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K^+-independent Actions of Diazoxide Question the Role of Inner Membrane K_{ATP} Channels in Mitochondrial Cytoprotective Signaling*[S]

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Activation by diazoxide and inhibition by 5-hydroxydecanoate are the hallmarks of mitochondrial ATP-sensitive K^+ (K_{ATP}) channels. Opening of these channels is thought to trigger cytoprotection (preconditioning) through the generation of reactive oxygen species. However, we found that diazoxide-induced oxidation of the widely used reactive oxygen species indicator 2′,7′-dichlorodihydrofluorescein in isolated liver and heart mitochondria was observed in the absence of ATP or K^+ and therefore independent of K_{ATP} channels. The response was blocked by stigmatellin, implying a role for the cytochrome bc_1 complex (complex III). Diazoxide, though, did not increase hydrogen peroxide (H_2O_2) production (quantitatively measured with Amplex Red) in intact mitochondria, submitochondrial particles, or purified cytochrome bc_1 complex. We confirmed that diazoxide inhibited succinate oxidation, but it also weakly stimulated state 4 respiration even in K^+-free buffer, excluding a role for K_{ATP} channels. Furthermore, we have shown previously that 5-hydroxydecanoate is partially metabolized, and we hypothesized that fatty acid metabolism may explain the ability of this putative mitochondrial K_{ATP} channel blocker to inhibit diazoxide-induced flavoprotein fluorescence, commonly used as an assay of K_{ATP} channel activity. Indeed, consistent with our hypothesis, we found that decanoate inhibited diazoxide-induced flavoprotein oxidation. Taken together, our data question the "mitochondrial K_{ATP} channel" hypothesis of preconditioning. Diazoxide did not evoke superoxide (which dismutates to H_2O_2) from the respiratory chain by a direct mechanism, and the stimulatory effects of this compound on mitochondrial respiration and 2′,7′-dichlorodihydrofluorescein oxidation were not due to the opening of K_{ATP} channels.

Short ischemic insults have been shown to protect various cell types from subsequent prolonged ischemia, an extensively investigated phenomenon known as ischemic preconditioning (1–3). Diazoxide has been shown to be equally effective as short bouts of ischemia in affording cellular protection, and both effects are blocked by the substituted fatty acid 5-hydroxydecanoate (5-HD).[2] In 1996, Garlid *et al.* (4) reported that diazoxide, with half-maximal effect (EC_{50}) at <3 μM, is a selective and potent opener of mitochondrial K_{ATP} channels. An independent group (5) showed that diazoxide, with an EC_{50} value of 27 μM, increases flavoprotein fluorescence in intact rabbit ventricular myocytes superfused with substrate-free solution. The authors inferred that the diazoxide-induced flavoprotein oxidation, widely used to assay mitochondrial K_{ATP} channel activity, is attributable to the uncoupling effect of opening K^+ channels (K_{ATP} channels) in the mitochondrial inner membrane.

The diazoxide-induced K^+ flux and flavoprotein fluorescence responses measured by the Garlid and Marban laboratories were blocked by 5-HD (5, 6), which was thought to block selectively mitochondrial K_{ATP} channels. However, the reliability of 5-HD as a selective blocker is questionable because it is now known that this substituted fatty acid is activated and metabolized by the matrix β-oxidation pathway (7–10). Nevertheless, when diazoxide is introduced in the presence of either 5-HD or glibenclamide, a nonselective K_{ATP} channel blocker, and subsequently is washed out, cardioprotection is abrogated (11, 12). These observations suggest that diazoxide may trigger preconditioning through the opening of mitochondrial K_{ATP} channels.

Recently, Forbes *et al.* (13), as well as Oldenburg *et al.* (14), found that diazoxide, in a 5-HD-sensitive manner, increases the rate of production of reactive oxygen species (ROS) in cardiac myocytes, measured using ROS-sensitive fluorescent indicators. These studies suggested that diazoxide may trigger preconditioning via ROS, which are known to activate various kinases, including protein kinase C (1, 15).

To test the hypothesis that diazoxide mediates cytoprotection by opening mitochondrial K_{ATP} channels and evoking ROS, we investigated under controlled conditions the effects of this compound on respiration and ROS production using intact liver and heart mitochondria, submitochondrial particles, and purified cytochrome bc_1 complex. In addition, we tested whether fatty acid metabolism, an alternative mechanism,

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[2] The abbreviations used are: 5-HD, 5-hydroxydecanoate; ROS, reactive oxygen species; H_2DCF, 2′,7′-dichlorodihydrofluorescein; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DNP, 2,4-dinitrophenol; RHM, rat heart mitochondria; DCF, 2′,7′-dichlorofluorescein.

could explain the ability of 5-HD to reverse diazoxide-induced flavoprotein oxidation in isolated ventricular myocytes.

EXPERIMENTAL PROCEDURES

Isolation of Intact Rat Liver Mitochondria—Coupled mitochondria were isolated from rat liver as described previously (16) and suspended in solution containing 250 mM sucrose, 2 mM Tris-HCl, and 1 mM EDTA (pH 7.4). The rate of respiration was monitored at 25 °C using an Oxygraph-2k system (Oroboros, Innsbruck, Austria) with DatLab software. Mitochondria, used at a final concentration of ~1.5 mg/ml, were added to buffer containing 200 mM sucrose, 10 mM potassium phosphate, 10 mM Tris-HCl, 10 mM $MgSO_4$, and 2 mM EDTA (pH 7.2). K^+-free solutions were prepared by replacing potassium phosphate with sodium phosphate.

State 4 respiration was measured using sodium malate (5 mM) and sodium glutamate (5 mM) as substrates. Where indicated, 100 μM ATP was added in the presence of the ATP synthase inhibitor oligomycin (1 μM) to inhibit mitochondrial K_{ATP} channels (17).

Isolation of Intact Rat Heart Mitochondria (RHM)—Heart mitochondria were isolated along the lines described by Jacobus and Saks (18). Diced ventricular tissue was minced and washed with solution containing 300 mM sucrose, 10 mM HEPES, and 0.2 mM EDTA (pH 7.2). 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 HEPES, 0.2 mM EDTA, and 1 mg/ml bovine serum albumin (pH 7.4). The above procedures were performed at 4 °C.

Isolation of Bovine Heart Submitochondrial Particles—Submitochondrial particles were isolated from bovine heart as described previously (19). In brief, mitochondria were isolated essentially as described by Smith (20) and resuspended in solution containing 250 mM sucrose, 10 mM potassium phosphate, 10 mM Tris-HCl, 2 mM $MgCl_2$, and 2 mM EGTA (pH 7.4). The mitochondria were subsequently disrupted in an ice bath using a Branson 250 sonifier (Branson Ultrasonics, Danbury, CT). The sonicated suspension was centrifuged at 10,000 × *g* for 10 min, then the supernatant was centrifuged at 100,000 × *g* for 45 min at 4 °C, and finally, the pellet was suspended in solution containing 75 mM sodium phosphate, 1 mM EDTA, and 1 mM $MgCl_2$ (pH 7.4). The preparation was stored in liquid nitrogen.

Isolation of Rat Ventricular Myocytes—Myocytes were isolated from collagenase digested hearts as described previously (7). Rats were anesthetized with desflurane in air and decapitated, and the hearts were excised and perfused via the aorta at 37 °C. The coronary arteries were initially perfused with solution containing 115 mM NaCl, 5.4 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 15 mM $NaHCO_3$, 5 mM HEPES, 16 mM taurine, 5 mM sodium pyruvate, 1 mM $CaCl_2$, and 5 mM glucose (pH 7.4) and then perfused with Ca^{2+}-free solution containing 1 mM EGTA followed by perfusion for 6–8 min with solution containing collagenase (type I, Sigma), 0.1% bovine serum albumin, and ~50 μM Ca^{2+}. After enzyme digestion, hearts were cut into small pieces and washed, and cells were dispersed into a solution containing 45 mM KCl, 70 mM potassium glutamate, 3 mM $MgSO_4$, 15 mM KH_2PO_4, 16 mM taurine, 10 mM HEPES, 0.5 mM EGTA, and 10 mM glucose (pH 7.4). Experiments were performed at room temperature using a solution containing 140 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 5 mM HEPES, 1 mM $CaCl_2$, and 10 mM glucose (pH 7.4). Glucose was omitted from the solution in selected experiments.

Flavoprotein Fluorescence Measurements—Isolated myocytes were placed on glass coverslips, which formed the base of a Perspex bath (volume ~ 100 μl), located on the stage of an inverted microscope (Diaphot 300, Nikon). A single cell was continually excited at 488 nm, and flavoprotein fluorescence was recorded at 530 ± 15 nm. The sampling rate was either 1 or 2.5 Hz.

Measurement of Reactive Oxygen Species—Isolated mitochondria were loaded with 2′,7′-dichlorodihydrofluorescein (H_2DCF) using its diacetate ester (H_2DCF-DA), which was dissolved in dimethyl sulfoxide. Mitochondria (500 μg of protein in the case of RHM or 60–80 μg of protein in the case of rat liver mitochondria) were incubated with 10 μM H_2DCF-DA at 30 °C in 2 ml of buffer containing 10 mM Tris-HCl, 10 mM potassium phosphate (or sodium phosphate), 10 mM $MgSO_4$, 100 μM ATP (unless stated otherwise), and 2 mM EDTA (pH 7.0). Oxidation of H_2DCF was monitored using a Shimadzu RF-5001 PC spectrofluorophotometer with the following settings: excitation 488 nm (3-nm slit), emission 525 nm (5-nm slit), and 1-Hz readout rate. The intactness of mitochondria was assessed by determination of respiratory control.

Generation of superoxide ($O_2^{\cdot-}$) was detected indirectly by quantitatively measuring [H_2O_2] with Amplex Red (Molecular Probes, Leiden, The Netherlands). Superoxide dismutates to H_2O_2, a reaction that occurs spontaneously, but it is also catalyzed by matrix superoxide dismutase. H_2O_2 reacts with Amplex Red in the presence of horseradish peroxidase and produces the fluorescent oxidation product resorufin, which has maximum absorption at 563 nm (21). To measure [H_2O_2], submitochondrial particles were used at a final concentration of 0.4 mg/ml, and the 200-μl reaction mixture contained 50 μM Amplex Red, 0.1 units/ml horseradish peroxidase, and buffer solution containing 75 mM sodium phosphate, 1 mM EDTA, and 1 mM $MgCl_2$ (pH 7.4). Absorbance at 560 nm was measured in a SpectraMax Plus^{384} microplate reader (Molecular Devices, Ismaning, Germany). In another series of experiments, 60 μl (~320 μg of protein) of rat heart mitochondria suspension was added to a 2-ml (total) reaction mixture containing 20 μM Amplex Red, 0.01 units/ml horseradish peroxidase, 200 mM sucrose, 10 mM Tris-HCl, 10 mM potassium phosphate, 10 mM $MgSO_4$, 100 μM ATP (unless stated otherwise), and 2 mM EDTA (pH 7.0). The mixture was excited at 560 nm (3-nm slit), and fluorescence was detected at 590 nm (3-nm slit). Standard curves were made by adding known amounts of H_2O_2 (supplemental Fig. 1). Substrates were added from concentrated stocks, and experiments were performed at 30 °C.

Cytochrome bc_1 Complex—Cytochrome bc_1 complex was purified from bovine heart mitochondria as described by Engel *et al.* (22), and the concentration was determined spectroscopically using a specific absorption coefficient for cytochrome *b* (at 562 nm) of 28.5 $mM^{-1} \cdot cm^{-1}$. Before ROS were measured from cytochrome bc_1 complex (complex III), the enzyme was

mixed with solubilized phospholipids (75% phosphatidylcholine (99% type III-E), 20% phosphatidylethanolamine (98% type IV-S), and 5% cardiolipin (from bovine heart, purity > 80%) dissolved in 1.7% sodium cholate and 2.4% *n*-octyl-β-D-glucopyranoside) at a molar ratio of 1:3000. To avoid interference from cytochrome *c* reduction, the oxidation product of Amplex Red (resorufin) was detected at 540 nm.

Succinate Oxidase Activity—Submitochondrial particles (10.2 μg of protein) were added to a 2-ml reaction mixture containing 75 mM sodium phosphate, 1 mM $MgCl_2$, 1 mM EDTA, and 5 mM sodium succinate (pH 7.4). Oxygen consumption was measured at 25 °C using an Oxygraph-2k system (Oroboros).

Statistical Analysis—An analysis of variance was used to determine statistical significance at the 0.95 level of confidence. Data are expressed as mean ± S.D., unless stated otherwise.

RESULTS

Weak Uncoupling Action of Diazoxide and Inhibition of Succinate Oxidase—Diazoxide is thought to activate mitochondrial K_{ATP} channels, causing accelerated respiratory chain activity as assessed by flavoprotein fluorescence measurements (5). We therefore examined the effects of diazoxide on state 4 respiration of intact liver mitochondria in the presence of Mg^{2+} and ATP, inhibitors of mitochondrial K_{ATP} channels (Fig. 1*A*). To circumvent the confounding inhibitory effect of diazoxide on succinate dehydrogenase, we used malate/glutamate as substrate in these experiments, which supplies NADH to complex I of the respiratory chain. In addition, we included the ATP synthase inhibitor oligomycin because the partial degradation of ATP to ADP might stimulate respiration. Diazoxide induced a concentration-dependent increase in state 4 respiration (Fig. 1*A*). The addition of valinomycin further increased the respiratory rate (Fig. 1*A*).

The observed weak stimulatory effect of diazoxide on respiration could, in principle, be attributed to activation of mitochondrial K_{ATP} channels. In that case, the stimulatory effect of diazoxide should be absent in K^+-free medium. However, as shown in Fig. 1*B*, diazoxide similarly stimulated respiration when K^+ was replaced with Na^+. Hence, the weak stimulatory effect of diazoxide does not fit with the activation of K^+-selective channels, but rather, it is compatible with a protonophoric uncoupling action, consistent with the earlier observations of Portenhauser *et al.* (23) and more recent reports (24, 25).

To scale the uncoupling action of diazoxide, we compared it with the protonophoric uncouplers carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and 2,4-dinitrophenol (DNP) under ATP-free conditions, when the mitochondrial K_{ATP} channel should be fully activated and not responsive to K^+ channel openers (17, 26). Diazoxide produced a weak concentration-dependent increase in respiratory rate (Fig. 1*C*), whereas FCCP (Fig. 1*C*) or DNP (not shown) produced a much stronger increase in the respiratory rate.

Using bovine heart submitochondrial particles, we confirmed that diazoxide, a blocker of succinate dehydrogenase (7), inhibited succinate oxidase (Fig. 1*D*). In comparison with the inhibitor malonate (not shown) or oxaloacetate (Fig. 1*D*), which

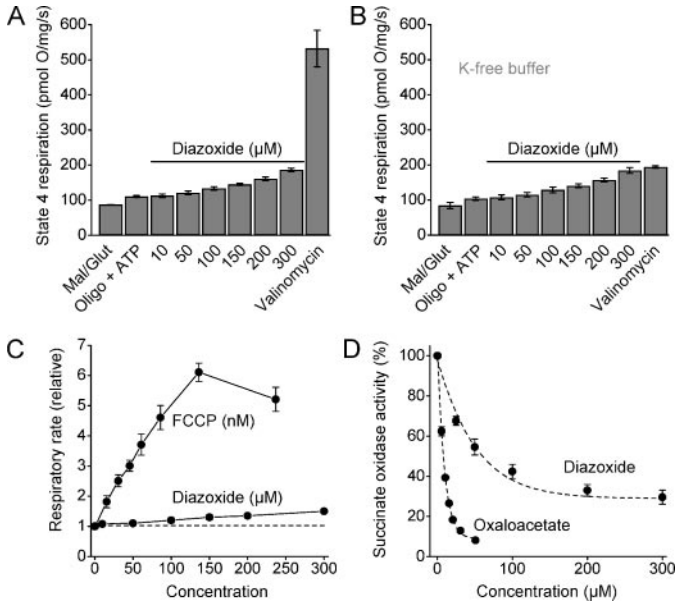

FIGURE 1. **Diazoxide increases state 4 respiration independent of K^+ influx and inhibits succinate dehydrogenase.** *A*, in the presence of oligomycin (ATP synthase inhibitor) and Mg^{2+}/ATP, the putative mitochondrial K_{ATP} channel opener diazoxide weakly increased state 4 respiration in coupled mitochondria isolated from rat liver. The K^+ ionophore valinomycin induced a strong respiratory response. *Mal/Glut*, malate/glutamate. *B*, cumulative concentration-response relation of diazoxide in K^+-free medium. Diazoxide increased respiration, and as expected, addition of valinomycin caused no further increase in respiration. *C*, diazoxide weakly (compared with the protonophore FCCP) increased respiration in ATP-free medium. *D*, diazoxide partially inhibited succinate dehydrogenase of submitochondrial particles, whereas oxalacetate was essentially a full inhibitor. In all cases, data are means of duplicate or quadruplicate determinations and are representative of at least three independent preparations.

almost completely inhibited catalytic turnover, 300 μM diazoxide only reduced succinate oxidase activity to ~30% (Fig. 1*D*).

Decanoate Reverses Diazoxide-induced Flavoprotein Oxidation in Glucose-free Medium—When diazoxide (100 μM) was introduced to an isolated rat ventricular myocyte, a small increase in flavoprotein fluorescence was observed (Fig. 2*A*), consistent with a weak uncoupling action. To calibrate the response, the potent uncoupler DNP was used to elicit maximal flavoprotein fluorescence (5). Note that oxidized, but not reduced, flavoproteins are strongly fluorescent. Because the diazoxide-induced flavoprotein oxidation reported by Liu *et al.* (5) was greatly potentiated in glucose-free medium,[3] we reexamined the effects of diazoxide using glucose-free superfusates. In accordance with Liu *et al.* (5), we found that diazoxide-induced flavoprotein oxidation was markedly potentiated in glucose-free medium (Fig. 2). On average, diazoxide increased flavoprotein fluorescence to 9.2 ± 1.8% ($n = 5$) of the maximum in the presence of glucose (Fig. 2*A*). In the absence of glucose, diazoxide increased flavoprotein fluorescence to 24 ± 5% ($n = 5$) of the maximum after 3-min exposure. Because the effect of diazoxide on flavoprotein fluorescence did not appear to have saturated at this time (Fig. 2*B*), we had probably underestimated the extent of potentiation under glucose-free conditions.

Liu *et al.* (5) also reported that 5-HD inhibits diazoxide-induced flavoprotein oxidation. However, we now know that

[3] Brian O'Rourke, personal communication.

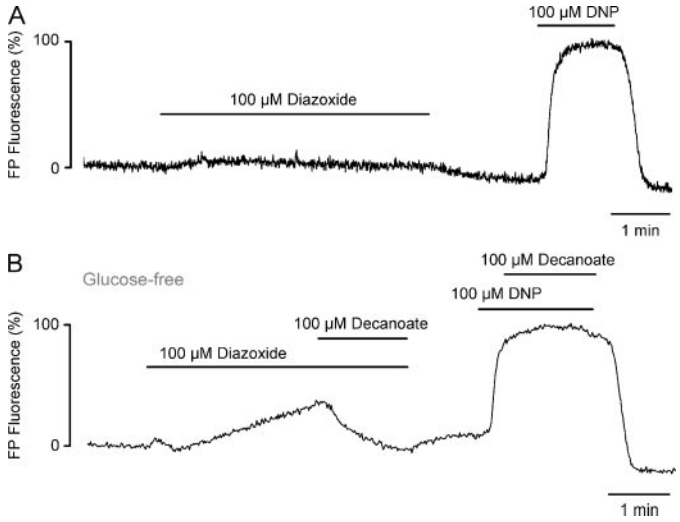

FIGURE 2. **Diazoxide-induced flavoprotein oxidation is inhibited by decanoate.** *A*, diazoxide (100 μM) caused a small increase in flavoprotein fluorescence, measured at 2.5 Hz in a single myocyte. DNP was used to scale the response. *B*, diazoxide-induced flavoprotein oxidation was potentiated in glucose-free medium (fluorescence was recorded at 1 Hz rather than 2.5 Hz, accounting for the increased signal-to-noise ratio). Addition of 100 μM decanoate inhibited the diazoxide-induced response but not DNP-induced flavoprotein oxidation.

5-HD is partially metabolized by the β-oxidation pathway (9, 10), which could affect the redox state of various flavoproteins. Indeed, we found that the increase in flavoprotein fluorescence produced by diazoxide was reversed by decanoate (Fig. 2*B*), a medium-chain fatty acid, which is more efficiently metabolized than 5-HD (10).

Independent of K_{ATP} Channels, Diazoxide Induces H_2DCF Oxidation in Liver Mitochondria—Isolated mitochondria were loaded with the nonfluorescent probe H_2DCF using its membrane-permeable diacetate form, which was subjected to hydrolysis by matrix esterases. After loading, a nonlinear increase in fluorescence intensity as a function of time was observed (Fig. 3*A*), presumably because of the basal production of ROS, which oxidized H_2DCF to the fluorescent compound 2′,7′-dichlorofluorescein (DCF). Addition of diazoxide increased DCF fluorescence in a concentration-dependent fashion, even in the absence of ATP (Fig. 3*A*). Similar results were obtained in four different mitochondrial preparations.

To test the possibility that weak protonophoric uncoupling by diazoxide might induce H_2DCF oxidation, we added the uncoupler FCCP at a concentration (15 nM) that stimulated respiration by about 2.4-fold (see *inset*, Fig. 3*B*). As shown in Fig. 3*B*, weak protonophoric uncoupling decreased, rather than increased, DCF fluorescence. We next investigated whether mitochondrial K^+ influx was necessary for diazoxide-induced H_2DCF oxidation. This was not the case because diazoxide similarly increased DCF fluorescence in the presence (Fig. 3*C*) or absence (Fig. 3*D*) of K^+ in the medium. Similar results were obtained in three different mitochondrial preparations.

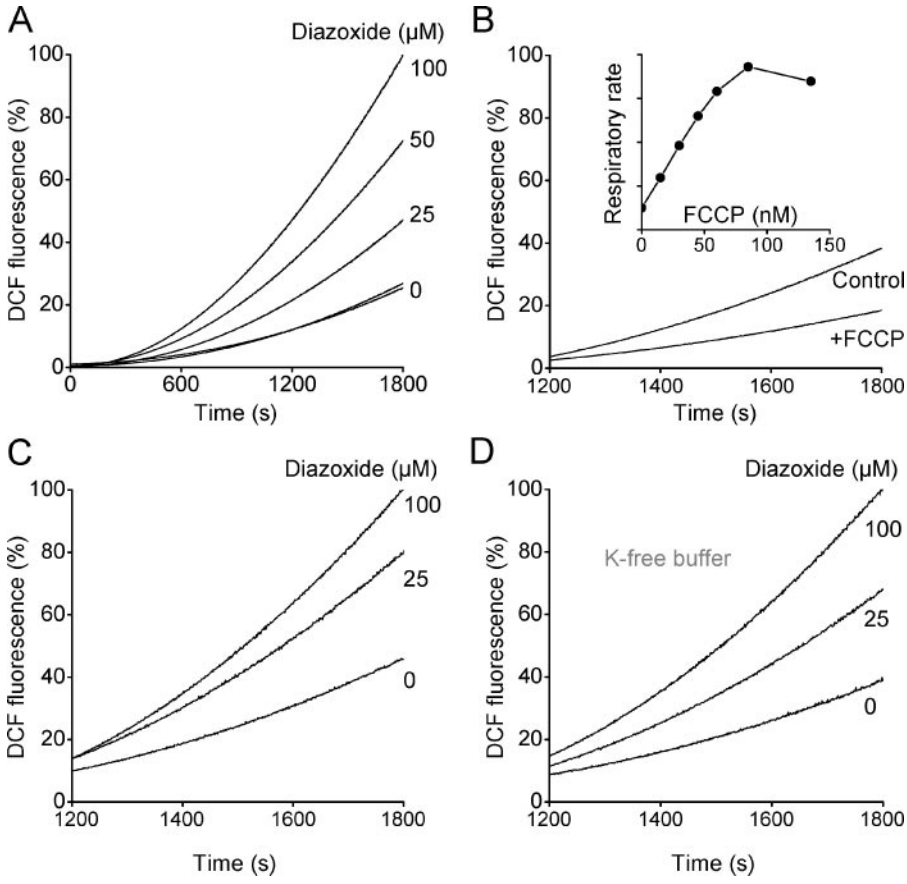

FIGURE 3. **The effect of diazoxide on H_2DCF oxidation in isolated rat liver mitochondria.** *A*, diazoxide increased H_2DCF oxidation, indexed as DCF fluorescence, in a concentration-dependent fashion (the medium was ATP-free). *B*, compared with control conditions, addition of 15 nM FCCP (indicated by *+FCCP*) decreased DCF fluorescence. In parallel experiments, we found that this concentration (15 nM) of FCCP stimulated respiration 2.4-fold (see *inset*). *C* and *D*, using the same batch of mitochondria, diazoxide increased DCF fluorescence similarly in the presence (*C*) or absence (*D*) of K^+ in the medium, which contained 10 mM Mg^{2+} and 100 μM ATP. Note that each of the above recordings (experimental protocols) in *A–D* are representative of 6–8 independent experiments (using 3–4 separate mitochondrial preparations).

Diazoxide Increases H_2DCF Oxidation in Heart Mitochondria in a Stigmatellin-sensitive Fashion—Similar to rat liver mitochondria, diazoxide increased H_2DCF oxidation in intact rat heart mitochondria (Fig. 4*A*). The cytochrome bc_1 complex inhibitor stigmatellin, which blocked the stimulatory effect of antimycin A (not shown), also blocked the oxidative activity induced by diazoxide (Fig. 4*A*). Addition of diazoxide to purified cytochrome bc_1 complex, though, did not cause H_2DCF oxidation (not shown). Interestingly, the increased H_2DCF oxidation induced by antimycin A and diazoxide in intact mitochondria was additive. As shown in Fig. 4*B*, the heart mitochondria used in these experiments were well coupled. Addition of ADP stimulated respiration 8-fold, and 15 nM FCCP increased respiration

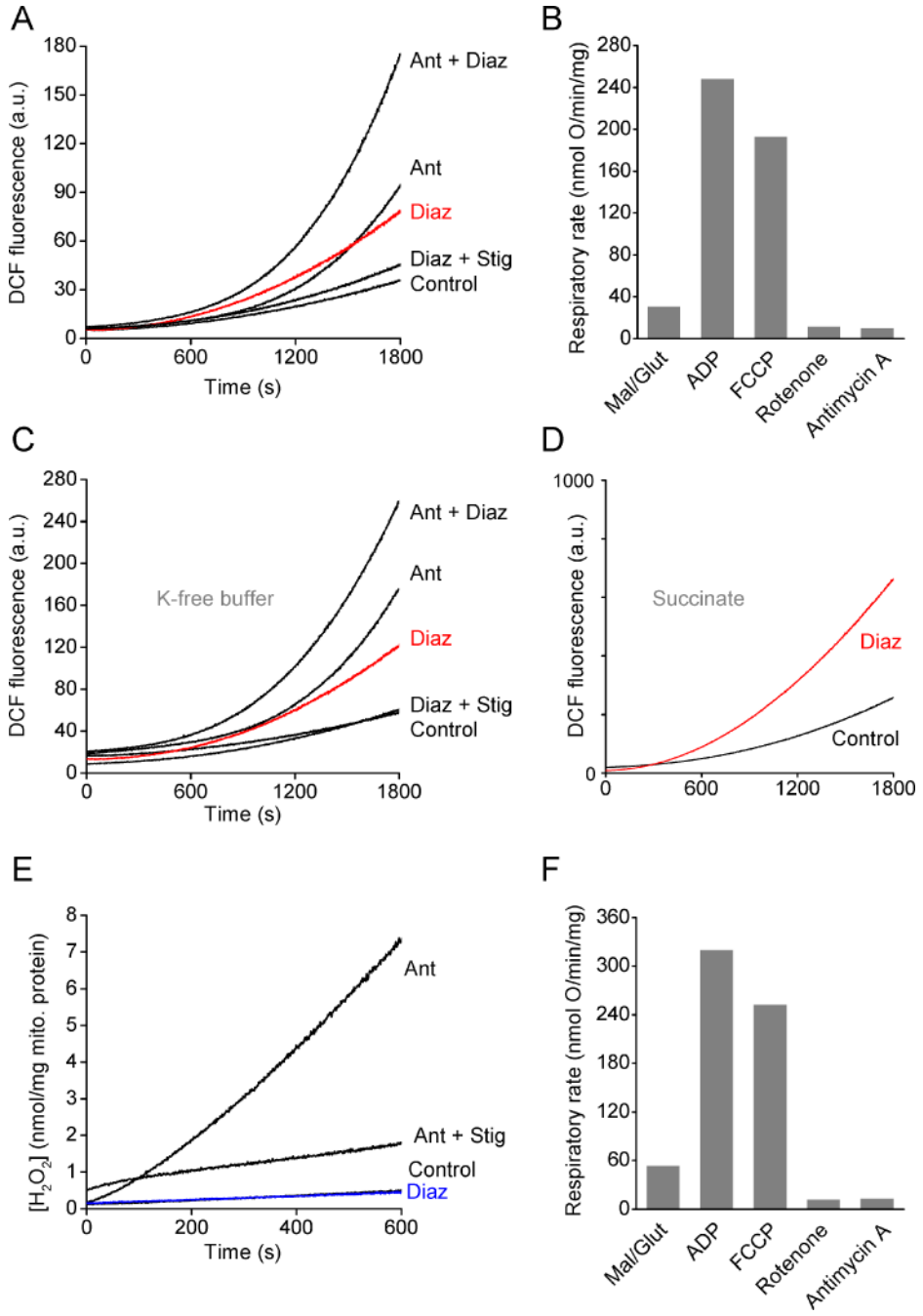

FIGURE 4. **Diazoxide induces H_2DCF oxidation but not H_2O_2 production in rat heart mitochondria.** *A*, the increase in DCF fluorescence evoked by 100 μM diazoxide (*Diaz*) was blocked by 10 μM stigmatellin (*Stig*). Antimycin A (*Ant*; 10 μM), in contrast, had an additive effect on diazoxide-induced DCF fluorescence. Sodium malate/sodium glutamate (5 mM/5 mM) was used as substrate. *B*, in the same mitochondrial preparation as in *A*, with malate/glutamate (*Mal/Glut*) as substrate, addition of 0.5 mM ADP or 15 nM FCCP strongly increased the rate of respiration, indicating that the mitochondria were well coupled. Note that sodium succinate was supplied (not shown) before addition of antimycin A. *C*, diazoxide-induced H_2DCF oxidation was also observed in K^+-free buffer. *D*, diazoxide-induced oxidation of H_2DCF was similarly observed when sodium succinate was used instead of malate/glutamate as substrate. *E*, diazoxide (100 μM) did not increase H_2O_2 production (quantified using Amplex Red) by mitochondria, whereas antimycin A produced a strong positive response, which was sensitive to stigmatellin (substrate, sodium succinate; similar results were obtained with malate/glutamate as substrate). *F*, oxygen consumption measurements performed using the same mitochondrial preparation as in *E*. Note that sodium succinate was supplied (not shown) before addition of antimycin A. In each case, the data (experimental protocols) in *A–F* are representative of duplicate or triplicate recordings performed using 2–5 separate mitochondrial preparations.

6-fold. Moreover, Fig. 4*C* shows that the stimulatory effect of diazoxide on H_2DCF oxidation persisted in K^+-free buffer and was therefore independent of the opening of K_{ATP} channels.

When malate/glutamate was substituted for succinate, diazoxide similarly induced the oxidation of H_2DCF (Fig. 4*D*).

H_2DCF primarily detects hydroxyl radicals and also peroxynitrite anions (27), but it is insensitive to H_2O_2. We therefore determined the effect of diazoxide on H_2O_2 production, which is upstream to hydroxyl radical generation (via the Fenton or Haber-Weiss reactions).

Diazoxide Does Not Increase H_2O_2 Production in Intact Rat Heart Mitochondria—When malate/glutamate was used as a substrate, the addition of 10 μM antimycin A to intact rat heart mitochondria increased extramitochondrial [H_2O_2], and this response was blocked by stigmatellin (Fig. 4*E*). However, neither 100 μM diazoxide (Fig. 4*E*) nor 500 nM valinomycin (not shown) increased H_2O_2 production. Similar results were obtained when sodium succinate was used as substrate; antimycin A increased H_2O_2 production, whereas diazoxide and valinomycin had no effect (not shown). Parallel measurements of oxygen consumption (Fig. 4*F*) indicated that the mitochondria used for H_2O_2 measurements were well coupled.

Diazoxide Does Not Evoke H_2O_2 from Submitochondrial Particles and Purified Cytochrome bc_1 Complex—We tested whether diazoxide stimulated ROS production when substrates were delivered directly to the respiratory chain of bovine heart submitochondrial particles. The basal rate of H_2O_2 production, measured using Amplex Red in the presence of horseradish peroxidase, was low using substrate for complex II (succinate), whereas the addition of antimycin A increased H_2O_2 production from 8 ± 8 (n = 9) to 560 ± 60 pmol/mg/min (n = 9), a 70-fold increase (Fig. 5*A*). The stimulatory effect of antimycin A was blocked by stigmatellin but unaffected by the complex I blocker rotenone, confirming that cytochrome bc_1 complex was the source of the ROS. As shown in Fig. 5*A*, addition of diazoxide (n = 9) had no significant effect on the basal rate of production of ROS. Malonate, which com-

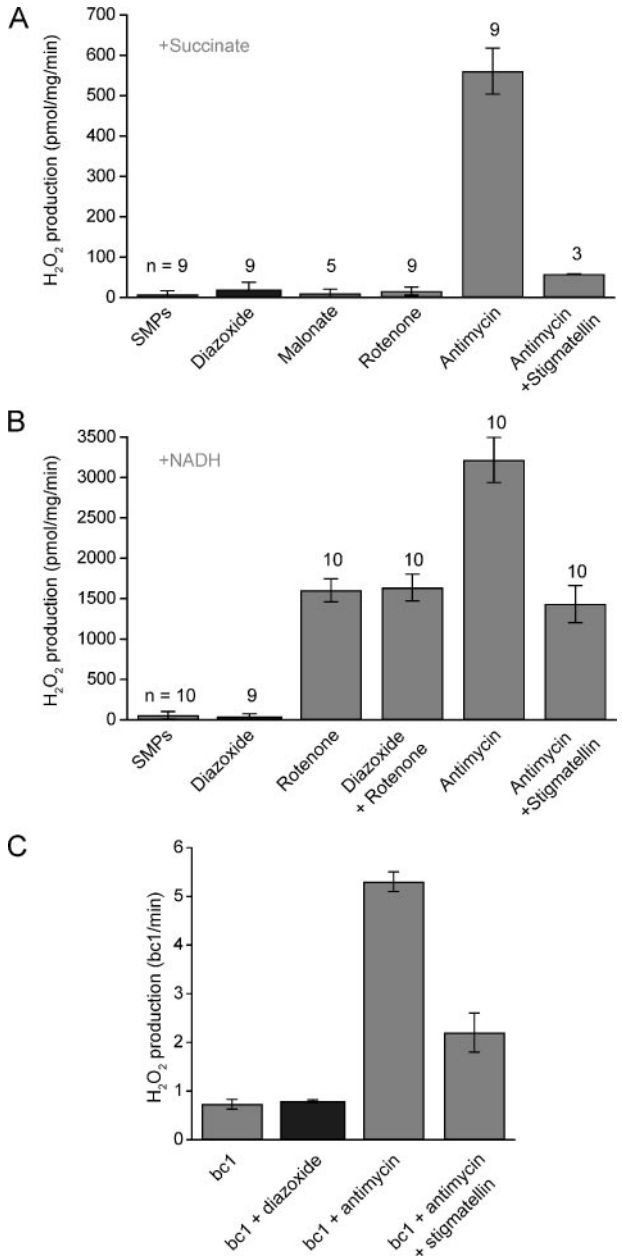

FIGURE 5. **Diazoxide does not evoke H_2O_2 in submitochondrial particles and purified cytochrome bc_1 complex.** *A*, when sodium succinate (substrate for complex II) was supplied to bovine heart submitochondrial particles (*SMPs*), H_2O_2 production was markedly increased by antimycin A in a stigmatellin-sensitive manner. Diazoxide (100 μM) had no significant effect on H_2O_2 production. *B*, NADH was supplied as substrate for complex I. Inhibition of complex I (rotenone) or complex III (antimycin A) evoked a profound increase in H_2O_2 production, whereas diazoxide had no effect. Diazoxide did not potentiate rotenone-induced H_2O_2 production. *C*, diazoxide did not directly evoke H_2O_2 from cytochrome bc_1 (complex III) of the respiratory chain (data are the means of three independent measurements).

petitively inhibits substrate binding of complex II, also did not generate ROS ($n = 5$).

When the complex I substrate NADH was used (Fig. 5*B*), rotenone increased H_2O_2 production from 57 ± 45 ($n = 10$) to 1604 ± 143 pmol/mg/min ($n = 10$), and antimycin A produced an even stronger response, increasing the basal rate about 56-fold ($n = 10$). Diazoxide (100 μM) had no effect ($n = 9$), and moreover, the presence of diazoxide did not affect rotenone-induced ROS production ($n = 10$).

In another series of experiments, we tested whether diazoxide could elicit ROS from cytochrome bc_1 complex in submitochondrial particles when electrons were supplied to this complex directly by decylubiquinol. Antimycin A increased H_2O_2 production from 108 ± 17 to 14,506 ± 101 pmol/mg/min ($n = 3$) when decylubiquinol was supplied as substrate, whereas diazoxide had no effect on H_2O_2 production (supplemental Fig. 2). Consistent with experiments using submitochondrial particles, diazoxide had no effect on H_2O_2 production by purified cytochrome bc_1 complex, whereas antimycin A elicited a strong (positive control) response (Fig. 5*C*).

DISCUSSION

ATP-sensitive K^+ channels in the mitochondrial inner membrane are thought to play a central role in the signal transduction mechanisms of ischemic and pharmacological preconditioning. In this study, our data obtained using various mitochondrial preparations and appropriate controls bring into question the paradigm that diazoxide triggers preconditioning by activating mitochondrial K_{ATP} channels.

Flavoprotein Oxidation—Flavoprotein fluorescence measurements have been widely used to assay mitochondrial K_{ATP} channel activity in intact myocytes (2, 5). However, this experimental approach has many limitations (28), and in the case of diazoxide, the increase in flavoprotein fluorescence can be attributed to inhibition of succinate dehydrogenase (7), protonophoric uncoupling (25), opening of mitochondrial K_{ATP} channels, or even transient opening of the mitochondrial permeability transition pore (29). The stimulatory effect of diazoxide on flavoprotein oxidation is reversed by 5-HD, supporting the conjecture that 5-HD is a selective mitochondrial K_{ATP} channel blocker (1–3, 5). However, we and others have shown that 5-HD is activated and metabolized by the β-oxidation pathway (7–10), and now we report that decanoate, a medium-chain fatty acid structurally related to 5-HD, reverses diazoxide-induced flavoprotein oxidation. We speculate that the ability of 5-HD to reverse flavoprotein oxidation is not because of K_{ATP} channel inhibition, but rather 5-HD induces a switch from glucose to fatty acid metabolism.

Uncoupling Action of Diazoxide—In the presence of Mg^{2+} and ATP, cardioprotective concentrations of diazoxide have been reported to stimulate mitochondrial oxygen consumption by about 20%, secondary to the opening of mitochondrial K^+ channels (24, 30). However, we found that 100 μM diazoxide increased state 4 respiration by about 20% in intact mitochondria, whether or not Mg^{2+} and ATP were present to inhibit mitochondrial K_{ATP} channels. The stimulatory effect of diazoxide was also observed in K^+-free medium. These data essentially rule out involvement of K_{ATP} channels. Diazoxide probably acts simply as a weak protonophoric uncoupler in accordance with previous work (23–25).

Diazoxide and ROS Production—Diazoxide is thought to activate survival kinase pathways through the generation of ROS, evoked by the opening of mitochondrial K_{ATP} channels (17, 26, 30). In support of this hypothesis, diazoxide has been shown to increase H_2DCF oxidation (indexed as DCF fluorescence) in rat ventricular myocytes, and 5-HD or antioxidants have been shown to abolish diazoxide-induced preconditioning

(13). We found, indeed, that diazoxide increased H_2DCF oxidation in intact liver and heart mitochondria. However, diazoxide-induced H_2DCF oxidation was observed in K^+-free medium, ruling out a mechanistic link with K^+ influx. Moreover, diazoxide increases H_2DCF oxidation in ATP-free media, when mitochondrial K_{ATP} channels should be already opened (31).

Diazoxide-induced oxidation of H_2DCF was observed when malate/glutamate was used as substrate, which bypassed inhibition at succinate dehydrogenase, ruling out an obvious role for complex II. The effect of diazoxide, however, was almost completely blocked by stigmatellin, suggesting a role for the cytochrome bc_1 complex. However, when we used purified enzyme in the presence of decylubiquinol, we found that diazoxide did not increase the rate of oxidation of H_2DCF.

It is important to note that H_2DCF does not detect superoxide or H_2O_2, but instead, it is oxidized by hydroxyl radicals derived from H_2O_2 via the Fe^{2+}-dependent Fenton reaction and to a lesser extent by peroxynitrite anions (derived from nitric oxide) (32). Moreover, H_2DCF can be readily oxidized directly by reduced heme-containing proteins such as cytochrome *c* (33, 34). Using a sensitive assay to quantify H_2O_2 production, we found that diazoxide did not increase H_2O_2 production by purified cytochrome bc_1 complex, submitochondrial particles, or intact mitochondria, whereas the appropriate controls produced robust responses. These data indicate that diazoxide does not generate superoxide from the respiratory chain. Thus, the mechanism by which diazoxide induces H_2DCF oxidation remains a question for future studies.

Conclusions—Data obtained at the level of isolated mitochondria, submitochondrial particles, and purified enzyme indicate that diazoxide does not evoke superoxide (which dismutates to H_2O_2) from electron transport complexes by a direct mechanism, and the stimulatory effects of this compound on mitochondrial respiration and H_2DCF oxidation are not because of the opening of K_{ATP} channels. Moreover, the inhibitory effect of decanoate on diazoxide-induced flavoprotein oxidation supports the notion that 5-hydroxydecanoate acts as a metabolic substrate rather than a K_{ATP} channel inhibitor. Thus, our data bring into question the paradigm that mitochondrial K_{ATP} channels are necessarily involved in the signal transduction mechanisms of preconditioning.

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