# Selective Inhibition of Deactivated Mitochondrial Complex I by Biguanides

Satoshi Matsuzaki[†] and Kenneth M. Humphries*[,†,‡,§]

[†]Free Radical Biology and Aging Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104, United States

[‡]Department of Biochemistry and Molecular Biology and [§]Reynolds Oklahoma Center on Aging, University of Oklahoma Health Science Center, Oklahoma City, Oklahoma 73104, United States

ABSTRACT: Biguanides are widely used antihyperglycemic agents for diabetes mellitus and prediabetes treatment. Complex I is the rate-limiting step of the mitochondrial electron transport chain (ETC), a major source of mitochondrial free radical production, and a known target of biguanides. Complex I has two reversible conformational states, active and de-active. The deactivated state is promoted in the absence of substrates but is rapidly and fully reversed to the active state in the presence of NADH. The objective of this study was to determine the relative sensitivity of active/de-active complex I to biguanide-mediated inhibition and

resulting superoxide radical ($O_2^{\bullet-}$) production. Using isolated rat heart mitochondria, we show that deactivation of complex I sensitizes it to metformin and phenformin (4- and 3-fold, respectively), but not to other known complex I inhibitors, such as rotenone. Mitochondrial $O_2^{\bullet-}$ production by deactivated complex I was measured fluorescently by NADH-dependent 2-hydroxyethidium formation at alkaline pH to impede reactivation. Superoxide production was 260.4% higher than in active complex I at pH 9.4. However, phenformin treatment of de-active complex I decreased $O_2^{\bullet-}$ production by 14.9%, while rotenone increased production by 42.9%. Mitochondria isolated from rat hearts subjected to cardiac ischemia, a condition known to induce complex I deactivation, were sensitized to phenformin-mediated complex I inhibition. This supports the idea that the effects of biguanides are likely to be influenced by the complex I state *in vivo*. These results demonstrate that the complex I active and de-active states are a determinant in biguanide-mediated inhibition.
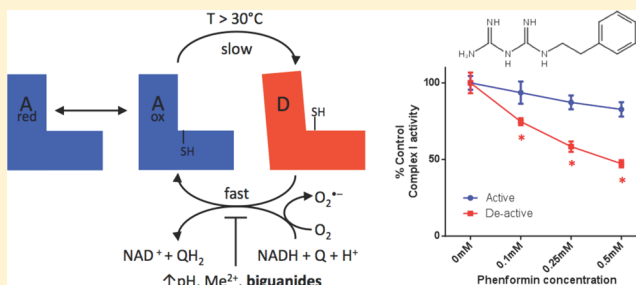
Biguanides are water-soluble, antihyperglycemic agents used in the treatment of diabetes mellitus[1] and for potential anticancer pharmacotherapy.[2−5] The mitochondrial electron transport chain (ETC) complex I was the first identified target of metformin.[6−10] This inhibition of complex I was hypothesized to enhance the production of AMP and activation of downstream signaling pathways.[10−12] However, metformin is a poor inhibitor of complex I at physiologically active concentrations.[13−15] More recently, phenformin, a lipophilic derivative of metformin, has been shown to inhibit complex I more effectively at a lower concentration.[7,16]

Mitochondrial complex I catalyzes the oxidation of NADH, a reaction that is the rate-limiting step in electron transport, and is a major site of superoxide anion ($O_2^{\bullet-}$) generation.[17] Inhibition of complex I activity is known to increase this capacity to generate $O_2^{\bullet-}$. Complex I is found in two reversible states: the active form (A-form) and the de-active form (D-form).[18−21] The transition from the A-form to the D-form is called thermal deactivation because the *in vitro* process requires a high activation energy (270 kJ/mol)[22] and physiologically relevant temperatures (>30 °C).[23] The deactivation process is slow, and the first-order decay rate constant has been reported ($k = 0.034$ min^{-1}).[24] In contrast, the D-form undergoes an enzyme turnover-dependent and rapid reactivation (D-form →

A-form) in the presence of NADH. However, reactivation is retarded in the presence of divalent cations, high pH, and/or sulfhydryl-modifying reagents such as *N*-ethylmaleimide (NEM).[25] This indicates the D-form undergoes conformational changes that expose the enzyme's reactive sulfhydryl moieties. The reactive thiols of several complex I subunits have been identified[26,27] as targets for post-translational modifications.[27−32] However, the effect of such conformational changes on the sensitivity of complex I inhibitors and their resulting effects on $O_2^{\bullet-}$ production have not been well scrutinized.

Mitochondrial function is essential for the maintenance of cellular energy status and for the production of free radicals that influence redox-regulated processes. Inhibitors of complex I, such as biguanides, are expected to have strong effects on cellular bioenergetics and oxidative stress. It is therefore critical to understand the conditions that affect the magnitude of this inhibition. Recent work suggests that complex I in the absence of NADH is sensitized to biguanide-mediated inhibition.[7] However, the direct effect of biguanides on active versus de-

active complex I is unknown. The goal of this study was to determine if complex I is inhibited by lower concentrations of biguanides in the de-active state. Furthermore, we sought to identify how deactivation of complex I affects $O_2^{\bullet-}$ production.

■ MATERIALS AND METHODS

Reagents and Animals. Antimycin A, metformin, NADH, phenformin, rotenone, ubiquinone-1, and superoxide dismutase (CuZn-SOD) were purchased from Sigma. Hydroethidine was purchased from Life Technologies. CMH was purchased from Enzo Life Sciences. Male Sprague-Dawley rats (250−300 g) were obtained from Harlan Laboratories. All animal procedures were in accordance with OMRF (Oklahoma Medical Research Foundation) Institutional Animal Care and Use Committee guidelines.

Preparation and Perfusion of Isolated Rat Hearts. Male Sprague-Dawley rats were decapitated, and hearts were excised and placed in 37 °C modified Krebs−Henseleit buffer (120 mM NaCl, 4.8 mM KCl, 2.0 mM CaCl$_2$, 1.25 mM MgCl$_2$, 1.25 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, and 5 mM glucose) to remove blood. Extraneous tissue was rapidly removed; the aorta was cannulated, and the heart was perfused in retrograde fashion according to the method of Langendorff with modified Krebs−Henseleit buffer, at 37 °C, saturated with a 95% O$_2$/5% CO$_2$ mixture. Hearts were placed in a water-jacketed chamber (37 °C), and the perfusion rate was maintained at 10 mL/min. The elapsed time between isolation of the heart and perfusion was approximately 1.0 min. Experiments consisted of the following protocols: (a) a 60 min normoxic perfusion or (b) a 30 min perfusion followed by a 45 min no-flow global ischemia.

Isolation of Mitochondria and Cardiac Submitochondrial Particles (SMPs). Subsarcolemmal mitochondria were isolated from hearts as previously described.[33] The isolation protocol was completed at 4 °C in the absence of respiratory substrates, which would be expected to minimize oxygen-induced changes following the ischemic period. Hearts were immersed and rinsed in ice-cold isolation buffer containing 210 mM mannitol, 70 mM sucrose, 10 mM MOPS, and 1.0 mM EDTA (pH 7.4). The hearts were then minced and homogenized in 20 mL of isolation buffer with a Polytron homogenizer (3 × 2 s passes). The homogenate was then centrifuged at 500*g* for 5.0 min, and the supernatant was collected. The supernatant was then filtered through cheese cloth and centrifuged at 10000*g* for 10 min. The resulting mitochondrial pellets were washed, resuspended in 25 mM MOPS (pH 7.4), and immediately snap-frozen in liquid N$_2$ for analysis of superoxide anion production and electron transport chain activities. For the preparation of SMPs, hearts were snap-frozen in liquid N$_2$ and pulverized. Pulverized tissue was placed into 20 mL of 25 mM MOPS and 1.0 mM EDTA (pH 7.4) and homogenized by 4 × 4 s passes using a Polytron homogenizer followed by 15 passes with a Potter-Elvejem homogenizer. The homogenate was then centrifuged at 750*g* for 5.0 min, and the supernatant was collected. The supernatant was centrifuged at 10000*g* for 20 min, and the resulting pellet was resuspended in 12 mL of 25 mM MOPS and 1.0 mM EDTA (pH 7.4) (2.0−5.0 mg/mL). Following two cycles of freezing (in liquid N$_2$) and thawing, the solution was sonicated on ice (8 × 15 s with 30 s intervals, sonic dismembrator output of 20 W). The sonicated preparation was then centrifuged at 10000*g* for 7.0 min to remove unbroken mitoplasts. The supernatant was centrifuged at 40000*g* for 60 min. The resulting pellet, containing SMPs, was resuspended in 25 mM MOPS (pH

7.4) at a protein concentration of 1.0 mg/mL. Protein concentrations were determined by the BCA method (Thermo), with BSA as a standard. All the preassay thermal deactivation procedures were performed as previously described,[34] with minor modifications. In brief, solubilized mitochondria and/or SMPs were incubated at 37 °C for 45 min in a dry heating block in the absence of substrates. Subsequently, the reactivation of the thermally deactivated sample was achieved by preincubation of the mitochondria with 10 μM NADH for 2.0 min at room temperature.

Where indicated, experiments were performed on solubilized mitochondria, defined as isolated mitochondria that are broken open to allow access to ETC substrates. Briefly, mitochondria were resuspended at a concentration of 1.0 mg/mL in 25 mM MOPS (pH 7.4), snap-frozen in liquid N$_2$, and thawed three times.

Electron Transport Chain Enzymatic Analyses. Mitochondrial electron transport chain enzymatic activity assays were performed as previously described.[33] Briefly, all assays were performed in 25 mM KCl and 25 mM MOPS (pH 7.4, 25 °C) with 25 μg/mL frozen−thawed mitochondria except where noted. For the NADH oxidase activity, an assessment of overall electron transport through complexes I, III, and IV was measured spectrophotometrically utilizing an Agilent 8453 diode array UV−vis spectrophotometer. Activity was measured as the rate of NADH oxidation ($\varepsilon_{340} = 6200$ M^{-1} cm^{-1}) following addition of 200 μM NADH. Activity was completely inhibited by antimycin A. For complex I, following addition of 50 nM antimycin A and 100 μM ubiquinone-1, complex I activity was initiated with NADH (200 μM). Activity was measured spectrophotometrically as the rate of NADH oxidation ($\varepsilon_{340} = 6200$ M^{-1} cm^{-1}). Activity required the presence of ubiquinone-1 and was inhibited by rotenone (100 nM), indicating that NADH utilization was dependent on complex I activity. For the combination of complexes III and IV (III−IV), the flow of electrons from complex III through complex IV was initiated upon addition of ubiquinol-1 (100 μM). Activity was measured spectrophotometrically as the rate of oxidation of ubiquinol-1 to ubiquinone-1 ($\varepsilon_{280} = 13700$ M^{-1} cm^{-1}). Antimycin A or CN$^-$ inhibited activity, indicating transport of electrons from complex III through complex IV.

Superoxide Anion Production Measurements. The NADH-supported rate of superoxide anion ($O_2^{\bullet-}$) production was measured as described previously.[35] Briefly, the oxidation of hydroethidine by $O_2^{\bullet-}$ to the fluorescent product 2-hydroxyethidium was measured utilizing a Shimadzu RF-5301 spectrofluorometer. The fluorescent signals were recorded (excitation, 480 nm; emission, 567 nm) over time utilizing 10 μM hydroethidine and 500 μM NADH. CuZn-SOD (8.0 units/mL) was used to test the specificity of the measurement for superoxide anion. Where indicated, the complex III inhibitor, antimycin A (50 nM), was included. It should be noted that oxidation of hydroethidine by $O_2^{\bullet-}$ resulted in a product with properties (reverse phase high-performance liquid chromatography elution profile and fluorescence properties) consistent with 2-hydroxyethidium and not ethidium as reported previously.[36,37] Nevertheless, the structurally related compound ethidium was used as a standard to estimate the relative rate of $O_2^{\bullet-}$ production because of the absence of a commercially available 2-hydroxyethidium standard.

EPR Spin-Trapping Experiments. The EPR spectra were obtained by using a Bruker (Billerica, MA) EMX spectrometer operating at X-band (∼9.78 GHz) with a 100 kHz modulation
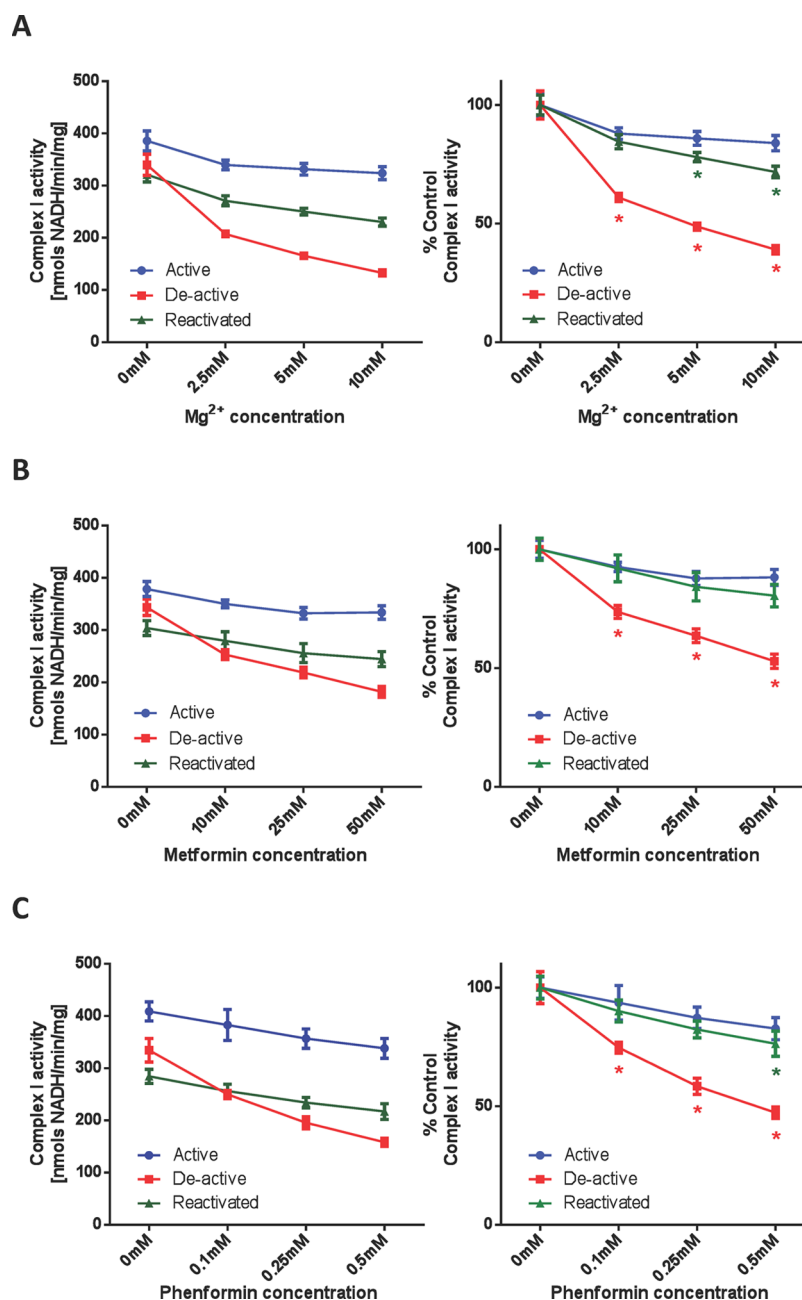
Figure 1. Mitochondrial complex I is sensitized to Mg^{2+} and biguanides after thermal deactivation. Complex I activities, in active, de-active, and reactivated states, were measured spectrophotometrically as the rate at which solubilized mitochondria at 25 μg/mL oxidized NADH (200 μM) in the presence of 50 nM antimycin A and 100 μM ubiquinone, in the absence or presence of indicated concentrations of (A) Mg^{2+}, (B) metformin, or (C) phenformin. Values are expressed as specific activities (left panels) and as percentages of the untreated basal activities (right panels). Values represent means $\pm$ SD ($n = 5$). Asterisks indicate statistical significance between active (control) and de-active groups ($p < 0.005$) using a Student's t test.

frequency and an ER 4125SHQ high-sensitivity cavity. Typical settings for the spectrometer are as follows: microwave power, 6.325 mW; modulation amplitude, 1.5 G; scan range, 50 G; time constant, 82 ms. The reaction mixtures indicated in the figure legend were assembled in microcentrifuge tubes, and after the indicated incubation time, the mixtures were transferred to a quartz flat cell for EPR determination. For the kinetic experiment shown in Figure 5B, the reaction was initiated in the flat cell in the cavity and time course (fixed-field) measurement was performed. All the EPR experiments were performed in 25 mM MOPS (pH 7.4), 25 mM KCl, 1 mM EDTA, and 50 μM DTPA, at room temperature.

Statistical Analysis. Data are presented as means $\pm$ the standard deviation (SD) or standard error of the mean (SEM), as indicated. The data were evaluated utilizing a two-tailed Student's t test.

■ RESULTS

Characterization of Thermally Deactivated (D) Complex I and Phenformin Sensitivity. Initial experiments were undertaken to determine the conditions that deactivate complex I in rat heart mitochondria. Solubilized mitochondria were heated for 45 min at 37 °C, and then complex I activity was measured in the presence of increasing concentrations of
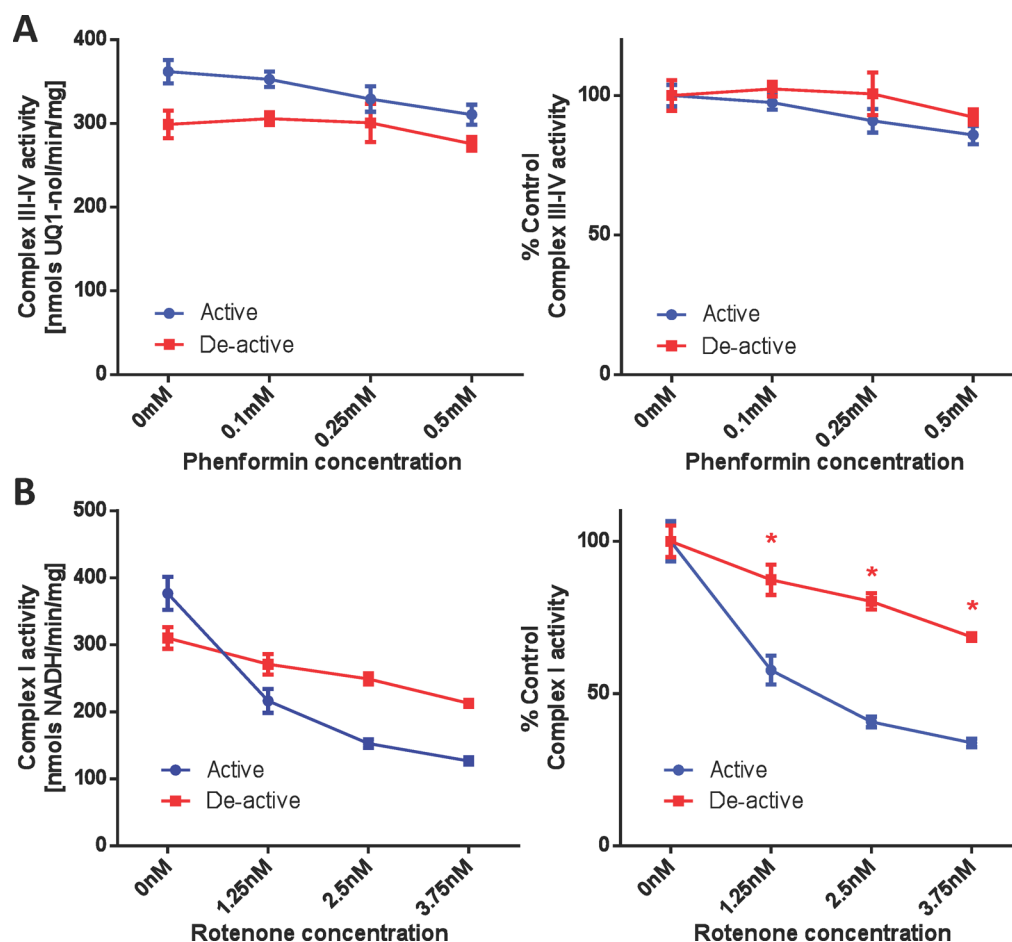
Figure 2. (A) Phenformin has no effect on complex III−IV activity. Complex III−IV activities, in nontreated (active) and thermally deactivated (deactive) mitochondria, were measured spectrophotometrically as the rate at which solubilized mitochondria at 5 μg/mL oxidized 100 μM ubiquinol in the absence or presence of indicated concentrations of phenformin. (B) Complex I is desensitized to rotenone after deactivation. Complex I activities, in nontreated (active) and thermally deactivated (de-active) mitochondria, were measured in the absence or presence of the indicated concentration of rotenone. Values are expressed as specific activities (left panels) and as percentages of the untreated activities (right panels). Values represent means ± SD (n = 5). Asterisks indicate statistical significance ($p < 0.005$) using a Student's t test.

Mg^{2+}. Divalent cations, such as Mg^{2+}, interact with and stabilize the de-active form of complex I.[25] As shown in Figure 1A, complex I activity was sensitive to Mg^{2+}-mediated inhibition following heating while control samples were largely unaffected. Pretreatment of thermally deactivated samples with NADH, which reactivates complex I, attenuated this sensitivity to Mg^{2+} (Figure 1A). This demonstrates deactivation is reversible. Heating alone induced a 12% decrease in basal complex I activity in the absence of any added Mg^{2+}, suggesting additional mechanisms are involved in the thermal process that include nonreversible inactivation. Furthermore, the overall electron transport chain (ETC) activity, as determined by NADH oxidase activity, exhibited a similar modest decline with a 12.3% decrease (943.8 ± 20.1 and 827.6 ± 39.3 nmol min^{-1} mg^{-1} for active and de-active forms, respectively; n = 5) following heating. These results demonstrate thermal treatment deactivates complex I while having little effect on the overall ETC.

Biguanides inhibit complex I activity in intact mitochondria.[9,10] However, the molecular mechanisms of biguanide-mediated complex I inactivation are unknown, and we hypothesized that deactivation of complex I sensitizes it to inhibition. Thus, the relative sensitivity of active versus de-active complex I to inhibition by biguanides was next assessed. As shown in Figure 1B, the sensitivity of metformin to complex

I was greatly enhanced by thermal deactivation. Similar to the profile with Mg^{2+}, the sensitivity was reversed by reactivation of complex I. It should be noted, however, that the effective concentration range of metformin required to observe complex I inhibition in vitro also exhibited nonspecific inhibition of other ETC components. Specifically, 25 mM metformin inhibits 12.1% of active complex I activity (Figure 1B), whereas the same concentration of metformin suppresses 44.6% of complex III−IV activity (ubiquinol oxidase activity) and 59.0% of NADH oxidase activity (the combination of complex I−III−IV activities). In contrast to metformin, the related biguanide, phenformin, exhibited inhibition of complex I in the submillimolar range after thermal deactivation (Figure 1C). A similar concentration range has been reported recently for phenformin-mediated inhibition of complex I activities.[7] This inhibition was attenuated by reactivation of the enzyme. In this concentration range, phenformin exhibited specificity toward inhibition of complex I. For example, the sensitivity to complex III−IV activity was minimal and did not change after thermal treatment (Figure 2A).

We next sought to assess whether thermal deactivation of complex I nonselectively sensitizes it to complex I inhibitors. As shown in Figure 2B, the linear concentration range of inhibition of complex I by rotenone lies in a narrow range (0−3 nM).
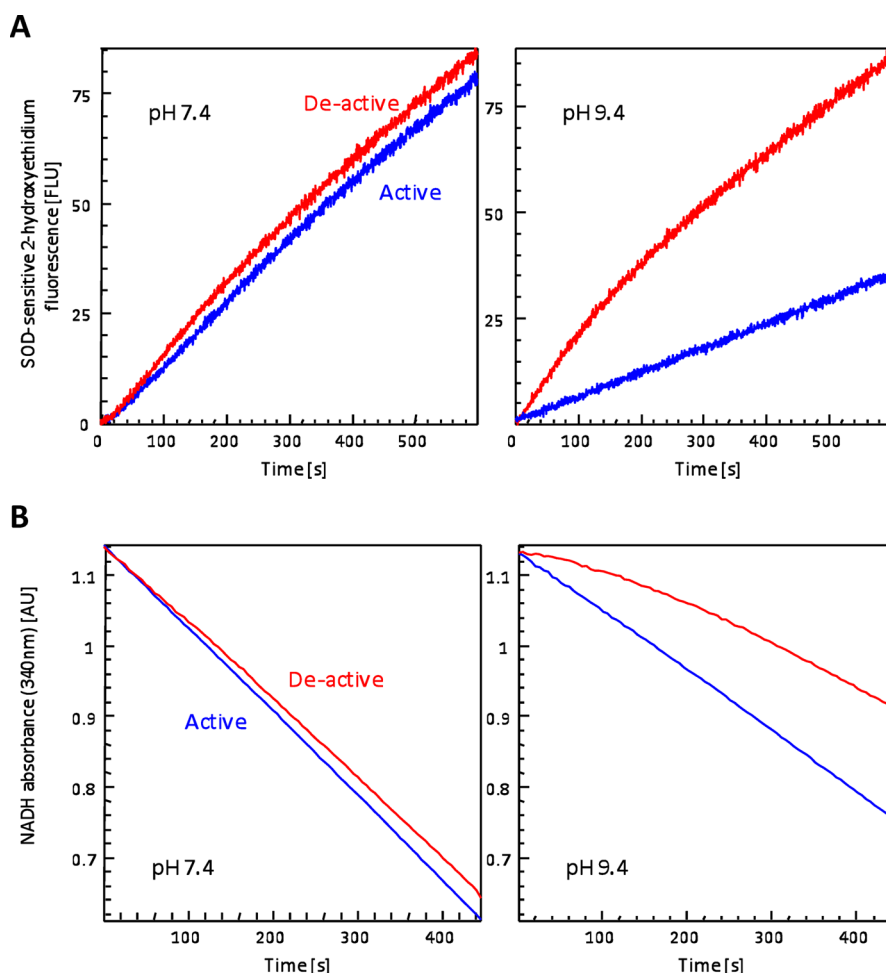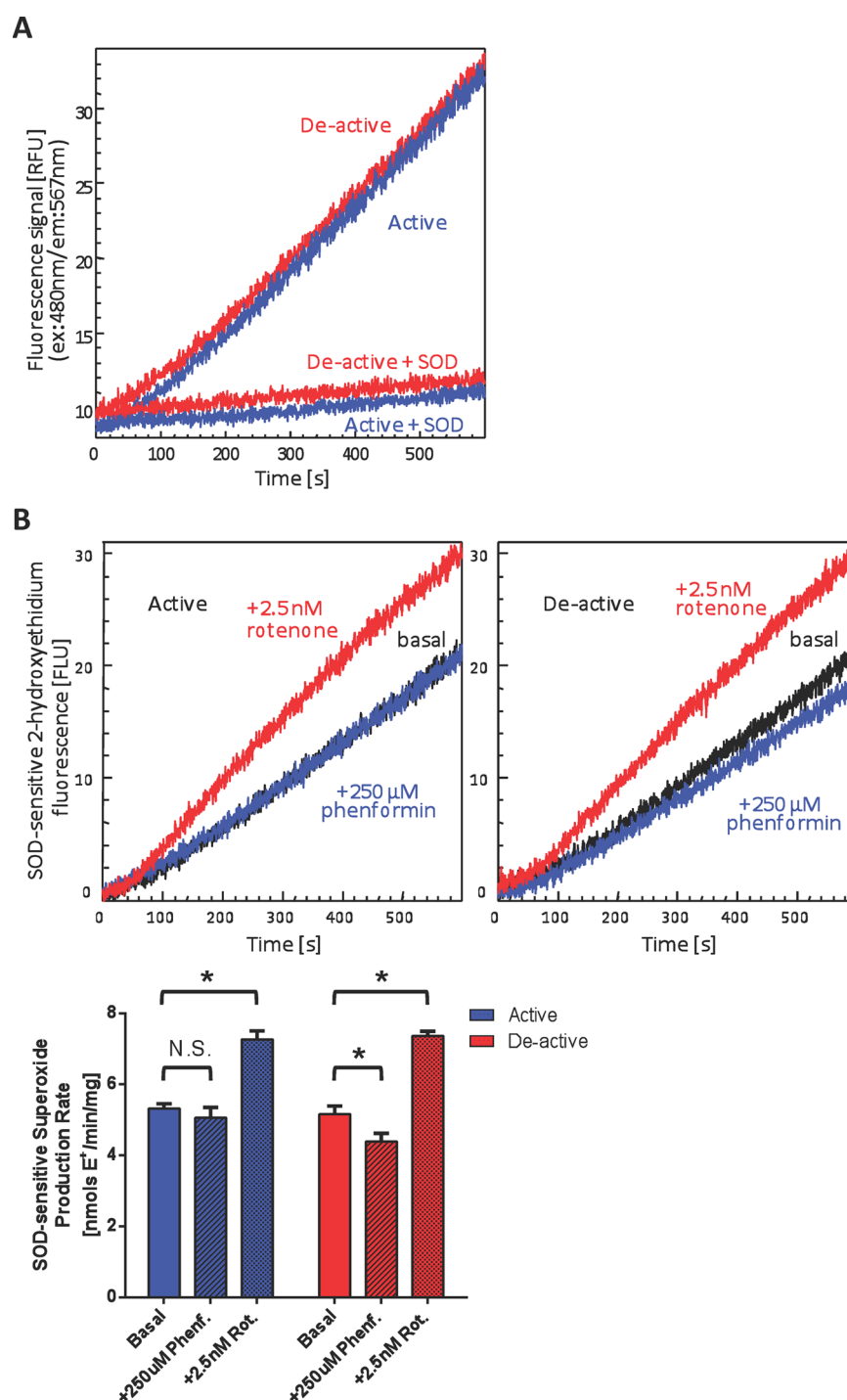
Figure 3. Increased mitochondrial superoxide production by thermal deactivation is observed at alkaline pH. (A) Superoxide production kinetics were monitored fluorimetrically as the rate at which nontreated (active) or thermally deactivated (de-active) 25 μg/mL SMPs oxidize hydroethidine in the presence of NADH, in 25 mM MOPS (pH 7.4, left) or in 25 mM CHES (pH 9.4, right). Traces are representatives of $O_2^{\bullet-}$-specific 2-hydroxyethidium formation (SOD-insensitive contributions were subtracted; see Figure 4 for typical +SOD traces). (B) NADH oxidase activity was measured spectrophotometrically as the rate at which nontreated (active) or thermally deactivated (de-active) SMPs at 25 μg/mL oxidized NADH (200 μM), in 25 mM MOPS (pH 7.4, left) or in 25 mM CHES (pH 9.4, right). Traces are representative of reduction in NADH absorbance at 340 nm. These traces are representative figures from five separate experiments.

There was a general trend of decreased sensitivity to rotenone after thermal deactivation. This suggests that the mode of inhibition of biguanides is distinct from that of rotenone, a semiquinone antagonist,[38] and does not universally occur for complex I inhibitors.

Modulation of Mitochondrial Free Radical Production by Deactivation and Phenformin-Mediated Inhibition of Complex I. Complex I is a major site of mitochondrial superoxide anion ($O_2^{\bullet-}$) production.[17] How this production is affected by deactivation is not well-understood. This is partly because complex I rapidly reactivates in the presence of NADH, where the turnover $t_{1/2} \sim 10$ s,[21] thus making it difficult to assess the deactivation effect on NADH-dependent $O_2^{\bullet-}$ production. To address this difficulty, the following experiment was performed. The effect of thermal deactivation on the basal capacity of $O_2^{\bullet-}$ production in control submitochondrial particles (SMPs) was measured as the NADH-dependent, SOD-sensitive increase in 2-hydroxyethidium fluorescence. SMPs, which are essentially devoid of matrix proteins, were used for these experiments to minimize endogenous anti-oxidant activities. As shown in Figure 3A, the stimulated rate of $O_2^{\bullet-}$ production by deactivation is not observed *in vitro* at

physiological pH 7.4 likely because of the aforementioned rapid reactivation. In contrast to pH 7.4, the rate of reactivation is significantly decreased at alkaline pH.[25] By exploiting this property, we were able to observe a significant increase (260.4 $\pm$ 21.7%; $n = 5$) in the substrate-dependent rate of $O_2^{\bullet-}$ production at pH 9.4. This stimulated rate of $O_2^{\bullet-}$ production diminished as complex I returned to the active state. Figure 3B demonstrates the effect of pH on the reactivation of NADH oxidase activity under the same conditions that $O_2^{\bullet-}$ production was measured. At pH 7.4, complex I reactivates quickly, and there is essentially no difference in the rate of NADH oxidation between active and deactivated samples. In contrast, a decrease in the initial rate of NADH oxidation can be observed at alkaline pH (56.4 $\pm$ 2.4%). These results indicate the complex I active versus de-active status regulates mitochondrial $O_2^{\bullet-}$ production, and the rate of $O_2^{\bullet-}$ production inversely correlates to ETC activity.

Next we determined the effect of phenformin-mediated complex I inhibition on $O_2^{\bullet-}$ production in the active and de-active states. The deactivation of complex I in solubilized mitochondria led to a minimal effect on the basal capacity of NADH-supported $O_2^{\bullet-}$ production (Figure 4A) at pH 7.4,

Figure 4. (A) Thermal deactivation has minimal effect on basal superoxide production at physiological pH. Superoxide production was measured fluorimetrically as the rate at which solubilized mitochondria oxidize hydroethidine in the presence of NADH in 25 mM MOPS (pH 7.4). Traces are representatives of 2-hydroxyethidium formation for nontreated (active) and thermally deactivated (de-active) mitochondria in the absence or presence of CuZn-SOD (8.0 units/mL). (B) The effect of phenformin-mediated complex I inhibition on superoxide production is distinct from that of rotenone-induced complex I inhibition. Traces were SOD-sensitive 2-hydroxyethidium formation monitored utilizing nontreated (active) or thermally deactivated (de-active) 25 μg/mL solubilized mitochondria in the absence or presence of 250 μM phenformin or 2.5 nM rotenone. SOD-sensitive rates are presented in the bar graph (bottom). Values represent means $\pm$ SD ($n = 5$). Asterisks indicate statistical significance vs respective basal rates ($p < 0.001$) using a Student's t test.

similar to our results in SMPs. This result supports the possibility that thermal deactivation does not impose irreversible damage on the ETC components to modulate $O_2^{\bullet-}$ production. We next examined the effect of phenformin on $O_2^{\bullet-}$ production in active and thermally deactivated solubilized mitochondria. Phenformin decreased $O_2^{\bullet-}$ production under basal conditions by 4.9% and following thermal deactivation by 14.9%. This result was unexpected given that inhibition of complex I is known to increase complex I-mediated free radical production. For example, rotenone (2.5
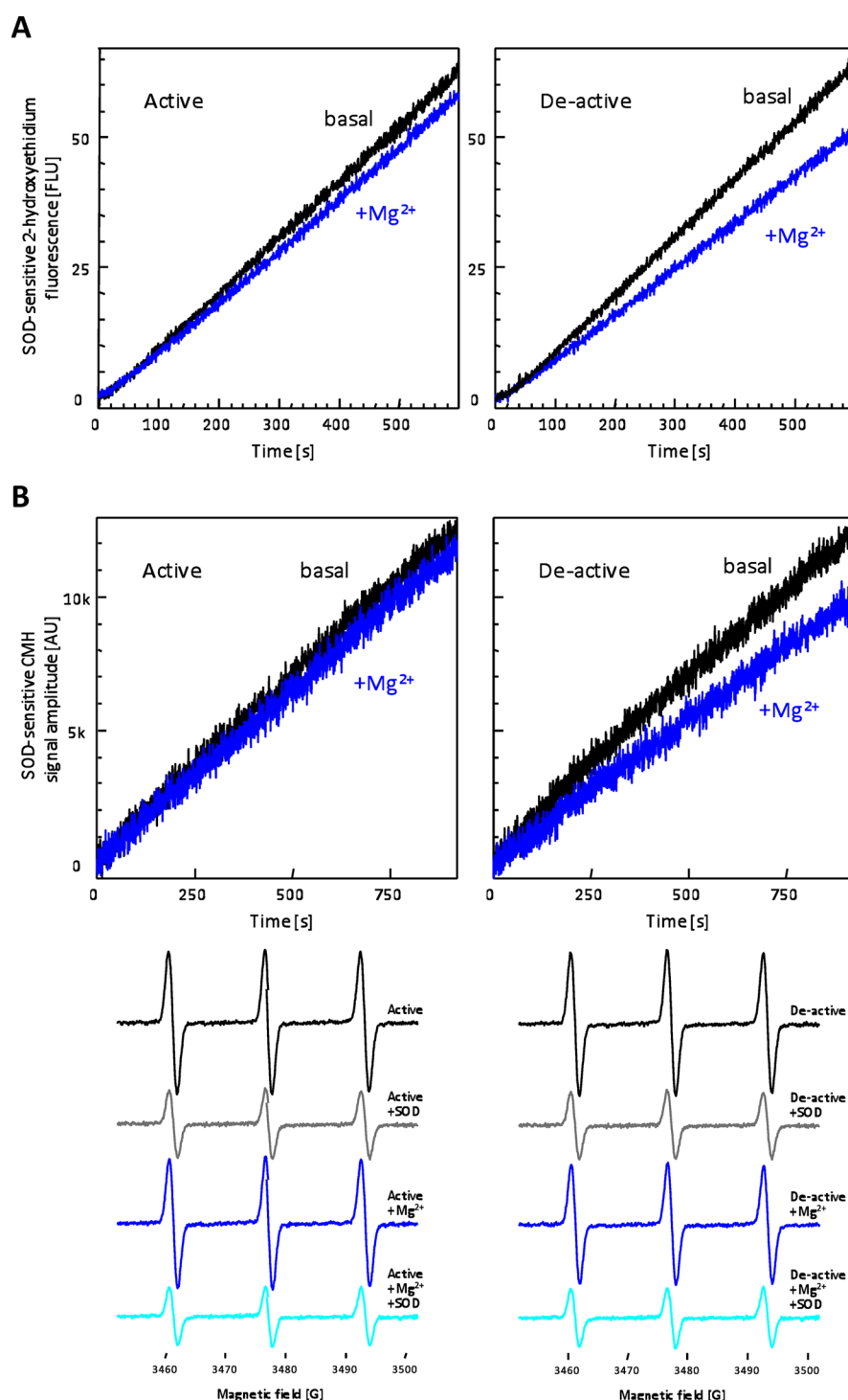
Figure 5. Complex I locked in the deactivated state produces less superoxide. (A) Representative traces are SOD-sensitive 2-hydroxyethidium formation by nontreated (active) and thermally deactivated (de-active) solubilized mitochondria incubated with 500 μM NADH and 50 nM antimycin A in the absence or presence of 5 mM Mg^{2+}. (B) Superoxide production kinetics were assessed by EPR spin-trapping utilizing a cyclic hydroxylamine, CMH.[55] Active or de-active solubilized mitochondria were incubated with 500 μM NADH, 50 nM antimycin A, and 500 μM CMH, in the absence and presence of 10 mM Mg^{2+}. Traces represent SOD-sensitive CMH signal formation. The reaction was initiated in a flat cell in cavity, and the peak intensity was monitored over time. End-point spectra were measured 20 min after the reaction was initiated. These traces and spectra are representative figures from five separate experiments.

nM) increased the rate of $O_2^{\bullet-}$ production (Figure 4B), thus further demonstrating phenformin inhibits via a distinct mechanism. Furthermore, because the concentration of phenformin utilized (250 μM) had no effect on downstream electron transport activity, it suggests this decrease in $O_2^{\bullet-}$

production is largely because of its effect on complex I. A similar decrease in the rate of mitochondrial free radical production was observed in thermally deactivated mitochondria in the presence of Mg^{2+} (Figure 5A), suggesting reagents that impede reactivation of complex I may unexpectedly decrease
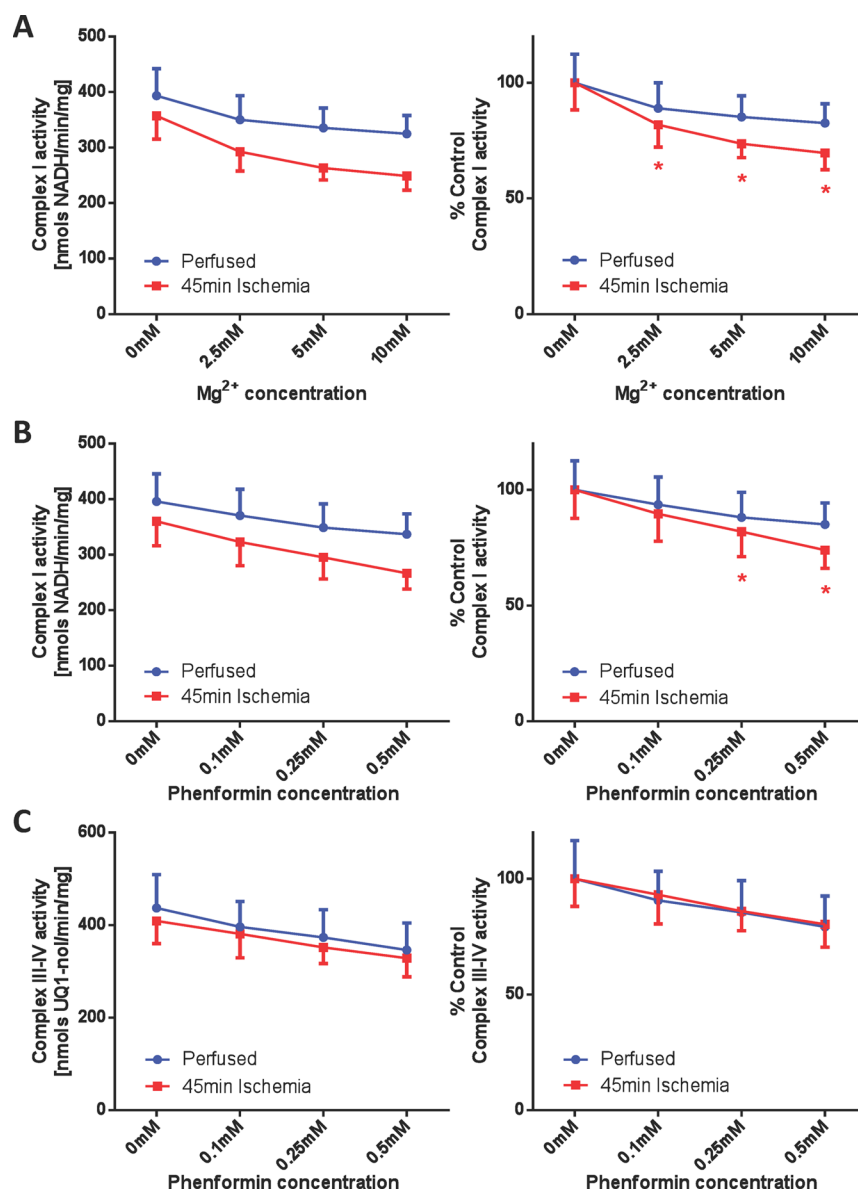
Figure 6. (A) Cardiac ischemia sensitizes complex I to magnesium. Mitochondria were isolated from perfused control hearts, and hearts underwent a 45 min ischemic period as described in Materials and Methods. Complex I activities were measured spectrophotometrically as the rate at which solubilized mitochondria at 25 μg/mL oxidized NADH (200 μM) in the presence of 50 nM antimycin A and 100 μM ubiquinone, in the absence or presence of the indicated concentrations of Mg^{2+}. (B) Cardiac ischemia sensitizes complex I to phenformin. Similarly, complex I activities were measured in the absence or presence of the indicated concentration of phenformin. (C) Cardiac ischemia does not affect complex III−IV sensitivity to phenformin. Complex III−IV activities were measured spectrophotometrically as the rate at which solubilized mitochondria at 5 μg/mL oxidized 100 μM ubiquinol in the absence or presence of the indicated concentrations of phenformin. Values are expressed as raw (left) and as percentages of the untreated basal rates (right). Values represent means ± SEM ($n = 5$). Asterisks indicate statistical significance ($p < 0.05$) using a Student's t test.

$O_2^{\bullet-}$ production. To support our hydroethidine fluorescence data, EPR spin-trapping experiments utilizing a membrane permeable cyclic hydroxylamine, CMH, were performed. Similar results were obtained (Figure 5B) using this technique, demonstrating the observed changes in $O_2^{\bullet-}$ production were not fluorescent artifacts.

Ischemia Sensitizes Cardiac Mitochondria to Phenformin-Mediated Inhibition. Given the rapid reactivation of complex I, it is difficult to observe deactivated complex I *in vivo*. However, evidence supports the idea that cardiac ischemia induces deactivation of complex I *in vivo*.[39−41] We therefore employed a Langendorff perfusion model to determine whether ischemia sensitizes cardiac mitochondria to biguanide-mediated complex I inhibition. As shown in panels A and B of Figure 6,

ischemia sensitized isolated mitochondria to both Mg^{2+}- and phenformin-mediated inhibition. Similar to the results derived from *in vitro* thermal treatment, the ischemic effect on complex III and IV was minimal, and more importantly, ischemia did not sensitize complex III−IV to phenformin (Figure 6C).

Next we examined the magnitude of ischemia-induced complex I deactivation by *in vitro* thermal deactivation. Solubilized mitochondria isolated from perfused hearts or hearts that underwent ischemia for 45 min were heated to induce complete deactivation. Following this thermal treatment, the differential sensitivity of complex I to Mg^{2+} or phenformin between perfused and ischemic samples was diminished. For example, 10 mM Mg^{2+} inhibited basal complex I in perfused hearts by 17.1 ± 4.6% before thermal deactivation

and 59.3 ± 3.7% after. In contrast, 10 mM Mg^{2+} inhibited complex I from ischemic hearts by 30.2 ± 2.7% before thermal deactivation and 64.2 ± 1.4% after. These data support the possibility that *in vivo* deactivation is occurring under the ischemic state and that this causes an increased sensitivity to biguanide-mediated inhibition. Thus, conditions that promote deactivation of complex I under physiological conditions may enhance biguanide-mediated inhibition.

■ DISCUSSION

It has been previously shown that in intact mitochondria complex I is a major target site of biguanides.[6−10] This report demonstrates that biguanides selectively inhibit the deactivated form of complex I. Recently, Hirst and colleagues reported that biguanides inhibit ubiquinone reduction in a noncompetitive manner.[7] The same report further suggested the deactivation of complex I may enhance the binding of biguanides to complex I,[7] which is conceivable because the ubiquinone binding site may undergo conformational change[42] by complex I deactivation. NADH dehydrogenase activity, assessed as NADH:ferricyanide oxidoreductase activity, was found to be unchanged after thermal deactivation and by submillimolar concentrations of phenformin (not shown). It is therefore postulated that this selective inactivation of deactivated complex I stems from modification of the ubiquinone binding site. The exact binding site for biguanides and the mechanism of interaction remain to be elucidated. More recently, the direct interaction of biguanides with isolated complex I or functionally isolated complex I has been questioned. It has been suggested that the energized state and/or the interaction of biguanides with ATP synthase is important for biguanide-mediated inhibition.[43] Our results presented in this report, however, indicate the deactivation greatly enhances the sensitivity of complex I toward biguanides, leading to observable inhibition of complex I activity at lower than previously reported concentrations. One of the deactivation requirements for complex I is the absence of electron transfer activity within the enzyme. Therefore, this study demonstrates the direct inhibition of complex I by biguanides does not require an energized state. Additionally, the early studies of the temperature-dependent biguanide sensitization in intact mitochondria, where incubation at 37 °C drastically increase biguanide-induced inhibition in respiration,[9] can be explained in part by the deactivation of complex I.

Our results demonstrate that complex I "locked" into the deactivated state by Mg^{2+} or biguanides decreases the ETC-mediated superoxide $(O_2^{\bullet-})$ production capacity. The properties of this locked de-active state have not been well characterized. It is partly because most of the known reactivation-impeding agents (NEM and divalent cations) affect downstream ETC components nonspecifically, and thus, modulation in $O_2^{\bullet-}$ production by the locked de-active state is difficult to assess. Specific inhibition of complex I by phenformin, therefore, is a valuable tool for evaluating mechanisms of $O_2^{\bullet-}$ production upstream of the ubiquinone binding site. It should be noted though that millimolar concentrations of phenformin, where downstream inhibition becomes evident, increased the observed level of $O_2^{\bullet-}$ production especially in the active state (not shown). This may explain in part the previous observations that biguanide-treated cells or intact mitochondria stimulate the production of ROS $(O_2^{\bullet-}$ or $H_2O_2)$.[44−46] What we present here is a direct mechanism of modulation of $O_2^{\bullet-}$ production excluding secondary contributions of overall oxidative stress *in vivo*.

In intact mitochondria, it has been widely reported that a major mechanism of production of $O_2^{\bullet-}$ from the mitochondrial ETC is by reverse electron transfer (RET). This is the complex II-mediated reduction of NAD^+ that can occur in the presence of a high membrane potential.[17,47] It can be speculated that at less than complete inhibition of complex I, the large RET-mediated increases in $O_2^{\bullet-}/H_2O_2$ production likely overwhelm the ∼15% suppression of NADH-supported $O_2^{\bullet-}$ production that we report here (Figure 4B), resulting in increased overall ROS production. However, consistent with our results, there is evidence that metformin decreases RET-mediated complex I ROS production in intact mitochondria.[48]

Phenformin and buformin carry an elevated risk of lactic acidosis. However, the physiological accumulation of these biguanides in mitochondria may facilitate their antineoplastic use. Because biguanides are positively charged, they are expected to accumulate in response to the mitochondrial energetic status. Specifically, lower respiratory activity and increased membrane potential induced by respiratory inhibitors may help increase the level of accumulation of biguanides into the mitochondrial matrix. It has been considered that organic cation transporter 1 (OCT1) is the main plasma membrane biguanide transporter.[49−51] In the case of phenformin, it has been determined that it could accumulate to roughly 200 μM[7] in the mitochondrial matrix *in vivo*, which is sufficient to induce ∼10 and ∼35−40% decreases in complex I activity on active and de-active states in our study, respectively.

A low-glucose environment has recently been shown to increase the potency of phenformin and other biguanides in cancer cell lines.[52] The synergistic effect of biguanides with agents that modulate glucose metabolism, such as oxamate[53] and dichloroacetate,[45,46] has been also reported. As presented in Figure 6, ischemia represents a condition that promotes deactivation of complex I *in vivo*, thereby increasing the sensitivity to biguanide-mediated inhibition. The increased sensitivity of complex I to biguanide-mediated inhibition may contribute to the observation that metformin improves cardiac function in a long-term postmyocardial infarction model.[54] Furthermore, it can be hypothesized that local hypoxic or ischemic environments found in certain cancers may thus represent a mechanism of increased biguanide activity. It will therefore be important to identify the physiological conditions that favor deactivation of complex I *in vivo*.

■ AUTHOR INFORMATION

Corresponding Author

*Free Radical Biology and Aging Research Program, Oklahoma Medical Research Foundation, 825 N.E. 13th St., Oklahoma City, OK 73104. E-mail: kenneth-humphries@omrf.org. Phone: (405) 271-7584. Fax: (405) 271-1437.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

CMH, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine; CHES, *N*-cyclohexyl-2-aminoethanesulfonic acid; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; ETC, electron transport chain; HE, hydroethidine; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NADH, nicotinamide adenine dinucleotide (reduced); SOD, superoxide dismutase; SMP, submitochondrial particle.

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