Free Radical Biology and Medicine 96 (2016) 22–33

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

# Reactive oxygen species production in cardiac mitochondria after complex I inhibition: Modulation by substrate-dependent regulation of the NADH/NAD$^+$ ratio

Paavo Korge [a,b], Guillaume Calmettes [a,b], James N. Weiss [a,b,c,*]

[a] UCLA Cardiovascular Research Laboratory, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA
[b] Department of Medicine (Cardiology), David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA
[c] Department of Physiology, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA

ARTICLE INFO

*Article history:*
Received 28 January 2016
Received in revised form
11 March 2016
Accepted 6 April 2016
Available online 9 April 2016

*Keywords:*
Mitochondria
NADH generation/oxidation
Complex I inhibition
ROS production

ABSTRACT

Reactive oxygen species (ROS) production by isolated complex I is steeply dependent on the NADH/NAD$^+$ ratio. We used alamethicin-permeabilized mitochondria to study the substrate-dependence of matrix NADH and ROS production when complex I is inhibited by piericidin or rotenone. When complex I was inhibited in the presence of malate/glutamate, membrane permeabilization accelerated $O_2$ consumption and ROS production due to a rapid increase in NADH generation that was not limited by matrix NAD(H) efflux. In the presence of inhibitor, both malate and glutamate were required to generate a high enough NADH/NAD$^+$ ratio to support ROS production through the coordinated activity of malate dehydrogenase (MDH) and aspartate aminotransferase (AST). With malate and glutamate present, the rate of ROS production was closely related to local NADH generation, whereas in the absence of substrates, ROS production was accelerated by increase in added [NADH]. With malate alone, oxaloacetate accumulation limited NADH production by MDH unless glutamate was also added to promote oxaloacetate removal via AST. α-ketoglutarate (KG) as well as AST inhibition also reversed NADH generation and inhibited ROS production. If malate and glutamate were provided before rather than after piericidin or rotenone, ROS generation was markedly reduced due to time-dependent efflux of CoA. CoA depletion decreased KG oxidation by α-ketoglutarate dehydrogenase (KGDH), such that the resulting increase in [KG] inhibited oxaloacetate removal by AST and NADH generation by MDH. These findings were largely obscured in intact mitochondria due to robust $H_2O_2$ scavenging and limited ability to control substrate concentrations in the matrix. We conclude that in mitochondria with inhibited complex I, malate/glutamate-stimulated ROS generation depends strongly on oxaloacetate removal and on the ability of KGDH to oxidize KG generated by AST.



## 1. Introduction

Isolated mitochondria energized with physiologically relevant NAD$^+$-related substrates produce very little reactive oxygen species (ROS) as long as the NADH generated by the tricarboxylic acid (TCA) cycle is rapidly oxidized to maintain a low NADH/NAD$^+$ ratio. Consensus exists that for electron transport in the forward direction, ROS production by isolated complex I depends on the degree of flavin mononucleotide (FMN) reduction, the latter increasing with an elevated NADH/NAD$^+$ ratio, indicating a close relationship between ROS production and the redox potential of the NADH/NAD$^+$ couple [1–3]. Fully reduced flavin is an electron donor capable reducing $O_2$ at its substrate binding site. Indeed, in the absence of any other electron acceptors except $O_2$, NADH oxidation by purified complex I results in superoxide production that at [NADH] > 1 μM becomes directly proportional to concentrations of complex I and $O_2$. An increase in [NAD$^+$] that lowers the NADH/NAD$^+$ ratio strongly inhibits ROS production. Because only the nucleotide-free binding site of reduced flavin is able to react with $O_2$, ROS production also depends on nucleotide concentrations as well as the NADH/NAD$^+$ ratio. Importantly, ROS production by isolated complex I does not require addition of

*Abbreviations:* Ala, alamethicin; AOA, aminooxyacetate; Ant, antimycin; AST, aspartate aminotransferase; Asp, aspartate; AA5, atpenin A5; CDNB, 1-chloro-2,4-dintrobenzene; CoA, coenzyme A; FMN, flavin mononucleotide; Glu, glutamate; KG, α-ketoglutarate; KGDH, α-ketoglutarate dehydrogenase; Mal, malate; MDH, malate dehydrogenase; Mito, mitochondria; PDH, pyruvate dehydrogenase; Pier, piericidin; Rot, rotenone; SP, succinyl phosphonate

* Corresponding author at: Department of Medicine (Cardiology), David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA.
*E-mail address:* jweiss@mednet.ucla.edu (J.N. Weiss).

http://dx.doi.org/10.1016/j.freeradbiomed.2016.04.002
0891-5849/

ubiquinone pocket inhibitors like piericidin or rotenone, unless an electron acceptor to stimulate NADH consumption and generate $NAD^+$ is present [2,4]. Because ubiquinone is extremely hydrophobic, amphiphilic ubiquinone analogs have been used as electron acceptors for isolated complex I, but they facilitate unphysiological side reactions that do not occur with endogenous ubiquinone [5].

Although rotenone- or piericidin-induced ROS production by cardiac mitochondria energized with $NAD^+$-related substrates is known to require high matrix NADH/$NAD^+$ ratio [6], experiments with intact mitochondria have certain limitations that make it difficult to study substrate-dependent regulation of NADH and ROS production. These limitations include rapid ROS scavenging in the matrix, low dicarboxylate carrier activity in cardiac mitochondria [7], decrease in matrix [KG] and [aspartate] in response to malate and glutamate addition and dependence of glutamate uptake on membrane potential [8,9]. Given these factors, it is not surprising that rotenone addition to cardiac mitochondria energized with NAD-related substrates typically results in a relatively small increase in NADH fluorescence and ROS production [10,11]. It remains still unclear why ROS production by complex I-inhibited mitochondria energized with $NAD^+$-related substrates is so variable and relatively low, generally reaching about 5-10% of that induced by succinate-supported reverse electron transport (for review see [12]). A reasonable assumption could be that low values and variability in ROS production are connected with substrate-dependent variations in NADH generation. However, for reasons presented above controlled manipulation of matrix [substrates] in intact mitochondria is very difficult if not impossible.

One way to study the importance of substrates in the regulation of NADH-supported $H_2O_2$ production in mitochondria with inhibited complex I is to use alamethicin-treated mitochondria. Inner membrane permeabilization with alamethicin allows the matrix NADH/$NAD^+$ ratio to be rapidly manipulated by exogenous pyridine nucleotides, and has been previously used to demonstrated a close dependence of cardiac mitochondrial $H_2O_2$ production on the NADH/$NAD^+$ ratio [13]. In this study, we used this technique to explore the substrate-dependence of matrix NADH and ROS production when complex I is inhibited by piericidin or rotenone. Specifically, we tested the hypothesis that the NADH/$NAD^+$ ratio, and hence ROS production by mitochondria with inhibited Complex I in the presence of malate and glutamate, is regulated via the coordinated activity of malate dehydrogenase (MDH) and aspartate aminotransferase (AST), and that α-ketoglutarate (KG), either added or generated by AST, adds more complexity to this regulation. These findings may be relevant to ROS generation under pathophysiological conditions in which Complex I is inhibited or damaged, such as ischemia/reperfusion.

## 2. Material and methods

### 2.1. Experimental techniques

This study was approved by the UCLA Chancellor's Animal Research Committee (ARC 2003-063-23B) and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication no. 85-23, revised 1996) and with UCLA Policy 990 on the Use of Laboratory Animal Subjects in Research (revised 2010).

All measurements were carried out using customized Fiber Optic Spectrofluorometer (Ocean Optics) in a partially open continuously stirred cuvette at room temperature (22–24 °C) [14]. Mitochondria were isolated from rabbit hearts as described previously [15]. Intact mitochondria were added (0.3–0.6 mg/ml) to incubation buffer containing 250 mM sucrose, 10 mM Hepes, pH 7.4 with Tris. Mitochondria were permeabilized with addition of alamethicin (20 μg/ml). Alamethicin creates pores which allow equilibration of low-molecular weight components across the inner membrane, while high-molecular weight proteins are retained in the matrix and intermembrane space [16]. Electron microscopy of alamethicin-treated mitochondria reveals no major disruption of membranes [16] and a consensus exists that enzymes, including those involved in ROS production, remain fully functional and have enhanced access to exogenously-delivered substrates [13,16–18]. Piericidin was obtained from Cayman Chemical Company (Sapphire Bioscience) and succinyl phosphonate from MedChem Express (MCE). All other chemicals, including enzymes, were obtained from Sigma-Aldrich (St. Louis, MO). Mitochondria were pretreated with 1-chloro-2,4-dintrobenzene (CDNB) exactly as described previously [11].

$H_2O_2$ release from mitochondria was measured using 5 μM Amplex Red and 0.2 U Horse Radish Peroxidase (HRP) in the buffer (excitation/emission, 540/590 nm). The increase in resorufin fluorescence was calibrated by adding $H_2O_2$ in known concentrations.

Mitochondrial $O_2$ consumption was measured continuously by monitoring buffer $O_2$ content using a fiber optic oxygen sensor FOXY-AL300 (Ocean Optics) [14].

NADH fluorescence was recorded at 366/460 nm excitation/emission wavelengths and calibrated by adding NADH in known concentrations. In experiments where $NAD^+$ or NADH were added to permeabilized mitochondria to enhance the fluorescence signal or stimulate $H_2O_2$ production, NAD(H) concentration was chosen based on reports that $H_2O_2$ production by rotenone-inhibited permeabilized cardiac mitochondria exhibits a non-hyperbolic dependency on [NADH], with production close to maximum at about 100 μM, and decreasing at $>200$ μM [13].

### 2.2. Statistical analysis

For each data set, the mean and accompanying 95% confidence intervals (CIs) are reported. The conventional percentile bootstrap-resampling approach with 10,000 replications was used for estimating 95% CI as well as examining the significant difference between groups (effect size statistics) [19–21]. A $P$ value $<0.05$ was considered statistically significant. All analyses were performed by subroutines for bootstrapping developed in the Python programming language [21].

## 3. Results

### 3.1. NADH production/oxidation by the coupled MDH and AST reactions regulates ROS production when complex I is inhibited

When mitochondria are exposed to piericidin or rotenone, ROS production by inhibited complex I or matrix dehydrogenases depends on the ability of the mitochondria to elevate the NADH/$NAD^+$ ratio [6]. Because the level of endogenous NAD(H) was too low to give a robust fluorescence signal, we added exogenous $NAD^+$ to Complex I-inhibited mitochondria to assess NADH generation after substrates were added. Using piericidin to inhibit complex I in alamethicin-permeabilized mitochondria, 5 mM malate caused only a small increase in NADH (Fig. 1A, blue trace), consistent with rapid inhibition of the MDH reaction due to oxaloacetate accumulation (D). Addition of 5 mM glutamate to remove oxaloacetate via the forward AST reaction led to a rapid and sustained increase in NADH, which was then rapidly oxidized by adding KG and aspartate to reverse the directions of the coupled AST and MDH reactions as indicated in Fig. 1D. Fig. 1B shows