Winter 3-31-2014

Mitochondrial Reactive Oxygen Species (ROS): Which ROS is Responsible for Cardioprotective Signaling?

Anders Olav Garlid
Portland State University

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by

Anders Olav Garlid

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biology

Thesis Committee:
Jason Podrabsky, Chair
Stanley Hillman
Suzanne Estes

Portland State University
2014

Abstract

Mitochondria are the major effectors of cardioprotection by procedures that open the mitochondrial ATP-sensitive potassium channel (mitoK$_{ATP}$), including ischemic and pharmacological preconditioning. MitoK$_{ATP}$ opening leads to increased reactive oxygen species (ROS), which then activate a mitoK$_{ATP}$-associated PKCε, which phosphorylates mitoK$_{ATP}$ and leaves it in a persistent open state (Costa, ADT and Garlid, KD. Am J Physiol 295, H874-82, 2008). Superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$) have each been proposed as the signaling ROS but the identity of the ROS responsible for this feedback effect is not known. Superoxide was excluded in earlier work on the basis that it does not activate PKCε and does not induce mitoK$_{ATP}$ opening (20). To further examine the identity of the signaling ROS, respiring rat heart mitochondria were preincubated with ATP and diazoxide to induce the phosphorylation-dependent open state, together with agents that may interrupt feedback activation of mitoK$_{ATP}$ by ROS scavenging or by blocking ROS transformations. Swelling assays of the preincubated mitochondria revealed that dimethylsulfoxide (DMSO), dimethylformamide (DMF), deferoxamine, trolox, and bromoenol lactone (BEL) each blocked the ROS-dependent open state but catalase did not interfere with this step. The lack of a catalase effect and the inhibitory effects of agents acting downstream of $HO^\bullet$ excludes H_2O_2 as the endogenous signaling ROS and focuses attention on $HO^\bullet$. In support of the hypothesis that $HO^\bullet$ is required, we also found that $HO^\bullet$-scavenging by DMF

blocked cardioprotection by both ischemic preconditioning and diazoxide in the Langendorff perfused rat heart. HO$^{\bullet}$ itself cannot act as a signaling molecule, because its lifetime is too short and it reacts immediately with nearest neighbor phospholipids and proteins. Therefore, these findings point to a product of phospholipid peroxidation, such as hydroperoxy-fatty acids. Indeed, this hypothesis was supported by the finding that hydroperoxylinoleic acid (LAOOH) opens the ATP-inhibited mitoK$_{ATP}$ in isolated mitochondria. This effect was blocked by the specific PKCε inhibitor peptide εV$_{1-2}$, showing that LAOOH activates the mitoK$_{ATP}$-associated PKCε. During ischemia, catabolism of mitochondrial phospholipids is accelerated, causing accumulation of plasmalogens and free fatty acids (FA) in the heart by the action of calcium-independent phospholipases A2 (iPLA2). We first assessed the role of FAs and hydroxy FAs on mitoK$_{ATP}$ opening and cardioprotection. Swelling assays of isolated rat heart mitochondria showed that naturally formed free FAs inhibit mitoK$_{ATP}$ opening and that they are more potent inhibitors of the pharmacological open state of mitoK$_{ATP}$ than the phosphorylation-dependent open state. That is, sustained mitoK$_{ATP}$ opening induced by the phosphorylation-dependent feedback loop is more resistant to FA inhibition than direct mitoK$_{ATP}$ opening by a potassium channel opener. Moreover, rat hearts perfused with micromolar concentrations of FA were resistant to cardioprotection by diazoxide or ischemic preconditioning. Racemic bromoenol lactone (BEL), a selective inhibitor of iPLA2, confers protection to otherwise untreated Langendorff perfused hearts by

preventing ischemic FA release. To bring this story full circle, BEL blocks

protection afforded by preconditioning and postconditioning by preventing the

iPLA2-mediated release of FAOOH generated in the conditioned heart. $HO^{\cdot}$

resulting from $mitoK_{ATP}$ opening oxidizes polyunsaturated fatty acid components

of the membrane phospholipids, resulting in a peroxidized side chain. FAOOH

must be released in order to act on the mitochondrial $PKC\varepsilon$, and this is achieved

by the action of iPLA2. iPLA2 is essential for most modes of cardioprotection

because it catalyzes the release of FAOOH. This fully supports the hypothesis

that the second messenger of cardioprotective ROS-mediated signaling is

hydroperoxy fatty acid (FAOOH), a downstream oxidation product of $HO^{\cdot}$.

Dedication

I dedicate this thesis to my late mentor and friend, William Stanley, and to my parents, Keith Garlid and Randi Brannan, all of whom inspired me, kept me energized and put up with me through it all.

Acknowledgments

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Glossary List/Abbreviations

ETS	electron transport system
F_1,F_o-ATPase	ATP synthase
K^+/H^+ antiporter	inner membrane mitochondrial protein for potassium and hydrogen exchange
$MitoK_{ATP}$	mitochondrial ATP-sensitive K^+ channel
Na,K-ATPase	sodium-potassium adenosine triphosphatase (Na^+-K^+ pump)
$\Delta\Psi$	membrane potential
VDAC	voltage-dependent anion channels
ANT	adenine nucleotide translocator
Kir	inward-rectifying potassium channel
SUR	sulfonyl-urea receptor
ROS	reactive oxygen species
$O_2^{\bullet-}$	superoxide
H_2O_2	hydrogen peroxide
$HO^{\bullet}$	hydroxyl radical
FA	fatty acid
FAOOH	hydroperoxy fatty acid
PKC	protein kinase C
GPCR	G_i-protein coupled receptor
SOD	superoxide dismutase (converts $O_2^{\bullet-}$ into H_2O_2)
iPLA2	calcium-independent phospholipase A2
KCO	potassium channel opener
IPC	ischemic preconditioning
MPG	mercaptopropionyl glycine (thiolreductant, $HO^{\bullet}$ scavenger)
Dzx	diazoxide (potassium channel opener)
5-HD	5-hydroxy decanoate ($mitoK_{ATP}$ channel inhibitor)
εV_{1-2}	epsilon-V_{1-2} (PKCε inhibitor)
CCCP	carbonyl cyanide m-chlorophenyl hydrazine (protonophore)
BEL	bromoenol lactone (iPLA2 inhibitor)
Dfo	deferoxamine (iron scavenger)
DMF	dimethyl formamide (hydroxyl radical scavenger)
DMSO	dimethylsulfoxide (hydroxyl radical scavenger)
Trolox	chain breaking antioxidant, peroxyl radical scavenger
Catalase	decomposes H_2O_2 into water and oxygen

Chapter 1: Literature Review

"From the Chemiosmotic Theory to Signaling in the Heart"

Mitochondria

Mitochondria produce ATP by oxidative phosphorylation, which uses the chemical energy of substrate oxidation. The mechanism by which substrate oxidation is coupled to phosphorylation remained elusive for many years. The prevailing assumption held that there must be a high-energy chemical intermediate that couples these processes, but no such intermediate could be found. In 1961, Peter Mitchell proposed a novel hypothesis to explain the coupling phenomenon, completely independent of experimental evidence (98). Within a decade, his model was widely accepted, and in 1978 he was awarded the Nobel Prize for this massive accomplishment.
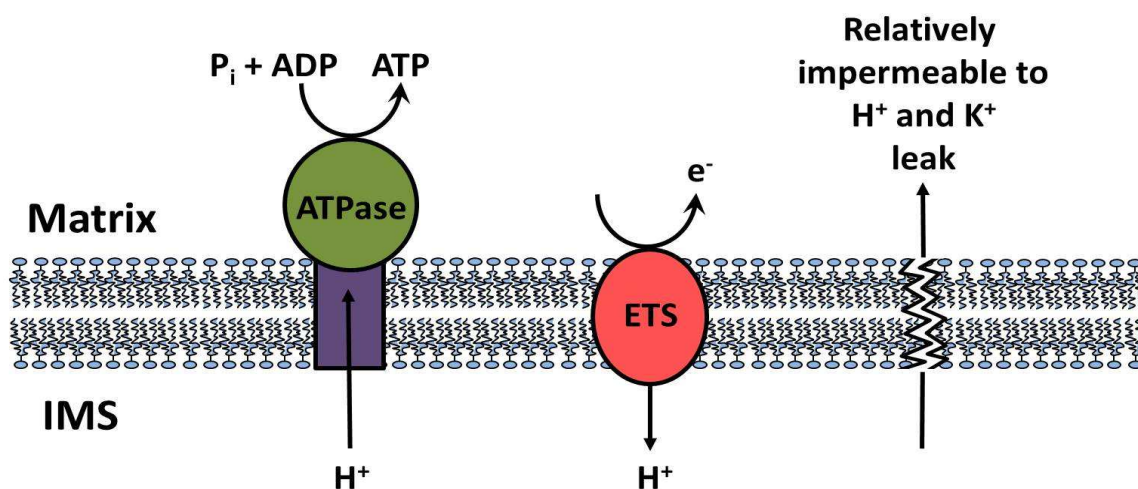
Fig. 1.1: *The first three postulates of Mitchell's chemiosmotic theory. 1)* The vectorially-oriented F_1, F_0-ATPase pumps hydrogen ions (H^+) out of the matrix to produce ATP from ADP with the addition of phosphate (P_i). *2)* The electron transport system (ETS) harnesses the chemical energy of substrate oxidation and drives protons out of the matrix. *3)* The mitochondrial inner membrane is relatively impermeable to protons. Taken together, these three properties establish a kind of protonic battery that generates an electricochemical gradient across the membrane in the form of membrane potential ($\Delta\Psi$).

Chemiosmotic Theory

Mitchell's hypothesis is comprised of four basic postulates, three of which define the systems of the battery required for ATP synthesis and a fourth which describes how mitochondria must deal with the physiological stresses imposed by the chemiosmotic mechanisms.

Mitchell's first three postulates regarding ATP synthesis are as follows, and are depicted in **Fig. 1.1**: (Postulate 1) The F_1,F_0-ATPase is a vectorially oriented enzyme that pumps hydrogen ions out of the mitochondrial matrix, using the energy of ATP hydrolysis. (Postulate 2) The electron transport system (ETS) is a series of vectorially oriented enzymes that harness the chemical energy of substrate oxidation and, through a series of oxidation-reduction reactions, drives protons out of the matrix. This action creates an electrochemical gradient for protons, which Mitchell called the protonmotive force. Because the F_1,F_0-ATPase is reversible, the protons pumped outward by the ETS can flow inward through the enzyme, coupling the synthesis of ATP to the inward translocation of protons. Thus, the protonmotive force is the missing connection that couples oxidation to phosphorylation.

In order for this system to synthesize ATP (Postulate 3), the membrane containing the F_1,F_0-ATPase and the ETS enzymes must be relatively impermeable to ions, and especially to protons. The protonic battery would be short-circuited and no ATP would be synthesized if protons were permitted to leak across the membrane. The vectorial nature of the ETS and F_1,F_0-ATPase and a relatively impermeable inner membrane were completely novel concepts at

the time but they form the basis of our understanding of oxidative-

phosphorylation today (97).

The protonic battery described by the first three postulates generates a

very high membrane potential ($\Delta\Psi$) due to the constant extrusion of protons by

the ETS. This will drive uptake of K^+ ions into the mitochondrial matrix so as to

balance the charge between the IMS and the matrix. The influx of K^+ ions draws

water into the matrix due to osmotic demands, causing its volume to increase at

such a rate that the mitochondrion would rupture within minutes (48). Moreover,

the tricarboxylic acid cycle requires uptake of substrate anions against their

electrochemical gradient. To address these inherent physiological requirements,

Mitchell proposed (Postulate 4) the existence of cation/H^+ antiporters and anion

exchangers that operate electroneutrally, thereby allowing essential metabolites

to enter and excess cations to exit in order to maintain volume homeostasis (38,

97).

Ion diffusion across the mitochondrial inner membrane

Ions, such as K^+ and H^+, diffuse across the mitochondrial inner membrane

at rates largely determined by the electrical gradient. The phospholipid bilayer

presents a large energy barrier that ions must cross to gain access to the other

side of the membrane and the permeability coefficient of the membrane is a

function of this energy barrier. Experiments are consistent with the location of the

energy barrier peak at the center of the inner membrane such that only ions with

a high enough energy get access to the other side (44). This is the rate-limiting step of ion diffusion across the membrane (44). The probability of crossing this energy barrier is given by the Boltzmann distribution function, $e^{\left(-\frac{\Delta\tilde{\mu}_p}{RT}\right)}$, where $\Delta\tilde{\mu}_p \equiv \tilde{\mu}_p - \tilde{\mu}_{aq}$ is the Gibbs energy of the ion at the peak of the energy barrier (μ_p) relative to its value in the energy well at the surface of the membrane (μ_{aq}). The flux expression $J = fP(C_{1o}e^{\frac{u}{2}} - C_{2o}e^{-\frac{u}{2}})$ is derived from this probability function to quantify the diffusion of cations across the inner membrane. Here, u is the reduced voltage ($zF\Delta\psi / RT$), divided by 2 to account for the fact that the barrier is at the center of the membrane, C_{1o} and C_{2o} are bulk aqueous concentrations at the outer and inner surfaces of the inner membrane, respectively, f is the surface partition coefficient, and P is the permeability constant. The second term in this equation accounts for backflux of cations out of the matrix. But the high membrane potential renders backflux negligible and the second term can be dropped, leaving a simpler function, $J = fP(C_{1o}e^{\frac{u}{2}})$ (44).

Note that at high membrane potentials such as those seen in mitochondria, ion-diffusion is minimally affected by concentration gradients across the membrane. Note as well that all diffusing cations face the same rate limiting step of crossing the energy barrier. These predictions were confirmed by the Garlid lab with the demonstration that TEA$^+$ and H$^+$ diffusion across the mitochondrial inner membrane are quantitatively identical, differing only in their permeability coefficient. Therefore, H$^+$ ions are transported in the same way as

cations in general and there is apparently no unique mechanism for their diffusion across the inner mitochondrial membrane (44).

Potassium cycle (K$^+$-cycle)

The K$^+$-cycle depicted in **Fig. 1.2** describes the flux of potassium across the inner membrane and the associated processes of electron transport (ETS) and anion flux via the Pi$^-$/H$^+$ symporter. Although the inner membrane is relatively impermeable to ions, inward leak of K$^+$ does occur at significant rates. Net uptake of K$^+$ at the expense of proton ejection by the ETS will drive net uptake of P$_i$ and other ions. This salt uptake, in turn, will drive net uptake of water, with matrix swelling. Therefore, this inward K$^+$ leak, if left unchecked, will result in excessive matrix swelling and eventual lysis of the mitochondrion (37, 48).

The inward flux of K$^+$ driven by the extrusion of H$^+$ ions during electron transport alkalinizes the matrix, and the resulting pH gradient causes P$_i$$^-$ and H$^+$

Fig. 1.2: *The mitochondrial potassium cycle.* The K$^+$-cycle is comprised of the electron transport system (ETS), which sets up the electrochemical gradient in the form of the protonmotive force. The P$_i$$^-$/H$^+$ symporter (P$_i$$^-$/H$^+$) provides the matrix with necessary anions and the K$^+$/H$^+$ antiporter ejects K$^+$ to avoid excessive matrix swelling due to potassium leak (K$^+$ leak). The mitochondrial ATP-sensitive K$^+$ channel (mitoK$_{ATP}$) is responsible for preventing matrix contracture during high work states by importing K$^+$ into the matrix.

to enter through the P_i^-/H^+ symporter. Additionally, as ion concentrations change with the rush of K^+ into the matrix, water is osmotically obligated to accompany the K^+ salt, causing matrix swelling. This sets up the physiological necessity of the ion exchange systems described by Mitchell's fourth postulate. Thus, the K^+/H^+ antiporter must eject excess K^+ in exchange for a proton to maintain electroneutrality (48). Moreover, it must eject only the excess K^+, because matrix K^+ is the primary osmolyte of the matrix.

Mitchell and Moyle initially uncovered evidence for Na^+/H^+ and K^+/H^+ antiport by measuring swelling in sodium acetate (NaOAc) and potassium acetate (KOAc) media (48). However, while Na^+/H^+ exchange was robust, K^+/H^+ exchange was barely detectable and not sufficient to compensate for K^+ leak. Direct evidence for K^+/H^+ antiport was provided by Garlid (42), who showed that swelling induces electroneutral potassium efflux (42). Mitochondria retain matrix K^+ in isotonic sucrose, which is too large to cross the inner membrane. In the hypotonic sucrose media, water enters the matrix in an attempt to dilute solute concentrations and achieve an osmotic balance, causing matrix swelling. The mitochondria respond by rapidly ejecting K^+ from the matrix in a passive and electroneutral manner (42, 48).

Several possible mechanisms exist for the regulation of this antiporter. The Mg^{2+} Carrier Brake Hypothesis states that the mitochondrial K^+/H^+ antiporter is always partially inhibited by Mg^{2+} on the matrix side of the enzyme and that it uses small changes in free Mg^{2+} concentrations to regulate matrix volume (39). Matrix Mg^{2+} content is relatively stable but ion concentrations decrease by

dilution when the matrix swells and Mg^{2+} activity is further decreased by the formation of complexes with incoming anions such as citrate and phosphate. This lowers the inhibition on the K$^+$/H$^+$ antiporter, allowing ejection of K$^+$ and a return to normal volume. This mechanism provides high sensitivity to changes in matrix volume and prevents futile cycling of K$^+$ by tuning the K$^+$/H$^+$ antiporter to match K$^+$ uniport.

Fig. 1.3: *Flux (J/J$_o$) vs. membrane potential (ΔΨ).*The exponential relationship between ΔΨ and *J/J$_o$* means that a very small change in ΔΨ results in a very large change in flux.

K$^+$/H$^+$ antiport is also inhibited by protons on the matrix side; H$^+$ concentration decreases below inhibitory concentration as the matrix swells and is alkalinized by the K$^+$ for H$^+$ exchange during electron transport (39). Under experimental conditions with no Mg^{2+} present, matrix volume still regulates K$^+$/H$^+$ exchange by an unknown mechanism. This may be due to other inhibitory ions or conformational changes experienced by the enzyme as the membrane is stretched (39, 48).

Mitochondrial ATP-sensitive potassium channel (mitoK$_{ATP}$)

A K$^+$ uniporter was reconstituted from mitochondria by Mironova et al. (96) and Diwan et al. (26) but its regulation and amino acid sequences were not

determined. This raised a new problem – mitochondria were already having to deal with K$^+$ leak, why would they need a K$^+$ channel?

It was proposed that a K$^+$ channel is needed for volume homeostasis in high work states (48). ATP production and oxygen consumption in the heart can increase up to 8-fold of their normal levels in a high work state (40). Higher rates of ATP synthesis arise from greater H$^+$ ejection and the greater H$^+$ current will cause electrical potential ($\Delta\Psi$) to drop based on the internal resistance of the electron transport system. The relationship between flux and $\Delta\Psi$ is exponential, as seen in **Fig. 1.3**. A small change in membrane potential results in a very large change in flux (48, 107). Therefore, under high work states and reduced $\Delta\Psi$, K$^+$ flux is greatly diminished and the mitochondrial matrix will contract, that is, the matrix volume will decrease. The K$^+$/H$^+$ antiporter senses the reduced volume by the mechanisms discussed in the previous section and slows K$^+$ ejection to establish a lower steady state matrix volume.

While this prevents matrix collapse, the matrix is still contracted, and this compromises the efficiency of energy transfer in heart due to the larger intermembrane space. This limits nucleotide transport through voltage-dependent anion channels (VDAC) across the outer membrane (28), and VDAC has been shown to control outer membrane permeability to ADP and ATP (122). VDAC exists in a low-conductance state in the heart that is not highly permeable to nucleotides. Creatine and creatine phosphate mediate energy transfers between mitochondria and cytosol through mitochondrial creatine kinase (123). Creatine

kinase spans the intermembrane space in an octameric arrangement, bridging the gap between the outer membrane VDAC and inner membrane adenine nucleotide translocator (ANT), as shown in **Fig. 1.4** (127, 147). Creatine kinase has been shown to bind to VDAC (126) and Garlid et al. hypothesized that it is this interaction that confers the low-conductance state to VDAC, preventing the outer membrane from being overly permeable to nucleotides (46). This binding requires a narrow intermembrane space to allow for a tight interaction between the octamer and VDAC. However, high work states lead to matrix contraction due to the decrease in $\Delta\Psi$ and creatine kinase would dissociate from VDAC, leading to higher ATP and ADP conductance at the outer membrane (46). The exponential relationship between flux and membrane potential sets up a situation in which effective energy transfer is compromised by matrix contraction at precisely the moment that it is needed most.

The exponential relationship between K^+ flux and its driving force, $\Delta\Psi$, demonstrates the need for another volume regulation channel that imports K^+ into the matrix to prevent contraction when $\Delta\Psi$ is decreased under high work states. The mitochondrial ATP-sensitive potassium channel (mitoK$_{ATP}$) adds a conductance pathway that is parallel to K^+ leak to restore matrix volume (48). It might be expected that $\Delta\Psi$ would decrease with this additional pathway for K^+ transport, reducing K^+ flux even further and exacerbating the problem of matrix contraction. However, mitoK$_{ATP}$ opening has been shown to reduce $\Delta\Psi$ by only about 1 mV, which is relatively insignificant in the normal physiological state;

mitochondria typically experience membrane potentials around 140 -180 mV (37, 40).

MitoK$_{ATP}$ was reconstituted by the Garlid lab, showing that the inner membrane contains an ATP-dependent K$^+$ channel (62, 66, 116). K$_{ATP}$ channels are comprised of a complex of two different proteins, including an inwardly-rectifying potassium channel (Kir) subunit and the sulfonylurea receptor (SUR) (69). Four of the Kir proteins are thought to form the K$_{ATP}$ channel pore and the SUR is responsible for regulation of the channel and its sensitivity to ATP and pharmacologic agents (1, 60). In 2012, Brian Foster et al. showed that the ROMK channel, another ATP-dependent potassium channel (Kir1.1), is a molecular

Fig. 1.4: *MitoK$_{ATP}$ regulation of VDAC permeability to nucleotides during ischemia and during high rates of ATP production.* $\Delta\Psi$ is supported by ATP hydrolysis during ischemia and by electron transport during the high work-state. Both of these stresses will cause a decrease in $\Delta\Psi$, resulting in reduced uptake of K+ salts and water, contraction of the matrix, and expansion of the IMS. This structural change can be prevented by a compensatory increase in K+ conductance mediated by opening mitoK$_{ATP}$. If mitoK$_{ATP}$ is blocked or does not open, the IMS expansion results in increased outer membrane permeability to ATP and ADP. During ischemia, this means that all of cellular ATP is available for hydrolysis by mitochondria, with consequent degradative loss of adenine nucleotides, and, ultimately, unavailability of ADP for rephosphorylation upon reperfusion. During the high work state, increased VDAC permeability to ADP and ATP constitutes a diversionary leak in the system away from the more efficient metabolic channeling through mitochondrial creatine kinase ("CK"). Consequently, if mitoK$_{ATP}$ is blocked, mitochondria cannot supply ATP at the high rates required, and the heart cannot respond to inotropic stress. From Garlid et al., 2003 (46).

subcomponent of the mitoK$_{ATP}$ channel (34).

MitoK$_{ATP}$ activity can be measured by light scattering, taking advantage of volume changes resulting from ion and water flux across the inner membrane (9, 43). The amount of light scattered by suspended mitochondria is a function of matrix volume (136), where an increase in volume accompanies a decrease in the light scattering signal intensity (9). Mitochondria were first reported to take up water with decreasing osmolality of their surrounding solution by Claude in 1946. They have since been shown to act as good osmometers, following the equation

$$W_m = W_b + \frac{S_o}{\phi}$$

where W_m is matrix water content (mg of H$_2$O/mg of protein), W_b is osmotically inactive water, S_o is water solute content (nosmol/mg), and ϕ is the osmolality of the containing medium (nosmol/mg of H$_2$O) (41). Direct measurement of matrix volume is an impractical method for solving this equation for rates of solute transport. Light scattering provides a reliable method by which to address this issue.

Ischemia and cardioprotection

Cardiovascular diseases are the leading causes of morbidity and mortality in man and ischemic heart disease is the leading cause of death in developed countries. Ischemia is the loss of blood flow to an organ, and extended periods of ischemia result in a large amount of cell death in the affected area, known as infarction, due to the lack of necessary nutrients (29). However, the damage

does not present itself during the ischemic phase but rather upon reperfusion. The influx of fresh blood to the heart following a heart attack brings with it a large concentration of oxygen that quickly forms damaging reactive oxygen species. These proceed to overload antioxidant systems and cause irreparable damage to cardiac tissue. Preventive measures or medications that could render the heart resistant to ischemia or reperfusion injury following myocardial infarction would be an ideal mechanism for reducing the morbidity from heart disease. Our main method for reducing damage is to reperfuse the heart as quickly as possible, but more work must be done to salvage the myocardium during the acute phase of ischemia.

Cardioprotection by ischemic preconditioning and postconditioning

In1986, Murry et al. demonstrated that the heart could be conditioned with a brief period of ischemia prior to a longer period of ischemia, thus rendering the myocardium less susceptible to ischemic injury (102). A typical perfused heart can present up to 80% infarction of the myocardium after being subjected to an experimentally-induced heart attack or "index ischemia". "Ischemic preconditioning" (IPC) drastically reduced the infarct size to just 25% compared to untreated hearts and has since been studied extensively to determine the mechanism behind this intrinsic form of cardioprotection (29). Ischemic postconditioning (155) operates in a similar manner, restricting the flow of blood to the heart upon reperfusion through short bursts of reperfusion and ischemia prior to a full reperfusion.

Cardioprotection by potassium channel openers: Cardioprotection can be achieved through preconditioning with potassium channel openers (KCOs) such as cromakalim, which mimic the protective effects of IPC. This was first observed in 1987 by Gross et al., using the KCO nicorandil, although it had not yet been classified as such (56). This class of drugs was first intentionally explored for their ability to confer protection by Grover et al. (61) and many more were demonstrated to have these effects in all animal models. K_{ATP} channel blockers such as glibenclamide and 5-hydroxydecanoate (5-HD) abolish the protection afforded by cromakalim. That KCOs mimic the cardioprotective effect of IPC implicates K_{ATP} channels as the mechanism of action of IPC. Accordingly, Gross et al. demonstrated that KCO inhibitors block the protective effects of IPC. These results imply that K_{ATP} channels are necessary for IPC and, presumably, for cardioprotection in general.

KCO protection by mitoK_{ATP}: The protective effects of KCOs were universally attributed to sarcolemmal K_{ATP} channel (sarcK_{ATP}) opening and a resultant "cardioplegic" effect (49). SarcK_{ATP} channel opening was thought to mimic IPC by causing a reduction in action potential duration (APD) that would reduce Ca^{2+} influx into the cytosol and result in cardioplegia, a temporary reduction or cessation of cardiac activity. However, KCOs do not effect the contractility of the heart muscle or result in any form of negative isotropy seen in the cardiodepression associated with a cardioplegic effect (57). It was soon demonstrated that the cardioprotective effect of KCOs is not correlated to APD

shortening (58, 59, 151). Furthermore, vasodilation using KCOs is minimal, suggesting that this effect is not important for cardioprotection.

The lack of cardiodepression, APD shortening and vasodilation suggests that the target of KCOs is distinct from sarcolemmal K_{ATP} (49). There are K_{ATP} channels expressed in the mitochondrial membrane, mitoK_{ATP}, which regulate mitochondrial volume and energetics (70) and are opened by KCOs in their cardioprotective concentration range (52). Garlid proposed that mitoK_{ATP} and not sarcK_{ATP} is responsible for cardioprotection (37).

The role of mitoK_{ATP} channels in cardioprotection was confirmed by Garlid et al. with a series of experiments using diazoxide and cromakalim (49). Cromakalim is an effective cardioprotective agent that opens both sarcolemmal K_{ATP} (sarcK_{ATP}) and mitochondrial K_{ATP} (mitoK_{ATP}) with equal sensitivities. Diazoxide, on the other hand, opened bovine cardiac mitoK_{ATP} at a concentration 2000 times lower than in sarcK_{ATP}, making it a very useful tool for separating the effects of sarcK_{ATP} and mitoK_{ATP} (50). To that end, the same degree of cardioprotection using cromakalim was attained with diazoxide at concentrations well below its $k_{1/2}$ for sarcK_{ATP} opening, strongly suggesting mediation by mitoK_{ATP} (49). Furthermore, diazoxide is a potent K_{ATP} opener and effective cardioprotectant, but it has a weak APD shortening effect and it is a less potent vasodilator than cromakalim. These results support the conclusion that vasodilation and APD shortening are not correlated with the cardioprotective effects of KCOs or IPC (49).

Further evidence for mitochondrial involvement in cardioprotection was obtained using the K_{ATP} channel inhibitor 5-HD. While 5-HD is an effective inhibitor of mitoK_{ATP} opening by cromakalim, it does not inhibit sarcK_{ATP} opening (94), providing another useful tool with which to distinguish the two K_{ATP} channels. Protection by cromakalim and diazoxide were inhibited by 5-HD with no effect on their APD shortening activity (94). Taken together, these results demonstrate that it is not sarcolemmal but mitochondrial K_{ATP} channels that mediate cardioprotection by KCOs (49). Ischemic preconditioning is also blocked by KCO inhibitors, including 5-HD, indicating that the endogenous signaling mechanism of IPC is mediated by mitoK_{ATP} as well (46, 49, 50, 52). The discovery that mitoK_{ATP} is the target for potassium channel openers was a breakthrough that brought the focus of cardioprotection and its signaling mechanisms to the level of the mitochondrion.

Cardioprotective signaling and G_i protein-coupled receptors (GPCR)

All forms of cardioprotection operate via mitochondrial K_{ATP}, including the intrinsic signaling mechanisms behind ischemic preconditioning. This process involves receptor-mediated signal formation at the cell membrane, leading to an intracellular signaling cascade that opens mitoK_{ATP}. Ischemic preconditioning utilizes adenosine (90), bradykinin (146), and opioid (128) signaling acting in concert to confer protection to the ischemic heart. IPC can be mimicked pharmacologically by perfusing the heart with any one of these trigger substances and inhibition of any one of the adenosine, bradykinin, or opioid

receptors blocks the protective effects of IPC (29). However, adding subsequent cycles of IPC can restore the protective effects, leading Goto et al. to propose that the three pathways operate in an additive manner to achieve a hypothetical protective threshold (55). If one receptor is inhibited and the heart is given further cycles of IPC, the two active receptors are able to reach this threshold and protect the heart from the injury that results from a longer period ischemia.

Each of these trigger substances acts through G_i protein-coupled receptors (GPCR) and, according to the multiple trigger theory, all three must converge on a single target enzyme (29). Downey et al. determined that protein kinase C (PKC) inhibitors reversed the protection afforded by all of these substances and proposed that the common target may be PKC (29). Activation of nearly any GPCR in the heart with agonists such as catecholamines, angiotensin II, or endothelin can mimic IPC. However, inhibiting any one of these other GPCRs does not block IPC as with adenosine, bradykinin, and opioid inhibitors (29). These three G_i-protein coupled receptors are the major players in signaling for ischemic preconditioning. Adenosine interacts directly with PKC through phospholipases while bradykinin and opiods trigger more complex pathways, ending up at the same target (29).

Costa et al. determined that protein kinase G (PKG) is the terminal kinase in the GPCR signaling cascade triggered by IPC as well as the pharmacological agents that mimic protection (17). PKG and cyclic GMP (cGMP) added together to isolated mitochondria opens the mitoK$_{ATP}$ channel with the same effect as a KCO such as diazoxide or cromakalim.

MitoK$_{ATP}$ has been invoked by Pain et al. to be involved in the "trigger phase" of the IPC signaling pathway (111). Receptor stimulation leads to mitoK$_{ATP}$ opening, resulting in the formation of mitochondrial reactive oxygen species (ROS) which activate PKC. This leads to a form of protective "memory" that remains for about an hour after the preconditioning is complete or after the trigger substance has been washed out (20, 144). ROS are typically viewed as a damaging substance and, in many cases, they are. However, ROS also serve a valuable role in signaling as second messengers of many systems; they are required, for example, for cardioprotective signaling. The protection from diazoxide and ischemic preconditioning is abolished when a ROS scavenger is included (6, 32, 139). Exposure to ROS also mimics the protective effects of IPC in a PKC-dependent manner (6, 139).

The mechanisms behind cardioprotective signaling and the increase in reactive oxygen species were still not well understood, but identifying mitoK$_{ATP}$ as the target for signaling provided a focus for continued research.

MitoK$_{ATP}$ and ROS

All forms of cardioprotection operate through mitoK$_{ATP}$. Opening mitoK$_{ATP}$ causes an influx of K$^+$ and osmotically-obligated water, leading to mitochondrial matrix swelling and an increase in matrix pH, as shown in **Fig. 1.5**. MitoK$_{ATP}$ opening also leads to an increase in the level of ROS. Andrukhiv et al. demonstrated that mitoK$_{ATP}$ opening by the KCOs diazoxide and cromakalim increased ROS production by 40-50%. This effect can be blocked by the

mitoK$_{ATP}$ channel blocker 5-HD (2). This increase in ROS was also shown to

inhibit the mitochondrial permeability transition (MPT), thus preventing cell death

(18). The high levels of calcium, phosphate and damaging ROS that persist after

prolonged ischemia cause MPT to open, resulting in pathogenic necrotic cell

death (22, 25, 79). ROS arising from mitoK$_{ATP}$ opening activate a PKCε pool on

the inner side of the inner membrane that is closely associated with MPT and

prevents it from opening, thus preventing cell death.

While it was evident that mitochondrial ROS were necessary for

Fig. 1.5: *Intramitochondrial signaling and ROS generation.* The unidentified receptor R1 on the mitochondrial outer membrane (MOM) is phosphorylated by an intracellular GPCR signal. R1 then activates a pool of PKCε on the outer face of the inner membrane (MIM), which opens mitoK$_{ATP}$ by phosphorylation. Potassium channel openers (KCOs) such as diazoxide or cromakalim activate mitoK$_{ATP}$ directly. MitoK$_{ATP}$ opening causes an influx in K$^+$ ($\uparrow$K$^+$) leading to swelling ($\uparrow\Delta$V) and alkalinization ($\uparrow$pH) of the mitochondrial matrix. The increase in pH slightly inhibits the ETC at complex I and leads to superoxide formation, which is rapidly transformed into a signaling reactive oxygen species (ROS) in the matrix. This ROS activates a second pool of PKCε on the inner face of the MIM (PKCε2), blocking the mitochondrial permeability transition (MPT) and preventing cell death.

cardioprotective signaling and that they arise due to mitoK$_{ATP}$ opening, the direct

cause and location of the ROS production was unknown. Membrane potential

($\Delta\Psi$) decreases upon influx of K$^+$, but this effect is known to decrease ROS

production and can be excluded. Swelling causes about 20% increase in matrix

water; Andrukhiv et al. demonstrated that this has no effect on ROS production

by lowering the osmolality of the assay medium to induce a 50% increase in

mitochondrial matrix volume. Suspending mitochondria in a more acidic or

alkaline medium, however, had significant effects, with a decrease in pH lowering

ROS production and an increase in pH raising it. The researchers proposed that

mitochondria produce ROS due to the alkalinization of the matrix upon potassium

influx through mitoK$_{ATP.}$ They further separated the effects of extramitochondrial

and matrix pH, demonstrating that matrix alkalinization from K$^+$ influx is directly

responsible for the increased ROS production (2).

Valinomycin, the K$^+$-specific ionophore, also increases ROS production by

the same amount as the KCOs when it is administered at a concentration that

matches the K$^+$-flux of the open mitoK$_{ATP}$ channel. This effect is not blocked by 5-

HD, however, because valinomycin operates independently of mitoK$_{ATP}$. At low

concentrations, valinomycin exhibits a dose dependent increase in ROS

production until that concentration that matches mitoK$_{ATP}$ K$^+$-flux. At any higher

concentration ROS production is quickly diminished. This demonstrates that

mitoK$_{ATP}$ opening has been finely tuned for the exact amount of K$^+$-flux

necessary to induce maximal ROS production. It can be inferred that mitoK$_{ATP}$

opening is designed to produce ROS for secondary messaging (2).

ROS is generated in the form of superoxide (O$_2$$^{\bullet-}$) when a single electron

is transferred directly from a redox center in the electron transport system to

molecular oxygen (141). "Superoxide of mitochondrial origin arise from three

major sites (mechanisms): *1)* the o-site of complex III, *2)* redox centers in

complex I that have been reduced by reverse electron transport from the Q-pool,

and *3)* redox centers in complex I that have been reduced by NADH" (Andrukhiv

et al., 2006). Inhibitors of redox centers in the electron transport system can be

used to identify the location of ROS formation. Myxothiazol inhibits the coenzyme

Q-cytochrome c
reductase complex and
blocks ROS arising from
complex III. It also
stimulates ROS arising
at complex I due to the
downstream block on
complex III and was
indeed shown to cause a
significant increase in
ROS production,
eliminating contribution
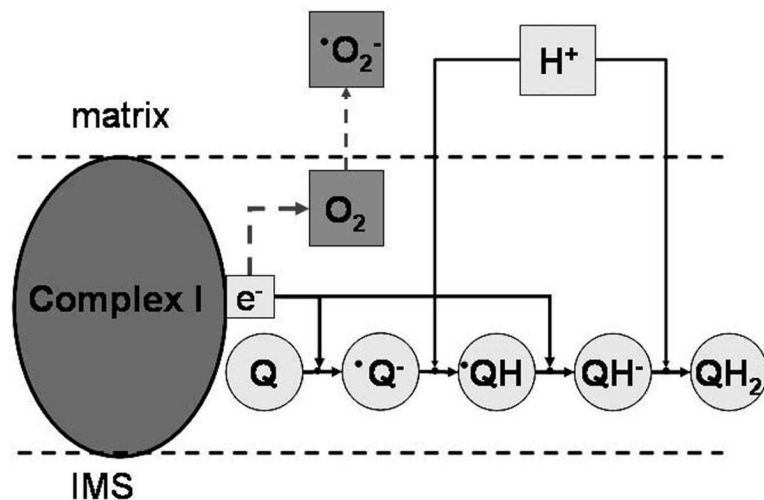
Fig. 1.6.*Effect of matrix pH on ROS production from complex I.* Electrons are passed to ubiquinone (Q) from complex I (NADH: ubiquinone oxidoreductase) of the electron transport chain. Although the site of superoxide production has been controversial, recent data suggest that the single electrons are delivered to oxygen from the FMNH$_2$ or FMN semiquinone (13). It can be seen in the diagram that the reduction of ubiquinone requires two matrix protons. Electron flow will therefore be retarded at these points as matrix pH increases, causing increased reduction at the flavin site and consequent increase in steady-state superoxide production. IMS, intermembrane space.

by *mechanism 1* (2). Rotenone inhibits complex I, but it did not have any significant effect, suggesting that *mechanism 2* is not a major contributor. ROS production by valinomycin was amplified by the addition of myxothiazol, suggesting that the ROS formed due to the increased K^+ influx is produced upstream of complex III. The positive effects of valinomycin and myxothiazol and the lack of effect of rotenone on ROS production point to *mechanism 3* and superoxide production at complex I. The transfer of electrons to molecular oxygen to form superoxide is shown in **Fig. 1.6**.

Mitochondrial ROS as second messengers

Superoxide is quickly transformed by superoxide dismutase into hydrogen peroxide (H_2O_2), which, is further transformed by enzymatic action or redox catalyzed reactions to other downstream ROS. The oxidation products of mitochondrial ROS act as second messengers in cardioprotective signaling and a host of other signaling processes. Mitochondrial ROS have a further role in triggering gene transcription at the nucleusand protein synthesis at the endoplasmic reticulum (46).

While these signaling ROS clearly play a crucial role in signaling throughout the cell and in numerous processes, the particular species operating in cardioprotective signaling has yet to be identified. The aim of this project is to accomplish this goal and determine the ROS acting as a second messenger in cardioprotective signaling.

Chapter 2: Mitochondrial Reactive Oxygen Species —

Which ROS Signals Cardioprotection?

Anders O. Garlid[1], Martin Jaburek[2], Jeremy P. Jacobs[1], and Keith D. Garlid[1]*

[1]Department of Biology, Portland State University, Portland, OR, 97201-0751,
[2]Department of Membrane Transport Biophysics, Institute of Physiology, v.v.i.,
Prague, Czech Republic, 14220

Running Head: Mitochondrial ROS Signaling

Keywords: mitochondria; cardioprotection; reactive oxygen species; K-ATP channels; cardiac ischemia; ROS signaling

2-A: Abstract

Mitochondria are the major effectors of cardioprotection by procedures that open

the mitochondrial ATP-sensitive potassium channel (mitoK$_{ATP}$), including

ischemic and pharmacological preconditioning. MitoK$_{ATP}$ opening leads to

increased reactive oxygen species (ROS), which then activate a mitoK$_{ATP}$-

associated PKCε, which phosphorylates mitoK$_{ATP}$ and leaves it in a persistent

open state (Costa, ADT and Garlid, KD. Am J Physiol 295, H874-82, 2008). The

ROS responsible for this effect is not known. The present study focuses on

superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$), each of

which has been proposed as the signaling ROS. Feedback activation of mitoK$_{ATP}$

provides an ideal setting for studying endogenous ROS signaling. Respiring rat

heart mitochondria were preincubated with ATP and diazoxide, together with an

agent being tested for interference with this process, either by scavenging ROS

or by blocking ROS transformations. The mitochondria were then assayed to

determine whether or not the persistent phosphorylated open state was

achieved. Dimethylsulfoxide (DMSO), dimethylformamide (DMF), deferoxamine,

Trolox, and bromoenol lactone (BEL) each interfered with formation of the ROS-

dependent open state. Catalase, the H_2O_2 scavenger, did not interfere with this

step. We also found that DMF blocked cardioprotection by both ischemic

preconditioning and diazoxide. The lack of a catalase effect and the inhibitory

effects of agents acting downstream of $HO^{\bullet}$ excludes H_2O_2 as the endogenous

signaling ROS. Taken together, the results support the conclusion that the ROS

message is carried by a downstream product of HO$^{\bullet}$ and that it is probably a product of phospholipid oxidation.
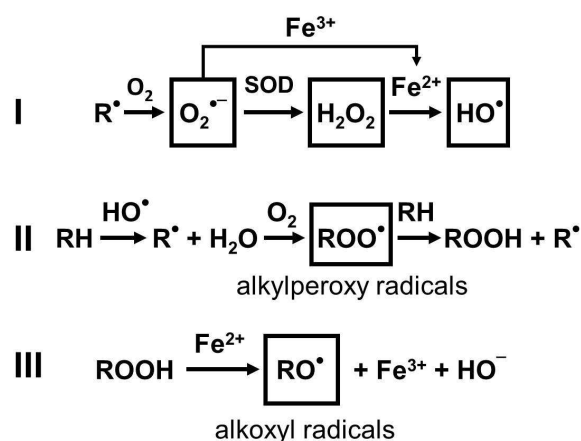
2-B: Introduction

Reactive oxygen species (ROS) are second messengers of preconditioning (50) and have long been known to be required for cardioprotective signaling (6, 15, 40, 103, 135, 153). The mechanism of increased ROS is reasonably well understood: signaling from the plasma membrane leads to opening of the mitochondrial ATP-sensitive K^+ channel (mitoK$_{ATP}$) (45), and the increased K^+ influx into the matrix causes an increase in ROS, which derive during normoxia from Complex I of the respiratory chain (2).

The ROS transformations that take place in mitochondria are summarized in **Figs. 2.1** and **2.2**. The primary ROS produced by the mitochondrial respiratory chain is superoxide ($O_2^{\bullet -}$), which is formed by single-electron reduction of oxygen. The majority of $O_2^{\bullet -}$ undergoes dismutation to hydrogen peroxide (H_2O_2) both spontaneously and through the action of superoxide dismutases located in the matrix and the intermembrane space (12, 75, 101). Much of the H_2O_2 generated is

$$\text{I} \quad R^{\bullet} \xrightarrow{O_2} \boxed{O_2^{\bullet -}} \xrightarrow{SOD} \boxed{H_2O_2} \xrightarrow{Fe^{2+}} \boxed{HO^{\bullet}}$$

(with Fe^{3+} feeding back)

$$\text{II} \quad RH \xrightarrow{HO^{\bullet}} R^{\bullet} + H_2O \xrightarrow{O_2} \boxed{ROO^{\bullet}} \xrightarrow{RH} ROOH + R^{\bullet}$$
alkylperoxy radicals

$$\text{III} \quad ROOH \xrightarrow{Fe^{2+}} \boxed{RO^{\bullet}} + Fe^{3+} + HO^{-}$$
alkoxyl radicals

Fig. **2.1.** *Mitochondrial electron transport chain-derived ROS and formation of alkyl peroxylalkoxyl radicals.***(I)** Superoxide ($O_2^{\bullet -}$) is formed by single electron transfer to molecular oxygen (O_2) from a reduced group ($R^{\bullet}$) in the respiratory chain and converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). Hydroxyl radical ($HO^{\bullet}$) is formed from H_2O_2 in the presence of a transition metal ion such as iron ($Fe^{2+/3+}$) (the Fenton reaction). **(II)** Subsequent reactions include the formation of alkyl peroxyl radicals ($ROO^{\bullet}$) and alkyl hydroperoxides (ROOH). $HO^{\bullet}$ acts on an alkyl side chain (RH) to form a reduced group ($R^{\bullet}$) that leads to the formation of $ROO^{\bullet}$, and these react with RH to produce ROOH. **(III)** Alkoxyl radicals ($RO^{\bullet}$) are formed in the decomposition of hydroperoxides (ROOH) by transition metal ions (Fe^{2+}).

reduced by the glutathione and thioredoxin reductase antioxidant systems, which prevent the emission of excess H_2O_2 to the cytosol (132). H_2O_2 is also decomposed by catalase, which is present in the matrix of heart mitochondria (121). Some of the H_2O_2 leads to formation of hydroxyl radical (HO$^\bullet$) through the Fenton reaction of H_2O_2 with transition metal ions (83). The highly reactive HO$^\bullet$ oxidizes proteins and lipids in diffusion limited reactions (78).

The consequences of ROS signaling for mitochondrial physiology are that two mitochondrial protein kinase C epsilons (PKCε) are activated by oxidation of their thiol groups (82). Activation of PKCε1, which is associated with mitoK$_{ATP}$ at the mitochondrial inner membrane, leads to opening of mitoK$_{ATP}$ (20, 71). Activation of PKCε2 leads to inhibition of the mitochondrial permeability transition (MPT) (18). Progress is being made in these areas and in the molecular identification of mitoK$_{ATP}$ (34). However, the ROS responsible for activating these PKCεs *in vitro* or *in vivo* is still not known. Because the signaling ROS not only originates in mitochondria but also acts on mitochondria, it should be possible to narrow the search for the signaling ROS by studies on isolated rat heart

$$\text{I} \quad LH \xrightarrow{RO^\bullet} L^\bullet + RHO \xrightarrow{O_2} LOO^\bullet$$

$$\text{II} \quad LOO^\bullet \xrightarrow{LH} \boxed{LOOH} + L^\bullet$$

Lipid hydroperoxide

$$\text{III} \quad LOOH \xrightarrow{iPLA2} \boxed{FAOOH} + \text{lyso-phospholipid}$$

Fatty acid hydroperoxide

Fig. 2.2.*Lipid peroxidation and release of hydroperoxy fatty acids.***(I and II)**alkoxyl (RO$^\bullet$), hydroxyl (RHO), and alkyl peroxyl radicals (LOO$^\bullet$) can initiate the non-enzymatic chain reaction leading to lipid peroxidation (LOOH). **(III)** The lipid hydroperoxides (LOOH) can be released by the action of mitochondrial phospholipases, such as Ca^{2+}-independent phospholipases PLA2 (iPLA2), which have been found to modulate the function of mitochondria (see Cedars et al (13) for a review). The action by iPLA2 results in a hydroperoxy fatty acid (FAOOH).

mitochondria. The objective of these studies was to identify the point in the reaction sequence of ROS transformations at which the ROS signal is formed and, ultimately, to determine the identity of the ROS signal itself.

We previously described feedback activation of mitoK$_{ATP}$, in which mitoK$_{ATP}$ opening by a K$_{ATP}$ channel opener leads to endogenous ROS formation and a persistent open state of mitoK$_{ATP}$. The phenomenon was shown to involve PKCϵ1 and an inner membrane phosphorylation event (20). This persistent open state of mitoK$_{ATP}$ is thought to be responsible for "memory," which is seen with all preconditioning (20).

We employed a preincubation protocol to probe the feedback activation system for the identity of the signaling ROS. Diazoxide was used to induce mitoK$_{ATP}$ opening, which leads to endogenous ROS formation and activation of PKCϵ1. This process establishes the prolonged phosphorylation-dependent open state of mitoK$_{ATP}$ (20). We then re-isolated the mitochondria and probed them for this state by measuring the resulting steady-state mitochondrial matrix volume using the light scattering technique (9, 17, 21, 47). Agents or conditions that interfered with any step in the generation of the signaling ROS would prevent formation of the phosphorylation dependent open state. Two additional assays were carried out to eliminate agents that interfered either with mitoK$_{ATP}$-dependent K$^+$ uptake or with ROS-activation of PKCϵ1. Together, these procedures allowed identification of agents that specifically interfered with formation of the ROS signal.

The agents that were found to block formation of the ROS signal were dimethylsulfoxide (DMSO) and dimethylformamide (DMF), scavengers of $HO^{\bullet}$ and $RO^{\bullet}$ (5, 99), trolox, a chain-breaking antioxidant and peroxyl radical scavenger (95), deferoxamine, an iron chelator (83, 131), and bromoenol lactone (BEL), which is an inhibitor of Ca^{2+}-independent phospholipase A2 (iPLA2) (13). Based on the site of action of these agents, we conclude that the ROS signal arises downstream of $HO^{\bullet}$ formation. Because H_2O_2 is produced upstream of $HO^{\bullet}$, these results exclude H_2O_2 as the signaling ROS. This conclusion is supported by the finding that catalase did not interfere with formation of the phosphorylation-dependent open state of $mitoK_{ATP}$.

We performed experiments on MPT, in which diazoxide was used to induce inhibition of MPT via ROS activation of PKCε2 (18). This effect was blocked by DMSO, also implicating a ROS signal downstream from $HO^{\bullet}$. Finally, we found that the $HO^{\bullet}$ scavenger DMF blocked cardioprotection by ischemic preconditioning and diazoxide. Taken together, each of these results supports the conclusion that the ROS message is carried by a downstream product of hydroxyl radical ($HO^{\bullet}$). The actions of BEL suggest a product of phospholipid oxidation.

2-C: Research Design and Methods

Langendorff perfused hearts

Hearts from male Sprague-Dawley rats (200-240g) were perfused as previously described (28, 51, 115, 120) with Krebs Henseleit buffer containing (in mM) 118 NaCl, 5.9 KCl, 1.75 $CaCl_2$, 1.2 $MgSO_4$, 0.5 EDTA, 25 $NaHCO_3$, 16.7 glucose at 37°C and pH 7.4 and gassed with 95% O_2 - 5% CO_2. Treatment protocols are described in **Fig. 2.3**. Hearts were stabilized for 25 min with Krebs Henseleit buffer prior to treatment with buffer containing drugs or agents then

subjected to 25 minutes global ischemia followed by 2 hours of reperfusion and measurement of infarct size. Ischemic preconditioning (IPC) was established by two cycles of 5 min global ischemia followed by 5 min reperfusion prior to the index ischemia. Ischemic postconditioning was performed with 6 cycles of 10 s ischemia plus 10 sec reperfusion (140). Hearts were not paced and mechanical performance was evaluated as the
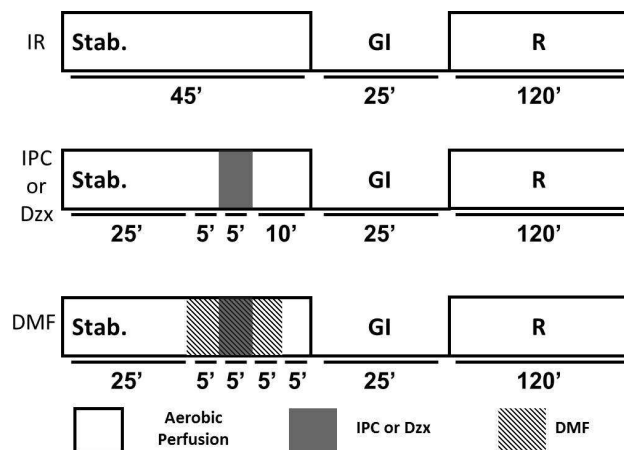
Fig. 2.3. *Isolated heart protocols.* Hearts were perfused with buffer, as described in "Methods." After 45 min of stabilization, hearts were subjected to 25 minutes global ischemia ("GI"), followed by 2 hours of reperfusion ("R") and, finally, processing for infarct size estimation. The protocol for ischemic reperfusion without additional treatment is labeled "IR." Ischemic preconditioning ("IPC") was established by 5 min global ischemia followed by 10 min reperfusion prior to the index ischemia. Diazoxide ("Dzx") (50 µM) was perfused for 5 min, followed by 10 min reperfusion with buffer prior to the index ischemia. Dimethylformamide ("DMF") (1% v/v) was administered 5 min before the first IPC ischemia and before diazoxide; it was continued for 5 min after these treatments and this was followed by 5 min reperfusion with buffer prior to the index ischemia.

product of heart rate and left ventricular developed pressure (LVDP) and reported as a percentage of rate pressure product at stabilization, t=0 (RPP % at t=0). Infarct size (IS) was determined by the method of Ytrehus et al. (152) whereby the treated hearts were stained with triphenyltetrazolium chloride (TTC) after reperfusion, fixed in formaldehyde and sliced into thin sections. Infarct size is reported as a percentage of the total cross-sectional area of the heart slices at risk. Experimental protocols complied with the Guiding Principles in the Use and Care of Animals published by the National Institutes of Health and were approved by IACUC at Portland State University.

Mitochondrial isolation

Male Sprague-Dawley rats (200 - 240 g) were anesthetized with CO_2, immediately decapitated, and the hearts were removed for mitochondrial isolation exactly as described previously (21). Hearts were washed and finely minced in ice-cold isolation buffer containing 250 mM sucrose, 10 mM HEPES at pH 7.2 and 0.5 mM K^+-EGTA. The suspension was diluted 3-fold with isolation buffer supplemented with 1% fatty acid-free BSA. The time between decapitation and completion of homogenization was kept as brief as possible and was completed within 2 min; mitoK_{ATP} activity shows a sharp dependence on the length of this period. The suspension was homogenized with a motorized Teflon pestle and centrifuged for 3 min at 1,500 *g*. The supernatant was centrifuged for 5 min at 9,000 *g*, and the resulting pellets were resuspended in isolation buffer lacking BSA and centrifuged for 3 min at 2,300 *g*. This supernatant was centrifuged for 5

min at 9,000 *g*. The final mitochondrial pellet was resuspended with isolation

buffer to 35–40 mg protein/ml and kept on ice. Where indicated, rat liver

mitochondria were isolated using a similar protocol, as previously described (9).

Mitochondrial protein concentration was estimated using the Biuret reaction (54).

Measurements of mitoK$_{ATP}$ activity

MitoK$_{ATP}$ opening causes mitochondrial swelling due to respiration-driven

uptake of K$^+$ salts and water. These volume changes were followed by light

scattering, as previously described (9, 17, 21, 47). Light scattering changes of

0.1 mg/ml mitochondrial suspensions were followed at 520 nm and 30ºC.

Mitochondria were suspended in a buffered salt assay medium containing K$^+$

salts of Cl$^-$ (120 mM), HEPES (10 mM), EGTA (0.1 mM), succinate (10 mM),

MgCl$_2$ (0.5 mM), phosphate (5 mM), ATP (200 μM), rotenone (2.5 μM), and

oligomycin (1 μg/ml) at pH 7.2. Osmolality ranged between 275 to 280 mOsm.

Data summarized in bar graphs as "MitoK$_{ATP}$ activity (%)," were calculated from

$$100 \times \frac{A^{-1}(x) - A^{-1}(ATP)}{A^{-1}(0) - A^{-1}(ATP)}$$

where $A^{-1}(x)$ is the observed inverse absorbance at 120 s under the given

experimental condition, and $A^{-1}(ATP)$ and $A^{-1}(0)$ are values independently

measured in the presence and absence of ATP, respectively.

Preincubation protocol

A preincubation protocol was used to trigger activation of PKCε by

endogenous ROS. As illustrated in **Fig. 2.4**, mitochondria were incubated in the

light scattering assay medium (described above) for 3 minutes at $30^{\circ}C$ with 30 μM diazoxide (Dzx). The preincubation medium was supplemented with various agents to explore the mechanisms of endogenous ROS signaling. Each sample was equally divided and preincubated in the presence or absence of diazoxide. The treated mitochondria were then isolated from the incubation medium and resuspended in a small volume (15
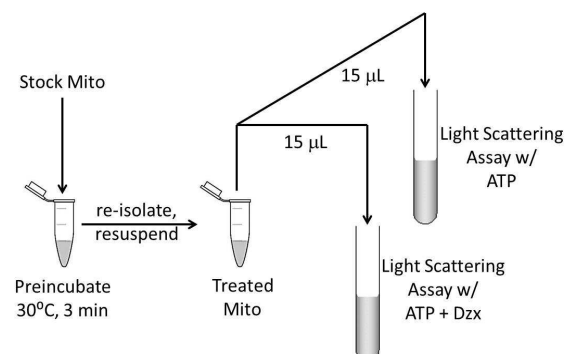
Fig. 2.4. *Preincubation protocol.* Isolated rat heart mitochondria suspended in 250 mM sucrose and 10 mM HEPES at 30 mg/mL ("Stock Mito") was incubated at 30ºC for 3 minutes in assay medium containing 200 μM ATP and 30 μM diazoxide (Dzx), in a total volume of 0.5 mL. The treated mitochondria were reisolated and resuspended, and mitoK$_{ATP}$ activity was measured using the light scattering assay, with a final protein concentration of 0.1 mg/mL. Half of the treated mitochondria were assayed with ATP and the other half with ATP and Dzx. The incubation was supplemented with various agents including 5-HD (300 μM), εV_{1-2} (0.5 μM), catalase (250 U/mL), DMSO (1% v/v), DMF (1% v/v), Trolox (100 μM), deferoxamine (1 mM), MPG (1 mM), or BEL (10 μM).

μl) of ice cold sucrose buffer containing 250 mM sucrose and 10 mM HEPES at pH 7.2. MitoK$_{ATP}$ activity of the treated mitochondria was then assayed using light scattering. When preincubated without diazoxide, the mitochondria behaved normally in the subsequent assay. That is, mitoK$_{ATP}$ activity was inhibited by ATP, and inhibition was released by diazoxide. When preincubated with diazoxide, mitoK$_{ATP}$ activity in the subsequent assay was not inhibited by ATP, and diazoxide had no effect. Thus, these conditions cause mitoK$_{ATP}$ to remain open due to its phosphorylation by PKCε (20). Any agent that blocks any step in the process will block feedback activation of mitoK$_{ATP}$.

Measurements of mitochondrial permeability transition

Mitochondrial permeability transition (MPT) activity was monitored by light scattering in medium containing 200 μM ATP and lacking EGTA. MPT opening was synchronized by sequential additions of $CaCl_2$ (100 μM free Ca^{2+}), ruthenium red (0.1 μM, to block further Ca^{2+} uptake), and carbonyl cyanide m-chlorophenyl hydrazine (CCCP) (250 nM), at 20, 40 and 60 sec, respectively (18). These experiments used mitochondria from livers taken from the animals supplying the heart.

Statistical analysis

Data are presented as mean ±standard deviation (SD) of the mean. One-way ANOVA followed by Tukey's post hoc test or Student's t-test of the means were used as required to analyze the data using Microcal Origin software (Northampton, MA, USA). A value of $p < 0.05$ was considered statistically significant.

Chemicals

Peroxynitrite was acquired from Cayman Chemical. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). N-2-mercaptopropionylglycine (MPG) was always freshly prepared and adjusted to neutral pH by Tris base. Diazoxide and εV_{1-2} were prepared in DMSO and bromoenol lactone (BEL) was always freshly prepared in DMSO. Uric acid,

trolox, deferoxamine, catalase, 5-hydroxydecanoate (5-HD) and ruthenium red were prepared in distilled water.

2-D: Results

Endogenous ROS signaling and PKCε1-dependent feedback activation of mitoK$_{ATP}$. Feedback activation of mitoK$_{ATP}$ can be divided into three steps, described in **Fig. 2.5**. In *Step 1*, K$^+$ channel openers such as diazoxide open mitoK$_{ATP}$ (50), and the resulting K$^+$ influx and matrix alkalinization leads to increased generation of superoxide (2).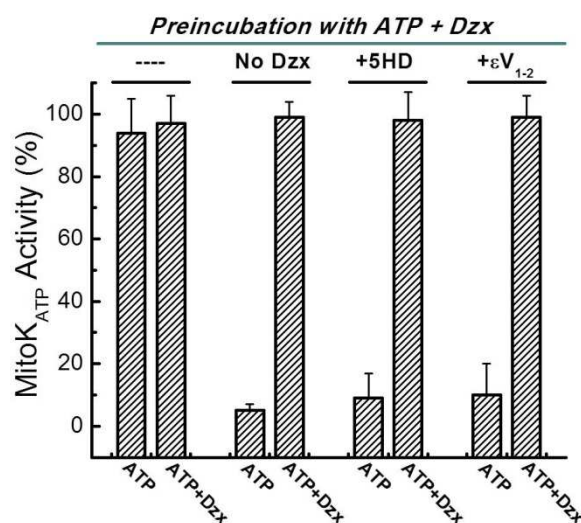 *Step 2* encompasses the ROS transformations that result in formation of the signaling ROS, i.e., the ROS that activates PKCε1. In *Step 3*, the activated PKCε1 establishes the phosphorylation dependent open state of mitoK$_{ATP}$ (20). The rationale for this division is that we can study the effects of agents on the overall cycle and, independently, on steps 1 and 3. In this way, we can identify agents that interfere specifically with
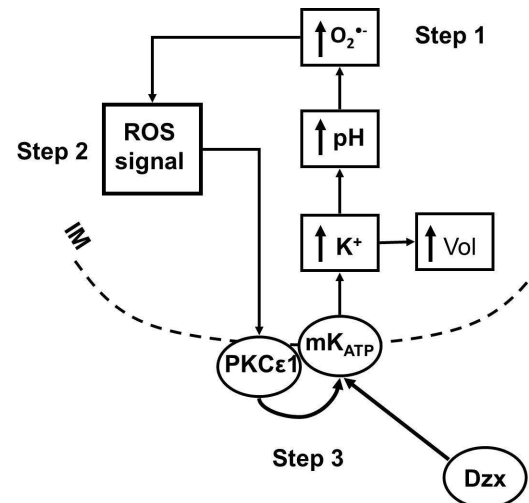
Fig. **2.5**. *ROS-dependent feedback activation of mitoK$_{ATP}$.* **Step 1** of the process encompasses the sequence from mitochondrial ATP-sensitive K$^+$ channel ("mitoK$_{ATP}$") opening to superoxide (O$_2^{\bullet-}$) formation. MitoK$_{ATP}$ is opened by K$_{ATP}$ channel openers such as diazoxide ("Dzx") (50). The consequent uptake of K$^+$ and anions leads to increased matrix volume ("↑Vol"), which is the basis of the light scattering assay for mitoK$_{ATP}$ activity (21). The cytosolic concentration difference between [K$^+$] and [phosphate] means that more K$^+$ than phosphate will be taken up, leading to matrix alkalinization ("↑pH") (21). Matrix alkalinization, in turn, inhibits Complex I, leading to increased production of superoxide ("↑O$_2^{\bullet-}$") (2). **Step 2** encompasses the many ROS transformations that take place and lead to the signaling ROS, which activates protein kinase C-epsilon 1 (PKCε1). In **Step 3**, the activated PKCε1 phosphorylates a protein, possibly mitoK$_{ATP}$ itself, which leads to the phosphorylated open state of mitoK$_{ATP}$(20). In parallel (not represented here), the ROS signal activates PKCε2, leading to inhibition of MPT opening (18).

formation of the signaling ROS (*Step 2*).

Fig. 2.6 contains results of control experiments using the preincubation protocol described in "Methods." When mitochondria were preincubated with diazoxide and ATP, they exhibited maximum volume change in the normal assay medium containing ATP and no further effect was seen with the addition of diazoxide (first two bars of **Fig. 2.6**). This result is characteristic of conditions that allow mitoK$_{ATP}$ to achieve the phosphorylation-dependent open state. When *Step 1* was blocked by omission of diazoxide (second pair of bars) or inclusion of 5-hydroxydecanoate (5-HD) (third pair of bars) in the preincubation, mitoK$_{ATP}$ behaved as if there were no preincubation; that is, mitoK$_{ATP}$ activity was inhibited by ATP and opened by diazoxide. When *Step 3* was blocked by inclusion of the specific PKCε inhibitor εV$_{1-2}$ in the preincubation (last pair of bars), feedback

Fig. 2.6. *Feedback activation of mitoK$_{ATP}$ is blocked by 5-hydroxydecanoate (5-HD) and εV$_{1-2}$.*Shown are the effects of various agents on mitoK$_{ATP}$ activity after preincubation with ATP (200 μM) plus diazoxide (Dzx) (30 μM). Mitochondria were preincubated with the agents indicated at the top of the figure, then assayed in K$^+$ medium with ATP or ATP + Dzx as indicated below (described in "Methods"). With no further additions to the preincubation ("----"), mitoK$_{ATP}$ remains in the phosphorylated open state with full activity, and diazoxide ("ATP+Dzx") has no further effect in the subsequent light scattering assay. When mitoK$_{ATP}$ opening during preincubation was prevented by omission of diazoxide ("No Dzx") or inclusion of the mitoK$_{ATP}$ blocker 5-HD (300 μM) ("+5-HD"), the phosphorylation-dependent open state was blocked. When feedback activation of PKCε1 was prevented by inclusion of εV$_{1-2}$ (0.5 μM) ("+εV$_{1-2}$"), the open state was also blocked. Data are means ±SD of at least 3 independent experiments. * p < 0.05.

activation was also prevented and mitoK$_{ATP}$ responded normally to ATP and diazoxide.

Fig. 2.7 contains results of preincubation experiments in which we evaluated agents that may block ROS signaling (Step 2) in the feedback loop shown in **Fig. 2.5**. Dimethylsulfoxide (DMSO), and dimethylformamide (DMF), and N-2-mercaptoprionylglycine (MPG) have all been described as reactants with HO$^{•}$, RO$^{•}$ (alkoxyl radicals), and ROO$^{•}$ (alkylperoxyl radicals) (5, 99), each of which may play a role in endogenous ROS signaling. Trolox, a vitamin E analogue, acts as a chain-breaking antioxidant and peroxyl radical scavenger (95). **Fig. 2.7A** demonstrates that each of these agents prevented formation of the phosphorylation-dependent open
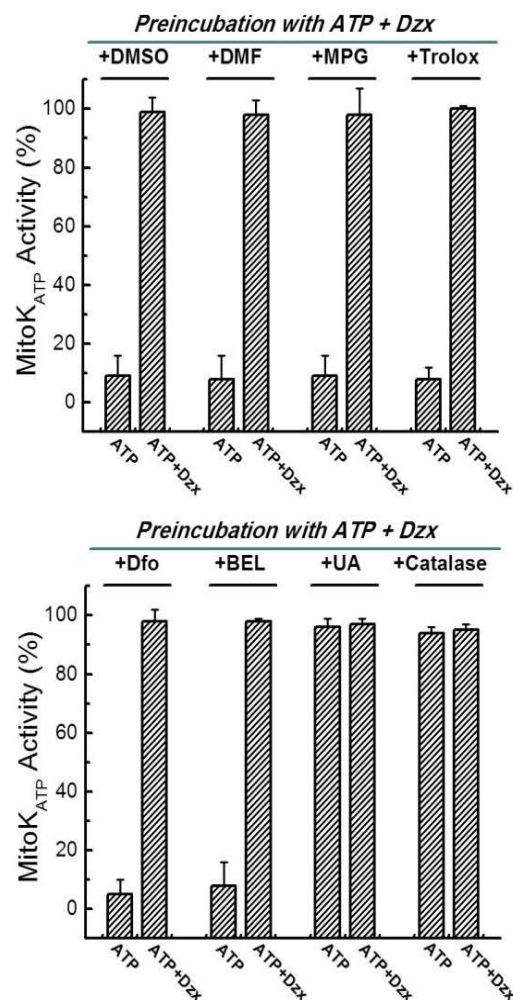
Fig. 2.7. *Effects of agents that may block formation of the signaling ROS on feedback activation of mitoK$_{ATP}$*. Shown in **(A)** are the effects of various ROS and radical scavenging agents and antioxidants on mitoK$_{ATP}$ activity after preincubation with ATP plus diazoxide (Dzx) in the presence of dimethylsulfoxide (DMSO) (1 %v/v), dimethylformamide (DMF) (1 %v/v), N-2-mercaptopropionylglycine (MPG) (1 mM), and Trolox (100 μM). Each of these agents blocked formation of the phosphorylation-dependent open state of mitoK$_{ATP}$. In **(B)**, deferoxamine (Dfo) (1 mM) and bromoenol lactone (BEL) (10 μM) also blocked this state, but uric acid (100 μM) ("+UA") (peroxynitrite scavenger) and catalase (250 U/mL) (H$_2$O$_2$ scavenger) did not interfere with feedback activation of mitoK$_{ATP}$. Mitochondria were preincubated with the agents indicated in the figure, then assayed in K^{+} medium, as described in "Methods." Data are means of mitoK$_{ATP}$ activity ± SD of at least 3 independent experiments. * $p < 0.05$.

state, suggesting that HO$^•$ is involved in the generation of the signaling ROS.

Deferoxamine is a chelator of iron (83, 131) and acts as a preventive antioxidant by reducing the rate of iron-dependent hydroxyl and peroxyl radical chain initiation. BEL (racemic bromoenol lactone) is an inhibitor of Ca^{2+}-independent phospholipase (iPLA2) (13) and it prevents the release of oxidized phospholipids such as hydroperoxy fatty acids (FAOOH) from the bilayer, a possible downstream oxidation product in the reaction sequence. **Fig. 2.7B** shows that both deferoxamine and BEL blocked feedback activation of mitoK$_{ATP}$, further suggesting that hydroxyl radicals and their downstream oxidation products are involved in the generation of the signaling ROS. When uric acid (UA), a peroxynitrite scavenger (137), was included in the preincubation, the phosphorylation-dependent open state was preserved, indicating that peroxynitriteis not involved with any of the steps and, therefore, is not the signaling ROS. Catalase is an enzyme that catalyzes the decomposition of H_2O_2 and is present in the matrix of heart mitochondria (121). However, catalase was also unable to block the process of achieving the phosphorylated open-state, suggesting that H_2O_2 is not the signaling ROS.

The mechanisms of action of these agents suggests that they are acting on *Step 2*, the formation of the ROS signal; however it is necessary to demonstrate that they are not acting elsewhere in the loop, i.e. on *Step 1* or *Step 3*. Accordingly, we tested each of the agents for their effect on mitoK$_{ATP}$ activity in the straight light scattering assay with no preincubation and found that none of them interfered with diazoxide opening of mitoK$_{ATP}$ (data not shown). Note that

this assay also detects respiratory inhibition or uncoupling, which inhibit K^+

uptake by reducing the driving force.

To evaluate their effect on S*tep 3* (induction of the phosphorylated open

state by PKCε1), we examined whether the agents were able to block mitoK$_{ATP}$

opening induced by H_2O_2, which works by activating PKCε1 (20). **Fig. 2.8**

contains representative light scattering traces using mitochondria without

preincubation. Here, we find that Trolox had no effect. MPG did block H_2O_2-

induced mitoK$_{ATP}$ opening, which means that we cannot distinguish whether

MPG acts on *Step 2* or *Step 3* from these experiments. The results of the S*tep 3*

assays are summarized in **Fig. 2.9** and show that deferoxamine, DMSO, DMF,
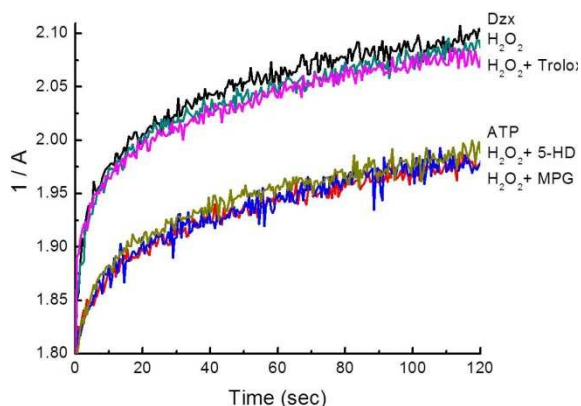
Fig. 2.8. *Differential effects of MPG and Trolox on H_2O_2-dependent mitoK$_{ATP}$ opening.* Shown are representative light scattering traces (1/A vs. time) of rat heart mitochondria respiring on succinate in K^+ medium. Mitochondria were suspended at 0.1 mg/ml and assayed as described in "Methods." ATP (200 μM) was present in all experiments. Where indicated, Trolox, MPG, 5-HD or no additions ("ATP") were present in the assay medium prior to the addition of mitochondria. Mitochondrial swelling was initiated by the addition of hydrogen peroxide (H_2O_2) (2 µM) or diazoxide 2 seconds after the addition of mitochondria to trigger PKCε1-dependent mitoK$_{ATP}$ opening.
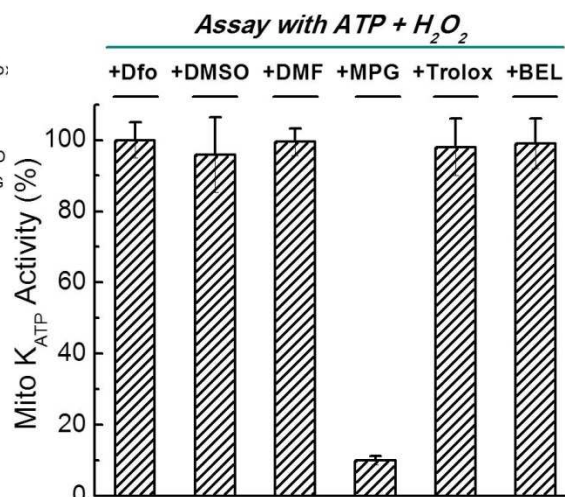
Fig. 2.9. *Effects of agents on activation of PKCε1 by H_2O_2.* The agents that were utilized in the preincubation experiments of **Fig. 2.6** were examined for their effects on H_2O_2-induced PKCε-dependent mitoK$_{ATP}$ opening in the straight light scattering assay described in "Methods." MPG was the only agent that interfered with this step in the process. Deferoxamine, DMSO, DMF, Trolox, and BEL had no effect on H_2O_2 activation. Data are means ±SD of at least 3 independent experiments.
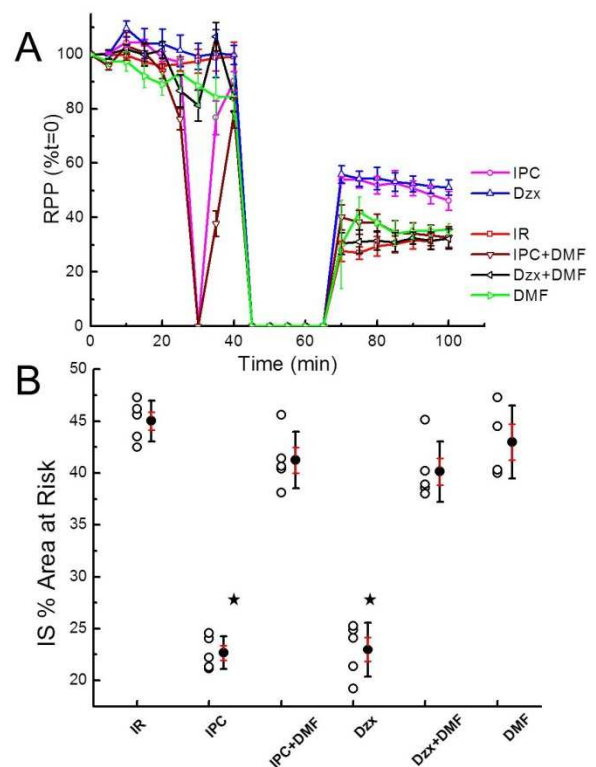
Trolox, and BEL did not inhibit H_2O_2-induced mitoK$_{ATP}$ opening (*Step 3*). Because they also did not interfere with diazoxide opening of mitoK$_{ATP}$ (*Step 1*), we infer that these agents are acting to prevent formation of the endogenous ROS signal that activates PKCε1 (*Step 2*).

These findings may be summarized as follows. *1)* Deferoxamine, DMSO, DMF, Trolox, and BEL each prevent formation of the endogenous ROS signal. These results imply that the ROS signal arises downstream from HO˙ radical. *2)* H_2O_2 is produced upstream of HO˙ radical, and catalase, the H_2O_2 scavenger, had no effect on the

Fig. 2.10. *Dimethylformamide (DMF) blocks protection of the perfused heart by diazoxide and IPC.***(A)** Shown are measurements of rate-pressure product ("RPP (% at t=0)") with time from perfusion of the ex vivo heart. Diazoxide (50 μM) ("Dzx") and ischemic preconditioning ("IPC") improved functional recovery, and this effect was blocked by dimethylformamide ("Dzx + DMF" and "IPC + DMF"), a free radical scavenger. DMF alone had no effect. **(B)**Infarct size as a percent of area at risk ("IS % Area at Risk") is plotted for the various treatments. Treatment with diazoxide (Dzx) and IPC reduced infarct size, and this effect was blocked by DMF. Data are means ±SD of at least 3 independent experiments. * p < 0.05.

formation of the phosphorylation-dependent open state. Therefore, H_2O_2 can be excluded as the signaling ROS. *3)* The action of MPG is independent of ROS scavenging. Like all thiols, MPG can scavenge HO˙, but HO˙ is not involved in the pathway responsible for the results in **Fig. 2.8**. Moreover, MPG has little or no H_2O_2 scavenging ability (10, 91). We propose that this action of MPG is due to its
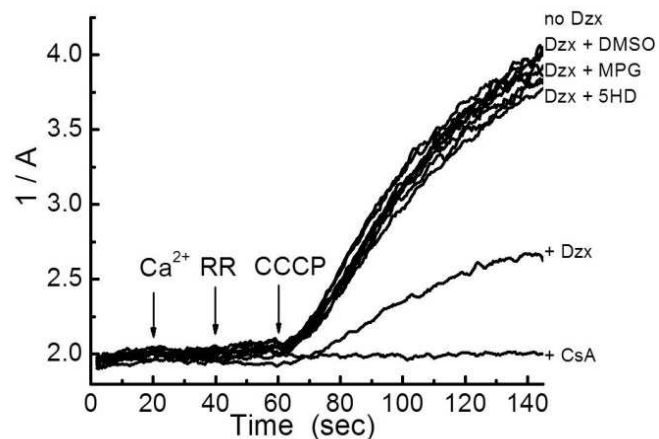
strong thiol reductant properties, holding PKCε in a reduced state and thereby preventing its activation.

DMF blocks cardioprotection by ischemic preconditioning and diazoxide. The implication of the previous mitoK$_{ATP}$ experiments is that intercepting the HO$^•$-dependent reactions will block all modes of ROS-dependent cardioprotection. To examine this question, we perfused the heart with 1% DMF, the HO$^•$-scavenger, following the protocols described in **Fig. 2.3**. As demonstrated in **Fig. 2.10**, DMF inhibited the improvement in post-ischemic functional recovery (**Fig. 2.10A**) and blocked the infarct-size reduction (**Fig. 2.10B**) normally observed with ischemic preconditioning (IPC) and

diazoxide (Dzx). DMF alone had no effect on infarct size in these conditions (**Fig. 2.10B**). Again, the effect of DMF on cardioprotective signaling implicates a downstream oxidation product of HO$^•$ as the signaling ROS.

Fig. 2.11. *Effects of free radical reactants on mitoK$_{ATP}$-dependent MPT inhibition.* Shown are light scattering traces (1/A) of rat liver mitochondria respiring on succinate in K$^+$ medium, and assayed as described in "Methods." Free calcium (100 μM) ("Ca^{2+}"), ruthenium red (0.1 μM) ("RR"), and carbonyl cyanide m-chlorophenylhydrazone (0.25 μM) ("CCCP") were added sequentially at 20 sec intervals, as shown. MPT inhibition by diazoxide (30 μM) was blocked by 5-HD (300 μM) (mitoK$_{ATP}$ blocker), MPG (1 mM) (thiolreductant), and DMSO (1%, 14 M) (HO$^•$/RO$^•$ reactant). The data presented are representative of three independent experiments with p < 0.05.

Endogenous ROS signaling and mitoK$_{ATP}$-dependent MPT inhibition. MPT synchronization,

described in "Methods," was employed to determine the role of HO$^•$, RO$^•$ and/or ROO$^•$ in diazoxide induced MPT inhibition, which depends on ROS activation of a second mitochondrial PKCε, PKCε2 (18). Results of a representative experiment are contained in **Fig. 2.11**. CsA completely blocked MPT activity, and diazoxide ("Dzx") inhibited MPT opening by about 60%, which is the customary extent observed in these experiments (20). This inhibition was reversed by 5-HD ("5-HD"). MPG, DMSO, and DMF (not shown) also reversed MPT inhibition by diazoxide. These results also imply that the signaling ROS that activates PKCε2 must be a downstream oxidation product of HO$^•$.

2-E: Discussion

The major findings of the present study are *1)* that the signaling reactive oxygen species (ROS) responsible for activating mitochondrial PKCεs is a downstream oxidation product of hydroxyl radical, not superoxide or H_2O_2, and *2)* that the main action of submillimolar MPG on mitochondria and perfused heart is inactivation of PKCε by thiol reduction rather than ROS scavenging.

MitoK$_{ATP}$ governs the ROS signaling that is essential for cardioprotection (40). MitoK$_{ATP}$ opening has been found to increase levels of ROS in perfused heart (113), cardiomyocytes (109) and in the mitochondrial matrix (2). The ionophore valinomycin, at a concentration yielding the same K^+ flux as diazoxide, caused the same ROS increase as diazoxide, so mitoK$_{ATP}$-dependent increase in ROS is due specifically to K^+ influx into the matrix (2). The primary mitochondrial targets of ROS signaling are PKCε1, which activates mitoK$_{ATP}$, and PKCε2, which inhibits MPT (reviewed in (45)). Mitochondrial PKCε1 and mitoK$_{ATP}$ co-purify and co-reconstitute to form a functioning signaling module in proteoliposomes (71). Thus, mitoK$_{ATP}$ in either mitochondria or proteoliposomes can be opened by all PKCε activators, including phorbol esters, the specific peptide activator, ΨεRACK, and a variety of thiol oxidizing agents.

Our objective is to identify the mitoK$_{ATP}$-dependent endogenous ROS that activates PKCε1 to leave mitoK$_{ATP}$ in a phosphorylated open state. Superoxide is the first ROS that increases due to increased K^+ uptake into the matrix (see **Fig. 2.1**) and has been proposed to be the ROS signal (119). Xanthine/xanthine oxidase (X/XO) induced mitoK$_{ATP}$ opening in isolated mitochondria; however,

superoxide rapidly dismutates to H_2O_2 in aqueous solution, and Costa et al. (20) showed that X/XO did not open mitoK$_{ATP}$ in the presence of catalase. Therefore, the observed effect of X/XO was mediated by H_2O_2, and superoxide can be excluded as a PKCε activator or mitoK$_{ATP}$ opener. Queliconi, et al. (119) repeated the X/XO experiment and obtained the same results; however they failed to control for H_2O_2 production and erroneously concluded that superoxide opens mitoK$_{ATP}$. PKCε is activated by nitric oxide (NO) (7) and H_2O_2 (82), which oxidize the thiols in the zinc finger of PKCε, leading to its activation. Consistent with this biochemistry, H_2O_2 and NO cause mitoK$_{ATP}$ opening (20, 71) that is blocked by the PKCε peptide inhibitor εV$_{1-2}$. The absence of an effect of H_2O_2 and NO in the presence of εV$_{1-2}$ demonstrates that these agents have no direct effect on mitoK$_{ATP}$. Again, Quelconi, et al. (119) repeated these experiments and obtained the same result; however they failed to control for PKCε involvement and erroneously concluded that H_2O_2 and NO open mitoK$_{ATP}$ directly.

Liu, et al. (91) concluded that <u>hydroxyl radical</u> (HO$^\bullet$) is responsible for ROS signaling in the heart, but this conclusion was based on experiments showing that MPG blocks ROS signaling and the assumption that this block was due to HO$^\bullet$ scavenging. This assumption may be incorrect. The data In **Fig. 2.8** show that MPG blocks H_2O_2-induced mitoK$_{ATP}$ opening. HO$^\bullet$ is not involved in this process, and MPG has little or no H_2O_2 scavenging ability (10, 91). Moreover, eliminating HO$^\bullet$ requires very high concentrations of scavenger due to the transitory nature of HO$^\bullet$, and it is unlikely that sub-millimolar concentrations of MPG would be effective. Thus, most scavengers "will never be present at levels

remotely approaching those of endogenous molecules that react at diffusion-controlled rates with HO$^{\bullet}$" (63). Therefore, we infer that MPG prevents signaling, not by scavenging ROS, but rather by virtue of its powerful thiol reduction properties, keeping PKCε in a reduced, inactive state. Conclusions drawn from experiments in which MPG blocks ROS signaling, including those of Liu, et al. (91), should be re-evaluated in view of this new finding related to the mechanism of action of MPG.

To investigate the location giving rise to the endogenous ROS signal, we employed the preincubation protocol described in "Methods" and "Results". This protocol leads to feedback activation of mitoK$_{ATP}$, which depends on endogenous ROS signaling to PKCε1 (20). The results presented in **Figs. 2.4** through **2.7** provide important clues to the identity of the ROS messenger. They are best understood in the context of **Figs. 2.1** and **2.2**. The antioxidants deferoxamine and Trolox each prevented mitoK$_{ATP}$ opening. Deferoxamine is an iron chelator, and Trolox, a water soluble derivative of vitamin E, is a peroxyl radical scavenger and lipid peroxidation chain-breaking antioxidant. These data indicate that the redox-sensitive opening of mitoK$_{ATP}$ is dependent on both iron and peroxyl radicals and are consistent with transition metal-catalyzed ROO$^{\bullet}$ - dependent signaling. These steps occur downstream of HO$^{\bullet}$ production and strongly suggest that HO$^{\bullet}$ is the precursor of the signaling molecule. This is supported by the finding that inclusion of DMSO or DMF, which react with HO$^{\bullet}$, RO$^{\bullet}$, and/or ROO$^{\bullet}$ (5), in the preincubation prevented mitoK$_{ATP}$ opening.

Many workers have proposed that $\underline{H_2O_2}$ is the messenger of ROS-dependent mitoK$_{ATP}$ opening (2, 145). H_2O_2 would appear to be a good candidate, not least because it is effective in activating mitochondrial PKCε (17, 20, 71, 154) and in protecting ischemic heart (150, 153) and hypoxic cardiomyocytes(31). However, a major finding of the present study is that H_2O_2 can be excluded as the signaling ROS in mitochondria and that the signaling ROS originates downstream of hydroxyl radical. The evidence is *1)* the ROS signal arises downstream from HO$^•$ radical, whereas H_2O_2 is produced upstream of HO$^•$ radical (**Fig. 2.1**); *2)* catalase, the H_2O_2 scavenger, had no effect on the formation of the phosphorylation-dependent open state, whereas DMF and DMSO, which have neither H_2O_2 scavenging nor thiolreductant properties, do block PKCε-dependent mitoK$_{ATP}$ activation. Circumstantial evidence also argues against H_2O_2 as the signaling ROS: H_2O_2 is produced in the matrix, whereas PKCε1 faces the intermembrane space. The matrix of heart mitochondria contains glutathione, thioredoxin reductase and catalase (121), which may prevent sufficient $H_2O_2$from reaching its target (132).

The conclusion that the ROS message is formed downstream from HO$^•$ is further supported by the effects of DMSO and DMF on endogenous ROS-dependent MPT inhibition (**Fig. 2.10**). Finally, the finding that DMF prevents cardioprotection by both ischemic preconditioning (IPC) and diazoxide (**Fig. 2.11**) supports extension of the conclusion to the ex vivo and in vivo heart. That HO$^•$ is necessary for cardioprotective signaling should not be taken to mean that HO$^•$

itself is the signaling ROS. HO$^{\bullet}$ is too reactive for signaling, with a half-life that is diffusion-limited at about one nanosecond (63). HO$^{\bullet}$ will react with its nearest neighbors almost immediately after it is formed, allowing for the formation of downstream oxidation products that are most likely responsible for signaling.

Finally, the finding that BEL interrupted the ROS signal (**Fig. 2.7**) implicates hydroperoxy fatty acids (FAOOH) as the ROS signal. A full investigation of the effects of fatty acids and hydroperoxy fatty acids on mitoK$_{ATP}$ and on ischemia-reperfusion injury will be presented in a subsequent communication.

Limitations. *1)* The conclusions are based largely on *in vitro* experiments carried out on isolated mitochondria. The rationale for this approach is that the signaling ROS not only originates in mitochondria but also acts on mitochondria. We have no reason to believe that the ROS transformation reaction sequence is any different in vivo. *2)* We stress that the conclusions relate specifically to ROS signaling secondary to mitoK$_{ATP}$ opening. The reverse sequence - mitoK$_{ATP}$ opening caused by ROS - may also be physiologically important. For example, it is possible that the increased ROS that occurs during ischemia opens mitoK$_{ATP}$, and this ROS may arise from a different location, such as Complex III. The identity of the ROS signal in this setting has not yet been addressed. *3)* BEL has been shown to inhibit activation of store-activated Ca^{2+} channels; however this is mediated by inhibition of iPLA2 and is therefore a pharmacological effect (130). It has recently been shown that BEL inhibits voltage gated Ca^{2+} and transient receptor potential canonical channels independently of iPLA2 (14). We consider

it unlikely that these effects play a role in the effect of BEL on isolated
mitochondria.

**Conclusions and potential conceptual/pragmatic values of these
findings.** We conclude that the signaling ROS responsible for activating
mitochondrial PKCεs is a downstream oxidation product of hydroxyl radical and
that superoxide and H_2O_2 are not the signaling ROS. We further conclude that
the main action of submillimolar MPG on mitochondria and perfused heart is not
ROS scavenging, but rather thiol reduction, causing inactivation of PKCε and
other ROS-dependent kinases. MitoK$_{ATP}$-dependent ROS signaling is a central
process in cardioprotection and cellular signaling generally. Determining the
identity of the mitochondrial signaling ROS is important for future research in
these areas.

ACKNOWLEDGMENTS

The authors express gratitude for the excellent technical and administrative assistance of Craig Semrad, Caitlin E. Pesout and Beau Sober.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

AOG, MJ and KDG conceived and designed experiments and wrote the paper.

AOG, MJ and JPJ performed the experiments and analyzed the results.

Chapter 3: The role of fatty acids and phospholipase iPLA2 in mitochondrial ATP-sensitive K⁺ channel activity and cardioprotection

Martin Jaburek[1], Anders O. Garlid[2], Petr Jezek[1] and Keith D. Garlid[2]*

[2]Department of Biology, Portland State University, Portland, OR, 97201-0751,

[1]Department of Membrane Transport Biophysics, Institute of Physiology, v.v.i.,

Prague, Czech Republic, 14220

Running Head: Hydroperoxy fatty acids are Second Messengers of

Cardioprotection

Keywords: mitochondria; cardioprotection; hydroperoxy fatty acids; K-ATP

channels; cardiac ischemia; ROS signaling

3-A: Abstract

During ischemia, catabolism of mitochondrial phospholipids is accelerated, causing accumulation of plasmalogens and free fatty acids (FA) in the heart. Calcium-independent phospholipases A2 (iPLA2) are the major phospholipases in myocardium and the role of iPLA2 as well as the effects of various fatty acids and fatty acid analogues during ischemia are not completely understood. Because mitochondrial ATP-sensitive K^+ channel (mitoK$_{ATP}$) is known to be involved in cardioprotection, here we tested whether FAs affect mitoK$_{ATP}$ opening and cardioprotection. We also studied whether there is a protective role of iPLA2-mediated FA release and tested our hypothesis that hydroperoxy fatty acids (FAOOH) are the second messengers of cardioprotective reactive oxygen species (ROS)-mediated signaling. Following the changes in mitoK$_{ATP}$-dependent changes in mitochondrial matrix volume, our results indicate that natural fatty acids and their hydroxy derivatives inhibit the pharmacologically open state of mitoK$_{ATP}$. Our results also show that fatty acids inhibit the phosphorylation-dependent open state of mitoK$_{ATP}$ with lower affinity than the pharmacologically open state. We found that hydroperoxylinoleic acid opens the ATP-inhibited mitoK$_{ATP}$ and that its effect is blocked by the specific PKCε inhibitor peptide εV$_{1-2}$. These data support our hypothesis of direct activation of PKCε by FAOOH. Using Langendorff perfused hearts, our studies indicate that iPLA2 is essential for most modes of cardioprotection. We show that a selective inhibitor of iPLA2, racemic bromoenol lactone (BEL), is cardioprotective when added before the ischemia and reperfusion. These results are consistent with BEL protecting against

ischemic reperfusion injury by preventing ischemic release of free fatty acids. However, our result also show that BEL blocks protection by preconditioning and postconditioning when added with the treatment. We interpret these results as the ability of BEL to prevent the iPLA2-dependent release of FAOOH, which are necessary for protection.

3-B: Introduction

Mitochondria are effectors of both ischemia-reperfusion injury (IRI) and cardioprotection. Ischemia causes profound and immediate mitochondrial derangements, including cessation of ATP synthesis, inhibition of respiration, and a drop in membrane potential ($\Delta\Psi$). This is accompanied during ischemia by increases in Ca^{2+} and phosphate, and during reperfusion, by large increases in reactive oxygen species (ROS) (30, 141). Together, these factors promote opening of the mitochondrial permeability transition (MPT), a high-conductance pore in the inner mitochondrial membrane, which is the main cause of necrotic cell death in IRI (22, 24, 25, 65, 148).

In addition to their role as mediators of cell death in IRI, mitochondria are major effectors of self-defense mechanisms, including ischemic pre- and post-conditioning (3, 4, 53, 65). These and other conditioning protocols have been shown to require opening of the mitochondrial ATP-sensitive K^+ channel (mitoK$_{ATP}$). Considerable progress has been made in understanding the mechanisms by which mitoK$_{ATP}$ opening increases ROS production (2) and the mechanisms by which ROS open mitoK$_{ATP}$ (20) and inhibits MPT (18). ROS activate key protein kinases (19), making them the second messengers of preconditioning (23). We described data in myocytes supporting the hypothesis that mitoK$_{ATP}$ opening was responsible for the increased ROS of cardioprotection (40), and this was confirmed by several laboratories (32, 40, 84, 108, 110, 111, 138).

During ischemia, catabolism of mitochondrial phospholipids is accelerated, causing accumulation of plasmalogens and fatty acids (FA) in the heart (89, 142, 143). Mitochondrial cardiolipin suffers the most profound change, its level decreasing by 30-50% (112). Several reports have highlighted the negative role that fatty acids may play in cardioprotection. For example, Ford, et al. (33) found that cromakalim did not protect working rat hearts that were perfused with fatty acids. Ischemia itself causes fatty acid accumulation in the heart, caused by net degradation of membrane phospholipids by phospholipases (142, 143). Prasad, et al. (118) and Harris, et al. (64) found that inhibition of phospholipase was cardioprotective. In important work, Sargent, et al (124) and Williams and Gottlieb (149) found that perfusion of rabbit hearts with bromoenol lactone (BEL) prior to ischemia reduced the infarct size almost to that of controls not subjected to ischemia. Protection by BEL was reversed by the simultaneous perfusion of 5-hydroxydecanoic acid (5-HD), implicating participation of $mitoK_{ATP}$ (125). Importantly, BEL is a specific inhibitor of calcium-independent phospholipase A2 (iPLA2), and iPLA2γ is the major phospholipase A2 in myocardium (13, 73).

Almost all of the attention on iPLA2 has focused on its deleterious effects (149), due to its releasing free fatty acids into the heart during ischemia. Thus, the iPLA2 inhibitor bromoenol lactone (BEL), administered before global ischemia, is cardioprotective in rat (124) and rabbit (149), causing marked reduction of infarct size. Protection by BEL was reversed by the simultaneous perfusion of 5-HD, implicating participation of $mitoK_{ATP}$. FA have long been known to promote MPT opening (129, 133), and BEL was also found to inhibit

Ca^{2+}-dependent MPT opening, due to inhibition of iPLA2-mediated arachidonic acid release (80).

There are several studies, however, that point to the protective effect of fatty acids, namely the protective effects of the products of lipoxygenase metabolism. Lipoxygenase inhibitors were shown to block the protective effect of preconditioning and elevated levels of 12-hydroxyeicosatetraenoic acid, a stable product of the lipoxygenase pathway of arachidonic acid metabolism, were detected following the preconditioning protocol (100). There is a correlation between functional recovery after ischemia and stimulation of the lipoxygenase pathway before the sustained period of ischemia (16) and experiments using leukocyte-type 12-lipoxygenase (12-LOX) deficient mice demonstrated the importance of 12-LOX in preconditioning (35).

Given the complex effects of various fatty acids during ischemia, we investigated the interactions of selected fatty acids with $mitoK_{ATP}$. We tested whether fatty acids other than
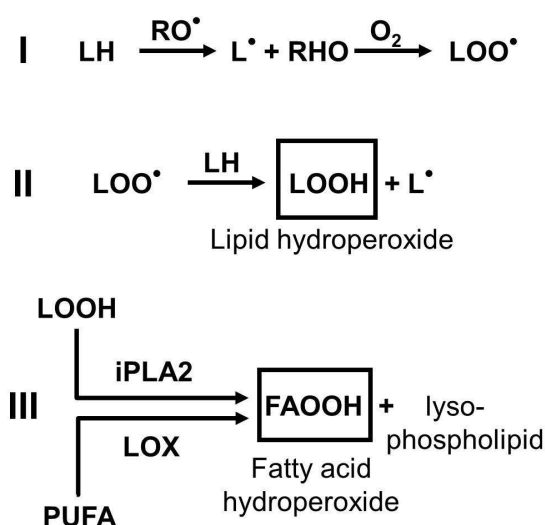
Fig 3.1. *Lipid peroxidation and release of hydroperoxy fatty acids.* **(I and II)** alkoxyl (RO$^{\bullet}$) and alkyl peroxyl radicals (LOO$^{\bullet}$) and alkyl hydroxyl molecules (RHO) can initiate the non-enzymatic chain reaction with fatty acid side chains (LH), leading to lipid hydroperoxides (LOOH). **(III)** LOOH can be released by the action of mitochondrial phospholipases, such as Ca^{2+}-independent phospholipases PLA2 (iPLA2), which have been found to modulate the function of mitochondria (see Cedars et al (13) for a review). The action on LOOH by iPLA2 results in a hydroperoxy fatty acid (FAOOH). Alternatively, FAOOH are formed from polyunsaturated fatty acids (PUFA) by the action of lipoxygenases (LOX).

5-HD affect mitoK$_{ATP}$ opening and cardioprotection. We have further studied the possible protective role of iPLA2-mediated FA release and tested our hypothesis that hydroperoxy fatty acids (FAOOH) are the second messenger of ROS signaling. Using the light scattering technique, our results indicate that natural fatty acids and their hydroxy derivatives behave similarly to 5-HD and inhibit the pharmacologically open state of mitoK$_{ATP}$. Our results also show that fatty acids inhibit the phosphorylated open state of mitoK$_{ATP}$ with lower affinity than the pharmacologically open state. We found that hydroperoxylinoleic acid, LAOOH, opens the ATP-inhibited mitoK$_{ATP}$ and that its effect is blocked by the specific PKCε inhibitor peptide εV$_{1-2}$. These data support our hypothesis of direct activation of PKCε by FAOOH. Our studies using Langendorff perfused heart indicate that iPLA2 is essential for most modes of cardioprotection. We show that BEL inhibits both ischemic preconditioning (IPC) and postconditioning (PC) when added with the treatment. These results are consistent with BEL protecting against ischemic reperfusion injury by preventing ischemic release of free fatty acids. However, our results also show that BEL blocks protection by IPC and postconditioning. We interpret these results as the ability of BEL to prevent the iPLA2-dependent release of FAOOH, which are necessary for protection.

3-C: Research Design and Methods

Langendorff perfused hearts

Hearts from male Sprague-Dawley rats (200-240g) were perfused for 50-55 min as previously described (51, 115, 120) with Krebs Henseleit buffer containing (in mM) 118 NaCl, 5.9 KCl, 1.75 $CaCl_2$, 1.2 mM $MgSO_4$, 0.5 EDTA, 25 $NaHCO_3$, 16.7 glucose at pH 7.4 and gassed with 95% O_2 - 5% CO_2. Hearts were stabilized for 25 min with Krebs Henseleit buffer, then with buffer containing drugs or agents, and then subjected to 25 minutes global ischemia followed by 2 hours of reperfusion and measurement of infarct size. Ischemic preconditioning (IPC) was established by two cycles of 5 min global ischemia followed by 5 min reperfusion prior to the index ischemia. Ischemic postconditioning was performed with 6 cycles of 10 s ischemia plus 10 s reperfusion (140). Cross-sectional slices were stained with TTC after each treatment, scanned, and analyzed to report infarct size as the percentage of damaged tissue relative to the area at risk (IS % Area at Risk). Treatments deemed protective were found to exhibit a reduced infarct size to an extent similar to that reported elsewhere (85, 114, 140). Experimental protocols were treated in accordance with the Guiding Principles in the Use and Care of Animals published by the National Institutes of Health.

Mitochondrial isolation

Male Sprague-Dawley rats (200 - 240 g) were anesthetized with CO_2 and immediately decapitated. The hearts were removed and washed in ice-cold Buffer A (250 mM sucrose, 10 mM HEPES pH 7.2, and 2 mM K-EGTA). The

tissue was finely minced in the presence of 1 mg/ml protease (type XXIV Sigma), and the suspension was diluted 3-fold with Buffer A supplemented with 1 mg/ml fatty acid-free BSA. We observed that mitoK_{ATP} activity depends critically on the time between sacrifice and completion of homogenization. This period was kept as brief as possible and was completed within 2 min. The suspension was homogenized with a motorized Teflon pestle and centrifuged for 3 min at 1500 g. The supernatant was centrifuged for 5 min at 9000 g, and the resulting pellets were resuspended in Buffer A lacking BSA and centrifuged for 3 min at 2300 g. This supernatant was centrifuged for 5 min at 9000 g. The final mitochondrial pellet was resuspended at 35 - 40 mg protein/ml and kept on ice. Mitochondrial protein concentration was estimated using the Biuret reaction (54). This procedure is in accordance with the Guiding Principles in the Care and Use of Animals and was approved by IACUC at Portland State University.

Bromoenol lactone treatment of mitochondria

Where indicated, the mitochondrial pellet was treated with racemic bromoenol lactone (BEL) to inhibit mitochondrial phospholipase iPLA2 and avoid unappreciated accumulation of free fatty acids during the course of a given experiment. The pellet was resuspended in 1 ml of Buffer A lacking BSA in the presence of 5 µM BEL. The suspension was diluted to 30 ml with Buffer A supplemented with 0.5% fatty-acid free BSA to remove residual fatty acids and centrifuged 9000 g for 5 minutes. The resulting pellet was resuspended and the protein concentration was determined as stated above.

Preincubation protocol of isolated mitochondria

Where indicated, isolated mitochondria were treated by an additional preincubation procedure aimed to investigate mitochondria under the conditions of endogenous signaling ROS (20). The mitochondria were incubated in assay medium for 3 minutes at 30°C with 200 μM ATP and 30 μM diazoxide (Dzx). The mitochondria were then isolated from the incubation medium and resuspended in fresh assay medium. MitoK$_{ATP}$ activity was observed using the light scattering assay, as described below, with ATP in the presence or absence of diazoxide.

Measurements of mitochondrial ATP-sensitive K^{+} channel

Changes in mitochondrial matrix volume, which accompany net salt transport into mitochondria, were followed using a quantitative light scattering technique (9, 43). Mitochondria were suspended in a buffered salt assay medium containing K^{+} salts of Cl^{-} (120 mM), HEPES (10 mM), EGTA (0.1 mM), succinate (10 mM) and phosphate (P$_i$) (5 mM), pH 7.2. The osmolality of these media ranged between 275 to 280 mOsm. All media also contained 0.5 mM MgCl$_2$, 2.5 μM rotenone and 1 μg/ml oligomycin.

Light scattering changes of 0.1 mg/ml mitochondrial suspensions were followed at 520 nm and 30°C. The data collection was initiated by addition of the mitochondrial suspension. Data summarized in bar graphs as "MitoK$_{ATP}$ activity (%) @ t=0s" are given by

$$100 \times \frac{[V(x) - V(ATP)]}{[V(0) - V(ATP)]}$$

where $V(x)$ is the observed volume at 120 s under the given experimental condition and $V(ATP)$ and $V(0)$ are observed values in the presence and absence of ATP, respectively.

Statistical analysis

Data are presented as mean ± standard deviation (SD) of the mean. Where appropriate, one-way ANOVA or repeated measures *t*-test were used to analyze the data using Microcal Origin software (Northampton, MA, USA). A value of $p < 0.05$ was considered statistically significant.

Chemicals

All fatty acids, fatty acid derived compounds and bromoenol lactone were from Cayman Chemical. All other chemicals used in the study were from Sigma Chemical Co. (St. Louis, MO, USA).

3-D: Results

5-hydroxydecanoic acid (5-HD) is a relatively simple fatty acid which is considered a specific inhibitor of the mitochondrial ATP-sensitive potassium channels (mitoK$_{ATP}$) (125). However, its chemical structure suggests that its action should not differ greatly from that of other fatty acids. We undertook to investigate this, because the actions of fatty acids and their hydroxy and hydroperoxy derivatives on mitoK$_{ATP}$ have not been previously investigated. **Fig. 3.2** shows light scattering traces from heart mitochondria respiring in K$^+$ medium. The control traces in the absence of ATP and in the presence of ATP indicate the open and the inhibited states of mitoK$_{ATP}$. Addition of 2 μM linoleic acid (LA) had no effect on the control traces in the absence or presence of ATP respectively (data not shown), but had an effect on the open trace in the presence of ATP and the Pharmacological opener diazoxide, similar to the known properties of 5-HD. Because 5-HD, the known inhibitor of mitoK$_{ATP}$, is a fatty acid containing a hydroxy group, we tested whether the hydroxy derivative of linoleic acid exhibits similar effects.

Fig. 3.2. *13-(S)-HODE inhibits mitoK$_{ATP}$.* Light scattering traces from heart mitochondria respiring in K$^+$ medium. The control trace in the in the presence of 0.2 mM ATP indicates the inhibited (closed) state of mitoK$_{ATP}$ and the addition of 30 μM diazoxide (ATP + Dzx) establishes the open state. Addition of 1 μM 13-(S)-HODE had no effect on the control traces in the absence or presence of ATP respectively (not shown), but blocked the diazoxide-induced open state in the presence of ATP (ATP + Dzx + HODE). These are representative traces of at least 3 independent experiments.

The data in **Fig. 3.2** show that the hydroxy derivative of linoleic acid, 13-hydroxyoctadecanoic acid (13-(S)-HODE, 0.5 µM), inhibits mitoK$_{ATP}$ in the presence of ATP and diazoxide. Similar sets of data were obtained with other fatty acids, including lauric acid, 12-hydroxylauric acid, and oleic acid (data not shown). The data indicate that natural fatty acids and their hydroxy derivatives behave similar to 5-HD and inhibit the pharmacologically open state of mitoK$_{ATP}$.

Linoleic acid inhibited the pharmacologically-opened state of mitoK$_{ATP}$ in the presence of ATP and diazoxide with a half-maximal inhibition of 0.46 +/- 0.06 µM (n = 3). To avoid unappreciated changes in the mitochondrial matrix volume due to adenine nucleotide translocase (ANT) - dependent, fatty acid-mediated decrease in membrane potential, similar sets of data were obtained in the presence of carboxyatractyloside (CAT), an inhibitor of the ANT. In the presence of CAT, the half-maximal inhibition was 0.6 +/- 0.05 µM linoleic acid (n = 3), indicating that the ANT-dependent processes did not significantly contribute to the changes in mitochondrial matrix volume under the given experimental conditions.

Hydroxy fatty acids are the detectable stable byproducts of fatty acid peroxidation that are formed by the enzymatic action of lipid glutathione peroxidases (68). Therefore, the stimulating effect of hydroperoxy fatty acids (FAOOH) on mitoK$_{ATP}$ would be hindered by the inhibitory effect of inherently formed hydroxy fatty acids, if the affinity of mitoK$_{ATP}$ to inhibitory fatty acids remains unaltered. On that account we speculated that the phosphorylated open state of mitoK$_{ATP}$ must be less prone to the inhibition by fatty acids and hydroxy fatty acids. To explore this hypothesis, we tested the effect of 13-(S)-HODE on
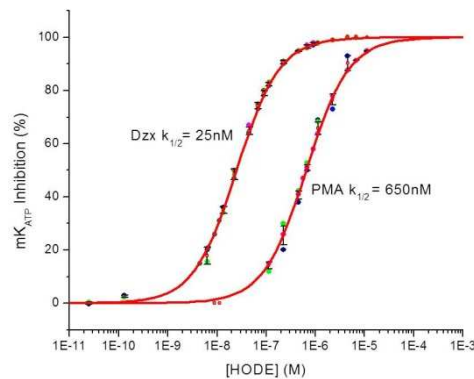
Fig. 3.3. *Diazoxide exhibits a ~25 fold greater sensitivity to 13-(S)-HODE than PMA.* The dose response curves demonstrate that pharmacological mitoK$_{ATP}$ opening is much more sensitive to inhibition by fatty acids than physiological, PKCε-dependent opening. HODE exhibited a K$_{1/2}$ of against the general PKC activator phorbol 12-myristate-13-acetate (PMA) while it exhibited a K$_{1/2}$ of with the K$^+$-channel opener diazoxide. Data are representative of at least 5 independent experiments.

the mitoK$_{ATP}$ in the presence of the pharmacological opener diazoxide or in the presence of general PKC activator phorbol 12-myristate 13-acetate (PMA). As seen in **Fig. 3.3**, 13-(S)-HODE inhibits the pharmacological open state of mitoK$_{ATP}$ induced by diazoxide and **Fig. 3.3** demonstrates that it inhibits diazoxide with ~25-fold greater affinity than the PMA.

We have also preincubated the mitochondria using our previously described preincubation protocol (20), except the pharmacological opener diazoxide was replaced by the general PKC activator phorbol myristate acetate (PMA). After preincubating mitochondria with PMA, 13-(S)-HODE inhibits mitoK$_{ATP}$ with significantly lower affinity than observed for its inhibition of the pharmacologically open state. Similar data were obtained when using LA and arachidonic acid as the inhibitory FA (data not shown). These experiments indicate that fatty acids inhibit the phosphorylated open state of mitoK$_{ATP}$ with significantly lower affinity than the pharmacologically opened state.

Figs. **3.5A** and **3.5B** show the effects of 13-(S)-HODE in Langendorff-perfused hearts. **Fig. 3.4** depicts the Langendorff perfusion protocols used. Both ischemic preconditioning (IPC) and diazoxide (50 µM), the pharmacological mitoK$_{ATP}$ opener, significantly reduce myocardial infarct sizes and improve functional recovery compared with untreated ischemic hearts. The protective effects of both were abolished by concurrent treatment
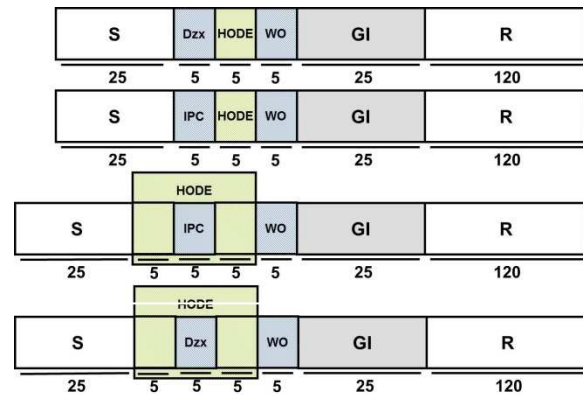
Fig. 3.4. *Isolated heart protocol – HODE studies.* Isolated perfused rat hearts were stabilized (S) for 25 minutes and treated as described in "Methods", following these protocols. After treatment, each heart is subjected to 25 minutes of global ischemia (GI) and 120 minutes of reperfusion (R). In the first two protocols, the 5 minute treatment with 50 µM diazoxide (Dzx) or ischemic preconditioning (IPC) is immediately followed by a 5 minute treatment with HODE and a 5 minute washout. The following two protocols have a bracketed administration of HODE such that it is perfused for 5 minutes before, during, and for 5 minutes after the IPC or Dzx treatments, then washed out for 5 minutes before the general ischemia.
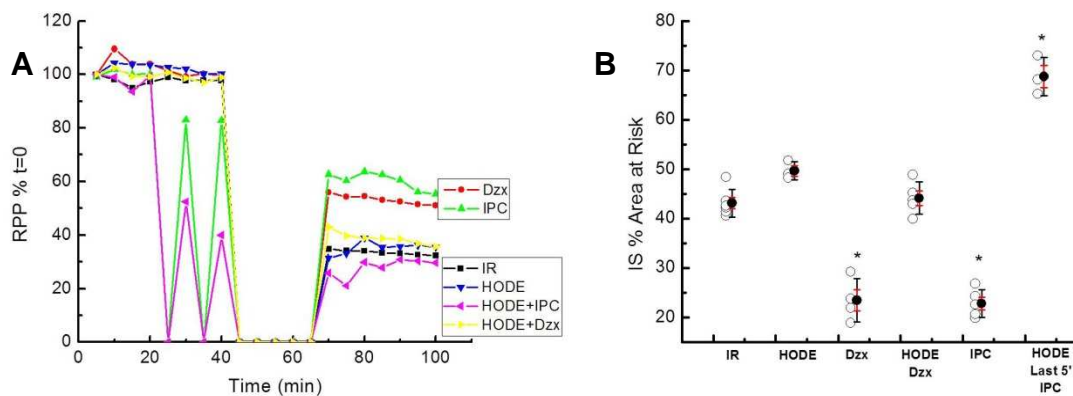
Fig. 3.5. *13-(S)-HODE blocks IPC and Dzx protection in isolated perfused rat heart.* The graph in **(A)** shows that HODE blocks the improvement in functional recovery afforded by both diazoxide (Dzx) and IPC. These are representative traces of at least 3 independent experiments. The chart in **(B)** shows that the infarct size reduction from diazoxide and IPC is also blocked by HODE. This data contains results from at least 3 independent experiments and data are means ±SD; * $p < 0.05$.

with 2 µM 13-(S)-HODE.

Our previous results have established HO˙ as a key component of the cardioprotective signaling (36). HO˙ is very unlikely to play a direct role in signaling, however, because its half-life is short and it reacts with everything it meets. Therefore, we have hypothesized that a downstream product that may arise from an increase in HO˙ is responsible for the signaling (36). Hydroperoxy fatty acids (FAOOH) are transient, non-radical and reactive species (27) that are known to activate protein kinases (134). They have the distinct advantage of being able to flip-flop across the inner membrane bilayer to

Fig. 3.6. *Hydroperoxy fatty acid-dependent opening of mitoK$_{ATP}$ is mediated via PKCε.* Light scattering traces (1/A) of rat heart mitochondria respiring on succinate in K$^+$ medium. With the exception of the trace ˈno ATPˈ, ATP was present in all traces. FAOOH opens mitoK$_{ATP}$ in a PKCε-dependent manner, as demonstrated by its inhibition by εV$_{1-2}$. Mitochondria were suspended at 0.1 mg/ml and assayed as described in ˈMethodsˈ. LAOOH (1 µM) was added 2 seconds after the addition of mitochondria. ATP (0.2 mM) and εV$_{1-2}$ (1 µM) were present in the assay medium prior to the addition of mitochondria. These results are representative of at least 3 independent experiments.

reach PKCε on either side of the membrane (72). In the following sets of experiments, we aimed to determine whether FAOOH are competent ROS messengers.
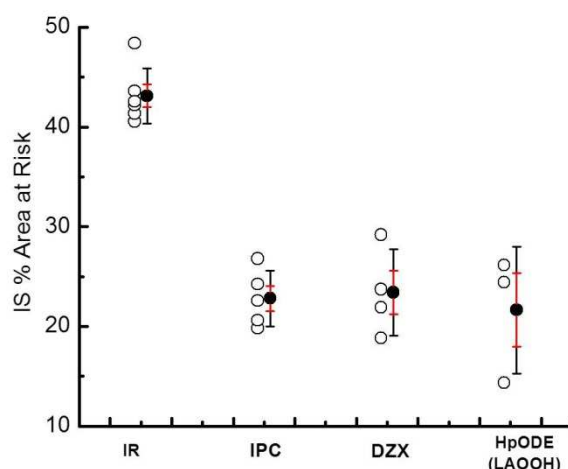
Fig. 3.6 contains light scattering traces from heart mitochondria respiring in K$^+$ medium showing that 1 µM hydroperoxy linoleic acid (13-

hydroperoxyoctadecanoic acid, or LAOOH), a product of cardiolipin peroxidation

and cleavage, had no effect on mitoK$_{ATP}$ under the conditions used to

demonstrate that fatty acids inhibit the channel. That is, in the presence of ATP

and the pharmacological opener diazoxide, LAOOH was ineffective. To the

contrary, LAOOH was able to open the ATP-inhibited mitoK$_{ATP}$. The data also

show that LAOOH acts via PKCε, because its effect was blocked by the specific

inhibitor peptide εV$_{1-2}$. The data support our hypothesis of direct activation of

PKCε by hydroperoxy fatty acids in general.

That hydroperoxy fatty acids open mitoK$_{ATP}$ suggests that they also

protect the heart. Indeed, **Fig. 3.7** demonstrates that the high infarct size

observed with an ischemia-reperfusion (IR) is abolished by diazoxide (Dzx) and

ischemic preconditioning (IPC) and that LAOOH causes a nearly identical reduction in infarct size; each of these treatments also showed significant improvement in the functional recovery of the hearts (data not shown).

Fig. 3.7. *FAOOH confer protection to the same degree as IPC and diazoxide.* FAOOH perfused at 2 μM were cardioprotective in the isolated perfused rat heart, to the same degree as ischemic preconditioning (IPC) and diazoxide (Dzx). Each treatment resulted in an improvement in functional recovery (not shown) and a significant reduction in infarct size. Data are means ±SD of at least 3 independent experiments and significant with $p < 0.05$.

We are not certain which of the many products of phospholipid oxidative modifications and breakdown are responsible for the signaling *in vivo*. For example, 4-hydroxynonenal

(HNE), a non-enzymatically produced breakdown product of lipid peroxidation, also opens mitoK$_{ATP}$ via PKCε, but it does so at about 10-fold lower potency than FAOOH (data not shown). In addition, nitration of unsaturated fatty acids yields electrophilic nitroalkene derivatives (FANO$_2$) known to function as pluripotent endogenous signaling molecules (67) and nitroalkenes were shown to be formed in mitochondria during IPC (105). Therefore, we have also tested the effect of nitro oleic acid (OANO$_2$) on mitoK$_{ATP}$. 1 μM nitro oleic acid exhibited properties similar to the tested linoleic acid hydroperoxide. Namely, the OANO$_2$ had no effect on the pharmacologically opened mitoK$_{ATP}$, indicating that FANO$_2$ do not inhibit mitoK$_{ATP}$. However, OANO$_2$ was able to reverse the inhibitory effect of ATP to the level of control open trace and this opening effect was inhibited by 5-HD, indicating that the effect OANO$_2$ was mediated by mitoK$_{ATP}$. The main difference between the opening effects of LAOOH and OANO$_2$ is supported by the data showing that the effect of OANO$_2$ is not

Fig. 3.8. *Dual role of iPLA2 action in mitochondria and cardioprotection.* **(I)** When a fatty acid side chain (L) interacts with the Ca^{2+}-independent phospholipase A2 (iPLA2), free fatty acids (FA) are formed, which inhibit mitoK$_{ATP}$ and cause damage to the untreated heart. In this case, bromoenol lactone (BEL), the iPLA2 inhibitor, acts as a protective agent because it blocks the formation of FA and allows mitoK$_{ATP}$ opening to occur. **(II)** When iPLA2 interacts with a lipid hydroperoxide, hydroperoxy fatty acids (FAOOH) are formed, which open mitoK$_{ATP}$ and confer protection. In this case, BEL blocks both diazoxide and IPC protection because the formation of the proposed signaling ROS is blocked.

sensitive to the specific inhibitor peptide $\varepsilon V_{1\text{-}2}$, indicating that the effect of $OANO_2$ acts independently of PKCε.

To determine the effect of fatty acids on mitoK$_{ATP}$ under the conditions of endogenous ROS production by mitochondria, we utilized a previously established preincubation protocol. This triggers a feedback activation mechanism that keeps mitoK$_{ATP}$ in its open state by increasing ROS production at Complex I of the respiratory chain (2) in a PKCε-dependent manner (20). The endogenous open state of mitoK$_{ATP}$ was prevented by both LA and 13-(S)-HODE, which were tested at a concentration sufficient to fully inhibit the pharmacologically open mitoK$_{ATP}$. In addition, the phosphorylated open state was blocked by racemic bromoenol lactone (BEL), the inhibitor of phospholipases iPLA2 (8). The inhibition of the ROS-induced open state by BEL implies the participation of the mitochondrial phospholipases iPLA2 in the regulation of mitoK$_{ATP}$. These experiments indicate that a compound responsible for the ROS-induced activation of PKCε is dependent upon the enzymatic function of iPLA2.

FAOOH arise from peroxidation of polyunsaturated fatty acids (PUFA) derived from membrane phospholipids or alternatively from the enzymatic action of lipoxygenases (LOX). As shown in **Fig. 3.8**, both mechanisms require iPLA2. In the following sets of experiments, we tested the hypothesis that iPLA2 activity is essential for intramitochondrial signaling and for cardioprotection. In order to
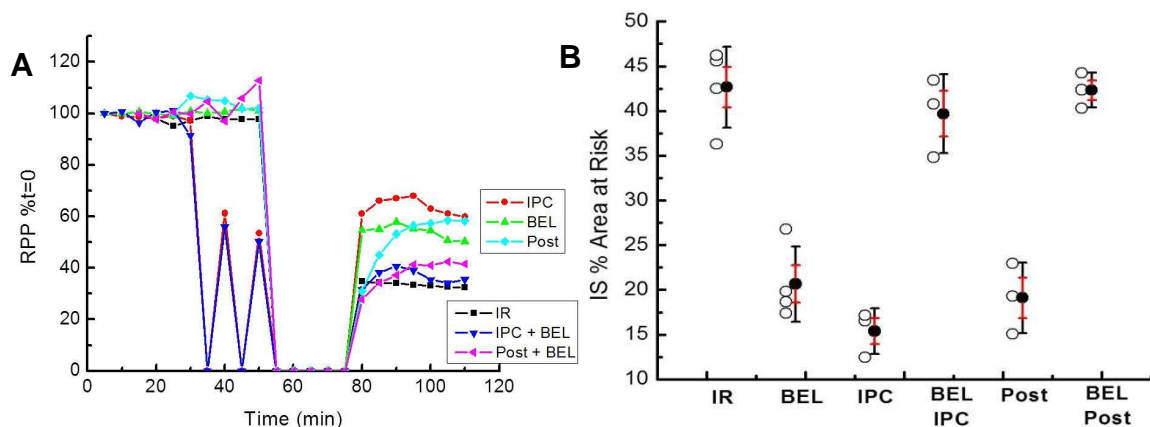
Fig. 3.9. *Inhibition of Ca2+-independent phospholipase A2 (iPLA2) demonstrates that FAOOH are necessary for signaling.* **(A)** BEL is shown here to block IPC ("IPC+BEL") and ischemic postconditioning ("Post+BEL") improvements in functional recovery but also to confer protection when administered alone ("BEL"). Data representative of at least 3 independent experiments. **(B)** Infarct size was significantly reduced with the administration of IPC and postconditioning and this was matched by treatment with BEL alone. However, BEL inhibited the protective effects of both IPC and postconditioning. Data are means ±SD, representative of at least 3 independent experiments. Not shown is BEL's ability to block diazoxide as well.

approach the hypotheses, the ability of the iPLA2 inhibitor bromoenol lactone

(BEL) to block cardioprotection was tested in the perfused heart. **Fig. 3.9** shows

that BEL protects the heart and reduces infarct size when added alone,

consistent with previously published findings (149). These results are consistent

with BEL protecting against ischemic reperfusion injury by preventing ischemic

release of free fatty acids, which are known to accumulate during myocardial

ischemia (81, 142). However, BEL blocks protection by both ischemic

preconditioning (IPC) and postconditioning (PC) when added with either

treatment. We interpret these results as the ability of BEL to prevent the iPLA2-

dependent release of FAOOH, which are necessary for protection.

3-E: Discussion

Fatty acids are the preferred metabolic substrates of the heart, but when elevated they are harmful to the heart. Mitochondria play a key role in both ischemia-reperfusion injury (IRI) and cardioprotection and the opening of mitoK$_{ATP}$ was shown to be obligatory in all tested protocols of cardioprotection. 5-HD is a simple hydroxy fatty acid which is frequently used in many studies as the specific inhibitor of mitoK$_{ATP}$ (125). Here we studied whether other fatty acids have a similar ability to inhibit the pharmacologically opened mitoK$_{ATP}$. We focused on linoleic acid and its metabolites in this study because these are the detected products of mitochondrial cardiolipin cleavage, which, during ischemia, suffers the most profound change (112). Linoleic acid inhibited the pharmacological open state of mitoK$_{ATP}$ in the presence of ATP and diazoxide. The half-maximal inhibition observed under the given experimental condition yielded a mean value of 0.46 µM. This value is based on total fatty acid added to the assay and will vary with experimental conditions, primarily with the concentration of phospholipids in the assay. The corresponding value in free fatty acid concentration can be calculated based on the known extinction coefficient for linoleic acid and the calculation based on our experimental conditions yields an approximate value of 250 nM. Our data in **Fig. 3.2** further show that not only linoleic acid, but the hydroxy derivative 13-hydroxydecadenoic acid (13-(S)-HODE), a stable product of linoleic acid peroxidation, also inhibits mitoK$_{ATP}$. These data indicate that natural fatty acids and their hydroxy derivatives behave similar to 5-HD and inhibit the pharmacologically open state of mitoK$_{ATP}$.

FA become elevated in two distinct ways: in association with obesity and/or metabolic syndrome, and in association with ischemia itself, due to activation of phospholipases. Pirro, et al (117) showed that elevated plasma FA concentrations are associated with an increased risk of ischemic heart disease, and Lopaschuk et al (92) noted that the risk for cardiovascular disease such as myocardial infarction and heart failure is drastically increased with obesity. Obesity has also been shown to be harmful experimentally. IPC failed to protect leptin-deficient ob/ob mice (11), and both IPC and diazoxide failed to protect Zucker obese rats (77). These studies, however, do not address the question as to whether FA have <u>acute</u> effects on cardioprotection, because compensatory adaptations may have occurred in man or genetically altered mice. In an important study, Ford, et al (33) found that the working rat heart was no longer protected from ischemia-reperfusion injury by cromakalim when the perfusate included 1.2 mM palmitate (as well as glucose and insulin). Our data shown in **Fig. 3.5** support the view that elevated FA directly and acutely interfere with cardioprotection. Fatty acids, including linoleic acid and its hydroxy derivative, 13-(S)-HODE, acutely block protection by IPC and diazoxide.

Our previous results have established hydroxyl radicals (HO$^\bullet$) as a key component of cardioprotective signaling (36). Hydroxyl radicals readily oxidize the side chains of polyunsaturated phospholipids, rendering them more susceptible to cleavage by phospholipases and resulting in formation of hydroperoxy fatty acids, FAOOH (Reaction II, **Fig. 3.8**). FAOOH are transient, non-radical and highly reactive species (72) and are known to activate protein

kinases (134). We also showed some years ago that FAOOH can readily flip-flop across the inner membrane bilayer (72) and thus reach its receptor on the other side of the membrane.

Fig. 3.6 contains results showing that hydroperoxy linoleic acid (LAOOH) is a potent $mitoK_{ATP}$ opener. **Fig. 3.6** also shows that LAOOH acts via $PKC\varepsilon$, because its effect is blocked by the specific peptide εV_{1-2}. LAOOH is the expected product of non-enzymatic peroxidation due to the action of hydroxyl radical on the side chains of polyunsaturated phospholipids, which are represented in mitochondria mainly by cardiolipin (88). Alternatively, LAOOH is a product of enzymatic peroxidation of linoleic acid cleaved from cardiolipin due to the action of lipoxygenases (Reaction III, **Fig. 3.8**). The role of lipoxygenase metabolites in ischemic preconditioning was studied previously with the focus on leukocyte type 12-lipoxygenase (12-LOX) metabolites of arachidonic acid (16, 100). The data suggested that 12-LOX metabolism plays an important role in cardioprotection. The results, however, did not address the role of mitochondria and also could not distinguish between the actions of hydroperoxy fatty acids and the subsequently produced hydroxy fatty acids, as only the corresponding elevated levels of hydroxy fatty acid analogue of arachidonic acid were measured as the stable metabolite indicating the actions of activated 12-LOX. Nevertheless, 12-LOX deficient mice show impaired ischemic preconditioning-induced cardioprotection (35). In our view, these results indicate the intriguing possibility that 12-LOX is co-localized to mitochondria, a hypothesis which awaits further

investigation. In support of the view that FAOOH are cardioprotective, we show in **Fig. 3.7** that exogenous LAOOH confers cardioprotection in isolated perfused rat heart comparable to the protection achieved by diazoxide.

Ischemic preconditioning is also known to yield elevated levels of nitric oxide with consequent formation of nitroalkenes, such as nitro fatty acids (105). We have therefore also investigated whether nitro oleic acid ($OANO_2$) acts in a manner similar to LAOOH and activates $mitoK_{ATP}$ via PKCε - dependent pathway. $OANO_2$ induces 5-HD-sensitive opening of ATP-inhibited $mitoK_{ATP}$ but it is not sensitive to the specific PKCε inhibitor $εV_{1-2}$, indicating that the action of $OANO_2$ is PKCε-independent. This finding agrees with recent observations that $mitoK_{ATP}$ was activated by nitrolinoleate ($LANO_2$) (119) and also with the finding that the cardioprotective effects of $LANO_2$ were not attributable to protective kinase signaling (105).

If hydroperoxy fatty acids (FAOOH) are the ROS species essential for intramitochondrial signaling, then it follows that phospholipase iPLA2 is essential for cardioprotection. iPLA2 was shown to be localized to mitochondria (13, 93)and also to be activated by elevated exogenous ROS in isolated mitochondria (74). The enzymatic actions of iPLA2 lead to release of either the FAOOH from the oxidized phospholipid side chains, or to release of FA that are oxidized to FAOOH by the enzymatic action of lipoxygenase, enabling FAOOH to diffuse to their PKCε targets. Our following new findings summarized in **Fig. 3.9** support this hypothesis: *1)* bromoenol lactone (BEL) blocks PKCε-dependent $mitoK_{ATP}$

opening; and *2)* BEL blocks protection by IPC and ischemic postconditioning. Note that BEL *protects* the unconditioned heart as shown previously by Williams and Gottlieb (149). This occurs because BEL blocks ischemic release of FAs that inhibit mitoK$_{ATP}$. This dual role of iPLA2 has not previously been described and is due to the fact that iPLA2 acts on both un-oxidized and oxidized phospholipids. We show that FA and FAOH inhibit mitoK$_{ATP}$ (**Figs. 3.2** and**3.3**) and block protection (**Fig. 3.5**). FAOOH released from oxidized lipids or formed by the enzymatic action of lipoxygenases are potent activators of the mitochondrial PKCεs that regulate mitoK$_{ATP}$ (**Fig. 3.6** through **3.8**). The phosphorylated open state of mitoK$_{ATP}$ is less sensitive to the inhibitory effect of FA, as demonstrated by the preincubation experiments involving LA and 13-(S)-HODE. The BEL experiments shown in **Fig. 3.9** establish that products of lipid peroxidation and cleavage provide the ROS signal and that FAOOH or one of its products is the ROS signal. It leads to the further hypothesis that mitochondrial iPLA2 is an essential component of cardioprotection.

Chapter 4: Conclusions

The primary aim of this project is to identify the reactive oxygen species (ROS) that acts as the second messenger in cardioprotective signaling by ischemic and pharmacological preconditioning. The results of this study lead to two conclusions: *1)* neither hydrogen peroxide (H_2O_2) nor hydroxyl radical ($HO^\bullet$) is responsible for feedback activation of mitoK_{ATP} and *2)* the signaling ROS is a downstream oxidation product of $HO^\bullet$, namely a hydroperoxy fatty acid (FAOOH) released by the calcium-independent phospholipase A2 (iPLA2). These results further the understanding of signaling in cardioprotection and dispel widely-held misunderstandings about the nature and identity of ROS signaling molecules. Furthermore, signaling ROS are crucial to processes across cell types and this research provides a location of ROS signal formation, a model for their transformation and a likely downstream candidate to seek in other systems.

H_2O_2 and $HO^\bullet$ are products of oxidation-reduction reactions beginning with superoxide ($O_2^{\bullet-}$) generated at complex I of the electron transport chain. Brookes et al. claimed that $O_2^{\bullet-}$ is the signaling ROS but failed to take into account the production of H_2O_2 (119). By adding catalase to their media as a control, Costa et al. demonstrated that $O_2^{\bullet-}$ does not directly activate mitoK_{ATP} but that H_2O_2 is responsible for the mitoK_{ATP} effect in Brookes' system (20). Schumacker et al. proposed that H_2O_2 is the signaling ROS and this was widely accepted without question (86, 145). However, the matrix of heart mitochondria contains large amounts of catalase and other antioxidants (121), which would likely consume the small amounts of H_2O_2 produced (132), indicating that H_2O_2 may not be a

viable signaling ROS in cardioprotection. Indeed, the data presented here demonstrate that H_2O_2 is not the signaling ROS, namely due to the lack of an effect by catalase on the feedback-activated state of mitoK$_{ATP}$ and the collection of scavengers and chain-breaking antioxidants indicating a signal downstream of HO$^\bullet$ (36).

Downey et al. proposed HO$^\bullet$ as the signaling ROS (6), but it is far too short lived to serve as a transportable signaling species (87). Furthermore, this conclusion was based on experiments utilizing MPG, a purported HO$^\bullet$-scavenger with strong thiol-reductant properties that confound experiments on mitoK$_{ATP}$ due to its effect on PKCε. The HO$^\bullet$-scavenging capabilities of MPG cannot be tested here because mitoK$_{ATP}$ will be inhibited whether or not HO$^\bullet$ is present with MPG keeping PKCε in its reduced state. Accordingly, our data indicate that the signaling ROS is a downstream oxidation product of HO$^\bullet$ that acts as the second messenger in cardioprotective signaling and we have identified this ROS as a hydroperoxy fatty acid.

We have demonstrated here that FAOOH are responsible for feedback activation of mitoK$_{ATP}$ and MPT inhibition and that they are necessary in cardioprotection by ischemic and pharmacological preconditioning. Fatty acid side chains are peroxidized by the ROS generated from mitoK$_{ATP}$ opening and FAOOH are released by the action of iPLA2 on these peroxidized side chains. FAOOH are then able to flip-flop across membranes (76) and could serve as a transportable form of ROS for a variety of signaling processes. Further studies

exploring the life-time and transportability of FAOOH as well as their role in other signaling processes are needed to complete this project and gain a greater understanding of ROS signaling from the mitochondrion.

While this study shows that FAOOH are a protective ROS, their non-peroxidized counterparts, fatty acids (FA), have been demonstrated to present dangers in a number of ways. FA become elevated in association with obesity and during ischemia itself by the action of phospholipases that release FA from phospholipid side chains. Elevated plasma FA is associated with a greater risk of ischemic heart disease (117) and the risk for cardiovascular disease such as myocardial infarction and heart failure is drastically increased with obesity (92). There are many avenues to pursue for therapeutic and preventative treatments of these conditions, but an in-depth understanding of the signaling processes involved is certainly necessary.

MitoK$_{ATP}$-dependent ROS formation has been shown to trigger gene transcription (46)and is also necessary in endogenous cardioprotective signaling via bradykinin, opioid and adenosine G$_i$ protein-coupled receptor (GPCR) pathways (29). GPCR signaling has recently been found to operate via a 'signalosome', a unique signaling model that transports the receptor-agonist complex formed at the cell membrane along with the entire signaling cascade in a caveolar raft (120). These signalosomes likely travel along microtubules as opposed to relying on diffusion, and inhibitors of microtubules have been shown to block preconditioning (106). ROS is required for activation of the adenosine

signalosome and it is also necessary for the feedback activation that establishes cardioprotective 'memory' witnessed in preconditioning (20, 144).

ROS signaling has been invoked by research labs across nearly all fields. Notably, Muscari et al. recently published a paper involving hypoxic pretreatment of adult stem cells for the purpose of developing more effective regenerative medicines by accomplishing greater survival rates for grafted stem cells (104). While multipotent stem cells could be of great use for in-vivo tissue repair, they suffer from a poor post-engraftment survival rate that makes the clinical treatment unviable. Ischemic preconditioning (IPC) certainly has the potential to serve as an important treatment in improving stem-cell survival and signaling ROS play a vital role in this process. As stated in the review, "mitochondrial ROS are consistently involved in the causative mechanisms of IPC" (104) and identifying the species behind these mechanisms is necessary for a full understanding of protective signaling.

The pragmatic value or application of this research rests in the future development of therapeutic and preventative treatment against heart disease and the damage resulting from heart attacks. While an immediate benefit from these studies are unavailable, uncovering the pathways and crucial players in signaling pathways of cardioprotection is vital to our future understanding of and ability to treat these diseases. Furthermore, the signaling processes at play in cardioprotection are by no means unique to the heart; $mitoK_{ATP}$ and ROS signaling play integral roles in signaling throughout all cell types.

With so much of the population at risk of heart disease, heart attacks, diabetes, obesity and a variety of mitochondrial diseases, it is of utmost importance that we explore these questions and undertake the efforts in basic research necessary to lay the foundation for treatment and prevention.

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Chapter 2 **"**Mitochondrial Reactive Oxygen Species (ROS): Which ROS Signals Cardioprotection?" has been published in the American Journal of Physiology: Heart and Circulatory Physiology section, and the full citation is found in the References section of this thesis, as follows:

36.   **Garlid AO, Jaburek M, Jacobs JP, and Garlid KD**. Mitochondrial Reactive Oxygen Species - Which ROS Signals Cardioprotection? *Am J Physiol Heart Circ Physiol* 2013.

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