Effects of Dipyridamole on Heart Muscle Mitochondria

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SUMMARY

Previously reported beneficial effects of dipyridamole on "damaged" mitochondria have not been confirmed in these experiments. Dipyridamole, in fact, has specific stimulatory or inhibitory effects on mitochondrial respiration depending on which segment of the electron transport chain is predominant. It is postulated that dipyridamole inhibits respiration by accepting electrons from NADH, thus competing with the main electron transport pathway for electrons. The drug is also capable of interacting with a high-energy intermediate when succinate is the substrate, resulting in an "uncoupling-like" action.

INTRODUCTION

In 1959 Hockerts and Bogelman (1) reported that dipyridamole,[1] a coronary vasodilator, partially reversed the decrease in myocardial ATP levels caused by hypoxia. Subsequent studies by Lamprecht (2) demonstrated that dipyridamole elevated ATP and phosphocreatine levels in damaged canine hearts. In 1961, Laudahn (3) reported a direct *in vitro* "beneficial" effect of dipyridamole on mitochondria from heart muscle. In these studies, Laudahn showed that dipyridamole at 10^{-6} M increased phosphorylation in "damaged" heart mitochondria without affecting oxygen consumption. He reported an 84% increase in P:O ratios compared to controls. Higher concentrations of dipyridamole produced an uncoupling of oxidative phosphorylation. In this study, Laudahn "damaged" the mitochondria by water lysis and equated these mitochondria to those isolated from hypoxic myocardium. No data on respiratory control were reported.

This study is concerned with the detailed effects of dipyridamole on mitochondrial respiratory control and oxidative phosphorylation from normal rat hearts, osmotically shocked mitochondria, and mitochondria obtained from rats with experimentally induced myocardiopathy (4).

[1] Dipyridamole: 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-(5,4-*d*)pyrimidine (Persantin®)

METHODS AND MATERIALS

Isolation of heart mitochondria. Rat heart mitochondria were isolated by a modification of the method of Tarjan and Von Korff (5). Rats were decapitated, the ventricles quickly excised and placed in an ice-cold solution of: 0.18 M KCl, 10 mM EDTA,[2] 1% bovine serum albumin (Fraction V, Sigma Chemical Co.), pH 7.4. This solution (Isolation Medium) was used throughout the isolation procedure and in the final suspension of the mitochondria. The ventricles were blotted, weighed, and placed in a homogenizing vessel (A. H. Thomas, size C) with 12 volumes of isolation medium per gram of tissue. The tissue was homogenized for 3–4 seconds with a

[2] Abbreviations used: ethylenediamine tetraacetate (EDTA), tris(hydroxymethyl)aminomethane (Tris), adenosine diphosphate (ADP), 2,4-dinitrophenol (2,4-DNP), reduced dihydronicotinamide adenine dinucleotide (NADH).

Polytron PT-20 tissue processor[3] at a rheostat setting of two. This instrument combines a shearing action with sonication. A motor-driven Teflon pestle was then passed through the homogenate twice to ensure complete cellular disruption. The homogenate was centrifuged at 700 *g* for 10 min to remove heavier particles. The supernatant was centrifuged at 6000 *g* for 12 min. The resulting mitochondrial pellet was then resuspended in the isolation medium and centrifuged again at 6000 *g* for 8 min. This washing procedure was repeated once. The mitochondria were finally suspended in Isolation Medium at a protein concentration of 20–30 mg/ml.

Assay procedure. Mitochondrial oxygen consumption was measured with an Oxygraph (Gilson Medical Electronics). The assay medium contained: 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 8.5 mM K_2HPO_4, and 5 mM Tris-glutamate or Tris-succinate; 0.1 ml of mitochondrial suspension was added yielding a final protein concentration of 1.0–1.5 mg/ml in the reaction chamber. Approximately 500 mμ-moles of ADP in 0.1-ml aliquots was added to produce state 3 respiratory "bursts" as described by Chance and Williams (6). The concentrations of the ADP solutions were estimated by measurement at 260 mμ in a Beckman DU-2 spectrophotometer, utilizing a millimolar extinction coefficient of 13.5. An enzymatic analysis (7) of ADP concentration was also done and found to be in good agreement with the direct spectrophotometric determinations. Dipyridamole was added as a 95% methanolic solution; appropriate methanol controls were employed throughout the experiments.

Isoproterenol treatment. Experimental myocardiopathy in 180–200 g white male rats was produced by the procedure of Rona *et al.* (4). This involves subcutaneous injections of isoproterenol in doses of 80 mg/kg twice daily for 2 days. The resulting myocardial damage varied from necrosis at the apex of the heart to extensive necrosis in the walls of both ventricles. Saline-injected animals were used as controls.

[3] Polytron PT-20 tissue processor, Brinkmann Instruments, Westbury, New York.

Materials. Biochemical reagents were obtained from the Sigma Chemical Co., St. Louis, Missouri. Dipyridamole was generously supplied by Geigy Pharmaceuticals, Ardsley, New York. Isoproterenol was purchased from Winthrop Laboratories, New York. All other chemicals were of analytical reagent grade quality.

RESULTS

Mitochondrial Oxidative Phosphorylation

The results of two experiments (I and II) employing normal heart mitochondria and mitochondria from rats with myocardial necrosis (isoproterenol-treated) are shown in Table 1. The respiratory control index (RCI) and ADP:O ratios of mitochondria from the isoproterenol-treated animals appear to be slightly depressed. The reasons for this isoproterenol effect are as yet unclear; further work is in progress. Respiration, with glutamate as substrate, in the presence of ADP (state 3) is reduced approximately 40–50% as compared to the control mitochondria. The subsequent *in vitro* addition of dipyridamole in concentrations reported (3) to be beneficial to impaired mitochondria (10^{-6} M–10^{-5} M) served only to further depress the respiratory control index as well as the rate of oxygen consumption. The effects of various concentrations of dipyridamole on normal mitochondria are indicated in Table 1, experiment III, and in Table 2. The unusual increase in RCI at 10^{-5} M dipyridamole in experiment III, Table 1, is due to depression of the state 4 oxidation rate. There is a striking difference of "high" concentrations of dipyridamole on the NADH-linked (glutamate) side of the electron transport chain as compared to the succinate-linked segment. The latter becomes *uncoupled* while NADH-linked respiration appears to be *inhibited.* In an attempt to duplicate the results reported by Laudahn (3), normal mitochondria were suspended in water at 2° and allowed to stand for 5 min. They were then centrifuged at 8000 *g* for 15 min, and the resulting pellet was resuspended in Isolation Medium. Figure 1 shows an oxygen-elec-

TABLE 1

Effect of dipyridamole on oxidative phosphorylation in mitochondria from normal and isoproterenol-treated rats

Assay described under Methods. Respiratory control index (RCI) is the ratio of oxygen uptake in the presence of ADP (state 3) to that in the absence of ADP (state 4). ADP:O is the ratio of added ADP to the amount of oxygen consumed during phosphorylation of the ADP. The Q_{O_2} is expressed as oxygen uptake in mμ atoms O/min/mg protein during state 3 respiration. Glutamate and succinate are the substrates. Dipyridamole (Dipy.) additions are indicated as the final concentration in the reaction chamber. The figures in parentheses indicate the number of determinations; data are averaged.

Experiment + final molarity in samples	Glutamate			Succinate		
	RCI	ADP:O	Q_{O_2}	RCI	ADP:O	Q_{O_2}
I						
A. Control (2)	12.2	2.76	32.4	4.4	1.94	29.5
B. Isoproterenol-treated (2)	11.8	2.44	16.1	3.9	1.85	18.0
$+10^{-6}$ M Dipy.	5.6	2.46	17.0	—	—	—
$+10^{-4}$ M Dipy.	2.8	2.41	10.6	—	—	—
II						
A. Control (2)	15.7	3.12	38.9	3.4	2.14	39.6
B. Isoproterenol-treated (2)	10.0	2.94	17.5	3.1	2.16	20.0
$+5 \times 10^{-6}$ M Dipy.	6.8	2.95	15.3	3.2	1.77	28.1
III						
A. Control (4)	14.3	2.94	37.8	3.7	1.63	48.0
$+5 \times 10^{-6}$ M Dipy.	14.7	2.87	44.0	—	—	—
$+10^{-5}$ M Dipy.	32.0	2.95	32.5	3.3	1.8	35.0
$+10^{-4}$ M Dipy.	3.0	1.84	13.5	0	0	39.0
$+5 \times 10^{-4}$ M Dipy.	0	0	0	0	0	39.0

trode tracing of these "damaged" mitochondria with glutamate as substrate. A tracing of respiratory activity prior to water treatment is shown in the box in Fig. 1. It can be seen that the respiratory activity and the ADP:O ratios are depressed in the osmotically shocked mitochondria as compared to the control. However, the addition of dipyridamole has no stimulatory effect. Similar results are obtained when succinate is used as substrate. The respiratory control index (RCI) could not be calculated for the osmotically shocked mitochondria because state 4 oxygen consumption was essentially zero. In another experiment, rats were injected with 20 mg/kg of dipyridamole daily for 10 days. Heart and liver mitochondria were then isolated and assayed. No differences in the mitochondria from these animals as compared to controls were found.

Effects on Electron Transport

The marked difference in NADH-linked and succinate-supported respiration, respectively, in the presence of "high" concentrations of dipyridamole prompted a further investigation into the phenomenon. Figure 2A is an oxygen-electrode tracing, with glutamate as substrate, of normal mitochondrial respiration and the subsequent addition of an "inhibitory" amount of dipyridamole. It can be seen that the addition of dipyridamole slightly increases the state 4 rate. Subsequent additions of ADP and 2,4-DNP, however, cause no further increase in oxygen consumption. In Fig. 2B the addition of dipyridamole with succinate as substrate causes a marked *increase* in respiratory rate, and subsequent additions of ADP and 2,4-DNP do not further increase the rate. It appears that dipyridamole produces both a "rotenone-like" inhibition of NADH-linked electron transport and a "2,4-DNP-like" uncoupling with succinate-supported respiration. Figure 3A shows the inhibitory effect of dipyridamole addition during a glutamate-supported state 3 "burst" of respiration. Dipyridamole does not completely inhibit

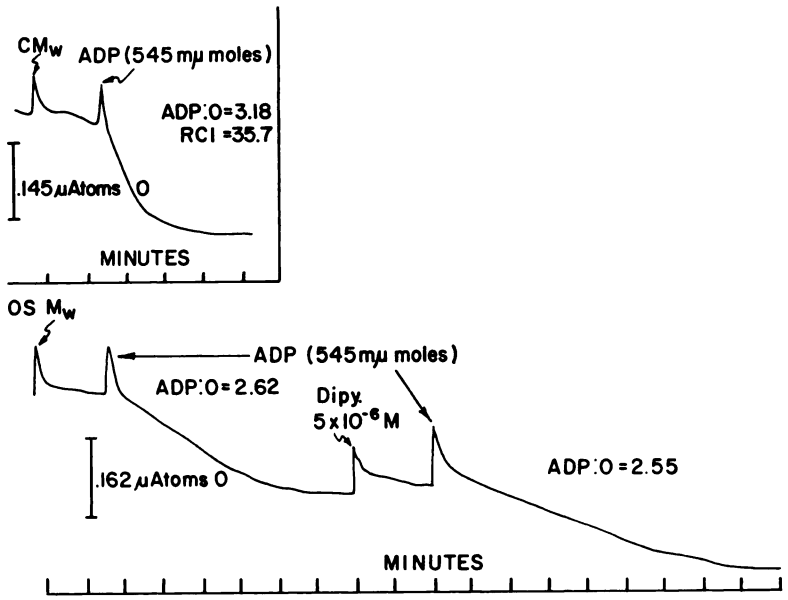

FIG. 1. *Effect of dipyridamole on normal mitochondria treated by water lysis*

Oxygen-electrode tracing of mitochondria subjected to water lysis as described in text. Assay medium described in Methods. ADP:O and RCI defined in Table 1. Dipyridamole (*Dipy.*) addition is expressed as final concentration in reaction chamber. The figure in the upper box is a tracing of the same mitochondrial preparation prior to water treatment. The protein concentrations were identical. Glutamate is the substrate. Abbreviations: control mitochondria (CM_w), osmotically shocked mitochondria ($OS\ M_w$).

respiration as would rotenone (indicated by the dotted line). However, the system loses respiratory control and subsequent additions of ADP have no further effect, as shown in Fig. 2A. Addition of dipyridamole to succinate-supported state 3 respiration has no inhibitory effect; the system becomes uncoupled and subsequent additions of ADP have no further effect. When 2,4-DNP is used to uncouple glutamate-supported respiration, dipyridamole addition inhibits the respiratory rate slightly, but not completely as would rotenone (Fig. 3B). Figure 3C shows the uncoupling effects of dipyridamole when succinate is used as substrate. Subsequent additions of ADP, oligomycin, and 2,4-DNP do not increase or reduce this rapid respiratory rate.

Titration of Dipyridamole

In numerous experiments it was noted that dipyridamole had no effect on mitochondrial respiration below a final concentration of 10^{-5} M. Table 2, experiment I, indicates that respiratory control, ADP:O ratio, and oxygen consumption of normal mitochondria in glutamate-supported state 3 respiration decreases with increasing concentrations of dipyridamole. The most sensitive parameter is respiratory rate. Experiments II and III of Table 2 show that preincubation of the mitochondria with dipyridamole, prior to the first addition of ADP, yields results which are similar to the above. Respiratory control index and ADP:O ratio are measurably affected at higher concentrations of the drug. When succinate is the substrate, changes in all three measured functions are not clearly evident until "high" concentrations (10^{-4} M) of dipyridamole are reached, at which time the mitochondria exhibit complete uncoupling.

DISCUSSION

Under the conditions of the present experiments, we have been unable to dupli-

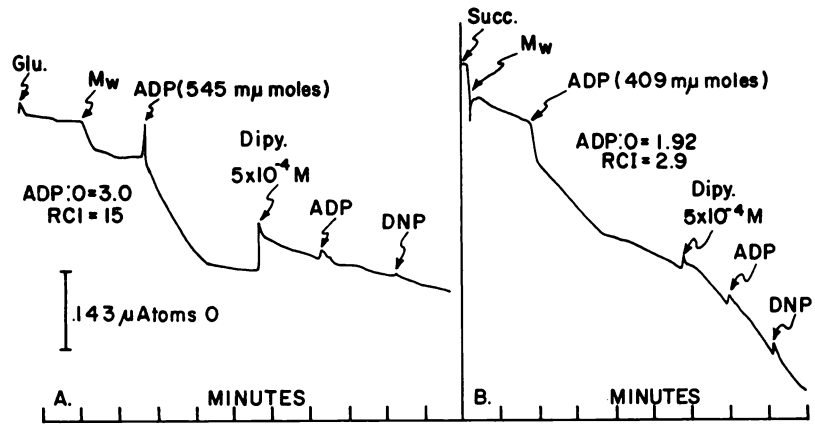

FIG. 2. *Effect of high concentrations of dipyridamole on mitochondrial respiration with glutamate and succinate as substrates*

Oxygen-electrode tracings of normal mitochondria with subsequent additions of dipyridamole. Assay medium described under Methods. ADP:O and RCI defined in Table 1. Dipyridamole additions are indicated as final concentrations in reaction chamber. Abbreviations: glutamate (*Glu.*), succinate (*Succ.*), mitochondria (M_W), dipyridamole (*Dipy.*), 4.75×10^{-5} M 2,4-dinitrophenol (*DNP*).

cate the *in vitro* stimulatory effects of dipyridamole on "damaged" mitochondria as reported by Laudahn (3). We are in agreement with Laudahn, however, that higher concentrations ($>10^{-4}$ M) of dipyridamole lead to a decrease in oxygen consumption and phosphorylation. Laudahn (3) observed that as the dipyridamole concentration was increased from "low" (10^{-6} M) to higher levels (10^{-5} M) the oxygen rate decreased more rapidly than phosphorylation, with NADH-linked substrates. This too is consistent with the observed rapid decrease in state 3 respira-

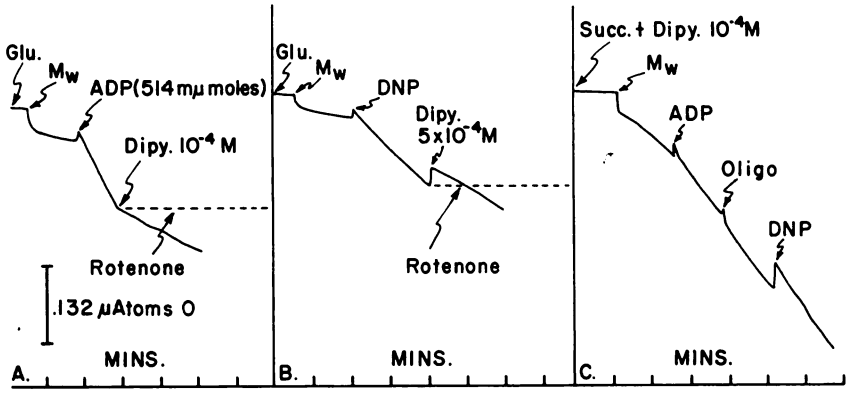

FIG. 3. *Inhibitory effects of dipyridamole on active glutamate-supported respiration and uncoupling of succinate-supported respiration.*

Tracings A and B show active mitochondrial respiratory states produced by ADP and 2,4-DNP, respectively, and the effect of subsequent additions of inhibitory amounts of dipyridamole with glutamate as substrate. The inhibition of respiration if rotenone were added is indicated by the dotted lines. Tracing C demonstrates the uncoupling effect of dipyridamole on succinate-supported respiration. Dipyridamole additions are expressed as final concentrations in the reaction chamber. Assay conditions are described under Methods. Abbreviations: glutamate (*Glu.*), succinate (*Succ.*), dipyridamole (*Dipy.*), mitochondria (M_W), 4.75×10^{-5} M 2,4-dinitrophenol (*DNP*), 1 μg/mg protein oligomycin (*Oligo.*). Protein concentrations were the same in A, B, and C.

TABLE 2

Effect of increasing concentrations of dipyridamole on normal mitochondria

Assay conditions are described under Methods. Measurement of the RCI, ADP:O ratio, and Q_{O_2} are described in Table 1. The protein concentration was the same in all experiments. Dipyridamole (Dipy.) additions were made serially; final concentrations in the reaction chamber are indicated. In experiments II and III, mitochondria (M_w) were preincubated with dipyridamole prior to subsequent additions of ADP and dipyridamole. Glutamate is the substrate.

Experiment + final molarity of dipyridamole	RCI	ADP:O	Q_{O_2} mμAO/min
I. Control	21.0	2.94	57.5
$+2.5 \times 10^{-5}$ M Dipy.	11.7	2.94	48.0
$+5 \times 10^{-5}$ M Dipy.	3.7	2.72	30.2
$+7.5 \times 10^{-5}$ M Dipy.	4.0	2.40	21.9
II. $M_w + 10^{-5}$ M Dipy.	9.8	2.74	67.0
$+2.5 \times 10^{-5}$ M Dipy.	9.5	2.97	52.0
$+5 \times 10^{-5}$ M Dipy.	4.7	2.63	35.6
III. $M_w + 5 \times 10^{-5}$ M Dipy.	5.4	2.5	37.0
$+10^{-4}$ M Dipy.	4.0	2.2	21.9

tory rates with less rapid decreases in the ADP:O ratios. However, the reduction in respiration does not appear to result from an *improvement* of phosphorylation efficiency, but rather an *inhibition* of electron transport in the case of the NADH-linked substrate. With succinate linked respiration there may be some increase of respiration due to uncoupling and there is no evidence for any direct inhibitory effect.

The different effects of dipyridamole on the NADH-linked and succinate-supported segments of the electron transport chain are of interest. At "high" concentrations of dipyridamole, the succinate-supported respiration is uncoupled. Uncoupling of oxidative phosphorylation by organic nitrates which are active coronary dilators has previously been reported (8). The observation that oligomycin produced no inhibition of the maximal uncoupled respiratory rate with succinate as substrate is similar to 2,4-DNP uncoupling. Apparently then, dipyridamole in sufficient concentrations *in vitro* can uncouple succinate-linked respiration, but exerts a somewhat different effect on NADH-supported oxidative phosphorylation. The effects of this compound on the latter appear to be more complicated. The fact that 2,4-DNP did not "release" respiration after dipyridamole (Fig. 2A) is indicative of its possible action more proximal to the electron transport chain than 2,4-DNP. Dipyridamole, even in "high" concentrations ($>10^{-4}$ M), cannot completely inhibit NADH-linked electron transport as does rotenone. Dipyridamole only slightly inhibits 2,4-DNP-uncoupled respiration whereas rotenone produces complete inhibition (9). The term "rotenone-like" is used only to indicate an apparent inhibition of electron transport near site I. A recent review (10) has indicated that dipyridamole has a potential (E'_o) between +0.01 and +0.23 V which places it in the same reactive range as the flavoprotein associated with NADH. The chemical evidence (10) regarding dipyridamole indicates that it might be capable of accepting electrons from NADH. If this is so then the drug might compete with the main electron transport chain for electrons from NADH.

It is possible that the "low" concentrations of dipyridamole used in these experiments may still be higher than those found *in vivo*. Therefore, no extrapolations from the present data to the *in vivo* state can be made. The *in vivo* effects of dipyridamole on mitochondrial function in cardiomyopathic rats is currently under investigation in this laboratory. In a recent report (11), Lozada and Laguens have presented electron micrographs suggesting that dipyrida-

mole prevents the morphological changes of heart mitochondria induced by hypoxia.

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