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Mitochondrial Reactive Oxygen Species at the Heart of the Matter: New Therapeutic Approaches for Cardiovascular Diseases

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Abstract

Reactive oxygen species (ROS) have been implicated in a variety of age-related diseases including multiple cardiovascular disorders. However, translation of ROS scavengers (anti-oxidants) into the clinic has not been successful. These anti-oxidants grossly reduce total levels of cellular ROS including ROS that participate in physiological signaling. In this review, we challenge the traditional anti-oxidant therapeutic approach that targets ROS directly with novel approaches that improve mitochondrial functions to more effectively treat cardiovascular diseases.

Keywords

Mitochondria; reactive oxygen species (ROS); fission; mitophagy; protein kinase C (PKC)

INTRODUCTION

The heart is a tissue rich in mitochondria with approximately 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

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the mitochondrial permeability transition pore (mPTP) as well as the subsequent collapse of the proton gradient and matrix swelling lead to the release of pro-apoptotic proteins. Such opening of the mPTP is implicated during ischemia/reperfusion (IR) injury.[4] Finally, not only are mitochondria major producers of ROS, they are also 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.

SECTION I – 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 ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and others.[5] ROS accumulate both inside and outside of the cell. For example, ROS are generated in the cytoplasm and the plasma membrane by nicotinamide adenine dinucleotide phosphate (NADPH) 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 upon release can affect neighboring cells from the outside. All of these ROS production sites have implications in cardiovascular disease. Interested readers are directed to Sugamura and Keaney's review.[6] However, our discussion focuses only on ROS generated by the mitochondria.

Around 0.1-0.2% of oxygen consumed is associated with leakage of electrons and production of ROS.[7] At least ten 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, branched-chain 2-oxoacid dehydrogenase, mitochondrial glycerol phosphate dehydrogenase, ETF (electron-transferring-flavoprotein) dehydrogenase, and dihydroorotate dehydrogenase.[8] 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, while the quinol site in 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.[8] The dynamic nature of ROS production at the ten 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 1940's.[9] 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 as well as 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.[10] 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 ten days with dilated cardiomyopathy.[11] It is therefore not surprising that multiple anti-oxidants have been tested in clinical trials to reduce the oxidative burden of cardiovascular diseases.

In 2011, Sugamura and Keaney summarized clinical trials for anti-oxidants in the context of cardiovascular disorders and concluded that targeting oxidative stress using ROS scavengers is an ineffective therapeutic strategy. While ROS scavengers are effective at reducing cellular ROS levels, they are in general ineffective and sometimes harmful in the context of cardiovascular pathology.[6] Table 1 provides an update of their summary. ROS scavengers, such as N-acetylcysteine (NAC), have mixed efficacy outcomes. For example, intravenous NAC prior to angioplasty and orally delivered NAC following 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.[12,13] NAC oral supplement given twice daily for four weeks increased forearm blood flow but did not affect patient outcome following both heart and renal failure.[14] A promising effect for NAC in improving cardiovascular function has only been shown in the study involving 354 patients undergoing angioplasty following acute MI.[13] A follow-up clinical study for the effect of NAC in patients undergoing angioplasty on adverse outcomes is currently recruiting patients.[15]

In addition to NAC, the anti-oxidant 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 (MDA), and increased the expression of anti-oxidant enzymes including catalase, glutathione peroxidase, and superoxide dismutase.[16] In patients with non-ST elevation MI, L-carnitine reduced the release of both creatine-MB and troponin-I.[17] However, the effect of L-carnitine on overall clinical outcome has not been determined. Other anti-oxidants such as α-lipoic acid (the level of which significantly declines with age)[18] and melatonin (an anti-oxidant and a neurohormone produced by the pineal gland)[19] are currently being tested in clinical trials for cardiovascular-related indications.[20–22] 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.[23]

Treatment of cardiovascular diseases with general ROS scavengers has multiple theoretical limitations that may play a role in their apparent failure in the clinic (Figure 1A). One limitation of ROS scavengers is that they must be provided in stoichiometric (greater than 1:1 ratio) levels relative to cellular ROS. In addition, ROS scavengers, such as NAC, are unstable; they can undergo auto-oxidation prior to treatment, effectively rendering them as oxidants.[24] As a result, anti-oxidants 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.[25]) For example, in the context of development, overexpression of the anti-oxidant GTPx-1 suppressed differentiation of multipotent hematopoietic progenitors in

Drosophila.[26] 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, *i.e.,* 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 as well as the sites in which ROS-activated signaling pathways converge.

SECTION II – 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.[27] An additional TPP^+-modified ROS scavenger, SkQ, increases the life-span of male versus female mice.[28] In addition to TPP^+-modified ROS scavengers, SS31 (Bendavia), a four amino acid synthetic peptide (phenylalanine-darginine-phenylalanine-lysine), selectively reaches the mitochondrial inner membrane, scavenges ROS through a dimethyltyrosine group, reduces mitochondrial ROS production, and prevents mPTP opening.[29] SS31 inhibits hydrophobic cytochrome *c* interactions with cardiolipin [1,3-bis(sn-3'-phosphatidyl)-snglycerol; a component of the inner mitochondrial membrane], freeing cytochrome *c* to function as an electron carrier.[30] SS31 has a cardioprotective effect in response to IR damage in multiple animal models of MI and heart failure, including in mice, rats, guinea pigs, rabbits, and sheep.[31–33] Furthermore, SS31 selectively improves multiple functional parameters in mitochondria isolated from old versus young mouse skeletal muscle as measured *in vivo*.[34] In addition, SS31's selective effect on old over young mice suggests that SS31 may better target pathological ROS production that occurs in aged mitochondria. SS31 is currently in a phase IIa clinical trial for patients with ST-segment elevation MI.[35] Finally, mitochondria-targeted enzymatic anti-oxidants have cardioprotective effects as well. For example, overexpression of catalase specifically in the mitochondria (mCat) reduces mitochondrial oxidative damage, increases lifespan of mice, and protects against angiotensin II-induced cardiac hypertrophy, fibrosis, and heart failure.[36,37] Anti-oxidants 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 anti-oxidants solve the localization limitation of ROS scavengers, they may still eliminate physiologically-relevant ROS as well (Figure 1B).

How can pathological and physiological ROS be distinguished and can pathological ROS generation be specifically targeted? One mechanism focuses on the removal of damaged mitochondria, which cause further damage by producing excessive or pathological ROS.[38] Perhaps specifically targeting damaged mitochondria or mechanisms that sequester and remove damaged mitochondria is an effective strategy to eliminate the production of pathological ROS.

Before considering elimination of damaged mitochondria, we should discuss normal mitochondrial fate. Mitochondria continuously undergo changes in morphology and size through processes including fission and fusion that are collectively referred to as "mitochondrial dynamics." Mitochondrial fission is the division of a single mitochondrion into two or more mitochondria, whereas mitochondrial fusion is the joining of two or more mitochondria to form a single mitochondrion. Mitochondrial fission is mediated by a large GTPase called Drp1 (dynamin-related protein 1) that is recruited to the outer mitochondrial membrane (OMM) by multiple protein adaptors, including Fis1 (fission 1), Mff (mitochondrial fission factor), as well as MiD49 and MiD51 (mitochondrial dynamics protein of 49 and 51 kDa). Once on the OMM, Drp1 polymerizes and constricts the mitochondria to induce division. During mitochondrial fusion, joining of the OMM is mediated by two GTPases, Mfn1 (mitofusin) and Mfn2, and stitching of the inner mitochondrial membrane (IMM) is mediated by Opa1 (optic atrophy 1), another large GTPase and a homolog of dynamin.[38–40] A balance between fission and fusion must be kept to maintain healthy mitochondria and cellular homeostasis (Figure 2A).

Mitochondrial fission/fusion does not seem to occur in isolated cardiomyocytes to as great of an extent as in cultured cells.[41] Nevertheless, an imbalance between fission and fusion is observed in multiple cardiovascular disorders as thoroughly reviewed by Dorn.[42] One example of particular interest is the observation that Opa1 is down-regulated in hearts from both humans with heart failure (HF) and a rat model of HF. In addition, mitochondria in HF are fragmented, suggesting the occurrence of excessive, pathological, and unopposed fission.[43] Mitochondrial fragmentation is found in other pathologies as well, including pulmonary arterial hypertension, which is associated with both reduced Mfn2 expression and up-regulation of Drp1.[44,45] Furthermore, excessive fragmentation occurs in other non-cardiac disorders, such as cancer and in a number of 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.[46] 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.[47] However, Drp1 may have multiple functions, depending on its interaction with each of its adaptor proteins, Fis1, Mff, MiD49 and MiD51 (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.[48] 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.[49,50] Therefore, a more specific inhibitor of Drp1, a so-called 'separation of function' inhibitor,[51] 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 lab 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 Fis1 only and does not affect Drp1 interaction with Mff and MiD51. This separation of function inhibitor was derived using a rational approach that examines sequences of homology between Fis1 and Drp1.[52] P110 treatment diminishes excessive mitochondrial fission and reduces neurotoxicity in cells derived from Parkinson's patients but does not affect the recruitment of Drp1 to the mitochondria under physiological conditions.[52] It is therefore hypothesized that Fis1-mediated 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 three models of IR in rats, including primary cardiomyocytes, *ex vivo* heart model, and an *in vivo* MI model.[53] In primary cardiomyocytes that were not exposed to IR stress, P110 does not affect ROS production and mitochondrial function nor does it induce cell death. Importantly, P110 treatment reduced infarct size and increased ATP production in the MI *in vivo* model.[53] Further, eight weeks of P110 treatment shows no adverse effects in wild-type mice, but improves behavior and reduced mortality in Huntington's mice.[54] 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.[55] Autophagy has long been thought to non-specifically 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.[56,57] 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 anti-oxidant capacity), autophagy provides limited mitochondrial pruning, thus promoting mitochondrial quality control to meet the cell's energy requirement.[58] When exposed to cellular insults, such as oxidative stress caused by IR, mitochondrial pruning through autophagy (mitophagy) is up-regulated by diverse molecular machinery.[59,60]

Depending on the way mitochondria are delivered to lysosomes, two distinct molecular pathways contribute to mitochondrial elimination: macro- and micro-mitophagy. Macro-mitophagy, 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.[56] A number of 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 C. elegans) is involved in initiation of autophagosome formation, which is primed by ULK1 (unc-51 like autophagy activating kinase 1) and AMBRA1 (activating molecule in beclin 1-regulated autophagy).[61–63] A group of Atg proteins and LC3 (microtubule-associated protein 1 light chain) then promote elongation and maturation of the autophagosome.[64] 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 protein 1, PINK1 (PTEN-induced putative kinase 1), Parkin (a component of an E3 ubiquitin ligase complex), and NIP1-like protein X (NIX, a pro-apoptotic protein).[65,66] PINK1, initially implicated in the pathogenesis of Parkinson's disease, is a molecular sensor to induce mitophagy in heart.[60,67] Upon depolarization, PINK1 accumulates on the outer mitochondrial membrane and recruits the cytosolic E3 ubiquitin ligase Parkin. Parkin recruitment leads to increased ubiquitination of mitochondrial proteins, which induces removal of impaired mitochondria.[56,66,68] Although the exact process has not been clearly elucidated, a number of molecular factors promoting mitophagy have been discovered. In addition to Parkin, several other substrates of PINK1 have recently been identified. For example, Mfn2 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.[69,70] Phosphorylation of MIRO (mitochondrial membrane rho GTPase protein mediating mitochondrial motility)[71] by PINK1 and subsequent degradation in a Parkin-dependent manner helps quarantine damaged mitochondria by arresting their motility and thus preventing their re-fusion with healthy mitochondria.[72] VDAC (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 VDAC is necessary for a mitophagic process.[73,74] Following ubiquitination of these and other mitochondrial proteins, molecular linkers, such as p62 (also called sequestosome 1 or SQSTM1), are recruited to the mitochondria and link ubiquitinated proteins on damaged mitochondria to autophagosomes *via* LC3.[73]

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).[65,75] NIX localizes to the OMM and directly binds autophagy machinery components LC3/GABARAP (γ-aminobutyric acid receptor-associated protein) and targets mitochondria to autophagosomes for degradation under a stressed condition (induced by mitochondrial uncoupler CCCP).[76] In addition, NIX exerts multiple functions on mitochondria, such as stimulating cardiomyocyte apoptosis, inducing clearance of damaged mitochondria, and regulating the mPTP.[77] Similar to NIX-mediated mitophagy, another mitochondrial membrane protein FUNDC1 (FUN14 domain containing 1) also mediates hypoxia-induced mitophagy by interacting with LC3.[78]

Independently of macro-mitophagy, micro-mitophagy, 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 micro-mitophagy in hepatocytes occurring in the presence of 3-methyladenine, an inhibitor of autophagosome formation, has been reported.[79] Following that study, our lab recently identified a new role for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), one of the common housekeeping metabolic enzymes, in micro-mitophagy (Figure 3B)[80]. Proteins readily undergo oxidation, such as S-nitrosylation, in response to ROS, thus changing their conformation and functions.[81] 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.[82] 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) has higher levels of oxidized GAPDH.[83] Mitochondria-associated iGAPDH promotes direct uptake of damaged mitochondria into a lysosomal-like (LL) structure, a hybrid organelle of late endosome and lysosome.[80] Exogenously-expressed, catalytically-dead iGAPDH is sufficient to induce LL structures to engulf damaged mitochondria for degradation.[80] The mechanism by which mitochondria-associated iGAPDH promotes mitochondrial uptake into the LL is still unknown, but may be triggered by the fusogenic property of GAPDH.[84,85] iGAPDH-bound mitochondria may also be eliminated by chaperone-mediated autophagy (CMA), whereby oxidized GAPDH is recognized by chaperone heat shock cognate protein and subsequently targeted to lysosome.[86,87] 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 micro-mitophagy 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 (UPR) and the upregulation of the proteasome machinery maintain mitochondrial quality control. These mechanisms are discussed in greater detail in the referenced reviews.[88,89] 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.[90] Therefore, components of the UPR and the proteasome machinery pose as attractive therapeutics targets, as well.

SECTION III – 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 ischemic preconditioning (IPC) of the heart, in which sublethal cycles of IR bursts prior to a prolonged ischemic event protect the heart from reperfusion-mediated injury, suggesting a

beneficial [91]. IPC and multiple pharmacological preconditioning tools trigger a cascade of signaling events that are at least partially mediated by the release of mitochondrial ROS.[92–95]

One cardioprotective mechanism triggered by ROS involves protein kinase C epsilon (PKCε) 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.[95] Furthermore, treatment of isolated cardiomyocytes with the PKCε agonist, ψεRACK, as well as the expression of this octapeptide in postnatal transgenic mice increases PKCε translocation to the mitochondria and promotes cardioprotection following IR injury.[96,97] 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.[98] It is not surprising that PKCε phosphorylates members of the electron transport chain, in particular members of complexes I,II, and III, as these sites have been previously implicated as locations of ROS production through superoxide leakage.[99] PKCε co-immunoprecipitates with cytochrome oxidase subunit IV (COIV) and this interaction contributes to ROS-induced preconditioning.[100] In addition, IR damage is associated with a loss of cytochrome oxidase subunit I (COI). Whereas preconditioning prevents the loss of COI, treatment with the PKCε translocation inhibitor, εV1-2, exacerbates COI loss.[101] The underlying hypothesis stemming from these two studies suggests that ROS-mediated preconditioning is dependent upon 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.[102] 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 co-immunoprecipitation studies in mitochondria isolated from mouse heart suggested that mPTP components, including the voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), and hexokinase II (HKII), are PKCε targets during IPC.[103] Since these studies, work by the Bernardi group has elucidated a more plausible structure of the mPTP, which is composed of an F_0F_1 ATP synthase dimer.[104] Interestingly, ATP synthase is also a PKCε substrate.[98] 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 pro-apoptotic factors (such as cytochrome *c*, Smac/DIABLO [second mitochondria-derived

activator of caspases /direct IAP binding protein with low pI], AIF [Apoptosis-inducing factor], and Omi/HtrA2 [mitochondrial serine protease]), dissipates the mitochondrial membrane potential, deregulates Ca^{2+} homeostasis, and releases pathological ROS.[105] 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 (CsA), which was initially discovered as part of a screen for anti-fungal antibiotics.[106] CsA is an effective inhibitor of cyclophilin D (CypD), a central regulator of mPTP dynamics that resides in the mitochondria.[107] CsA treatment prevents myocardial necrosis during reperfusion (initially demonstrated by Di Lisa and co-workers).[106,108] Although CsA successfully prevents mPTP opening through inhibiting CypD, its off-target effects make it a less ideal drug candidate. Most notably, CsA forms a complex with cyclophilin that binds and inhibits calcineurin, resulting in immunosuppressive effects.[109] CsA's off-target effects highlight the need to develop more specific inhibitors of CypD that inhibit mPTP opening.

Multiple strategies have been employed to harness CsA's mPTP closing effects while minimizing its off-target effects. Additional screens for inhibitors of CypD that do not have corresponding immunosuppressive effects yielded compounds such as alisporivir, SCY-635, and NIM811 (as reviewed elsewhere).[110] NIM811, for example, is as effective as CsA in preventing mitochondrial swelling and loss of mitochondrial membrane potential due to CypD inhibition, yet it lacks CsA's immunosuppressive effects.[111,112] Treatment of streptozotocin-induced diabetic and control rat hearts with NIM811 at post-ischemia reduces infarct size.[113] Another inhibitor of CypD, Debio-025, potently blocks mPTP opening, reduces infarct size and left-ventricle dilation, and improves survival following IR injury.[114,115] More recently, a new class of cinnamic anilides were found to inhibit mPTP opening of isolated mitochondria in response to calcium overload, to electron transport chain uncoupling, and to oxidative stress.[116] In addition, one of the lead compounds in the class is cardioprotective in a rabbit heart model of myocardial infarction. Interestingly, using a calcium retention capacity assay, this lead compound has additive effect to CsA, suggesting that cinnamic anilides inhibit mPTP opening using an alternative target to CypD.[116] However, it is also possible that CsA does not completely inhibit CypD, due to its poor selectivity, and so treatment with an additional CypD inhibitor would have an additive effect. Furthermore, mitochondrial pore opening has been observed in the presence of CsA and the absence of CypD due to excess Ca^{2+} in the matrix, posing a limitation on CypD as a therapeutic target.[104] 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 adenosine triphosphate-dependent potassium, mitoK_{ATP}, channel is also a potential target of PKCε phosphorylation upon ischemic reperfusion injury (Figure 4C). Initial work suggesting the mitoK_{ATP} channel's cardioprotective role showed that inhibitors of mitoK_{ATP} channel opening, such as glibenclamide and sodium 5-hydroxydecanoate (5-HD), prevent the effect observed during ischemic pre-conditioning in a canine heart

model.[117,118] The mitoK$_{ATP}$ channel is a downstream of PKCε-mediated preconditioning; pharmacological inhibition of mitoK$_{ATP}$ channel opening does not affect PKCε translocation to the mitochondria.[119] In addition, using a reconstituted liposome system, PKCε associates with activated mitoK$_{ATP}$. In this reconstituted system, addition of ROS in the form of H_2O_2 induces potassium flux through the channel, which is abolished in the presence of the selective PKCε inhibitor εV$_{1-2}$ and the mitoK$_{ATP}$ inhibitor 5-HD.[120] In isolated heart mitochondria, treatment with H_2O_2 but not superoxide activates the mitoK$_{ATP}$ channel in a PKCε-dependent manner, as treatment with εV$_{1-2}$ reduces the potassium flux.[121] These results suggest that physiological ROS induce PKCε phosphorylation and open the mitoK$_{ATP}$ channel, which contributes to cardioprotection during (IPC). Although the mechanism by which mitoK$_{ATP}$ opening contributes to cardioprotection is not completely understood, mitoK$_{ATP}$ activation is associated with a reduction of pathological ROS, demonstrated by using 2',7'-dichlorofluorescein as a fluorescent ROS indicator in isolated rat myocytes.[122] Thus, therapeutics that specifically activate the mitoK$_{ATP}$ channel can trigger the downstream cardioprotective preconditioning effects of physiological ROS and prevent the overproduction of pathological ROS.

Several therapeutics aimed at the mitoK$_{ATP}$ channel activation have been tested. Diazoxide is both an opener of mitoK$_{ATP}$ and sarcolemmal K$_{ATP}$ channels, with a much stronger preference for mitoK$_{ATP}$. Diazoxide also elicits a cardioprotective effect, measured by the reduction of cytosolic LDH (lactate dehydrogenase) release (a marker of cell necrosis) in an isolated rat heart model of MI; diazoxide's effect is abolished by the mitoK$_{ATP}$ inhibitor 5-HD.[123] Interestingly, it was suggested that diazoxide activates mitoK$_{ATP}$ channel opening 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 electrical coupling between cardiac cells).[124] However, because diazoxide also has vasodilator and hyperglycemic effects, it is not an ideal pharmacological preconditioning mimetic.[125] A recent review discusses the benefits of additional mitoK$_{ATP}$ activators, such as BMS 180448, BMS 191095, KR31466, and F163.[125] Since the review's publication, two other drugs proposed to target the mitoK$_{ATP}$ 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 effect in an IR rat heart model, measured either by the infarct area or by LDH release. The effect of either drug is abolished in the presence of 5-HD.[126,127] However, in both cases, it is unclear whether the mitoK$_{ATP}$ channel is the direct target of these drugs. Lonidamine, a mPTP opener, abolishes atorvastatin's cardioprotective effect,[127] whereas treatment with an NO synthase blocker, L-nitro-arginine methyl ester (L-NAME), abolished diosgenin's cardioprotective effect.[126] Although both diosgenin and atorvastatin induce pharmacological pre-conditioning that results in mitoK$_{ATP}$ channel opening, neither have been shown to specifically bind and open the mitoK$_{ATP}$ channel.

Another mitochondrial PKCε substrate is the mitochondrial aldehyde dehydrogenase 2 (ALDH2). The major function of ALDH2 is to detoxify reactive aldehyde substrates to their corresponding acids.[128] One of the deleterious effects of pathological ROS is the generation of toxic and reactive aldehydes, such as unsaturated alkynals, 4-hydroxy-2-nonenal (4HNE) and malondialdehyde (MDA), by peroxidation of the mitochondrial lipid membrane.[129]

These reactive aldehydes can lead to mitochondrial dysfunction through covalently binding to and inactivating proteins, lipids and DNA.[130] Notably, reactive aldehyde-mediated mitochondrial dysfunction has long been suspected to be a culprit of many human cardiovascular diseases, such as atherosclerosis, hypertension, peripheral artery disease, cardiomyopathy, MI and heart failure.[131] Our group showed that PKCε phosphorylation of ALDH2 leads to enhanced aldehyde dehydrogenase activity and reduction of toxic aldehydes under oxidative stress.[132] Physiological ROS-mediated activation of PKCε can lead to a protective effect through ALDH2 activation. Therefore, increasing ALDH2's aldehyde detoxification activity directly should also mimic the function of physiological 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 activity due to a single point mutation, resulting in an amino acid substitution of glutamic acid for lysine, in ALDH2 (ALDH2*2).[133] The mutation behaves in an over-dominant fashion so that ALDH2 enzymatic activity is reduced to 20-35% of wild type enzyme in heterozygous individuals. Recent genome-wide association and epidemiological studies provide strong evidence linking ALDH2*2 genotype with the risk of coronary artery disease (CAD),[134,135] MI,[136] hypertension,[137] dyslipidemia,[138] and hyperglycemia.[139] The elevated health risk of cardiovascular diseases that many East Asians may face due to the ALDH2*2 further demonstrates the relevance 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 pathological ROS-induced lipid peroxidation is damaging under stressed conditions. Our group first discovered a class of ALDH2 selective activators, represented by Alda-1 (for **Al**dehyde **d**ehydrogenase **a**ctivator).[140] Alda-1 enhances ALDH2 enzymatic activity by binding to and accelerating the clearance of its aldehyde substrates, such as acetaldehyde and 4HNE.[140–142] Administration of Alda-1 or its analog causes significant cardioprotection against IR injury.[132,140] Targeting ALDH2 by an Alda-like compound is therefore pursued as a potential therapeutic target. In this review, we focus only on more recent advances 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 models. 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 function preservation after IR.[143] Alda-1 treatment is also effective in preventing MI-induced heart failure in rats. Alda-1 given continuously for 4 weeks at 24 hours immediately following the LAD occlusion surgery significantly improves mitochondrial and left ventricular function with concomitant reduction of aldehydic load in cardiomyocytes.[144] Separately, a 6-week delivery of Alda-1 to rats during the heart failure stage results in a significant improvement in heart failure outcome.[145] In both cases, long-term, systemic treatment of Alda-1 has no observable adverse effect. Furthermore, prolonged oral ingestion of Alda-1 together with a high fat-diet for 4 months reduces atherosclerotic lesion by 25% and alleviates liver steatosis, as compared with high-fat diet

alone in an Apo-E knockout mice model.[146] Similar to MI-induced IR injury, in a rat stroke model employing middle cerebral artery occlusion, Alda-1 enhances ALDH2-mediated clearance of 4HNE, MDA and reduces infarct volume, adverse neurological score, and mortality rate.[147] In type I diabetes-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 (AMPK)-dependent autophagy regulation.[148] Alda-1 also improves the viability of induced human pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) after ischemia.[149] In this study, we showed that after an ischemic insult in culture, iPSC-CMs derived from the fibroblasts of ALDH2-deficient human subjects (carrying the inactive mutant ALDH2*2 allele) generate more toxic aldehydes and exhibit increased apoptosis as well as cell cycle arrest as compared to iPSC-CMs 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 iPSC-CMs. These results indicate that ROS-induced ADLH2 activation can be mimicked by direct activation of ALDH2 by a small molecule (such as Alda-1) to produce not only increased cell survival, but also to enhance cardiac repair and regeneration after ischemia.

The case study of ROS-mediated PKCε activation to promote signaling pathways that protect the cell from stress reveals multiple mitochondrial therapeutic targets for cardiovascular disorders, including the mPTP, mitoK_{ATP}, and ALDH2. Pharmacological interventions that mimic ROS-dependent PKCε activation by closing the mPTP, opening the mitoK_{ATP}, and activating ALDH2 are effective therapeutic strategies in the pre-clinical 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.

SECTION IV – Targeting Mitochondria to Prevent Harmful ROS Signaling Through Protein Kinase Cδ and its Downstream Effectors

TPPPKCδ, another member of the PKC family, is implicated in a number of pathological conditions including cancer, stroke, diabetes, neurodegenerative diseases and ischemic heart disease.[150–153] ROS can directly activate PKCδ *via* oxidative modification or/and tyrosine phosphorylation[154] as well as by inducing caspase 3-mediated PKCδ proteolysis.[155,156] For example, glutathione depletion-induced ROS generation of heart-derived H9C2 cells is triggered by caspase 3 and PKCδ activation.[157] Following ischemia, right at the onset of reperfusion, the rise in ROS[158] triggers PKCδ translocation to the mitochondria,[159] 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.[160,161] PKCδ inhibition also reduces endothelial vascular dysfunction, due to eNOS-mediated ROS levels, by increasing survival of coronary endothelial cells.[162] Endoplasmic reticulum (ER) oxidative stress, induced by tunicamycin or by IR in cardiac myocytes, triggers PKCδ localization to the ER and causes cell death that is inhibited by δV1-1.[163] Therefore, in addition to its negative effect at the mitochondria, ROS-induced PKCδ activation disrupts ER homeostasis causing cell death. Together, these results suggest that a selective PKCδ inhibitor might be a useful therapeutic agent against cell injury due to 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.[164] 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 as well as organelles). The binding between those two proteins regulates PKCδ translocation and inhibiting this interaction with a small molecule, JTV519,[165] leads to cardioprotection in a model of MI.[166] 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 following IR damage when administered at the time of reperfusion in an isolated rat heart model.[165] 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 pyruvate dehydrogenase kinase (PDK, a key enzyme leading to the Krebs cycle).[164] Thirty years ago, Patel *et al.* reported a decline in mitochondrial pyruvate dehydrogenase (PDH) activity in ischemic rat heart.[167] Subsequent work demonstrated that once phosphorylated by PKCδ, activated PDK 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.[164] Therefore, specific inhibitors of PKCδ-mediated PDK 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_1F_0ATP synthase.[168] However, cardiac ATP levels do not recover for a long time after IR, resulting in tissue injury,[169] 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 PKCε and the closing of the mPTP. PKCδ activation was also reported to regulate ATP synthase.[170] The study demonstrated that PKCδ's direct interaction with F_1F_0 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 F_1F_0ATP 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 following IR injury, where it mediates mitochondrial elimination by the lysosomal machinery.[80] 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 due to pathological-ROS disruption of micro-mitophagy.

CONCLUSION

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 as well as 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 due to 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 anti-oxidants 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 micro-mitophagy 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 PDK 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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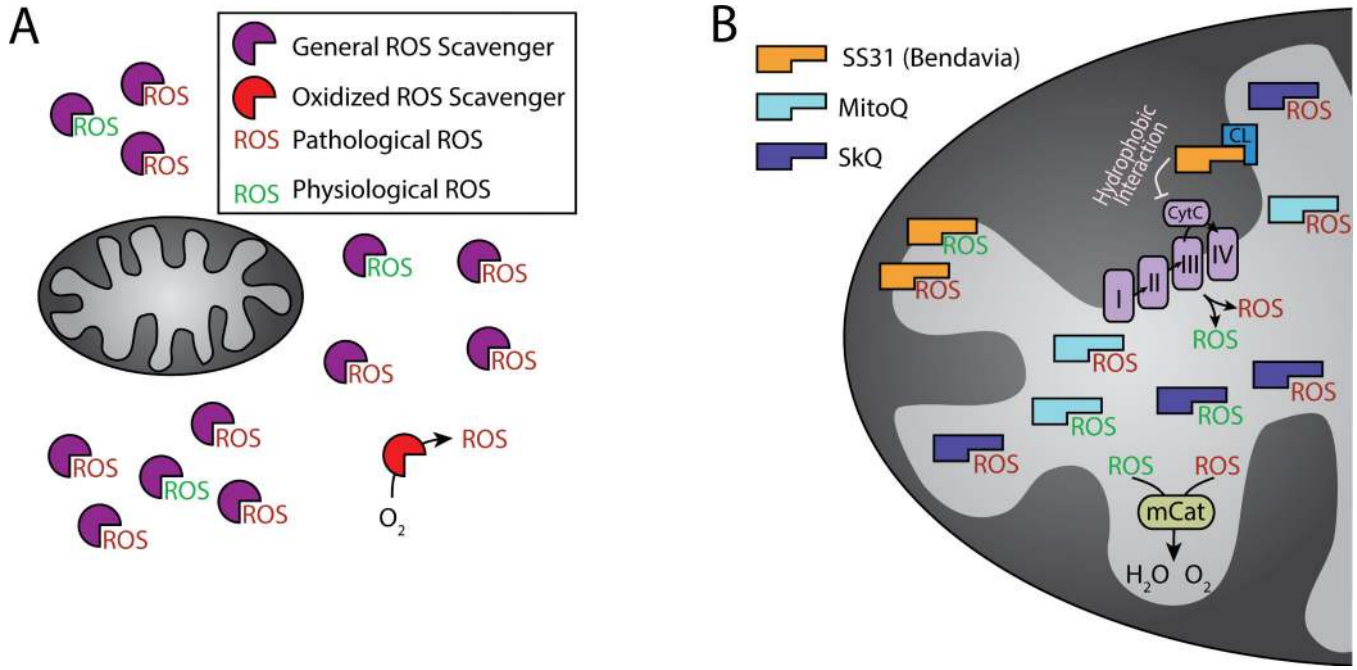

Figure 1.

Targeting ROS using scavengers. A. General ROS scavengers (purple symbol) target and reduce both pathological (in red) and physiological (in 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 anti-oxidants (*e.g.,* 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 pathologic ROS. The electron transport machinery (complexes I-IV) and cytochrome *c* are depicted in the inner mitochondrial membrane/matrix interface. CL = Cardiolipin.

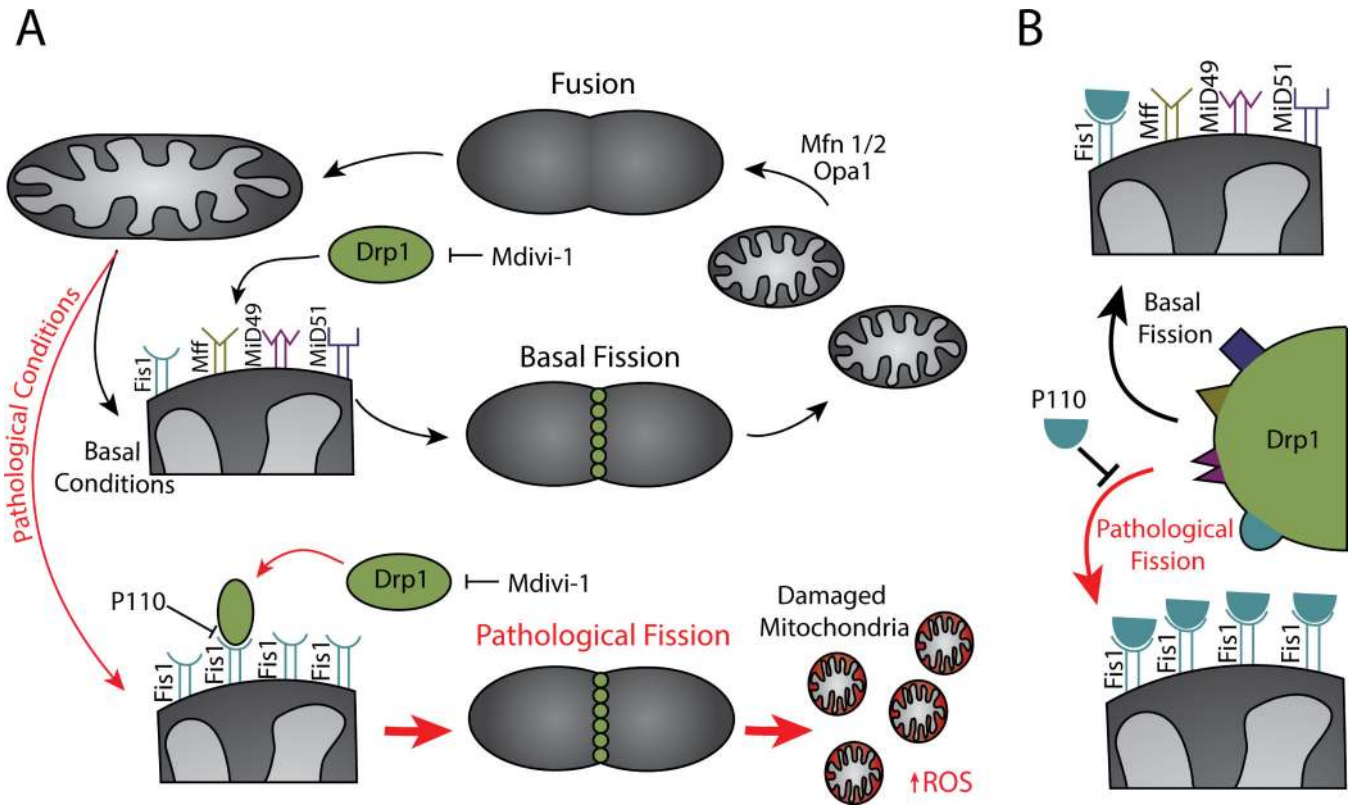

Figure 2.

Targeting defective mitochondria to prevent production of excessive ROS. A. Mitochondrial fusion opposed by either basal or pathological fission including the relevant players. Fission inhibitors Mdivi-1 and P110 inhibit either Drp1 directly or Drp1 recruitment to the mitochondrial membrane, respectively. B. Mechanism of pathological fission inhibition by P110. Depicted are the four mitochondrial adaptors of Drp1 in mammalian cells including Fis1, Mff, 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).

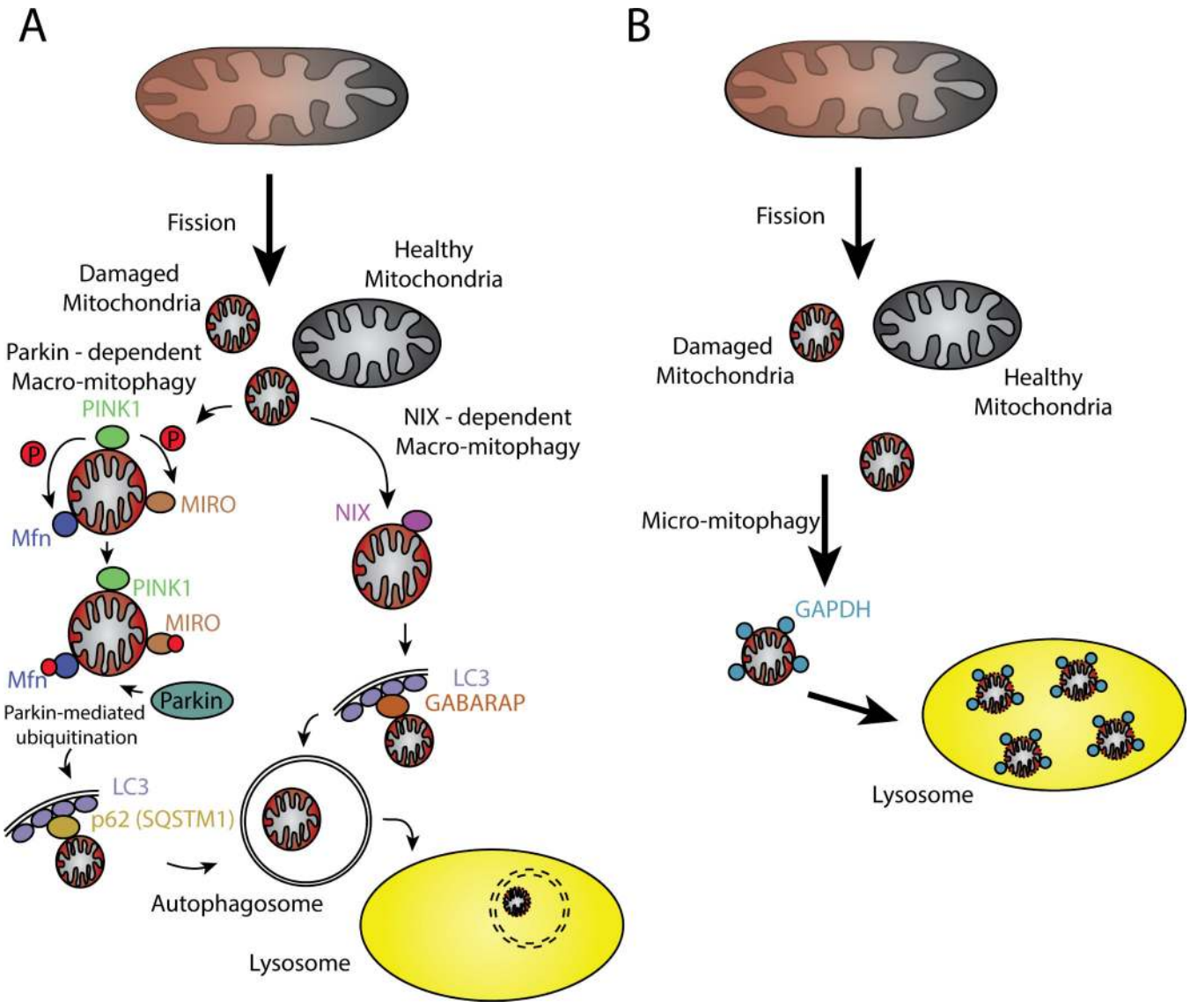

Figure 3.

Targeting damaged mitochondrial clearance to prevent production of excessive ROS. A. Parkin- and NIX-dependent macro-mitophagy. B. GAPDH-dependent micro-mitophagy. See text for details.

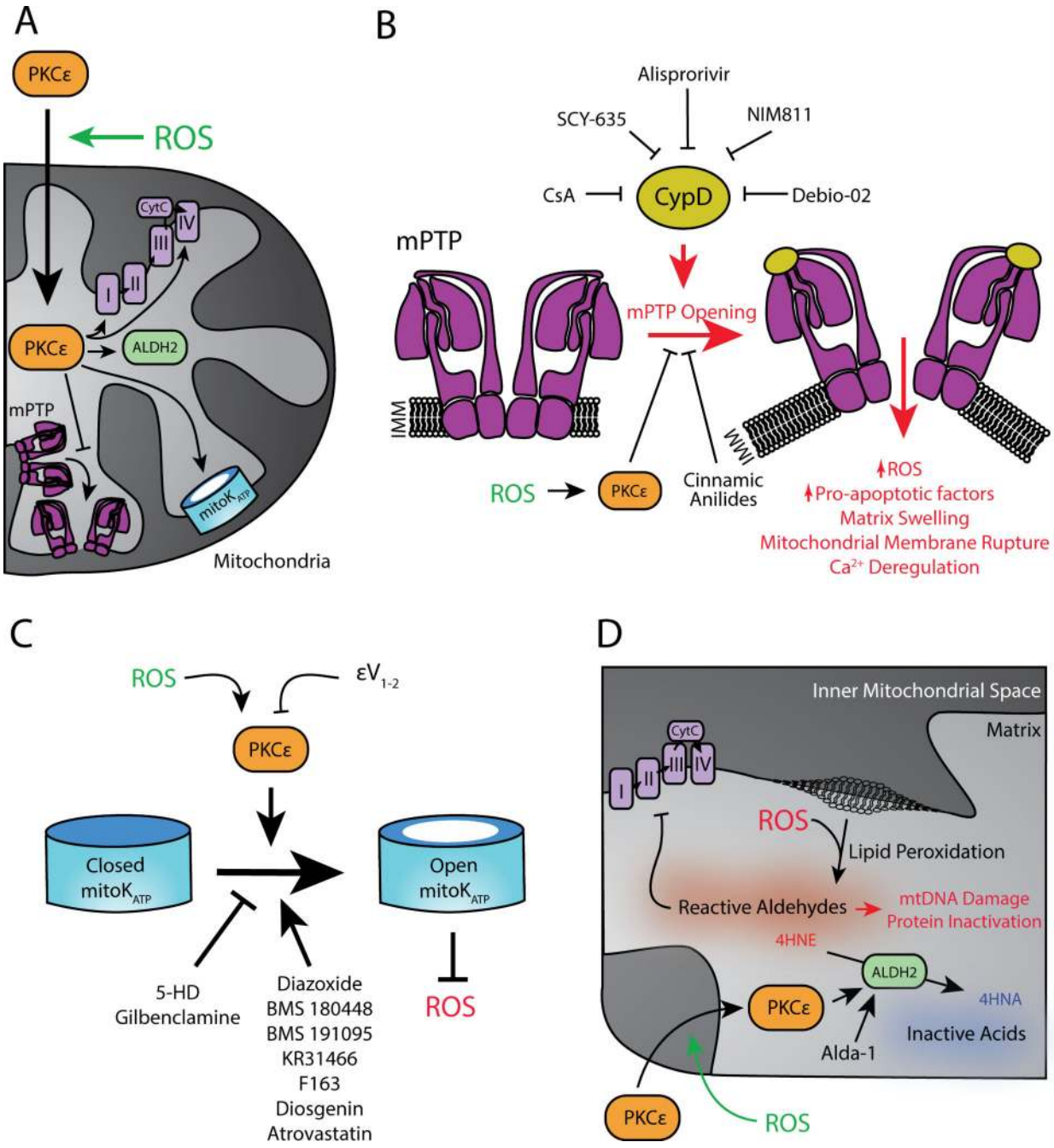

Figure 4.

Targeting downstream ROS signaling pathways that are beneficial for the cell. A. Summary of physiological ROS-mediated PKCε activation and translocation to the mitochondria and its mitochondrial substrates. B. Pharmacological inhibition of mPTP opening as a therapeutic strategy. C. Activation of the mitoK$_{ATP}$ channel as a therapeutic strategy. D. Activation of the mitochondrial ALDH2 as a therapeutic strategy and the molecular events affected by it. See text for details. IMM = inner mitochondrial membrane.

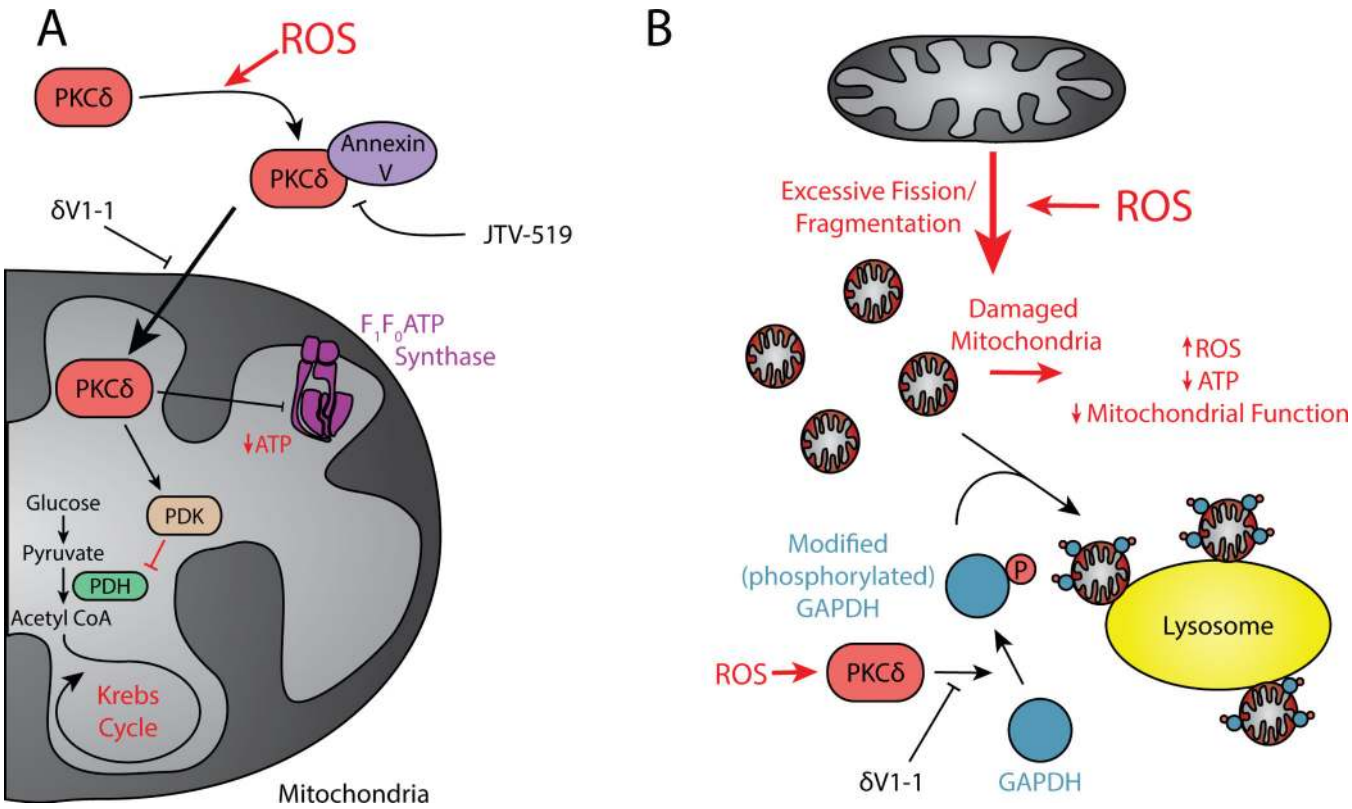

Figure 5.

Targeting downstream ROS signaling pathways that are harmful for the cell. A. Pathological ROS-mediated PKCδ activation and translocation to the mitochondria. Once at the mitochondria, PKCδ activates PDK, which in turn inhibits PDH and entrance to the Krebs cycle. In addition, PKCδ reduces ATP production by targeting F_1F_0 ATP synthase. B. Pathological ROS-mediated PKCδ disruption of micro-mitophagy. See text for details.

Table 1

Summary of selected clinical trials using anti-oxidants as therapeutics for cardiovascular diseases (based on the review by Sugamura and Keaney).[6]

Anti-oxidant	Indication	Outcome	Comments	Ref.
Primary Prevention				
α-lipoic acid		N/A	Recruiting	21
β-carotene		Ineffective or harmful	Completed (higher incidence of lung cancer)	6 *
Vitamin C		Effective	Lowered cardiovascular disease risk	6 *
		Ineffective	Completed	6 *
Vitamin E		Effective	Associated with lower cardiovascular disease risk	6 *
		Ineffective or harmful	Completed (increased HF)	6 *
Secondary Prevention				
α-lipoic acid	Type II diabetes / CAD	N/A	Recruiting	20
β-carotene	MI	Harmful	Increased CAD risk	6 *
Allopurinol	HF	Effective	Reversed endothelial dysfunction	6 *
	HF	Ineffective	Completed	6 *
Co-enzyme Q10	Idiopathic dilated cardiomyopathy - Children	Effective	Improved diastolic function	171
Edaravone	MI	Effective	Decreased infarct size and reperfusion arrhythmia	6 *
L-carnitine	CAD	Effective	Increase anti-oxidant activity	16
L-carnitine	MI	Effective	Reduced level of cardiac MI markers	17
Melatonin	MI	N/A	Recruiting	22,172
	MI	Effective	Reduced nephrotoxicity	173
N-acetylcysteine	HF & renal failure	Ineffective	Increased blood flow to forearm	14
	ACS	Ineffective	Completed	12
	Post angiography	N/A	Recruiting	15
Oxypurinol	HF	Ineffective	Completed	6 *
Probucol	Post PCI	Effective	Prevented restenosis	6 *
	CAD - Women	Harmful	Increased mortality	6 *
Vitamin C	CAD	Effective	Improved endothelial function	6 *
	Variant angina	Effective	Attenuated vasomotor dysfunction	6 *
	CAD	Effective	Reduced CAD events	6 *
	Hemodialysis	Effective	Reduced cardiovascular events	6 *
Vitamin E	MI	Ineffective	Completed	6 *
	Variant angina	Effective	Reversed endothelial dysfunction	6 *
	HF	Ineffective	Completed	6 *

Anti-oxidant	Indication	Outcome	Comments	Ref.
Succinobucol	ACS	Ineffective or harmful	Completed (increased onset atrial fibrillation and bleeding events)	6 *

HF=heart failure; MI=myocardial infarction; CAD=coronary artery disease; ACS=acute coronary syndrome; PCI= Percutaneous coronary intervention.

* Referenced trials can be found in Table 1 of Sugamura and Keaney's review.