Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) Phosphorylation by Protein Kinase Cδ (PKCδ) Inhibits Mitochondria Elimination by Lysosomal-like Structures following Ischemia and Reoxygenation-induced Injury*

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Background: How damaged mitochondria are removed by mitophagy is not fully described.
Results: Ischemia and reoxygenation (I/R)-induced injury triggers mitochondria association of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and mitophagy, and protein kinase Cδ (PKCδ) activation inhibits it.
Conclusion: PKCδ-mediated phosphorylation of GAPDH inhibits mitophagy.
Significance: GAPDH/PKCδ is a signaling switch, which is activated during ischemic injury to regulate the balance between cell survival by mitophagy and cell death by apoptosis.

After cardiac ischemia and reperfusion or reoxygenation (I/R), damaged mitochondria propagate tissue injury by promoting cell death. One possible mechanism to protect from I/R-induced injury is the elimination of damaged mitochondria by mitophagy. Here we identify new molecular events that lead to mitophagy using a cell culture model and whole hearts subjected to I/R. We found that I/R induces glyceraldehyde-3-phosphate dehydrogenase (GAPDH) association with mitochondria and promotes direct uptake of damaged mitochondria into multior-ganellar lysosomal-like (LL) structures for elimination independently of the macroautophagy pathway. We also found that protein kinase Cδ (PKCδ) inhibits GAPDH-driven mitophagy by phosphorylating the mitochondrially associated GAPDH at threonine 246 following I/R. Phosphorylated GAPDH promotes the accumulation of mitochondria at the periphery of LL structures, which coincides with increased mitochondrial permeability. Either inhibition of PKCδ or expression of a phosphorylation-defective GAPDH mutant during I/R promotes a reduction in mitochondrial mass and apoptosis, thus indicating rescued mitophagy.

Following myocardial infarction and injury induced by ischemia, the return of blood flow to the heart and subsequent reoxygenation produces further oxidative damage to the heart (1).

This ischemia-reoxygenation or reperfusion (I/R)5-induced injury is mediated by reactive oxygen species, generated primarily by damaged mitochondria, which leads to opening of the mitochondrial permeability transition pore and the onset of cell death by apoptosis and necrosis (1, 2). Hence, ensuring proper elimination of damaged mitochondria is imperative to cell survival.

One possible way to promote cardiomyocyte survival during I/R-induced injury is to eliminate damaged mitochondria by targeting them for lysosomal degradation by autophagy, a process often referred to as mitophagy (3). One major form of mitophagy is macroautophagy, where mitochondria are targeted to lysosomes after sequestration into autophagosomes and fusion of the autophagosomes with the lysosome (4, 5). A number of proteins, including a ubiquitin ligase (Parkin), a serine threonine kinase (ULK1), and a BH3 family member (NIX), have been shown to be required for macroautophagy (6–8). Several recent studies in yeast have reported that mitochondria can also be degraded by micromitophagy, where the lysosomes in mammals or vacuoles in plant and fungi directly engulf mitochondria (9–13). Macroautophagy-independent mitophagy has also been observed in hepatocytes following photoradiation in the presence of the macroautophagy inhibitor, 3-methyl-lalanine (3MA) (14, 15). The molecular mechanisms of macromitophagy have been well described (16–18). However, little is known about the molecular regulation of micromitophagy and its role in cell fate after an ischemic insult.

We have previously shown in mouse, rat, and porcine models of myocardial infarction that translocation and activation of protein kinase Cδ (PKCδ) from the cytosol to the mitochondria at the onset of reoxygenation promotes irreversible damage to the myocardium by impairing multiple mitochondrion-mediated

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The abbreviations used are: I/R, ischemia and reoxygenation or reperfusion; iGAPDH, inactive glyceraldehyde-3-phosphate dehydrogenase; VDAC, voltage-dependent anion channel; LAMP-1 (or LAMP-2), lysosome-associated membrane proteins 1 (or 2); LC3, microtubule-associated protein 1 light chain 3; TEM, transmission electron microscopy; 3MA, 3-methyl-lalanine; MEF, mouse embryonic fibroblast; PO, propylene oxide; LL, lysosomal-like; PS, phosphatidylserine.
processes (19–22). I/R-induced translocation and activation of mitochondrial PKCβ is associated with reduced mitochondrial ATP production, increased mitochondrial-mediated apoptosis, and coincides with the appearance of mitochondria that are swollen and structurally in disarray (19, 20, 23, 24). In contrast, inhibition of PKCβ translocation to mitochondria by a selective PKCβ peptide inhibitor, δV1–1, during I/R inhibits mitochondrial dysfunction (20, 23, 24). Here, we identified a pathway of mitochondrial elimination following I/R-induced injury and the role of PKCβ in the regulation of this process using isolated whole hearts and cells in culture.

EXPERIMENTAL PROCEDURES

Cell Cultures—The murine HL1 cardiomyocyte cell line was kindly obtained from Dr. William Claycomb (Louisiana State University Health Sciences Center) and expanded in Claycomb medium (Sigma), supplemented with 10% (v/v) fetal bovine serum (FBS), 4 mM L-glutamine, 100 μM norepinephrine and antibiotics. HL1 cells were subcultured at high density (typically a 1:3 split) on 10-cm² plates or glass coverslips pre-coated with 0.02% gelatin and 25 μg/ml of fibronectin (Sigma). HL1 cell culture medium was replaced daily to maintain their differential growth. Ischemia-mimetic solution (125 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.25 mM MgSO₄, 1.2 mM CaCl₂, 6.25 mM NaHCO₃, 5 mM sodium lactate, 20 mM HEPES, pH 6.6) and placing the cells in hypoxic pouches (GasPak EZ Anaerobe Gas Generating Pouch System with indicator, BD Biosciences). After 2.5 h of simulated ischemia, reperfusion injury was initiated by removing the ischemia buffer and immediately replacing with pre-warmed normoxic Krebs-Henseleit solution (110 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.25 mM MgSO₄, 1.2 mM CaCl₂, 25 mM NaHCO₃, 15 mM glucose, 20 mM HEPES, pH 7.4) and incubated at 95% room air, 5% CO₂ for 15 min or as indicated. Control groups were incubated for the equivalent amount of time in normoxic Krebs-Henseleit solution.

Peptide-mediated Inhibition of PKCβ Translocation—PKCβ isoform translocation to mitochondria was inhibited by the isozyme-specific translocation inhibitor peptide, δV1–1 (amino acid residues 14–21 of PKCβ). The peptide was designed as previously described (21, 26). The peptide was dissolved in sterile water and used at a final concentration of 1 μM in cultured cells throughout I/R-induced injury.

Expression of Recombinant Active (GAPDH-V5) and Inactive GAPDH (iGAPDH-V5)—Catalytically active and inactive V5-tagged human GAPDH cDNAs were kindly obtained from Dr. Douglas Green (St. Jude Children’s Research Hospital) (27). Retrovirus production in 293T cells was performed by co-transfecting 293T cells with cDNA encoding GAPDH-V5 or iGAPDH-V5, the VSVG envelope protein, and GAG-POL polypeptide and collecting the virus-containing supernatant after 48 h. HL1 cells or Atg5⁻/⁻ MEFs were transduced with freshly harvested GAPDH-V5 or iGAPDH-V5 virus-containing supernatant for 16 h in the presence of 8 μg/ml of Polybrene and analyzed 48 h later.

Expression of Recombinant WT iGAPDH and Mutant T246A iGAPDH—The catalytically inactive V5-tagged human GAPDH cDNA was kindly obtained from Dr. Douglas Green (St. Jude Children’s Research Hospital) (27). Threonine at position 246 was mutated to alanine (T246A) to inhibit phosphorylation using a QuikChange site-directed mutagenesis kit (Stratagene). The following primers were used: Ala-246 sense, 5’-ccc act gcc agc acg gac ggt gct gtc cgt cta gaa aaa cct gcc gaa tat-3’; Al-246 antisense, 5’-ata ttg gcc agg att ttc ttc gac ggc gag cag gtc cac cac gtt gag gcc gat ccc-3’. Mutants were sequenced to ensure that no additional mutations were introduced and then transfected into 293T cells using FuGENE6 transfection reagent (Roche Applied Science). Retrovirus production was performed as described above.

Mitochondria Enrichment by Differential Centrifugation—Mitochondria were isolated from HL1 or Atg5⁻/⁻ cells by differential centrifugation. Confluent monolayers of cells were rinsed with PBS and then scraped from plates using mannitol-sucrose buffer (MS; 210 mM mannitol, 70 mM sucrose, 5 mM MOPS, 1 mM EDTA, pH 7.4). Cells were triturated 16 times on ice using a 27-gauge needle, followed by microcentrifugation at 800 × g to pellet nuclei and cell debris. The postnuclear supernatant was microcentrifuged at 10,000 × g for 15 min to obtain a mitochondrial pellet, which was surface washed three times with MS buffer prior to downstream applications. It should be noted that the amount of mitochondria removed by the 800 × g spin or the amount of mitochondria obtained in the 10,000 × g spin was unaffected by I/R treatments (data not shown).

Mitochondria and Lysosomal Enrichment Using Density Gradients—In a parallel approach, density gradients were employed to enrich for mitochondria and lysosomes. Cells were subjected to hypobaric shock 5 times, pelleted at 500 × g to remove nuclei, and the released organelles were separated on density gradients by ultracentrifugation using a lysosome enrichment kit for tissues and cultured cells (Thermo Scientific). Fractions were collected from each gradient from the top to the bottom, combined with 2 volumes of PBS, and pelleted at 10,000 × g for 30 min. The organelle pellets were surface washed twice with buffer A (supplied in the kit) prior to downstream applications.

Immunofluorescence—HL1 cells or Atg5⁻/⁻ cells, exponentially growing on glass coverslips, were fixed with freshly prepared PBS containing 4% (w/v) paraformaldehyde for 15 min at 4 °C, permeabilized with PBS containing 3% (w/v) BSA and 0.1% (w/v) Triton X-100 at room temperature for 15 min, and then blocked overnight at 4 °C with PBS containing 3% (w/v) BSA (blocking buffer). In some instances, cells were labeled with 0.5 μM LysoTracker Red DND-99 (Molecular Probes) for 30 min as recommended by the supplier, prior to fixation. Fixed cells were then incubated with primary antibodies diluted in blocking buffer for 3 h at room temperature in a humidified chamber followed by 1 h of incubation with Alexa Fluor 488-conjugated.
or Alexa Fluor 568-conjugated secondary antibodies (Molecular Probes). Samples were washed with PBS and counterstained with Hoechst (Molecular Probes) to visualize nuclei and analyzed using a DeltaVision OMX SR wide field deconvolution fluorescence microscope (Applied Precision Inc). Consecutive images were acquired at ×100 magnification using the oil objective with the red, green, and blue channels, pseudo colored, and overlaid using SoftWoRx 3.4.5 software (Applied Precision).

Transmission Electron Microscopy (TEM)—Isolated organelles or intact HL1 and Atg5−/− MEFs growing on glass coverslips were fixed in Karnovsky’s fixative (2% (v/v) glutaraldehyde and 4% (v/v) formaldehyde in 0.1 M sodium cacodylate, pH 7.4) for 1 h at room temperature. Sections were postfixed in 1% osmium tetroxide for 1 h at room temperature, washed with ultrafiltered water, and stained for 2 h at room temperature. Samples were then dehydrated in a series of ethanol washes for 15 min at 4 °C beginning at 50, 70, and 95% ethanol in water. Samples were then equilibrated to room temperature and washed with 100% ethanol twice, then propylene oxide (PO) for 15 min. Samples were infiltrated with EMBed-812 resin (EMD) that was mixed with PO. Samples were placed into EMBed-812 for 2 to 4 h, then placed into molds with fresh resin, and incubated overnight at 65 °C. Sections were cut at 75- and 90-nm intervals, picked up on formvar-carbon-coated slot copper grids, and stained for 15 to 20 min in 1:1 saturated uranyl acetate (~7.7%) and 100% ethanol followed by staining in 0.2% lead citrate for 3 to 4 min. Images were acquired using a JEOL1230 Gatan 967 CCD transmission electron microscope at 80 kV and a Gatan Orius 4k digital camera. Images were acquired at ×1000, 3000, or 10,000 magnifications from cells of approximately the same size.

Determination of GAPDH Activity—GAPDH activity was determined using a KDalert GAPDH assay kit as instructed by the manufacturer (Applied Biosystems). Briefly, samples were diluted in KDalert lysis buffer along with a series of GAPDH enzyme dilutions for the standard curve. After 20 min of incubation on ice, samples were combined with the KDalert master mixture in 96-well plates and read at 615 nm using a UV-visible plate reader. GAPDH activity was calculated and expressed as units of GAPDH/mg of total protein.

RESULTS

PKCθ Translocates to HL1 Mitochondria during I/R-induced Injury and Inhibits Mitochondrial Elimination—We have previously shown that PKCθ translocates to mitochondria during I/R-induced injury and mediates mitochondrial dysfunction by inhibiting ATP production and promoting the release of cytochrome c to the cytosol (20, 23, 24). Here we studied the effects of PKCθ on mitochondrial elimination. I/R was modeled in HL1 cardiomyocytes using a modified method of Gottlieb and collaborators (25), where cells were subjected to 2.5 h of ischemia followed by a short 30-min reoxygenation. I/R-induced injury promoted a robust translocation of PKCθ to HL1 mitochondria, whereas ischemia alone (I) did not (Fig. 1B). As expected, PKCθ translocation to mitochondria during I/R-induced injury was inhibited by the selective inhibitor of PKCθ translocation and function, δV1–1 (Fig. 1B) (21). Furthermore, as previously shown in ex vivo and in vivo models of rat, mouse, and porcine of I/R-induced injury (20, 23, 24), PKCθ translocation to HL1 mitochondria coincided with increased cytosolic levels of cytochrome c, which were reduced in the presence of δV1–1 (Fig. 1C and D).

Together, these results show that I/R-induced injury in HL1 cardiomyocytes recapitulates the translocation of PKCθ to mitochondria and the onset of apoptosis seen in other models of I/R.

Interestingly, we observed that I/R-induced injury in the presence of δV1–1 significantly reduced the amount of the mitochondrial matrix protein, Grp75, in total cell extracts, whereas I/R-induced injury alone did not (Fig. 1E and F). These data suggest that PKCθ translocation to mitochondria is potentially involved in the pathway leading to mitochondrial elimination during I/R-induced injury.

I/R-induced Injury Triggers the Formation of Lysosomal-like (LL) Structures—To assess whether PKCθ contributes to cell death by inhibiting the elimination of damaged mitochondria, HL1 cells were subjected to I/R-induced injury, and analyzed by immunofluorescence using the mitochondrial marker, Tom20. We noticed that I/R-induced injury triggers the formation of vacuolated structures of varying size (Fig. 2A). Furthermore, inhibition of PKCθ translocation by δV1–1 during I/R-induced injury promoted the appearance of Tom20-positive (Tom20+) material inside the lumen of these vacuoles, suggesting mitochondrial internalization into the vacuolated structures when PKCθ is inhibited (Fig. 2A, right panel).

The primary organelle thought to be responsible for uptake and elimination of mitochondria in mammalian cells is the lysosome (4, 28). Thus, we reasoned that I/R-induced injury triggers a rapid expansion of the lysosomes seen as large vacuolated structures to facilitate the elimination of damaged cellular components (Fig. 2A). To test this hypothesis directly, organelles were isolated by density-gradient centrifugation. Under normoxic conditions, mitochondria were found at the bottom of density gradients, as indicated by the presence of the mitochondrial protein, VDAC (voltage-dependent anion channel) in that fraction. Lysosomes were present at the top of gradients, as evidenced by the presence of the lysosomal protein, LAMP-1 (Fig. 2B). We noticed that I/R-induced injury in the absence or presence of δV1–1 promoted only a modest expansion of the lysosomal system as determined by comparing the relative amounts of LAMP-1 (Fig. 2B, middle and bottom panels as compared with the top panel). LAMP-2 has been known to be predominantly associated with lysosomes (29, 30), but recently it was detected in a late endosomal compartment of dendritic cells that mediates microautophagy of specific cytosolic proteins, by invaginations of the endosomal membrane (31). As such, under normoxic conditions or following I/R in the absence or presence of δV1–1, LAMP-2 was detected at the top of gradients along with LAMP-1 lysosomes. Interestingly, I/R-induced injury resulted in a 3–4-fold increase in lysosomal LAMP-2 protein in the mitochondrial fraction as indicated by co-fractionation with the mitochondrial marker protein, VDAC. The increased amount of the lysosomal marker, LAMP-2, following I/R likely indicates expansion of the lysosomal machinery for degradation of damaged mitochondria. Consistently, immunofluorescence studies showed that LAMP-2-positive material is partially localized to the vacuolated structures triggered by I/R-induced injury in the absence
or presence of δV1–1 (Fig. 2C). Additional immunofluorescence studies confirmed that these structures were also positive for LysoTracker Red and Rab9, markers used to detect acidic organelles, and lysosome and late endosomes, respectively (Fig. 2D). Taken together, our studies suggest that the vacuolated structures observed after I/R-induced injury are of a late endosomal and lysosomal origin and that they internalize mitochondrial morphology was noticeably improved in the presence of dense bodies around the vacuolated structures, whereas the mitochondria in intact HL1 cells and in isolated rat hearts following I/R in the presence of δV1–1 (Fig. 2E–G), which coincides with a reduction in mitochondrial mass that we observed (Fig. 1E). In addition, I/R-induced injury promoted the accumulation of mitochondria containing fragmented cristae and amorphous dense bodies around the vacuolated structures, whereas the mitochondrial morphology was noticeably improved in the presence of δV1–1 (Fig. 2G). These data suggest roles for the vacuolated structures and PKCδ in mitochondrial elimination following I/R-induced injury. Based on our observations, we termed these distinct vacuolated structures as LL structures.

Catalytically Inactive GAPDH (iGAPDH) Associates with Mitochondria during I/R-induced Injury—We next focused on the molecular mechanism by which I/R-induced mitophagy occurs. We often used glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control on Western blots but consistently observed a time-dependent increased level of GAPDH accumulating in the mitochondrial enriched fraction from HL1 cells following I/R-induced injury (Fig. 3, B and C). We also found that the mitochondrially damaging agents, antimycin A and hydrogen peroxide (H₂O₂), promoted the rapid translocation of GAPDH to mitochondria in HL1 cells, suggesting that the mitochondrial-mediated damage triggers the association of GAPDH with mitochondria (Fig. 3, E–G). Cytosolic GAPDH catalyzes the sixth step of glycolysis, where glucose is converted to pyruvate. In addition to its crucial role in glycolysis, GAPDH has also been shown to be involved in several nonglycolytic cellular processes, including DNA repair, membrane fusion and transport, cytoskeletal dynamics, and cell death (32). Furthermore, both the nuclear and cytosolic forms of overexpressed GAPDH are implicated in the clearance of mitochondria by macroautophagy (27). Based on these previously reported functions of GAPDH in macroautophagy and our observation of GAPDH accumulation in the mitochondrial fraction during I/R, we set out to determine whether GAPDH contributes to the regulation of I/R-induced mitophagy.

Interestingly, despite a noticeable I/R-induced increase in the mitochondrially associated GAPDH protein in HL1 cells,
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Figure A shows images of normoxia (C), I/R (C), and I/R δPKC (C) with Tom20 and LAMP-2 labels. Figure B presents Western blot analysis for VDAC, LAMP-1, and LAMP-2 under normoxia and I/R conditions.

Figure D displays images of HL1 cells labeled for Tom20, Lysotracker, and Merge under normoxia and I/R conditions with Rab9 and Lysotracker labels.

Figure E shows images of HL1 cells under I/R (C) and I/R δPKC conditions.

Figure F presents images of isolated rat heart under I/R (C) and I/R δPKC conditions.

Figure G displays images of isolated rat heart under normoxia, I/R (C), and I/R δPKC conditions.
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FIGURE 2. I/R-induced injury triggers the formation of LL structures. A, HL1 cells were treated with a control peptide (C) and the PKCδ translocation inhibitor peptide (ΔV1–1, indicated by a downward arrow) and subjected to normoxia (N) or I/R. The cells were fixed and analyzed by immunofluorescence (>100 magnification) using an antibody for the mitochondrial marker, Tom20 (green channel). An indicated portion of each image is expanded to illustrate the uptake of mitochondria (Tom20, white arrowheads) into the large vacuolated structures in the presence of ΔV1–1 (white arrowheads). B, HL1 cells subjected to normoxia or I/R with a control peptide (C), or I/R with ΔV1–1 were fractionated on density gradients. Fractions were collected from the top (T) to the bottom (B) of each gradient. Organelles were pelleted and analyzed by Western blotting for the presence of lysosomes, mitochondria, and late endosomes using anti-LAMP-1, anti-VDAC, and anti-LAMP-2 antibodies, respectively. LAMP-2 levels in the mitochondrion-enriched fractions (at the bottom of each gradient) are indicated as a percentage of LAMP-2 levels present in the entire gradient. C, HL1 cells subjected to normoxia or I/R were labeled with LysoTracker Red, fixed, and analyzed by immunofluorescence with a deconvolution microscope using anti-Tom20 or anti-Rab9 to visualize mitochondria and late endosomal membranes, respectively. Images were acquired at >100 magnification and represent maximum intensity projection of image stacks. White arrows indicate LL structures. The boxed portion in each I/R group is expanded, showing representative LL structures. E and F, TEM analysis of intact HL1 cells (E) or isolated rat hearts (F) following I/R-induced injury in the absence or presence of the PKCδ translocation inhibitor, ΔV1–1. Yellow arrows indicate the LL structures, red arrows indicate the peri-LL-associated mitochondria, and blue arrows the internalized mitochondria. G, TEM images of normoxic control rat hearts (left panel) or hearts subjected to 30 min of ischemia followed by 30 min of reperfusion in the presence of a control peptide (middle panel) or ΔV1–1 (right panel). Direct engulfment of mitochondrial material into a vacuolated structure is shown in the right panel. Yellow arrows indicate the I/R-induced appearance of vacuolated structures, in the vicinity of mitochondria following I/R injury. Red arrows indicate segregated portions of mitochondria engulfed by vacuolated structures.

FIGURE 3. Catalytically inactive GAPDH associates with mitochondria during I/R-induced injury. A, Western blot showing the enrichment of mitochondrial (Mito) and cytosolic (Cyto) fractions from HL1 cells using antibodies to the mitochondrial marker, Tom20, and cytosolic marker, enolase. B, HL1 cells were subjected to 2.5 h of ischemia followed by 30 min of reoxygenation for the indicated times. The mitochondrion- and cytosol-enriched fractions obtained by differential centrifugation were pooled and analyzed for GAPDH protein levels by Western blotting. C, the relative amount of mitochondrially associated GAPDH present was calculated using ImageJ software. D, measurement of GAPDH activity in total extract (n = 3). There was no significant increase in GAPDH enzymatic activity. E, GAPDH Western blots of mitochondrial- and cytosol-enriched fractions from HL1 cells incubated with DMSO (control), antimycin A (Ant A), or H2O2 for 30 min. F, quantification of Western blots shown in E (n = 3 per group, *p < 0.05 versus control group). G, Western blot showing the enrichment of mitochondrial (Mito) and cytosolic (Cyto) fractions from isolated rat hearts using antibodies to the mitochondrial marker, VDAC, and cytosolic marker, enolase. H, isolated rat hearts were subjected to normoxia or 30 min of ischemia, followed by 30 min of reoxygenation (I/R) ex vivo. The mitochondrion- and cytosol-enriched fractions were pooled and GAPDH protein levels were determined by Western blotting. Shown are analyses of three cardiac extracts per treatment. I, quantification of mitochondrial GAPDH translocation described in H (*, p < 0.05). J, measurement of GAPDH activity in the mitochondrial (Mito) and cytosolic (Cyto) fractions described in H. There was no detectable (nd) enzymatic activity in the mitochondrial fraction (n = 6 per group).
there was no significant increase in GAPDH enzymatic activity (Fig. 3D). We also observed an increase in GAPDH protein accumulating in the mitochondrially enriched fraction from isolated rat hearts subjected to I/R-induced injury without an increase in GAPDH enzymatic activity in the mitochondrial fraction (Fig. 3, H–J). These data suggest that I/R-induced injury promotes the mitochondrial accumulation of GAPDH and that the catalytic activity of the enzyme is not required for its mitochondrial role.

Exogenously Expressed iGAPDH Associates with Mitochondria and Induces the Formation of LL Structures—To determine whether indeed catalytically inactive GAPDH is involved in the formation of LL structures for mitochondrial elimination, we expressed both catalytically active and inactive GAPDH tagged with V5 sequence (GAPDH-V5 and iGAPDH-V5) in HL1 cells. We observed association of iGAPDH with the mitochondrial fraction under normoxic conditions; mitochondrial association of iGAPDH was much greater as compared with active GAPDH (Fig. 4, A and B). Because catalytically inactive GAPDH preferentially associates with mitochondria under basal conditions, we further characterized the morphology of cells expressing iGAPDH by immunofluorescence.

We observed that iGAPDH expression under normoxic conditions was sufficient to promote the formation of large vacuolated structures, some of which contained Tom20+ material (Fig. 4C), suggestive of mitochondrial uptake. These organelles in iGAPDH-V5-expressing cells were also positive for a marker labeling acidic compartments, LysoTracker, and for a lyso-
Mitochondrially Associated GAPDH Is Phosphorylated by PKCδ during I/R-induced Injury—Because both PKCδ and GAPDH associate with the mitochondria following I/R, we next determined whether the mitochondrially associated GAPDH is affected by PKCδ activity. We observed that I/R-induced injury promoted the phosphorylation of the mitochondrially associated iGAPDH in isolated rat hearts and HL1 cells, which was reduced in the presence of the PKCδ translocation inhibitor, δV1–1 (Fig. 5, A and B, upper blots). These results suggested that iGAPDH is phosphorylated following I/R, either directly or indirectly by PKCδ.

In vitro kinase assays using endogenous GAPDH immunoprecipitated from isolated rat hearts (Fig. 5C), or purified GAPDH-GST (Fig. 5D) confirmed that GAPDH is directly phosphorylated by PKCδ, but not by PKCe (Fig. 5, C and D), a related PKC isozyme that is also activated in the myocardium during I/R-induced injury (21). Phosphopeptide mapping of the products of the kinase assays showed that PKCδ phosphorylates GAPDH at Thr-246 alone (peptide 2) and GAPDH phosphorylated at Ser-241 only (peptide 3). Peptide 2 (Thr-246 alone) comprised over 70% of the entire phosphorylated material detected (Fig. 5E). Importantly, Thr-246 (SVVDLT246CRLE) is within the consensus sequence for PKC substrates and is evolutionary conserved in GAPDH from a variety of species, including human, rat, and mouse (data not shown). Collectively, these results suggest that PKCδ translocates to mitochondria following I/R and phosphorylates GAPDH, predominantly at Thr-246.

Inhibition of PKCδ-mediated iGAPDH Phosphorylation during I/R Promotes Mitochondrial Uptake into LL Structures, Which Coincides with Reduced Mitochondrial Mass—To determine the effects of GAPDH phosphorylation on GAPDH-driven mitophagy during I/R-induced injury, we mutated threonine at position 246 of catalytically inactive human iGAPDH-V5 (WT iGAPDH-V5) to an alanine (T246A iGAPDH-V5) and expressed both proteins in HL1 cells.

Using confocal microscopy, we found that expression of WT iGAPDH-V5 (Fig. 6A, cell outlined in dashed line) or T246A iGAPDH-V5 (Fig. 6B, cell outlined in dashed line) under normoxic conditions promoted the appearance of large LL structures, which were surrounded by Tom20+ mitochondria and varied in size and number per cell (Fig. 6, A and B, arrows). Furthermore, these vacuolated structures also contained Tom20+ material inside their lumen, suggestive of mitochondrial uptake (Fig. 6, A and B, arrowheads). Following I/R, Tom20+ staining increased inside the LL structures of T246A iGAPDH-V5 expressing cells, but not in WT iGAPDH-V5 expressing cells, suggestive of a reduced mitochondrial mass and increased mitophagy.

somomal and late endosomal marker, Rab9 (Fig. 4, D and E), suggesting that these vacuolated structures are LL structures that we observed in HL1 cells during I/R-induced injury. Therefore, these results suggest that exogenous iGAPDH expression is sufficient to promote the formation of LL structures under normoxic conditions. Furthermore, iGAPDH expression coincides with the recruitment of mitochondria into these structures.
expressing cells (Fig. 6, C and D, respectively). These results suggest that increased phosphorylation of the mitochondrially associated iGAPDH-V5 during I/R prevents the uptake of mitochondria into LL structures.

We hence reasoned that the I/R-induced recruitment and uptake of mitochondria into LL structures act as a mechanism to rapidly eliminate damaged mitochondria during I/R. Indeed, the mitochondrial mass in HL1 cells expressing the phosphorylation-defective T246A iGAPDH-V5 mutant during I/R-induced injury was lower relative to WT iGAPDH-V5 and T246A iGAPDH-V5 and subjected to normoxic (N) or I/R injury. Blots were stripped and re-probed for enolase. Quantification of Grp75 levels in three independent experiments to determine mitochondrial mass is provided (expressed as a ratio of Grp75: enolase, *, p < 0.05). F, Western blotting for cytochrome c levels in mitochondrion (Mito) and cytosol (Cyt)-enriched fractions isolated from control vector-transfected HL1 cells (C) or HL1 cells transiently expressing WT iGAPDH-V5 or T246A iGAPDH-V5 and subjected to normoxic (N) or I/R injury. Quantification of cytosolic cytochrome c levels in three independent experiments is provided. Significant differences (p < 0.05) to normoxic control group (*), I/R control group (#), and I/R WT iGAPDH-V5 group (†).

FIGURE 6. Expression of phosphorylation defective iGAPDH (T246A) during I/R promotes mitochondrial uptake into LL structures, which coincides with reduced mitochondrial mass. A–D, HL1 cells transiently expressing WT iGAPDH-V5 and T246A iGAPDH-V5 were maintained under normoxic (N) conditions or subjected to I/R injury. Cells were fixed and stained with anti-V5 (red channel) and anti-Tom20 (green channel) antibodies. Confocal images were acquired at ×100 magnification. Arrows indicate the presence of LL structures in a GAPDH-V5-expressing cell (outlined with a dashed line). Arrowheads in the expanded images indicate the presence of Tom20 material inside the expanded LL structures. E, representative anti-Grp75 Western blot of total cell lysates prepared from control HL1 cells or HL1 cells transiently expressing WT iGAPDH-V5 and T246A iGAPDH-V5 and subjected to normoxic (N) or I/R injury. Blots were stripped and re-probed for enolase. Quantification of Grp75 levels in three independent experiments to determine mitochondrial mass is provided (expressed as a ratio of Grp75: enolase, *, p < 0.05). F, Western blotting for cytochrome c levels in mitochondrion (Mito)- and cytosol (Cyt)-enriched fractions isolated from control vector-transfected HL1 cells (C) or HL1 cells transiently expressing WT iGAPDH-V5 or T246A iGAPDH-V5 and subjected to normoxic (N) or I/R injury. Quantification of cytosolic cytochrome c levels in three independent experiments is provided. Significant differences (p < 0.05) to normoxic control group (*), I/R control group (#), and I/R WT iGAPDH-V5 group (†).
arrowheads). However, in HL1 cells that transiently express iGAPDH-V5, inhibition of macroautophagy with 3MA did not prevent the formation of LL structures (Fig. 7, A, C, and D, indicated by white arrows); the vacuolated structures contained the late endosome and lysosomal markers, Rab9 and LysoTracker, within their lumen. These results suggest that iGAPDH promotes a redistribution of the lysosomal system into distinct degradation centers in the cell and does so inde-
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...dependently of macroautophagy. Furthermore, the presence of Tom20+ material within LL structures of 3MA-treated iGAPDH expressing cells suggests that mitochondria are recruited to these structures independently of macroautophagy (Fig. 7C). To further examine a potential role for macroautophagy in GAPDH-mediated mitophagy, we also used macroautophagy-deficient, Atg5−/− MEF cells (33). I/R-induced injury in these macroautophagy-deficient cells also promoted a time-dependent translocation of inactivated and endogenous GAPDH to mitochondria (Fig. 7F). Furthermore, as in HL1 cells, iGAPDH preferentially associated with the mitochondrial fraction (Fig. 7G) and the phosphorylation-defective T246A iGAPDH-V5 mutant showed increased mitochondrial elimination and reduced cytochrome c release relative to WT iGAPDH-V5 under I/R-induced injury (Fig. 7, H and I). These data suggest that GAPDH-mediated mitophagy is independent of the macroautophagy process. In support of this, we observed by TEM many instances of mitochondria being directly engulfed by LL structures in T246A iGAPDH-V5 expressing Atg5−/− MEF cells during I/R but not in WT iGAPDH-V5 Atg5−/− MEF cells (Fig. 7, J and K).

DISCUSSION

In this study, we identified a rapidly mounted mitophagy process that is mediated by mitochondrial association of GAPDH but is negatively regulated by PKCζ during I/R-induced injury (see the proposed model in Fig. 8). We show that mitochondrial uptake by LL structures is regulated by the association of the mitochondria with mitochondrial injuries during I/R-induced injury. This, in turn, mediates direct recruitment and uptake of damaged mitochondria into LL structures. We also show that mitochondrial associated GAPDH is a direct substrate for PKCζ, a known mediator of mitochondrial dysfunction and cell death in cardiomyocytes (19, 20, 23, 24). As I/R-induced injury increases, PKCζ translocation to mitochondria may reach a critical threshold, where it phosphorylates enough mitochondrial GAPDH at Thr-246 to inhibit GAPDH-driven mitophagy and instead, promotes the peri-LL accumulation of damaged mitochondria, the cytosolic release of cytochrome c from these mitochondria, and the onset of mitochondrion-mediated cell death by apoptosis and likely also by necrosis.

PKCζ-mediated phosphorylation of GAPDH mostly occurred at Thr-246, with a smaller amount of phosphorylation occurring at Ser-241. It is possible that either site or both can be phosphorylated also by other kinases such as casein kinase II or Ca2+/calmodulin-dependent protein kinase II (34, 35), although the exact amino acid modified by them is unknown. Nonetheless, Thr-246 substituted by alanine (T246A) was sufficient to abolish PKCζ-mediated inhibition of mitophagy during I/R-induced injury, which coincided with a reduction in mitochondrial mass. These results suggest that GAPDH phosphorylation at Thr-246 by PKCζ is a critical regulator of GAPDH-driven mitophagy by LL structures. Phosphorylation at Thr-246 by other kinases may propagate other signaling cascades. Additionally, we noticed that the catalytic activity of GAPDH is not required for mitochondrial elimination by mitophagy following I/R.

To date, several molecular machineries have been identified for the elimination of mitochondria, including autophagy-related 32 (Atg32) in yeast, and NIP1-like protein X (NIX) during red blood cell differentiation, unc51-like kinase (ULK1), and p62/SQSTM1-ubiquitinated protein complexes in mammalian cells (6, 7, 36, 37). Phosphatase and tensin homolog-induced putative kinase protein 1 (PINK1) and Parkin have been found to be involved in mitophagy as well (6, 38). In this model, VDACs and mitofusin 2 (Mfn2) were very recently identified as mitochondrial docking sites for recruitment of Parkin from cytosol to mitochondria (39, 40). Although most of these studies focus on macroautophagy, our studies suggest that GAPDH-mediated mitophagy occurs by what appears to be a micromitophagy process and is independent of macroautophagy. This is consistent with recent studies showing that photodamage-induced mitophagy in hepatocytes can occur in the presence of 3MA, suggesting that mitochondrial elimination in mammalian cells can also occur independently of macroautophagy (14, 15).

GAPDH-mediated mitophagy described here was initiated by oxidative stress during I/R-induced injury. Under oxidative stress during I/R-induced injury, GAPDH induces an increase in mitochondrial mass and mitophagy. The mechanism by which GAPDH induces mitophagy is not well understood, but it is known that GAPDH can interact with mitochondria and regulate mitochondrial function through a direct interaction with VDACs and Mfn2 (34, 35). It is possible that GAPDH-mediated mitophagy occurs by what appears to be a micromitophagy process and is independent of macroautophagy. This is consistent with recent studies showing that photodamage-induced mitophagy in hepatocytes can occur in the presence of 3MA, suggesting that mitochondrial elimination in mammalian cells can also occur independently of macroautophagy (14, 15).

FIGURE 7. GAPDH-induced mitophagy occurs independently of the macroautophagy pathway. A, HL1 cells maintained under normoxic conditions (left, top panel) or subjected to I/R-induced injury (right, top panel), I/R-induced injury in the presence 100 µM 3MA (left, bottom panel), or I/R-induced injury in the presence of 100 µM 3MA and the PKCζ translocation inhibitor, δV1–1 (right, bottom panel), were fixed and analyzed by immunofluorescence using a rabbit anti-LC3 antibody (green channel). Cells were counterstained with Hoechst (blue channel) to visualize nuclei. Yellow arrowheads indicate LC3+ autophagosomes and white arrows indicate the formation of LC3+ autophagosomes. Images were acquired at ×100 magnification, and the number of punctate LC3+ autophagosomes per cell was scored. Results are expressed as the mean ± S.D. (n = 40). C and D, HL1 cells transiently expressing iGAPDH-V5 or control vector (C) were incubated with 3MA for 4 h to inhibit macroautophagy. During the last 30 min, cells were labeled with LysoTracker Red, fixed, and analyzed by immunofluorescence with a deconvolution microscope using anti-Tom20 (A, green channel) or anti-Rab9 (B, green channel) antibodies. LL structures are indicated within white arrows. Images were acquired at ×100 magnification and represent maximum intensity projection of image stacks. The boxed area is expanded in the lower panels and representative LL structures are indicated by white arrows. E, Atg5 Western blot of Atg5+/− and Atg5−/− MEF cells. F, representative GAPDH Western blot of mitochondrial (Mito) and cytosol (Cyto) enriched fractions from Atg5−/− MEFs subjected to normoxia (N) or 2.5 h of ischemia, followed by 0, 5, or 30 min of reoxygenation (I/R). The blot was stripped and re-probed with anti-Tom20 and anti-enolase as indicators of mitochondrial and cytosolic enrichment, respectively. GAPDH activity in mitochondrial and cytosolic fractions was measured (n = 4 per group). There was no significant enzymatic activity of GAPDH in mitochondrial fractions. G, representative Western blot showing that inactive GAPDH (iGAPDH-V5) preferentially associates with mitochondria in Atg5−/− MEFs. H, Atg5+/− MEFs transiently expressing control vector (C), GAPDH-V5 (active), or iGAPDH-V5 (inactive) under normoxic conditions were enriched for mitochondria and cytosol and analyzed by Western blotting with anti-GAPDH. I, Atg5−/− MEFs transiently expressing control vector (C), iGAPDH-V5, or T246A iGAPDH-V5 were subjected to normoxia or I/R injury. Total cell extracts were analyzed by Western blotting with anti-Tom20. The blot was re-probed for enolase and V5. Quantification of the Western blots was provided. Significant differences (p < 0.05) to the normoxic control group (*) and WT I/R group (#) are indicated. L, Western blotting for cytochrome c levels in the mitochondrial- and cytosol-enriched fractions isolated from mock transfected Atg5−/− MEFs (C) or Atg5−/− MEFs transiently expressing WT iGAPDH-V5 or T246A iGAPDH-V5 and subjected to normoxia or I/R injury. Images were acquired at ×3,000 magnification. Red arrows indicate mitochondria accumulated around LL structures. Yellow arrows indicate perivacuole-associated mitochondria in the process of being directly engulfed, whereas blue arrows indicate mitochondria internalized into LL structures.

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stress conditions, many proteins can undergo oxidative modifications, which may further modulate their activities. It has been reported that oxidative stress by H₂O₂ induces tyrosine phosphorylation of PKC isozymes, including PKCδ/H9254, by tyrosine kinases such as Lck and Syk, thereby activating PKC (41–45). Additionally, oxidative stress results in translocation of PKCδ/H9254 to mitochondria where it promotes cytochrome c release and apoptosis (19, 46). GAPDH was also reported to be modified under oxidative stress conditions. Specifically, a cysteine residue in the active site of GAPDH was oxidized to cysteic acid, which makes this enzyme inactive (47, 48). Although the precise mechanism by which GAPDH is translocated to mitochondria has not yet been determined, oxidative stress in I/R potentially regulates the onset of GAPDH and PKCδ/H9254-mediated mitophagy.

How mitochondrially associated GAPDH promotes the formation of LL structures and the uptake of mitochondria into these structures still needs to be investigated. However, based on previous studies, it is possible that GAPDH can act as a fusogen, promoting the association and fusion of negatively charged phospholipids, in particular plasmenylethanolamine and phosphatidylycerine (PS) (49, 50). Although PS is present in all organelle membrane, it is likely to confer a negative charge to organelles with a single limiting membrane, such as plasma membrane, endosomes, and lysosomes, because PS present in mitochondria, Golgi, and ER is confined to their luminal leaflets (51). It is therefore possible that GAPDH may promote fusion of the outer mitochondrial membrane with the LL membrane, thereby facilitating direct uptake of damaged mitochondria during I/R-induced injury. In support of this possibility, TEM analysis in isolated rat hearts subjected to I/R injury shows the presence of mitochondria, with their outer membrane directly associated and in some cases, fused with LL membranes. Another possibility is that GAPDH-induced mitophagy under oxidative stress conditions may depend on chaperone-mediated autophagy and Hsc70. GAPDH contains a KFERQ motif that is selectively recognized by this chaperone heat shock cognate protein (52). This interaction targets the complex to the lysosomal membrane, where it binds to the LAMP-2 originated LL structures, which acts as a receptor for this pathway (53).

What is the relative contribution and importance of mitochondrial clearance by GAPDH-driven mitophagy and macroautophagy in cardiac cell survival after I/R-induced injury? Cell-based and animal-based studies have shown that macroautophagy is down-regulated in cardiomyocytes during I/R-induced injury and that pharmacological up-regulation of this process ensures maximal recovery from cardiac I/R-induced injury (28). Therefore, it appears that cardiomyocytes deploy two complimentary autophagy responses to cull damaged mitochondria and both of these are attenuated during I/R-induced injury. We show that a GAPDH-driven mitophagy response is rapidly blunted by PKCδ activation and phosphorylation of GAPDH. A
macroautophagy response can also be deployed to remove damaged mitochondria, along with other cytosolic components in cardiomyocytes. However, like GAPDH-driven mitophagy, macroautophagy is rapidly inactivated upon I/R-induced injury in cardiomyocytes (25). Therefore, pharmacological up-regulation of GAPDH-driven mitophagy (e.g. by inhibiting PKCδ translocation to mitochondria) or macroautophagy (e.g. with rapamycin or chloramphenicol succinate (25, 54)) would promote the clearance of damaged mitochondria and therefore prevent the onset of mitochondrialy mediated cell death following I/R. Altogether, these two independent autophagy systems could contribute to the removal of damaged mitochondria and the preservation of a healthy mitochondrial population. In summary, we report that mitochondrialy associated, inactive GAPDH promotes cell protective mitophagy by LI structures in cardiomyocytes. This is a tightly regulated process, which can be readily switched to mitochondrialy mediated cell death by PKCδ-mediated phosphorylation of mitochondrially associated, inactive GAPDH and inhibition of the cytotoxicative mechanism of damaged mitochondrialy elimination by mitophagy (Fig. 8).

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REFERENCES

1. Prasad, A., Stone, G. W., Holmes, D. R., and Gersh, B. (2009) Reperfusion injury, microvascular dysfunction, and cardioprotection. The “dark side” of reperfusion. Circulation 120, 2105–2112

2. Honda, H. M., and Ping, P. (2006) Mitochondrial permeability transition in cardiac cell injury and death. Cardiovasc. Drugs Ther. 20, 425–432

3. Youle, R. J., and Narendra, D. P. (2011) Mechanisms of mitophagy. Nat. Rev. Mol. Cell Biol. 12, 9–14

4. Klionsky, D. J. (2007) Autophagy. From phenomenology to molecular understanding in less than a decade. Nat. Rev. Mol. Cell Biol. 8, 931–937

5. Rabinowitz, J. D., and White, E. (2010) Autophagy and metabolism. Science 330, 1344–1348

6. Wild, P., and Dikic, I. (2010) Mitochondria get a Parkin’ ticket. Nat. Cell Biol. 12, 104–106

7. Kundu, M., Lindsten, T., Yang, C. Y., Wu, J., Zhao, F., Zhang, J., Selak, M. A., Ney, P. A., and Thompson, C. B. (2008) Ulk1 plays a critical role in mammalian cells. Revisiting a 40-year-old conundrum. Autophagy 4, 333–345

8. Cuervo, A. M., and Dice, J. F. (2000) Regulation of lamp2a levels in the lysosomal membrane. J. Biol. Chem. 275, 5802–5806

9. Sahu, R., Kaushik, S., Clement, C. C., Cannizzo, E. S., Scharf, B., Follenzi, A., Potolicchio, I., Nieves, E., Cuervo, A. M., and Santambrogio, L. (2011) Microautophagy of cytosolic proteins by late endosomes. Proc. Natl. Acad. Sci. U.S.A. 108, 18394–18400

10. Hart, H., Hoischen, A., and Rehmann, S. (2008) Autophagy in neuronal disease. Annu. Rev. Neurosci. 31, 99–123

11. Andjelkovic, M. A., and Klionsky, D. J. (2004) Selective autophagy. Curr. Opin. Cell Biol. 16, 53–59

12. Cuervo, A. M., and Dice, J. F. (2003) Molecular mechanisms of selective autophagy. Annu. Rev. Biochem. 72, 343–370

13. Cuervo, A. M., and Dice, J. F. (2000) Regulation of lamp2a levels in the lysosomal membrane. J. Biol. Chem. 275, 5802–5806

14. Kim, I., and Lemasters, J. J. (2011) Mitophagy selectively degrades individual damaged mitochondria after photoirradiation. Antioxid. Redox Signal. 15, 1919–1928

15. Kim, I., Rodriguez-Enriquez, S., and Lemasters, J. J. (2007) Selective degradation of mitochondria by mitophagy. Arch. Biochem. Biophys. 462, 245–253

16. Gottlieb, R. A., Mentzer, R. M., Jr., and Linton, P. J. (2011) Impaired mitophagy at the heart of injury. Autophagy 7, 1573–1574

17. Huang, C., Andres, A. M., Ratliff, E. P., Hernandez, G., Lee, P., and Gottlieb, R. A. (2011) Preconditioning involves selective mitophagy mediated by Parkin and p62/SQSTM1. PLoS One 6, e20975

18. Dorn, G. W., 2nd. (2010) Mitochondrial pruning by Nix and BNip3. An essential function for cardiac-expressed death factors. J. Cardiovasc. Transl. Res. 3, 374–383

19. Churchill, E. N., Murril, C. L., Chen, C. H., Mochly-Rosen, D., and Szewda, L. I. (2005) Reperfusion-induced translocation of PKCδ to cardiac mitochondria prevents pyruvate dehydrogenase reactivation. Circ. Res. 97, 85–95

20. Inagaki, K., Chen, L., Ikono, F., Lee, F. H., Imahashi, K., Bouley, D. M., Rezae, M., Yock, P. G., Murphy, E., and Mochly-Rosen, D. (2003) Inhibition of protein kinase Cδ protects against reperfusion injury of the ischemic heart in vivo. Circulation 108, 2304–2307

21. Chen, L., Hahn, H., Wu, G., Chen, C. H., Liron, T., Schechtman, D., Cavallaro, G., Banci, L., Guo, Y., Bolli, R., Dorn, G. W., 2nd, and Mochly-Rosen, D. (2001) Opposing cardioprotective actions and parallel hyperthermic effects of PKCδ and PKCe. Proc. Natl. Acad. Sci. U.S.A. 98, 11114–11119

22. Churchill, E. N., and Szewda, L. I. (2005) Translocation of PKCδ to mitochondria during cardiac reperfusion enhances superoxide anion production and induces loss in mitochondrial function. Arch. Biochem. Biophys. 439, 194–199

23. Murriel, C. L., Churchill, E., Inagaki, K., Szewda, L. I., and Mochly-Rosen, D. (2004) Protein kinase Cδ activation induces apoptosis in response to cardiac ischemia and reperfusion damage. A mechanism involving BAD and the mitochondria. J. Biol. Chem. 279, 47985–47991

24. Ikono, F., Inagaki, K., Rezae, M., and Mochly-Rosen, D. (2007) Impaired perfusion after myocardial infarction is due to reperfusion-induced PKCδ-mediated myocardial damage. Cardiovasc. Res. 73, 699–709

25. Hamacher-Brady, A., Brady, N. R., and Gottlieb, R. A. (2006) Enhancing macroautophagy protects against ischemia/reperfusion injury in cardiac myocytes. J. Biol. Chem. 281, 29776–29787

26. Qvit, N., and Mochly-Rosen, D. (2010) Highly specific modulators of protein kinase C localization. Applications to heart failure. Drug Discov. Today Dis. Mech. 7, e87–e93

27. Colell, A., Ricci, J. E., Tait, S., Milasta, S., Maurer, U., Boucher-Hayes, L., Fitzgerald, P., Guio-Carrion, A., Waterhouse, N. J., Li, C. W., Mari, B., Barbry, P., Newmeyer, D. D., Beere, H. M., and Green, D. R. (2007) GAPDH and autophagy preserve survival after apoptotic cytochrome c release in the absence of caspase activation. Cell 129, 983–997

28. Gottlieb, R. A., and Carreia, R. S. (2010) Autophagy in health and disease. S. Mitophagy as a way of life. Am. J. Physiol. Cell Physiol. 299, C203–210

29. Cuervo, A. M., and Dice, J. F. (2000) Regulation of lamp2a levels in the lysosomal membrane. Traffic 1, 570–583

30. Cuervo, A. M., Mann, L., Bonten, E. J., d’Azzo, A., and Dice, J. F. (2003) Cathepsin A regulates chaperone-mediated autophagy through cleavage of the lysosomal receptor. EMBO J. 22, 47–59

31. Sahu, R., Kaushik, S., Clement, C. C., Cannizzo, E. S., Scharf, B., Follenzi, A., Potolicchio, I., Nieves, E., Cuervo, A. M., and Santambrogio, L. (2011) Microautophagy of cytosolic proteins by late endosomes. Dev. Cell 20, 131–139

32. Tristan, C., Shahani, N., Sedlak, T. W., and Sawa, A. (2011) The diverse functions of GAPDH. Views from different subcellular compartments. Cell. Signal. 23, 317–323

33. Hosokawa, N., Harai, Y., and Mizushima, N. (2006) Generation of cell lines with tetracycline-regulated autophagy and a role for autophagy in controlling cell size. FEBS Lett. 580, 2623–2629

34. Gao, Y., and Wang, H. Y. (2006) Casein kinase 2 is activated and essential for Wnt/β-catenin signaling. J. Biol. Chem. 281, 18394–18400
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35. Ashmarina, L. I., Louzenko, S. E., Severin, S. E., Jr., Muronetz, V. I., and Nagradova, N. K. (1988) Phosphorylation of d-glyceraldehyde-3-phosphate dehydrogenase by Ca²⁺/calmodulin-dependent protein kinase II. FEBS Lett. 231, 413–416
36. Okamoto, K., Kondo-Okamoto, N., and Ohsumi, Y. (2009) A landmark protein essential for mitophagy. Autophagy 5, 1203–1205
37. Narendra, D., Kane, L. A., Hauser, D. N., Fearnley, I. M., and Youle, R. J. (2010) p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy. VDAC1 is dispensable for both. Autophagy 6, 1090–1106
38. Sun, Y., Vashisht, A. A., Tchieu, J., Wohlschlegel, J. A., and Dreier, L. J. (2013) PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. Science 340, 471–475
39. Scheif, L. S., Kirihara, J. M., Burg, D. L., Geahlen, R. L., and Ledbetter, J. A. (1993) p72tyk2 tyrosine kinase is activated by oxidizing conditions that induce lymphocyte tyrosine phosphorylation and Ca²⁺ signals. J. Biol. Chem. 268, 16688–16692
40. Qin, S., Inazu, T., and Yamamura, H. (1995) Activation and tyrosine phosphorylation of p72tyk2 as well as calcium mobilization after hydrogen peroxide stimulation in peripheral blood lymphocytes. Biochem. J. 308, 347–352
41. Hardwick, J. S., and Sefton, B. M. (1995) Activation of the Lck tyrosine protein kinase by hydrogen peroxide requires the phosphorylation of Tyr394. Proc. Natl. Acad. Sci. U.S.A. 92, 4527–4531
42. Gopalakrishna, R., and Anderson, W. B. (1989) Ca²⁺- and phospholipid-independent activation of protein kinase C by selective oxidative modification of the regulatory domain. Proc. Natl. Acad. Sci. U.S.A. 86, 6758–6762
43. Konishi, H., Tanaka, M., Takemura, Y., Matsuzaki, H., Ono, Y., Kikkawa, U., and Nishizuka, Y. (1997) Activation of protein kinase C by tyrosine phosphorylation in response to H₂O₂. Proc. Natl. Acad. Sci. U.S.A. 94, 11233–11237
44. Majumder, P. K., Pandey, P., Sun, X., Cheng, K., Datta, R., Saxena, S., Kharbanda, S., and Kufe, D. (2000) Mitochondrial translocation of protein kinase Cθ in phorbol ester-induced cytochrome c release and apoptosis. J. Biol. Chem. 275, 21793–21796
45. Butterfield, D. A., Hardas, S. S., and Lange, M. L. (2010) Oxidatively modified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Alzheimer’s disease. Many pathways to neurodegeneration. J. Alzheimers Dis. 20, 369–393
46. Glaser, P. E., and Gross, R. W. (1995) Rapid plasmeneylethanolamine-selective fusion of membrane bilayers catalyzed by an isoform of glyceroldehyde-3-phosphate dehydrogenase. Discrimination between glycolytic and fusogenic roles of individual isoforms. Biochemistry 34, 12193–12203
47. Yeung, T., Gilbert, G. E., Shi, J., Silivius, J., Kapus, A., and Grinstein, S. (2008) Membrane phosphatidylserine regulates surface charge and protein localization. Science 319, 210–213
48. Cuervo, A. M., Tocilescu, M. A., Liu, W., Ko, H. S., Magrané, J., Moore, D. J., Dawson, V. L., Grailhe, R., Dawson, T. M., Li, C., Tieu, K., and Przedborski, S. (2010) p62/SQSTM1 is required for Parkin-induced mitochondrial clumping but not mitophagy. VDAC1 is dispensable for both. Autophagy 6, 1090–1106
49. Sun, Y., Vashisht, A. A., Tchieu, J., Wohlschlegel, J. A., and Dreier, L. J. (2013) PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. Science 340, 471–475
50. Yeung, T., Gilbert, G. E., Shi, J., Silivius, J., Kapus, A., and Grinstein, S. (2008) Membrane phosphatidylserine regulates surface charge and protein localization. Science 319, 210–213
51. Cuervo, A. M., Tocilescu, M. A., Liu, W., Ko, H. S., Magrané, J., Moore, D. J., Dawson, V. L., Grailhe, R., Dawson, T. M., Li, C., Tieu, K., and Przedborski, S. (2010) p62/SQSTM1 is required for Parkin-induced mitochondrial clumping but not mitophagy. VDAC1 is dispensable for both. Autophagy 6, 1090–1106
52. Cuervo, A. M., and Dice, J. F. (1996) A receptor for the selective uptake and degradation of proteins by lysosomes. Science 273, 26374–26380
53. Cuervo, A. M., and Dice, J. F. (1996) A receptor for the selective uptake and degradation of proteins by lysosomes. Science 273, 26374–26380
54. Sala-Mercado, J. A., Wider, J., Undyala, V. V., Jahania, S., Yoo, W., Men- tzer, R. M., Jr., Gottlieb, R. A., and Przyklenk, K. (2010) Profound cardio- protection with chloramphenicol succinate in the swine model of myocardial ischemia-reperfusion injury. Circulation 122, S179–184
55. Cuervo, A. M., and Dice, J. F. (1996) A receptor for the selective uptake and degradation of proteins by lysosomes. Science 273, 26374–26380
56. Sala-Mercado, J. A., Wider, J., Undyala, V. V., Jahania, S., Yoo, W., Men- tzer, R. M., Jr., Gottlieb, R. A., and Przyklenk, K. (2010) Profound cardio- protection with chloramphenicol succinate in the swine model of myocardial ischemia-reperfusion injury. Circulation 122, S179–184