Diazoxide attenuates ROS generation and exerts cytoprotection under conditions of ROS overproduction in rat uterus cells

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Abstract: Reactive oxygen species (ROS) overproduction may severely affect cell functions and even provoke cell death. Diazoxide, an opener of ATP-sensitive potassium channels, induces pharmacological preconditioning in different types of cells. However, the target of action of diazoxide is not clear enough because this substance can activate ATP-sensitive potassium channels localized in mitochondria (mitoK<sub>ATP</sub>) as well as in plasma membranes (K<sub>ATP</sub> channels). It has not yet been established if diazoxide activates mitoK<sub>ATP</sub> in uterus cells. Our objective was to examine the influence of diazoxide on ROS production and on the viability of rat uterus cells under oxidative stress conditions. Using an ROS-sensitive fluorescent probe, we found that diazoxide enhances ROS production under normal conditions, but attenuates this process under conditions of ROS overproduction. Cells pretreated with diazoxide demonstrated lower levels of ROS production and of cell death under oxidative stress, in comparison with conditions where diazoxide was not present. The effects of diazoxide were eliminated by 5-hydroxydecanoate (5-HD), a blocker of mitoK<sub>ATP</sub>. The principal conclusion of the present study is that decreased ROS production and increased cell survival in the presence of diazoxide under oxidative stress conditions are mediated by the activation of mitoK<sub>ATP</sub> in rat uterus cells.

Key words: Diazoxide, 5-hydroxydecanoate, mitoK<sub>ATP</sub>, reactive oxygen species, uterus

1. Introduction

Under normal physiological conditions the balance between reactive oxygen species (ROS) generation and ROS scavenging is highly controlled. During oxidative stress, however, production of ROS is strongly increased and the cellular systems of antioxidant defense are not able to eliminate them (Tsutsui et al., 2009; Al-Gubory et al., 2010; Javadov et al., 2013). Excessive accumulation of ROS causes cellular dysfunction, facilitates lipid peroxidation and DNA damage, and can lead to irreversible cell damage and death. Oxidative stress is particularly destructive for muscle cells because excessive ROS can directly impair contractile function by modifying proteins participating in excitation-contraction coupling, which provides ground for the onset of various pathological conditions in muscle tissues (Al-Gubory et al., 2010). Thus, investigating possible mechanisms that help muscle cells withstand negative consequences of oxidative stress is an important field of study.

ROS may not only destroy the cell, but may also regulate cell functioning and survival. Their role as signaling agents is a subject of increasing scientific interest. ROS can serve as signaling molecules and trigger signaling pathways involved in cell protection (Gibson, 2013). Over the past decade there is a growing body of evidence that confirms the participation of ROS in the phenomenon of preconditioning in muscle cells (Yellon et al., 2003; González et al., 2010; Garlid et al., 2013). This phenomenon was described for the first time by Murry et al., who demonstrated that the heart muscle could be protected from severe ischemic injury by pre-treatments with short-time ischemic periods (Murry et al., 2006). It was subsequently found that some pharmacological agents could mimic this phenomenon, which became known as pharmacological preconditioning (Loubani, 2004; González et al., 2010). Despite the growing amount of data, our knowledge of this subject is still limited. Loubani et al. showed cytoprotection in human myocardium by protein kinase C (PKC) activator, phorbol myristate acetate, and diazoxide (Loubani et al., 2004). There is also enough evidence that the K<sub>ATP</sub> channel could be another participant of preconditioning. Diazoxide, a known opener of K<sub>ATP</sub> channels, diminishes necrotic damage to the heart tissue under ischemic conditions and facilitates survival of cardiac cells (González et al., 2010). Nevertheless, it is still uncertain whether the plasma membrane K<sub>ATP</sub> channels or the mitoK<sub>ATP</sub> play a
dominant role in preconditioning (Suzuki, 2002; González et al., 2010). Interest in the cytoprotective role of mitoK<sub>ATP</sub> developed following the publication of Garlid’s data (1997). Due to the evidence presented there (Garlid et al., 1997), it became obvious that diazoxide exerts cardioprotection without influencing the action potential duration on the plasma membrane of heart cells, and therefore the target of pharmacological preconditioning of cardiomyocytes by diazoxide should be mitoK<sub>ATP</sub> and not plasma membrane K<sub>ATP</sub> channels. Furthermore, González et al. showed that the protective effect of diazoxide and other openers of K<sub>ATP</sub> channels was eliminated by the mitoK<sub>ATP</sub> selective blocker 5-HD (González et al., 2010). Andrukhiv et al. directly showed an increase of ROS production in isolated heart mitochondria following the activation of mitoK<sub>ATP</sub> (Andrukhiv et al., 2006). They postulated that ROS mediate the cytoprotective effect exerted by diazoxide (González et al., 2010). Andrukhiv et al. assumed that ROS mediate the cytoprotective effect of PKC, which in turn phosphorylates mitoK<sub>ATP</sub> and thus turns it into a permanently opened state (Murry et al., 2001). Besides the enhancement of ROS production, the opening of the mitoK<sub>ATP</sub> by pharmacological or physiological activators results in moderate mitochondrial matrix swelling, which leads to enhancement of respiration and ATP synthesis (Hanley et al., 2005). This may totally improve mitochondrial functioning. Activation of the mitoK<sub>ATP</sub> has beneficial consequences on mitochondrial physiology under both normal and stress conditions. Ozcan et al. provided direct evidence that diazoxide maintains the functional and structural integrity of isolated cardiac mitochondria exposed to anoxia/reoxygenation (Ozcan et al., 2001).

Taking into consideration the harmful consequences of oxidative stress on smooth muscles, particularly uterine smooth muscle (Al-Gubory et al., 2010), our objective was to examine the influence of the opener of K<sub>ATP</sub> channels diazoxide on ROS production and on the viability of uterus cells under oxidative stress conditions. To this end we used fluorescence spectroscopy with an ROS-sensitive fluorescent probe to detect changes in ROS production. We also used propidium iodide (PI) staining to examine the cytoprotective effect of diazoxide in rat uterus cells. Using appropriate substances, such as rotenone, diazoxide, 5-HD, cyclosporine A (CsA), we examined the impact of oxidative stress conditions on the functioning of rat uterus cells and tried to answer the main question of the present study: whether activation of mitoK<sub>ATP</sub> channels is involved in pharmacological preconditioning in rat uterus cells.
2.4. Measurements of ROS generation

Measurements were conducted using the fluorescence technique on a spectrofluorometer (QuantaMaster 40, PTI, Canada), with an ROS-sensitive fluorescent probe, 2,7-dichlorodihydrofluorescein-diacetate (DCFH-DA), in a thermostated cuvette with a magnetic stirrer at 37 °C. The detection of excitation and emission of fluorescence was set at wavelengths of 500 nm and 525 nm, respectively. A nonfluorescent and membrane permeable form of DCFH-DA easily penetrates membranes. Once inside the cytoplasm, the acetate group of DCFH-DA is cleaved by esterase, yielding a nonfluorescent product 2,7-dichlorofluorescein (DCFH) that accumulates inside the cell. Oxidation by ROS yields the fluorescent product 2,7-dichlorofluorescein (DCF) (Gomes et al., 2005). Small aliquots of freshly prepared stock solution of DCFH-DA were added to the sample solutions right before measurements to a final concentration of 5 μM. This small concentration of DCFH-DA was chosen in order to avoid self-quenching of the fluorescent probe. Registration of a fluorescence signal started immediately following the addition of the DCFH-DA solution.

2.4.1. Measurements of ROS generation under normal conditions

Aliquots of cells suspension were added to Hank's solution to the final amount of 5 × 10^5 of cells per milliliter. Diazoxide (50 μM), 5-HD (200 μM), glybenclamide (1 μM), and CsA (10 μM) were added just before the addition of DCFH-DA (5 μM). Measurements were conducted under continuous stirring at 37 °C.

2.4.2. Measurements of ROS generation following incubation with rotenone

Samples containing 5 × 10^5 of cells per milliliter were treated for 2.5 h as indicated (see section 2.3). At the end of incubation, each sample solution was placed into a glass cuvette, an aliquot of DCFH-DA solution was added and fluorescence measurements immediately started. The final concentration of DCFH-DA was 5 μM. Measurements were conducted under continuous stirring at 37 °C.

2.5. Cytofluorimetric measurements

Measurements were conducted on flow cytometer (Backman Coulter Epics XL, USA) equipped with an argon laser (excitation wavelength of 488 nm), using an emission filter at 620 nm. Forward and side scatter were gated on the major population of normal-sized myocytes. Cell viability was determined with PI staining. PI is an intercalating agent that can penetrate only through the permeable plasma membrane and binds to nucleic acids. The fluorescence intensity of PI increases by an order of 30 after its binding to DNA; therefore there is no need to wash cells from unbound dye. This dye is commonly used for identifying dead cells in a population (Krysko et al., 2008). In order to confirm the credibility of this method for detection of cell death, we added 150 μM digitonin to a sample with intact cells to cause total permeabilization of the plasma membrane and observed more than 95% of PI positive cells.

Samples were treated as indicated in section 2.3, and then were incubated with PI (2 μM) in the dark at room temperature for 5 min and analyzed.

2.6. Statistical analysis

Analysis of the results and graph generation were conducted with Microcal Origin, version 5.0 (Microcal Software Inc., USA). Data presented in columns were analyzed using a one-way ANOVA test; the differences were statistically significant at the 0.05 level; n = 5 for each group. Data presented in graphs and histograms were repeated in at least four independent experiments.

2.7. Drugs and chemicals

HEPES, KCl, CaCl₂ (1 M solution), rotenone, cyclosporine A, diazoxide, glybenclamide, 5-HD, 2,7-DCFH-DA, propidium iodide, collagenase, bovine serum albumin, and soybean trypsin inhibitor were provided by Sigma-Aldrich (USA). Tris, EDTA, and NaCl were provided by Fluka (Switzerland). The remaining chemicals were provided by domestic manufacturers (Ukraine).

3. Results and discussion

Using an ROS-sensitive fluorescent probe DCF, we have shown that isolated rat uterus cells demonstrate moderate ROS generation under normal conditions (Figure 1). The evidence that the mitochondrial permeability transition pore (MPTP) blocker CsA (10 μM) has no effect on this process is the absence of mitochondrial damage and MPTP induction under normal conditions (Figure 1). It is well known that ROS are the normal by-products of electron-transporting chains in mitochondria and may

![Figure 1](https://example.com/image1.png)
not only deteriorate cell functioning under oxidative stress, but may also serve as cell messengers. The mode of their action strongly depends on the intensity of their production (Tsutsui et al., 2009). Under normal conditions slight increases in ROS production may regulate smooth muscle tone and improve many physiological processes. It was shown that ROS production can be modulated by transporting K ions inside the cell (Andrukhiv et al., 2006). Thus, it was interesting to establish whether the activation of K\textsuperscript{+} channels in uterus cells may change the generation of ROS. The addition of diazoxide (50 µM), a known opener of K\textsuperscript{+} channels, to the incubation medium with freshly isolated myocytes caused a mild increase in ROS production (Figure 1). It has been shown on different cell types that diazoxide may activate K\textsuperscript{+} channels in the plasma membrane and in the inner mitochondria membrane as well (Pi et al., 2007; Hibino et al., 2010). The existence of K\textsuperscript{ATP} channels in the plasma membrane of uterus myocytes is well established (Xu et al., 2011). However, the functioning of mitoK\textsuperscript{ATP} channels in the uterus had not been definitely demonstrated. We used the selective mitoK\textsuperscript{ATP} channel blocker 5-HD to distinguish between the activation of plasmalemmal and mitochondrial K\textsuperscript{ATP} channels. It was shown that 5-HD effectively blocks the mitochondrial form of K\textsuperscript{ATP} channels and does not affect the plasma membrane form of the channel (Liu et al., 2001). Thus, the effect of 5-HD may serve as proof of the involvement of mitoK\textsuperscript{ATP} channels. In our experiments 5-HD (200 µM) fully prevented an increase in ROS production by diazoxide and the same results were obtained with another blocker of K\textsuperscript{ATP} channels (glybenclamide, 1 µM, data not shown). Therefore, we may assume that modulation of ROS production by diazoxide and 5-HD in rat uterus myocytes under normal conditions is due to the activation and deactivation of K\textsuperscript{ATP} channels in mitochondria and not in the plasma membrane. Our results correlate with those reported by Andrukhiv et al. (2006), who demonstrated that the activation of K\textsuperscript{+} current in the mitochondrial membrane by diazoxide increases ROS production in cardiomyocytes.

Oxidative stress, mediated by massive ROS generation in the cell, may deteriorate cell functioning and cause cell death via necrosis or apoptosis (Tsutsui et al., 2009; Wu et al., 2014). Thus, the search for a mechanism that would help prevent or diminish the effect of oxidative stress in cells is an important field of study. As a model of oxidative stress we used the inhibition of electron-transporting chains with rotenone because it has been shown that inhibition of the first complex of an electron-transporting chain results in oxidative stress that is mediated by massive ROS generation (Li et al., 2003). In our experiments rotenone (an inhibitor of the first complex of an electron-transporting chain) increased ROS generation. We showed that incubation of uterus cells with rotenone (50 µM) for 2 h resulted in a substantial increase in ROS generation compared with the controls (as measured by the fluorescence technique using fluorescence probe DCF) (Figure 2). CsA (10 µM) added before the addition of rotenone mainly suppressed the effect of rotenone (Figure 2). Therefore, we assume that the prevailing of oxidative processes in the presence of rotenone may mean that a prolonged inhibition of the first complex of an electron-transporting chain may cause damage to mitochondria and the activation of the CsA-sensitive MPTP.

An opener of K\textsuperscript{ATP} channels, diazoxide has beneficial effects on the mitochondrial physiology in cardiomyocytes, which is mediated by the modulation of ROS production (Garlid et al., 2003). However, it is not well established whether this substance can influence ROS production under stress conditions in smooth muscle cells, and uterus cells in particular. Thus, research on the influence of diazoxide on ROS production under stress conditions in rat uterus cells is one of practical importance. It appeared that preincubation of the cells suspension with diazoxide (50 µM) for 30 min before the addition of rotenone resulted in lowering of ROS production as compared with the parallel probe incubated under the same conditions, but in vehicle (DMSO) instead of diazoxide (Figure 3). On the other hand, when diazoxide was added along with 5-HD, the protective effect of diazoxide on ROS production was eliminated (Figure 3). The same results were obtained with another K\textsuperscript{ATP} channels blocker (glybenclamide) (data not shown).

Our data demonstrate that a drastic increase of ROS generation in the presence of rotenone results from
Figure 3. Preliminary incubation of rat uterus cells with diazoxide 50 µM (RoDz) attenuated ROS generation induced by rotenone 50 µM as compared with the conditions where diazoxide was substituted by an equal aliquot of vehicle (Rotenone); the protective effect of diazoxide was abolished when 5-HD 200 µM was added along with diazoxide (RoDzHD); in the control conditions (Cont), samples were incubated in vehicle instead of rotenone (see section 2.4 for details). The relative fluorescence of DCF is plotted against time (M ± m, n = 5).

mitochondrial damage following MPTP activation, because MPTP inhibitor CsA fully reversed this effect. Similar effects of CsA and diazoxide in our experiments gave us grounds to think that the mode of protective action of diazoxide may involve the inhibition of MPTP as well. This protection may be mediated by ROS because the activation of mitoK\textsubscript{ATP}-channels by diazoxide slightly increased ROS production under normal conditions in our experiments. The protective effect of diazoxide was demonstrated also by Wu et al. (2006), who showed that diazoxide blocked hypoxic cell injury by preventing the induction of MPTP opening. Ozcan et al. (2001) also showed that the activation of mitoK\textsuperscript{+}-channels by diazoxide helps maintain normal mitochondrial morphology and physiology during anoxia/reoxygenation, and preserves the oxidative phosphorylation and ATP production in anoxic mitochondria, while the mitochondria of cells that underwent oxidative stress tend to lose the normal morphology (Wilson et al., 2005). Thus, if MPTP, in response to oxidants, may cause mitochondrial volume deregulation, rupture of the outer mitochondrial membrane, and release of cytochrome c, mitoK\textsubscript{ATP}-channels activation, unlike MPTP, has beneficial effects on mitochondrial bioenergetics. The opening of mitoK\textsuperscript{+}-channels slightly decreases ΔΨm, stimulates respiration, and reduces mitochondrial Ca\textsuperscript{2+} uptake (Murata et al., 2001; Costa et al., 2006; Javadov et al., 2013). Therefore, improving the mitochondrial physiology by the activation of mitoK\textsuperscript{ATP}-channels may prevent MPTP opening. Thus, we assume that a decrease in ROS production in the presence of diazoxide may be a result of prevention of MPTP induction following the activation of mitoK\textsubscript{ATP}-channels in rat uterus cells.

We also admit the possibility of involvement of some protein kinases, the activation of which might improve cell survival under conditions of oxidative stress. It has been shown that mitoK\textsubscript{ATP} acts in cooperation with PKC to help cells to overcome deteriorating consequences of stress conditions (Garlid et al., 2013). Lebuffe et al. (2003) showed that the activation of PKC conferred cell survival. Townsend et al. (2007) assumed the possibility that activation of PKC prevents cell death by reducing the burst of ROS. A slight enhancement of ROS production following the activation of mitoK\textsuperscript{+}-channels may evoke ROS-dependent activation of PKC, which inhibits MPTP and thus improves cell survival (Costa et al., 2008). Such mechanisms of involvement of mitoK\textsuperscript{ATP}-channels in cardioprotection have been described by Garlid et al. (2003). The authors speculate that the opening of mitoK\textsuperscript{+}-channels results in matrix alkalinization and the subsequent generation of moderate ROS levels, which may trigger the activation of multisignaling pathways responsible for cardioprotection (Garlid et al., 2003). This hypothesis is yet to be confirmed in our experimental model on smooth muscle uterus cells.

It has been shown that prolonged unfavorable conditions, such as inhibition of electron-transporting chains and massive ROS generation, may impair cell survival (Li et al., 2003). That is why it was important to study whether a sustained incubation of rat uterus cells with rotenone would impair their viability. Two possible ways of cell death are apoptosis or necrosis (oncosis). Both processes may occur simultaneously in a cell population, although they have different time scales. It is well known that apoptosis involves the activation of caspases, the rearrangement of plasma membrane lipids, cell blebbing, and finally fragmentation of the nucleus. It is a sustained process, the very first signs of which are hardly detectable (Krysko et al., 2008). Unlike apoptosis, necrotic cell death may occur in a few hours and its detectable features, such as cell swelling and plasma membrane permeability, can be easily visualized using PI cell staining and measuring of forward light scattering (FS) (Krysko et al., 2008). The latter parameter is inversely proportional to cell size, so that its decrease correlates with an increase of single cell size. This can be observed as the first indicator of necrotic (oncotic) cell death. Therefore, we used FS measurements by flow cytometry to detect changes in cell size following the inhibition of electron-transporting chains. Moreover, we measured the percentage of cells stained with PI. The membrane-impermeant dye PI is generally excluded from viable cells and thus the percentage of cells stained with PI correlates with the percentage of dead cells (Krysko et al., 2008).
As shown in Figure 4, immediately following the isolation procedure (see section 2.2 for details) the cells are mostly (more than 85%) unstained and thus have impermeable membranes. More than 70% of the cells before the treatment had both normal size and impermeable membranes (Figure 4, panel “intact”, the left

Figure 4. Propidium iodide staining (x axis) vs forward scatter (FS) of myometrium cells (y axis). Cells were treated as follows: Panel “control”—the sample was preincubated in vehicle (DMSO), followed by incubation with vehicle for 2 h. “Rotenone”—the sample was preincubated in vehicle, followed by incubation with 50 µM of rotenone for 2 h. “RoDz”—the sample was preincubated with 50 µM of diazoxide, followed by incubation with 50 µM of rotenone for 2 h (see section 2.3 for details). Immediately following treatment, probes were analyzed by flow cytometry. Panel “intact”—cells without any treatments were measured immediately following isolation (see section 2.5 for details). The upper left quadrant of each panel contains viable cells of a normal size and intact plasma membranes; the lower left quadrant contains swollen but still unstained, presumably dying cells. Both right quadrants contain dead cells stained with PI. Results of typical experiments are shown. * significantly different from control.
upper quadrant refers to unstained cells of a normal size; the left lower quadrant refers to swollen, presumably dying cells with still impermeable plasma membranes). For the control conditions, we incubated cells in vehicle (2.5 µL of DMSO) instead of rotenone for 2 h at 37 °C and detected a small decrease in the percentage of unstained cells of a normal size (Figure 4, panels “control” and “intact”, compare the upper left quadrants), and correspondingly a small increase in the percentage of PI positive cells compared with the intact cells (Figure 4, panels “control” and “intact”, both right quadrants). In the control samples incubated under control conditions, without rotenone, unstained cells of a normal size made up no less than 60% in each experiment. Incubation of myocytes suspension with rotenone (50 µM) for 2 h at 37 °C (see section 2.3 for details) resulted in a drastic lowering of the percentage of unstained cells of a normal size (Figure 4, panel “rotenone”; upper left quadrant). On the other hand, cell suspension preincubated for 30 min with 50 µM diazoxide at room temperature before incubation with rotenone had an increased percentage of unstained cells of a normal size in comparison with conditions without preincubation with diazoxide (Figure 4, panel “RoDz”, panel “rotenone”, upper left quadrants). Thus, our data allow us to suggest that swelling of rat uterus myocytes occurs as a result of rotenone inhibition of the mitochondrial electron-transporting chain, which may prove our assumption that under such unfavorable conditions cells may undergo necrosis (oncosis). The results in Figure 4 (panel “RoDz”) demonstrate that diazoxide (50 µM) moderately increased the amount of viable cells, preventing them from swelling (Figure 4, panel “RoDz”).

Further, we have shown that cell swelling following the addition of 50 µM rotenone was accompanied by an increase in the percentage of dead cells, which we calculated using cell staining with PI (Figure 4, upper and lower right quadrants on each panel). There was a moderate increase in the percentage of dead cells under the control conditions, where cells were incubated for 2 h in vehicle instead of in rotenone (Figure 4, panel “control” and “intact”, both right quadrants). The mean percentage of dead cells under control conditions was 24.3%, whereas following incubation with 50 µM rotenone the fraction of dead cells reached 59.6% (Figure 5). It is apparent that preincubation with 50 µM diazoxide for 30 min at room temperature has a moderate protective effect, which results in a smaller percentage of dead cells: 47.42% in comparison with conditions without diazoxide. The protective effect of the K⁺<sub>ATP</sub>-channels opener diazoxide on cell viability was blocked by 5-HD (Figure 5). When

![Figure 5. Diazoxide decreases rat uterus cell death under oxidative stress conditions. Presented are the averaged data of five separate experiments. Preliminary incubation with diazoxide 50 µM decreased the percentage of dead cells induced by rotenone 50 µM (RoDz) as compared with conditions where diazoxide was substituted for an equal aliquot of vehicle (Rotenone). The protective effect of preincubation of cells with diazoxide was abolished when 5-HD 200 µM was present along with diazoxide (RoDzHD); in the control conditions (control), samples were incubated in vehicle instead of rotenone (see section 2.3 for details). n = 5 for each group; at the 0.05 level, the means are significantly different. #, significantly different from control; &, significantly different from Rotenone; *, significantly different from RotDz.](image-url)

5-HD (200 µM) was added along with 50 µM diazoxide, no protection was observed.

Thus, we showed that prolonged inhibition of the mitochondrial electron-transportation chain with 50 µM rotenone enhances ROS production and considerably reduces cell viability as a result of MPTP opening. Conversely, when diazoxide was added before the onset of oxidative stress, both a decrease in ROS production and an increase in cell viability were observed. The reversal of its action by 5-HD, a known blocker of mitoK⁺<sub>ATP</sub>-channels, allowed us to conclude that the protective action of diazoxide involves the activation of K⁺<sub>ATP</sub>-channels in the mitochondrial, but not in the plasma membranes of rat uterus cells.

The main conclusion of the present study is that a decrease in ROS production and an enhancement of cell survival under conditions of ROS overproduction is mediated by the activation of mitoK⁺<sub>ATP</sub>-channels in rat uterus cells by diazoxide.

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References


