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Opening of the MitoK_{ATP} Channel and Decoupling of Mitochondrial Complex II and III Contribute to the Suppression of Myocardial Reperfusion Hyperoxygenation

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Abstract

Diazoxide, a mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channel opener, protects the heart from ischemia-reperfusion injury. Diazoxide also inhibits mitochondrial complex II-dependent respiration in addition to its preconditioning effect. However, there are no prior studies of the role of diazoxide on post-ischemic myocardial oxygenation. In the current study, we determined the effect of diazoxide on the suppression of post-ischemic myocardial tissue hyperoxygenation *in vivo*, superoxide (O₂^{-•}) generation in isolated mitochondria, and impairment of the interaction between complex II and complex III in purified mitochondrial proteins. It was observed that diazoxide totally suppressed the post-ischemic myocardial hyperoxygenation. With succinate but not glutamate/malate as the substrate, diazoxide significantly increased ubisemiquinone-dependent O₂^{-•} generation, which was not blocked by 5-HD and glibenclamide. Using a model system, the super complex of succinate-cytochrome c reductase (SCR) hosting complex II and complex III, we also observed that diazoxide impaired complex II and its interaction with complex III with no effect on complex III. UV-visible spectral analysis revealed that diazoxide decreased succinate-mediated ferricytochrome *b* reduction in SCR. In conclusion, our results demonstrated that diazoxide suppressed the *in vivo* post-ischemic myocardial hyperoxygenation through opening the mitoK_{ATP} channel and ubisemiquinone-dependent O₂^{-•} generation via inhibiting mitochondrial complex II-dependent respiration.

Keywords

Mitochondria; Diazoxide; Superoxide; Ischemia Reperfusion; Oxygenation

Introduction

Preconditioning by one or several intermittent episodes of ischemia protects the heart from subsequent ischemia/reperfusion injury [1, 2]. Ischemic preconditioning (IPC) also

suppressed post-ischemic myocardial hyperoxygenation and preserved tissue oxygen consumption [3]. The effect of IPC can be mimicked by a variety of drugs, a phenomenon called pharmacological preconditioning (PPC). Among these drugs, diazoxide, a mitochondrial ATP-dependent potassium (mitoK_{ATP}) channel opener, has been widely studied in hearts, cultured cells, or isolated mitochondria [4-6].

In addition to opening of the mitoK_{ATP} channels, diazoxide has a number of mitoK_{ATP}-independent effects on mitochondria. It was reported as early as in 1969 that diazoxide inhibited succinate oxidation and slightly stimulated NADH oxidation [7]. More recent studies have demonstrated that diazoxide inhibited succinate-supported respiration and glibenclamide failed to block the effect. Mitochondrial respiration was not affected by diazoxide in the presence of glutamate/malate [8]. Hanley et al. [9] found that diazoxide inhibited succinate oxidation without affecting complex I-dependent respiration. Kim et al. showed that diazoxide inhibited state 3 succinate oxidation and hypothesized that formation of reactive oxygen species (ROS) from inhibition of the mitochondrial respiratory chain by both ischemia and diazoxide was important for preconditioning, rather than opening the mitoK_{ATP} channels [10]. Dröse and co-workers [11] confirmed that diazoxide inhibited succinate oxidation and the stimulatory effect of diazoxide on 2',7'-dichlorodihydrofluorescein (H₂DCF) oxidation was not due to the opening of the mitoK_{ATP} channels. In digitonin-permeabilized C2C12 myotubes, diazoxide attenuated succinate-supported respiration independent of the mitoK_{ATP} channels [12]. These studies unambiguously demonstrated the mitoK_{ATP} channel-independent effect of diazoxide, in addition to its opening of the channel and protection of the ischemic heart. However, the pharmacological preconditioning effect of diazoxide on the post-ischemic myocardial hyperoxygenation and tissue oxygen consumption has not been studied. Furthermore, the inhibition site of diazoxide in complex II remains unclear.

Inhibition of electron transport chain may result in increase or decrease of superoxide (O₂^{-•}) generation depending on the inhibition sites. Other complex II inhibitors, such as 3-nitropropionic acid (3-NPA) and malonate, can induce ROS production in mitochondria [13, 14]. For example, 3-NPA increases antimycin A-induced O₂^{-•} generation in the presence of complex I substrate, but it decreases antimycin A-induced O₂^{-•} generation in mitochondria respiring on complex II substrates. The O₂^{-•} generation has been localized to a site between the ubiquinol pool and the 3-NPA block in respiratory complex II [13]. It has been demonstrated that complex III appears to be responsible for most of the O₂^{-•} formation in heart mitochondria as a result of the auto-oxidation of ubisemiquinone (Q^{-•}) [15]. Therefore, it is important to study the effect of diazoxide on mitochondrial O₂^{-•} formation at the ubiquinone site.

The development of electron paramagnetic resonance (EPR) techniques using oxygen sensitive probes such as lithium phthalocyanine (LiPc) has provided a fast and accurate method for monitoring tissue Po₂ in various organs and tissues *in vivo* and *in vitro* [16-19]. EPR spin trapping technique has also provided a powerful means for ROS detection and finger-printing [20].

In this study, we determined the effect of diazoxide on post-ischemic myocardial tissue hyperoxygenation using EPR oximetry with LiPc as the oxygen sensitive probe. In order to further dissect the mechanisms in the protection of the ischemic heart by diazoxide, we measured O₂^{-•} generation in antimycin A-inhibited mitochondria (simulating the ischemic condition *in vivo*) using 5, 5-dimethyl-1-pyrroline *N*-oxide (DMPO) as the spin probe. We also monitored the impairing effect of diazoxide on the interaction between mitochondrial complex II and complex III using purified proteins. We demonstrated that diazoxide totally suppressed the post-ischemic myocardial hyperoxygenation through opening the mitoK_{ATP}

channels as well as the $\text{mitoK}_{\text{ATP}}$ -independent effect on ubisemiquinone-dependent $\text{O}_2^{\cdot-}$ generation in mitochondria and inhibition of complex II-dependent respiration.

Methods

Chemicals

Diazoxide, 5-hydroxydecanoate (5-HD), and glibenclamide (Sigma-Aldrich, USA) were prepared in PBS with less than 0.5% dimethyl sulfoxide (DMSO) as stock solution and were given to the mice 10 min before ischemia or preconditioning stimuli at dosages of 6 mg/kg and 10 mg/kg i.v. respectively [21]. DMPO was purchased from Dojindo Laboratories (Kumamoto, Japan). Catalase was obtained from Roche Diagnostics (Mannheim, Germany). All other chemicals were from Sigma Chemical (St. Louis, MO). The final concentration of DMSO was less than 0.1% in the reaction mixture and same amount of DMSO was introduced to the control experiments.

Myocardial Ischemia Reperfusion (I/R) Model and IPC Protocol

Male C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, Me). All procedures were performed with the approval of the Institutional Animal Care and Use Committee at The Ohio State University, Columbus, Ohio, and conformed to the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 85-23, revised 1996).

The surgical protocol was similar to methods described previously [3, 18, 22]. Mice were anesthetized with ketamine (55 mg/kg) plus xylazine (15 mg/kg). Animals were orally intubated with PE-90 tubing and connected to a mouse mini-ventilator (Model 845, Harvard Apparatus). Core body temperature was maintained at 37°C with a thermo heating pad. In all groups of mice, similar basal heart rates were observed, with values of 300 to 350 bpm, typical for anesthetized mice.

A thoracotomy was performed. The left anterior descending coronary artery (LAD) was visualized and completely ligated for 30 min in the I/R mice by tightening an 8-0 silk suture after passing it over a length of PE-10 tubing beneath the LAD at points 1 to 2 mm inferior to the left auricle. Ischemia was confirmed visually by the appearance of pale and bulging myocardium in the area at risk. The ligature was removed and reperfusion was visually confirmed after 30 min of LAD occlusion. Reperfusion was maintained for 60 min to obtain oximetry measurements.

IPC was introduced by three cycles of 5 min ischemia, followed by 5 min reperfusion. The final reperfusion period of the IPC protocol was extended to 15 min in order to ensure the stabilization of tissue Po_2 . The IPC + I/R mice were then subjected to 30 min LAD occlusion followed by 60 min of reperfusion for EPR oximetry measurements.

Isolation of Mitochondria

Male Sprague-Dawley rats (250-300 g) were housed under a 12:12-h light-dark cycle and were provided with water and food ad libitum. Intact mitochondria were isolated from rat heart with differential centrifugation [23]. Briefly, rats were anesthetized with i.p. injection of sodium pentobarbital (100 mg/kg). Hearts were removed and washed in ice-cold isolation buffer containing 250 mM sucrose, 0.5 mM EGTA and 3 mM HEPES (pH 7.2). Then hearts were finely minced in the presence of 1 mg/ml proteinase (type XXIV, Sigma), and the suspension was diluted (1:3) with isolation buffer supplemented with 0.5% fatty acid-free BSA. The minced tissue was homogenized by a Potter-Elvehjem Teflon pestle and then centrifuged at 1,500 g for 3 min. The supernatant was centrifuged at 9,000 g for 5 min. The obtained pellet was suspended in isolation buffer and re-centrifuged at 2,300 g for 3 min.

The pellet was discarded and the supernatant was centrifuged at 9,000 g for 5 min. The isolated mitochondria were then suspended at a concentration of 30-40 mg protein/ml and kept on ice. In the present study, we did not check any contamination during the isolation process further since differential centrifugation has been widely used for mitochondria isolation to study the effect of diazoxide on mitochondrial function or ROS generation [11, 23-25].

Mitochondrial respiration and integrity were assessed as previously described [26]. The respiration buffer contained 230 mM mannitol, 70 mM sucrose, 30 mM Tris-HCl (pH 7.4), 5 mM KH_2PO_4 , 1 mM EDTA, and 0.1% BSA. Mitochondria were prepared at a concentration of 0.25 mg protein/ml with either 5 mM glutamate/5 mM malate or 10 mM succinate as the substrate. Rotenone (2 μM) was added when succinate was used. Oxygen consumption was measured with a Clark-type electrode (WPI, Sarasota, Florida) in a gas-tight chamber at 30 °C. State 3 respiration was induced by introducing 500 μM ADP. The isolated mitochondria showed a respiratory control ratio of >4 using glutamate/malate and 2.5 using succinate as the substrate, which was consistent with a previous report [26].

Preparation of Sub-Mitochondrial Particles (SMP)

Sub-mitochondrial particles from rat heart were prepared from the mitochondrial suspension as described [27]. The concentration of mitochondria was adjusted to 20 mg protein/ml with isolation buffer and then the suspension was diluted (1:4) with 25 mM Tris-HCl (pH 8.0). The mitochondria were sonicated in an ice bath (8×10 s with 1 min intervals) using a Branson 450 sonifier (Branson, Danbury, CT). The resulting suspension was first centrifuged at 10,000 g for 10 min at 4 °C and the pellet was discarded. The supernatant was further centrifuged at 105,000 g for 30 min at 4 °C. Finally, the obtained pellet was washed and suspended in a buffer containing 230 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 5 mM HEPES (pH 7.0).

Preparation of Mitochondrial Succinate Cytochrome c Reductase (SCR)

Mitochondrial SCR was prepared from bovine heart SMP and assayed as described [28]. The pellet obtained after the last centrifugation was dissolved in a buffer containing 50 mM sodium/potassium phosphate (pH 7.4) and 0.25 M sucrose. The suspension was dialyzed overnight against the same buffer before being stored at -80 °C. The purified SCR exhibited an activity of ~ 9.1 μM of cytochrome *c* reduced/min/mg of protein. Protein concentration was estimated with the Lowry method [29] using BSA as the standard.

EPR Oximetry

After thoracotomy, about 10 μg of LiPc was loaded in a 27-gauge needle and implanted into the mid-myocardium of the area at risk. The location of the LiPc was confirmed by histology. Then the mouse was moved to a home-built *in vivo* EPR spectrometer and EPR oximetry was performed after a 30 min equilibration period with the following parameters: frequency 1.1 GHz, incident microwave power 16 mW, modulation field 0.045 G. The EPR line-width of LiPc linearly responds to tissue Po_2 in the range of 0 to 150 mmHg with a sensitivity of 5.8 mG/mmHg [22].

EPR Spin Trapping Measurements

EPR spin trapping measurements were performed on a Bruker EMX spectrometer operating at 9.86 GHz with 100 kHz modulation at room temperature [26]. Measurement of $\text{O}_2^{\cdot-}$ generation in mitochondria was performed in buffer A containing 120 mM KCl, 10 mM HEPES (pH 7.2), 0.1 mM EGTA, 5 mM phosphate, 0.5 mM MgCl_2 and 200 μM ATP [23]. For K^+ -free medium, K^+ in buffer A was replaced with 120 mM tetraethyl ammonium

(TEA⁺) as described [23]. ATP in buffer A was omitted to obtain ATP-free medium. O₂^{-•} generation in SMP was detected in a buffer containing 230 mM mannitol, 70 mM sucrose, 30 mM Tris-HCl (pH 8.2). Since DMPO has a low reactivity with O₂^{-•} ($K_{app}=50\text{--}60\text{ M}^{-1}\text{S}^{-1}$) [20], we used a relatively high concentration of 160 mM as reported [26]. All the reaction mixtures contained 0.2 mM diethylenetriaminepentaacetic acid (DTPA) and 2600 units/ml catalase. Antimycin A was added at the concentration of 1 μg/mg protein in all the measurements to simulate the *in vivo* ischemic conditions in the EPR oximetry experiments. Reaction was initiated by introducing mitochondria or SMP into the reaction mixture which was vortexed and transferred to a 50-μl capillary tube. The EPR spectrum was recorded 1 min afterwards and was averaged over 10 scans for 7 min. The instrumental settings were as following: modulation amplitude, 0.1 mT; receiver gain, 2×10^5 ; microwave power, 12.8 mW; sweep width, 8 mT; time constant, 81.92 ms; conversion time 20.48 ms. The amount of O₂^{-•} was represented by the relative intensity of the second peak to the left in the EPR spectrum.

UV-Visible Spectrophotometry

Optical spectra were recorded in a Shimadzu 2401 UV-visible spectrophotometer with corresponding wavelength.

Measurement of O₂^{-•} Generation with Ferricytochrome c

O₂^{-•} generation in SMP was also measured with ferricytochrome *c* according to the published method [30] with modifications. The kinetics of cytochrome *c* reduction was initiated by SMP and the absorption increase at 550 nm was monitored at room temperature ($\epsilon_{550\text{nm}}=18.5\text{ mM}^{-1}\text{cm}^{-1}$). The reaction mixture contained 230 mM mannitol, 70 mM sucrose, 30 mM Tris-HCl (pH 8.2), 30 μM ferricytochrome *c*, 1 mM DTPA, 0.1 mM KCN, 0.5 μg/ml antimycin A, and 0.1 mg protein/ml with either 0.7 mM NADH or 7 mM succinate as the substrate. When succinate was used as the substrate, rotenone (1 μM) was added to the medium. The rate of O₂^{-•} generation was determined as the SOD-sensitive rate of cytochrome *c* reduction. In the present study, SOD was replaced by an efficient SOD mimetic, M40403 [31] (50 μM), as KCN could inhibit the activity of Cu,Zn-SOD [32].

Assay of SCR, Succinate Ubiquinone Reductase (SQR) and Ubiquinol Cytochrome c Reductase (QCR) Activities

The electron transfer activities of SCR, SQR, and QCR were measured with a Shimadzu 2401 UV-visible spectrophotometer using the corresponding electron donors and acceptors.

The electron transfer activity of SCR was assayed by measuring cytochrome *c* reduction [33]. SCR was added at a final concentration of 10 nM heme *b* to an assay mixture (1 ml) containing 50 mM phosphate buffer, pH 7.4, 0.3 mM EDTA, 19.8 mM succinate, and 50 μM ferricytochrome *c*. The SCR activity was determined by measuring the increase in absorbance at 550 nm ($\epsilon_{550\text{nm}}=18.5\text{ mM}^{-1}\text{cm}^{-1}$).

The electron transfer activity of SQR in the super complex of SCR was measured as described [28]. SCR was added at a final concentration of 10 nM heme *b* to an assay mixture containing 50 mM phosphate buffer (pH 7.4), 0.1 mM EDTA, 75 μM dichlorophenolindophenol (DCPIP), 50 μM ubiquinone-2 (Q₂), and 20 mM succinate. SQR activity was determined by measuring the decrease in absorbance at 600 nm. The specific activity of SQR (nM of DCPIP reduction/min/mg of SQR) was calculated using a molar extinction coefficient $\epsilon_{600\text{nm}}=21\text{ mM}^{-1}\text{cm}^{-1}$.

The electron transfer activity of QCR in the super complex of SCR was assayed as reported [28]. Briefly, SCR was added at a final concentration of 2.5 nM heme *b* to 1 ml assay

mixture (50 mM sodium/potassium phosphate buffer, pH 7.0, 1 mM EDTA, 50 μ M ferricytochrome *c*, and 25 μ M Q₂H₂). QCR activity was determined by measuring the increase in absorbance at 550 nm ($\epsilon_{550\text{nm}}=18.5 \text{ mM}^{-1}\text{cm}^{-1}$).

Statistics Analysis

A one-way ANOVA was used for data analysis of Po₂ at 60 min of reperfusion. For O₂•⁻ and enzyme activity measurements, comparisons were conducted using the paired *t*-test. A value of *p* < 0.05 was considered significant. Data shown represent mean $\pm$ SE of at least 3 identical experiments.

Results

Diazoxide and IPC Totally Suppressed Post-Ischemic Myocardial Tissue Hyperoxygenation

Mice were treated with diazoxide 10 min before ischemia and EPR oximetry was performed on the treated mice (Diazoxide + I/R group) as well as the I/R only mice (I/R group) as described in the Methods section. The baseline values of myocardial tissue Po₂ in the pre-ischemic states were 16.3 ± 0.8 and 15.1 ± 0.4 mmHg in the I/R and Diazoxide + I/R mice, as shown in Fig. 1A. There is no significant difference between the two groups at this time point. After ischemia, tissue Po₂ decreased rapidly in both groups during the first 10 min of ischemia and then gradually thereafter and finally reached values of 1.0 ± 0.2 and 1.8 ± 0.2 mmHg in the I/R and Diazoxide + I/R mouse hearts. Upon reperfusion, tissue Po₂ increased quickly in the first 10 min. In the I/R group, tissue Po₂ continued to rise to a value of 25.4 ± 1.0 mmHg at 60 min reperfusion which has been reported as the hyperoxygenation phenomenon in the post-ischemic myocardium [3, 18, 22]. However, in the Diazoxide + I/R group, tissue Po₂ reached a plateau with a value of 15.1 ± 0.9 mmHg at 60 min reperfusion which is comparable to that in the pre-ischemic state. Clearly, diazoxide treatment before ischemia totally suppressed the post-ischemic myocardial tissue hyperoxygenation (**p* = 0.001, Diazoxide + I/R vs. I/R).

Since diazoxide mimics preconditioning in protecting the ischemic heart, next we measured tissue Po₂ in the IPC + I/R group. In order to determine if opening of the mitoK_{ATP} channels in the preconditioned heart suppressed the hyperoxygenation, we compared the measurement in the IPC + I/R group with that in the 5-HD + IPC + I/R group. As shown in Fig. 1B, baseline values of myocardial tissue Po₂ in the preischemic states were 15.4 ± 0.8 and 13.6 ± 0.6 mmHg in the IPC + I/R and 5-HD + IPC + I/R mice. Again, there was no significant difference between the two groups at this time point. After the onset of preconditioning, decreases of tissue Po₂ were clearly observed during each 5 min ischemic period followed by increases in each reperfusion phase. At the end of last reperfusion of the stimuli, Po₂ values restored back to 13.3 ± 0.9 and 12.7 ± 0.6 mmHg which were comparable to the pre-ischemic values respectively. In both groups, tissue Po₂ values dropped rapidly following the onset of ischemia to less than 2 mmHg within 10 min and then gradually decreased to values of 1.2 ± 0.3 and 1.5 ± 0.4 mmHg in the IPC + I/R and 5-HD + IPC+I/R mice. Upon reperfusion, the values of Po₂ increased rapidly in both groups. In the IPC + I/R mice, tissue Po₂ increased to a plateau with a value of 15.3 ± 1.2 mmHg at the end of 60 min reperfusion which is consistent with our previous report ([†]*p* = 0.001, IPC + I/R vs. I/R) [3]. However, in the 5-HD + IPC + I/R mice, tissue Po₂ continued to rise over the preischemic value and reached 21.2 ± 0.2 mmHg at the end of 60 min reperfusion (***p* = 0.006, 5-HD + IPC + I/R vs. IPC + I/R; ^{††}*p* = 0.011, 5-HD +IPC +I/R vs. I/R). It is clearly demonstrated that diazoxide mimics the IPC effect and totally suppressed the post-ischemic myocardial hyperoxygenation as in the treatment with diazoxide and IPC + I/R groups (no significant difference between these two groups). The reappearance of the overshoot of tissue Po₂ in the 5-HD + IPC + I/R group demonstrated that opening of the mitoK_{ATP}

channels contributed to the suppression of the post-ischemic myocardial hyperoxygenation. Interestingly, at 60 min reperfusion, the value of tissue P_{O_2} in the 5-HD + IPC + I/R group was not as high as that in the I/R group (21.2 ± 0.2 vs. 25.4 ± 1.0 mmHg). As indicated by the p values of 0.001 and 0.011 between 5-HD + IPC + I/R and Diazoxide + I/R vs. I/R groups, 5-HD could not totally abrogate the IPC effect on the attenuation of post-ischemic myocardial hyperoxygenation. Since diazoxide and IPC can both open the mitoK_{ATP} channels, and 5-HD is a specific mitoK_{ATP} channel inhibitor [34, 35], these data indicated that there may be other mechanisms involved in the total suppression of the post-ischemic myocardial hyperoxygenation in addition to the opening of the mitoK_{ATP} channel by diazoxide and IPC.

Diazoxide-Induced $O_2^{\bullet-}$ Formation in Intact Mitochondria Measured with EPR

In order to identify additional mechanisms involved in the total suppression of the post-ischemic myocardial hyperoxygenation from diazoxide and considering the limitations of *in vivo* free radical measurements, isolated mitochondria were used as the model system. In order to mimic the *in vivo* ischemic conditions, antimycin A was used to inhibit the mitochondrial respiration on complex III [26]. It has been reported that $O_2^{\bullet-}$ can be released into matrix as well as cytoplasm from mitochondria and plays an important role in cell signaling [26, 36]. In order to determine whether diazoxide induces $O_2^{\bullet-}$ formation therefore exerts additional effect on the total suppression of the post-ischemic hyperoxygenation *in vivo*, the isolated mitochondria were also treated with diazoxide and EPR spin trapping was applied for the measurement of $O_2^{\bullet-}$.

To measure $O_2^{\bullet-}$ generation, mitochondria were prepared at a concentration of 0.5 mg protein/ml, and 5 mM glutamate/5 mM malate or 10 mM succinate was applied as the substrate. Rotenone (2 μ M) was present to prevent electron back flow from complex II to complex I when succinate was used. Antimycin A, which blocks the transfer of the second electron of ubiquinol (QH₂) from cytochrome *b_H* to ubiquinone (Q) and increases the steady-state levels of ubisemiquinone at the Q_O site (Q_O^{•-}) in complex III, was added with a concentration of 1 μ g/mg protein [26]. As shown in Fig. 2, only a trace EPR signal was detected from mitochondria in the absence of substrate (Fig. 2a). The intensity of the signal was low in the presence of glutamate/malate (Fig. 2b) or succinate (Fig. 2e). The EPR signal intensity was enhanced prominently in the presence of antimycin A with either glutamate/malate (Fig. 2c) or succinate as the substrate (Fig. 2f). The observed spectra can be readily ascribed to the DMPO-OH adduct (quartet signal with intensity ratios of 1:2:2:1; $A_N=A_H=1.49$ mT). As DTPA and catalase were added in the system to prevent the formation of hydroxyl radical ($\bullet$ OH), it can be assured that the signal of DMPO-OH adduct was derived from the $O_2^{\bullet-}$ spin adduct (DMPO-OOH) [26]. The EPR signal was totally abolished by Cu, Zn-SOD (Fig. 2d and 2g), which further verified $O_2^{\bullet-}$ as the source of DMPO-OH. Since the outer mitochondrial membrane is impermeable to Cu, Zn-SOD, the SOD-quenchable EPR signal indicated that the spin adduct was formed outside or close to the outer membrane, probably due to the diffusion of $O_2^{\bullet-}$ from inside. In addition, we used 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-Oxide (DEPMPO), which can form a longer half-life $O_2^{\bullet-}$ adduct [37], as the spin trap under the same conditions and only the spectra of DEPMPO-OH adduct were obtained (data not shown), indicating that the $O_2^{\bullet-}$ adduct was converted to the hydroxyl radical adduct by components in the mitochondria. This is consistent with the detected DEPMPO-OH spin adduct in the presence of glutathione peroxidase [37]. These results clearly demonstrated that ubisemiquinone-dependent $O_2^{\bullet-}$ was released from intact mitochondria and was detected with spin trapping EPR spectroscopy.

Diazoxide-Induced $O_2^{\bullet-}$ Formation in Antimycin A-Inhibited Mitochondria Was Substrate-Dependent, but not $mitoK_{ATP}$ -Dependent

$O_2^{\bullet-}$ can function as a precursor to other types of ROS, such as hydrogen peroxide and hydroxyl radical. Using EPR spin trapping technique, we measured $O_2^{\bullet-}$ generation in the intact mitochondria as shown in Fig. 3. Diazoxide was added to the reaction medium at a concentration of 100 μ M. This relatively high concentration was used to be relevant to the *in vivo* diazoxide dosage (6 mg/kg i.v.) in a live mouse with body-weight about 25 - 30 g. 5-hydroxydecanoate (5-HD, 500 μ M) or glibenclamide (10 μ M) was used as inhibitors to determine if diazoxide-induced $O_2^{\bullet-}$ generation was $mitoK_{ATP}$ -dependent. Diazoxide also has a weak protonophoric uncoupling effect on mitochondrial respiration [11, 38, 39]. Therefore, a mitochondrial uncoupler, carbonyl cyanide-*p*-tri-fluoromethoxyphenyl-hydrazone (FCCP) was used to test whether an uncoupler can affect $O_2^{\bullet-}$ generation in the same manner as diazoxide. To test the substrate dependence, 5 mM glutamate/5 mM malate or 10 mM succinate were used. Representative EPR spectra are shown in Fig. 3A. The intensity of the control spectra obtained with the corresponding substrate was defined as 100% for statistic analysis. The percentage change of $O_2^{\bullet-}$ generation in comparison to the control is shown in Fig. 3B. With glutamate/malate as the substrate (Fig. 3Ba), $O_2^{\bullet-}$ generation in mitochondria was not altered by diazoxide ($1.7 \pm 4.58\%$ vs. 0% in control; $P > 0.05$, $n=6$). Neither 5-HD ($-3.97 \pm 4.25\%$ vs. $1.7 \pm 4.58\%$ with diazoxide; $P > 0.05$, $n=6$), a specific $mitoK_{ATP}$ channel blocker, nor glibenclamide ($-1.5 \pm 1.21\%$ vs. $1.7 \pm 4.58\%$ with diazoxide; $P > 0.05$, $n=3$), a non-specific blocker, altered the $O_2^{\bullet-}$ generation. Furthermore, 5-HD ($-3.55 \pm 2.91\%$; $P > 0.05$ vs. 0% in control, $n=6$) or glibenclamide ($-0.17 \pm 0.29\%$; $P > 0.05$ vs. 0% in control, $n=3$) alone did not change $O_2^{\bullet-}$ generation with glutamate/malate as the substrate. However, when succinate was used (Fig. 3Bb), $O_2^{\bullet-}$ generation was significantly increased by diazoxide ($23.2 \pm 2.27\%$ vs. 0% in control; $P < 0.05$, $n=6$), and this effect was not blocked by either 5-HD ($23.7 \pm 2.20\%$ vs. $23.2 \pm 2.27\%$ with diazoxide; $P > 0.05$, $n=6$) or glibenclamide ($22.7 \pm 1.30\%$ vs. $23.2 \pm 2.27\%$ with diazoxide; $P > 0.05$, $n=3$). Once again, 5-HD ($0.9 \pm 1.10\%$; $P > 0.05$ vs. 0% in control, $n=6$) or glibenclamide ($0.4 \pm 2.95\%$; $P > 0.05$ vs. 0% in control, $n=3$) alone did not have any effect on $O_2^{\bullet-}$ generation. Interestingly, FCCP induced a significant increase of $O_2^{\bullet-}$ generation in both substrate-supported mitochondria ($29.4 \pm 5.92\%$ vs. 0% in control; $P < 0.05$ with glutamate/malate and $25.0 \pm 3.99\%$ vs. 0% in control; $P < 0.05$ with succinate, $n=4$), which is obviously different from that of diazoxide. Therefore, we speculate that this substrate- but not $mitoK_{ATP}$ -dependent effect on the formation of $O_2^{\bullet-}$ may account for the additional contribution of diazoxide to the total suppression of the post-ischemic myocardial hyperoxygenation since $O_2^{\bullet-}$ can trigger the IPC effect.

To further study the effective concentration of diazoxide on $O_2^{\bullet-}$ generation in antimycin A-inhibited mitochondria, dose-dependent experiments were performed with diazoxide concentrations ranging from 10-300 μ M. Considering a mouse with body weight of ~30 g and the fact that diazoxide injected acutely to the circulation system, an *in vivo* dosage of 6 mg/kg would render an equivalent blood concentration of diazoxide in the range of 250 μ M or less. Therefore, the concentration range chosen for diazoxide is relevant to *in vivo* diazoxide dosage used in a living mouse. It was observed that $O_2^{\bullet-}$ generation was increased with the increase of diazoxide concentration in a dose-dependent manner (Fig. 4). The $O_2^{\bullet-}$ generation was $150.6 \pm 6.8\%$ of the control at 300 μ M of diazoxide. An EC_{50} of 45 μ M was obtained from the simulation curve. This result confirmed that diazoxide increased $O_2^{\bullet-}$ generation from complex II-dependent respiration of mitochondria in the concentration range we used.

Diazoxide-Induced $O_2^{\cdot-}$ Formation in ATP- and K^+ -Free Media

To further prove that this portion of diazoxide-induced $O_2^{\cdot-}$ formation is mitoK_{ATP}-independent, we measured mitochondrial $O_2^{\cdot-}$ generation with succinate as the substrate in both ATP- and K^+ -free media. The mitoK_{ATP} channels are already opened in ATP-free medium. In K^+ -free medium, diazoxide is not able to exert its function on the mitoK_{ATP} channels. It has been reported that opening mitoK_{ATP} channels increased $O_2^{\cdot-}$ generation [40]. If this is also true for antimycin A-inhibited mitochondria, diazoxide should not induce any $O_2^{\cdot-}$ generation in K^+ -free medium or in ATP-free medium. As shown in Fig. 5, $O_2^{\cdot-}$ generation was increased by diazoxide ($141.3 \pm 1.65\%$ vs. 100% in control; $P < 0.05$, $n = 4$) when $200 \mu\text{M}$ ATP was omitted in the medium. In addition, diazoxide induced increase of $O_2^{\cdot-}$ generation when $120 \text{ mM } K^+$ was replaced with 120 mM TEA^+ ($190.0 \pm 2.44\%$ vs. $164.0 \pm 8.57\%$ in control; $P < 0.05$, $n = 4$). These results confirmed that diazoxide-induced ubisemiquinone-dependent $O_2^{\cdot-}$ generation is independent of the mitoK_{ATP} channels in the presence of antimycin A.

Diazoxide-Induced $O_2^{\cdot-}$ Formation in Antimycin A-Inhibited SMP Was Also Substrate-Dependent

In order to eliminate the complexity from the matrix as well as the intermembrane space, we measured ubisemiquinone-dependent $O_2^{\cdot-}$ generation in antimycin A-inhibited SMP with both EPR spectrometry and cytochrome *c* reduction methods. For EPR measurement, SMP ($0.5 \text{ mg protein/ml}$) were added to the buffer with either 1 mM NADH or 10 mM succinate as the substrate. The intensity of the control EPR spectra obtained with succinate was defined as 100% . As shown in Fig. 6, when NADH was used, $O_2^{\cdot-}$ generation was not altered by diazoxide ($131.2 \pm 4.63\%$ vs. $135.9 \pm 1.65\%$ in control; $P > 0.05$, $n = 4$). However, when succinate was used, $O_2^{\cdot-}$ generation was significantly increased to $120.2 \pm 2.99\%$ of control ($P < 0.05$, $n = 4$).

In addition to EPR spin trapping, we also used the classic cytochrome *c* reduction assay for measuring $O_2^{\cdot-}$ generation. It has been reported that some drugs can bind to mitochondrial cytochrome *c*, reduce the heme, and lead to enhanced ROS levels in mitochondria [41]. Therefore, we determined firstly if there was a direct interaction between diazoxide and ferricytochrome *c*. As indicated in Fig. 7A, trace a shows the UV-visible absorbance spectrum of ferricytochrome *c*, which consists of the unresolved absorption band with a maximum at 528 nm . There was no change in the absorbance spectrum when diazoxide was introduced to the ferricytochrome *c* solution (Trace b). Addition of ascorbate resulted in a distinguishable spectrum with Soret α and β bands at 550 and 520 nm , indicating that ferricytochrome *c* was reduced by ascorbate (Trace c). These results indicated that the redox status of ferricytochrome *c* was not affected by diazoxide.

The effect of diazoxide on SMP-mediated $O_2^{\cdot-}$ generation was evaluated with ferricytochrome *c* and the statistic results are shown in Fig. 7B. The rate of $O_2^{\cdot-}$ generation was $31.6 \pm 1.45 \text{ nM ferrocycytochrome } c/\text{min/mg protein}$ for control SMP and $30.4 \pm 1.74 \text{ nM ferrocycytochrome } c/\text{min/mg protein}$ in the presence of diazoxide ($P > 0.05$ vs. control, $n = 4$) when NADH was used. The rate of $O_2^{\cdot-}$ generation was significantly increased by diazoxide when succinate was used ($11.5 \pm 1.22 \text{ nM ferrocycytochrome } c/\text{min/mg protein}$ vs. $7.6 \pm 0.58 \text{ nM ferrocycytochrome } c/\text{min/mg protein}$ in control; $P < 0.05$, $n = 4$). In consistent with EPR spin trapping measurements, these results demonstrated that diazoxide-induced $O_2^{\cdot-}$ generation is controlled by succinate-dependent respiration. Since the effect of diazoxide on $O_2^{\cdot-}$ generation is substrate dependent, we hypothesize that diazoxide-induced $O_2^{\cdot-}$ generation is mediated through its interaction with complex II-supported respiration.

The Effect of Diazoxide on the Electron Transfer Activities in SCR

Our results with intact mitochondria showed that diazoxide inhibited succinate-supported respiration in a dose-dependent manner (data not shown), which is consistent with other reports [7-9, 11]. To understand how diazoxide affects the FADH₂-dependent respiration, we isolated SCR, a super complex hosting complex II (SQR) and complex III (QCR), from bovine hearts, and measured the electron transfer activities of succinate to cytochrome *c* for SCR, of Q₂-mediated DCPIP reduction for SQR, and of Q₂H₂ to cytochrome *c* for QCR respectively in the presence of diazoxide. The enzymatic activities in the absence of diazoxide were defined as 100%. As indicated in Fig. 8A, diazoxide inhibited the electron transport activity of SCR in a dose-dependent manner. Significant inhibition of electron transfer activity was detected in SCR (~80% inhibition at 200 μ M diazoxide) and SQR (~50% inhibition at 200 μ M diazoxide). The inhibitory effect of diazoxide was not detected in the QCR, suggesting that the effect of diazoxide on the activity of electron transfer from succinate to cytochrome *c* (by SCR) is due to the partial inhibition of electron transfer from succinate to ubiquinone (by SQR). Further, the more severe inhibition of diazoxide on the SCR than SQR is presumably due to the inhibitory effect on the interaction between SQR and QCR since the electron transfer activity from ubiquinol to cytochrome *c* in SCR was not affected by diazoxide (Fig. 8A). 5-HD did not show any effect on the electron transport activities of SCR, SQR, and QCR in the presence or absence of diazoxide (data not shown).

To further understand how diazoxide affects the interaction between SQR and QCR, UV-visible spectra of diazoxide-inhibited SCR following the addition of succinate (20 μ M) were obtained as in Fig. 8B. The obtained UV-visible absorption spectrum indicated a decrease in the ferricytochrome *b* reduction of SCR in the presence of diazoxide (100 μ M); suggesting diazoxide partially inhibited the electron transfer from SQR to QCR through reaction with the heme *b* of SCR.

Discussion

Diazoxide opens the mitoK_{ATP} channels potently and mimics the preconditioning effect pharmacologically in the protection of ischemic heart [1, 2, 10]. Recently we have demonstrated that IPC suppresses the post-ischemic myocardial hyperoxygenation after ischemia reperfusion and protects the post-ischemic heart by preserving tissue oxygen consumption during reperfusion, a critical time window when ATP is needed [3, 18, 22]. However, there are no prior studies of the role of diazoxide on the post-ischemic myocardial oxygenation.

In the current study, using an *in vivo* EPR oximetry technique, we determined that diazoxide, given 10 min before ischemia, totally suppressed the post-ischemic myocardial tissue hyperoxygenation, which mimicked the effect of IPC. Interestingly, 5-HD only partially abolished the suppression, which implies that opening of the mitoK_{ATP} channels by diazoxide or IPC only contributed partially to the total suppression of post-ischemic myocardial hyperoxygenation. These *in vivo* observations led us to speculate that other mechanisms must also have been involved in the total suppression effect of diazoxide or IPC in addition to the opening of the mitoK_{ATP} channels. Given the literature context that diazoxide has had a number of mitoK_{ATP}-independent effects [9, 11], as well as the limitations with *in vivo* experiments in detecting free radicals, we used isolated mitochondria as our model system in order to dissect these additional mechanisms involved in the total suppression of the *in vivo* myocardial hyperoxygenation. Since diazoxide was present during ischemia in the ischemic heart for 30 min in the *in vivo* experiments, to be consistent, we prepared our isolated mitochondria in the presence of antimycin A which inhibits mitochondrial respiration on complex III [26].

Since EPR spectroscopy is a powerful technique that can measure free radicals directly and specifically as it detects the presence of unpaired electrons [42], in the isolated mitochondria, we measured generation of $O_2^{\bullet-}$ in antimycin A-supplemented mitochondria, using an EPR spin trapping technique with DMPO as the spin trap. The detected EPR signal was quenched by Cu, Zn-SOD indicating that only $O_2^{\bullet-}$ escaping from the intact mitochondria but not the total ROS production was detected. As none of the probes react with all of the ROS [43], the measurement of released $O_2^{\bullet-}$ should provide important information regarding ROS generation in the intact mitochondria.

The effect of diazoxide on $O_2^{\bullet-}$ generation in normal mitochondria has been extensively studied [11, 12, 24, 25, 40]. However, there is no prior EPR spin-trapping measurement of diazoxide-induced $O_2^{\bullet-}$ generation in inhibited mitochondria which mimics the *in vivo* ischemic condition. With EPR, we determined that diazoxide induced ubisemiquinone-dependent $O_2^{\bullet-}$ generation from antimycin A-supplemented mitochondria and SMP. Our results indicated that diazoxide increased $O_2^{\bullet-}$ release from mitochondria when respiring on the substrate for complex II. Neither 5-HD nor glibenclamide abrogated this effect. In addition, $O_2^{\bullet-}$ generation was not altered by diazoxide when mitochondria were respiring on the substrate for complex I. Based on these pharmacological interventions, we concluded that in the presence of antimycin, diazoxide-induced ubisemiquinone-dependent $O_2^{\bullet-}$ generation is $mitoK_{ATP}$ -independent. While it is difficult to quantitatively correlate the *in vitro* and *in vivo* experiments, it is reasonable to speculate that the modest increase of $O_2^{\bullet-}$ may contribute to the additional effect of diazoxide on the total suppression of the post-ischemic myocardial hyperoxygenation in addition to the opening of the $mitoK_{ATP}$ channels.

However, all the pharmacological agents have non-specific effect on mitochondria. For example, 5-HD was rapidly converted to 5-HD-CoA in mitochondria and acted as a weak substrate or inhibitor of respiration depending on the conditions [10]. Glibenclamide was found to interfere with mitochondrial bioenergetics by inducing changes on membrane ion permeability [44] and to affect mitochondrial respiration [45]. In order to confirm that diazoxide-induced ubisemiquinone-dependent $O_2^{\bullet-}$ generation is independent of the $mitoK_{ATP}$ channels, we measured $O_2^{\bullet-}$ generation in K^+ - or ATP-free medium. We observed diazoxide-induced ubisemiquinone-dependent $O_2^{\bullet-}$ generation even without K^+ or K^+ flux and this effect was dependent on the substrate used. Furthermore, the diazoxide-induced $O_2^{\bullet-}$ generation can be observed in SMP preparations. These results clearly demonstrated that enhancement of ubisemiquinone-dependent $O_2^{\bullet-}$ by diazoxide may contribute additionally to $O_2^{\bullet-}$ signaling which is important in pharmacologically preconditioning.

Diazoxide is also a weak protonophoric uncoupler of mitochondria [11, 38, 39]. Uncoupling of mitochondria decreases ROS, but uncoupling of inhibited mitochondria enhances ROS generation [46, 47]. We evaluated the effect of FCCP, a mitochondrial uncoupler, on $O_2^{\bullet-}$ generation in mitochondria in the presence of antimycin A and found that $O_2^{\bullet-}$ generation was increased with substrates for both complex I and complex II. In contrast, diazoxide, at the concentration of 100 μ M, only enhanced $O_2^{\bullet-}$ generation in the presence of substrate for complex II. These results indicated that enhancement of $O_2^{\bullet-}$ generation by diazoxide is not due to its uncoupling effect on mitochondria.

It was reported that diazoxide inhibited succinate oxidation [7], which has been confirmed by recent studies [8, 9, 11]. However, the underlying mechanisms are not completely understood. Our results are consistent with those reports that diazoxide inhibited succinate-supported respiration. By measuring the electron transfer activities of SCR, SQR and QCR, we demonstrated that diazoxide impaired the interaction between complex II (SQR) and complex III (QCR) through modest inhibition on complex II activity. As revealed by UV-

visible spectral analysis, diazoxide partially inhibited succinate-mediated cytochrome *b* reduction in the SCR. The detected inhibition of ferricytochrome *b* reduction is involved in the cytochrome *b*₅₆₀ of complex II since diazoxide has no effect on the electron transfer activity of complex III. The cytochrome *b*₅₆₀ has been reported to be part of ubiquinone-binding sites in the SQR, thus it may play an important role in the protein-protein interaction between SQR and QCR. It is known that ubiquinone reduction by SQR during enzyme turnover is mediated by ubiquinone-binding site. It is likely that the interaction of diazoxide with cytochrome *b*₅₆₀ results in incomplete reduction of ubiquinone. In the intact mitochondria, antimycin A increases the steady-state levels of QO^{•-} in complex III resulting in prominent O₂^{•-} production which can be detected by EPR spin trapping. With succinate as the substrate, diazoxide impairs protein-protein interaction between complex II and complex III, which may potentially increase the electron leakage for O₂^{•-} production by further increasing the steady-state concentration of Q₂^{•-} in complex III under the conditions of enzyme turnover of SCR. Therefore, higher level of O₂^{•-} production is detected in the presence of diazoxide.

The activity of complex III is partially inhibited during cardiac ischemia [48, 49]. It has been found that O₂^{•-} is generated in cardiomyocytes during ischemia mainly from the ubisemiquinone site of complex III [50]. Our *in vitro* data suggested an additional mechanism of diazoxide on the protection of the ischemic heart in suppressing the post-ischemic myocardial hyperoxygenation.

In summary, by using EPR oximetry and spin trapping techniques, we have demonstrated that diazoxide totally suppressed the *in vivo* post-ischemic myocardial tissue hyperoxygenation that mimics the IPC in the protection of the ischemic heart. The fact that 5-HD only partially abolished this effect indicates that in addition to the opening of the mitoK_{ATP} channels, there may be other mechanisms involved in the process. Our *in vitro* data showed that diazoxide also increased succinate-supported ubisemiquinone-dependent O₂^{•-} generation in antimycin A-supplemented mitochondria, which may provide an additional mechanism independent of the opening of the mitoK_{ATP} channels. Our results also demonstrated that diazoxide-induced O₂^{•-} generation is substrate dependent and the impairment of the electron transfer activity and interaction between complex II and complex III by diazoxide results in the modest increase of the ubisemiquinone-dependent O₂^{•-} formation.

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Abbreviations

DCPIP	dichlorophenolindophenol
DEPMPO	5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline <i>N</i> -Oxide
DMPO	5,5-Dimethyl-1-pyrroline <i>N</i> -oxide
DTPA	diethylenetriaminepentaacetic acid
EPR	electron paramagnetic resonance
FCCP	carbonyl cyanide- <i>p</i> -tri-fluoromethoxyphenyl-hydrazone
5-HD	5-hydroxydecanoate

H₂DCF	2',7'-dichlorodihydrofluorescein
IPC	ischemic preconditioning
mitoK_{ATP}	mitochondrial ATP-sensitive potassium channel
3-NPA	3-nitropropionic acid
O₂^{-•}	superoxide
•OH	hydroxyl radical
Po₂	tissue oxygen tension
PPC	pharmacological preconditioning
Q	ubiquinone
Q^{-•}	ubisemiquinone
Q₂	ubiquinone-2
QCR	ubiquinol cytochrome c reductase or complex III
QH₂	ubiquinol
QO^{-•}	ubisemiquinone at the Q _O site
ROS	reactive oxygen species
SCR	succinate-cytochrome c reductase or super complex containing complex II and complex III
SMP	sub-mitochondrial particles
SOD	Superoxide dismutase
SQR	succinate ubiquinone reductase or complex II
TEA⁺	tetraethyl ammonium

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Fig. 1.

In vivo measurement of myocardial tissue Po_2 with EPR oximetry. Mice were subjected to 30 min LAD occlusion followed by 60 min reperfusion (I/R) or 3 cycles of 5 min LAD occlusion followed by 5 min reperfusion (the last reperfusion was prolonged to 15 min), and 30 min ischemia and 60 min reperfusion (IPC + I/R). A, tissue Po_2 in the I/R as well as diazoxide-treated mouse hearts (Diazoxide + I/R, bolus i.v. injection at a dose of 6 mg/kg 10 min before ischemia); B, tissue Po_2 in the IPC+I/R as well as 5-HD-treated mouse hearts (5-HD + IPC + I/R, bolus i.v. injection at a dose of 10 mg/kg 10 min before the ischemic stimuli). It was observed that diazoxide and IPC totally suppressed the post-ischemic myocardial hyperoxygenation, but 5-HD only partially abolished the suppression. *p = 0.001, Diazoxide + I/R vs. I/R; +p = 0.001, IPC + I/R vs. I/R; **p = 0.006, 5-HD + IPC + I/R vs. IPC + I/R; and ++p = 0.011, 5-HD + IPC + I/R vs. I/R. There is no significant difference between IPC + I/R and Diazoxide + I/R groups. n = 7.

Fig. 2.

Representative EPR spectra of $O_2^{\bullet-}$ formation in mitochondria. EPR spectra were recorded with an X-band spectrometer and were averaged over 25 scans. Other details are described in the Materials and Methods. a: no treatment; b: a plus 5 mM glutamate (Glu)/5 mM malate (Mal); c: b plus 1 μ g/mg protein antimycin A; d: c plus 400 units/ml SOD; e: a plus 10 mM succinate (Suc) and 2 μ M rotenone; f: e plus 1 μ g/mg protein antimycin A; g: f plus 400 units/ml SOD. These results clearly demonstrated that inhibition of mitochondrial respiration by antimycin A induced $O_2^{\bullet-}$ formation.

Fig. 3.

Diazoxide (DZX)-induced $O_2^{\bullet-}$ generation in intact mitochondria with succinate as the substrate. A: representative EPR spectra obtained with 5 mM glutamate/5 mM malate as the substrate (a-g) and with 10 mM succinate as the substrate (h-n). The spectra were recorded in the presence of vehicle (a, h), diazoxide (b, i), diazoxide plus 5-HD (c, j), diazoxide plus glibenclamide (Glib; d, k), 5-HD (e, l), glibenclamide (f, m) and FCCP (g, n). The

concentrations of diazoxide, 5-HD glibenclamide, and FCCP were 100, 500, 10 and 1 μ M respectively. Antimycin A was added at the concentration of 1 μ g/mg protein in all the measurements. EPR experimental conditions were the same as in Fig. 2 except that the number of scans was changed to 10. B: statistic analysis of the EPR signal intensity. The relative intensity of the control spectra obtained with the corresponding substrate was defined as 100% and the increase of $O_2^{\cdot-}$ generation comparing with the control is shown in the figure. a: with glutamate/malate as the substrate, the $O_2^{\cdot-}$ generation was not altered by diazoxide ($1.7 \pm 4.58\%$ vs. 0% in control; n=6), diazoxide plus 5-HD ($-3.97 \pm 4.25\%$; n=6), diazoxide plus glibenclamide (-1.5 ± 1.21 ; n=3), 5-HD ($-3.55 \pm 2.91\%$; n=6) or glibenclamide ($-0.17 \pm 0.29\%$; n=3). b: with succinate as the substrate, $O_2^{\cdot-}$ generation was significantly increased by diazoxide ($23.2 \pm 2.27\%$ vs. 0% in control. n=6), while this effect was not blocked by either 5-HD ($23.7 \pm 2.20\%$; n=6) or glibenclamide ($22.7 \pm 1.30\%$; n=3). Neither 5-HD ($0.9 \pm 1.10\%$; n=6) nor glibenclamide ($0.4 \pm 2.95\%$; n=3) alone has any effect on $O_2^{\cdot-}$ generation. FCCP induced a significant increase of $O_2^{\cdot-}$ generation despite the substrate used ($29.4 \pm 5.92\%$ vs. 0% in control with glutamate/malate as the substrate and $25.0 \pm 3.99\%$ vs. 0% in control with succinate as the substrate; n=4). These results demonstrated that diazoxide enhanced $O_2^{\cdot-}$ generation in antimycin A-supplemented mitochondria in a mitoK_{ATP} channel-independent manner. * $P < 0.05$ vs. control with the corresponding substrate.

Fig. 4.

Dose-dependent effect of diazoxide on mitochondrial $O_2^{\bullet-}$ generation with succinate as the substrate. Diazoxide was added at the concentration ranging from 10 to 300 μ M. The relative intensity of the control spectra obtained with 0 μ M of diazoxide was defined as 100%. Other conditions are the same as in Fig. 3. Simulated curve is shown in solid line. The EC_{50} of diazoxide to induce $O_2^{\bullet-}$ generation was 45 μ M. These data showed that diazoxide increased $O_2^{\bullet-}$ generation in antimycin A-supplemented mitochondria in a dose-dependent manner. * $P < 0.05$; $n = 5$.

Fig. 5.

Effect of diazoxide on $O_2^{\cdot-}$ generation in intact mitochondria in ATP- and K^+ -free media with succinate as the substrate. The relative intensity of the control spectra obtained in ATP-free medium in the absence of diazoxide was defined as 100%. Other conditions are the same as in Fig. 3. $O_2^{\cdot-}$ generation was increased by diazoxide ($141.3 \pm 1.65\%$ vs. 100% in control) in ATP-free medium, and diazoxide also induced increase of $O_2^{\cdot-}$ generation in K^+ -free medium ($190.0 \pm 2.44\%$ vs. $164.0 \pm 8.57\%$ in control). These results indicated that diazoxide induced $O_2^{\cdot-}$ generation in antimycin A-supplemented mitochondria when the $mitoK_{ATP}$ channels were already opened and when there was no K^+ in the medium. * $P < 0.05$, DZX vs. control in ATP-free medium, and # $P < 0.05$, DZX vs. control in K^+ -free medium; $n = 4$.

Fig. 6.

Effect of diazoxide on $O_2^{\cdot-}$ generation in SMP. The reaction medium contained 0.5 mg protein/ml SMP with either 1 mM NADH or 10 mM succinate as the substrate. Rotenone (2 μ M) was added when using succinate as the substrate. The relative intensity of the control spectra obtained with succinate as the substrate was defined as 100%. The EPR settings were the same as in Fig. 3. With NADH as the substrate, $O_2^{\cdot-}$ generation was not changed by diazoxide (131.2 $\pm$ 4.63% vs. 135.9 $\pm$ 1.65% in control) but with succinate as the substrate, $O_2^{\cdot-}$ generation was significantly increased to 120.2 $\pm$ 2.99% of control. * P < 0.05; n = 4.

Fig. 7.

The effect of diazoxide on $O_2^{\cdot-}$ generation in SMP measured by cytochrome *c* reduction. A: UV-visible absorbance spectrum of cytochrome *c* in 50 mM phosphate buffer (pH 7.4) in the wavelength range of 400-800 nm. Trace a: 50 μ M ferricytochrome *c* (Ferricyt *c*); Trace b: 50 μ M ferricytochrome *c* plus 100 μ M diazoxide; Trace c: 50 μ M ferricytochrome *c* plus 100 μ M ascorbate. Diazoxide did not change the redox status of ferricytochrome *c*. B: the effect of diazoxide on $O_2^{\cdot-}$ generation in SMP measured with ferrocyanochrome *c*. Experimental details are described in the section of Materials and Methods. The $O_2^{\cdot-}$ generation rate was 31.6 ± 1.45 nM ferrocyanochrome *c*/min/mg protein in control and 30.4 ± 1.74 nM ferrocyanochrome *c*/min/mg protein in the presence of 100 μ M diazoxide with NADH as the substrate. The rate of $O_2^{\cdot-}$ generation was significantly increased by diazoxide with succinate as the substrate (11.5 ± 1.22 nM ferrocyanochrome *c*/min/mg protein vs. 7.6 ± 0.58 nM ferrocyanochrome *c*/min/mg protein in control). These results demonstrated that diazoxide induced $O_2^{\cdot-}$ generation in antimycin A-supplemented SMP when succinate was used as the substrate. * $P < 0.05$; $n = 4$.

Fig. 8.

The effect of diazoxide on electron transfer activities in the super complex of SCR. A: the effect of diazoxide on electron transfer activities of SCR, SQR, and QCR. See Material and Methods for experimental details. Electron transport activities of both SCR (●) and SQR (○) were decreased by diazoxide in a dose-dependent manner with SCR activity inhibited to a greater extent. Diazoxide did not have any effect on QCR (▼) activity. Symbols represent the means of at least 2 independent experiments, and the SE is less than 5%. These results indicated that diazoxide impaired the interaction between complex II and complex III with modest inhibition on complex II and no effect on complex III. B: the effect of diazoxide on heme *b* reduction in SCR. The reaction mixture contained 4 μM SCR, 0.05% (w/v) lauryl maltoside and 20 μM succinate in PBS. Visible absorption spectra were recorded from 500–600 nm in the presence of 100 μM diazoxide (solid line) or vehicle (control, dotted line) repeatedly at intervals of 60 s for 5 min after the addition of succinate. For control, only the spectrum obtained from the 5th scan is shown in the figure. These results showed that diazoxide partially inhibited heme *b* reduction in SCR.