Review
Mitochondrial potassium channels and reactive oxygen species
Dominika Malinska a, Sandra R. Mirandola b, Wolfram S. Kunz b,*

a Laboratory of Intracellular Ion Channels, Nencki Institute of Experimental Biology, 3 Pasteur st., 02-093 Warsaw, Poland
b Department of Epileptology and Life & Brain Center, University Bonn, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany

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ABSTRACT
Pretreatment of tissues with potassium channel openers (KCO's) has been observed to be cytoprotective in a broad variety of insults. This phenomenon has been proposed to be intimately linked to activation of mitochondrial potassium channels which apparently modulate the mitochondrial production of reactive oxygen species (ROS). This critical review summarizes literature findings about the mitochondrial production of ROS, the action of KCO's on mitochondrial ROS production and the putative link to the cytoprotective action of these drugs.

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1. Introduction. Mitochondrial generation of reactive oxygen species

Reactive oxygen species (ROS) are highly reactive molecules originating from molecular oxygen which have been implicated to be important for a broad variety of pathologies and ageing, as well as for intracellular signaling [1]. The superoxide anion – the product of one-electron reduction of oxygen – is the precursor of most ROS. Its dismutation reaction produces hydrogen peroxide, which in turn can be split by catalase to water and oxygen or within the Fenton reaction partially reduced to the extremely reactive hydroxyl radical. Well known cell specific enzymatic sources of superoxide include NADPH oxidases, cytochrome P450-dependent oxidases and xanthine oxidase. In most tissues the primary source of superoxide are certain redox centers of the mitochondrial respiratory chain [2], which are able for single electron transfer at the appropriate redox potential enabling reduction of molecular oxygen, having a standard redox potential of –160 mV [3]. Several sites within the mitochondrion have been implicated for donating these electrons, including FMNH 2 of respiratory chain complex I [4,5], and semiquinone at center ‘o’ of respiratory chain complex III [6,7], which both are probably the most relevant sites for brain and muscle tissue [8,9]. Moreover, several mitochondrial flavoproteins, like the α-lipoamide dehydrogenase moiety of the α-ketoglutarate dehydrogenase complex [10], the electron-transfer flavoprotein of the β-oxidation pathway [11] and α-glycerophosphate dehydrogenase [12] have been also suggested as possible additional sites for mitochondrial ROS production.

As depicted in Scheme 1, the major sites at complex I and III release the membrane impermeable superoxide anion to different compartments – the FMNH 2 – site of complex I to the mitochondrial matrix space and the semiquinone center ‘o’ of complex III to the intermembrane space [7,8,11]. While most experimental data suggest that the complex I-produced superoxide remains trapped in the matrix space [8,11], the complex III-produced superoxide apparently can escape the intermembrane space trough the voltage gated anion channel of the outer mitochondrial membrane (VDAC; porin pore) [13]. On the other hand, the dismutation product of superoxide – hydrogen peroxide – can cross the mitochondrial inner membrane presumably by aquaporin 9 channels [14]. There have been also reports assuming matrix superoxide release via the mitochondrial chloride channel [15] but direct experimental evidence for efficient superoxide release by this pathway is so far lacking.

Concerning the velocity of superoxide formation, it is important to note, that this rate is controlled by two factors: (i) the...
concentration of the electron donors – FMNH$_2$ or SQo – and (ii) the concentration of oxygen [16]. As shown in Scheme 1, this implies for the complex I site that processes which enhance the supply of NADH or diminish its oxidation (inhibition of electron flow through respiratory chain) would increase the concentration of FMNH$_2$ thus increasing the rate of superoxide formation. In line with this concept for complex I-dependent superoxide formation the unique determinant appears to be the mitochondrial NAD-redox state [9]. An important, pathology relevant process, proposed to increase complex I-dependent ROS formation, is PTP opening, leading to inhibition of NADH oxidation due to dissociation of cytochrome c [17]. This mechanism is particularly important to explain increased ROS formation during reperfusion injury. Since the redox state of the mitochondrial NAD system is controlled by substrate supply (reducing equivalent influx) and respiratory chain activity (reducing equivalent efflux), it is clear that stimulation of respiratory chain electron flow by decrease of mitochondrial membrane potential always would decrease the rate of complex I-dependent superoxide formation. The respiratory activity is strongly activated when uncouplers of the oxidative phosphorylation increase the proton permeability of mitochondrial membrane and waste the redox energy by means of a futile proton cycle [18]. A physiological process which uses this principle is ‘mild mitochondrial uncoupling’, which has been proposed to ameliorate the complex I-dependent ROS production by reducing reverse electron flow [18,19]. These changes in mitochondrial energy metabolism are promoted by uncoupling proteins [20] but the redox energy can be also dissipated by rotenone-insensitive external and internal NAD(P)H dehydrogenases or the alternative, cyanide-resistant, terminal oxidases (AOX) of plant, fungal and protozoan mitochondria [21,22]. Both mechanisms decrease efficiency of oxidative phosphorylation and increase electron transport, resulting in a physiological control of complex I-dependent ROS formation [23].

On the other hand, the concentration of the semiquinone at center ‘o’ of bc$_1$ complex, which is the direct donor of electrons for superoxide formation at complex III – SQo – is kinetically controlled by the electron transfer reaction within the Q-cycle (Scheme 1). Since the concentration of this relevant semiquinone species is the highest at intermediate levels of CoQ reduction, this implies – in contrast to complex I-dependent superoxide production – for complex III a bell-shaped dependency of superoxide production rate on the reduction state of the entire coenzyme Q pool [24]. That explains experimental findings in presence of antimycin that at a high initial state of CoQ reduction inhibitors of succinate oxidation, like malonate or TTFA [24], but also protonophores and ionophores enhanced superoxide formation rates from this particular site [25], while on the other hand, complete inhibition of succinate dehydrogenase inhibited ROS production [25]. This site is also due to the limited activity of intermembrane space SOD1 [26] potentially able to liberate superoxide to the cytosolic space (cf. Scheme 1). Therefore, complex III-dependent ROS formation, which can potentially be activated under conditions of a highly reduced coenzyme Q pool by depolarization of mitochondrial inner membrane, but also by inhibition of succinate dehydrogenase, could be important for the activation of ROS-dependent signal transduction pathways. For this reason it is plausible to suggest that this particular site might be involved in the preconditioning effects observed upon application of potassium channel openers (for details, see Section 3).

2. Effects of potassium channel openers on mitochondrial ROS production

After the functional identification of potassium channels in the inner mitochondrial membrane [27] and the demonstration, that cytoprotective properties of certain potassium channel openers (KCO’s) apparently result from stimulation of mitochondrial ROS
generation by these chemicals [28] there grew an interest in the relation between mitochondrial potassium fluxes and ROS generation by the respiratory chain. The earliest and most intensively studied mitochondrial potassium channel is the ATP regulated channel (mitoKATP). Recently, also a mitochondrial large conductance calcium activated potassium channel (mitoBKCa) is gaining increasing attention [29]. It is widely accepted that activation of potassium channels in the inner mitochondrial membrane and, in consequence, increasing potassium influx into the matrix leads to a decrease in mitochondrial membrane potential and consecutive stimulation of respiration rate leading to matrix alkalization (Scheme 2, [30,31]). In contrary to mitochondrial calcium uptake, where a decrease in membrane potential is a secondary effect resulting from intracellular calcium buffering role played by mitochondria, potassium uptake by these organelles can be a convenient mechanism for adjusting the mitochondrial metabolic state to the needs of the cell. Yet, there is not much agreement on how mitochondrial potassium fluxes influence mitochondrial superoxide generation. Studies on heart mitochondria provided so far conflicting results: ROS production increased [32,33] or decreased [34,35] upon activation of potassium transport by application of various potassium channel openers. Such inconsistency can result from the complexity of responses of the different mitochondrial superoxide-producing sites, described in the first part of this review. Additionally, the individual contributions of the different superoxide-producing sites appear to be tissue dependent [2,8]. The predicted effects of increased potassium influx into the matrix on ROS production by complexes I and III are summarized in Table 1. This possible site dependency of the observed effect on superoxide generation has been also discussed by Heinen et al. [35], who observed upon application of mitoBKCa opener NS 1619 a decrease of reverse electron flow dependent ROS generation (which is exclusively complex I-dependent), while forward electron flow-dependent ROS generation (at least partially complex III-dependent) was apparently stimulated by the same potassium channel opener [33]. More recent observations made with isolated brain mitochondria [36] indicated however a 20% decrease of hydrogen peroxide production upon application of the mitoBKCa openers CGS 7184 and NS 1619, irrespectively of the respiratory substrates (succinate or glutamate + malate) applied. These effects were observed to be strictly potassium selective. Moreover, the specific inhibitors of the BKCa channel, charybdotoxin and iberiotoxin, applied in nanomolar concentrations prevented the observed inhibitory action of the potassium channel openers CGS 7184 and NS 1619 [36]. The differences in the effects observed by Heinen et al. [33] and by Kulawiak et al. [36] might be related to different contributions of the complex I- and complex III-dependent superoxide generating sites in heart and brain mitochondria [8]. Since in brain tissue the complex I-dependent superoxide generation has a higher contribution than the complex III-dependent superoxide generation [8,9], the observed decrease in superoxide production is in line with the already mentioned effects of opening of potassium channels in inner mitochondrial membrane under resting state conditions of highly reduced NAD-system and of high resting state membrane potential. The net flux of potassium ions from the cytosol into the mitochondrial matrix space would lead to a ‘mild uncoupling’, resulting in a lowered mitochondrial membrane potential and lowered redox state of the NAD system leading to a diminished concentration of FMNH2. This in turn would decrease the production of reactive oxygen species by respiratory chain complex I (cf. Schemes 1 and 2).

It also needs to be mentioned that studying the mitochondrial potassium channels is aggravated by the poor selectivity of available potassium channel openers. It appeared that the most widely used opener of ATP-regulated potassium channel (KATP), diazoxide, apart from stimulating potassium influx into the matrix can inhibit succinate dehydrogenase [37], NS1619, the popular opener of calcium-activated potassium channel (BKCa), was shown to inhibit succinate dehydrogenase [38]. Of course, such direct modulation of the activity of respiratory complexes by KCO’s strongly affects mitochondrial superoxide generation. In case of the BKCa channel, the application of iberiotoxin and charybdotoxin, highly selective peptide inhibitors with effective concentrations in nanomolar range, allows quite reliable inhibitor-based controls. About the most widely used inhibitor of the mitoKATP channel, 5-hydroxydecanoic acid (5-HD) there is much more controversy. In contrary to the BKCa inhibitors, where the interaction of the drug with the channel is well described, the mechanism of the mitoKATP inhibition by 5-HD is more elusive. Some observations suggest even, that 5-HD can counteract the consequences of channel-unrelated effects of diazoxide [37,39]. Thus, while investigating the physiological role of mitochondrial potassium fluxes with the use of KCO’s a special attention should be paid to the potassium- and inhibitor-sensitivity of the observed effects and to the applied opener concentrations. Still, experiments with isolated mitochondria showing that many of the KCO effects can be channel-unrelated effects of diazoxide [37,39]. Thus, while investigating the physiological role of mitochondrial potassium fluxes with the use of KCO’s a special attention should be paid to the potassium- and inhibitor-sensitivity of the observed effects and to the applied opener concentrations. Still, experiments with isolated mitochondria showing that many of the KCO effects can be.

![Scheme 2. Potential effects of KCO's on mitochondrial ROS production and there putative link to cytoprotection. Thick red arrows indicate the expected effects of KCO's on the mitochondrial ROS producing sites at complexes I and III via mitoKATP and mitoBKCa, dashed arrows indicate potential side effects. IMM – inner mitochondrial membrane, OMM – outer mitochondrial membrane.](Image)

**Table 1**

Synopsis of the expected effects of potassium channel openers on mitochondrial ROS generation.

<table>
<thead>
<tr>
<th>Documented biochemical action of KCO’s</th>
<th>Expected action on ROS-generating sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complex I (ROS species liberated to cytosol: hydrogen peroxide)</td>
</tr>
<tr>
<td>Mild uncoupling [31]</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Matrix swelling [55]</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Inhibition of complex I of respiratory chain [38]</td>
<td>Stimulation</td>
</tr>
</tbody>
</table>

* The effect depends on supply of reducing equivalents and applied mitochondrial substrates.


[5] The relative amount of released superoxide depends on activity of SOD1 in the mitochondrial intermembrane space (cf. [26]).

3. Potential impact of potassium channel mediated alteration of ROS production on cellular survival

The cytoprotective effect obtained by pre-treatment of tissue with certain potassium channel openers, like diazoxide, before an insult is a well known phenomenon [40]. Application of KCO's before exposing the tissue to ischemia and reperfusion strongly reduces the ischemic injury. A similar effect is obtained by ischemic preconditioning (IPC), i.e., exposing the tissue to short ischemic periods before the appearance of longer ischemia and reperfusion. After the additional discovery, that IPC can be abolished by potassium channel inhibitors, it was suggested that opening of potassium channels is an event appearing in the preconditioning phase and essential for the observed protective effect.

The beneficial action of KCO's was mostly studied in cardiac tissue exposed to ischemia and reperfusion. However, similar cytoprotective effects were observed also in brain and kidney [41,42] and under different insults, like exposure to hydrogen peroxide or glutamate [43]. Since potassium channels are present both in plasma membrane and in inner mitochondrial membrane both sites can be the targets of KCO action. At present the prevailing view is that the activation of channels located in mitochondria is crucial for KCO-mediated cytoprotection [34,40]. Still, the link between modulation of mitochondrial function by KCO's and cytoprotection is not clear. Different hypotheses explain it as a consequence of matrix volume modulation, of ‘mild uncoupling’ resulting in limited calcium uptake into the mitochondria and diminished superoxide generation by the respiratory chain [34] or, in contrary, of stimulation of mitochondrial ROS production, which leads to the activation of pro-survival signaling pathways [40]. The last assumption is supported by observations, that the KCO-induced preconditioning is abolished by co-administration of antioxidants and, in consequence, prevention of the opener-induced stimulation of ROS generation [28,44,45]. The KCO-mediated cytoprotection was often accompanied by stimulation of redox sensitive kinases involved in pro-survival signaling pathways, like ERK [44,46] or PI3K [43]. In these studies the protective effects of KCO's were abolished by both kinase inhibitors and ROS scavengers. Moreover, scavenging free radicals prevented ERK activation, showing that ROS generation is necessary for activation of this kinase during preconditioning. The relation between decreasing mitochondrial membrane potential, increased ROS generation and preconditioning was demonstrated by Brennan et al. [47]. In this study the preconditioning was achieved by application of low doses of the uncoupler FCCP and the protective effect was abolished by ROS scavengers.

As discussed in the previous part of this review, the Q\textsubscript{0} site of complex III is the most probable superoxide-releasing site at which superoxide production can be stimulated by increased potassium influx and subsequent decrease in membrane potential. The assumption that ROS responsible for the preconditioning are originating from complex III is supported by observations, that the cytoprotective effects are abolished by application of SOD mimetics, which stimulate dismutation of superoxide to hydrogen peroxide [43,45]. Thus, the signaling molecule activating cytoprotective pathways seems to be superoxide, which is released by complex III to the cytosolic compartment (Schemes 1 and 2).

Preconditioning can be also achieved by exposing the tissue to moderate doses of hydrogen peroxide before the ischemic insult and interestingly, this H\textsubscript{2}O\textsubscript{2} induced protection was sensitive to the mitoKATP inhibitor 5-HD [48]. It seems then that in the preconditioning cascade of events, increased ROS levels are not only the consequence of activation of mitochondrial potassium channels but, in addition, can be responsible for the channel opening.

This view is supported by demonstrations of redox sensitivity of mitochondrial potassium fluxes [49]. Other studies show the dependence of mitoKATP activation on protein kinase C\textsubscript{e} (PKC\textsubscript{e}). Activation and translocation of this kinase to mitochondria is observed during preconditioning [50] and PKC\textsubscript{e} is in turn known to phosphorylate the ATP-regulated potassium channel, leading to increased activation of the channel and further stimulation of potassium influx into the matrix [32].

During preconditioning a moderate increase in superoxide generation induced by KCO's seems to be responsible for the beneficial effects of the openers. In contrary, the massive ROS production observed during reperfusion and responsible for tissue damage appeared to be limited by KCO pre-treatment [39,40]. Re-introduction of oxygen after the hypoxic period, when respiratory chain is highly reduced, results in strong stimulation of mitochondrial superoxide generation and this in turn leads to oxidative damage of the cells. Antioxidant treatment can limit the reperfusion-induced tissue damage, confirming the crucial role of ROS in this type of cell injury [51]. Thus, diminishing the reperfusion ROS levels by KCO's can therefore result from (i) activation of pro-survival signaling pathways during the preconditioning phase (e.g., activation of PKD leading to increased expression of SOD [52], as well as (ii) from diminished complex I-dependent ROS generation due to decreased mitochondrial membrane potential (Scheme 2).

As discussed previously, ‘mild uncoupling’ as potential mechanism to reduce ROS production has been proposed to explain tissue protection against ROS toxicity [18,19]. Therefore, the data of Facundo et al. [34], Heinen et al. [35] and Kulawiak et al. [36] reporting upon application of BK channel openers a decrease of mitochondrial ROS production suggest that openers of BK channels could in principle deploy their cytoprotective action by this particular mechanism.

The assumption that pharmacological preconditioning with KCO's is caused by opening of mitochondrial potassium channels can be questioned due to doubtful specificity of the available potassium channel modulators. Therefore, it has been suggested that the diazoxide-mediated cardioprotection can be attributed rather to inhibition of succinate dehydrogenase (SDH) [53] or the protonophoric properties of the drug [54] than to activation of potassium channels. Nevertheless, both mentioned side effects of KCO's would modulate mitochondrial ROS production in a similar way (cf. Scheme 2, dashed arrows). They would lead to inhibition of complex I-dependent ROS production – important for protection against oxidative stress in the reperfusion phase – and at saturation of substrate supply potentially to a stimulation of complex III-dependent ROS production, which could be beneficial for activation of pro-survival signaling pathways in the preconditioning phase.

4. Concluding remarks

The cytoprotective actions of KCO's are very likely related to two different effects on mitochondrial ROS production: (i) Their potential stimulation of complex III-dependent ROS production which might be relevant for ROS triggered signal transduction to the cytosol in the precondition phase and (ii) their established inhibition of complex I-dependent ROS production in the matrix compartment occurring in the reperfusion phase. Whether these effects are directly caused by opening of mitochondrial potassium channels, like mitoKATP or mitoBKCa, or are linked to possible side
effects of the applied drugs remains to be elucidated yet. Additionally, the potential impact of KCO-induced changes of ROS levels for intracellular processes should be much better estimated applying more precise quantitative measurements of the KCO-induced changes of mitochondrial ROS release. Paradoxically, during ischemia and reperfusion ROS are responsible for both tissue injury and preconditioning. Thus, it still remains an important goal to determine which types and which sources of ROS are involved in the particular, beneficial or deleterious effect. Since the efficacy of general antioxidant treatments, giving promising results in some experimental systems of ischemia and reperfusion, was so far not confirmed in clinical practice, there is still a need for the development of cytoprotective treatments targeted to the relevant sources of ROS.

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