary Fig. 2), which is a well-known activator of PKC, that palmitate activates various PKC isoforms in diverse cell types (37, 38), that PKC activation often parallels Nox activation, and that PKC inhibition impairs Nox activity (39, 40). H9C2 CMs were incubated with different PKC inhibitors. Incubation with the nonspecific PKC isoform inhibitors (rottlerin or GF109203x) increased LC3-II to levels that equalled or exceeded those of CMs that were incubated with palmitate alone, and addition of palmitate did not further increase LC3-II (supplementary Fig. 7A). However, inhibition of classical PKCs with Gö6976 did not influence basal LC3-II levels, but blunted palmitate-induced LC3-II accumulation and partially reduced p62 levels (Fig. 8A, B). CQ increased LC3-II levels but did not influence p62 accumulation in Gö6976-treated CMs exposed to palmitate, suggesting that PKC may modulate lipid-induced lysosomal dysfunction. Palmitate treatment increased membrane translocation of phospho-PKCβ and its downstream target p47phox, which are required for PKCβ and Nox2 activation, respectively. Interestingly, Gö6976 markedly reduced the membrane acidity and reversed palmitate-induced impairment in lysosomal CatL and LAP activities (Fig. 7A–C). Improved lysosomal function accompanied a significant reduction in LC3-II levels (~31%), normalization of p62 accumulation, and lowering of GFP-LC3-positive autophagosome accumulation (48%) in palmitate-treated CMs depleted of p47phox (Fig. 7D–F). Collectively, these data support the hypothesis that Nox2-derived superoxides impair lysosomal acidification and enzyme activity leading to autophagosome accumulation in palmitate-treated CMs and in HFD-fed murine hearts.

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Fig. 5. Palmitate impairs lysosomal acidification and enzyme activity in a superoxide dependent manner.

A: CMs were incubated with vehicle (Veh) or palmitate (Palm) in the presence or absence of 10 mM tiron for 4 h and subsequently incubated with LysoSensor yellow/blue dye during the last 10 min. Graphs represent the ratio of LysoSensor blue to yellow fluorescence ± SEM quantified from the confocal fluorescence micrographs (n = 40 CMs). B: CMs treated with vehicle or palmitate and with or without tiron were incubated with MR-CatL substrate during the last 1 h of treatment and visualized by confocal microscopy. Graphs represent MR-fluorescence intensity per CM ± SEM (n = 80, 69, 51, and 72 CMs for vehicle-saline, palmitate-saline, vehicle-tiron, and palmitate-tiron, respectively). Scale bar, 20 μm. C: In vitro enzymatic activities of lysosomal enzymes, β-galactosidase and β-hexosaminidase, measured in crude lysosomal preparations from vehicle- or palmitate-treated H9C2 CMs as described in the Materials and Methods (n = 3 independent samples per group). D: H9C2 CMs incubated with vehicle or palmitate were analyzed by quantitative real-time PCR for mRNA levels of genes involved in lysosomal biogenesis, acidification, and enzyme activity (n = 5). E: H9C2 CMs treated with vehicle or palmitate were analyzed for cysteine S-nitrosylation of proteins in the lysosomal fraction by mass spectrometry. Amino acid sequence alignment of ATP6V1A1 subunits from mouse (GenBank accession number: NP_031534), rat (NP_001101788), human (NP_001681), xenopus (NP_001089571), zebrafish (NP_957429), Caenorhabditis elegans (NP_506559), and yeast (NP_010096), and archaea (YP_007823039), respectively, showing the sequence of interest. The cysteine residues characterized previously are shaded gray whereas the S-nitrosylated cysteine residues identified in this study are shaded black. The numbers represent the position of the cysteine residues on human ATP6V1A1. For panel E, ***, identical; **, conserved; and *, semi-conserved amino acid substitution. For other panels, *P < 0.05; ***P < 0.001.
LC3-II and p62 in CQ-treated HFD hearts supports the conclusion that increased autophagosome abundance may be secondary to impaired autophagosome turnover. This study indicates that defects in cardiac autophagy may be an early response of the heart to lipid oversupply that occurs despite the preserved cardiac function at baseline. However, in the presence of an additional stress such as ischemia and reperfusion (I/R), high fat-fed hearts become more prone to cardiac injury due to impaired autophagy (15). In the long-term, HFD mice eventually develop contractile dysfunction in parallel with impaired autophagy (41). These data suggest that impairment in autophagic flux may heighten sensitivity to stress and may progressively contribute to contractile dysfunction in diet-induced obesity. Therefore, an intervention to maintain normal autophagic flux in a diet-induced obese heart could provide potential benefits against an additional cardiac stress.

Because the molecular events regulating autophagosome accumulation in HFD hearts were unclear, we utilized an in vitro cell culture model to elucidate the mechanism of lipid overload-induced regulation of autophagy in CMs. localization of both proteins (Fig. 8C) and prevented Nox2-derived superoxide production in palmitate-treated CMs (Fig. 8D). Furthermore, inhibition of pan-PKCγ, or specifically PKCβII, activity with isoform-specific peptide inhibitors reversed palmitate-induced LC3-II accumulation to basal levels (Fig. 8E, F). In contrast, siRNA-mediated silencing of PKCα led to a small increase in LC3-II levels in both vehicle- and palmitate-treated CMs (supplementary Fig. 7B). Together, these data suggest that PKCβ is the major PKC isoform mediating the activation of Nox2 and impairment of autophagic flux in response to palmitate.

**DISCUSSION**

Obesity is associated with tissue-specific modulation of autophagic flux. Although obesity promotes cardiac dysfunction, studies examining the regulation of cardiac autophagy in obesity are limited. This study demonstrates that HFD increases cardiac autophagosome abundance independently of canonical autophagy initiation signaling via insulin or mTOR. Lack of additional accumulation of LC3-II and p62 in CQ-treated HFD hearts supports the conclusion that increased autophagosome abundance may be secondary to impaired autophagosome turnover. This study indicates that defects in cardiac autophagy may be an early response of the heart to lipid oversupply that occurs despite the preserved cardiac function at baseline. However, in the presence of an additional stress such as ischemia and reperfusion (I/R), high fat-fed hearts become more prone to cardiac injury due to impaired autophagy (15). In the long-term, HFD mice eventually develop contractile dysfunction in parallel with impaired autophagy (41). These data suggest that impairment in autophagic turnover may heighten sensitivity to stress and may progressively contribute to contractile dysfunction in diet-induced obesity. Therefore, an intervention to maintain normal autophagic flux in a diet-induced obese heart could provide potential benefits against an additional cardiac stress.

**Fig. 6.** Nox2-derived superoxides mediate palmitate-induced impairment in lysosomal enzyme activity and autophagosome turnover. A: Immunoblots of p47phox in the plasma membrane fraction and total lysate of hearts from mice on NCD or HFD for 12 weeks (n = 3 for NCD, n = 4 for HFD). B: H9C2 CMs treated with vehicle (Veh) or 500 μM palmitate (Palm) for 4 h in the presence or absence of 10 mM apocynin (ACN) were incubated with MR-CatL substrate for the last 1 h of treatment and analyzed by confocal microscopy as described in Fig. 5B (n = 63, 92, 62, and 88 CMs for vehicle-control, palmitate-control, vehicle-ACN, and palmitate-ACN, respectively). Scale bar, 20 μm. C: Immunoblot analysis of LC3 in CMs treated with vehicle or palmitate as described in (B) (n = 3). D: Superoxide production in H9C2 CMs transfected with scramble (Scr) or p47phox siRNA (si-p47phox) and treated with vehicle or palmitate as described in (B) was measured by ESR using CMH as a spin trap probe (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.
The data provided evidence for superoxide-dependent impairment in lysosomal acidification and enzyme activity as a primary mechanism for defective autophagic turnover in CMs. Consistent with our in vivo observations, 4 h palmitate treatment significantly increased autophagosome abundance in H9C2 CMs in part by impairing autophagic turnover. Impaired autophagic turnover has been observed in pancreatic β cells following prolonged treatment with palmitate or oleate (34). However, it is not clear whether the effect was specific to autophagy or secondary to lipotoxicity, because lipid overload also led to cell death, mitochondrial dysfunction, and ATP depletion in these cells. We observed impairment in autophagic turnover as an early response to lipid overload in CMs that precedes lipotoxic effects such as ER stress and apoptosis. Notably, palmitate withdrawal following acute treatment

**Fig. 7.** Inhibition of Nox2 reverses lipid overload-induced impairment in lysosomal enzyme activity and autophagosome turnover. A: H9C2 CMs were transfected with scramble (Scr) or p47phox siRNA (si-p47phox). CMs treated with vehicle (Veh) or palmitate (Palm) for 4 h were stained with LysoSensor yellow/blue dye (1 μM) for the last 10 min. Graphs represent blue to yellow fluorescence ratio ± SEM (n = 144, 148, 152, and 167 CMs for vehicle-scramble, palmitate-scramble, vehicle-si-p47phox, and palmitate-si-p47phox, respectively). Scale bars, 20 μm. B, C: H9C2 CMs described in (A) were either incubated with MR-CatL substrate (B) or with LysoLive Phosgreen-LAP substrate (C) for the last 1 h of vehicle or palmitate treatment. Graphs represent mean MR-fluorescence intensity ± SEM (n = 28, 51, 28, and 76 CMs for vehicle-scr, palmitate-scramble, vehicle-si-p47phox, and palmitate-si-p47phox, respectively) or mean LysoLive Phosgreen fluorescence intensity ± SEM (n = 96 CMs). Scale bar, 20 μm. D: Immunoblots of p47phox, LC3 (D) and p62 (E) in H9C2 CMs transfected with scramble (Scr) or p47phox siRNA (si-p47phox) and treated with vehicle or palmitate for 4 h (n = 3). F: Confocal images of GFP-LC3 in adenoviral GFP-LC3 expressing CMs transfected with scramble or p47phox siRNA and incubated with vehicle or palmitate for 4 h (n = 27 CMs for vehicle-scr, palmitate-scr, and vehicle-si-p47phox, and 25 CMs for palmitate-si-p47phox). Scale bar, 20 μm. *P < 0.05; **P < 0.01; ***P < 0.001.
Fig. 8. Classical PKCs mediate palmitate-induced activation of Nox2. A, B: Analysis of LC3-II (A) and p62 (B) levels in H9C2 CMs treated with vehicle (Veh) or palmitate (Palm) for 4 h in the presence or absence of 2 μM Gö6976 (n = 3). C: Protein levels of phosphorylated PKCα/βII (pPKCα/βII) (S638/641) and p47phox assessed by Western blot in the plasma membrane and cytosolic fractions isolated from H9C2 CMs described in (A) (n = 3). D: Levels of superoxide in total cell lysates from H9C2 CMs described in (A) (n = 5, 5, 3, and 3 samples for vehicle-control, palmitate-control, vehicle-Gö6976, and palmitate-Gö6976, respectively, from two independent experiments). E: H9C2 CMs were pretreated with control or 10 μM pan-PKC pseudosubstrate (PKCα) for 90 min and subsequently incubated with vehicle or palmitate for 4 h in the presence or absence of the pseudosubstrate. Immunoblots showing pPKCα/βII (S638/641) and LC3 levels in CMs. F: H9C2 CMs were pretreated with control or 2 μM PKCα peptide inhibitor (PKCαi) and subsequently with vehicle or palmitate as described in (E). Immunoblots of pPKCα/βII (S638/641) and LC3 are shown. G: A model for lipid overload-induced impairment in autophagic flux. Lipid overload by palmitate increases intracellular DAG levels that activate PKCα. Activated PKCα promotes membrane translocation of the p47phox-containing regulatory subcomplex that induces Nox2 complex activity. Nox2-derived superoxide overproduction leads to oxidative modification of lysosomal vATPases, which inhibits their ability to acidify lysosomal vesicles. Under basal conditions (inset), autolysosome formation (step a) and autophagic substrate degradation (step b) occurs at a normal rate. When lysosomal pH is increased, although autolysosome formation is unaffected, lysosomal enzyme activity to degrade autophagosome cargo is significantly impaired. This leads to the accumulation of autolysosomes in lipid-overloaded CMs indicating impaired autophagic flux. *P < 0.05; **P < 0.01; ***P < 0.001.
reversed autophagosome accumulation in H9C2 CMs. It appears that while defective autophagic turnover induced by acute lipid overload can be reversed, prolonged impairment in autophagic turnover may represent a secondary effect of lipotoxicity that could ultimately lead to ER stress and apoptosis following long-term palmitate treatment.

Growing evidence suggests that the effect of lipid oversupply on myocardial dysfunction is mediated by toxic lipid intermediates, which increase ROS generation, oxidative stress, and ultimately cell death (3, 42). Consistent with this notion, palmitate significantly increased superoxide levels in CMs that paralleled autophagosome accumulation, which was attenuated by scavenging ROS with tiron. Notably, the monounsaturated fatty acid oleate completely reversed palmitate-induced autophagosome accumulation. Previous studies showed that oleate exerts different cellular effects than palmitate (43). It prevents lipotoxic effects of palmitate presumably by channelling toxic lipid intermediates of palmitate metabolism to the relatively benign triacylglycerol depot, which we also observed in H9C2 CMs, and may explain its effect on the reversal of palmitate-induced autophagy (44). We observed accumulation of ceramides, a toxic byproduct of lipid metabolism that was independent of palmitate-induced autophagosome accumulation. Recent studies suggest that other lipid intermediates, such as DAG, may regulate palmitate-induced autophagy, albeit via unclear mechanisms (45). The possibility remains that the increased DAG levels observed could directly modulate autophagy induction in palmitate-treated H9C2 CMs, independently of the ability of DAG to activate PKC.

An important finding of this study is the superoxide-mediated impairment of lysosomal acidification and pH-dependent enzyme activity. ROS-dependent impairment of cardiac autophagosome clearance was also observed following I/R (46). Whether ROS overproduction in a lipid-loaded heart modulates autophagic flux in response to I/R is unknown, and will be pursued in future studies. Our study indicates that superoxide-mediated lysosomal dysfunction inhibits autophagosome clearance in lipid-loaded CMs. Lipid overload impairs lysosomal function by multiple mechanisms, which appear to be tissue specific. HFD alters membrane composition of mouse liver autophagosomes and lysosomes, which impairs autophagolysosome formation (47). In pancreatic β cells, prolonged palmitate treatment results in ATP depletion, which impairs acidification of lysosomes (34). Thus, impaired lysosomal function may represent an important general mechanism of defective autophagosome clearance in response to lipid overload.

The present study observed Nox2 activation in both HFD-fed murine hearts and palmitate-treated H9C2 CMs. Inhibition of Nox2 significantly reduced superoxide levels in parallel with normalizing lysosomal pH and enzyme activity and lowering autophagosome abundance induced by palmitate. Nox2-derived ROS are known to regulate phagososomal pH, which is required for antigen cross-presentation by dendritic cells and for targeting phagosomes for autophagic degradation (48, 49). Our study identified a novel function of Nox2, which supports its role in regulating the pH of lysosomes involved in autophagy in non-inflammatory cells. A recent study identified Nox1 in the regulation of cardiac autophagy, particularly upon glucose deprivation (50), which suggests that the activation of specific Nox isoforms may depend on the specific stress.

Palmitate-induced Nox2 activation is mediated by the activation of classical PKCs, possibly by DAG. Inhibition of classical PKCs blunted Nox2 activity and reduced LC3-II levels. Particularly, inhibition of PKCβII completely normalized palmitate-induced LC3-II accumulation. Activation of classical PKCs was observed in skeletal muscles of obese humans and in high fat-fed murine hearts (51, 52). Consistent with our findings, activation of Nox2 by PKCβII was also observed in CMs exposed to hyperglycemia (53). Although Nox2 activation by PKC contributes to oxidative stress, its role in autophagy mediated by lipid-induced oxidative stress is unknown. This study shows, we believe for the first time, that lipid-overload-induced activation of PKC-Nox2 signaling impairs lysosomal acidification and enzyme activity leading to defective autophagosome clearance.

Our study supports a mechanism whereby palmitate leads to PKCβ-mediated Nox2 activation, which generates ROS that inhibit lysosomal function. A key remaining question is the mechanism by which ROS may impair lysosomal function. Although an association between Nox and lysosomal membranes has been described leading to a mechanism by which locally generated superoxide might modulate lysosomal acidification (54, 55), we found no evidence of Nox enrichment in lysosomal membranes in a palmitate-dependent manner. We therefore examined the possibility that oxidative modifications of the lysosomal vATPases could impact its function. These initial studies provide preliminary evidence for oxidative S-nitrosylation of lysosomal vATPases that may inhibit the ability of these proton pumps to acidify lysosomes (56). Specifically, we observed increased S-nitrosylation of a highly conserved Cys-277 residue of the Atp6v1a1 subunit in palmitate-treated CMs. A free Cys-277 thiol has been proposed to be required for reducing the inhibitory disulphide bond between two cysteines (Cys-254 and Cys-353) to maintain vATPase activity (35, 57). S-nitrosylation of Cys-277 could therefore impair its reducing function, keeping vATPase in its inactive conformation, potentially leading to defective lysosomal acidification and autophagic clearance. Further study is underway to define the role of oxidative cysteine modifications in regulating vATPase function in response to lipid overload.

Lipids play a central role in autophagy as essential constituents of the autophagosomal membrane, as signaling molecules regulating multiple steps in autophagy, and as substrates for a selective process called lipophagy (45). Future studies will determine whether altered lipophagy may contribute to the pathophysiology of cardiac lipotoxicity. Here, we report that nonoxidative palmitate metabolism in the face of increased palmitate availability results in the generation of ROS and impairment of autophagosome
turnover in CMs. Dysregulated autophagy is observed in various cardiac pathologies, including myocardial I/R and heart failure. HFD worsens ischemic injury in an autophagy-dependent manner (12, 15). However, the precise mechanism and functional consequences of altered autophagy in HFD hearts remains elusive. Several studies have shown that Nox-derived ROS contribute to numerous pathologies, including cardiac hypertrophy, I/R injury, diabetes, and CVD (58). Induction of multiple Nox2 subunits and ROS production have been reported in experimental models of obesity and diabetes (59, 60) and in patients with metabolic syndrome (61). Here, we have uncovered a novel role for Nox2 in regulating autophagosome turnover. Our study suggests that lipid overload might impact autophagy by mechanisms that impinge on lysosomal acidification and activity. Therefore, targeting lysosomal dysfunction might provide a novel therapeutic approach for reactivating autophagy in lipotoxic cardiovascular pathology associated with obesity and the metabolic syndrome (62).

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