The heart is a tissue rich in mitochondria with ≈30% of cardiomyocyte volume occupied by these ATP-generating organelles.\(^1\) The large mitochondrial mass also orchestrates a myriad of signaling pathways related to metabolite generation, stress response, and cell death. Thus, it is not surprising that mitochondrial dysfunction is implicated in numerous cardiovascular-related diseases,\(^2\) making mitochondria attractive therapeutic targets.

As hubs of cellular signaling cascades, mitochondria participate in a diverse array of pathways that are clinically relevant. For example, the contribution of mitochondria to intracellular Ca\(^{2+}\) is necessary for proper myocardial contraction and relaxation.\(^3\) In addition, mitochondria are master regulators of cell life versus death decisions. Opening of the mitochondrial permeability transition pore (mPTP) and the subsequent collapse of the proton gradient and matrix swelling lead to the release of proapoptotic proteins. Such opening of the mPTP is implicated during ischemia/reperfusion (IR) injury.\(^4\) Finally, not only are mitochondria major producers of reactive oxygen species (ROS) but also are the main target of ROS-induced signaling. In this review, we focus on mitochondria and ROS-related mechanisms as novel targets for therapeutics in cardiovascular diseases.

**ROS as a Clinical Target**

ROS are a class of reactive molecules that were originally ascribed a pathological role in the cell. In addition to increasing the oxidative environment and thus irreversibly modifying organelles and macromolecules, ROS also trigger important signaling events. Members of this class include the hydroxyl...
radical (HO•), peroxynitrite (OONO−), superoxide (O2−), hydrogen peroxide (H2O2), and others. ROS accumulate both inside and outside of the cell. For example, ROS are generated in the cytoplasm and the plasma membrane by nitric oxide. Adenine dinucleotide phosphate oxidases and by xanthine oxidase, as well as uncoupled nitric oxide synthase in the cytoplasm. In addition, monocytes and neutrophils use myeloperoxidase to produce ROS, which on release can affect neighboring cells from the outside. All of these ROS production sites have implications in cardiovascular disease. Interested readers are directed to the review of Sugamura and Keaney. However, our discussion focuses only on ROS generated by the mitochondria.

Around 0.1% to 0.2% of oxygen consumed is associated with leakage of electrons and production of ROS. At least 10 sites of mitochondrial ROS production that are not only limited to the electron transport chain have been identified using isolated mitochondria from rat skeletal muscle. These include complex I, complex II, complex III, 2-oxoglutarate, pyruvate dehydrogenase (PDH), branched-chain 2-oxoacid dehydrogenase, mitochondrial glycerol phosphate dehydrogenase, electron-transferring-flavoprotein dehydrogenase, and dihydroorotate dehydrogenase. The relative contribution of ROS by each of these sites was overestimated in in vitro studies, which masked the dynamic changes in contribution of each site under different cellular conditions. For example, although the mitochondrial complex I and the flavin site in complex II equally contribute to the majority of ROS production under basal conditions, the flavin site in complex I dominates ROS production under conditions that mimic aerobic exercise. The dynamic nature of ROS production at the 10 mitochondrial sites is yet to be investigated under various conditions mimicking cardiovascular disorders. An understanding of the sites that contribute the majority of ROS accumulation under pathological conditions would allow for a more directed therapeutic approach for targeting pathological ROS production.

Experiments demonstrating the damaging effects of ROS date back to the 1940s. The role of ROS has been since implicated in a variety of pathological processes and conditions, including aging, DNA mutagenesis, inflammation, and multiple cell death pathways. In 1956, Harman proposed the free radical theory, which suggested that ROS accumulate spontaneously and in response to the environment. Diseases associated with aging, such as many cardiovascular disorders, can be traced to the effects that these ROS have on normal cellular functions. Much support for these early studies has accumulated. One example is the use of knockout mice of manganese superoxide dismutase (SOD2); mice lacking SOD2, an enzyme that converts superoxide within the mitochondria to hydrogen peroxide, die within 10 days with dilated cardiomyopathy. It is therefore not surprising that multiple antioxidants have been tested in clinical trials to reduce the oxidative burden of cardiovascular diseases.

In 2011, Sugamura and Keaney summarized clinical trials for antioxidants in the context of cardiovascular disorders and concluded that targeting oxidative stress using ROS scavengers is an ineffective therapeutic strategy. Although ROS scavengers are effective at reducing cellular ROS levels, they are in general ineffective and sometimes harmful in the context of cardiovascular pathology. The Table provides an update of their summary. ROS scavengers, such as N-acetylcysteine (NAC), have mixed efficacy outcomes. For example, intravenous NAC before angioplasty and orally delivered NAC after the procedure reduced nephrotoxicity in patients with acute myocardial infarction (MI), whereas an intravenous NAC treatment was ineffective at reducing contrast-induced nephropathy in patients with acute coronary syndrome. NAC oral supplement given twice daily for 4 weeks increased forearm blood flow but did not affect patient outcome after both heart and renal failure. A promising effect for NAC in improving cardiovascular function has only been shown in the study involving 354 patients undergoing angioplasty after acute MI. A follow-up clinical study for the effect of NAC in patients undergoing angioplasty on adverse outcomes is currently recruiting patients.

In addition to NAC, the antioxidant l-carnitine (4-N-trimethylammonium-3-hydroxybutyric acid) has been tested in the clinic using biomarkers for oxidative stress and heart damage with promising results. In patients with coronary artery disease, l-carnitine reduced the levels of the toxic aldehyde, malondialdehyde, and increased the expression of antioxidant enzymes including catalase, glutathione peroxidase, and superoxide dismutase. In patients with non-ST-segment–elevation MI, l-carnitine reduced the release of both creatine-MB and troponin-I. However, the effect of l-carnitine on overall clinical outcome has not been determined. Other antioxidants such as α-lipoic acid (the level of which significantly declines with age) and melatonin (an antioxidant and a neurohormone produced by the pineal gland) are currently being tested in clinical trials for cardiovascular-related indications. Finally, other ROS scavengers that have been used in other indications such as NXY-059 (Cerovive, a hydrophilic free radical spin trap agent) in stroke are yet to be tested in the context of cardiovascular disorders.
Limitation of ROS scavengers is that they must be provided in stoichiometric (>1:1 ratio) levels relative to cellular ROS. In addition, ROS scavengers, such as NAC, are unstable; they can undergo auto-oxidation before treatment, effectively rendering them as oxidants. As a result, antioxidants may exert an opposite effect to the one intended. Finally, ROS also have physiological functions at lower amounts as regulators of autophagy, immunity, differentiation, and longevity (these pathways are described in detail by Sena and Chandel). For example, in the context of development, overexpression of the antioxidant GTPx-1 suppressed differentiation of multipotent hematopoietic progenitors in *Drosophila*. In addition, ROS activate signaling cascades that enable responses to stress conditions (more details are provided later in the review). In this review, we define lower levels of ROS involved in signaling pathways as physiological ROS and excessive levels of ROS that induce cell damage as pathological ROS. Therefore, targeting ROS elimination at a particular source, ie, the mitochondria, may overcome the limitations of generalized ROS scavengers. This is of particular importance in the context of cardiovascular disorders because mitochondria are a significant source of pathological ROS production in the heart and the sites in which ROS-activated signaling pathways converge.

### Targeting Defective Mitochondria to Prevent Production of Excessive ROS

Targeting ROS scavengers to the mitochondria eliminates some of the localization and stoichiometric difficulties that general ROS scavengers must overcome (Figure 1B). An example of such ROS scavenger is MitoQ. MitoQ is an ubiquinol with a
lipophilic triphenylphosphonium cation (TPP⁺) modification, which enables mitochondrial delivery of the compound. In a rat heart Langendorff model of IR damage, MitoQ protects against cardiac function loss, as well as tissue and mitochondrial damage.30 An additional TPP⁺-modified ROS scavenger, SkQ, increases the life span of male versus female mice.31 In addition to TPP⁺-modified ROS scavengers, SS31 (Bendavia), a 4 amino acid synthetic peptides (phenylalanine-d-arginine-phenylalanine-lysine), selectively reaches the mitochondrial inner membrane, scavenges ROS through a dimethyltyrosine group, reduces mitochondrial ROS production, and prevents mPTP opening.32 SS31 inhibits hydrophobic cytochrome c interactions with cardiolipin (1,3-bis(sn-3′-phosphatidyl)-sn-glycerol, a component of the inner mitochondrial membrane), freeing cytochrome c to function as an electron carrier.33 SS31 has a cardioprotective effect in response to IR damage in multiple animal models of MI and heart failure (HF), including in mice, rats, guinea pigs, rabbits, and sheep.34–36 Furthermore, SS31 selectively improves multiple functional parameters in mitochondria isolated from old versus young mouse skeletal muscle as measured in vivo.37 SS31 selectively improves multiple functional parameters in mitochondria isolated from old versus young mouse skeletal muscle as measured in vivo.37 In addition, SS31’s selective effect on old versus young mice suggests that SS31 may better target pathological ROS production that occurs in aged mitochondria. SS31 is currently in a phase Ia clinical trial for patients with ST-segment–elevation MI.38 Finally, mitochondria-targeted enzymatic antioxidants have cardioprotective effects as well. For example, overexpression of catalase specifically in the mitochondria (mCat) reduces mitochondrial oxidative damage, increases life span of mice, and protects against angiotensin II–induced cardiac hypertrophy, fibrosis, and HF.39,40 Antioxidants that directly target the mitochondria seem to be more efficacious than generalized ROS scavengers in animal models, yet their benefit in humans is unknown. Whereas mitochondria-targeted antioxidants solve the localization limitation of ROS scavengers, they may still reduce both pathological and physiological ROS. The electron transport machinery (complexes I–IV) and cytochrome c are depicted in the inner mitochondrial membrane/matrix interface. CL indicates cardiolipin.

Figure 1. Targeting reactive oxygen species (ROS) using scavengers. A, General ROS scavengers (purple symbol) target and reduce both pathological (red) and physiological (green) ROS. These ROS scavengers do not have localization specificity and must be administered at stoichiometric (at least 1:1) amounts. In addition, ROS scavengers may get oxidized (red symbol) and instead exert an opposite effect than intended. B, Mitochondria-targeted antioxidants (eg, SS31, MitoQ, SkQ, and mCat; see text for details) aim at ROS-producing sites. Whereas some, such as SS31 (orange symbol), do not have to be administered at stoichiometric doses; they may still reduce both pathological and pathological ROS. The electron transport machinery (complexes I–IV) and cytochrome c are depicted in the inner mitochondrial membrane/matrix interface. CL indicates cardiolipin.
Mitochondrial fission/fusion does not seem to occur in isolated cardiomyocytes to as great of an extent as in cultured cells. Nevertheless, an imbalance between fission and fusion is observed in multiple cardiovascular disorders as thoroughly reviewed by Dorn. One example of particular interest is the observation that optic atrophy 1 is downregulated in hearts from both humans with HF and a rat model of HF. In addition, mitochondria in HF are fragmented, suggesting the occurrence of excessive, pathological, and unopposed fission. Mitochondrial fragmentation is found in other pathologies as well, including pulmonary arterial hypertension, which is associated with both reduced mitofusin 2 expression and up-regulation of Drp1. Furthermore, excessive fragmentation occurs in other noncardiac disorders, such as cancer and in many neurodegenerative diseases. Therefore, fission and fusion proteins are attractive therapeutic targets. The ability to maintain a balanced fission/fusion cycle can prevent the accumulation of damaged and fragmented mitochondria and thus eliminate the production of pathological ROS (Figure 2A).

Recent work on pharmacological intervention of mitochondrial dynamics focused on the ability to inhibit mitochondrial fission though targeting Drp1. Cassidy-Stone et al screened for inhibitors of mitochondrial fission using a yeast fzo1-1 mutant that have excessive fragmentation and failure to grow on glycerol. From this screen, mdivi-1 was identified as an inhibitor of Drp1. Mdivi-1 treatment in mice undergoing IR reduces myocardial infarct size. In addition, mdivi-1 decreases cell death, normalizes mitochondrial membrane potential, and reduces excessive fragmentation in adult rat cardiomyocytes exposed to simulated IR injury. However, Drp1 may have multiple functions, depending on its interaction with each of its adaptor proteins, fission 1, mitochondrial fission factor, and mitochondrial dynamics protein of 49 and 51 kDa (Figure 2A), as well as other functions independent of mitochondrial fission; inhibition of all these functions may have undesired effects. Recent work on mdivi-1 in porcine embryos and primary cells showed that mdivi-1 reduces cell growth, blastocyst production, and mitochondrial membrane potential, while increasing ROS production. Whereas mdivi-1 inhibits excessive fission under stress conditions as with the IR model, mdivi-1 may also inhibit other functions of Drp1, including healthy physiological fission (Figure 2A). This is consistent with the observation that a Drp1 knockout interferes with mitochondrial clearance and is lethal. Therefore, a more specific inhibitor of Drp1, a so-called separation of function inhibitor, that is selective for pathological fission and spares physiological fission is needed.

To overcome the potential toxic effects of mdivi-1 through the complete inhibition of Drp1 function, our laboratory identified a small separation of function peptide inhibitor that selectively prevents recruitment of Drp1 to the mitochondria only under pathological conditions. The peptide, P110, hinders the interaction between Drp1 and fission 1 only and does not affect Drp1 interaction with mitochondrial fission factor and mitochondrial dynamics protein of 51 kDa. This separation of function inhibitor was derived using a rational approach that examines sequences of homology between fission 1 and Drp1. P110 treatment diminishes excessive mitochondrial fission and reduces neurotoxicity in cells derived from patients with Parkinson disease but does not affect the recruitment of Drp1 to the mitochondria under physiological conditions. It is therefore hypothesized that fission 1–mediated

Figure 2. Targeting defective mitochondria to prevent production of excessive reactive oxygen species (ROS). A. Mitochondrial fusion opposed by either basal or pathological fission including the relevant players. Fission inhibitors Mdivi-1 and P110 inhibit either dynamin-related protein 1 (Drp1) directly or Drp1 recruitment to the mitochondrial membrane, respectively. B. Mechanism of pathological fission inhibition by P110. Depicted are the 4 mitochondrial adaptors of Drp1 in mammalian cells including fission 1 (Fis1), mitochondrial fission factor (Mff), mitochondrial dynamics protein of 49 kDa and 51 kDa (Mid49 and Mid51). To illustrate the specific inhibition of Fis1-dependent pathological fission, the mitochondria at the bottom left and right show only Fis1 on the outer mitochondrial membrane. P110, a selective inhibitor of only Drp1 binding to Fis1, acts as a separation of function inhibitor, separating the Fis1-dependent function (likely pathological) from the other Drp1 functions (including physiological fission).
Drp1 recruitment to the mitochondrial membrane occurs under stress conditions, and that treatment with P110 selectively inhibits pathological fragmentation without interfering with normal Drp1 functions (Figure 2B). Relevant to this review, treatment with P110 decreases mitochondrial fragmentation, improves mitochondrial function, and reduces ROS production in 3 models of IR in rats, including primary cardiomyocytes, ex vivo heart model, and an in vivo MI model.66 In primary cardiomyocytes that were not exposed to IR stress, P110 does not affect ROS production and mitochondrial function or does it induce cell death. Importantly, P110 treatment reduced infarct size and increased ATP production in the MI in vivo model.66 Further, 8 weeks of P110 treatment shows no adverse effects in wild-type mice but improves behavior and reduced mortality in Huntington mice.67 Thus, targeting of mitochondrial fragmentation by selectively inhibiting pathological fission may be an effective strategy for reducing pathological ROS release and inducing a cardioprotective effect.

Another mitochondrial dynamics mechanism that prevents the production of pathological ROS occurs through a process of eliminating damaged mitochondria. Autophagy is a vital catabolic mechanism degrading unnecessary, excessive, or dysfunctional cellular components by the lysosome and recycling them to sustain cellular metabolism.68 Autophagy has long been thought to nonspecifically mediate the bulk degradation of cytosolic components in response to acute stress, such as energy deprivation, to meet energy demand and maintain homeostasis and viability.59,60 However, multiple lines of evidence suggest that autophagy selectively mediates removal of specific targets. Under physiological conditions (in which mitochondrial ROS is balanced with the antioxidant capacity), autophagy provides limited mitochondrial pruning, thus promoting mitochondrial quality control to meet the cell’s energy requirement.61 When exposed to cellular insults, such as oxidative stress caused by IR, mitochondrial pruning through autophagy (mitophagy) is upregulated by diverse molecular machinery.62,63

Depending on the way mitochondria are delivered to lysosomes, 2 distinct molecular pathways contribute to mitochondrial elimination: macro- and micromitophagy. Macromitophagy, the better-studied form of mitophagy, is characterized by encapsulation of mitochondria into unique, double-membrane vesicles known as autophagosomes (Figure 3A). The subsequent fusion of autophagosomes with lysosomes leads to degradation of their content.69 Several molecular pathways regulate the formation of the autophagosome and lead to mitochondrial clearance. Beclin 1 (mammalian ortholog of the yeast autophagy-related gene, Atg 6, and BEC-1 in Caenorhabditis elegans) is involved in initiation of autophagosome formation, which is primed by unc-51 like autophagy activating kinase 1 and activating molecule in beclin 1-regulated autophagy.64–66 A group of Atg proteins and microtubule-associated protein 1 light chain (LC3) then promote elongation and maturation of the autophagosome.67 Damaged and depolarized mitochondria, previously segregated from healthy mitochondria by Drp1-mediated fission (a physiological role of Drp1), are recognized and flagged by sensing molecules, including phosphatase and tensin homolog-induced putative kinase 1 (PINK1) (Figure 3B). PINK1, together with Parkin (a component of an E3 ubiquitin ligase complex)– and NIP1-like protein X (NIX)–dependent macromitophagy, results in Parkin-mediated ubiquitination of Mfn, leading to recruitment of the mitofusin–interacting protein Miro, facilitating recruitment of the mitofusin–interacting protein Miro, facilitating autophagic clearance of damaged mitochondria.68,69

![Figure 3. Targeting damaged mitochondrial clearance to prevent production of excessive reactive oxygen species (ROS).](image-url)

A, Parkin (a component of an E3 ubiquitin ligase complex)– and NIP1-like protein X (NIX)–dependent macromitophagy. B, GAPDH– dependent micromitophagy (see text for details). GABARAP indicates γ-aminobutyric acid receptor–associated protein; LC3, microtubule-associated protein 1 light chain; Miro, mitochondrial membrane ρ GTPase protein–mediating mitochondrial motility; Mfn, mitofusin; NIX, NIP1-like protein X; and PINK1, PTEN-induced putative kinase 1.
kinase protein 1, PTEN-induced putative kinase 1 (PINK1), Parkin (a component of an E3 ubiquitin ligase complex), and NIP1-like protein X (NIX, a proapoptotic protein).56,69 PINK1, initially implicated in the pathogenesis of Parkinson disease, is a molecular sensor to induce mitophagy in heart.61,72 On depolarization, PINK1 accumulates on the OMM and recruits the cytosolic E3 ubiquitin ligase Parkin. Parkin recruitment leads to increased ubiquitination of mitochondrial proteins, which induces removal of impaired mitochondria.59,60,71 Although the exact process has not been clearly elucidated, several molecular factors promoting mitophagy have been discovered. In addition to Parkin, several other substrates of PINK1 have recently been identified. For example, mitofusin 2 phosphorylation by PINK1 mediates Parkin recruitment to damaged mitochondria in mouse cardiac myocytes, suggesting that mitochondrial fusion, in addition to fission, is also connected to mitophagy.72,73 Phosphorylation of mitochondrial rho GTPase 1, which mediates mitochondrial motility,74 by PINK1 and subsequent degradation in a Parkin-dependent manner helps quarantine damaged mitochondria by arresting their motility and thus preventing their refusion with healthy mitochondria.75 Voltage-dependent anion channel, controlling mitochondrial membrane permeability, functions as a molecular receptor that aids in Parkin recruitment to mitochondria, albeit it is not clear whether ubiquitination of voltage-dependent anion channel is necessary for a mitophagic process.76,77 After ubiquitination of these and other mitochondrial proteins, molecular linkers, such as p62 (also called sequestosome 1), are recruited to the mitochondria and link ubiquitinated proteins on damaged mitochondria to autophagosomes via microtubule-associated protein 1 light chain.78

Along with PINK1/Parkin-mediated mitophagy, NIX (a BH3-only member of the Bcl-2 family), originally identified for its role in apoptotic cell death, induces mitophagy during erythrocyte maturation (Figure 3A).68,79 NIX localizes to the OMM and directly binds autophagy machinery components.80,81 Mitochondria-associated protein 1 light chain /γ-aminobutyric acid receptor-associated protein and targets mitochondria to autophagosomes for degradation under a stressed condition (induced by mitochondrial uncoupler CCCP).79 In addition, NIX exerts multiple functions on mitochondria, such as stimulating cardiomyocyte apoptosis, inducing clearance of damaged mitochondria, and regulating the mPTP.82 Similar to NIX-mediated mitophagy, another mitochondrial membrane protein FUN14 domain containing 1 also mediates hypoxia-induced mitophagy by interacting with microtubule-associated protein 1 light chain.83

Independently of macromitophagy, micromitophagy, whereby damaged mitochondria are sequestered and degraded by direct invagination of lysosomal membranes, also contributes to the maintenance of mitochondrial quality control. Only photodamage-induced micromitophagy in hepatocytes occurring in the presence of 3-methyladenine, an inhibitor of autophagosome formation, has been reported.84 After that study, our laboratory recently identified a new role for GAPDH, one of the common housekeeping metabolic enzymes, in micromitophagy (Figure 3B).85 Proteins readily undergo oxidation, such as S-nitrosylation, in response to ROS, thus changing their conformation and functions.86 GAPDH is one such protein that is sensitive to oxidative post-translational modifications; particularly, oxidation of a cysteine residue (C152) in its active site results in the inactivation of the enzyme.87 This metabolically inactive GAPDH (iGAPDH) confers a novel role for the enzyme: inducing mitophagy. It is possible that high levels of ROS released by mitochondria inactivate nearby GAPDH, which, in turn, triggers its association with damaged mitochondria. Therefore, iGAPDH serves as a molecular sensor for detecting and tagging damaged mitochondria. Furthermore, mitochondrial fractions isolated from hearts subjected to myocardial ischemic preconditioning (IPC) have higher levels of oxidized GAPDH.88 Mitochondria-associated iGAPDH promotes direct uptake of damaged mitochondria into a lysosomal-like structure, a hybrid organelle of late endosome and lysosome.89 Exogenously expressed, catalytically dead iGAPDH is sufficient to induce lysosomal-like structures to engulf damaged mitochondria for degradation.90 The mechanism by which mitochondria-associated iGAPDH promotes mitochondrial uptake into the lysosomal-like is still unknown but may be triggered by the fusogenic property of GAPDH.87,88 iGAPDH-bound mitochondria may also be eliminated by chaperone-mediated autophagy, whereby oxidized GAPDH is recognized by chaperone heat shock cognate protein and subsequently targeted to lysosome.90,91 Removing damaged or excessively ROS-producing mitochondria by modulating mitochondrial GAPDH may be key in protecting cells from damage. Together, it appears that activators of macro- and micromitophagy that enhance removal of damaged mitochondria and spare healthy mitochondria may be beneficial in preventing ROS-mediated damages in cardiac diseases.

In addition to mitochondrial dynamics, other mechanisms such as the unfolded protein response and the upregulation of the proteasome machinery maintain mitochondrial quality control. These mechanisms are discussed in greater detail in the referenced reviews.91,92 In particular, inhibition of the proteasome using bortezomib and overexpression of K48R mutated ubiquitin induces mitochondrial damage, which consequently leads to accumulation of pathological ROS.93 Therefore, components of the unfolded protein response and the proteasome machinery pose as attractive therapeutics targets, as well.

Targeting Mitochondria to Promote Beneficial ROS Signaling Through Protein Kinase C ε and Its Downstream Substrates

In addition to the challenges related to providing stoichiometric amounts of scavengers to quench each ROS and the inherit instability of ROS scavengers, not all amounts of ROS are damaging; ROS production may induce beneficial effects as well. The best example is IPC of the heart, in which sublethal cycles of IR bursts before a prolonged ischemic event protect the heart from reperfusion-mediated injury, suggesting a beneficial.94 IPC and multiple pharmacological preconditioning tools trigger a cascade of signaling events that are at least partially mediated by the release of mitochondrial ROS.95–98 One cardioprotective mechanism triggered by ROS involves protein kinase C epsilon (PKCe) activation. Antimycin
A, which triggers preconditioning, induces ROS production and results in PKCε translocation to the mitochondria and subsequent reduction in cardiac infarct size; this ROS-induced cardioprotective effect is lost in PKCε knockout mice.98 Furthermore, treatment of isolated cardiomyocytes with the PKCε agonist, ψεRACK (ψε-receptor for activated C-kinase), as well as the expression of this octapeptide in postnatal transgenic mice increases PKCε translocation to the mitochondria and promotes cardioprotection after IR injury.99,100 The finding that PKCε translocation to the mitochondria underlies a mechanism behind ROS-induced cardioprotection during IPC suggests that downstream mitochondrial PKCε substrates could be powerful therapeutic targets (Figure 4A).

Multiple downstream substrates of PKCε that reside in the mitochondria have been identified. These include proteins involved in glycolysis, lipid metabolism, and the electron transport chain, as well as heat shock proteins.101 It is not surprising that PKCε phosphorylates members of the electron transport chain, in particular members of complexes I, II, and III, because these sites have been previously implicated as locations of ROS production through superoxide leakage.102 PKCε communoprecipitates with cytochrome oxidase subunit IV, and this interaction contributes to ROS-induced preconditioning.103 In addition, IR damage is associated with a loss of cytochrome oxidase subunit I. Whereas preconditioning prevents the loss of cytochrome oxidase subunit I, treatment with the PKCε translocation inhibitor, εv1-2, exacerbates cytochrome oxidase subunit I loss.104 The underlying hypothesis stemming from these 2 studies suggests that ROS-mediated preconditioning is dependent on PKCε activation and translocation to the mitochondria. Once at the mitochondria, stabilization of cytochrome oxidase subunits by PKCε elicits a cardioprotective effect, potentially through restoring ATP production or preventing pathological ROS release through electron leakage (Figure 4A). Alongside phosphorylation of electron transport chain components, other post-translational modifications elicit cardioprotective effects as well. For example, selective S-nitrosation of Cys39 on the ND3 subunit of complex I by MitoSNO reduces infarct size in mice exposed to IR damage.105 Therefore, targeting PKCε’s mitochondrial substrates to mimic ROS-induced preconditioning may serve as an effective therapeutic strategy for cardiovascular disorders.

One of PKCε’s substrates is the mPTP initial coimmunoprecipitations in mitochondria isolated from mouse heart suggested that mPTP components, including the voltage-dependent anion channel, adenine nucleotide translocase, and hexokinase II, are PKCε targets during IPC.106 Since these studies, work by Bernardi107 has elucidated a more plausible structure of the mPTP, which is composed of an F4F4 ATP synthase dimer. Interestingly, ATP synthase is also a PKCε substrate.108 Identifying the mPTP as a phosphorylation target of PKCε suggests that PKCε-mediated cardioprotection occurs by preventing the opening of the mPTP (Figure 4B). Pathological opening of this pore leads to matrix expansion and OMM rupturing, which releases proapoptotic factors (such as cytochrome c, Smac/DIABLO [second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with low isoelectric point], apoptosis-inducing factor, and Omi/HtrA2 [mitochondrial serine protease]), dissipates the mitochondrial membrane potential, deregulates Ca2+ homeostasis, and releases pathological ROS.109 Still, the mechanism by which ROS-induced PKCε translocation to the mitochondria and phosphorylation of mPTP components contributes to mPTP closing remains unclear.

Targeting the mPTP to inhibit pore opening and mimic the effects observed with IPC serves as a valuable therapeutic approach for cardioprotection against IR-induced injury (Figure 4B). Multiple mPTP inhibitors have been tested in the context of MI, including cyclosporin A, which was initially discovered as part of a screen for antifungal antibiotics.109 Cyclosporin A is an effective inhibitor of cyclophilin D, a central regulator of mPTP dynamics that resides in the mitochondria.110 Cyclosporin A treatment prevents myocardial necrosis during reperfusion (initially demonstrated by DiLisa et al109,111). Although cyclosporin A successfully prevents mPTP opening through inhibiting cyclophilin D, its off-target effects make it a less ideal drug candidate. Most notably, cyclosporin A forms a complex with cyclophilin that binds and inhibits calcineurin, resulting in immunosuppressive effects.112 Cyclosporin A’s off-target effects highlight the need to develop more specific inhibitors of cyclophilin D that inhibit mPTP opening.

Multiple strategies have been used to harness cyclosporin A’s mPTP closing effects, while minimizing its off-target effects. Additional screens for inhibitors of cyclophilin D that do not have corresponding immunosuppressive effects yielded compounds, such as alisporivir, SCY-635, and NIM811 (as reviewed elsewhere).113 NIM811, for example, is as effective as cyclosporin A in preventing mitochondrial swelling and loss of mitochondrial membrane potential because of cyclophilin D inhibition, yet it lacks cyclosporin A’s immunosuppressive effects.114,115 Treatment of streptozotocin-induced diabetic and control rat hearts with NIM811 at postischemia reduces infarct size.116 Another inhibitor of cyclophilin D, Debio-025, potently blocks mPTP opening, reduces infarct size and left-ventricle dilation, and improves survival after IR injury.117,118 More recently, a new class of cinnamic anilides was found to inhibit mPTP opening of isolated mitochondria in response to calcium overload, to electron transport chain uncoupling, and to oxidative stress.119 In addition, one of the lead compounds in the class is cardioprotective in a rabbit heart model of MI. Interestingly, using a calcium-retention capacity assay, this lead compound has additive effect to cyclosporin A, suggesting that cinnamic anilides inhibit mPTP opening using an alternative target to cyclophilin D.119 However, it is also possible that cyclosporin A does not completely inhibit cyclophilin D, because of its poor selectivity, and so treatment with an additional cyclophilin D inhibitor would have an additive effect. Furthermore, mitochondrial pore opening has been observed in the presence of cyclosporin A and the absence of cyclophilin D because of excess Ca2+ in the matrix, posing a limitation on cyclophilin D as a therapeutic target.120 By identifying the proteins that bind this novel class of inhibitors, we may find alternative protein targets to prevent mPTP opening and the corresponding pathological ROS release to benefit the heart.
The mitochondrial ATP-dependent potassium, mitoK\textsubscript{ATP} channel is also a potential target of PKC\textsubscript{ε} phosphorylation on ischemic reperfusion injury (Figure 4C). Initial work suggesting the mitoK\textsubscript{ATP} channel’s cardioprotective role showed that inhibitors of mitoK\textsubscript{ATP} channel opening, such as glibenclamide and sodium 5-hydroxydecanoate, prevent the effect observed during IPC in a canine heart model.\textsuperscript{120,121} The mitoK\textsubscript{ATP} channel is a downstream of PKC\textsubscript{ε}-mediated preconditioning; pharmacological inhibition of mitoK\textsubscript{ATP} channel opening does not affect PKC\textsubscript{ε} translocation to the mitochondria.\textsuperscript{122} In addition, using a reconstituted liposome system, PKC\textsubscript{ε} associates with activated mitoK\textsubscript{ATP} in this reconstituted system, addition of ROS in the form of H\textsubscript{2}O\textsubscript{2} induces potassium flux through the channel, which is abolished in the presence of the selective PKC\textsubscript{ε} inhibitor εV\textsubscript{1-2} and the mitoK\textsubscript{ATP} inhibitor 5-hydroxydecanoate.\textsuperscript{123} In isolated heart mitochondria, treatment with H\textsubscript{2}O\textsubscript{2} but not with superoxide activates the mitoK\textsubscript{ATP} channel in a PKC\textsubscript{ε}-dependent manner because treatment with εV\textsubscript{1-2} reduces the potassium flux.\textsuperscript{124} These results suggest that physiological ROS induce PKC\textsubscript{ε} phosphorylation and open the mitoK\textsubscript{ATP} channel, which contributes to cardioprotection during (IPC). Although the mechanism by which mitoK\textsubscript{ATP}
opening contributes to cardioprotection is not completely under-
stood, mitoK\textsubscript{ATP} activation is associated with a reduction of pathological ROS, demonstrated by using 2,7'-dichlo-
rofluorescein as a fluorescent ROS indicator in isolated rat
myocytes.\textsuperscript{125} Thus, therapeutics that specifically activate the
mitoK\textsubscript{ATP} channel can trigger the downstream cardioprotective
preconditioning effects of physiological ROS and prevent the
overproduction of pathological ROS.

Several therapeutics aimed at the mitoK\textsubscript{ATP} channel activa-
tion have been tested. Diazoxide is both an opener of mitoK\textsubscript{ATP}
and sarcolemmal K\textsubscript{ATP} channels, with a much stronger pre-
ference for mitoK\textsubscript{ATP}. Diazoxide also elicits a cardioprotective
effect, measured by the reduction of cytosolic lactate dehy-
drogenase release (a marker of cell necrosis) in an isolated rat
heart model of MI; diazoxide’s effect is abolished by the mito-
K\textsubscript{ATP} inhibitor 5-hydroxydecanoate.\textsuperscript{126} Interestingly, it was
suggested that diazoxide activates mitoK\textsubscript{ATP} channel open-
ing by generating ROS in a connexin 43–dependent manner,
which further solidifies an important physiological role for
ROS during preconditioning (connexin 43 is a gap junction
protein, providing electric coupling between cardiac cells).\textsuperscript{127}
However, because diazoxide also has vasodilator and hyper-
glycemic effects, it is not an ideal pharmacological precondition-
ing mimic.\textsuperscript{128} A recent review discusses the benefits of additional mitoK\textsubscript{ATP}
activators, such as BMS 180448, BMS 191095, KR31466, and F163.\textsuperscript{128} Since the review’s publica-
tion, 2 other drugs proposed to target the mitoK\textsubscript{ATP} channel have been tested in the context of IR damage: diosgenin
and atorvastatin. Two independent studies showed that diosgenin
and atorvastatin induce a cardioprotective preconditioning ef-
fact in an IR rat heart model, measured either by the infarct
area or by lactate dehydrogenase release. The effect of either
drug is abolished in the presence of 5-hydroxydecanoate.\textsuperscript{129,130}
However, in both cases, it is unclear whether the mitoK\textsubscript{ATP}
channel is the direct target of these drugs. Lonidamine, a
mPTP opener, abolishes atorvastatin’s cardioprotective ef-
fact,\textsuperscript{130} whereas treatment with an NO synthase blocker,
l-nitro-arginine methyl ester (L-NAME), abolished diosgenin’s
cardioprotective effect.\textsuperscript{129} Although both diosgenin and ator-
vasatin induce pharmacological preconditioning that results in
mitoK\textsubscript{ATP} channel opening, neither have been shown to specifi-
cally bind and open the mitoK\textsubscript{ATP} channel.

Another mitochondrial PKC\varepsilon substrate is the mitochondri-
al aldehyde dehydrogenase 2 (ALDH2). The major function
of ALDH2 is to detoxify reactive aldehyde substrates to their
 corresponding acids.\textsuperscript{131} One of the deleterious effects of patho-
logical ROS is the generation of toxic and reactive aldehydes,
such as unsaturated alkenals, 4-hydroxy-2-nonenal, and mal-
doinaldehyde, by peroxidation of the mitochondrial lipid mem-
brane.\textsuperscript{132} These reactive aldehydes can lead to mitochondrial
dysfunction through covalently binding to and inactivating
proteins, lipids, and DNA.\textsuperscript{133} Notably, reactive aldehyde-me-
diated mitochondrial dysfunction has long been suspected to
be a culprit of many human cardiovascular diseases, such as
atherosclerosis, hypertension, peripheral artery disease, car-
diomyopathy, MI, and HF.\textsuperscript{134} Our group showed that PKC\varepsilon
phosphorylation of ALDH2 leads to enhanced aldehyde de-
hydrogenase activity and reduction of toxic aldehydes under
oxidative stress.\textsuperscript{135} Physiological ROS-mediated activation of
PKC\varepsilon can lead to a protective effect through ALDH2 activa-
tion. Therefore, increasing ALDH2’s aldehyde detoxification
activity directly should also mimic the function of physiologi-
cal and protective ROS (Figure 4D).

ALDH2 deficiency is one of the most common genetic
mutations in humans. About 560 million East Asians or 8% of
the world population are deficient in ALDH2 enzyme ac-
tivity because of a single point mutation, resulting in an ami-
no acid substitution of glutamic acid for lysine, in ALDH2
(ALDH2*2).\textsuperscript{136} The mutation behaves in an overdominant
fashion so that ALDH2 enzymatic activity is reduced to 20%
to 35% of wild-type enzyme in heterozygous individuals.
Recent genome-wide association and epidemiological stud-
ies provide strong evidence linking ALDH2*2 genotype with
the risk of coronary artery disease,\textsuperscript{137,138} MI,\textsuperscript{139} hypertension,\textsuperscript{140}
dyslipidemia,\textsuperscript{141} and hyperglycemia.\textsuperscript{142} The elevated health
risk of cardiovascular diseases that many East Asians may
face because of the ALDH2*2 further demonstrates the rel-
evance of ALDH2 as a cardiovascular therapeutic target.

The use of an isozyme-selective small molecule activator of
ALDH2 is particularly attractive for its ability to accelerate the
removal of toxic aldehyde in the mitochondria, where patholo-
logical ROS-induced lipid peroxidation is damaging under stressed
conditions. Our group first discovered a class of ALDH2 selec-
tive activators, represented by aldehyde dehydrogenase activa-
tor (Alda-1).\textsuperscript{143} Alda-1 enhances ALDH2 enzymatic activity by
binding to and accelerating the clearance of its aldehyde sub-
strates, such as acetaldehyde and 4-hydroxy-2-nonenal.\textsuperscript{143–145}
Administration of Alda-1 or its analog causes significant car-
dioprotection against IR injury.\textsuperscript{135,143} Targeting ALDH2 by an
Alda-like compound is therefore pursued as a potential ther-
apeutic target. In this review, we focus only on more recent ad-
vances in the application of ALDH2 activators as a potential
mitochondria-based therapeutics for cardiovascular diseases.

The efficacy of Alda-1 has been demonstrated in many
oxidative stress-related cardiovascular conditions or mod-
els. Related to IR injury during MI, activation of ALDH2 by
Alda-1 in a cardioplegia solution, which is commonly used
for human open-heart surgeries, results in better cardiac func-
tion preservation after IR.\textsuperscript{146} Alda-1 treatment is also effective
in preventing MI-induced HF in rats. Alda-1 given continu-
ously for 4 weeks at 24 hours immediately after left anterior
descending coronary artery occlusion surgery significantly
improves mitochondrial and left ventricular function with
concomitant reduction of aldehydic load in cardiomyocytes.\textsuperscript{147}
Separately, a 6-week delivery of Alda-1 to rats during the HF
stage results in a significant improvement in HF outcome.\textsuperscript{148}
In both cases, long-term, systemic treatment of Alda-1 has no
observable adverse effect. Furthermore, prolonged oral in-
gestion of Alda-1 together with a high-fat diet for 4 months
reduces atherosclerotic lesion by 25% and alleviates liver ste-
atosis when compared with high-fat diet alone in an Apo-E
knockout mice model.\textsuperscript{149} Similar to MI-induced IR injury,
in a rat stroke model using middle cerebral artery occlusion,
Alda-1 enhances ALDH2-mediated clearance of 4-hydroxy-
2-nonenal, malondialdehyde and reduces infarct volume,
adverse neurological score, and mortality rate.\textsuperscript{150} In type I
diabetes mellitus–induced myocardial injuries and suppression of autophagy, administration of Alda-1 restores cardiac dysfunction and high-glucose–induced cardiotoxicity, most likely through 5′ AMP–activated protein kinase–dependent autophagy regulation. Alda-1 also improves the viability of induced human pluripotent stem cell–derived cardiomyocytes after ischemia. In this study, we showed that after an ischemic insult in culture, induced human pluripotent stem cell–derived cardiomyocytes derived from the fibroblasts of ALDH2-deficient human subjects (carrying the inactive mutant ALDH2*2 allele) generate more toxic aldehydes and exhibit increased apoptosis and cell cycle arrest when compared with induced human pluripotent stem cell–derived cardiomyocytes derived from normal ALDH2 human subjects (carrying the ALDH2*2 wild-type allele). Alda-1 treatment reduces toxic aldehyde production and rescues ischemic damage in the mutant induced human pluripotent stem cell–derived cardiomyocytes. These results indicate that ROS-induced ADLH2 activation can be mimicked by direct activation of ALDH2 by a small molecule (such as Alda-1) not only to produce increased cell survival but also to enhance cardiac repair and regeneration after ischemia.

The case study of ROS-mediated PKCe activation to promote signaling pathways that protect the cell from stress reveals multiple mitochondrial therapeutic targets for cardiovascular disorders, including the mPTP, mitoK<sub>A</sub><sup>ATP</sup> and ALDH2. Pharmacological interventions that mimic ROS-dependent PKCe activation by closing the mPTP, opening the mitoK<sub>A</sub><sup>ATP</sup> and activating ALDH2 are effective therapeutic strategies in the preclinical setting. Targeting these mitochondrial proteins directly not only preserves physiological ROS signaling pathways but also enhances them to aid the cell in its response to stress.

**Targeting Mitochondria to Prevent Harmful ROS Signaling Through Protein Kinase C δ and Its Downstream Effectors**

PKCδ, another member of the PKC family, is implicated in several pathological conditions including cancer, stroke, diabetes mellitus, neurodegenerative diseases, and ischemic heart disease. ROS can directly activate PKCδ via oxidative modification or tyrosine phosphorylation, as well as by inducing caspase 3–mediated PKCδ proteolysis. For example, glutathione depletion-induced ROS generation of heart-derived H9C2 cells is triggered by caspase 3 and PKCδ activation. After ischemia, right at the onset of reperfusion, the rise in ROS triggers PKCδ translocation to the mitochondria and enhances superoxide anion production, which induces mitochondrial dysfunction and subsequent potentiation of oxidative stress. This PKCδ activation can be blocked by a specific PKCδ translocation peptide inhibitor called δV1-1. PKCδ inhibition also reduces endothelial vascular dysfunction because of endothelial nitric oxide synthase–mediated ROS levels, by increasing survival of coronary endothelial cells. Endoplasmic reticulum oxidative stress, induced by tunicamycin or by IR in cardiac myocytes, triggers PKCδ localization to the endoplasmic reticulum and causes cell death that is inhibited by δV1-1. Therefore, in addition to its negative effect at the mitochondria, ROS-induced PKCδ activation disrupts endoplasmic reticulum homeostasis causing cell death. Together, these results suggest that a selective PKCδ inhibitor might be a useful therapeutic agent against cell injury because of ROS elevation.

The mechanisms involved in PKCδ translocation to the mitochondria and by which proteins shuttle the enzyme into mitochondrial matrix are unknown. PKCδ does not have a mitochondrial targeting sequence. However, we provided evidence for an interaction between activated PKCδ and annexin V, a calcium-dependent phospholipid-binding protein, which promotes the translocation of PKCδ from the cytosol to the cell membranes (a cell fraction composed with heavy membranes and organelles). The binding between these 2 proteins regulates PKCδ translocation and inhibiting this interaction with a small molecule, JTV519, leads to cardioprotection in a model of MI. This suggests that annexin V might serve as a shuttle protein moving PKCδ on the microtubules to its subcellular compartments. Treatment with JTV519, an inhibitor of annexin V, improves LV function and reduces calcium overload after IR damage when administered at the time of reperfusion in an isolated rat heart model. Therefore, annexin V may serve as an additional therapeutic target to inhibit pathological ROS-induced translocation of PKCδ to the mitochondria.

What are the downstream targets of PKCδ as mediators of apoptosis/necrosis at the mitochondria and can these be therapeutic targets to provide additional selective inhibition of ROS-induced cardiac injury? Electron microscopy studies demonstrated that activated PKCδ crosses the mitochondrial membrane to interact with specific substrates and mediates cardiac injury, at least in part, by phosphorylating the mitochondrial matrix enzyme PDH kinase (a key enzyme leading to the Krebs cycle). Thirty years ago, Patel and Olson reported a decline in mitochondrial PDH activity in ischemic rat heart. Subsequent work demonstrated that once phosphorylated by PKCδ, activated PDH kinase phosphorylates and inhibits PDH thus blocking glycolytic flux and ATP production by the mitochondria (Figure 5A). Thus, cells must rely on alternative mechanisms for ATP production, such as fatty acid oxidation. Inhibiting PKCδ with δV1-1 at reperfusion increases ATP levels and mitochondrial function, as well as reduces MI. Therefore, specific inhibitors of PKCδ-mediated PDH kinase activation will likely reduce pathological ROS-induced abrogation of metabolism through the Krebs cycle and subsequent cardiac injury.

ATP levels in the cell are crucial for survival. The big majority of cellular ATP is produced by the mitochondrial F<sub>F</sub>,F<sub>0</sub>-ATP synthase. However, cardiac ATP levels do not recover for a long time after IR, resulting in tissue injury, and it is still unclear whether PKC signaling directly affects ATP production. Evidence about the modulation of the mitochondrial ATP synthase was discussed earlier in the context of PKCe and the closing of the mPTP. PKCδ activation was also reported to regulate ATP synthase. The study demonstrated that PKCδ’s direct interaction with F<sub>F</sub>,F<sub>0</sub>-ATP synthase during IR prolongs hypoxia in cardiomyocytes, inhibits ATP synthesis, and contributes to cardiac injury. However, no
phosphorylation of the complex by PKCδ has been reported. Thus, it is not clear whether F1F0 ATP synthase is an additional PKCδ substrate at the mitochondria that modulates cardiac injury (Figure 5A).

As discussed earlier, we recently found that GAPDH associates with the mitochondria after IR injury, where it mediates mitochondrial elimination by the lysosomal machinery. In addition to oxidation modification, we demonstrated that in a rat IR model and in cardiomyocytes, PKCδ induces GAPDH phosphorylation, predominantly on threonine 246, which results in cardiac damage by inhibiting the GAPDH-mediated mitophagy process for damaged mitochondrial elimination (Figure 5B). Therefore, targeting the interaction between PKCδ and GAPDH may reduce cardiac damage caused by pathological ROS disruption of micromitophagy.

Conclusions
Beyond their canonical function as powerhouses of the cell, mitochondria act as integral hubs of signaling pathways that determine cell fate. Their role in regulating cell death and their abundance in heart tissue make mitochondria an attractive therapeutic target for cardiovascular disorders. Among the various signaling pathways that converge on the mitochondria, ROS-mediated cellular responses have been of particular interest because of their association with age- and cardiovascular-related diseases. Whereas traditional notion suggests that ROS is exclusively pathological, through its contribution to oxidative stress, the use of ROS scavengers in the clinic to treat cardiovascular disorders has not been successful. Emerging research suggests that ROS also hold physiological and protective functions that gross treatment with antioxidants may eliminate. Therefore, an effective therapeutic approach must take ROS’s pathological and physiological roles into account. Mitochondria are attractive targets for cardiovascular diseases because they are both a significant source of ROS and also the downstream ends of ROS-induced signaling pathways. Rather than eliminating all ROS directly, it is possible to pharmacologically target specific proteins in the mitochondria to selectively prevent the production of pathological ROS. In addition, inhibiting select downstream ROS pathways that result in cell damage and promoting ROS-induced cell survival pathways can involve the mitochondria.

A distinction between pathological and physiological mitochondrial ROS production can be achieved by selectively targeting damaged mitochondria. Selective inhibition of pathological fission prevents excessive fragmentation of mitochondria and reduces the production of ROS. Similarly, activating elimination of damaged mitochondria by macro- or micromitophagy will prune the dysfunctional mitochondria and avoid healthy nearby mitochondria to selectively reduce pathological ROS. Elucidation of the mechanisms that lead to enrichment of functional mitochondria, therefore, identifies important therapeutic targets to selectively reduce the production of cell-damaging ROS.

We also discussed additional therapeutic targets for heart disease that are downstream of ROS. These targets specifically activate cardioprotective pathways and inhibit damaging pathways. ROS activates PKCε signaling, which in turn phosphorylates multiple mitochondrial substrates that elicit cardioprotective effects. Pharmacological agents that target these substrates directly mimic these protective effects in multiple cardiovascular disorders. Finally, there is a therapeutic opportunity in opposing ROS-mediated PKCδ activation and translocation to the mitochondria, which results in a harmful
molecular cascade for the cell. Pharmacological inhibition of PKCδ translocation to the mitochondria is cardioprotective. Furthermore, inhibitors of mitochondrial substrates of ROS-activated PKCδ, including PDH kinase and GAPDH, are also attractive targets for cardiovascular disease intervention. In culmination, mitochondria-based therapeutics that modulate molecular events upstream and downstream of ROS production provide a new approach for diseases of the old in general, and of ischemic cardiovascular disease in particular.

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