Glucocorticoids decrease cytochrome c oxidase activity of isolated rat kidney mitochondria

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Abstract The importance of mitochondria is rising as a target in pathologic processes such as ischemia. We have investigated the effects of hydrocortisone, prednisolone, dexamethasone and triamcinolone on oxidative phosphorylation, Ca^{2+} fluxes, swelling and membrane potentials in isolated kidney mitochondria. The measurement of respiration state 3 showed a significant decrease in presence of glucocorticoids whereas the other respiration states were not modified. When mitochondria were uncoupled and either the complexes III and IV or the complex IV were stimulated, the O_2 consumption was decreased by glucocorticoids. These results suggest the cytochrome c oxidase is a target of the glucocorticoid effect on the respiratory chain. Indeed, the other mitochondrial functions investigated were unchanged, ruling out a direct effect on Ca^{2+} fluxes or swelling. A regulation of cytochrome c oxidase activity by glucocorticoids will be of particular interest in pathology involving metabolic insult.

Key words: Mitochondria; Glucocorticoid; Oxidative phosphorylation; Cytochrome c oxidase

1. Introduction

Glucocorticoids are potent immunosuppressive agents extensively used after organ transplantation [1]. Their relevant pharmacological properties are still not completely known. They inhibit the synthesis of almost all known cytokines and several cell surface molecules required for immune function. Recently, the nuclear factor kappa B (NF-KB) which activates many immunoregulatory genes was suggested to be a target for glucocorticoid-mediated immunosuppression [2,3]. Furthermore, dexamethasone is a well-known inducer of thymocyte apoptosis [4]. The exact mechanism of this effect is not well defined but involves alterations of mitochondrial structures and functions which suggest that these organelles are involved in the process [4]. Other mitochondrial effects have been suggested for glucocorticoids. Djouadi et al. [5] have shown a cell-specific regulation of rat kidney oxidative metabolism by glucocorticoids. The mechanism could be a direct effect on mitochondrial gene transcription [6]. Thus glucocorticoids could be involved in mitochondria biogenesis and in the development of respiratory chain enzymes [7]. However, the authors suggest that these activities do not regulate postnatal changes in mitochondrial density and mitochondrial DNA content.

The effects of steroid treatment on energy metabolism have already been described [8] but nothing is known on the comparative effects of glucocorticoids on oxidative phosphorylation, Ca^{2+} fluxes, membrane potentials and swelling. So, we investigated the mitochondrial effects of hydrocortisone, prednisolone, triamcinolone and dexamethasone in vitro using rat kidney mitochondria.

2. Materials and methods

2.1. Materials

Sucrose, EGTA, D-mannitol, rotenone, malonate, antimycin A, oligomycin, succinate, malate, pyruvate, ADP, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), sodium ascorbate, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), decylubiquinone (DUQ), and bovine serum albumin (BSA) were purchased from Sigma (Saint Quentin Fallavier, France). KCl and $MgCl_2$ were obtained from Prolabo (Paris, France). KH_2PO_4 was purchased from Merck (Paris, France).

Hydrocortisone, prednisolone, triamcinolone and dexamethasone were purchased from Sigma Chemical Company. They were solubilized in dimethylformamide (DMF) and distilled water (v:v) in order to obtain a stock solution at 10^{-3} M. All the controls were carried out using the same solvent mixture. The final solution contained no more than 0.05% DMF.

Decylubiquinol ($DUQH_2$) was immediately prepared from decylubiquinone (DUQ) as described by Veitch et al. [9]. The solution was evaporated to dryness under nitrogen gas, dissolved in 1 ml of DMF and stored at −70°C until used.

2.2. Methods

2.2.1. Isolation of kidney mitochondria. Mitochondria were extracted from a homogenate of one rat kidney by differential centrifugations according to Simon et al. [10]. Rats (male, Wistar, weighing 280–300 g) were killed by decapitation and the kidneys removed and placed quickly in an ice-bath. The kidneys were then minced in ice-cold isolation medium (Tris 20 mM, sucrose 250 mM, KCl 40 mM, EGTA 2 mM and bovine serum albumin 1 mg/ml; pH 7.2) and homogenized (6 ml/g of tissue) using a Potter-Elvejhem homogenizer. Mitochondria isolation was performed at 4°C without delay using differential centrifugation: the homogenate was centrifuged at $2000\times g$ for 8 min to remove cell debris and nuclei, mitochondria were separated from the supernatant by centrifugation at $12\,000\times g$ for 10 min. The pellet (mitochondria) was washed and resuspended in a medium containing sucrose 250 mM, KH_2PO_4 5 mM for the Ca^{2+} fluxes, membrane potentials and induced swelling experiments, or in a respiratory buffer (mannitol 300 mM, KH_2PO_4 10 mM, KCl 10 mM, $MgCl_2$ 5 mM, pH 7.2) for measuring respiratory activity. Protein concentrations of the mitochondrial suspension were determined by the method of Lowry. All assays were done on freshly isolated mitochondria. This protocol meets the guidelines of the french agency regarding animal experimentation (authorization No. 00748 delivered to Prof. J.P. Tillement).

2.2.2. Assay of mitochondrial oxygen consumption. Oxygen uptake was determined with a Clark-type microelectrode (Hansatech, UK). Each experiment was carried out as follows: 45 μl of mitochondria suspension were preincubated during 10 min at 4°C with (or without) the tested drug, then incubated 1 min at 37°C in 500 μl of the respiratory buffer (400 to 1200 μg/ml proteins) without or with the inhibitors, then the substrate was added and oxygen consumption was

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checked (state 2). To initiate state 3 respiratory activity, 200 μM ADP was added to the cuvette. When all ADP was converted to ATP the state 4 was measured. The following parameters were determined: the respiratory rates calculated as nanomoles of O_2 per min per mg of mitochondrial protein, the respiratory control ratio (RCR) expressed as the ratio of state 3/state 4 oxygen consumption and the P/O ratio.

The rate of oxygen consumption by the different complexes was determined according to Rustin et al. [11]. Briefly, rotenone (2 μM), malonate (10 mM), antimycin (1 μM), and oligomycin (10 μM) were used to inhibit complexes I, II, III and V, respectively and carbonylcyanure-*m*-chlorophenylhydrazone (CCCP) was added as decoupling agent (10 μM). Malate (10 mM) plus pyruvate (10 mM), succinate (10 mM), decylubiquinone (150 μM), ascorbate (5 mM) plus TMPD (1 mM) were used as substrates for complexes I, II, III and IV respectively.

2.2.3. Ca^{2+} fluxes. A specific Ca^{2+} electrode (Orion 9320) fitted to a Hansatech recorder via a 720A Orion ionometer was used to record Ca^{2+} movements in extramitochondrial medium in a thermostat-controlled reaction chamber (3.8 ml) at 37°C containing sucrose 250 mM, KH_2PO_4 5 mM, plus succinate 6 mM. The study was performed in presence of 25 μM of Ca^{2+}. Glucocorticoid effects were measured at 1 μM and were added prior to Ca^{2+} and mitochondria to ensure that they did not modify the electrode response. The Ca^{2+} concentration in extramitochondrial medium decreased rapidly when mitochondria (2 mg/ml of protein) were added, due to Ca^{2+} uptake into mitochondria. After all of the oxygen in the medium was consumed, anaerobiosis lead to Ca^{2+} release.

2.2.4. Measurement of swelling of energized mitochondria. Mitochondria (2 mg/ml of protein) were added to 1.8 ml of a medium containing sucrose 250 mM, KH_2PO_4 5 mM and rotenone 3 μM at 37°C. After mixing, swelling was initiated by addition of Ca^{2+} 25 μM plus succinate 6 mM to the sample cuvette. Swelling of energized mitochondria was determined by measuring the decrease of optical density at 520 nm, with a Hitachi spectrophotometer (U3000).

2.2.5. Measurement of membrane potentials. Absorbance measurements were made at high concentrations of rhodamine 123 (0.3 μM) with a Perkin Elmer luminescence spectrometer using 0.1 mm cuvettes. For each measure 1 ml of a medium containing sucrose (250 mM), KH_2PO_4 (5 mM), succinate (6 mM), rotenone (3 μM) and the drugs tested (1 μM) were added in the cuvette at 37°C. When the fluorescence was stabilized (10 s), mitochondria (0.5 mg/ml of protein) were added to the sample cuvette. Then, the fluorescence decreased according to rhodamine influx into mitochondria. We also investigated the effect of glucocorticoid on membrane potential in presence of high concentration of Ca^{2+} (50 μM).

The mitochondria membrane potential was calculated by the Nernst equation (Equation 1) according to Emaus et al. [12] and expressed in mV.

$$\Delta\Psi_m = 61.41 \times \log([\text{rhodamine}_{in}]/[\text{rhodamine}_{out}]). \quad (1)$$

2.2.6. Statistical analysis. For each experiment, mean values of RCR or percentages were compared with a one-way analysis of variance. EC_{50} was calculated by non-linear regression fit of effect-concentration curve to the equation:

$$E = (E_{max} \times C)/(C + EC_{50}),$$

where E_{max} and EC_{50} are the maximal effect and the concentration producing 50% effect respectively, using a commercially available software (Microparm) [13].

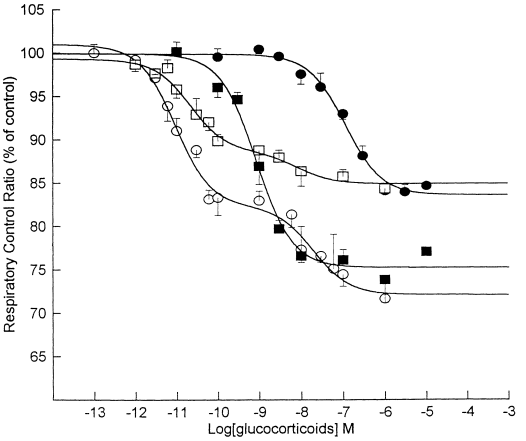

Fig. 1. Effect of hydrocortisone, prednisolone, triamcinolone and dexamethasone on respiratory control ratio (RCR) of rat kidney mitochondria. Concentration-response curves were expressed as the percentages of RCR, hydrocortisone (closed circles), prednisolone (open circles), triamcinolone (closed squares) and dexamethasone (open squares). All experiments were carried out at 37°C with succinate and rotenone, without EGTA in the respiratory buffer. Each point is the mean ± S.D. of eight determinations.

3. Results

3.1. Oxygen consumption

When the respiratory chain was activated by malate/pyruvate and ADP (complexes I to V), the RCR was significantly decreased from 12 to 21% for all the glucocorticoids (Table 1). Significant inhibition of state 3 was observed whereas the state 4 was not modified. So the decrease of RCR value was due to a decrease of state 3 except for hydrocortisone where a slight increase of state 4 occurred. The P/O ratio remained similar to the control.

When the respiratory chain was activated by succinate and complex I inhibited by rotenone, glucocorticoids decreased significantly RCR (Fig. 1). This effect was concentration-de-

Table 1
Glucocorticoid effects on oxydative respiration of isolated rat kidney mitochondria

	State 2 (nmol O_2 min^{-1} mg^{-1})	State 3 (nmol O_2 min^{-1} mg^{-1})	State 4 (nmol O_2 min^{-1} mg^{-1})	RCR	P/O	Complex III and IV (nmol O_2 min^{-1} mg^{-1})	Complex IV (nmol O_2 min^{-1} mg^{-1})
Control	6.74 (1.28)	46.65 (8.34)	7.13 (0.92)	6.54 (0.86)	2.27 (1.07)	76.59 (24.19)	348.61 (22.81)
Hydrocortisone	7.72 (1.06)	41.85 (8.91)	7.26 (1.39)	5.76 (0.33)*	2.23 (0.85)	50.52 (16.53)*	271.37 (72.24)*
Prednisolone	6.70 (1.43)	37.30 (8.11)*	7.07 (1.37)	5.27 (0.90)**	2.17 (0.68)	50.67 (13.25)*	262.67 (74.39)*
Triamcinolone	7.22 (1.34)	35.26 (7.41)**	6.34 (1.12)	5.56 (0.72)*	2.28 (0.69)	49.90 (17.41)*	263.63 (70.78)*
Dexamethasone	6.44 (0.99)	32.99 (6.04)**	6.40 (0.80)	5.15 (0.80)**	2.38 (0.56)	49.25 (18.49)*	264.57 (75.98)*

*$P < 0.05$ and **$P < 0.01$. Each value represents the mean (± S.D.) of six determinations. The duration time for glucocorticoid incubation (1 μM) was 15 min. The controls were carried out using an equivalent volume of dimethylformamide (0.05%). At the beginning to activate complexes I to V, the oxygen consumption was measured with malate (10 mM) plus pyruvate (10 mM) as substrates and ADP (0.2 mM) for a mitochondrial concentration of 1.2 mg/ml. To investigate complexes III and IV, the oxygen consumption was measured with rotenone (2 μM), malonate (10 mM) and oligomycine (10 μM) in order to inhibit complexes I, II and V, and decylubiquinone (150 μM) plus carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 150 μM). Then complex III was inhibited by antimycin (1 μM) and complex IV was stimulated by sodium ascorbate (5 mM) and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD, 1 mM). The mitochondrial concentration for these last experiments was 0.4 mg/ml.

pendent leading to a biphasic curve with two EC_{50} values for prednisolone and dexamethasone. The EC_{50} values were 9.22×10^{-12} M and 1.94×10^{-8} M for prednisolone; 2.13×10^{-11} M and 8.24×10^{-9} M for dexamethasone; 8.48×10^{-10} M for triamcinolone and 1.14×10^{-7} M for hydrocortisone. The maximal inhibitory effect varied from 14.31% to 28.85% with dexamethasone (14.31%) < hydrocortisone (16.25%) < triamcinolone (24.66%) < prednisolone (28.85%). These results show that glucocorticoids decrease oxidative phosphorylation without inhibiting complex I. Since EGTA did not modify the glucocorticoid effects, they could be considered Ca^{2+}-independent (data not shown).

When complexes III and IV were activated alone, glucocorticoids (1 μM) reduced significantly oxygen consumption with a similar 35% extent (Table 1). Finally, when complex IV operated alone, glucocorticoids (1 μM) still modified oxygen consumption (Table 1). The complex IV inhibition was similar between the glucocorticoids (22 to 25%).

3.2. Ca^{2+} fluxes through mitochondria

Ca^{2+} overloading experiments were carried out to confirm the Ca^{2+} dependency of the mitochondrial effects of glucocorticoids. None of the glucocorticoids (1 μM) tested modified Ca^{2+} fluxes (Fig. 2).

3.3. Swelling of energized mitochondria

Glucocorticoids (1 μM) did not modify the swelling of energized mitochondria induced by 25 μM Ca^{2+} plus inorganic phosphate (KH_2PO_4 5 mM). The rates of swelling were 112%, 106%, 113%, 119% versus control for hydrocortisone, prednisolone, triamcinolone and dexamethasone respectively.

3.4. Membrane potential of mitochondria

Glucocorticoid effects on membrane potential were investigated with or without high concentration of Ca^{2+} (50 μM). Without Ca^{2+} none of the glucocorticoids tested had a significant effect on membrane potential. However with 50 μM Ca^{2+}, they decreased the membrane potential and this decrease was significant for prednisolone and dexamethasone (Table 2).

4. Discussion

The effects of steroids on mitochondria and energy metabolism have already been investigated. Kerppola has previously described a decrease in state 3 oxygen consumption in rats treated during 7 days with cortisone acetate [14]. At the opposite, Wakat and Haynes have shown after a short-term administration of glucocorticoids to rats (3 h before experiment) an increase of substrate utilization by isolated hepatic mitochondria [8]. These effects were produced by cortisol, triamcinolone, and dexamethasone but not by deoxycorticosterone.

Table 2
Glucocorticoid effects on mitochondrial membrane potential

Drugs	Membrane potential (mV)	
	Without Ca^{2+}	With Ca^{2+} (50 μM)
Control	217.89 ± 7.46	196.75 ± 4.56
Hydrocortisone	215.63 ± 2.97	195.13 ± 4.75
Triamcinolone	216.56 ± 4.92	193.75 ± 7.64
Prednisolone	216.87 ± 6.16	190.42 ± 3.81*
Dexamethasone	215.07 ± 5.79	190.45 ± 4.78*

*$P < 0.01$. The comparisons were made versus control.
The membrane potential was measured with succinate (10 mM) as substrate for a mitochondrial protein concentration of 0.5 mg/ml. Each glucocorticoid concentration was 1 μM. The values are mean ± S.D. of five experiments.

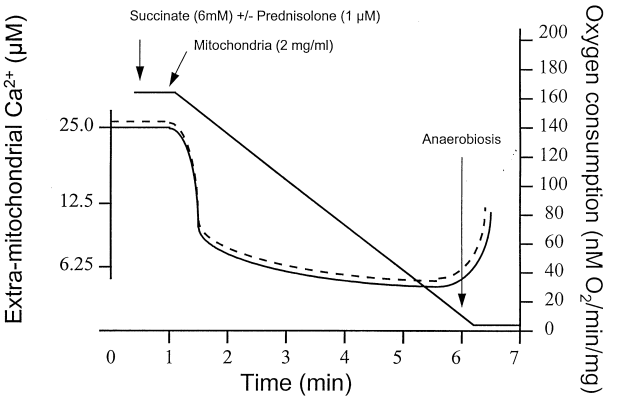

Fig. 2. Effect of prednisolone on Ca^{2+} fluxes of rat kidney mitochondria. At the beginning of the experiment, Ca^{2+} medium concentration was 25 μM. Then mitochondria (2 mg/ml) were added in the cuvette. The change of Ca^{2+} in medium was recorded with a Ca^{2+}-sensitive electrode system. A downward deflection corresponded to a decrease of Ca^{2+} concentration in the medium, i.e. an increase of mitochondrial Ca^{2+}. The medium contained succinate 6 mM and either control (dashed line) or prednisolone (solid line) 1 μM. The data shown are of a representative experiment.

Furthermore, the glucocorticoid effects on mitochondria seem to vary with age and sex of the rats. With such drugs we suggest that it is important to discriminate between the direct effect on mitochondria and the indirect effect involving nuclear pathway. So we investigated the effects of glucocorticoids on isolated mitochondria following a direct incubation. In order to understand the mechanism, we also studied Ca^{2+} fluxes, swelling and membrane potential.

Our results show that hydrocortisone, prednisolone, triamcinolone and dexamethasone decreased the respiratory control ratio with NAD linked substrates (pyruvate plus malate) or $FADH_2$ linked substrate (succinate). Interestingly, this decrease was concentration-dependent and occurred at low concentrations from 10^{-12} to 10^{-7} M. These modifications resulted from a decrease in state 3. The inhibition of oxygen consumption in decoupled conditions was also observed for the four drugs. These results suggest that glucocorticoid effects on oxidative respiration were not predominant on complexes I, II and V. Furthermore the oxygen consumption was still decreased after the inhibition of complex III indicating that complex III was not the main target. So, it seems that glucocorticoids had an inhibitory effect on cytochrome c oxidase (complex IV). The intensity of the inhibition was different between the glucocorticoids studied. When sorted according to EC_{50} value, prednisolone was the most potent inhibitor, followed by dexamethasone, triamcinolone, and hydrocortisone. When sorted according to complex IV inhibition, the most potent inhibitor was also prednisolone followed by triamcinolone, dexamethasone and hydrocortisone. Previous studies have already shown inhibition of complex IV. For example, L-DOPA inhibited complex IV activity in a human neuroblastoma cell line (NB69) in a dose-dependent way [15]. It has been suggested that complex IV inhibition by L-DOPA could contribute to the pathogenesis of Parkinson's disease

but this should be confirmed. Nitric oxide (NO) inhibited the complex IV by competing with oxygen [16,17]. The inhibition was reversible, indicating that NO did not damage the mitochondria. The authors suggested that NO could be a physiological regulator of the affinity of the respiratory chain for oxygen.

We found no effect of glucocorticoids on swelling induced by Ca^{2+}/Pi, and Ca^{2+} fluxes. These results are not in agreement with those of Campbell and Al-Nasser [18]. They observed that dexamethasone inhibited the opening of a Ca^{2+}-dependent, non-specific, pore. However, their observations were obtained with high concentrations of glucocorticoids (40–200 μM). In their experiments, the inhibition of the pore opening started at 40 μM, which could not explain the effects on respiratory chain we observed at lower concentrations (Table 2).

A direct glucocorticoid regulation on cytochrome c oxidase activity has been already suggested by Bennett et al. [19]. Indeed they found that low dose of corticosterone administered to rats, which did not increase serum corticosterone, acted synergistically with sodium azide to inhibit cytochrome c oxidase activity. Furthermore they showed that chronic corticosterone and sodium azide treatment produced a spatial learning deficit. So they concluded that glucocorticoid treatment may worsen diseases involving metabolic insults. This is in agreement with our previous observation that prednisolone could worsen a mitochondrial toxicity induced by cyclosporin A [10]. Moreover, glucocorticoids may exacerbate injury induced by hypoxia when cytochrome c oxidase activity is inhibited [20]. Many reports have established the damaging properties of glucocorticoids during ischemic injury. Endogenous corticosterone contributed to the basal level of brain injury resulting from cerebral ischemia and exocitotoxic seizure activity, and metapyrone, an inhibitor of glucocorticoid production, reduced the brain injury induced by focal and global ischemia and seizure [21]. Glucocorticoids have been shown to exacerbate both neuronal and astrocytes injury following hypoxia [22]. In a model of myocardial ischemia reperfusion, Scheuer and Mifflin (1997) have shown that the corticosterone-treated rats had an infarct size significantly higher compared to the controls [23]. They concluded that chronic elevations in plasma corticosterone concentrations could contribute to the increased risk of cardiovascular disease in clinical conditions associated with elevated glucocorticoid levels.

In general, the steroid molecule is required to penetrate the cellular membrane and demonstrate affinity for the steroid-binding site on the glucocorticoid receptor, leading to activation of the receptor and to the antiinflammatory activities. The receptor affinity is a useful parameter that compares the potency of different steroids [24]. The EC_{50} values for the affinity to the steroid-binding site of hydrocortisone, dexamethasone, prednisolone and triamcinolone are 1.30×10^{-8}, 1.02×10^{-9}, 7.21×10^{-9}, and 5.07×10^{-10} M respectively [25]. Interestingly, the EC_{50} for the mitochondrial effect is lower or similar, suggesting a mitochondrial effect in addition to the nucleus effect at therapeutic concentrations.

In summary, the major findings of this study are that glucocorticoids inhibit cytochrome c oxidase activity by a direct mechanism. There are two important issues for these results. First, glucocorticoids may regulate the cellular metabolism by a direct action on the respiratory chain. The mechanism suggested is a modulation of cytochrome c oxidative activity. Second, glucocorticoids may potentiate disease insult in which oxidative metabolism is altered (ischemia for example).

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