Journal of Steroid Biochemistry & Molecular Biology 117 (2009) 50–55

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Direct effects of corticosterone on ATP production by mitochondria from immortalized hypothalamic GT1-7 neurons

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ARTICLE INFO

Article history:
Received 19 March 2009
Received in revised form 7 July 2009
Accepted 13 July 2009

Keywords:
Corticosterone
Glucocorticoid
Mitochondria
ATP production
Stress

ABSTRACT

Glucocorticoids are known to decrease intracellular ATP levels in the brain. This study was performed to investigate whether corticosterone at physiological levels depresses mitochondrial ATP production by directly acting on mitochondria. Mitochondria were isolated from immortalized hypothalamic GT1-7 neurons. ATP levels were determined using a luciferase–luciferin assay. When malate, α-ketoglutarate or pyruvate was used as a respiration substrate, corticosterone at ≥100 nM decreased ATP production by 10%. In contrast, corticosterone did not affect ATP production when succinate or *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine + ascorbate were used. To investigate the specificity of corticosterone inhibition, we examined several steroids. All steroids tested suppressed mitochondrial ATP production by 10% at a concentration of 100 nM, in a manner similar to that of corticosterone. To examine the effects of corticosterone on GT1-7 cell physiology, we incubated GT1-7 cells with *t*-butyl hydroperoxide (*t*-BuOOH) with corticosterone. Corticosterone largely enhanced *t*-BuOOH-induced cell death. These results indicate that corticosterone non-specifically inhibits mitochondrial ATP production by suppressing electron transfer from NADH to the electron transfer chain through complex I. Partial inhibition of mitochondrial ATP production by corticosterone may contribute to oxidative stress-induced cell death.

1. Introduction

Mitochondrial dysfunction is frequently observed in cells affected by oxidative stress, toxin administration, or neurodegenerative diseases, etc. [1]. Mitochondria determine the energetic state of the cell by yielding a major proportion of the ATP, generate oxidative stress in the cells by producing reactive oxygen species (ROS), and trigger cell death by releasing proapoptotic proteins. Therefore, mitochondrial damage or modification has been considered to greatly influence the cell. For example, cells overexpressing uncoupling protein 2, which is localized in the inner mitochondrial membrane, have decreased ROS release in the mitochondrial matrix and are resistant to experimental stroke and brain trauma [2]. Cyclosporine A inhibits mitochondrial membrane permeabilization and suppresses cell death induced by Ca^{2+} overloading [3]. Hexokinase II binds to outer mitochondrial membranes and inhibits BAX-induced apoptosis by preventing BAX from binding to the outer membranes [4].

The concentration of glucocorticoid in the blood increases in response to stress, thereby affecting cellular and mitochondrial energy metabolism in various ways [5–7]. Glucocorticoids have been reported to decrease the energy supplied by mitochondria, especially in the brain. In hippocampal cultures, natural and synthetic glucocorticoids depress ATP levels and mitochondrial potential in conjunction with gp120 [8]. In addition, corticosterone – a physiological glucocorticoid – has been reported to cause long-lasting depolarization of the mitochondrial membrane through prolonged Ca^{2+} elevation in hippocampal neurons [9]. Rats injected with dexamethasone – a synthetic glucocorticoid – showed decreased concentrations of cytochromes aa3 and b as well as ATPase activity in their brain mitochondria [10]. In addition to these effects, some of glucocorticoids slightly suppress state 3 respiration of isolated rat brain mitochondria [11]. However, it is not clear whether mitochondrial ATP production is actually suppressed by a physiological glucocorticoid or whether corticosterone – the principal glucocorticoid in rodents – decreases ATP production by mitochondria in the same manner as do other steroids, because some steroids other than glucocorticoid are also reported to inhibit state 3 respiration [12,13].

The present study demonstrated that 100 nM corticosterone suppressed ATP production in mitochondria isolated from a

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doi:10.1016/j.jsbmb.2009.07.002

hypothalamic neuronal cell line GT1-7, which could be attributed to the inhibition of complex I. In addition to corticosterone, the effects of several other physiological steroid hormones were also examined.

2. Materials and methods

2.1. Materials

Corticosterone, dexamethasone, 17β-estradiol, progesterone, testosterone, pregnenolone, aldosterone, and RU486 (mifepristone) were purchased from Sigma–Aldrich (St. Louis, MO, USA); cortisol and the luciferase–luciferin assay kit were purchased from Wako (Osaka, Japan); and the Amplex Red Hydrogen Peroxide/Peroxidase assay kit was obtained from Invitrogen (Carlsbad, CA, USA). Antisera against rat glucocorticoid receptors (GRs) were obtained from Prof. M. Kawata (Kyoto Prefectural University of Medicine, Kyoto, Japan). Other chemicals used were of the highest purity that was available commercially.

2.2. Cell culture of GT1-7 cells

GT1-7 cells (a generous gift from Prof. R. Weiner, University of California, San Francisco, CA) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. 1 or 2 days before the measurements, the medium was replaced with DMEM without phenol red and fetal bovine serum. The cultures were maintained at 37 °C in a CO_2 incubator under a humidified atmosphere of 95% air and 5% CO_2.

2.3. Preparation of isolated mitochondria from GT1-7 cells and cytosolic fractions from rat brains

Prior to isolation of mitochondria from GT1-7 cells, the cells were washed twice with Tris buffer (10 mM Tris–HCl, 250 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA); pH 7.4) and bathed in the same buffer. The cells were then collected with a cell scraper and transferred to a glass-Teflon homogenizer. The samples were carefully homogenized and centrifuged for 10 min at 1000 × *g*. The supernatant was collected and the pellet was discarded. After centrifuging the supernatant for 10 min at 5000 × *g*, the obtained pellet was suspended in Tris buffer. Preparation of cytosolic fractions from rat brains was performed essentially as described elsewhere [14]. Briefly, the rat brain was homogenized with 0.32 M sucrose in 1 mM $NaHCO_3$, 1 mM $MgCl_2$, and 0.5 mM $CaCl_2$ with a protease inhibitor cocktail, and centrifuged at 1400 × *g* for 10 min. The supernatant was further subjected to centrifugation at 100,000 × *g* for 60 min, and the obtained supernatant was collected. All the procedures were performed at 4 °C. Protein content was determined using the bicinchoninic acid (BCA) protein assay with bovine serum albumin (BSA) as a standard.

2.4. Western immunoblot analysis

The mitochondria were suspended at 10 mg/ml protein in sodium dodecylsulfate (SDS) buffer (62.5 mM Tris–HCl, 3% sodium dodecylsulfate, 5% sucrose, 5 mM dithiothreitol, and 0.01% bromophenol blue; pH 6.8). The samples were denatured for 5 min at 100 °C and subjected to electrophoresis on 7.5% polyacrylamide gels. After gel electrophoresis and transfer to polyvinylidene fluoride membranes, the blots were probed with a 1:3000 dilution of antisera against GRs for 18 h and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG [15]. Protein bands were detected with ECL Plus Western blotting detection reagents (GE Healthcare, Buckinghamshire, England). To obtain high quality images of the chemiluminescence of the protein bands, an LAS3000 Image Analyzer (Fuji Film, Tokyo, Japan) with a 16-bit wide dynamic range was used.

2.5. Determination of ATP production rate

Before inducing ATP production, mitochondria were incubated at 0.02 mg protein/ml of ATP production buffer (10 mM Tris–HCl, 250 mM sucrose, 1 mM EDTA, 2 mM KH_2PO_4, and 0.98 mM $CaCl_2$; pH 7.4) with steroids or 0.05% ethanol, a solvent for steroids, for 10 min at room temperature. The calculated free Ca^{2+} concentration in the ATP production buffer was approximately 350 nM. Mitochondrial ATP production was induced by the addition of an appropriate amount of substrate and 0.5 mM adenosine diphosphate (ADP). After a 5-min incubation of the mitochondria with the substrate and ADP, the mitochondrial suspension was kept on ice and centrifuged at 10,000 × *g* for 10 min at 4 °C. The ATP concentration of the supernatant was assayed with luciferase and luciferin according to Wibom et al. [16]. Luminescence was measured with a luminometer (MLR-100 Micro Lumino Reader; Corona Electric, Ibaraki, Japan).

In mitochondria, ATP is produced by adenylate kinase as well as by F_oF_1-ATPase. Because the activity of adenylate kinase is suppressed by removing Mg^{2+} from the buffer [17], the concentration of Mg^{2+} in the buffer was decreased in this study to observe the effects of steroids on ATP production by F_oF_1-ATPase. In fact, the presence of 0.5 mM Mg^{2+} significantly increased the ATP production rate of the mitochondria and decreased the percentage of ATP produced by F_oF_1-ATPase. This made it difficult to determine the significant changes in the rate of ATP production by F_oF_1-ATPase. In addition, we decreased the K^+ concentration to 2 mM, because excess amounts of K^+ decreased the luminescence intensity and prevented the identification of slight changes in ATP concentrations.

2.6. Determination of ROS production from isolated mitochondria

The conversion of 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent) to resorufin catalyzed by horseradish peroxidase was used to detect ROS released from mitochondria [18]. Mitochondria were incubated for 10 min at room temperature at 0.2 mg protein/ml of H_2O_2 assay buffer (10 mM Tris–HCl, 250 mM sucrose, 1 mM EDTA, 2 mM KH_2PO_4, 0.98 mM $CaCl_2$, 10 U/ml of Cu–Zn superoxide dismutase, 20 μM Amplex Red reagent and 0.1 U/ml horseradish peroxidase; pH 7.4) with steroids or 0.05% ethanol. The changes in the fluorescence emitted by resorufin upon addition of 1 mM malate and 0.5 mM ADP were monitored for 15 min by using a fluorescence plate reader (Wallac ARVO SX 1420; PerkinElmer, Waltham, MA, USA); the fluorescence was measured at excitation and emission wavelength of 560 and 640 nm, respectively, and the fluorescence was calibrated with a standard solution of H_2O_2.

2.7. Detection of cell death

Prior to induction of cell death, GT1-7 cells were incubated for 3 h at 37 °C with 100 nM corticosterone or 0.05% ethanol, a solvent for corticosterone, in a HEPES-buffered medium (10 mM HEPES, 120 mM NaCl, 4 mM KCl, 0.5 mM $MgSO_4$, 1.2 mM $CaCl_2$, 1 mM NaH_2PO_4, 4 mM $NaHCO_3$, and 25 mM glucose; pH 7.4). After preincubation with corticosterone or ethanol, the medium was replaced with DMEM without phenol red and serum, and then cells were incubated with or without 100 μM *t*-butyl hydroperoxide (*t*-BuOOH) in the presence of corticosterone or ethanol for 3 h at 37 °C in a CO_2 incubator. Cell death was evaluated by measuring the fluorescence of propidium iodide (PI), added at 3 μM. PI fluorescence was monitored at wavelengths >580 nm by using

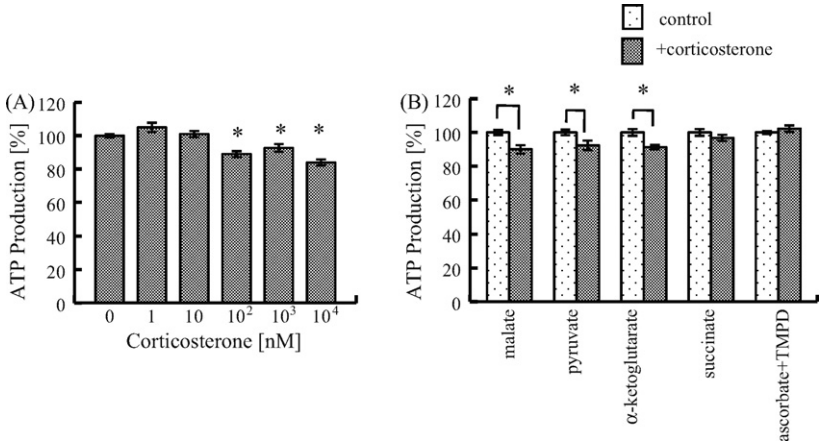

Fig. 1. Corticosterone affects ATP production by mitochondria isolated from GT1-7 cells. Data are expressed as ratios to the control without corticosterone. The ATP production rate for the control mitochondria was 21 ± 2 nmol/mg protein/min. (A) The effects of corticosterone at various concentrations. The respiration substrate used was malate (1 mM). Values represent the means ± SEM (n = 6). *P < 0.05 vs. control. (B) The effects of corticosterone (100 nM) in the presence of various respiration substrates. Malate (1 mM), pyruvate (0.3 mM), α-ketoglutarate (0.3 mM), succinate (1 mM), and ascorbate (2.5 mM) + TMPD (0.25 mM) were used as the respiration substrates. Succinate was added with rotenone (1 μM). Values represent the means ± SEM (n = 6). *P < 0.05 vs. control.

fluorescence microscopy. Fluorescence was elicited by illumination with a 75-W xenon lamp through a 15-nm bandpass filter centered at 535 nm.

2.8. Data analysis

The results were expressed as means ± SEM, and analyzed with ANOVA followed by the Bonferroni correction. Differences were considered statistically significant when P < 0.05.

3. Results

3.1. The effects of corticosterone on ATP synthesis and ROS generation by mitochondria isolated from GT1-7 cells

To examine the effects of corticosterone on mitochondria, the ATP production rate was measured in the presence of 1 mM malate. Under this condition, the ATP production rate was 21 ± 2 nmol/mg protein/min and was approximately 70% of the ATP production rate in the presence of excess malate. The rate of ATP production by the mitochondria significantly depended on the preparation. Therefore, for the precise evaluation of the effects of corticosterone, the ATP production rate in the presence of corticosterone was expressed as a ratio to the control measured with the same preparation in the presence of 0.05% ethanol, a solvent for steroids.

The corticosterone concentration was first increased from 0 to 10 μM, and malate was used as a respiration substrate (Fig. 1A). Corticosterone up to 10 nM had no effect on mitochondrial ATP production. When the concentration was increased from 10 to 100 nM, the ATP production rate by the mitochondria decreased to approximately 90%. No further decrease in ATP production was observed when the concentration was increased from 100 nM to 10 μM. In the presence of oligomycin or rotenone, the ATP production rate decreased to approximately 50% and was not affected by corticosterone (data not shown). These results indicate that corticosterone affects ATP production by F_oF_1-ATPase and not by adenylate kinase.

Subsequently, the suppression effects of corticosterone in the presence of various respiration substrates were examined. For this purpose, malate, pyruvate, α-ketoglutarate, succinate, and ascorbate + *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) were used as substrates. The concentrations of these substrates were adjusted so that the ATP production rates in the presence of these substrates without corticosterone were similar to the rates in the presence of 1 mM malate. Corticosterone suppressed the ATP production rate to 90% only when malate, pyruvate, and α-ketoglutarate were used as respiration substrates (Fig. 1B).

Similar to the pattern seen with ATP production, corticosterone decreased the rate of ROS generation in the presence of 1 mM malate (Fig. 2).

3.2. GR-independent suppression of ATP synthesis by corticosterone

Although most GRs are present in the cytosol and are translocated to the nucleus after glucocorticoid binding, some GRs are reported to bind to mitochondria in certain cells [19,20]. Therefore, GR involvement in the observed inhibition of ATP production by corticosterone was studied. For this purpose, the presence of GRs in the mitochondria and the effects of RU486, a potent antagonist of GRs, on mitochondrial ATP production were examined.

The presence of GRs in the mitochondria isolated from GT1-7 cells was examined using Western blotting analysis (Fig. 3A). GRs were not detected in the mitochondria isolated from GT1-7 cells, although they were observed in the cytosolic fraction of cells from rat brains. This result indicates that GRs were not present in the

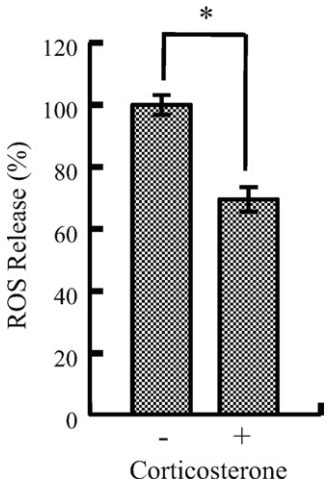

Fig. 2. Effects of corticosterone on ROS release from mitochondria. Data are expressed as the ratios to the control. Corticosterone was added at 100 nM and 1 mM malate was used as the respiration substrate. Values represent the means ± SEM (n = 6). *P < 0.05.

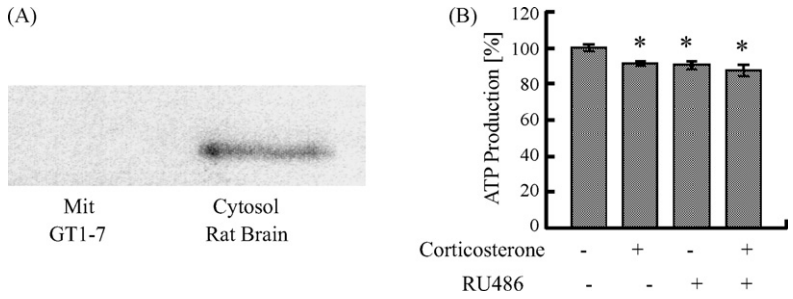

Fig. 3. GR-independent inhibition by corticosterone. (A) Western blot analysis of GRs. (Left) Mitochondria isolated from GT1-7 cells, (right) cytosolic fraction from rat brains. (B) Effects of a GR-inhibitor (RU486) on mitochondrial ATP suppression by corticosterone. Malate was used as the respiration substrate. The concentrations of corticosterone and RU486 used were 100 nM and 1 μM, respectively. Data are expressed as the ratios to the control without corticosterone and RU486. Values represent the means ± SEM ($n = 6$). $^*P < 0.05$ vs. control.

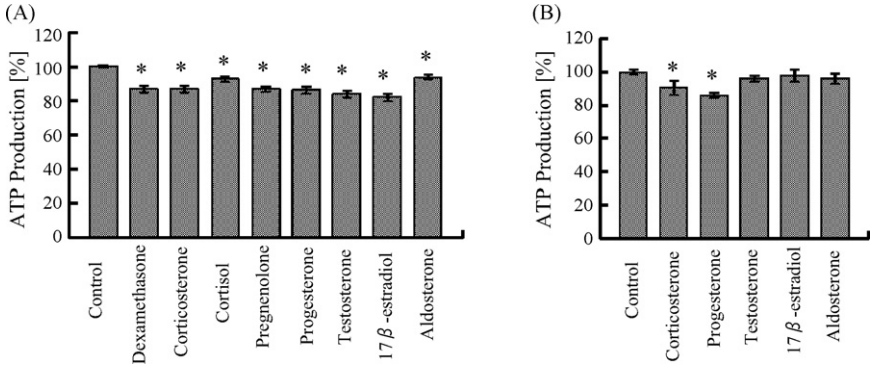

Fig. 4. The effects of various steroids on mitochondrial ATP production. Data are expressed as the ratios to the control without steroids. (A) All steroids were added at 100 nM. Malate was used as the respiration substrate. Values represent the means ± SEM ($n = 9$). $^*P < 0.05$ vs. control. (B) Effects of steroid hormones at physiological concentrations in the blood. The steroid concentrations used were 100 nM for corticosterone and progesterone, 20 nM for testosterone, 1 nM for 17β-estradiol, and 500 pM for aldosterone. Malate was used as the respiration substrate. Data are expressed as the ratios to the control. Values represent the means ± SEM ($n = 12$). $^*P < 0.05$ vs. control.

GT1-7 mitochondria or that the amount of GRs in the mitochondria was quite low. We then examined the effects of RU486 on mitochondrial ATP production. As shown in Fig. 3B, RU486 did not inhibit the effect of corticosterone on ATP production by mitochondria. RU486 alone also suppressed ATP production. These results suggest that the observed effects of corticosterone on ATP production were not mediated by GRs.

3.3. Effects of various steroid molecules on ATP production by mitochondria

To examine the specificity of corticosterone in the suppression of ATP synthesis, the effects of other steroids on ATP production in the presence of 1 mM malate were measured. All of the steroids examined were added at 100 nM, and they all decreased the ATP production to 90% (Fig. 4A). To further characterize the effects of these steroids on ATP production, ATP synthesis in the presence of 1 mM succinate was measured. Similar to the case of corticosterone, none of the steroids tested affected the ATP production rate of the mitochondria under these conditions (data not shown).

Some of these steroids were then added at concentrations identical to the physiological concentrations in the blood (Fig. 4B). Suppression of ATP production was observed for corticosterone and progesterone, but not for testosterone, 17β-estradiol, or aldosterone.

3.4. Effects of corticosterone on t-BuOOH-induced cell death

Oxidative stress induces overactivation of nuclear poly(ADP-ribose) polymerase (PARP), decreasing the cellular ATP level, which results in cellular dysfunction and cell death [21]. To investigate the effects of corticosterone on oxidative stress-induced cell death, GT1-7 cells were incubated in the presence of *t*-BuOOH and corticosterone (Fig. 5). Addition of *t*-BuOOH slightly increased the percentage of PI-positive cells to 12%. This increase was suppressed by 3-aminobenzamide (3AB), an inhibitor of PARP, indicating that *t*-BuOOH induced PARP-dependent cell death in GT1-7 cells. When both corticosterone and *t*-BuOOH were present in the medium, the percentage of PI-positive cells increased considerably to 29%, although corticosterone alone did not bring about this increase. These results indicate that corticosterone stimulated *t*-BuOOH-induced cell death through PARP activation.

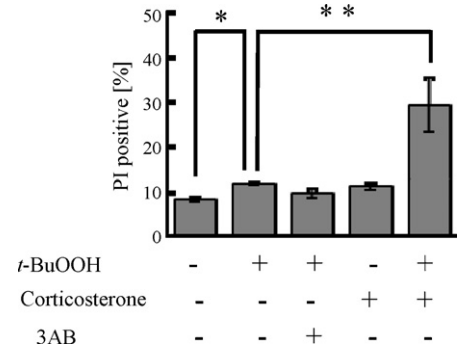

Fig. 5. The effects of corticosterone on *t*-BuOOH-induced cell death. The concentrations used were 100 nM for corticosterone, 100 μM for *t*-BuOOH, and 5 mM for 3AB. 3AB was added to the cells in HEPES-buffered medium 30 min before the addition of *t*-BuOOH and was also added during the 3-h incubation with DMEM. Values represent the means ± SEM ($n = 3$). $^*P < 0.05$ and $^{**}P < 0.01$.

4. Discussion

The results of this study indicated that corticosterone suppressed mitochondrial ATP production. Electrons are donated to the mitochondrial electron transfer chain through complex I by malate, pyruvate, or α-ketoglutarate; through complex II by succinate; and through cytochrome c by ascorbate + TMPD. The effects of corticosterone on these substrates were compared to identify the inhibition site. Suppression of ATP production by corticosterone was effective when mitochondria were energized by malate, pyruvate, or α-ketoglutarate, but not when they were energized by succinate or ascorbate + TMPD. These results indicate that corticosterone suppressed the donation of electrons to the electron transfer chain through complex I. Consistent with this result, corticosterone decreased the generation of ROS by mitochondria in the presence of malate. This is because ROS in mitochondria are primarily generated by components of the electron transfer chain in reduced states. The suppression by corticosterone would be due to the decreased generation of NADH and/or depression of complex I activity. The latter reasoning is more plausible, because NADH production from malate, pyruvate, and α-ketoglutarate is catalyzed by different enzymes. It must be noted that the suppression of mitochondrial respiration by corticosterone was not statistically significant, because the experimental error for the respiration rate was >10%.

The observed inhibition by corticosterone was independent of GRs, although the binding to GRs would affect mitochondria [19,20]. Further, the present findings on the suppression of ATP production were not specific to corticosterone. All of the steroids tested inhibited ATP production at 100 nM, probably through inhibition of complex I. RU486 also inhibited ATP production by mitochondria, and this may be because it has a similar structure to steroids. However, corticosterone and progesterone may be the only steroids that suppress mitochondrial ATP production at physiological concentrations, since the other major physiological steroid hormones (17β-estradiol, testosterone and aldosterone) did not directly affect ATP production by the mitochondria at physiological levels in the blood. To the best of our knowledge, this is the first report on the direct inhibition of complex I by corticosterone and progesterone at their physiological concentrations in the blood.

Corticosterone is one of the glucocorticoids, which have been reported to increase energy expenditure and metabolic rate [22]. Consistent with this effect, glucocorticoid-dependent increases in nuclear DNA transcripts that encode cytochrome c in mouse C2C12 myoblasts [23] as well as mitochondrial DNA transcripts that encode mitochondrial proteins and RNAs in rat hepatoma cell lines have been observed [24,25]. Because these proteins and RNAs are involved in oxidative phosphorylation, increased expression would augment the metabolic rate. In contrast, in the rat brain, glucocorticoids inhibited mitochondrial activity; they depressed ATP levels and the mitochondrial potential in conjunction with gp120 [8] and caused long-lasting depolarization of the mitochondrial membrane through prolonged Ca^{2+} elevation [9]. In addition, dexamethasone decreases cytochromes aa3 and b concentrations and ATPase activities [10].

In the present study, in order to investigate the mechanism by which corticosterone decreased intracellular ATP concentrations, we examined whether corticosterone decreased mitochondrial ATP production by directly acting on mitochondria. Previous studies have shown that glucocorticoids directly depress state 3 respiration of mitochondria through the inhibition of the activities of F_oF_1-ATPase [11] and complex IV [26]. However, it was not clear whether inhibition of respiration actually resulted in a decrease in ATP production in intact mitochondria, because the inhibition of the respiration was slight and the enzyme activities were measured in disrupted mitochondria under a hypoosmotic condition. Our results showed that corticosterone directly inhibited ATP production by intact mitochondria from immortalized mouse hypothalamic neurons. Further, we showed that all of the steroids tested inhibited complex I activity, consistent with previous reports on the inhibition of complex I by estrogen [13].

We have shown that corticosterone depresses the activity of complex I at physiological levels in the blood and inhibits ATP production by mitochondria. However, it remains unclear whether this inhibition is physiologically significant, because the inhibitory effect is slight. When GT1-7 cells were incubated with *t*-BuOOH, corticosterone greatly enhanced cell death. Because oxidative stress induces cell death by reducing intracellular ATP levels [21], corticosterone may reduce the cell viability by further decreasing ATP levels. In addition, corticosterone is reported to affect brain mitochondria by depolarizing mitochondria via increased Ca^{2+} influx [9] and by decreasing the concentrations of the components of the electron transfer chain [10]. Because these effects lead to a decrease in ATP production, the combination of the direct suppression of complex I observed in the present study might exacerbate the decrease in mitochondrial ATP production. In addition to glucocorticoids, dehydroepiandrosterone has recently been reported to suppress mitochondrial function [27]. Further studies are required to clarify the mechanisms by which steroids, including corticosterone, decrease energy production by mitochondria.

Acknowledgments

We thank Prof. R. Weiner (University of California, San Francisco, CA, USA) for generously providing GT1-7 cells and Prof. M. Kawata (Kyoto Prefectural University of Medicine, Kyoto, Japan) for kindly supplying the antisera against GR. We also thank Dr. M. Kato (Nippon Medical School, Tokyo, Japan) for kindly showing us how to handle GT1-7 cells and Dr. C. Nakamura (Tokyo University of Agriculture and Technology, Tokyo, Japan) for kindly permitting our usage of a fluorescence plate reader. This work was funded by grants from the Ministry of Education, Science, and Culture (17570151).

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