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Synergy between chronic corticosterone and sodium azide treatments in producing a spatial learning deficit and inhibiting cytochrome oxidase activity

(electron transport chain/mitochondria/memory/oxidative metabolism/glucocorticoids)

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ABSTRACT **Previously, we developed a rat model of persistent mitochondrial dysfunction based upon the chronic partial inhibition of the mitochondrial enzyme cytochrome oxidase (EC 1.9.3.1). Continuous systemic infusion of sodium azide at ≈1 mg/kg per hr inhibited cytochrome oxidase activity and produced a spatial learning deficit. In other laboratories, glucocorticoids have been reported to exacerbate neuronal damage from various acute metabolic insults. Therefore, we tested the hypothesis that corticosterone, the primary glucocorticoid in the rat, would potentiate the sodium azide-induced learning deficit. To this end, we first identified nonimpairing doses of sodium azide (≈0.75 mg/kg per hr) and corticosterone (100-mg pellet, 3-week sustained-release). We now report that chronic co-administration of these individually nonimpairing treatments produced a severe learning deficit. Moreover, the low dose of corticosterone, which did not elevate serum corticosterone, acted synergistically with sodium azide to inhibit cytochrome oxidase activity. The latter result represents a previously unidentified effect of glucocorticoids that provides a candidate mechanism for glucocorticoid potentiation of neurotoxicity induced by metabolic insult. These results may have the clinical implication of expanding the definition of hypercortisolism in patient populations with compromised oxidative metabolism. Furthermore, they suggest that glucocorticoid treatment may contribute to pathology in disease or trauma conditions that involve metabolic insult.**

The brain, with its disproportionate energy requirement, is among the organs most sensitive to damage from impaired oxidative metabolism. Vulnerability within the brain is heterogeneous, with structures such as the hippocampus, striatum, and regions of neocortex being among the most susceptible to metabolic insult (1–5). Various mitochondrial enzyme defects have been identified in several neurodegenerative diseases, although in most cases it is not known whether these defects have a role in disease etiology (6–15). Mitochondrial dysfunction occurring in progressive neurodegenerative diseases creates a condition of chronic partial impairment of oxidative phosphorylation, the biological significance of which is not well understood. However, this type of mitochondrial impairment is qualitatively different in duration and severity than that following an acute cessation of oxidative metabolism following a complete infarct or anoxic event.

A rat model of persistent cytochrome oxidase inhibition has been developed (16, 17) to induce conditions *in vivo* that more closely resemble chronic partial mitochondrial dysfunction than do hypoxia/ischemia models that employ severe acute insult. Cytochrome oxidase (EC 1.9.3.1) is the terminal and rate-limiting enzyme of the mitochondrial respiratory chain. In previous work, a regimen of systemic sodium azide infusion was identified that induces partial cytochrome oxidase inhibition, without significantly affecting other respiratory chain enzymes. Rats given this treatment for 1–4 weeks show severe impairment of spatial learning tasks that is not secondary to a sensory or motor dysfunction (16, 17).

Glucocorticoids, hormones that are released by the adrenal cortex and have numerous homeostatic and stress-response functions, have been shown to potentiate the neurotoxicity of a variety of acute metabolic insults (18–22). This interaction led us to predict that corticosterone, the primary glucocorticoid in the rat, would potentiate the learning impairment of sodium azide. In the present investigation we tested the hypothesis that co-administration of corticosterone and sodium azide, at doses that individually do not produce a behavioral impairment, would produce synergistic impairment of spatial learning and memory. The effect of these treatments, individually and in combination, on brain cytochrome oxidase activity was also evaluated.

MATERIALS AND METHODS

Subjects and Treatment. Adult male Sprague–Dawley rats (Harlan Laboratories) were group-housed in a facility approved by the American Association for the Accreditation of Laboratory Animal Care, with food and water available *ad libitum* (light:dark, 12:12, lights on at 0700). The rats, which were approximately 4 months of age and weighed between 375 and 425 g at the time of surgery, were given at least 1 week to acclimate to laboratory conditions prior to surgery. The experimental design was 2 × 2, with the independent variables being, 1, the presence or absence of sodium azide, and, 2, the presence or absence of exogenous corticosterone. Rats were anesthetized with secobarbital (50 mg/kg) and were each implanted with an osmotic minipump (Alzet 2ML4; 2.5 μl/hr, 2-ml reservoir) that contained either sodium azide at 120 mg/ml in 0.9% saline or the saline vehicle, and a 3-week duration, constant-release pellet (G-111; Innovative Research of America) that contained either 100 mg of corticosterone or the placebo matrix. The rats were divided into four groups (n = 8 per group): Control (saline pump/placebo pellet), Cort (saline pump/corticosterone pellet), Azide (sodium azide pump/placebo pellet), and Cort/Azide (sodium azide pump/corticosterone pellet). The sodium azide-containing pumps

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Abbreviation: ANOVA, analysis of variance.

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delivered a dose of 300 μg/hr, or approximately 0.75 mg/kg per hr. The corticosterone pellet delivered a dose totaling approximately 4.76 mg/day at a uniform infusion rate.

No mortality was observed as a result of the surgical manipulations. However, a single rat from the Azide group, and two rats from the Cort/Azide group, had wounds that became infected during the behavioral training. Training was discontinued in these animals and they were subsequently dropped from the study.

Spatial Learning. The rats were required to use information provided by extra-apparatus cues to learn the location of a round hidden escape platform (12 cm in diameter) located in a black circular tank, 1.5 m in diameter and 0.3 m high, which was filled with water maintained at 24–25°C. The platform was placed midway between the center and the wall of the tank, with its top submerged 1 cm below the water surface. Location of the platform within the tank remained constant during 5 consecutive days of spatial training. The tank was located in a room containing numerous sensory cues that were maintained in constant locations for the duration of all behavioral testing.

Training was begun 1 week after surgery. Prior to the first training trial, each rat was placed on the platform and allowed to remain there for 30 sec. Each animal was then given three trials per day with an intertrial interval of 10 min. A trial consisted of the rat's being placed into the water at one of three predetermined starting locations, each of which was used each day but the order of use varied by day. The rat was required to swim for 60 sec or until it located the hidden escape platform and climbed onto it. If the platform was not located within 60 sec, the rat was guided by hand to it. In either case, the animal was allowed to remain on the platform for 15 sec before being returned to its home cage. Swim times and pathlengths were recorded by using a computerized video tracking system (San Diego Instruments).

On day 6, each rat was given a 60-sec probe test of retention with the escape platform removed from the tank. Performance on the task was measured by (*i*) the amount of time spent in the quadrant of the tank that the platform previously occupied; (*ii*) latency to first crossing over the previous location of the platform; and (*iii*) total number of such crossings.

The following day, all rats were given six cued trials in which the platform was elevated 1 cm above the water surface and thus was visible to the swimming rats. During the cued task, the visible platform was moved to a new location for every trial so that place information was no longer relevant. Only swim times were recorded for the visible platform test.

Measurement of Cytochrome Oxidase Activity. During the week following behavioral testing, a time window during which drug exposure remained constant, mitochondria were isolated from brain tissue of the subjects. The rats were sacrificed by rapid decapitation, in an order randomized with respect to treatment. The brain was removed from the skull within 30 sec and subdivided by a coronal cut made at the level of the pons. Brain tissue anterior to the cut was used to isolate cell body and synaptic membrane mitochondria by using a modification of the method of Lai and Clark (23).

Cytochrome oxidase activity was assayed spectrophotometrically at 550 nm. Reagent volumes were calculated on the basis of a 1-ml total assay volume. Potassium phosphate buffer (20 mM, pH 7.0 at room temperature) was added to a cuvette that contained 20 μl of 10 mM dodecyl maltoside, a detergent used to solubilize cytochrome oxidase. A sufficient volume of the electron donor, reduced cytochrome *c*, was added to give a baseline absorbance reading of approximately 0.725, then the cuvette was equilibrated for 3 min at 30°C. The reaction was initiated by the addition of 10 μg of mitochondrial protein and was measured for 2 min. To determine the minimum absorbance at the completion of the reaction ($A_{550} \approx 0.22$), several crystals of potassium ferricyanide, a potent oxidant of ferrocytochrome *c*, were added after this time. The rate constant was calculated from the slope of the region where the linear correlation equaled 0.995. Cytochrome oxidase activity is reported as a first-order rate constant in units of $sec^{-1} \cdot mg^{-1}$ (mg referring to mitochondrial protein).

Measurement of Serum Corticosterone. One day after the final behavioral trials, a blood sample was obtained from each rat to allow measurement of serum corticosterone by a radioimmunoassay (24). Tail vein blood samples were drawn from the rats within a sampling time of less than 2 min from the time that the investigator first touched the home cage. All samples were taken between 1200 and 1400 hours. The intra-assay coefficient of variation in the procedure was determined to be 8.7%.

Statistics. Group performances for the hidden and visible platform training were analyzed by a repeated-measures two-way analysis of variance (ANOVA). One factor was treatment and the repeated-measures factor was days or trials of training (time). A one-way ANOVA was used to analyze probe trials of retention for hidden-platform learning, cytochrome oxidase activity, and serum corticosterone levels. A significant ANOVA test was followed by a *a posteriori* analysis for unequal sample numbers to identify the source(s) of significant differences either between or within groups. The α level was set *a priori* at 0.05.

RESULTS

Spatial Learning. Analysis of swim pathlength data over the 5 days of training revealed a significant effect of treatment [$F(3, 25) = 6.67$; $P < 0.002$] and of time [$F(4, 100) = 2.58$; $P < 0.001$] but no significant interaction [$F(12, 100) = 1.21$, $P > 0.1$] among the four treatment groups. Several significant differences were identified by the *a posteriori* analyses. The overall pathlength of the Cort group, collapsed over days and trials, was significantly less than the pathlengths of the other groups ($P < 0.01$, all comparisons). However, performance of the Cort group on the last day of training was not significantly different from either the Control or the Azide group ($P > 0.1$). Thus, the Cort group acquired the task faster, but asymptotic performance levels among these three groups did not differ significantly. In contrast, both overall performance and day 5 performance of the Cort/Azide group were significantly poorer than those performances by each of the other three treatment groups ($P < 0.01$, all comparisons). These data are shown in Fig. 1. There were no significant differences among the groups in swim speeds. Furthermore, swim times and pathlengths were highly correlated ($r > 0.9$).

Probe trial results paralleled the outcome of the training trials; that is, by all three measures the Cort/Azide group performed significantly worse than the other three groups (Fig. 2). There was a significant difference among the groups in total time spent in the quadrant of the tank where the escape platform had been located during training [$F(3, 25) = 7.1$; $P < 0.01$], with the Cort/Azide group spending less time in the quadrant than each of the other groups ($P < 0.05$). Time to first crossing over the place where the platform had been located was also significantly different among the groups [$F(3, 25) = 10.4$; $P < 0.001$], with the Cort/Azide group having significantly longer latency to first crossing than each of the other groups ($P < 0.001$, all comparisons). Finally, there was a significant difference among groups in total crossings [$F(3, 25) = 20.9$; $P < 0.001$], with the Cort/Azide group making significantly fewer crossings than each of the other groups ($P < 0.001$, all comparisons). No significant differences were identified between the Control, Cort, and Azide groups for any probe measure.

The analysis of the visible platform data indicated that there was no significant effect of treatment [$F(3, 25) = 2.01$; $P > 0.1$], a significant effect of trials [$F(4, 25) = 11.94$; $P < 0.001$], and no significant interaction [$F(5, 125) = 0.91$; $P > 0.1$]. Thus, in contrast with the results for the hidden-platform task, there

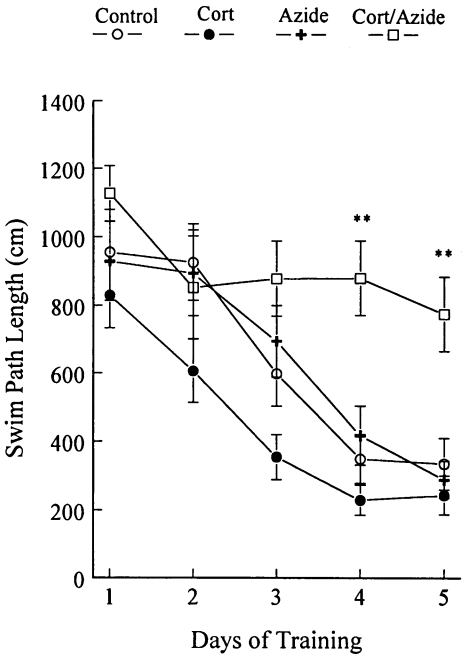

FIG. 1. Spatial learning in the Morris water maze. Control, Cort, and Azide rats all learned the location of the hidden escape platform over the 5-day training period, as indicated by a shorter swim path to find the platform. In contrast, the Cort/Azide rats were significantly impaired in this task. **, Days where the Cort/Azide animals were different ($P < 0.01$) from all other groups, while the other groups were not different from each other. Error bars indicate SEM.

were no significant differences in learning performance between groups when the platform was made visible. All groups showed significant improvement over the course of training ($P < 0.05$, all groups). These data are summarized in Table 1.

Cytochrome Oxidase Activity. Cytochrome oxidase activity in cell body mitochondria was significantly affected by treatment [$F(3, 22) = 19.32$; $P < 0.001$]. The *a posteriori* analyses revealed that cytochrome oxidase activity in cell body mitochondria of the Cort/Azide group was significantly lower than activity found in each of the other three treatment groups ($P < 0.01$, all comparisons). In contrast, no significant differences were found between cytochrome oxidase activities of the Control, Cort, and Azide groups ($P > 0.1$ for all pairs). These data are shown in Fig. 3*A*.

Cytochrome oxidase activity in synaptic membrane mitochondria also was affected significantly by treatment [$F(3, 22) = 14.56$; $P < 0.001$]. Cort treatment alone did not significantly affect cytochrome oxidase activity; however, the mean activity in synaptic mitochondria of this group was 25% lower than that in the Control group ($0.05 < P < 0.1$). In contrast with the case in cell body mitochondria, cytochrome oxidase activity in the synaptic membrane mitochondria of the Azide group was significantly lower than that of both the Control and the Cort groups ($P < 0.01$, both comparisons). The cytochrome oxidase activity in the synaptic fraction of the Cort/Azide group also was significantly lower than that in both the Control ($P < 0.01$) and the Cort group ($P < 0.05$), but activity in this group was not significantly different than that in the Azide group. These data are shown in Fig. 3*B*.

Serum Corticosterone. There was a significant overall effect of treatment on serum corticosterone [$F(3, 19) = 4.49$, $P < 0.05$]. The *a posteriori* analyses indicated that no group was significantly different from the Control group, but that the Azide group had significantly higher serum corticosterone concentrations than did the Cort group. These data are shown in Table 2.

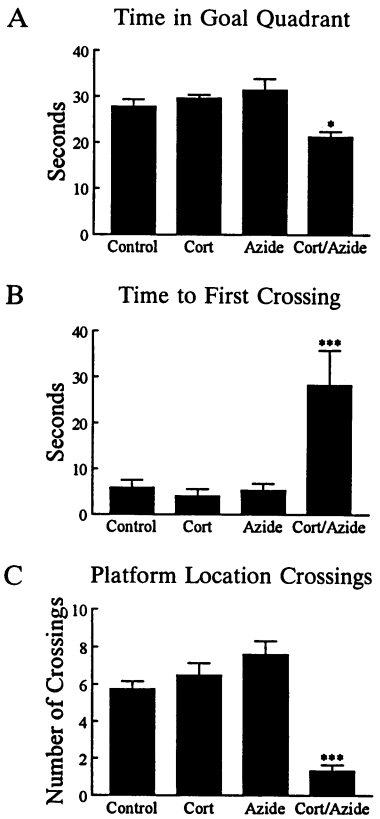

FIG. 2. Probe trial measures. Cort/Azide rats were significantly impaired in all measures of memory for the location of the hidden platform when tested 24 hr after the last training session. For this test the escape platform was removed from the tank and the rats were given a single 60-sec swim. The Cort/Azide group spent less time in the region of the tank where the platform had been located (*A*), took longer to first cross the location where the platform had been (*B*), and had fewer total crossings of this location (*C*). *, $P < 0.05$; ***, $P < 0.001$.

DISCUSSION

Treatment regimens of both sodium azide and corticosterone that individually did not impair learning of the swim maze task were selected on the basis of pilot studies to investigate potential synergy between these two treatments. Consistent with the pilot studies, the Azide treatment in the present study did not, by itself, alter acquisition or retention performance significantly compared with the Control treatment. This result contrasts with our previous finding that a higher-dose regimen of sodium azide produces a pronounced spatial learning deficit (16, 17). The low-dose Cort treatment significantly accelerated the rate of task acquisition; however, by the end of training, performance proficiencies of the Control, Cort, and Azide groups were statistically indistinguishable. In marked contrast,

Table 1. Visible platform training swim times

Group	Swim time, sec: Trials 1 and 2	Swim time, sec: Trials 5 and 6
Control	22.1 ± 2.6	9.3 ± 1.1
Cort	16.9 ± 3.0	8.4 ± 1.2
Azide	18.3 ± 3.5	9.9 ± 2.4
Cort/Azide	19.6 ± 4.7	8.8 ± 1.2

Results are presented as mean ± SEM. There were no significant differences between groups of the visible platform task performance (ANOVA). Comparisons of trial blocks 1 and 2 versus 5 and 6 showed significant differences within all groups, indicating that all groups learned this task.

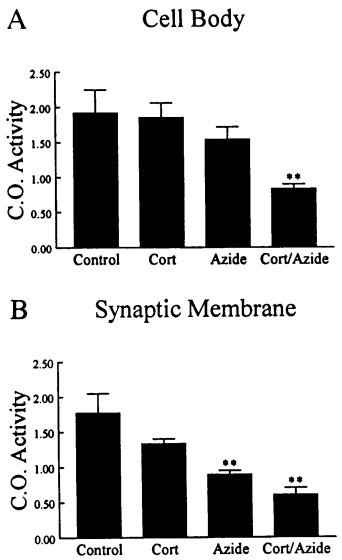

FIG. 3. Cytochrome oxidase activity expressed as a first-order rate constant in units of $sec^{-1} \cdot mg^{-1}$ (mg referring to mitochondrial protein). (*A*) In mitochondria extracted from the cell body fraction, cytochrome oxidase activity was significantly reduced in Cort/Azide rats compared with the other groups. (*B*) In mitochondria extracted from the synaptic membrane fraction, cytochrome oxidase activity was significantly reduced in both Azide and Cort/Azide rats compared with the Control or Cort groups. **, $P < 0.01$.

the Cort/Azide treatment induced a severe impairment in the performance of this task compared with each of the other three treatment groups.

Several observations indicate that the performance deficit of the Cort/Azide group reflected a learning deficit, rather than being a product of sensory, motor, or motivational changes. First, rats in all groups appeared healthy and maintained normal weight (data not shown). Second, the lack of significant differences in visible platform trials among the groups indicates that the rats in all groups could see the cues, were capable of swimming to the platform, and were motivated to do so. Finally, the lack of differences in group swim speeds, as well as the strong correlation between swim times and pathlengths, provides further evidence that swimming ability was not impaired by any treatment.

The potentiation of sodium azide-induced cytochrome oxidase activity inhibition by corticosterone was unpredicted on the basis of our reading of the experimental literature, as we found no reports of glucocorticoid effects on respiratory chain enzyme activity. Cytochrome oxidase activity in mitochondria isolated from the cell body fraction was severely inhibited by the Cort/Azide treatment, but neither the Cort nor the Azide individual treatments induced a significant inhibition of its activity in this fraction. Thus, the present result provides a demonstration of a biochemical synergy between glucocorticoid treatment and oxidative metabolic inhibition. Glucocorticoids have been shown to produce certain metabolic effects that may be related to the present findings; these include decreasing cellular glucose uptake (25, 26), accelerating ATP loss following metabolic insult (27, 28), and increasing extracellular accumulation of excitotoxic amino acids during both stress (29, 30) and chemical ischemia (22). The acceleration of ATP loss, in particular, would be predicted to be a direct consequence of the decrease in cytochrome oxidase activity found in the Cort/Azide group. The decrease in glucose uptake and the increase in excitatory amino acid-induced neuronal activity in response to glucocorticoid could each be contributory to a positive-feedback cascade leading to a cellular energy crisis. Taken together, these results, along with the present findings, provide demonstrations of interactions between glucocorticoids and metabolic insult that may be factors in their synergy of neurotoxicity (18).

Table 2. Serum corticosterone levels

Group	Conc., μg/dl
Control	6.2 ± 0.5
Cort	3.7 ± 0.9
Azide	7.9 ± 1.6
Cort/Azide	6.8 ± 2.8

ANOVA main effect ($P < 0.05$). No treatment group was significantly different from the Control group (Tukey–Kramer *a posteriori* comparison); Cort versus Azide, $P < 0.05$.

In synaptosomal mitochondria, the treatments altered cytochrome oxidase activity in a pattern somewhat different than in cell body mitochondria. Cort treatment alone reduced cytochrome oxidase activity in synaptic membrane mitochondria by approximately 25% below Control level, a difference that did not reach statistical significance. Further investigation is required to determine whether corticosterone has an inhibitory effect on synaptic membrane cytochrome oxidase activity. The Azide treatment alone produced a significant inhibition of cytochrome oxidase in synaptic membrane, in contrast with its effect in soma. For the synaptosomal mitochondria, the combination of Cort/Azide produced the largest inhibition of cytochrome oxidase activity found in any treatment group; however, the degree of inhibition was not greater than the sum of the two individual treatments.

Cytochrome oxidase activity was affected by Azide and Cort/Azide treatments in the synapse to a greater degree than in the soma, a finding that suggests oxidative metabolism in the synapse may be more vulnerable to disruption than that in the soma. Furthermore, preservation of oxidative metabolism in neuronal cell bodies appears to provide some protection of function, since learning was normal in the AZIDE group despite a significant decrease in synaptic cytochrome oxidase activity. Under experimental conditions of greater task complexity or higher metabolic demand, however, a learning deficit might have emerged in the Azide group.

The effects of the treatments on basal serum corticosterone were small at the time point measured. The most important result of the serum corticosterone measurements is that no treatment produced long-term elevation of corticosterone to levels approaching those that have been reported after classical stressors (31–38), nor would the serum corticosterone levels of any group be classified as hypercortisolemic. The clinical significance of this finding may be that it expands the window for glucocorticoid exposure that may be detrimental under some conditions. A similar suggestion has been made for the role of glucocorticoids in aging-related neuronal damage. Evidence from aging research indicates that chronic exposure to glucocorticoids—even at normal levels—leads to the gradual accumulation of deleterious neuronal effects (39–43). Mitochondrial dysfunction may render the brain more susceptible to glucocorticoid damage that typically accumulates over longer periods of time during normal aging.

The empirical finding that the continuous treatment with such a low dose of corticosterone produced such a powerful synergy of impairment with the sodium azide treatment is somewhat puzzling. Although not significantly different from the Control group, the Cort group had a mean serum corticosterone concentration that was the lowest of all four treatment groups. This result confirms that the Cort pellets provided very low-level chronic exposure to corticosterone, although this degree of exposure may have been sufficient to blunt the activity of the pituitary–adrenal axis in its regulation of corticosterone (44–46). We propose, however, that some direct or secondary effect of the corticosterone treatment, rather than a lack of corticosterone, mediates its synergy with sodium azide. Subsequent investigation of the neurotoxicity of

corticosterone and sodium azide supports this interpretation. Recently, a dose-dependent synergy of neurotoxicity was identified with co-administration of higher doses of corticosterone and sodium azide than were used in the present study (47). However, the Cort/Azide regimen described in this report produced little evidence of neuronal damage. Therefore, frank neurodegeneration is not likely to be the mechanism underlying the effects of these treatments.

In summary, the major findings of this study are that chronic low-dose corticosterone treatment potentiates a sodium azide-induced spatial learning deficit and cytochrome oxidase inhibition. There are two important clinical implications of this result. First, even low serum corticosterone levels may produce *functional* hypercortisolism when oxidative metabolism is impaired. For example, brain metabolism is severely reduced in Alzheimer disease (48); in addition, hypercortisolism has been identified as a late feature affecting approximately 50% of the patient population (49–53). Therefore, given the present results, functional hypercortisolism may affect a greater proportion of the patient population, and be clinically significant earlier in the course of this disease, than has been recognized previously. A second implication of these data is that even a subtle dysregulation of glucocorticoid metabolism may exacerbate cognitive decline in diseases in which oxidative metabolism is compromised. Under these conditions, even a modest hypercortisolism may accelerate the progression of that disease, regardless of whether the metabolic impairment has a role in disease etiology.

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