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In vitro effects of glucocorticoid on mitochondrial energy metabolism

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A systematic study of the effects of the synthetic glucocorticoid, methylprednisolone (MP), on respiration and energy coupling in tightly-coupled mitochondria isolated from rat tissues has been initiated. In intact rat skeletal muscle, liver and heart mitochondria, incubation, in vitro, with $\geq$ 0.1 mM MP caused inhibition of the state 3 respiratory rates with succinate and NAD-linked substrates. In skeletal muscle and heart mitochondria, the oxidation of succinate was significantly more sensitive to MP than was that of the NAD-linked substrates. No effects were seen at low concentrations (< 0.02 mM) of MP. In all three tissues, these data together with analysis of the partial reactions of the electron transport chain and steady-state kinetic analysis of cytochrome reduction indicated that in isolated mitochondria high concentrations of MP: (a) inhibit the oxidation of NAD-linked substrates at the level of the respiratory chain between the primary NADH dehydrogenase flavoprotein and coenzyme Q, most likely at the iron-sulfur centers or coenzyme Q-binding proteins of complex I; and (b) inhibit succinate oxidation in intact (but not disrupted) mitochondria, not by inhibiting electron transfer along the respiratory chain, but possibly at the level of succinate transport into the mitochondria. The results of these studies suggest that the therapeutic effects of MP in mitochondrial disease result from indirect effects rather than direct effects on the mitochondrial membrane. More importantly, the absence of an effect at low MP concentrations provides the baseline information needed for further studies to be carried out in vivo.

Introduction

It is widely appreciated that the glucocorticoids play an important role both as therapeutic agents and as metabolic regulators within the cell [1–7]. They are used pharmacologically in the treatment of such widely diverse conditions as arthritis, leukemia, infectious diseases, severe allergies and autoimmune disorders, to name only a few. In recent years, glucocorticoids also have been increasingly used in the treatment of neuromuscular diseases. For example, improved muscle function has been seen in patients with Duchenne muscular dystrophy upon treatment with glucocorticoids [8,9], and in the avian model of myopathy improvements in both muscle function and muscle histopathology have been reported [10,11].

In patients suffering from mitochondrial myopathies and encephalomyopathies, treatment with relatively low doses (8–60 mg/day) of the synthetic glucocorticoids, prednisone and methylprednisolone [12–21], has resulted in a dramatic increase in muscle strength, a decrease in serum lactate, and in one case in which a second biopsy was carried out following the steroid treatment [12], fewer abnormalities in mitochondrial ultrastructure. Improvement in the encephalopathic symptoms was seen in the patients with mitochondrial encephalomyopathy.

These beneficial effects of therapy with low doses of glucocorticoids in patients with neuromuscular diseases are in marked contrast to the muscle wasting and steroid-induced myopathy which are potential side-effects in patients receiving larger daily doses of these

Abbreviations: DCPIP, dichlorophenol-indophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; MP, methylprednisolone; PMS, phenazine methosulfate; RSM, rat skeletal muscle mitochondria; TMPD, tetramethyl-*p*-phenylenediamine.

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drugs (100–1000 mg/day) for the treatment of other steroid-responsive conditions [4]. This implies that there may be more than one mode of action of the glucocorticoids in skeletal muscle, depending on the tissue levels of the drug. This view is supported by ultrastructural studies which have shown a considerable difference in skeletal muscle morphology between rats subjected to treatment with low doses of glucocorticoid [22] and those subjected to treatment with high doses [23,24].

The mechanisms of neither the myopathy induced by high-dose glucocorticoid therapy nor the improvement seen in patients with neuromuscular diseases upon treatment with low-dose glucocorticoids are completely understood at the present time. However, in the case of the mitochondrial myopathies and encephalomyopathies it is not unreasonable to suspect, particularly in view of the decrease in serum lactate, that it is a metabolic effect and that it may occur at the level of mitochondrial structure and/or function. Therefore, in order to better understand the action of these drugs in muscle tissues, in general, and with respect to their therapeutic effects in mitochondrial diseases, we have initiated a systematic study of the effects of methylprednisolone (MP) on tightly-coupled mitochondria isolated from rat tissues. In this paper, we report the results of in vitro studies on the effects of incubation with varying concentrations of MP on respiration and oxidative phosphorylation in isolated rat skeletal muscle mitochondria, as well as (for comparison) heart and liver mitochondria. The results of these studies suggest that the therapeutic effects of MP in mitochondrial disease may result from indirect effects rather than direct effects on the mitochondrial membrane. More importantly, the results at low MP concentrations provides the baseline information needed for further studies to be carried out in vivo. Brief portions of this work have been reported in abstract form [25,26].

Materials and Methods

Isolation of mitochondria

Rat skeletal muscle mitochondria were isolated by the Nagarse method of Makinen and Lee [27] with the following modifications to the isolation media: the concentration of EDTA in the initial skeletal muscle homogenization medium was increased from 1 mM to 2 mM, and the concentrations of $MgCl_2$, ATP and EDTA in the first mitochondrial washing medium were increased from 1 mM to 2 mM, from 0.2 mM to 2 mM and from 0.2 mM to 1 mM, respectively.

Mitochondria were isolated from rat hearts according to the method of Tyler and Gonze [28].

Rat liver mitochondria were isolated by a slight modification of the method of Johnson and Lardy [29] as previously described [30].

Respiratory and phosphorylating activities

The respiratory rates and phosphorylating efficiencies of the isolated mitochondrial preparations oxidizing various substrates were measured polarographically at 30°C using a Clark oxygen electrode (Yellow Springs Instrument Co.) fitted into a thermostatted Delrin plastic chamber with a capacity of 1.0 ml (Instrument Shop, The Johnson Research Foundation, University of Pennsylvania) as previously described [30]. The incubation medium consisted of 150 mM sucrose, 25 mM Tris-HCl and 10 mM phosphate (pH 7.5).

Partial reactions of electron transport

The mitochondrial samples were disrupted for measurement of the partial reactions of electron transport by either sonicating on ice for three 10 s intervals at an output of 40 W using a Heat Systems Model W-225R sonifier (Heat Systems/Ultrasonics) (NADH-linked reactions) or freeze/thawing four or five times in a solid CO_2/ethanol bath (succinate-linked reactions). Intact mitochondria were activated for measurement of succinate-linked reactions by incubating at room temperature for 30–60 min in a medium consisting of 0.2 M sucrose, 75 mM Tris-HCl, 7.5 mM phosphate (pH 7.5.), 30 mM succinate, 15 mM pyruvate, 5 μM rotenone and 3 mM ATP. A 2–10-fold increase in the rate was achieved, depending on the type of mitochondrion.

The reaction rates were measured spectrophotometrically using a single-beam Zeiss recording spectrophotometer (Carl Zeiss Instruments) at the wavelengths indicated below. All assays were carried out at room temperature in a final volume of 3.0 ml.

NADH oxidase activity was measured at 340 nm as previously described [30] in a medium consisting of 0.15 M sucrose/25 mM Tris-HCl/10 mM phosphate (pH 7.5). In some cases, the Tris-HCl buffer was replaced with 50 mM phosphate (pH 7.5); this substitution had no effect on the experimental results. The reaction was initiated by the addition of 0.16 mM NADH. An extinction coefficient for NADH of 6.22 $mM^{-1}\,cm^{-1}$ was used for calculation.

The NADH-cytochrome *c* reductase (rotenone-sensitive) and succinate-cytochrome *c* reductase activities were assayed by following cytochrome *c* reduction at 550 nm in 0.05 M phosphate (pH 8.5.) according to King and Howard [31] and King [32], respectively. Other additions were 0.16 mM NADH (or 3.3 mM succinate), 1.7 mM KCN, and 0.05 mM cytochrome c^{3+}. Data were calculated using an extinction coefficient for cytochrome *c* of 19.1 $mM^{-1}\,cm^{-1}$.

The NADH-ferricyanide reductase (rotenone-insensitive) activity was measured by following ferricyanide reduction at 420 nm in a medium consisting of 0.15 M sucrose/0.05 M phosphate (pH 7.5.) according to King and Howard [31] using an extinction coefficient for ferricyanide of 1.0 $mM^{-1}\,cm^{-1}$. Other additions were

0.24 mM NADH, 1 mM $K_3Fe(CN)_6$ and 0.83 μM rotenone.

The succinate-phenazine methosulfate (PMS) reductase activity was measured in intact mitochondria in a medium containing 0.15 M sucrose/0.05 M phosphate (pH 7.5), by following the reduction of dichlorophenolindophenol (DCPIP) at 600 nm according to the method of King [33]. Other additions were 3.3 mM succinate, 0.5 mM PMS, 0.1 mM DCPIP, 1.5 mM KCN and 0.2% (w/v) defatted bovine serum albumin. An extinction coefficient for DCPIP of 21 $mM^{-1}\,cm^{-1}$ was used for quantitation.

Reduction of cytochromes

The substrate-reducible (under both steady-state and anaerobic conditions) and chemically-reducible (with dithionite) levels of the respiratory chain pigments were assessed by measuring the reduced minus oxidized difference spectra of the intact mitochondrial samples in each state, as previously described [30]. The medium was identical to that used in the polarographic studies. The substrate-reducible levels were measured in the presence of 5 mM succinate, 5 mM pyruvate, 2.5 mM malate, 0.6 mM ADP and 0.1 μM FCCP. The data were quantitated using the following extinction coefficients: cytochrome *a* (605 minus 630 nm), 24.0 $mM^{-1}\,cm^{-1}$; cytochrome a_3 (445 minus 455 nm), 80.0 $mM^{-1}\,cm^{-1}$ [34]; cytochrome *b* (562 minus 575 nm), 20.0 $mM^{-1}\,cm^{-1}$ [35]; cytochromes $c + c_1$ (551 minus 540 nm), 19.1 $mM^{-1}\,cm^{-1}$ [36].

Protein determination

Mitochondrial protein was determined by the method of Lowry et al. [37] using bovine serum albumin as standard.

Materials

6-α-Methylprednisolone was purchased from Sigma Chemical Company. Crystalline *B. subtilis* proteinase (Nagarse) was obtained from the Teikoku chemical Company, Osaka, Japan. Carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) was a gift of Dr. Peter Heytler, E.I. DuPont de Nemours & Co. All other chemicals of the highest grades were obtained commercially. Glass-redistilled water was used throughout the present investigation.

Results

Effects of MP on respiration and oxidative phosphorylation

Utilization of NAD-linked substrates. Fig. 1 shows typical polarographic tracings of isolated rat skeletal muscle mitochondria oxidizing pyruvate + malate in the absence and presence of 0.9 mM and 1.8 mM MP (dissolved in 100% dimethyl sulfoxide). As can be seen

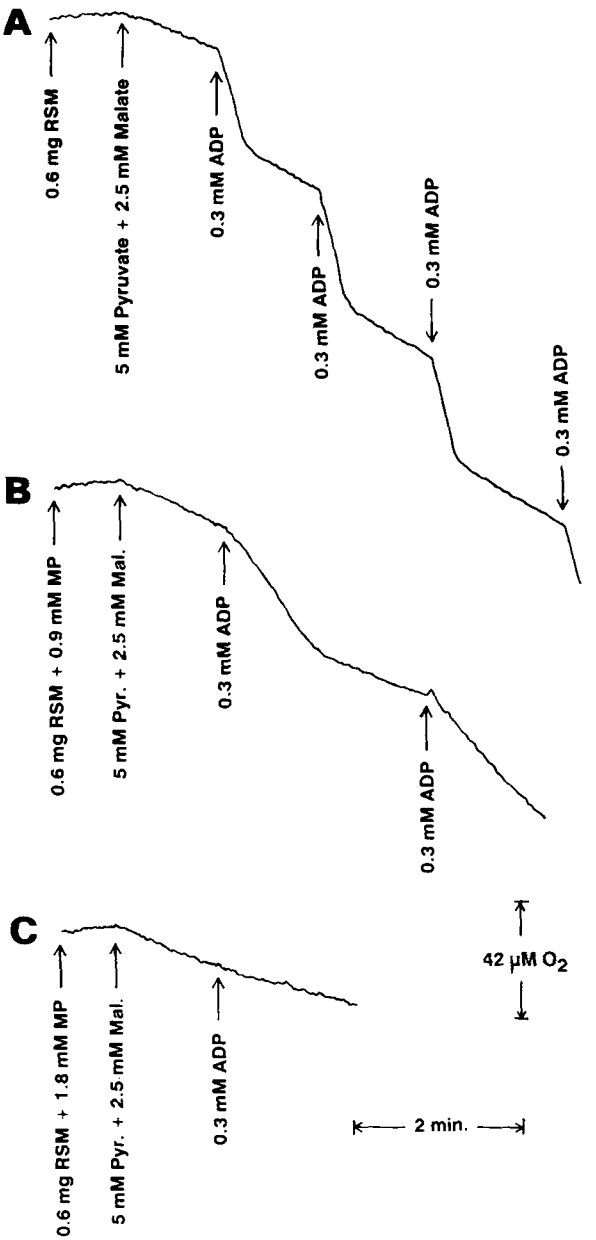

Fig. 1. Effects of MP on isolated rat skeletal muscle mitochondria oxidizing pyruvate + malate. Experimental conditions were as described in Materials and Methods. Other additions were as indicated. RSM = rat skeletal muscle mitochondria. (A) Control; (B) +0.9 mM MP; (C) +1.8 mM MP.

in panel A, the untreated mitochondria were tightly-coupled, exhibiting very good respiratory control. In the presence of 0.9 mM MP (panel B), a significant inhibition of the state 3 respiratory rate could be clearly seen. There was also a slight decrease in the efficiency of energy coupling under these conditions, as evidenced by a decreased ADP/O ratio (control = 3.5 ± 0.1; MP = 2.9 ± 0.1, $n = 3$, $P < 0.01$). Doubling the amount of MP to 1.8 mM (panel C) resulted in severe inhibition of the state 3 respiratory rate. The rates of respiration in the presence of uncoupler (FCCP) were inhibited by MP to

the same extent as the ADP-induced state 3 rates, and the inhibition of state 3 respiration could not be relieved by the addition of uncoupler. This indicates that the inhibition of the state 3 respiratory rates was not caused by inhibition of ATP synthesis. Neither could the respiratory inhibition seen in mitochondria oxidizing NAD-linked substrates be relieved by addition of either NAD^+ or NADH (data not shown). Thus the inhibition was not due to an increase in the permeability of the inner membrane to and leakage of these cofactors out of the mitochondrial matrix. Incubation of the mitochondria with dimethyl sulfoxide alone had no effect on respiration.

The effects of MP on intact mitochondria isolated from both heart and liver were qualitatively similar to those seen with intact skeletal muscle mitochondria when malate + pyruvate were used as the respiring substrates. In all three types of mitochondrion, the oxidation of other NAD-linked substrates (i.e., skeletal muscle and heart mitochondria using malate + glutamate, and liver mitochondria using β-hydroxybutyrate) were also similarly affected by the MP treatment (data not shown).

The magnitude of the inhibition of the state 3 respiratory rate depended, in all cases, on the concentration of MP in the incubation mixture (cf. titration profiles in Fig. 2 for skeletal muscle, heart and liver mitochondria utilizing malate + pyruvate), with no dependence on the amount of mitochondrial protein present (not shown). The inhibition of the state 3 respiratory rates in the mitochondria from all three tissues was greatly enhanced at increasing concentrations of MP, whereas the effect on the state 4 rates was minimal. In

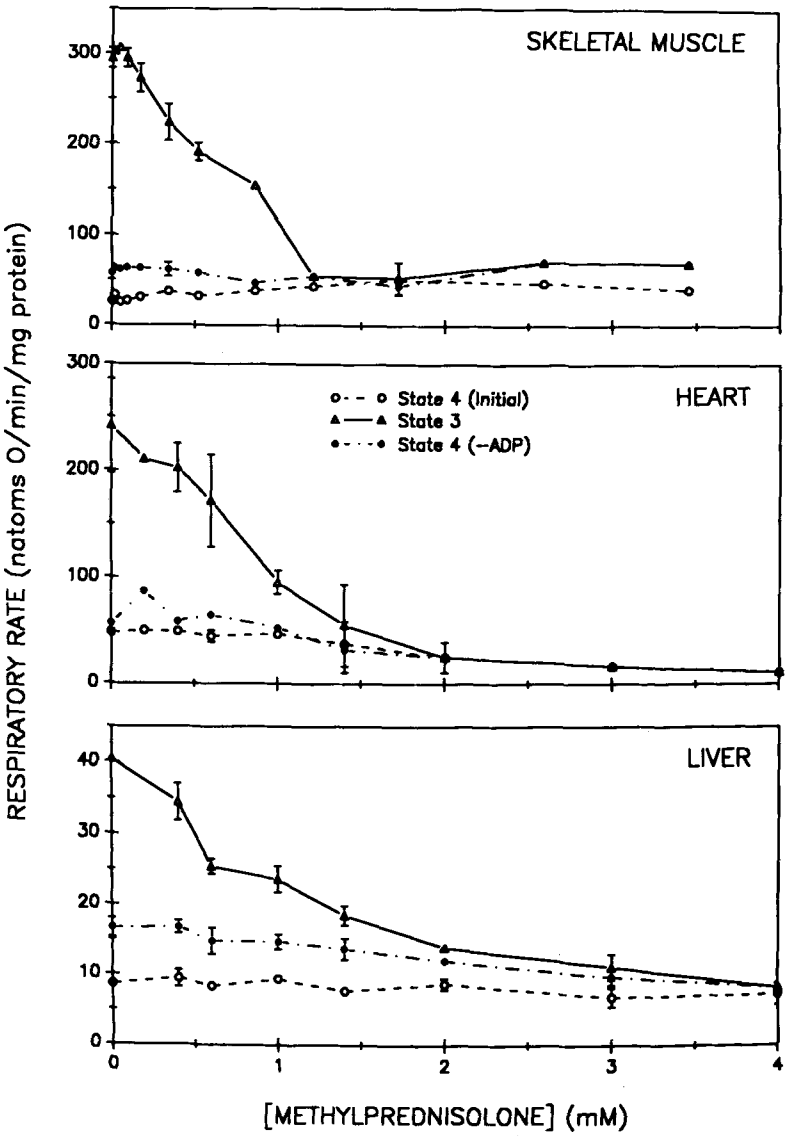

Fig. 2. Concentration dependence of the effects of MP on pyruvate + malate oxidation. Experimental conditions were as described in the legend to Fig. 1. Data are expressed as mean ± S.E. ($n = 3$ for skeletal muscle, heart, liver). State 4 (initial) = measured prior to the addition of ADP; State 4 (−ADP) = measured after the utilization of added ADP.

most cases, the slight uncoupling observed in earlier experiments (cf. Fig. 1) was overshadowed by the large inhibitory effect of the MP treatment and, therefore, could not be assessed readily.

The sensitivities of the state 3 respiratory rates with the NAD-linked substrates to the MP-induced inhibition were similar among mitochondria from skeletal muscle, heart and liver (cf. Fig. 2). The concentrations of MP that gave 50% inhibition were in the region of 0.8–1.0 mM for all three types of mitochondrial preparation when malate + pyruvate or β-hydroxybutyrate (liver only, data not shown) were used as the substrates. In all cases, there was no effect on respiration or oxidative phosphorylation at concentrations of MP less than 0.1 mM.

Utilization of succinate. The effects of MP on the respiratory and phosphorylating activities of intact skeletal muscle, heart and liver mitochondria oxidizing succinate (+ rotenone) were qualitatively similar to those seen with the NAD-linked substrates. That is, incubation of intact mitochondria with MP induced a severe inhibition of the state 3 respiratory rates. In the case of succinate oxidation, however (cf. titration profiles in Fig. 3), not only did the sensitivities of the mitochondria to the MP treatment differ dramatically from those seen with the NAD-linked substrates for any given type of mitochondrial preparation, but the sensitivities also differed among the three different types of mitochondrion examined. That is, in intact skeletal muscle and heart mitochondria, 50% inhibition of the state 3 respiratory rates with succinate (+ rotenone) as substrate was achieved at approx. 0.1 mM and 0.2 mM MP, respectively, whereas at least 2 mM was required for 50% inhibition in the mitochondria isolated from

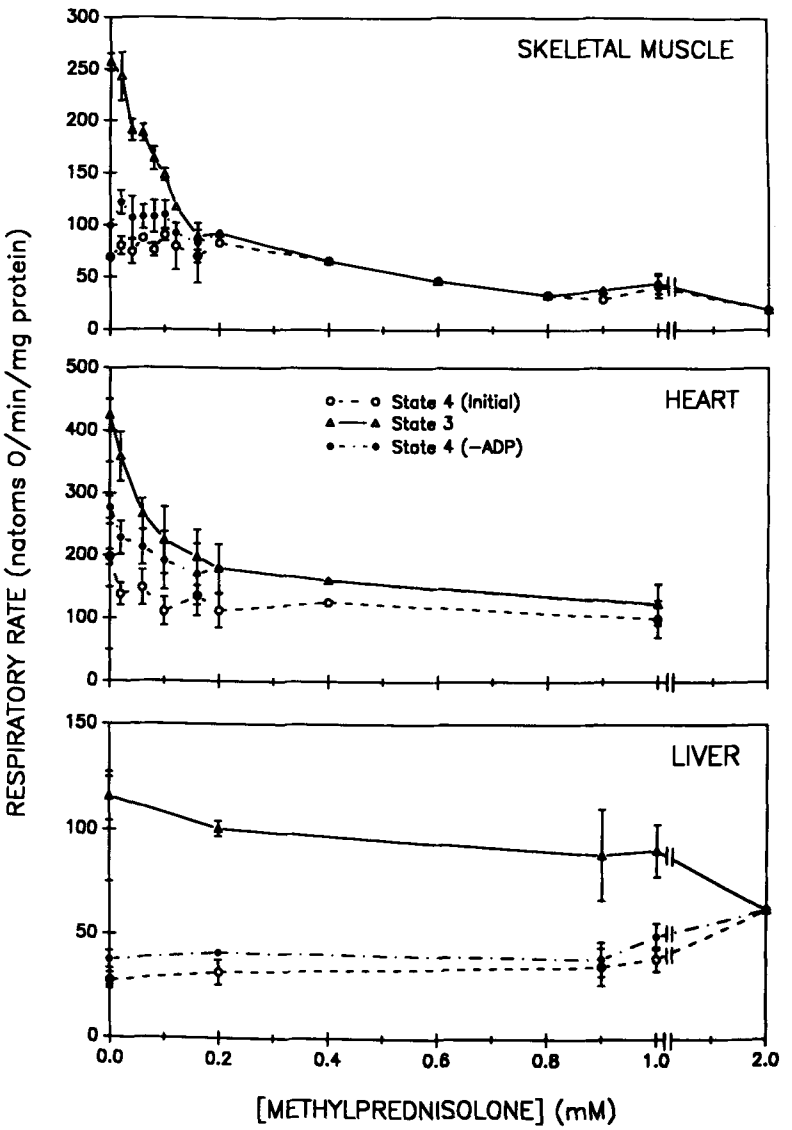

Fig. 3. Concentration dependence of the effects of MP on succinate oxidation. Experimental conditions were as described in the legend to Fig. 1. Other additions were 5 mM succinate, 2.5 μM rotenone, 200 μM ADP. Data are expressed as mean ± S.E. ($n = 4$ for skeletal muscle; $n = 3$ for heart, liver). State 4 (initial) = measured prior to the addition of ADP; State 4 (−ADP) = measured after the utilization of added ADP.

liver. As with the NAD-linked substrates, low concentrations ($\leqslant 0.02$ mM) of MP had no effect on either respiration or energy-coupling in any of the three types of mitochondrion.

The data show that the sensitivities of the mitochondrial preparations to MP-induced respiratory inhibition were not necessarily comparable among the three tissues tested (skeletal muscle, heart, liver). Although there were no significant differences among the three when pyruvate + malate were used as the respiratory substrates (cf. Fig. 2), in the case of succinate oxidation, skeletal muscle and heart mitochondria were considerably more sensitive to the inhibitory action of MP than were liver mitochondria (cf. Fig. 3). Thus, the response of mitochondrial energy metabolism to MP is at least partially tissue-specific, with the difference being between the muscle tissues (skeletal, heart) and the non-muscle tissue (liver).

Effects of MP on respiratory chain-linked partial reactions

NADH-linked reactions. Table I shows the effects of incubating skeletal muscle, heart and liver mitochondria with 0.9 mM MP on both the state 3 respiratory rates with NAD-linked substrates and the rates of electron transfer through the various segments of the respiratory chain associated with the oxidation of these substrates. Treatment with MP inhibited both the rotenone-sensitive NADH oxidase and NADH-cytochrome *c* reductase activities of disrupted (uncoupled) skeletal muscle, heart and liver mitochondria to an extent equal to or greater than was seen for the oxidation of NAD-linked substrates in intact mitochondria. Due to its higher pH optimum, NADH-cytochrome *c* reductase was assayed at pH 8.5 rather than pH 7.5 (assay pH for respiration and NADH oxidase). However, the extent of inhibition by MP was identical at pH 8.5 vs. pH 7.5 (data not shown). From these data we conclude that the impaired state 3 respiratory rates seen with these substrates in the presence of MP result from inhibition at the level of the respiratory chain rather than the substrate-linked dehydrogenases (e.g., pyruvate dehydrogenase). On the other hand, the NADH-ferricyanide reductase activities of the disrupted samples were not inhibited by the MP treatment, indicating that the site of inhibition of NAD-linked substrate oxidation was located on the cytochrome side of the primary NADH dehydrogenase flavoprotein.

TABLE I

Effect of MP on NADH-linked electron transfer reactions

Experimental conditions were as described in Materials and Methods. [MP] = 0.9 mM.

Reaction	Rate (nmol/min per mg mitochondrial protein) [a]		
	skeletal muscle	heart	liver
Intact mitochondria			
Respiratory rate (Mal + Pyr) [b]			
control	185 ± 17 (13)	256 ± 26 (5)	48 ± 3 (10)
+ MP	67 ± 13 (13) [c]	92 ± 10 (5) [c]	19 ± 2 (10) [c]
Disrupted mitochondria			
NADH oxidase			
control	297 ± 23 (13)	333 ± 83 (5)	110 ± 18 (9)
+ MP	178 ± 19 (13) [c]	197 ± 55 (5) [e]	45 ± 9 (9) [c]
NADH–cytochrome *c* reductase			
control	632 ± 119 (4)	1007 ± 155 (3)	148 ± 10 (5)
+ MP	157 ± 30 (4) [c]	483 ± 108 (3) [d]	90 ± 7 (5) [c]
NADH–ferricyanide reductase			
control	6189 ± 878 (3)	1490 ± 30 (2)	2675 ± 125 (2)
+ MP	7167 ± 742 (3)	2275 ± 445 (2)	3150 ± 150 (2)

[a] Data are expressed as the mean ± S.E. (the number of experiments is given in parentheses). [b] natoms O/min per mg mitochondrial protein. Statistical significance (paired *t*-test): [c] $P < 0.01$; [d] $P < 0.02$; [e] $P < 0.05$.

TABLE II

Effect of MP on succinate-linked electron transfer reactions

Experimental conditions were as described in Materials and Methods. [MP] = 0.9 mM.

Reaction	Rate (nmol/min per mg mitochondrial protein) [a]		
	skeletal muscle	heart	liver
Intact mitochondria			
Succinate oxidase [b]			
control	260 ± 11 (11)	439 ± 25 (8)	112 ± 13 (6)
+ MP	38 ± 5 (11) [c]	122 ± 28 (8) [c]	90 ± 10 (6) [e]
Succinate-PMS reductase			
control	363 ± 47 (5)	390 ± 12 (4)	188 ± 17 (4)
+ MP	180 ± 15 (5) [c]	216 ± 19 (4) [c]	183 ± 12 (4)
Disrupted mitochondria			
Succinate oxidase [b]			
control	275 ± 16 (5)	217 ± 2 (2)	155 ± 19 (4)
+ MP	204 ± 11 (5) [c]	163 ± 20 (2) [d]	146 ± 19 (4)
Succinate-cytochrome *c* reductase			
control	266 ± 33 (4)	219 ± 25 (3)	185 ± 13 (3)
+ MP	239 ± 25 (4)	229 ± 13 (3)	162 ± 13 (3)

[a] Data are expressed as the mean ± S.E. (the number of experiments is given in parentheses). [b] natoms O/min per mg mitochondrial protein. Statistical significance (paired *t*-test): [c] $P < 0.01$; [d] $P < 0.02$; [e] $P < 0.05$.

Succinate-linked reactions. The effect of 0.9 mM MP on the succinate-linked electron transfer reactions is shown in Table II. Qualitatively, the results were similar for all three types of mitochondrial preparations. That is, although the state 3 rates of succinate oxidation and the succinate-PMS reductase activities were inhibited in intact mitochondria. However, in the disrupted (uncoupled) suspensions the succinate oxidase activities were inhibited much less severely (75% of control for disrupted vs. 15–30% of control for intact) in skeletal muscle and heart mitochondria. The succinate-cytochrome *c* reductase activities were not inhibited by treatment with MP. In intact liver mitochondria, the succinate-PMS reductase activity was not significantly

inhibited at 0.9 mM MP, in line with the observation that succinate oxidation in intact liver mitochondria was very much less sensitive to inhibition than in skeletal muscle and heart mitochondria (cf. Fig. 3). Thus, in the case of succinate oxidation it is evident that the decrease in the state 3 rate of respiration induced by MP was not due to inhibition of electron transfer along the respiratory chain.

Effects of MP on electron transfer in the cytochrome region

Cytochrome oxidase activities. Methylprednisolone did not inhibit electron transfer through the cytochrome oxidase segment of the respiratory chain, as evidenced by its lack of an inhibitory effect on the respiratory rates with ascorbate + tetramethyl-*p*-phenylenediamine (TMPD) as substrates in intact skeletal muscle, heart or liver mitochondria. The measured rates were: 449 vs. 458 natoms O/min per mg (skeletal muscle), 868 vs. 719 natoms O/min per mg (heart), and 188 vs. 236 natoms O/min per mg (liver) for control vs. 0.9 mM MP-treated mitochondria, respectively. No decrease in the rate was seen, even at concentrations of MP that severely inhibited the oxidation of NADH or succinate.

Participation of the cytochromes in electron transfer. Measurements of the reduction kinetics of the cytochromes were made in order to determine whether the rate-limiting step of the inhibited respiration with NAD-linked substrates was in this region of the respiratory chain, and the effect of MP on the active participation of the cytochromes in electron transfer from NADH to oxygen. The reduction of cytochromes a, a_3, b, and $c + c_1$ by substrates (under steady-state and anaerobic conditions) and dithionite in intact rat skeletal muscle, heart and liver mitochondria was examined in the presence and absence of 0.9 mM MP (data not shown). No cross-over point (which would appear as an increase in the steady-state level of reduction of cytochrome(s) 'upstream' from the site of inhibition) was exhibited by the treated samples. In addition, the total substrate-reducible (measured at anaerobiosis) and chemically reducible (with dithionite) levels of the cytochromes were virtually identical in the treated and untreated mitochondria. These results indicate that the site of inhibition was not located in the cytochrome segment of the respiratory chain.

Steroid specificity

In order to determine whether the effects seen were specific to MP or could be produced by other steroids, isolated rat skeletal muscle mitochondria were incubated with a variety of structurally and functionally diverse steroids (MP, prednisone, dexamethasone, hydrocortisone, cortisone, testosterone, cholesterol). Table III shows the effects of these compounds on both the state 3 and state 4 respiratory rates with pyruvate + malate as substrates. As was seen with MP, all of the compounds tested significantly inhibited the state 3 rates at a concentration of 0.9 mM ($P < 0.01$ in each case), with the exception of cortisone-acetate ($P > 0.1$). However, both prednisone and cortisone-acetate were less inhibitory than MP ($P < 0.01$).

TABLE III

Effects of MP and other steroids on the respiratory rates of isolated rat skeletal muscle mitochondria oxidizing pyruvate + malate

Experimental conditions were as described in the legend to Fig. 1. Steroid concentrations were 0.9 mM. State 4 (initial) = measured prior to the addition of ADP; State 4 (−ADP) = measured after the utilization of added ADP.

	Respiratory rate (natoms O/min per mg mitochondrial protein) [a]		
	State 4 (initial)	State 3	State 4 (−ADP)
No additions	24 ± 2	152 ± 12	50 ± 5
+ MP	26 ± 5	54 ± 14 [b]	36 ± 6
+ Prednisone	21 ± 3	98 ± 10 [b]	56 ± 8
+ Dexamethasone	37 ± 5 [d]	72 ± 16 [b]	44 ± 4
+ Hydrocortisone	43 ± 2 [b]	70 ± 28 [c]	49 ± 9
+ Cortisone	27 ± 5	125 ± 15	57 ± 12
+ Testosterone	18 ± 3	32 ± 1 [b]	32 ± 1 [c]
+ Cholesterol	29 ± 8	82 ± 17 [b]	41 ± 5

[a] Data are expressed as mean ± S.E. ($n = 6$). Statistical significance: [b] $P < 0.01$; [c] $P < 0.02$; [d] $P < 0.05$.

Effects similar to those of MP on respiration with succinate (+ rotenone) and ascorbate + TMPD as substrates, and the NAD- and succinate-linked partial reactions were also seen with hydrocortisone. These data indicate that the steroid-induced inhibition of mitochondrial respiration is not specific to MP and appears to be independent of the glucocorticoid function of MP, since both cholesterol and testosterone (an androgen) had effects similar to those of MP, whereas steroids more closely related to MP (prednisone, cortisone-acetate) had less effect on mitochondrial function in these experiments.

Discussion

Our data show that incubation, in vitro, of isolated rat skeletal muscle, heart and liver mitochondria with high concentrations (≥ 0.1 mM) of the synthetic glucocorticoid, MP, caused significant inhibition of the state 3 respiratory rates of intact mitochondria utilizing either succinate or NAD-linked substrates. In the case of NAD-linked substrates, MP inhibited the rotenone-sensitive NADH oxidase and NADH-cytochrome *c* reductase activities of sonicated mitochondrial preparations from all three tissues to the same extent as the state 3 respiratory rates, but not the NADH-ferricyanide reductase or cytochrome oxidase activities. Steady-state kinetic analysis of cytochrome reduction

revealed no involvement of these pigments in the inhibitory effects of MP. The succinate data (see below) indicated that MP did not inhibit at a site common to the oxidation of NADH and succinate. We conclude from these data that MP inhibits isolated rat skeletal muscle, heart and liver mitochondria at the level of the respiratory chain between the primary NADH dehydrogenase flavoprotein and coenzyme Q, and suggest that the site of inhibition may be the iron-sulfur centers or coenzyme Q binding proteins associated with complex I.

In the case of succinate oxidation, concentrations of MP that severely inhibited both state 3 respiration and the succinate-PMS reductase activities in intact mitochondria did not inhibit either the succinate oxidase or succinate-cytochrome *c* reductase activities of disrupted preparations of skeletal muscle, heart or liver mitochondria. Thus, the site of inhibition was not at the level of the respiratory chain. Rather, the data suggest that MP may inhibit succinate oxidation in intact mitochondria by blocking the transport of succinate into the mitochondria.

The effects of a wide variety of glucocorticoids on the function of isolated liver mitochondria both in vitro and in vivo have been documented in the literature over the past 30 years [38–47]. Briefly, the results of the in vitro studies [38–41] showed that incubation of isolated liver mitochondria with high concentrations of glucocorticoid caused uncoupling and a decrease in the state 3 respiratory rates, in agreement with the data reported in this paper. However, the results of studies on liver mitochondria cannot, necessarily, be extrapolated to muscle or other tissues, as exemplified by the results shown in Fig. 3, above. It is well known that there is a considerable difference between the effects of glucocorticoid administration on the overall metabolism of liver as compared to skeletal muscle or other tissues [1–5,7]. In liver, the overall effect is anabolic, stimulating gluconeogenesis and glycogen storage, whereas in skeletal muscle and many other tissues it is catabolic, leading to increased lipolysis in adipose tissues and to protein breakdown in muscle with the release of amino acids to serve as precursors for gluconeogenesis.

Studies of the effects of glucocorticoids on the respiratory functions of isolated skeletal muscle mitochondria [48–50] have been relatively few (consisting of only three reports describing the effects following treatment of animals, in vivo) and have shown conflicting results. The results of the study reported here provide the first description of the direct in vitro effects of glucocorticoids on the respiratory and energy-coupling activities of isolated heart and skeletal muscle mitochondria. They also expand on the reported studies on liver mitochondria, (a) demonstrating that MP has effects on mitochondrial function qualitatively similar to those reported in the literature [38–41] for other glucocorticoids and that the dose-response curves for these effects are partially tissue-specific, and (b) providing a more precise description of the site of in vitro inhibition by glucocorticoids in all three types of mitochondria.

At low concentrations ($\leqslant 0.02$ mM) no effect of MP on mitochondrial function was observed in these in vitro experiments. These concentrations were within the range of those expected in patients with mitochondrial disease undergoing low-dose glucocorticoid therapy. Based on a maximum daily dose of 60 mg/day, the calculated total concentration of MP in plasma of a 70 kg adult would be approx. 0.03 mM. However, the actual concentration of free MP would be considerably less, as many patients receive much lower doses (as low as 8–10 mg/day) and a large proportion of the hydrophobic MP would be bound to plasma albumin.

This lack of effect at low concentrations of glucocorticoid, when considered together with the high concentration-requirement and the inhibitory and apparently non-specific nature of the in vitro effects reported here, indicate that direct effects of glucocorticoids on the mitochondrial membrane do not play a role in their therapeutic action, in vivo. Rather, the data suggest that the mechanism of action of these drugs in the mitochondrial myopathies and encephalomyopathies is an indirect one. It is well known that the effects of glucocorticoids on cellular metabolism result from a combination of actions including induction/repression of specific genes, inhibition of protein synthesis and 'permissive' effects on the action of other hormones [1–7]. Whether such mechanisms play a role in the therapeutic effects of MP in mitochondrial disease remains to be determined by systematic in vivo studies.

On the other hand, the possibility that direct inhibition of mitochondrial respiration may play a role in pathology cannot be ruled out. Glucocorticoid-induced myopathy, the mechanism of which is unknown, is a serious side-effect of prolonged high-dose therapy with these drugs [4,51]. We have shown that succinate oxidation in muscle and heart mitochondria was at least 10 times more sensitive to glucocorticoid-induced inhibition than in liver mitochondria, with inhibition occurring at as low as 20–40 μM MP (cf. Figs. 3 and 4). During chronic high-dose glucocorticoid therapy, cells would be exposed to MP for much longer periods than in our experiments. As yet, the possibility that long-term exposure of mitochondria to MP may enhance inhibition at lower doses cannot be ruled out. Under conditions in which blood levels of glucocorticoid rise high enough to saturate their intracellular receptors [4], one may suspect that direct inhibition of mitochondrial metabolism could contribute to the cellular damage seen in these myopathies, even if it is not the primary cause. Such a role can only be established or ruled out by carefully-designed systematic studies, in vivo.

The results of the in vitro studies reported here establish a foundation upon which future in vivo studies can be built. When examining drug mechanisms in complex systems, as would be encountered in vivo or even in cell culture, it is often difficult or impossible to distinguish between direct and indirect effects. Use of the simpler, in vitro, system can provide the information needed to differentiate between these two modes of action. In the case of MP effects on mitochondrial function, our results provide that information. In the case of low doses of MP in vivo, the direct inhibition of respiratory chain-linked electron transfer observed in this study can be ruled out as a mechanism for any mitochondrial changes that may be seen in future studies. In addition, the direct effects described here at high levels of MP provide information that can be applied to future studies of high-dose treatment, in vivo.

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