FORMATION OF ENLARGED MITOCHONDRIA IN A LIVER CELL LINE IN RESPONSE TO A SYNTHETIC GLUCOCORTICOID

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For a number of years it has been recognized that glucocorticoids cause alterations in liver cell morphology (6, 9). Several investigators have shown that in liver in vivo mitochondria can be enlarged to many times their normal volume by treatment with cortisone (13, 15). There is a concomitant decrease in mitochondrial number, and the results of Kimberg and Loeb suggest that this is due to mitochondrial fusion (7). However, the exact mechanism whereby mitochondrial volume is altered and whether in fact cortisone is the direct causal agent are not known due to the complexity of studying these questions in a whole animal system.

We have found that dexamethasone sodium phosphate (dex), a synthetic glucocorticoid, causes the formation of enlarged mitochondria in a liver cell line RLC-GAI, which grows in defined medium. In this paper we present our observations on the amount of enlargement that occurs after 5 days of treatment. The formation of enlarged mitochondria is reversible upon removal of the hormone from the medium, and we have attempted to determine whether "mitochondrial" or "nonmitochondrial" inhibitors are more effective in blocking the return of mitochondria to their normal size when the hormone is removed.

MATERIALS AND METHODS

A clonal variant of the RLC line, isolated by Dr. L. Gerschenson, which grows in defined medium (RLC-GAI), was used in these studies (3). 3 days after plating, 10^{-5} M dex was added to half the cultures for 5 days. The medium was changed each day of this 5-day period on all cultures. In experiments where the dex effect was reversed, cultures were washed three times with medium and then cultured an additional 2 days before use. Drugs added to the medium during the reversal period were 50 μg/ml hydroxyurea, 2.8 μg/ml cycloheximide, 0.1 μg/ml ethidium bromide, and 10 μg/ml chloramphenicol.

For electron microscopy, cells were fixed in 2% glutaraldehyde in 0.13 M Sorensen's phosphate buffer, postfixed in 1% OsO_4, and embedded on the culture dishes by a modification of the method of Brinkley et al. (2). With this modification, 100% alcohol is removed by three rinses with Luft's Epon, and capsules filled with Epon are placed on the dish before polymerization. Grids were stained for 30 min with alcoholic uranyl acetate and 5 min with lead citrate.

The general experimental design and the statistical measurements made on mitochondria were patterned on those of Loud (11). For each treatment, five experiments were performed; two capsules placed at random on the petri dishes were used for sectioning, yielding a total of 10 blocks for each treatment. Five micrographs were taken from each block and analyzed at a magnification of 18,500 with a 2 cm grid. For each treatment, 50 micrographs were analyzed, representing approximately 4,500 μm^2 of cytoplasm. The specific methods for calculating the various parameters given in the Tables were taken from another paper by Loud et al. (12). The following characteristics were measured: (*a*) percent of cytoplasm occupied by mitochondria ($\mu m/100\ \mu m^3$); (*b*) the surface-to-volume ratio of an average mitochondrion ($\mu m^2/\mu m^3$); (*c*) the number of mitochondria per 100 μm^3 of cytoplasm (μm^{-3}); and (*d*) the volume of an average mitochondrion (μm^3). The last parameter, the average mitochondrial volume, was calculated by dividing the percent of cytoplasm containing mitochondria by the number of mitochondria per 100 μm^3. In addition, the average nuclear size and the nucleo/cytoplasmic ratio were calculated as a measure of change in cell volume. These calculations were made from 0.5-μm sections of pelleted cells; scraping and pelleting of cells was necessary to achieve random orientation.

RESULTS

In cells treated with 10^{-5} M dex for 5 days, mitochondria were increased in size mainly due to an increase in length (Figs. 1–4). Branched mitochondria were increased in treated culture, representing 5% of the total population compared to 0.3% in untreated cultures. Table I contains a statistical summary of the changes produced by dex. The volume of an average mitochondrion was increased, number per unit volume decreased, and the surface per volume decreased all significantly ($P < 0.012$), although there was no change in the percent of the cytoplasm occupied by mitochondria. Not included in the Table but also calculated was the diameter of an average mitochondrion which showed a barely significant

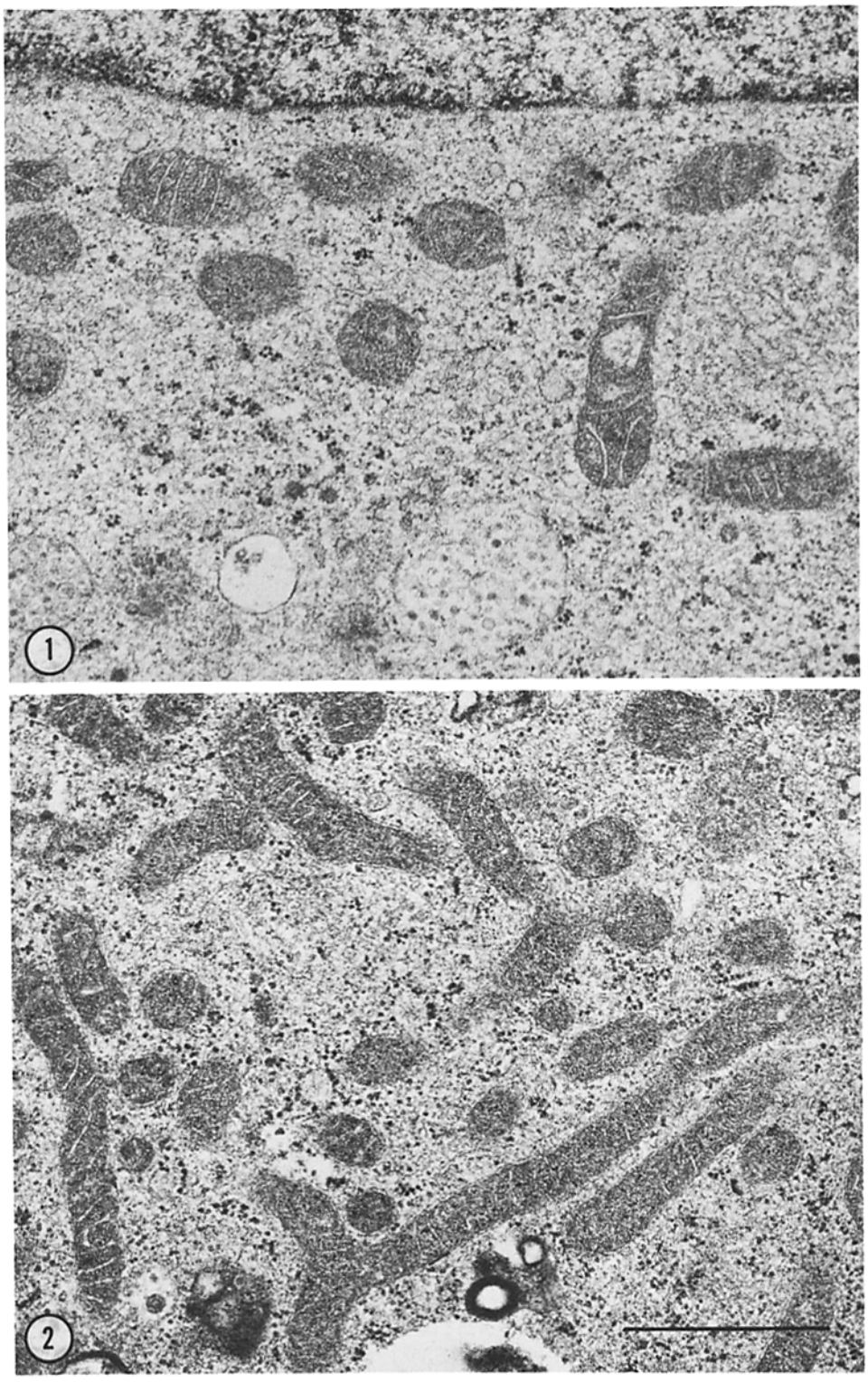


FIGURE 1 Mitochondria from cells cultured 5 days in control medium. × 30,000.

FIGURE 2 Mitochondria from cells cultured 5 days in medium containing 10^{-5} M dex. Branched mitochondria can be seen. × 30,000. Line represents 1 μm.

TABLE I

Mitochondrial Changes with Dexamethasone

Parameter	Units	Control	Dex	Medium reversal
Percent cytoplasm containing mitochondria	$\mu m^3/100\mu m^3$	7.11 ± 0.81	8.01 ± 0.92	8.10 ± 0.71
Mitochondrial surface-to-volume ratio	$\mu m^2/\mu m^3$	12.64 ± 1.21	9.09 ± 1.12*	11.64 ± 1.01
Number of mitochondria per 100 μm^3 cytoplasm	μm^{-3}	57.46 ± 13.21	28.08 ± 11.93‡	58.23 ± 10.10
Average mitochondrial volume	μm^3	0.13 ± 0.03	0.33 ± 0.09*	0.14 ± 0.04

± Standard error.

* Using a Student t test and comparing the mean shown to the mean in the control $P < 0.012$.

‡ $P < 0.025$.

Dex, dexamethasone sodium phosphate.

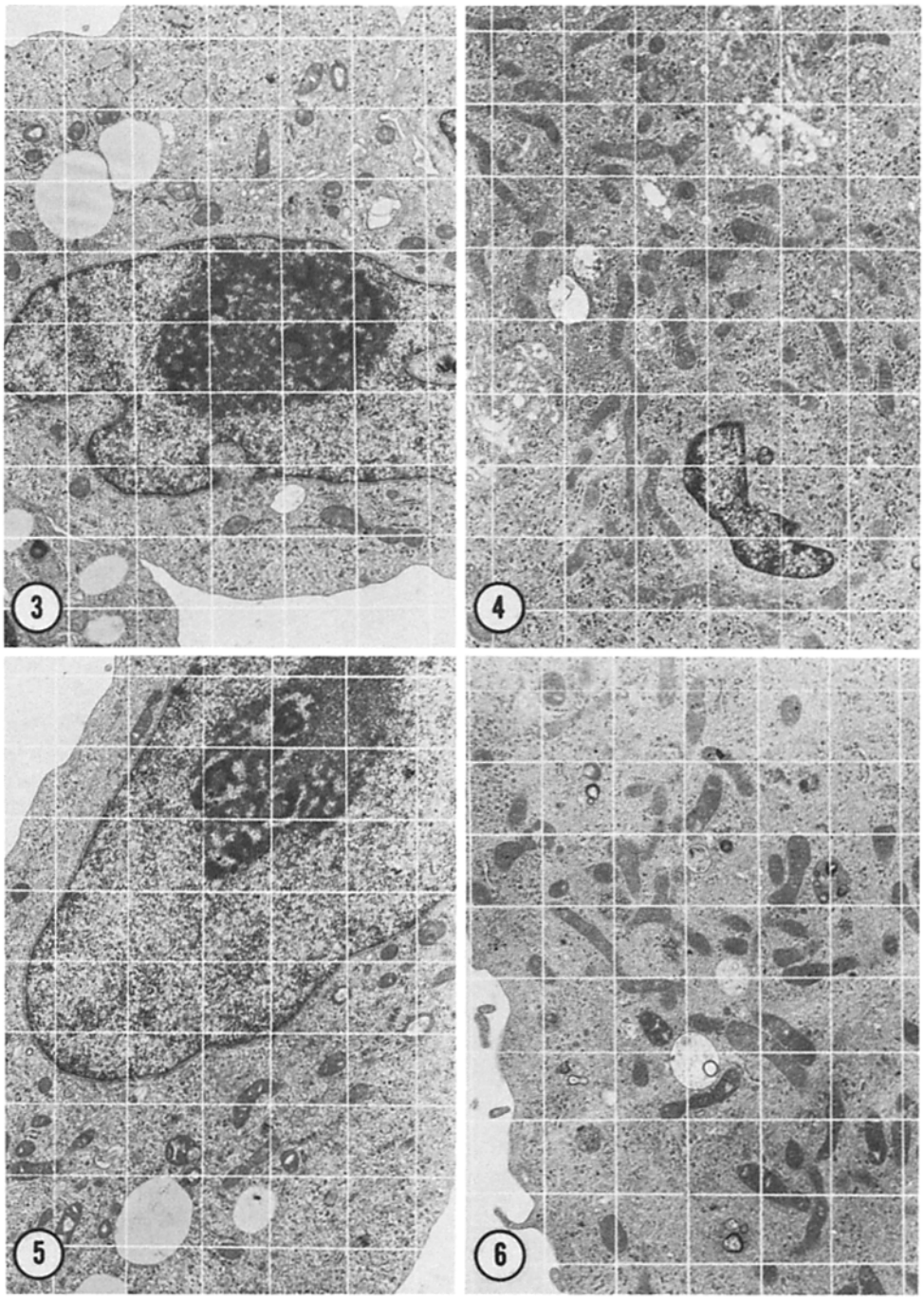
difference between control and dex-treated cells ($P < 0.10$), reinforcing the general observation from micrographs that mitochondria enlarge mainly by an increase in length. The decrease in number of mitochondria per unit volume of cytoplasm reflects an actual decrease in number of mitochondria per cell since there is no difference in the cell volume with dex treatment as reflected in the nucleus-to-cytoplasmic ratio (control = 3.70 ± 0.20, dex = 3.75 ± 0.24). This is a valid measure of altered cytoplasmic volume since the average nuclear diameter is not significantly different between control and dex cells (control = 7.0 ± 0.24, dex = 6.8 ± 0.26), and there is one nucleus per cell.

The effects of dex are reversible upon removal of the hormone from the medium for 2 days (Fig. 5). The statistical analysis of reversal in fresh medium is presented in Table I in the last column. The effects of four metabolic inhibitors on the reversal phenomenon were studied with inhibitor concentrations known to be effective either from studies on our cells or from studies of other investigators (5, 1, 4, 10). Two of the inhibitors utilized, hydroxyurea and cycloheximide, primarily block "nonmitochondrial" synthesis at the concentrations used and two primarily effect "mitochondrial" metabolism. The effects of these inhibitors alone on mitochondrial morphology when added for 2 days were examined (Table II). None of the four inhibitors altered the percent of the cytoplasm occupied by mitochondria. However, ethidium bromide alone caused the formation of enlarged mitochondria (Fig. 6), similar to the enlargement seen with dex. Some investigators using higher concentrations of ethidium bromide and chloramphenicol have found degenerative changes in mitochondria (8, 14) but we observed no such effects.

The effect of adding inhibitors to the medium during the reversal period is presented in Table III. Cycloheximide appears to have no effect on the return of mitochondria to normal size, whereas hydroxyurea does significantly alter slightly the number of mitochondria per unit volume of cytoplasm and the volume of an average mitochondrion ($P < 0.025$). Both chloramphenicol and ethidium bromide appear to block the return to normal size. With ethidium bromide the mitochondria are larger than mitochondria treated with dex alone (Table I), but the effects of the two agents are not additive.

DISCUSSION

With these studies we have shown that a synthetic glucocorticoid can cause mitochondrial enlargement in a liver cell line in culture. This suggests that glucocorticoids are also the direct causal agent in vivo. In RLC-GAI cells the mitochondrial changes are similar to, though less extensive than those seen in vivo; mitochondrial volumes are increased and numbers reduced while the percent of the cytoplasm occupied by mitochondria remains constant. These observations are consistent with the idea that enlarged mitochondria are formed by mitochondrial fusion as has been shown in vivo. There are several differences in mitochondrial enlargement in vitro and in vivo: (*a*) in cultured cells increases in volume appear to be due mainly to increased length, and (*b*) there is more branching in vitro. The significance of these differences is not known but could be related to the much greater division rates in cultured cells.



FIGURES 3–6 These photographs are × 0.5 reproductions of electron micrographs printed with the superimposed grid used for stereological analysis. The distance between grid lines is 1.1 μm. Fig. 3, Untreated culture as for Fig. 1. Fig. 4, Dex-treated culture as for Fig. 1. Fig. 5, Dex-treated culture from which Dex was removed and cells cultured an additional 2 days in control medium. Fig. 6, Cells cultured 3 days in control medium after seeding and then 2 days with 0.1 μg/ml ethidium bromide.

TABLE II

Effect of Drugs

Parameter	Units	Hydroxyurea	Cycloheximide	Chloramphenicol	Ethidium bromide
Percent cytoplasm containing mitochondria	$\mu m^3/100\mu m^3$	7.45 ± 0.87	7.91 ± 0.75	7.50 ± 0.71	8.01 ± 0.80
Mitochondrial surface-to-volume ratio	$\mu m^2/\mu m^3$	12.13 ± 0.98	12.41 ± 0.91	11.09 ± 0.81	9.11 ± 1.01*
Number of mitochondria per 100 μm^3 cytoplasm	μm^{-3}	52.45 ± 13.45	48.71 ± 14.21	50.13 ± 10.79	23.41 ± 11.31‡
Average mitochondrial volume	μm^3	0.12 ± 0.05	0.14 ± 0.07	0.14 ± 0.05	0.38 ± 0.12*

± Standard error.

* By comparison to the mean for the control from Table I $P < 0.012$.

‡ $P < 0.025$.

TABLE III

Reversal of Dex Changes in Drug-Containing Medium

Parameter	Units	Hydroxyurea	Cycloheximide	Chloramphenicol	Ethidium bromide
Percent cytoplasm containing mitochondria	$\mu m^3/100\mu m^3$	8.01 ± 0.82	8.51 ± 0.71	7.50 ± 0.91	8.13 ± 0.73
Mitochondrial surface-to-volume ratio	$\mu m^2/\mu m^3$	10.62 ± 0.71	11.66 ± 0.89	9.10 ± 0.74*	8.27 ± 0.97‡
Number of mitochondria per 100 μm^3 cytoplasm	μm^{-3}	40.12 ± 10.10	54.51 ± 12.20	30.64 ± 12.47§	20.51 ± 11.90*
Average mitochondrial volume	μm^3	0.19 ± 0.06	0.16 ± 0.08	0.24 ± 0.06*	0.48 ± 0.07‡

± Standard error.

* By comparison to the mean for the control from Table I $P < 0.012$.

‡ $P < 0.010$.

§ $P < 0.025$.

Inhibitor studies suggest that the mitochondrial volume decrease observed after corticoid withdrawal can occur without nuclear DNA or protein synthesis, but that mitochondrial protein synthesis and mitochondrial RNA and perhaps DNA synthesis are necessary.

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