NIH Public Access
Author Manuscript
Biochim Biophys Acta. Author manuscript; available in PMC 2012 July 1.

Published in final edited form as:
Biochim Biophys Acta. 2011 July ; 1813(7): 1309–1315. doi:10.1016/j.bbamcr.2010.11.005.

Redox Regulation of the Mitochondrial K_{ATP} Channel in Cardioprotection

Bruno B. Queliconi[a,*], **Andrew P. Wojtovich**[b,*], **Sergiy M. Nadtochiy**[b], **Alicia J. Kowaltowski**[a], and **Paul S. Brookes**[b,§]

[a]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brazil.

[b]Department of Anesthesiology, University of Rochester Medical Center, Rochester NY, USA.

Abstract

The mitochondrial ATP-sensitive potassium channel (mK_{ATP}) is important in the protective mechanism of ischemic preconditioning (IPC). The channel is reportedly sensitive to reactive oxygen and nitrogen species, and the aim of this study was to compare such species in parallel, to build a more comprehensive picture of mK_{ATP} regulation. mK_{ATP} activity was measured by both osmotic swelling and Tl^+ flux assays, in isolated rat heart mitochondria. An isolated adult rat cardiomyocyte model of ischemia-reperfusion (IR) injury was also used to determine the role of mK_{ATP} in cardioprotection by nitroxyl. Key findings were as follows: (i) mK_{ATP} was activated by $O_2^{\bullet-}$ and H_2O_2 but not other peroxides. (ii) mK_{ATP} was inhibited by NADPH. (iii) mK_{ATP} was activated by S-nitrosothiols, nitroxyl, and nitrolinoleate. The latter two species also inhibited mitochondrial complex II. (iv) Nitroxyl protected cardiomyocytes against IR injury in a mK_{ATP}-dependent manner. Overall, these results suggest that the mK_{ATP} channel is activated by specific reactive oxygen and nitrogen species, and inhibited by NADPH. The redox modulation of mK_{ATP} may be an underlying mechanism for its regulation in the context of IPC.

Keywords

K^+ channel; Ischemia; Preconditioning; Nitric Oxide; Redox

© 2010 Elsevier B.V. All rights reserved.

§Corresponding Author: Paul S. Brookes, PhD. Dept. of Anesthesiology, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642, USA Tel. 585-273-1626 paul_brookes@urmc.rochester.edu.
*These authors contributed equally to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

RESEARCH HIGHLIGHTS, Queliconi et al.

- Several redox active species have been proposed to modulate the activity of the mitochondrial ATP sensitive potassium channel (mK_{ATP}), but a comprehensive analysis is lacking. We show that:
- Superoxide and hydrogen peroxide activate mK_{ATP}, but other reactive oxygen species do not.
- Several reactive nitrogen species including S-nitrosothiols, nitroxyl, and nitrated lipids all activate the mK_{ATP} channel. Some of this activation is likely mediated via complex II of the mitochondrial respiratory chain.
- Reducing agents show variable ability to inhibit the mK_{ATP} channel, but NADPH is particularly effective, via a mechanism unrelated to its redox potential.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

1. INTRODUCTION

The past 25 years has witnessed much investigation into the phenomenon of ischemic preconditioning (IPC), in which short non-lethal periods of ischemia and reperfusion (IR) can elicit protection against prolonged ischemia-reperfusion (IR) injury [1]. Despite this effort, the mechanism by which IPC protects organs such as the heart and brain from IR injury is still debated.

One proposed mechanism of IPC-induced cardioprotection is the opening of a mitochondrial ATP-sensitive potassium channel (mK_{ATP}), which elicits mild swelling of the mitochondrial matrix. This in turn is thought to impact on mitochondrial Ca^{2+} loading, reactive oxygen species (ROS) generation, metabolic efficiency, and assembly of the permeability transition pore [2], and these downstream events bring about protection via unclear mechanisms. Although the molecular identity of the mK_{ATP} channel remains unknown, several pharmacologic mK_{ATP} modulators mimic IPC, and many IPC signaling pathways are thought to converge on mK_{ATP} as an end effector [3].

Mitochondria are a quantitatively significant source of ROS, which contribute to tissue damage during ischemia, but are also mediators of IPC signaling [4]. Accumulating evidence suggests that redox signaling pathways play an important role in IPC [5-9], and can promote mK_{ATP} activation [10-15]. The primary ROS generated by mitochondria is superoxide ($O_2^{\bullet-}$) [16,17], while hydrogen peroxide (H_2O_2) or lipid peroxides can be formed secondarily [17]. Both $O_2^{\bullet-}$ and H_2O_2 are thought to activate mK_{ATP} [13,18-20], although conflicting reports exist regarding $O_2^{\bullet-}$ [18,20]. The effect of other peroxides on mK_{ATP} is not known. Furthermore, it is apparent that some but not all types of antioxidants can inhibit IPC and mK_{ATP} activity [6,13], warranting further investigation. Table 1 summarizes the disparate results to date regarding redox regulation of the mK_{ATP} channel.

Nitric oxide ($NO^{\bullet}$) is also implicated in IPC, and elicits a large variety of cardioprotective effects [21]. $NO^{\bullet}$ has been detected in isolated mitochondrial preparations [22], and can secondarily generate many reactive nitrogen species (RNS) [23-25], which can serve either damaging or beneficial signaling roles [17,21,25,26]. mK_{ATP} is a potential target for such RNS, and while high doses (10 mM) of an S-nitrosothiol have been shown to activate the channel in intact mitochondria [20], evidence for more subtle physiologically relevant effects of $NO^{\bullet}$ has mostly relied on indirect measures of channel activity [27] or study of the channel removed from its mitochondrial environment [28]. Thus, it is not clear whether the levels of $NO^{\bullet}$ that would be experienced inside mitochondria are capable of modulating mK_{ATP} activity.

The one electron reduction product of $NO^{\bullet}$, nitroxyl (HNO) may also modulate the mK_{ATP} channel. Nitroxyl is protective in IR injury [29], and while it shares some signaling pathways with $NO^{\bullet}$, it also possesses distinct biochemistry from $NO^{\bullet}$, such as a direct interaction with thiols. In this regard, the nitroxyl donor Angeli's salt (AS) inhibits mitochondrial complex II in a manner sensitive to glutathione and independent of S-nitrosation [30]. mK_{ATP} activity is exquisitely sensitive to complex II modulation [31,32], and herein we explored the concept that nitroxyl may regulate mK_{ATP} via effects on complex II.

Despite a collection of studies to date examining the effect of single redox agents on mK_{ATP}, often at high doses, a comparative study across a wide range of doses is lacking. In addition, unique chemical properties of certain $NO^{\bullet}$ derived species have precluded their use to date in studying mK_{ATP}. The current study aimed to address such issues, and the collective results suggest mitochondrial redox state is an important regulator of mK_{ATP} channel activity, mitochondrial function, and cardioprotection in the context of IPC.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

2. MATERIALS AND METHODS

Full experimental details are in the online supplement.

2.1 Animals, chemicals and supplies

Male Sprague-Dawley rats, 200–300 g, were purchased from Harlan (Indianapolis, IN) or bred at the *Biotério do Conjunto das Químicas* (*Universidade de São Paulo*) housed on a 12 hr. light/dark cycle with food and water available *ad libitum*. All procedures were performed in accordance with the US National Institutes of Health "Guide for the care and use of laboratory animals" and the *Colégio Brasileiro de Experimentação Animal*, and were approved by the appropriate university animal ethics committees. Linoleic peroxide was a kind gift from Sayuri Miyamoto (São Paulo) and stored under argon in methanol [33]. Nitro-linoleate was synthesized and analyzed as previously described [34]. All other reagents used were analytical grade or higher, obtained from Sigma (St. Louis MO) or EMD (Gibbstown NJ).

2.2 Mitochondrial isolation, Cx-II, and mK_{ATP} assays

Heart mitochondria were rapidly isolated as previously described [31,35]. Cx-II activity was measured as previously described [31,32]. mK_{ATP} activity was measured by osmotic swelling as previously described [32,36]. All channel modulating agents (reactive oxygen and nitrogen species, antioxidants etc.) were present in the assay buffer prior to mitochondrial addition. The nature of the mK_{ATP} osmotic swelling assay, requiring mitochondrial addition last of all, precludes its use to study highly reactive species such as HNO [37]. In such cases a fluorescence-based Tl^+ flux assay for mK_{ATP} activity [38] was used, permitting incubation of mitochondria prior to assay initiation by Tl^+ addition.

2.3 Cardiomyocyte model of IR injury

Adult rat ventricular myocytes were isolated, and a model of simulated IR (SIR) injury was as previously described [32]. Cells were incubated in anoxic glucose-free Krebs Henseleit (KH) buffer at pH 6.5 for 30 min., followed by reoxygenation in glucose-replete KH at pH 7.4. Where indicated, compounds were present 20 min. prior to the onset of simulated ischemia. At the end of all protocols, viability was determined by Trypan blue exclusion.

2.4 Statistics

All experiments were performed on at least 3 independent mitochondrial or cell preparations, and results are presented as mean ± SEM. Statistical significance between groups was determined by ANOVA.

3. RESULTS & DISCUSSION

3.1 mK_{ATP} is activated by some but not all peroxides

While the ability of ROS to modulate mK_{ATP} activity has been reported [13,18-20], the differential action of various classes of ROS is less well understood. In particular, ROS such as H_2O_2 may initiate chemistry that generates secondary peroxides such as lipid hydroperoxides (LOOH) [17]. We therefore hypothesized that peroxides may modulate mK_{ATP} activity. In Figure 1A, the effects of H_2O_2, *t*-butyl hydroperoxide (*t*-BuOOH), and linoleic hydroperoxide (LOOH) on mK_{ATP} were tested. Interestingly, while H_2O_2 robustly opened the channel at 1 μM, higher concentrations of *t*-BuOOH and LOOH did not. The differential hydrophobicity of *t*-BuOOH, LOOH, and H_2O_2 suggests that the peroxide sensor of mK_{ATP} may be in a hydrophilic region of the molecule. Alternatively, it has been suggested that H_2O_2 activation of the mK_{ATP} may occur via a PKCε dependent mechanism

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

[20], although the location of the kinase in this particular mitochondrial preparation is unknown.

3.2 mK_{ATP} is activated by supra-physiological levels of superoxide

Activation of mK_{ATP} by $O_2^{\bullet-}$ has been reported previously [18], but at relatively high doses. It is unclear whether the amounts of $O_2^{\bullet-}$ made by mitochondria are capable of modulating mK_{ATP} activity. To investigate this, a xanthine/xanthine oxidase (X/XO) system was used to modulate ROS flux. Figures 1B and 1C show that XO levels as low as 1.4 U/ml (approximating to a $O_2^{\bullet-}$ flux of 1.2 μM/min) activated the channel. Reported maximal rates of $O_2^{\bullet-}$ generation by heart mitochondria range from 30 nM/min to 1 μM/min [39,40]. Thus, it is unlikely that mitochondrial $O_2^{\bullet-}$ generation under normal conditions approaches levels required for mK_{ATP} channel activation. This finding suggests that the channel would only be activated in situ with ROS originated from non-mitochondrial sources or conditions that increase ROS production such as pre- and post-conditioning. Also under conditions of reverse electron flow, $O_2^{\bullet-}$ in the mitochondrial microenvironment may reach levels capable of activating the channel [13]. No effect of H_2O_2 or X/XO on mitochondrial swelling was observed in Na^+ based media (supplemental Figure 1).

3.3 mK_{ATP} is inhibited by some but not all reductants/antioxidants

While IPC is inhibited by both thiol antioxidants and catalase (suggesting a role for H_2O_2 [12]), cardioprotection by mK_{ATP} agonists such as diazoxide is prevented only by thiol antioxidants [13], suggesting that antioxidant-sensitive proteins distinct from mK_{ATP} may be important in IPC (e.g. SERCA, [41]). Thus, a detailed understanding of the selective regulation of mK_{ATP} by reductants is a key step toward understanding its role in IPC.

The effect of several reducing agents on mK_{ATP} activity was tested under baseline conditions and conditions of maximal channel opening (presence of ATP and diazoxide). Figure 2 shows that while most reducing agents had a mild inhibitory effect on mK_{ATP} activity, NADPH was a strong inhibitor, almost completely preventing channel activity. Compiling these data with those from our previous study [13], supplemental Figure 2 shows the ability of reductants to inhibit mK_{ATP} channel activity correlated with redox potential ($r^2 = 0.73$). A notable outlier to this correlation was NADPH (inclusion of NADPH in the linear regression fit lowers r^2 to 0.43). The underlying mechanism for a difference in the effect of NADH vs. NADPH, despite their identical redox potentials, is elusive at this stage. Interestingly, while both surface K_{ATP} channels [42-45] and the mK_{ATP} [12,13,18,46,47] do have redox active thiols, NADPH does not directly reduce thiols, suggesting its direct redox activity is not the mechanism of channel regulation.

Nevertheless, NADPH does play an important role in overall mitochondrial redox status; transhydrogenases reduce mitochondrial $NADP^+$ using electrons from NADH and the electrochemical proton gradient as an energy source [48]. The resulting NADPH is used as an electron source for thiol peroxidase removal systems, including glutathione and thioredoxin peroxidase/reductase [17]. Thus, the finding that NADPH can regulate mK_{ATP} activity suggests this channel may play a role in sensing both energy metabolism and redox status [13,49].

Notably, surface K_{ATP} channel activity has been shown to be sensitive to pyridine nucleotides [50], possibly at the same site as adenine nucleotides modulate channel activity. Furthermore, insulin secretion in pancreatic β cells, which occurs secondarily to K_{ATP} closure, correlates with $NADPH/NADP^+$ ratios [51]. Thus, a direct modulation of the mK_{ATP} channel by NADPH binding may occur. Another possibility might be that NADPH competes with DZX for a binding site on the mK_{ATP} channel. However, we consider this

unlikely since NADPH was also able to inhibit channel opening by the structurally unrelated opener atpenin A5 [32] (supplemental Figure 3).

3.4 mK_{ATP} is activation by S-nitrosothiols and L-arginine

In addition to ROS, much recent interest has focused on mK_{ATP} as a possible target for $NO^{\bullet}$ or its redox congeners [20]. While $NO^{\bullet}$ effects on mK_{ATP} activity in intact cells are thought to be mediated via cGMP signaling [52,53], direct effects of $NO^{\bullet}$ on the purified channel have been measured in planar bilayer studies [28]. In addition $NO^{\bullet}$ was shown to activate mK_{ATP} in mitochondria by using flavoprotein fluorescence as a read-out [27]. However, the dose response of mK_{ATP} to $NO^{\bullet}$ in intact mitochondria is unknown. Figures 3A and 3B show that S-nitrosoacetylpenacillamine (SNAP) dose-dependently activated mK_{ATP}, in a glybenclamide and 5-HD sensitive manner. Notably, >10 μM SNAP led to mK_{ATP} inhibition, presumably due to $NO^{\bullet}$ inhibition of cytochrome *c* oxidase [54] leading to mitochondrial deenergization, removing the driving force for K^+ uptake. SNAP did not activate swelling in Na^+ based buffers (supplemental Figure 4). Notably, the optimal SNAP concentration for mK_{ATP} channel opening in this study (10 μM) is 3 orders of magnitude lower than previously reported (Table 1). We are unsure as to the origin of this very large discrepancy [20].

There has been much interest in the possibility that mitochondria may contain a nitric oxide synthase, termed “mtNOS”. Despite recent developments including retraction of some work [55-57], NOS is a common contaminant of isolated mitochondrial preparations [58]. This may be particularly applicable to mK_{ATP} studies, since a rapid and crude mitochondrial isolation is required to effectively measure channel activity (full experimental details are in the online supplement) [32,36]. The data in Figure 3C show that the NOS substrate L-arginine stimulates mK_{ATP} opening, in a manner sensitive to the NOS inhibitor L-NAME. No effect was seen with D-arginine, suggesting the origin of this effect resides at the level of a mitochondrially associated NOS or L-NAME-sensitive enzyme. Unfortunately, further purification of mitochondria that would be required to assert a mitochondria-resident NOS in mediating these effects, also results in loss of mK_{ATP} channel activity, in a manner mechanistically related to classical channel “run-down” [38].

3.5 Certain RNS can activate mK_{ATP}, via a mechanism involving mitochondrial complex-II

In addition to “classical” RNS such as $NO^{\bullet}$, RNS such as nitro-lipids and nitroxyl may regulate mK_{ATP}. Nitro-lipids are an emerging class of anti-inflammatory signaling lipids which can mediate $NO^{\bullet}$ signaling, [59,60] and are known to be generated during IPC [34,61]. One example of a nitro-lipid, nitrolinoleate ($LA\text{-}NO_2$), can elicit cardioprotection in a cGMP-independent manner [34,62]. Figure 4A shows that, low doses (0.5 μM) of $LA\text{-}NO_2$ opened the mK_{ATP} channel in a 5-HD- and glyburide-sensitive manner, while native linoleic acid (LA) did not. Complex II of the mitochondrial respiratory chain has been proposed as an important regulator of mK_{ATP} activity [31,32,63], and in this regard Figure 4B shows that $LA\text{-}NO_2$, but not LA, inhibited complex-II in a dose-dependent manner. Notably however, the amount of $LA\text{-}NO_2$ required to open the channel was significantly lower than that required to inhibit complex II (see below for discussion).

The disparate effects of $LA\text{-}NO_2$ (stimulation mK_{ATP} activity), and lipid hydroperoxide (no effect on mK_{ATP}, see section 3.1) highlight the different chemical properties of these species. While both are hydrophobic reactive lipids, only $LA\text{-}NO_2$ possesses an electrophilic moiety that can adduct thiols by Michael addition [61]. Thus it is suggested that the mechanism of $LA\text{-}NO_2$ mediated mK_{ATP} opening may involve modification of thiols on the channel (c.f. section 3.3).

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

The levels of LA-NO_2 generated inside mitochondria during IPC may reach 1 μM [34], raising the possibility that LA-NO_2 is an important endogenous mK_{ATP} regulator. However, we previously showed that cardioprotection induced by exogenously added LA-NO_2 was insensitive to mK_{ATP} blockers [34], suggesting that other mechanisms of LA-NO_2 action (e.g. mild uncoupling) may account for its cardioprotective effects.

Similar to nitro-lipids, the importance of nitroxyl in cardiovascular signaling has also been the subject of recent attention [29,37]. In agreement with previous findings, we showed that the nitroxyl donor Angeli's salt (AS) dose-dependently inhibited complex II in rat heart mitochondria (Figure 5A). Consistent with an interaction between complex II and the mK_{ATP} channel, we also found that Angeli's salt opened mK_{ATP} in a manner sensitive to 5-HD and glyburide (Figure 5B). Furthermore, in agreement with previous studies [29,64] Angeli's salt was protective in a cardiomyocyte model of IR injury. This protection was sensitive to 5-HD and glyburide, but insensitive to the guanylate cyclase inhibitor ODQ or the protein kinase G inhibitor KT-5823 (Figure 5C). The role of other known components of the preconditioning signaling pathway (e.g. PKC, ERK, PI3 kinase) in mediating the effects of Angeli's salt is currently unknown, although none of these signals has previously been linked to nitroxyl. Together, these data suggest that Angeli's salt mediated cardioprotection proceeds via non-PKG mediated activation of mK_{ATP}, possibly involving an inhibition of complex II.

Both nitroxyl and LA-NO_2 inhibit mitochondrial complex II, activate mK_{ATP}, and are known to react with complex II thiols [30,34]. This suggests that modification of complex II thiols may underlie the mechanism of mK_{ATP} activation. However, the concentrations of nitroxyl and LA-NO_2 which activated mK_{ATP} did not significantly inhibit complex II enzymatic activity (Figures 4 and 5). In this regard, nitroxyl and LA-NO_2 are similar to other species which activate the mK_{ATP} at doses far below those at which they inhibit complex II, including atpenin A5 [32], malonate [31] and diazoxide [65]. Thus, the regulation of mK_{ATP} activity appears to be mechanistically divorced from the bulk enzymatic activity of complex II itself. The fact that 5 unrelated compounds which inhibit complex II by distinct mechanisms all activate mK_{ATP} at lower concentrations, collectively suggests that a small sub-population of complex II may play an important role in regulating mK_{ATP} activity, while not impacting total complex II enzymatic activity.

4. CONCLUSIONS

In summary, a variety of redox active species, including ROS, RNS, antioxidants, and reductants, all act on the mK_{ATP}. While a broad conclusion of this work can be summarized as “oxidants activate, reductants inhibit”, it is apparent that many species do not conform to this simple model. Key examples include NADPH, which may regulates the channel via direct binding, and LA-NO_2 and nitroxyl, which are thought to mediate their effects via complex II. A summary of the potential mechanisms of mK_{ATP} channel modulation by various species is shown in Figure 6. Clearly, further work in this area, including the molecular identification of the mK_{ATP} itself, and the redox-sensitive residues within it, will facilitate a better understanding of the role that channel regulation by redox plays in events such as IPC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Acknowledgments

We thank Camille C. Caldeira da Silva (São Paulo) and Emily Redman (Rochester) for technical support, and Sayuri Miyamoto (São Paulo) for providing linoleic hydroperoxide. This work was supported by the National Institutes of Health [HL-071158 and GM-087483 to PSB]; the John Simon Guggenheim Memorial Foundation [AJK]; the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [AJK]; the Instituto Nacional de Ciência e Tecnologia de Processos Redox em Biomedicina (Redoxoma) [AJK]; the American Heart Association Founders Affiliate [0815770D to APW]; and FAPESP Predoctoral Fellowship [BBQ].

LITERATURE CITED

1. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation. 1986; 74:1124–1136. [PubMed: 3769170]
2. Facundo HT, Fornazari M, Kowaltowski AJ. Tissue protection mediated by mitochondrial K+ channels. Biochim. Biophys. Acta. 2006; 1762:202–212. [PubMed: 16026967]
3. Garlid KD, Dos SP, Xie ZJ, Costa AD, Paucek P. Mitochondrial potassium transport: the role of the mitochondrial ATP-sensitive K(+) channel in cardiac function and cardioprotection. Biochim. Biophys. Acta. 2003; 1606:1–21. [PubMed: 14507424]
4. Murphy E, Steenbergen C. Preconditioning: the mitochondrial connection. Annu. Rev. Physiol. 2007; 69:51–67. [PubMed: 17007587]
5. Vanden Hoek TL, Becker LB, Shao Z, Li C, Schumacker PT. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. J. Biol. Chem. 1998; 273:18092–18098. [PubMed: 9660766]
6. Chen W, Gabel S, Steenbergen C, Murphy E. A redox-based mechanism for cardioprotection induced by ischemic preconditioning in perfused rat heart. Circ. Res. 1995; 77:424–429. [PubMed: 7614726]
7. Becker LB, Vanden Hoek TL, Shao ZH, Li CQ, Schumacker PT. Generation of superoxide in cardiomyocytes during ischemia before reperfusion. Am. J. Physiol. 1999; 277:H2240–H2246. [PubMed: 10600842]
8. Penna C, Mancardi D, Rastaldo R, Pagliaro P. Cardioprotection: a radical view Free radicals in pre and postconditioning. Biochim. Biophys. Acta. 2009; 1787:781–793. [PubMed: 19248760]
9. Baines CP, Goto M, Downey JM. Oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium. J. Mol. Cell Cardiol. 1997; 29:207–216. [PubMed: 9040035]
10. Forbes RA, Steenbergen C, Murphy E. Diazoxide-induced cardioprotection requires signaling through a redox-sensitive mechanism. Circ. Res. 2001; 88:802–809. [PubMed: 11325872]
11. Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV, Downey JM. Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. Circ. Res. 2000; 87:460–466. [PubMed: 10988237]
12. Facundo HT, Carreira RS, de Paula JG, Santos CC, Ferranti R, Laurindo FR, Kowaltowski AJ. Ischemic preconditioning requires increases in reactive oxygen release independent of mitochondrial K+ channel activity. Free Radic. Biol. Med. 2006; 40:469–479. [PubMed: 16443162]
13. Facundo HT, de Paula JG, Kowaltowski AJ. Mitochondrial ATP-sensitive K+ channels are redox-sensitive pathways that control reactive oxygen species production. Free Radic. Biol. Med. 2007; 42:1039–1048. [PubMed: 17349931]
14. Yue Y, Qin Q, Cohen MV, Downey JM, Critz SD. The relative order of mK(ATP) channels, free radicals and p38 MAPK in preconditioning's protective pathway in rat heart. Cardiovasc. Res. 2002; 55:681–689. [PubMed: 12160965]
15. Pasdois P, Quinlan CL, Rissa A, Tariosse L, Vinassa B, Costa AD, Pierre SV, Dos SP, Garlid KD. Ouabain protects rat hearts against ischemia-reperfusion injury via pathway involving src kinase, mitoKATP, and ROS. Am. J. Physiol. 2007; 292:H1470–H1478.
16. Murphy MP. How mitochondria produce reactive oxygen species. Biochem. J. 2009; 417:1–13.
17. Kowaltowski AJ, de Souza-Pinto NC, Castilho RF, Vercesi AE. Mitochondria and reactive oxygen species. Free Radic. Biol. Med. 2009; 47:333–343. [PubMed: 19427899]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

18. Zhang DX, Chen YF, Campbell WB, Zou AP, Gross GJ, Li PL. Characteristics and superoxide-induced activation of reconstituted myocardial mitochondrial ATP-sensitive potassium channels. Circ. Res. 2001; 89:1177–1183. [PubMed: 11739283]
19. Fornazari M, de Paula JG, Castilho RF, Kowaltowski AJ. Redox properties of the adenoside triphosphate-sensitive K+ channel in brain mitochondria. J. Neurosci. Res. 2008; 86:1548–1556. [PubMed: 18189325]
20. Costa AD, Garlid KD. Intramitochondrial signaling: interactions among mitoKATP, PKCepsilon, ROS, and MPT. Am. J. Physiol. 2008; 295:H874–H882.
21. Burwell LS, Brookes PS. Mitochondria as a Target for the Cardioprotective Effects of Nitric Oxide in Ischemia-Reperfusion Injury. Antioxid. Redox. Signal. 2007; 10:579–600. [PubMed: 18052718]
22. Giulivi C, Poderoso JJ, Boveris A. Production of nitric oxide by mitochondria. J. Biol. Chem. 1998; 273:11038–11043. [PubMed: 9556586]
23. Ferrer-Sueta G, Radi R. Chemical biology of peroxynitrite: kinetics, diffusion, and radicals. ACS Chem. Biol. 2009; 4:161–177. [PubMed: 19267456]
24. Trostchansky A, Rubbo H. Nitrated fatty acids: mechanisms of formation, chemical characterization, and biological properties. Free Radic. Biol. Med. 2008; 44:1887–1896. [PubMed: 18395528]
25. Grisham MB, Jourd'Heuil D, Wink DA. Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites:implications in inflammation. Am. J. Physiol. 1999; 276:G315–G321. [PubMed: 9950804]
26. Rudolph V, Freeman BA. Cardiovascular consequences when nitric oxide and lipid signaling converge. Circ. Res. 2009; 105:511–522. [PubMed: 19745170]
27. Sasaki N, Sato T, Ohler A, O'Rourke B, Marban E. Activation of mitochondrial ATP-dependent potassium channels by nitric oxide. Circulation. 2000; 101:439–445. [PubMed: 10653837]
28. Ljubkovic M, Shi Y, Cheng Q, Bosnjak Z, Jiang MT. Cardiac mitochondrial ATP-sensitive potassium channel is activated by nitric oxide in vitro. FEBS Lett. 2007; 581:4255–4259. [PubMed: 17714708]
29. Pagliaro P, Mancardi D, Rastaldo R, Penna C, Gattullo D, Miranda KM, Feelisch M, Wink DA, Kass DA, Paolocci N. Nitroxyl affords thiol-sensitive myocardial protective effects akin to early preconditioning. Free Radic. Biol. Med. 2003; 34:33–43. [PubMed: 12498977]
30. Shiva S, Crawford JH, Ramachandran A, Ceaser EK, Hillson T, Brookes PS, Patel RP, Darley-Usmar VM. Mechanisms of the interaction of nitroxyl with mitochondria. Biochem. J. 2004; 379:359–366. [PubMed: 14723605]
31. Wojtovich AP, Brookes PS. The endogenous mitochondrial complex II inhibitor malonate regulates mitochondrial ATP-sensitive potassium channels: Implications for ischemic preconditioning. Biochim. Biophys. Acta. 2008; 1777:882–889. [PubMed: 18433712]
32. Wojtovich AP, Brookes PS. The complex II inhibitor atpenin A5 protects against cardiac ischemia-reperfusion injury via activation of mitochondrial KATP channels. Basic Res. Cardiol. 2009; 104:121–129. [PubMed: 19242645]
33. Miyamoto S, Martinez GR, Medeiros MH, Di MP. Singlet molecular oxygen generated from lipid hydroperoxides by the russell mechanism: studies using 18(O)-labeled linoleic acid hydroperoxide and monomol light emission measurements. J. Am. Chem. Soc. 2003; 125:6172–6179. [PubMed: 12785849]
34. Nadtochiy SM, Baker PR, Freeman BA, Brookes PS. Mitochondrial nitroalkene formation and mild uncoupling in ischaemic preconditioning: implications for cardioprotection. Cardiovasc. Res. 2009; 82:333–340. [PubMed: 19050010]
35. Cancherini DV, Queliconi BB, Kowaltowski AJ. Pharmacological and physiological stimuli do not promote Ca(2+)-sensitive K+ channel activity in isolated heart mitochondria. Cardiovasc. Res. 2007; 73:720–728. [PubMed: 17208207]
36. Kowaltowski AJ, Seetharaman S, Paucek P, Garlid KD. Bioenergetic consequences of opening the ATP-sensitive K(+) channel of heart mitochondria. Am. J. Physiol. 2001; 280:H649–H657.
37. Fukuto JM, Switzer CH, Miranda KM, Wink DA. Nitroxyl (HNO): chemistry, biochemistry, and pharmacology. Annu. Rev. Pharmacol. Toxicol. 2005; 45:335–355. [PubMed: 15822180]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

38. Wojtovich AP, Williams DM, Karcz MK, Lopes CM, Gray DA, Nehrke KW, Brookes PS. A Novel Mitochondrial KATP Channel Assay. Circ. Res. 2010; 106:1190–1196. [PubMed: 20185796]
39. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of reactive oxygen species by mitochondria: central role of complex III. J. Biol. Chem. 2003; 278:36027–36031. [PubMed: 12840017]
40. Tahara EB, Navarete FD, Kowaltowski AJ. Tissue-, substrate-, and site-specific characteristics of mitochondrial reactive oxygen species generation. Free Radic. Biol. Med. 2009; 46:1283–1297. [PubMed: 19245829]
41. Tong X, Evangelista A, Cohen RA. Targeting the redox regulation of SERCA in vascular physiology and disease. Curr. Opin. Pharmacol. 2010; 10:133–138. [PubMed: 20045379]
42. Islam MS, Berggren PO, Larsson O. Sulfhydryl oxidation induces rapid and reversible closure of the ATP-regulated K+ channel in the pancreatic beta-cell. FEBS Lett. 1993; 319:128–132. [PubMed: 8454044]
43. Trapp S, Tucker SJ, Ashcroft FM. Mechanism of ATP-sensitive K channel inhibition by sulfhydryl modification. J. Gen. Physiol. 1998; 112:325–332. [PubMed: 9725892]
44. Tricarico D, Camerino DC. ATP-sensitive K+ channels of skeletal muscle fibers from young adult and aged rats: possible involvement of thiol-dependent redox mechanisms in the age-related modifications of their biophysical and pharmacological properties. Mol. Pharmacol. 1994; 46:754–761. [PubMed: 7969056]
45. Coetzee WA, Nakamura TY, Faivre JF. Effects of thiol-modifying agents on KATP channels in guinea pig ventricular cells. Am. J. Physiol. 1995; 269:H1625–H1633. [PubMed: 7503258]
46. Szewczyk A, Wojcik G, Lobanov NA, Nalecz MJ. Modification of the mitochondrial sulfonylurea receptor by thiol reagents. Biochem. Biophys. Res. Commun. 1999; 262:255–258. [PubMed: 10448101]
47. Grigoriev SM, Skarga YY, Mironova GD, Marinov BS. Regulation of mitochondrial KATP channel by redox agents. Biochim. Biophys. Acta. 1999; 1410:91–96. [PubMed: 10076019]
48. Pedersen A, Karlsson GB, Rydstrom J. Proton-translocating transhydrogenase: an update of unsolved and controversial issues. J. Bioenerg. Biomembr. 2008; 40:463–473. [PubMed: 18972197]
49. Cardoso AR, Queliconi BB, Kowaltowski AJ. Mitochondrial ion transport pathways: Role in metabolic diseases. Biochim. Biophys. Acta. 2010
50. Dabrowski M, Trapp S, Ashcroft FM. Pyridine nucleotide regulation of the KATP channel Kir6.2/SUR1 expressed in Xenopus oocytes. J. Physiol. 2003; 550:357–363. [PubMed: 12766240]
51. Ronnebaum SM, Ilkayeva O, Burgess SC, Joseph JW, Lu D, Stevens RD, Becker TC, Sherry AD, Newgard CB, Jensen MV. A pyruvate cycling pathway involving cytosolic NADP-dependent isocitrate dehydrogenase regulates glucose-stimulated insulin secretion. J. Biol. Chem. 2006; 281:30593–30602. [PubMed: 16912049]
52. Cuong DV, Kim N, Youm JB, Joo H, Warda M, Lee JW, Park WS, Kim T, Kang S, Kim H, Han J. Nitric oxide-cGMP-protein kinase G signaling pathway induces anoxic preconditioning through activation of ATP-sensitive K+ channels in rat hearts. Am. J. Physiol. 2006; 290:H1808–H1817.
53. Costa AD, Garlid KD, West IC, Lincoln TM, Downey JM, Cohen MV, Critz SD. Protein kinase G transmits the cardioprotective signal from cytosol to mitochondria. Circ. Res. 2005; 97:329–336. [PubMed: 16037573]
54. Brunori M, Giuffre A, Forte E, Mastronicola D, Barone MC, Sarti P. Control of cytochrome c oxidase activity by nitric oxide. Biochim. Biophys. Acta. 2004; 1655:365–371. [PubMed: 15100052]
55. Zemojtel T, Frohlich A, Palmieri MC, Kolanczyk M, Mikula I, Wyrwicz LS, Wanker EE, Mundlos S, Vingron M, Martasek P, Durner J. Plant nitric oxide synthase: a never-ending story? Trends Plant Sci. 2006; 11:524–525. [PubMed: 17030145]
56. Guo F. Response to Zemojtel Et Al: Plant Nitric Oxide Synthase: AtNOS1 Is Just the Beginning. Trends Plant Sci. 2006; 11:527–528.
57. Crowford N, Galli M, Tischner R, Heimer YM, Okamoto M, Mack A. Response to Zemojtel Et Al: Plant Nitric Oxide Synthase: Back to Square One. Trends Plant Sci. 2006; 11:526–527.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

58. Venkatakrishnan P, Nakayasu ES, Almeida IC, Miller RT. Absence of nitric-oxide synthase in sequentially purified rat liver mitochondria. J. Biol. Chem. 2009; 284:19843–19855. [PubMed: 19372221]
59. Baker PR, Lin Y, Schopfer FJ, Woodcock SR, Groeger AL, Batthyany C, Sweeney S, Long MH, Iles KE, Baker LM, Branchaud BP, Chen YE, Freeman BA. Fatty acid transduction of nitric oxide signaling: multiple nitrated unsaturated fatty acid derivatives exist in human blood and urine and serve as endogenous peroxisome proliferator-activated receptor ligands. J. Biol. Chem. 2005; 280:42464–42475. [PubMed: 16227625]
60. Schopfer FJ, Baker PR, Giles G, Chumley P, Batthyany C, Crawford J, Patel RP, Hogg N, Branchaud BP, Lancaster JR Jr. Freeman BA. Fatty acid transduction of nitric oxide signaling. Nitrolinoleic acid is a hydrophobically stabilized nitric oxide donor. J. Biol. Chem. 2005; 280:19289–19297. [PubMed: 15764811]
61. Schopfer FJ, Batthyany C, Baker PR, Bonacci G, Cole MP, Rudolph V, Groeger AL, Rudolph TK, Nadtochiy S, Brookes PS, Freeman BA. Detection and quantification of protein adduction by electrophilic fatty acids: mitochondrial generation of fatty acid nitroalkene derivatives. Free Radic. Biol. Med. 2009; 46:1250–1259. [PubMed: 19353781]
62. Rudolph V, Rudolph TK, Schopfer FJ, Bonacci G, Woodcock SR, Cole MP, Baker PR, Ramani R, Freeman BA. Endogenous generation and protective effects of nitro-fatty acids in a murine model of focal cardiac ischaemia and reperfusion. Cardiovasc. Res. 2010; 85:155–166. [PubMed: 19666678]
63. Ardehali H, Chen Z, Ko Y, Mejia-Alvarez R, Marban E. Multiprotein complex containing succinate dehydrogenase confers mitochondrial ATP-sensitive K+ channel activity. Proc. Natl. Acad. Sci. 2004; 101:11880–11885. [PubMed: 15284438]
64. Tocchetti CG, Wang W, Froehlich JP, Huke S, Aon MA, Wilson GM, Di BG, O'Rourke B, Gao WD, Wink DA, Toscano JP, Zaccolo M, Bers DM, Valdivia HH, Cheng H, Kass DA, Paolocci N. Nitroxyl improves cellular heart function by directly enhancing cardiac sarcoplasmic reticulum Ca2+ cycling. Circ. Res. 2007; 100:96–104. [PubMed: 17138943]
65. Dzeja PP, Bast P, Ozcan C, Valverde A, Holmuhamedov EL, Van Wylen DG, Terzic A. Targeting nucleotide-requiring enzymes: implications for diazoxide-induced cardioprotection. Am. J. Physiol. 2003; 284:H1048–H1056.
66. Mironova GD, Skarga YY, Grigoriev SM, Negoda AE, Kolomytkin OV, Marinov BS. Reconstitution of the mitochondrial ATP-dependent potassium channel into bilayer lipid membrane. J. Bioenerg. Biomembr. 1999; 31:159–163. [PubMed: 10449243]
67. Beavis AD, Lu Y, Garlid KD. On the regulation of K+ uniport in intact mitochondria by adenine nucleotides and nucleotide analogs. J. Biol. Chem. 1993; 268:997–1004. [PubMed: 8380420]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

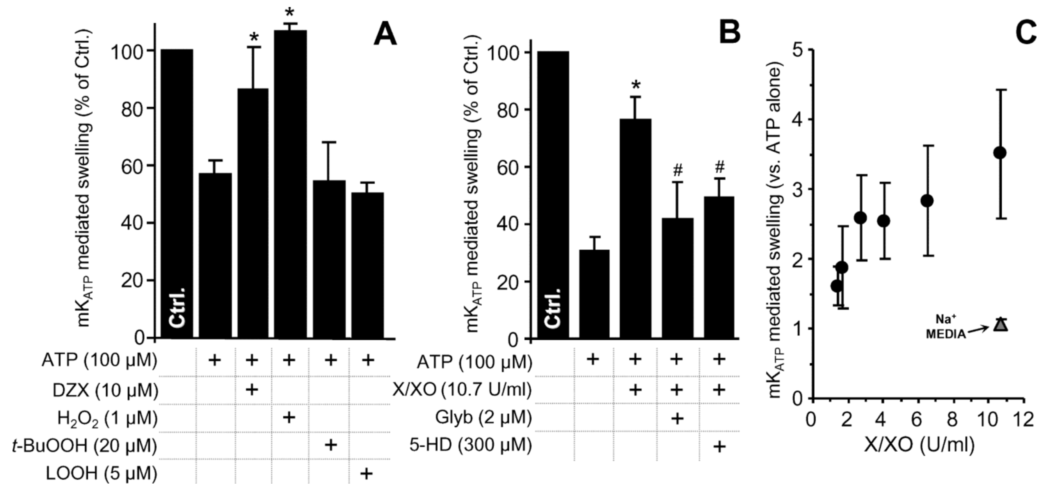

Figure 1. Selective mK_{ATP} Activation by ROS

mK_{ATP} activity was measured by osmotic swelling as detailed in the methods. Controls (Na^+ based media) are in Figure S1. **(A):** mK_{ATP} is activated by H_2O_2 but not by *t*-BuOOH or LOOH. Diazoxide (DZX) opening of mK_{ATP} was used as a positive control. **(B):** mK_{ATP} activation by $O_2^{\bullet -}$ generated by the X/XO system. Glyburide (Glyb) and 5-hydroxydecanoate (5-HD) are mK_{ATP} antagonists. **(C):** Dose response of mK_{ATP} activation by X/XO in K^+ (black circles) or Na^+ (gray triangles) based media. *$p<0.05$ vs. ATP alone. #$p<0.05$ vs. ATP + H_2O_2 or X/XO. Experimental conditions are listed below the X axis.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

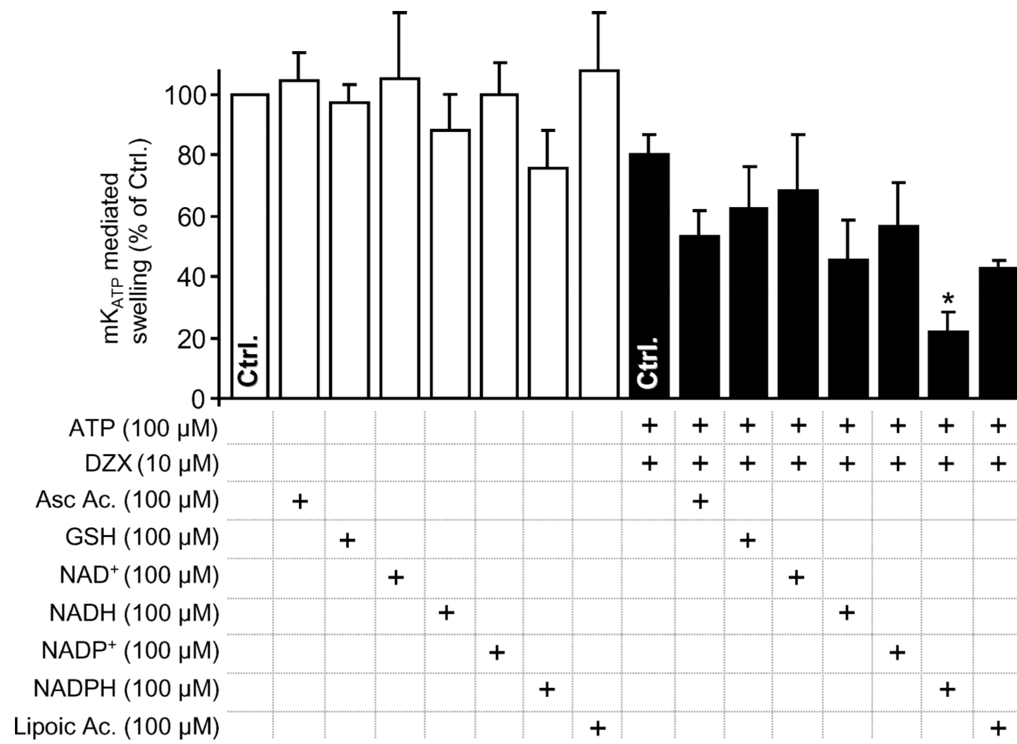

Figure 2. Inhibition of DZX-Activated mK$_{ATP}$ by Reductants

mK$_{ATP}$ activity was measured by osmotic swelling as detailed in the methods. Data are shown for the baseline condition (open bars) or maximal swelling in the presence of both ATP and DZX (filled bars). Reductants were present in the media before mitochondrial addition, at the concentrations indicated. *$p<0.05$ vs. the appropriate control (bar marked Ctrl.) in the absence of reductant.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

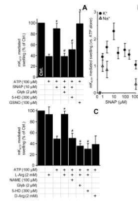

Figure 3. mK_{ATP} Activation by S-nitrosothiols and Mitochondrial-Associated NOS
mK_{ATP} activity was measured by osmotic swelling as detailed in the methods. Controls (Na^+ based media) are in Figure S2. **(A):** mK_{ATP} was activated by S-nitrosoacetylpenacillamine (SNAP) and S-nitrosoglutathione (GSNO), at the indicated doses. **(B):** Dose-response of mK_{ATP} activation by SNAP in K^+ (black circles) or Na^+ (gray triangles) based media. *p<0.05 vs. ATP alone. #p<0.05 vs. ATP plus SNAP. **(C):** mK_{ATP} activation by NOS modulators. L- or D-Arginine, L-nitroarginine methyl ester (NAME), Glyb and 5-HD were present at the concentrations indicated. *p<0.05 vs. *p<0.05 vs. ATP alone. #p<0.05 vs. ATP plus L-arginine.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

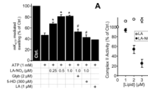

Figure 4. LA-NO_2 Opens mK_{ATP} and Inhibits Complex II

(A): mK_{ATP} activity was measured by osmotic swelling as detailed in the methods. LA-NO_2, native linoleate (LA), Glyb, and 5-HD were present at the indicated concentrations. *$p<0.05$ vs. ATP alone. #$p<0.05$ vs. ATP plus LA-NO_2. **(B):** Complex II activity in the presence of LA-NO_2 was determined as detailed in the methods. Values are expressed as percentage of control complex II rate (128 ± 26 nmols DCPIP · min^{-1} · mg $protein^{-1}$).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

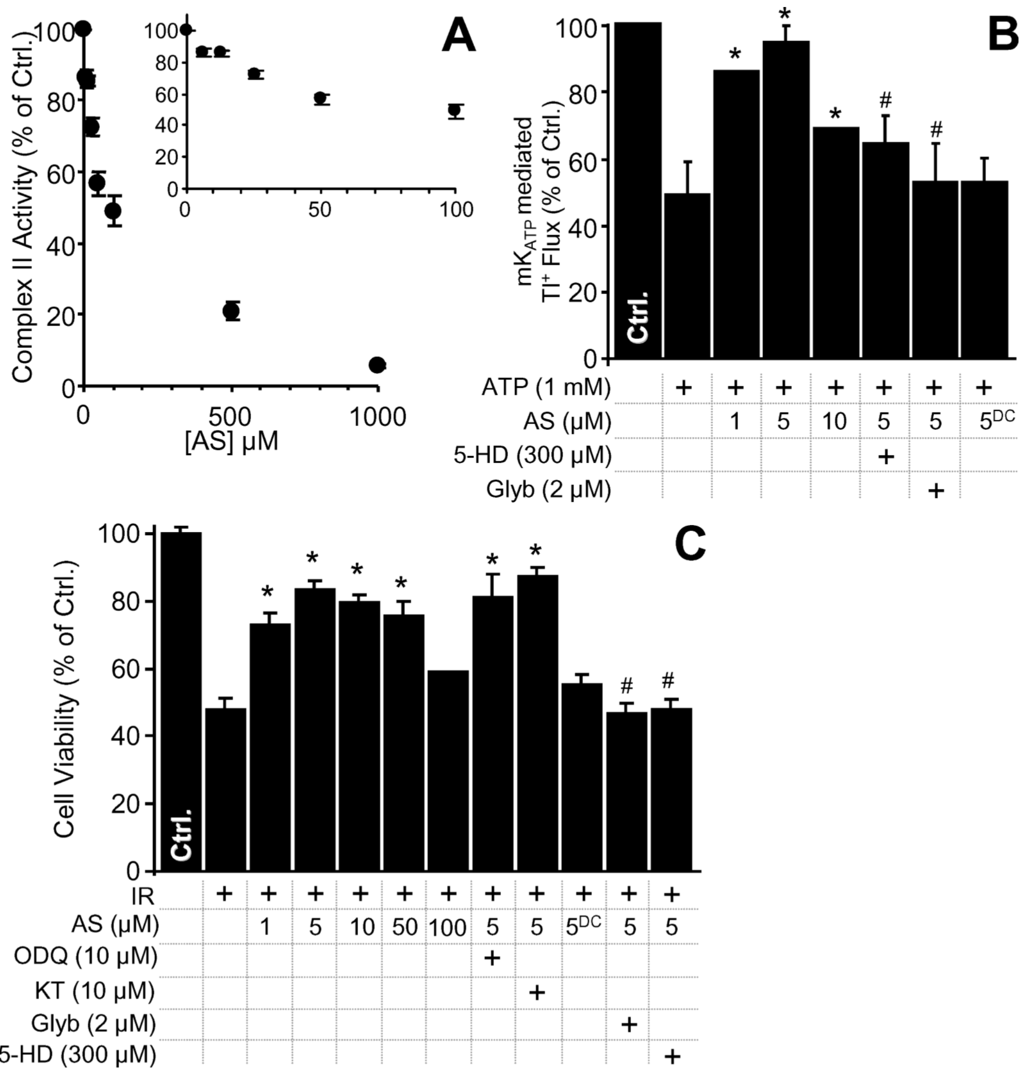

Figure 5. Nitroxyl Inhibits Complex II, Opens mK_{ATP} and is Cardioprotective

(A): Complex II activity was measured, following nitroxyl exposure of mitochondria, as described in the methods. Values are expressed as percentage of control complex II rate (108 ± 8 nmols DCPIP · min^{-1} · mg $protein^{-1}$). **(B):** Nitroxyl activation of mK_{ATP} was monitored using a novel Tl^+ flux assay as described in the methods. Data show the magnitude of change in intra-mitochondrial Tl^+ based fluorescence following Tl^+ addition, relative to control. 5-HD, Glyb, the nitroxyl donor Angeli's salt (AS) and decomposed AS (AS^{DC}) were present at the indicated concentrations. *p<0.05 vs. ATP alone. #p<0.05 vs. ATP plus 5 μM AS. **(C):** Nitroxyl protects against cardiomyocyte IR injury. Cell viability was measured via Trypan blue exclusion at the end of reoxygenation, as described in the methods, and expressed as percentage of control (normoxic) cell viability. 5-HD, Glyb, Angeli's salt (AS), decomposed AS (AS^{DC}), the PKG inhibitor KT-5823 (KT) or the soluble guanylate cyclase inhibitor ODQ were present at the indicated concentrations. *p<0.05 vs. IR alone. #p<0.05 vs. IR plus 5 μM AS.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

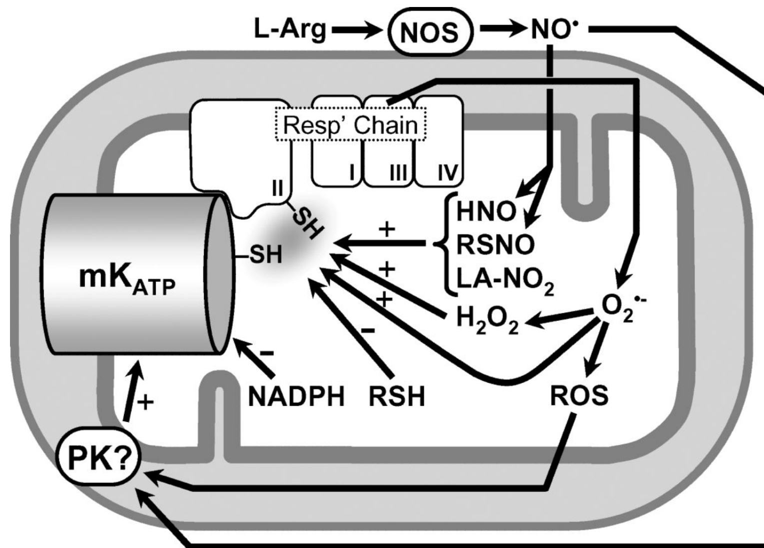

Figure 6. Schematic Showing Redox Regulation of mK_{ATP}

Nitroxyl (HNO), RSNO, and $LA\text{-}NO_2$ activate the channel, possibly via thiols on the channel itself or on complex II of the respiratory chain. The ability of low molecular weight thiols (RSH) to inhibit the channel may also be mediated via thiols on the channel or on complex II. In contrast, the effects of NADPH are likely no mediated via thiols. The ability of $NO^{\bullet}$ to activate the channel may be mediated via the generation of secondary RNS (e.g. RSNO, $LA\text{-}NO_2$, HNO), which can activate the channel via PKG-independent mechanisms, or via classical $NO^{\bullet}$ protein kinase signaling. ROS (in particular H_2O_2) can also activate the channel, via mechanisms that may include thiol modification or protein kinase signaling. The nature of the interaction between complex II and the subunits of the mK_{ATP} channel itself remains to be elucidated.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Table 1

Previous Studies on the Effects of Oxidants, Reactive Nitrogen Species, Antioxidants and Reducing Agents on mK_{ATP} Channel Activity.

Reagent	Conc.	Effects	Experimental conditions	References
Ascorbate	1 mM	No effect	Isolated mitochondria	[12]
DTE	10 mM	Inhibits glyburide binding	Submitochondrial particles	[46]
DTNB	500 µM	Inhibits glyburide binding	Submitochondrial particles	[46]
DTT	100 µM	Inhibits activation by DZX	Isolated mitochondria	[12,13]
	1 mM	Activates rundown channels Loss in selectivity	Reconstituted channels	[66]
	10 mM	Inhibits glyburide binding	Submitochondrial particles	[46]
Mersalyl	100 µM	Inhibits glyburide binding	Submitochondrial particles	[46]
MPG	200 µM	Inhibits activation by DZX	Isolated mitochondria	[12,13,31]
NAC	4 mM	Inhibits activation by DZX	Isolated mitochondria	[12,13]
NEM	2 mM	Inhibits activation by $O_2^{\cdot-}$	Reconstituted channels	[18]
		Inhibits glyburide binding	Submitochondrial particles	[46]
	60 nmol/mg	Decreases selectivity	Isolated mitochondria	[67]
Thimerosal	500 µM	Inhibits glyburide binding	Submitochondrial particles	[46]
X/XO	0.038 U/mL	Activates	Reconstituted channels	[18]
	6 mU/mL	No Effect	Isolated Mitochondria	[20]
H_2O_2	1 µM	Activates	Isolated Mitochondria	[13]
	1 µM	Activates	Isolated Mitochondria	[19]
	6 mU/mL X/XO + 30 U SOD	Activates	Isolated Mitochondria	[20]
SNAP	10 mM	Activates	Isolated Mitochondria	[20]