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Ambivalent effects of diazoxide on mitochondrial ROS production at respiratory chain complexes I and III

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ABSTRACT

Background: Reactive oxygen species (ROS) are among the main determinants of cellular damage during ischemia and reperfusion. There is also ample evidence that mitochondrial ROS production is involved in signaling during ischemic and pharmacological preconditioning. In a previous study we analyzed the mitochondrial effects of the efficient preconditioning drug diazoxide and found that it increased the mitochondrial oxidation of the ROS-sensitive fluorescent dye 2′,7′-dichlorodihydrofluorescein (H_2DCF) but had no direct impact on the H_2O_2 production of submitochondrial particles (SMP) or intact rat heart mitochondria (RHM).
Methods: H_2O_2 generation of bovine SMP and tightly coupled RHM was monitored under different conditions using the amplex red/horseradish peroxidase assay in response to diazoxide and a number of inhibitors.
Results: We show that diazoxide reduces ROS production by mitochondrial complex I under conditions of reverse electron transfer in tightly coupled RHM, but stimulates mitochondrial ROS production at the Q_o site of complex III under conditions of oxidant-induced reduction; this stimulation is greatly enhanced by uncoupling. These opposing effects can both be explained by inhibition of complex II by diazoxide. 5-Hydroxydecanoate had no effect, and the results were essentially identical in the presence of Na^+ or K^+ excluding a role for putative mitochondrial K_{ATP}-channels.
General significance: A straightforward rationale is presented to mechanistically explain the ambivalent effects of diazoxide reported in the literature. Depending on the metabolic state and the membrane potential of mitochondria, diazoxide-mediated inhibition of complex II promotes transient generation of signaling ROS at complex III (during preconditioning) or attenuates the production of deleterious ROS at complex I (during ischemia and reperfusion).

1. Introduction

Mitochondrial ROS production is usually regarded as a deleterious process and it has been linked to the pathophysiology of diabetes, neurodegenerative disorders such as Alzheimer disease or Parkinson disease, and the aging process. However, recent results from different research fields suggest that a transient increase in mitochondrial ROS generation can also be an intrinsic part of cellular signaling pathways [1]. For example, ROS generated at the Q_o site of the cytochrome bc_1 complex (complex III) seem to play an essential role in mediating stabilization of human hypoxia-inducible transcription factor-1α (HIF-1α) and promoting cellular survival during hypoxia [2,3]. There is also experimental evidence that ROS generated from mitochondria are involved in the signaling during ischemic and pharmacological preconditioning, interventions which protect the heart from ischemia and reperfusion injury (overview in [4–6]). This early ROS production during ischemic and pharmacological preconditioning subsequently reduces the deleterious ROS burst that occurs upon reperfusion after a longer ischemic period. Involvement of ROS production in ischemic preconditioning was originally proposed after observing an attenuating effect of free radical scavengers in a pig heart model [7], a rat heart model [8] and in cardiomyocytes [9]. Increased mitochondrial ROS production was also observed during pharmacological preconditioning. In cell cultures and isolated mitochondria, the application of the drug diazoxide, a known K_{ATP} channel opener, resulted in increased mitochondria-dependent oxidation of ROS sensitive fluorescence dyes [10–13] that was attenuated by the addition of radical scavengers or 5-HD. However, there are contradicting results indicating that mitochondrial ROS production is not enhanced or may even decrease upon addition of diazoxide [14–16]. To understand the molecular mechanism by which

Abbreviations: DBH, *n*-decylubiquinol; DBQ, *n*-decylubiquinone; DQA, 2-*n*-decyl-quinazolin-4-yl-amine; FCCP, carbonyl-cyanide-*p*-trifluoro-methoxy-phenylhydrazone; 5-HD, 5-hydroxydecanoate; HRP, horseradish peroxidase; (mito)K_{ATP} channel, (mitochondrial) ATP-sensitive K^+-channel; Q-pool, ubiquinone pool; RCF, respiratory control factor; RHM, rat heart mitochondria; ROS, reactive oxygen species; SMPs, submitochondrial particles; SOD, superoxide dismutase; TCA cycle, tricarboxylic acid cycle (Krebs cycle)

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diazoxide modulates mitochondrial ROS generation we previously performed detailed studies with bovine heart submitochondrial particles (SMP), and intact rat liver and heart mitochondria [17]. While we were not able to detect a direct effect of diazoxide on H_2O_2 generation by SMPs and rat heart mitochondria, we detected an increase in mitochondria-linked oxidation of the ROS-sensitive fluorescent dye 2′,7′-dichlorodihydrofluorescein (H_2DCF). The diazoxide-linked H_2DCF oxidation is additive to the effect of antimycin A, a Q_i site inhibitor of complex III that can stimulate superoxide production at the Q_o site [18,19] and is sensitive to the Q_o site inhibitor stigmatellin [17]. This implied involvement of complex III in this process. Since ROS delivered from the Q_o site of complex III may play an essential role in mediating the hypoxia-dependent stabilization of HIF-1α [3] and probably other redox signaling pathways, this is a significant observation. Remarkably, the Q_o site inhibitor myxothiazol also reduced diazoxide-dependent H_2DCF oxidation in cardiomyocytes [11] and ROS generation during hypoxia [9], respectively. How diazoxide can stimulate mitochondrial ROS generation is a matter of debate; while some groups favour the involvement of the putative mitochondrial K_{ATP} channel [10–12], we and others [13,17] could not show any K^+-dependence in this process, favouring other mitochondrial targets of the drug such as its known inhibitory effect on complex II (succinate: ubiquinone oxidoreductase) [20,21]. However, these mechanisms of action so far could not explain the discrepancies between the effect of diazoxide on the oxidation of H_2DCF and MitoTracker probes and the absence of an effect on H_2O_2 generation measured by the Amplex Red/HRP assay [16,17]. This leaves the possibility that the drug has undesirable interaction with these fluorescence dyes as suggested by Kowaltowski et al. [15,16,22] or that the stimulation of ROS generation occurs only under specific conditions.

Recently, we proposed a new mechanism for superoxide formation at the ubiquinol oxidation center (Q_o site, center P) of membrane-bound or purified complex III in which an electron is transferred in a reverse reaction from reduced cytochrome b_L via oxidized ubiquinone onto oxygen rather than from semiquinone formed as an intermediate in the forward ubiquinone cycle reaction [23]. Based on this mechanism we show here that diazoxide can indeed stimulate mitochondrial ROS production by complex III in vitro under conditions of oxidant-induced reduction via its inhibitory effect on complex II. On the other hand, diazoxide decreases complex I dependent ROS generation by complex I during 'reverse electron transfer' that might occur during reoxygenation due to the accumulation of succinate under hypoxia [24]. Our results provide a straightforward mechanistic explanation for the ambivalent effects of diazoxide recently observed by Pasdois et al. [25] in Langendorff-perfused rat hearts. In this study diazoxide was shown to induce a transient moderate H_2O_2 production during the preconditioning phase and a strong decrease in oxidant generation during subsequent ischemic and reperfusion phases.

2. Materials and methods

2.1. Materials

DQA (2-*n*-decyl-quinazolin-4-yl-amine, SAN 549) was obtained from AgrEvo (Frankfurt, Germany), amplex red (*N*-acetyl-3,7-dihydrophenoxazine) was purchased from Invitrogen/Molecular Probes (Eugene, OR) and decylubiquinone (DBQ) was from Alexis Biochemicals (Lausen, Switzerland). The reduced form (DBH) was produced by dithionite-reduction of DBQ according to the protocol of Wan et al. [26]. The concentration of an ethanolic dilution was determined spectrophotometrically using ε_{290}: 4.2 mM^{-1} cm^{-1}. Fatty acid free bovine serum albumin (BSA) was from SERVA (Heidelberg). Superoxide dismutase (SOD, from bovine liver), horseradish peroxidase (HRP) and all other chemicals were from Sigma-Aldrich. DBH, diazoxide (7-chloro-3-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide), antimycin A, DQA, stigmatellin, FCCP (carbonyl-cyanide-*p*-trifluoro-methoxy-phenyl-hydrazone), oligomycin and amplex red were dissolved in DMSO. 96-Well-plates for fluorescence measurements (FIA, black, non-binding) were purchased from Greiner Bio-One (Frickenhausen, Germany).

2.2. Preparation of submitochondrial particles

Submitochondrial particles from bovine heart mitochondria where prepared as previously described [17]. The preparation used in the experiments had a protein concentration of 60 mg/ml, the cytochrome content was 32 μM haem *b* and 36 μM haem aa_3.

2.3. Isolation of intact rat heart mitochondria

Heart mitochondria were isolated at 4 °C as previously reported [17]. Diced ventricular tissue was minced and washed with a solution containing 300 mM sucrose, 10 mM Na^+/Hepes (pH 7.2), and 0.2 mM EDTA. The tissue was treated with trypsin (~0.1 mg/ml) for 15 min and twice homogenized before adding soybean trypsin inhibitor (~0.3 mg/ml). The heart mitochondria were subsequently washed, centrifuged, and resuspended in solution containing 300 mM sucrose, 10 mM Na^+/Hepes (pH 7.4), 0.2 mM EDTA, and 1 mg/ml fatty acid free bovine serum albumin.

2.4. Detection of reactive oxygen species

ROS were detected by the amplex red/HRP assay [27]. The primary ROS superoxide is converted spontaneously or by the action of superoxide dismutases (SOD) into H_2O_2. Hydrogen peroxide is used by horseradish peroxidase (HRP) to oxidize the colorless substance amplex red into resorufin [27] that can be either detected by absorbance or fluorescence measurements. The hydrogen peroxide production of SMPs was measured spectrophotometrically following the published protocol [17]. However, we measured the resorufin formation at 571 nm (compare [28]), which is its absorption maximum in contrast to the value of 563 nm given by [27]. The assay was calibrated with known H_2O_2 concentrations. Resorufin formation was monitored in a SpectraMax Plus^{384} microplate reader (Molecular Devices, Germany) at 30 °C. Usually, 80 μg SMPs were used in a total volume of 200 μl reaction mixture, containing 50 μM amplex red, 0.1 U/ml HRP, 400 U/ml SOD, 75 mM sodium phosphate (pH 7.4), 1 mM EDTA, and 1 mM $MgCl_2$. The respiratory chain was fueled by 0.25 mM NADH, 5 mM succinate or 100 μM DBH, respectively. If not indicated otherwise, inhibitors were added in the following concentrations: 100 μM diazoxide, 1 μM DQA, 1 μM antimycin A, 1 μM stigmatellin and 1.5 mM malonate. The H_2O_2 generation of coupled RHM was measured in a SpectraMax M2^e multimode reader (Molecular Devices, Germany) at the following settings: sensitivity low; top read; excitation 540 nm, emission 600 nm (fixed bandwidth 9 nm for Excitation/Emission); cut off filter 590 nm. RHM (19–34 μg protein/cavity) were diluted in a reaction mixture containing 50 μM amplex red, 0.1 U/ml HRP, 200 mM sucrose, 10 mM Tris/HCl, 10 mM potassium or sodium phosphate (pH 7.0), 10 mM $MgSO_4$, 100 μM ATP (if not indicated otherwise), 2 mM EDTA and 0.5 mg/ml BSA (fatty acid free). RHM were fueled either by malate/glutamate (4.8/5.6 mM) or 5 mM succinate, respectively. Inhibitors/effectors were added in the following final concentrations: 1 μM antimycin A, 1 μM stigmatellin, 1 μM DQA, 100 μM diazoxide, 50 nM FCCP, 1.5 mM malonate, 2 μg/ml oligomycin and 500 μM 5-hydroxydecanoate. Note that the concentration of FCCP was chosen operationally to totally prevent ROS generation by reverse electron transfer, but being low enough to completely exclude inhibitory effects that could have significantly affected our results. The assay was calibrated with known H_2O_2 concentrations (0–5 μM).

2.5. Measurement of mitochondrial respiration

The rate of mitochondrial respiration was monitored at 25 °C using an Oxygraph-2k system (Oroboros, Innsbruck, Austria), equipped with