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Research Article

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CONCISE

PUBLICATIONS

Corticosteroids Increase Superoxide Anion Production by Rat Liver Microsomes

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ABSTRACT Superoxide anion production by liver microsomes from intact, adrenalectomized, and cortisol-treated adrenalectomized rats has been determined. The amount formed was roughly proportionate to the amount of cortisol given, and a similar response was seen in the activity of NADPH-cytochrome *c* reductase. The amount of measurable superoxide anion was markedly reduced by the addition of superoxide dismutase. The increased production of this potent free radical with cortisol therapy suggests that its formation may contribute to some of the harmful effects of corticosteroids given in more than physiologic amounts.

INTRODUCTION

Corticosteroids have been shown to have widespread effects on many enzymes and physiological processes. Among these are stimulation or inhibition of some of the enzymes that combine with molecular O_2: oxygenases and flavoprotein dehydrogenases (1–3). These enzymes have in common the production and/or utilization of superoxide anion, a powerful oxidant and reductant shown to be potentially harmful in a number of tissues (4–6). As the NADPH-cytochrome *c* reductase of rat liver microsomes has been shown to produce superoxide anion (7), this study was undertaken to see if cortisol influenced production of this free radical in this tissue.

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METHODS

A NADPH-dependent oxidation of epinephrine to adrenochrome that was inhibited by superoxide dismutase was followed as a measure of superoxide production, as described by Aust et al. (7) and as employed by Prough and Masters (8). Aust et al. found that the epinephrine oxidation activity copurified with NADPH-cytochrome *c* reductase. NADPH-cytochrome *c* reductase activity was also measured, therefore, to identify further the enzyme being influenced by cortisol treatment.

Adult female rats of the Sprague-Dawley strain were adrenalectomized for 3–14 days. Microsomes were prepared from control, adrenalectomized, and cortisol (pregn-4-ene-11β,17α,21-triol-3,20-dione)-treated adrenalectomized rats. Those animals receiving cortisol were given 0.25, 1.0, or 5 mg (Cortef, The Upjohn Company, Kalamazoo, Mich.) by daily subcutaneous injections for 2 days and one-fifth the daily dose of cortisol sodium succinate 60 min before sacrifice on the 3rd day. Animals were decapitated, and the livers were immediately removed, weighed, and placed in cold 0.3 M sucrose. 3 g of liver were homogenized in two approximately equal portions, each in 5 ml of cold 0.3 M sucrose with 10 strokes of a Ten Broeck homogenizer. The homogenate was centrifuged in the cold at 2,000 *g* for 10 min, and the supernate was then centrifuged at 12,500 *g* for 10 min. The final centrifugation at 105,000 *g* for 60 min at 4°C yielded a microsomal pellet that was resuspended in appropriate volumes of 0.3 M sucrose for addition to the assay cuvettes. Protein determinations were by the method of Lowry et al. (9). Superoxide anion was measured as a function of NADPH-cytochrome *c* reductase activity by following the conversion of epinephrine to adrenochrome at 480 nm in an Aminco Chance spectrophotometer in the split beam mode (American Instrument Co., Silver Spring, Md.), with an extinction coefficient of 4,020 $M^{-1}\,cm^{-1}$ (10). NADPH-cytochrome *c* reductase was also quantified by measuring the reduction of cytochrome *c* at 550

TABLE I

Effect of Adrenalectomy and Cortisol on Superoxide Anion Production by Rat Liver Microsomes

Incubation time	Group A	Adrenochrome Mean	Adrenochrome SE	Group B	Adrenochrome Mean	Adrenochrome SE	*P*
min		*nmol/mg protein*			*nmol/mg protein*		
10	Adrenalectomy	70.6	7.3	Intact	94.6	10.1	<0.1 (NS)
	Adrenalectomy	57.7	4.0	Cortisol	84.6	7.0	<0.01
15	Adrenalectomy	166.1	13.7	Intact	215.6	16.0	<0.05
	Adrenalectomy	143.9	7.8	Cortisol	223.3	14.2	<0.001
20	Adrenalectomy	265.4	13.2	Intact	318.6	19.8	<0.05
	Adrenalectomy	246.4	10.4	Cortisol	361.1	13.1	<0.001

Adrenochrome formation from epinephrine, as a measure of superoxide anion production, by female rat liver microsomes. Means represent values obtained from six to nine independent experiments involving different animals. Adrenalectomy was from 3 to 14 days before sacrifice. Cortisol groups were adrenalectomized and received 5 mg as Cortef for 2 days plus 1 mg cortisol sodium succinate 60 min before sacrifice. Time was measured from addition of epinephrine. *P* values represent comparisons of groups A and B by unpaired *t* test at incubation times shown (18).

nm in an Aminco Chance spectrophotometer in the split beam mode, with an extinction coefficient of 21,000 M^{-1} cm^{-1} (11). Superoxide dismutase was obtained from Diagnostic Data, Inc., Mountain View, Calif.

RESULTS

Adrenochrome production from epinephrine by rat liver microsomes as a measure of superoxide anion did not occur without added NADPH. The addition of superoxide dismutase blocked the formation of adrenochrome and thus by inference decreased superoxide anion (6). Although epinephrine will undergo spontaneous oxidation to adrenochrome at increased pH, the NADPH required for its formation, as well as the block by the superoxide dismutase, indicated the enzymatic character of the reaction being studied (7). NADH was much less effective than NADPH as an electron donor in the system.

Table I presents the adrenochrome formation (superoxide anion production) by liver microsomes from control, adrenalectomized, and cortisol-treated adrenalectomized animals. It is apparent that the absence of corticosteroids results in a decrease in the activity of the reductase and that therapy with cortisol significantly increased production of superoxide anion in the adrenalectomized animals. The means presented in each group are obtained from six to nine independent experiments with the values in adrenalectomized animals representing experiments run at the same time as the corresponding intact or cortisol-treated animals. Only at the shorter incubation time of 10 min was there no statistically significant differences between the rates in adrenalectomized and in intact animals. The values in cortisol-treated rats were significantly different at all incubation times.

NADPH-cytochrome *c* reductase activity of liver microsomes was measured by their ability to reduce cytochrome *c* in the presence of NADPH. Various dosage schedules for cortisol were followed to see if the response of cytochrome *c* reductase was dose dependent. Fig. 1 depicts cytochrome *c* reductase after 15-min incubations of liver microsomes from adrenalectomized female rats. The response appears to be dose dependent, with rates at 0- and 0.25-mg doses being statistically separable from the 5-mg dosage ($P < 0.05$ and < 0.025, respectively).

Superoxide anion production showed the same dose dependence when measured by epinephrine conversion to adrenochrome in 20-min incubations. A value of 368±36 nmol adrenochrome/mg protein was obtained from livers of 5-mg-cortisol-treated, adrenalectomized rats. This was statistically different from livers of animals receiving no steroid (221±21, $P < 0.025$) and also from those receiving 1 mg cortisol (244±28, $P < 0.025$).

DISCUSSION

A rather large number of reactions dependent upon the presence of molecular O_2 have been shown to generate superoxide anion (O_2^-). This free radical is known to undergo spontaneous dismutation to H_2O_2 and O_2, and this reaction has been demonstrated to be greatly accelerated by the enzyme superoxide dismutase (12). It has been postulated that the relative instability of the anion, as well as the presence of the dismutase, act under physiologic conditions to reduce its toxic effects.

Conditions that stimulate production of superoxide may produce serious tissue damage. The toxicity of 100% oxygen has been attributed to an increase in

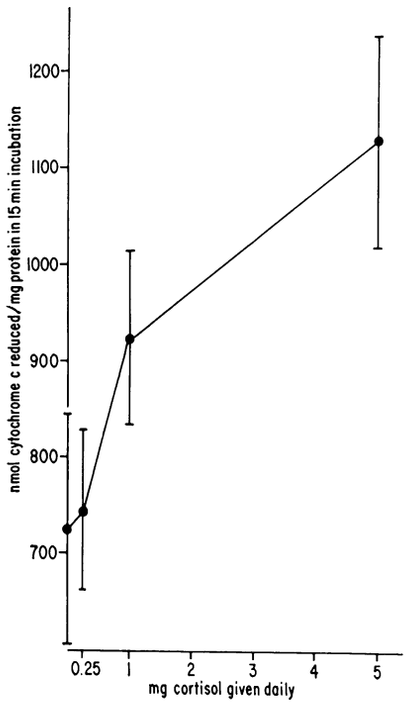

FIGURE 1 Cytochrome *c* reduced in 15-min incubations of rat liver microsomes from adrenalectomized female rats. Cortisol was given in doses of 0.25–5 mg daily for 2 days, and then one-fifth the dose was given on the 3rd day 60 min before sacrifice. Reaction mixtures contained approximately 5 μg/ml microsomal protein, 15 μM cytochrome *c*, and 0.11 mM NADPH in 0.15 M potassium phosphate, pH 8.5. Optical density was recorded in an Aminco Chance spectrophotometer in the split beam mode. Cytochrome reduction was calculated from changes in absorbance at 550 nm with the time measured from the point of addition of microsomes. Each point represents the mean and SE of five animals. *P* values calculated by Student's *t* test for unpaired samples (18) showed 0 and 0.25 mg to be significantly different from the 5-mg dosage. ($P < 0.05$ and < 0.025, respectively).

superoxide anion production (6), and the harmful effects of ionizing radiation are at least partially due to an increase in this free radical (13). Production of superoxide anion has also been implicated in the killing of bacteria by phagocytizing leukocytes (14). McCord has demonstrated the depolymerization of hyaluronic acid and bovine synovial fluid by superoxide anion (5). Any reaction that causes an increase in superoxide anion production might, therefore, be considered potentially harmful.

A decrease in drug metabolism by the adrenalectomized animal is well known. The clinician has long recognized the sensitivity of the Addisonian patient to morphine and other drugs, and a decreased rate of drug metabolism in the adrenalectomized animal has been demonstrated (15). Castro and co-workers, in a study of the liver mixed-function oxygenase system of rats that catalyzes ethylmorphine metabolism, concluded that the decreased activity in adrenalectomized animals was not due to a decrease in cytochrome P-450 content or to the ability of this cytochrome to bind drug substrates. They found that the activities of the NADPH-cytochrome *c* reductase and cytochrome P-450 reductase roughly paralleled changes in ethylmorphine demethylase activity after adrenalectomy and corticosteroid administration (16). Sasame et al. have suggested that superoxide formation acts as a mechanism of uncoupling between the oxidation of pyridine nucleotides and drug oxidation by rat liver microsomes (17).

The present studies confirm the action of cortisol to increase the activity of the NADPH-cytochrome *c* reductase of rat liver microsomes (16) and demonstrate that this hormone action is accompanied by a dose-dependent increase in superoxide anion production. The known harmful effects of superoxide anion as well as the widespread tissue damage known to occur when corticosteroids are administered in larger than physiologic doses for prolonged periods raises the question of whether superoxide anion production may contribute to this damage. Cortisol has been shown to influence the activity of a number of dehydrogenases and oxygenases that could produce excess superoxide anion. Further studies will have to be performed to demonstrate whether cortisol produces an increase in this radical in other systems and whether superoxide dismutase can prevent such an increase from harming the organism.

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