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REGULATORY EFFECT OF COPPER ON RAT ADRENAL CYTOCHROME P-450 AND STEROID METABOLISM*

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Abstract—Accumulation of Cu^{2+} in rat adrenal glands produced a biphasic response in concentrations of the mitochondrial cytochrome P-450 and heme which were, in turn, reflected in abnormal steroid biosynthesis and output. In the mitochondria, 1 day after Cu^{2+} treatment, when the concentration of the metal ion was increased by 2- to 3-fold over the control value, a significant increase in cytochrome P-450-dependent steroid 11 β-hydroxylase activity was observed. These effects were accompanied by a nearly 85% increase in concentrations of cytochrome P-450 and heme. In addition, the activity of δ-aminolevulinate synthetase was increased by 3-fold. In those animals lipid peroxidation, assessed by measuring concentrations of conjugated dienes, was reduced to approximately 50% of the control value. However, after 7 days of Cu^{2+} treatment (via a mini-osmotic pump), a significantly lowered rate of 11β-hydroxylase activity was noted, and the plasma concentration of corticosterone was also reduced significantly. Also, in the mitochondria, the concentrations of cytochrome P-450 and heme were decreased in comparison with the control values. These decreases were accompanied by elevated levels of the mitochondrial lipid peroxidation and a further increase in adrenal Cu^{2+} content (5-fold). At this time, δ-aminolevulinate synthetase activity remained elevated but to a lower extent than that observed after 1 day of Cu^{2+} treatment. In contrast to 11β-hydroxylase activity, the reduction in cytochrome P-450 content was not reflected in a decrease in the rate of cholesterol side-chain cleavage; rather this activity was increased in Cu^{2+}-treated animals. Adrenal heme oxygenase activity was unaffected by either Cu^{2+} treatment, as was the specific content of cytochrome P-450 in the microsomal fraction. The present findings suggest that the Cu^{2+}-mediated regulation of cytochrome P-450-dependent steroidogenic activity in the adrenal mitochondria is predominately a reflection of the metal ion affecting heme biosynthesis and lipid peroxidation in this organ. Moreover, these actions appear to differentially affect the mitochondrial cytochrome P-450 species catalyzing different hydroxylation reactions in the adrenal steroidogenesis pathway.

Copper is an essential element yet, when in excess, toxic effects are manifested. The latter could stem from accidental exposure to this metal ion or be caused by the genetic disorder of copper metabolism, i.e. Wilson's disease. In both cases, toxicity is expressed when the capacity of the liver to sequester and store this metal ion is surpassed [1].

The metabolism of Cu^{2+} appears to be closely linked to adrenocortical hormones. It follows that a considerable amount of information is available regarding the effects of adrenocortical hormones on Cu^{2+} metabolism. Little, however, is known concerning the effects of Cu^{2+} on steroidogenic processes in the adrenal cortex. Previous studies in the rat have demonstrated that the Cu^{2+} storage capacity of the liver and the concentration of Cu^{2+} which enters the circulation from the liver are influenced by adrenocortical hormones [2]. These phenomena also have been observed in patients with adrenocortical insufficiency [3] and in leukemic patients [4]. In the latter patients, the administration of ACTH or synthetic glucocorticoids has been shown to cause a reduction in elevated serum Cu^{2+} levels [4]. Moreover, an ability of the endogenously synthesized glucocorticoids to lower serum Cu^{2+} concentrations by inhibiting the biosynthesis of the primary Cu^{2+} transport protein, ceruloplasmin, has been demonstrated [5, 6]. In addition, glucocorticoids have been shown to promote the biosynthesis of metallothioneins, the cysteine-rich protein that binds and sequesters Cu^{2+} in the liver [7].

The ability of metal ions to alter heme and hemoprotein metabolism in nonsteroidogenic tissues, such as the liver [8], as well as steroid producing organs, such as the ovary and the testes [9], is well established. Metal ions in nonsteroidogenic organs induce the activity of heme oxygenase, the rate-limiting enzyme of the heme degradation pathway [9–11]. These agents also alter the activity of δ-aminolevulinate (ALA) synthetase, the rate-limiting enzyme in the heme biosynthesis pathway [11, 12]. Consequences of these actions include a reduction in the contents of microsomal heme and cytochrome P-450 and a suppression of the hemoprotein-dependent monooxygenase activities [8]. Recently, an interaction between metal ions and the metabolism of heme was also suggested in the adrenal glands [13]. Moreover, in rats lower plasma concentrations of glucocorticoids, following acute and subchronic administration of Cd^{2+}, have been reported [14].

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In the adrenals, several steps in steroid biosynthesis are catalyzed by cytochrome P-450. These include the mitochondrial cholesterol side-chain cleavage and 11 β-hydroxylation. These reactions, which are catalyzed by cytochrome P-450_{scc} and cytochrome P-$450_{11\beta}$, constitute the initiating and the terminating steps in glucocorticoid biosynthesis respectively. Therefore, alterations in heme metabolism in the adrenals could be expected to have ramifications in the steroidogenesis process.

The present study was undertaken to investigate the effects of Cu^{2+} on heme and hemoprotein metabolism and the cytochrome P-450-dependent steroidogenic reactions in rat adrenals.

MATERIALS AND METHODS

Materials. $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, corticosterone and deoxycorticosterone were obtained from the Sigma Chemical Co., St. Louis, MO. [7-^{3}H]-Pregnenolone was obtained from New England Nuclear, Boston, MA. 25-Hydroxycholesterol was purchased from Research Plus, Inc., Bayonne, NJ. Cyanoketone was a gift of the Sterling-Winthrop Laboratories, Rensselaer, NY. Pregnenolone antisera was obtained from Wein Laboratories, Succasunna, NJ. Supplies for atomic absorption spectrophotometry were obtained from Varian Inc., Des Plaines, IL. The high performance liquid chromatography (HPLC) supplies were purchased from Waters Associates Inc., Milford, MA, and the Fisher Scientific Co., Itasca, IL. All other chemicals were products of the Fisher Scientific Co.

Animals and tissue preparations. Male Sprague–Dawley rats (200–250 g) were purchased from Harlan Industries, Madison, WI, and allowed access to food and water *ad lib.* Rats were injected (s.c.) with 125 μmoles/kg of $CuSO_4 \cdot 5H_2O$ and killed 24 hr later. All injections were made between 8:30 and 9:30 a.m. Animals were treated subchronically with Cu^{2+} via a mini-osmotic pump which was implanted subcutaneously, and delivered a daily dose of 40 μmoles/kg of $CuSO_4 \cdot 5H_2O$ for 7 days. Control animals received saline. When plasma was required, rats were first anesthetized with Nembutal (5 mg/kg, i.p.), and blood samples were obtained by cardiac puncture; thereafter, the animals were decapitated. The adrenal glands were removed, defatted, and homogenized in 20 vol. of 0.01 M Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose. The mitochondrial and the microsomal fractions were prepared as described before [9]. The cellular homogenate was used for the determination of ALA synthetase activity, while the 9000 *g* supernatant fraction was used for the assessment of heme oxygenase activity. The mitochondrial and the microsomal fractions were utilized for measurements of concentrations of cytochrome P-450 and heme, analysis of Cu^{2+} content, and assessment of lipid peroxidation. The activities of 11 β-hydroxylase and cholesterol side-chain cleavage were determined in the mitochondrial fractions.

Assay procedures. The activity of ALA synthetase was measured by the spectrophotometric procedure of Marver *et al.* [15], as modified before [16]. This method was chosen over others [17] because of the direct manner in which the product can be distinguished from interfering aminoketopyrrole complexes that are formed in the ALA synthetase assay system. Heme oxygenase activity was determined by measuring the enzymatic conversion of the substrate, hematoheme, to hematobilirubin, as described earlier [9]. The formation of hematobilirubin was detected spectrophotometrically from the absorption between 350 and 550 nm, and was quantitated using the millimolar extinction coefficient of 30. The concentration of cytochrome P-450 was measured as described by Nozu *et al.* [18] using extinction coefficients of 91 $mM^{-1}\,cm^{-1}$ and 52 $mM^{-1}\,cm^{-1}$ [19] for the microsomal and the mitochondrial hemoproteins respectively. The pyridine hemochromogen method of Paul *et al.* [20] was used for determination of heme content.

Analysis of Cu^{2+} was performed by graphite furnace atomic absorption spectrophotometry [21]. Briefly, the mitochondrial and the microsomal fractions were digested in concentrated nitric acid and subsequently heated for 30 min at 60°. An injection volume of 5 μl was used for each analysis. Copper was detected at 324.8 nm and quantitated by comparing the peak area of the sample with that of the standard. The standard was prepared in the same matrix as the sample. Concentration of conjugated dienes, the end-product of *in vivo* lipid peroxidation, was measured in extracts from the mitochondrial and microsomal fractions according to the procedure described by Buege and Aust [22].

Adrenal mitochondrial cholesterol side-chain cleavage activity was determined using the procedure of Mason *et al.* [23]. The assay system (1.0 ml) consisted of the mitochondrial protein (200–250 μg), an NADPH-generating system, 25-hydroxycholesterol (1 mM) and cyanoketone (6 μM). The reaction mixture was preincubated for 10 min at 37°. The reaction was initiated by the addition of 10 mM DL-isocitrate. At 0, 2, 5 and 10 min, 200 μl aliquots were removed and used for extraction of the product, pregnenolone. This compound was subsequently measured by radioimmunoassay and quantitated using a standard curve ranging in pregnenolone concentration from 0.1 to 3.0 ng. 11 β-Hydroxylase activity was estimated by measuring the rate of conversion of the substrate, deoxycorticosterone, to corticosterone [24]. The product was analyzed by HPLC using a 5 μm Hypersil-ODS column (4 mm × 15 cm) and an isocratic elution of 60% methanol in water, at a flow rate of 2 ml/min. Detection was made spectrophotometrically at 254 nm, and quantitated by comparing the sample peak height with that of the external standard. The procedure of Stoks and Benraad [25] was employed for extraction of corticosterone from the plasma. The subsequent analysis by HPLC was identical to that described above, except that an isocratic elution of 65% methanol in water, at a flow rate of 2 ml/min, was used to provide a better separation and identification of corticosterone. In both analyses, corticosterone peaks were symmetrical and were well within the linear range of the standard curve. The retention time of corticosterone was about 5 min compared with 9 min for deoxycorticosterone. Steroid recovery routinely exceeded

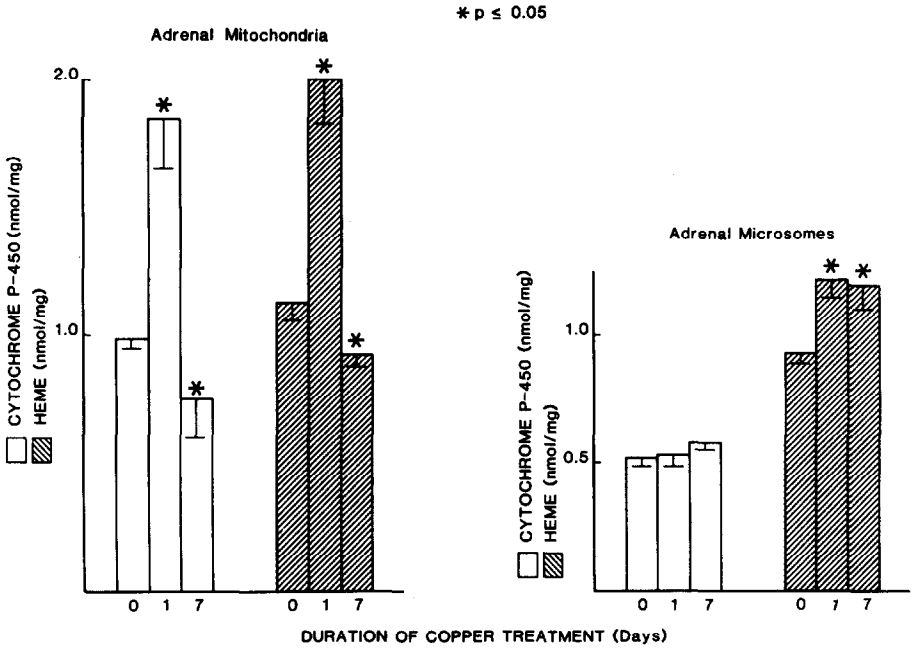

Fig. 1. Alterations in concentrations of heme and cytochrome P-450 in rat adrenal mitochondrial and microsomal fractions following 1 day and 7 days of copper treatments. Male Sprague–Dawley rats (200–250 g) were treated with Cu^{2+} either once, (125 μmoles/kg, s.c.) and killed 24 hr later, or for 7 days (40 μmoles/kg/day, mini-osmotic pump, s.c.). Control animals received saline. Thereafter, the adrenal mitochondrial and the microsomal fractions were prepared, and the concentrations of heme and cytochrome P-450 were determined according to the procedures detailed in Materials and Methods. Each value represents the mean ± S.D. for four determinations.

95% in the 11 β-hydroxylase assay and was about 85% with the plasma extracts.

The concentration of protein was assessed by the method of Lowry *et al.* [26]. All spectrophotometric analyses were conducted on an SLM-Aminco DW-2C dual beam spectrophotometer. Copper analysis was performed on a Varian AA475 atomic absorption spectrophotometer, equipped with a CRA 90 graphite furnace unit. Chromatography was carried out on a Waters System 201 HPLC apparatus which was linked with a Hewlett–Packard 3390A recording integrator. All results are expressed as the mean ± S.D. for four determinations. All data were analyzed using Student's *t*-test; a P value of ≤ 0.05 denoted significance.

RESULTS

The effects of 1 day and 7 days of Cu^{2+} treatment on the specific contents of cytochrome P-450 and heme in the mitochondrial and the microsomal fractions of rat adrenal glands are shown in Fig. 1. As shown, in the mitochondria 1 day after Cu^{2+} treatment a nearly 85% increase in concentrations of cytochrome P-450 and heme was observed. In contrast, in animals treated for 7 days with Cu^{2+} a significant decrease in the concentration of heme and the hemoprotein in this fraction was detected. The response elicited by these treatments in the microsomal fraction differed from that evoked in the mitochondria. In the microsomal fraction, no appreciable alterations in the concentration of cytochrome P-450 were observed after 1 day and 7 days of Cu^{2+} treatment. However, the microsomal heme content was increased significantly at these times.

The above described Cu^{2+}-mediated alterations in the mitochondrial heme and hemoprotein concentrations suggested an active role of Cu^{2+} in regulating the rate of heme biosynthesis and/or degradation in the adrenals. This possibility was investigated by examining the effects of 1 day and 7 days of Cu^{2+} treatment on the activity of ALA synthetase and heme oxygenase. As shown in Table 1, compared with controls, the treatment of rats with Cu^{2+} for 1 day caused a nearly 300% increase in ALA synthetase activity. This finding is consistent with the increases observed after 1 day of Cu^{2+} treatment in the concentrations of cytochrome P-450 and heme in the mitochondria. This table also shows that the activity of ALA synthetase remained elevated after treatment of rats with Cu^{2+} for 7 days, although in comparison with the 1-day treatment values the magnitude of increase was reduced substantially. The activity of heme oxygenase appeared refractory to both regimens of Cu^{2+} treatment. In the absence of a decrease in ALA synthetase activity, and presumably in heme biosynthesis, the mechanism of the above noted (Fig. 1) decrease in the mitochondrial concentrations of heme and cytochrome P-450 after 7 days of Cu^{2+} treatment appeared to involve factors other than an inhibited cellular heme biosynthetic activity. Therefore, the

Table 1. Copper-mediated alterations in the activities of rat adrenal δ-aminolevulinate synthetase and heme oxygenase

Treatment	ALA synthetase (pmoles ALA/mg/hr)	Heme oxygenase (nmoles hematobilirubin/mg/hr)
Control	141.0 ± 13.0	0.56 ± 0.07
Cu^{2+}, 1 day	419.5 ± 69.0*	0.55 ± 0.03
Cu^{2+}, 7 days	246.5 ± 37.0*†	0.58 ± 0.08

Male Sprague–Dawley rats (200–250 g) were treated with Cu^{2+} either once with 125 μmoles/kg (s.c.) or by means of implanted (s.c.) mini-osmotic pumps which delivered a daily dose of 40 μmoles/kg for 7 days. Control animals received saline. At the indicated intervals, the animals were killed, and the tissue homogenate and the 9000 *g* supernatant fraction were prepared. The activity of ALA synthetase was assessed using the tissue homogenate, and the rate of heme oxygenase was measured using the 9000 *g* supernatant fraction. Procedures are described in detail in Materials and Methods. Each value represents the mean ± S.D. for four determinations.

* $P \leq 0.05$ when compared with control values.

† $P \leq 0.05$ when compared with 1 day Cu^{2+}-treated rats.

possibility of involvement of lipid peroxidation in this process was investigated.

It is well known that the adrenal mitochondria actively sequester divalent cations [27]. Also, it has been demonstrated that large accumulation of Cu^{2+} in the mitochondria can promote lipid peroxidation in this organelle [28], while the presence of this metal ion inhibits lipid peroxidation in the microsomal fraction [29]. Similarly, lipid peroxidation-mediated destruction of cytochrome P-450 is a well recognized phenomenon [30]. Thus, the possibility was examined that the accumulation of Cu^{2+} in the adrenal subcellular fractions may have exerted a direct effect on the turnover rate of cytochrome P-450. Accordingly, concentrations of Cu^{2+} and tissue levels of conjugated dienes were measured in the mitochondrial and the microsomal fractions of animals treated with Cu^{2+} for 1 day and 7 days. The results of these studies are shown in Table 2. As shown, in both fractions the concentration of Cu^{2+} increased with the longer duration of exposure of animals to the metal ion. The greater magnitude of increase was noted in the mitochondria where, after 7 days of treatment, Cu^{2+} levels measured more than five times that of the controls. At this time, the concentration of the metal ion measured nearly twice that detected in the mitochondria of the 1 day Cu^{2+}-treated animals. Similarly, in the microsomal fraction the concentration of Cu^{2+} increased. Copper concentration in this fraction after 1 day and 7 days of treatment measured approximately two and three times, respectively, that of the control value.

The uptake of Cu^{2+} into the mitochondrial and microsomal fractions was accompanied by alterations in the concentration of conjugated dienes in these fractions. As also noted in Table 2, the pattern of alterations in the concentration of conjugated dienes was reciprocal to that observed for the contents of heme and cytochrome P-450 in the mitochondria and heme in the microsomal fraction (Fig. 1). Moreover, the accumulation of Cu^{2+} in the adrenal mitochondria appeared to elicit a bimodal effect on lipid peroxidation, i.e. the lower magnitude of increase in tissue Cu^{2+} content corresponded with a decrease in concentration of conjugated dienes, whereas a higher tissue concentration of Cu^{2+} corresponded with an

Table 2. Effect of 1 day and 7 days of copper treatment on copper concentration and *in vivo* lipid peroxidation in rat adrenal mitochondrial and microsomal fractions

	Mitochondria		Microsomes	
Treatment	Copper (nmoles/mg)	Conjugated dienes (nmoles/mg)	Copper (nmoles/mg)	Conjugated dienes (nmoles/mg)
Control	0.23 ± 0.03	2.92 ± 0.26	1.08 ± 0.40	5.50 ± 0.86
Cu^{2+}, 1 day	0.57 ± 0.05*	1.02 ± 0.38*	1.94 ± 0.13*	2.41 ± 0.43*
Cu^{2+}, 7 days	1.21 ± 0.15*†	5.60 ± 0.61*†	2.94 ± 0.50*†	3.02 ± 0.36*

Male Sprague–Dawley rats (200–250 g) were treated with Cu^{2+} as described in the legend of Table 1. At the indicated times, the animals were killed, and the mitochondrial and the microsomal fractions were prepared. The concentration of Cu^{2+} was determined in these fractions by graphite furnace atomic absorption spectrophotometry. *In vivo* lipid peroxidation was determined by measurement of conjugated dienes. The experimental details are described in Materials and Methods. Each value represents the mean ± S.D. for four determinations.

* $P \leq 0.05$ when compared with control values.

† $P \leq 0.05$ when compared with the 1 day Cu^{2+}-treated animals.

increased level of the dienes. As noted, 1 day after Cu^{2+} treatment the amount of extractable conjugated dienes was reduced to a mere 30% of that of the controls. In contrast, in animals treated 7 days with Cu^{2+} a doubling of the concentration of conjugated dienes was observed. On the basis of these findings, it appears reasonable to suggest that the greater rate of mitochondrial lipid peroxidation noted in the 7 day Cu^{2+}-treated animals could be, in part, responsible for the degradation of the mitochondrial cytochrome P-450 and heme at this time (Fig. 1). Conversely, in the microsomal fraction significant decreases in lipid peroxidation could contribute to increases noted in the levels of the microsomal heme following 1 day and 7 days of treatment (Fig. 1).

Although the presently observed decrease in lipid peroxidation in the microsomal fraction coincided with an increase in the Cu^{2+} content in this fraction, it is noteworthy that relative to the mitochondria of 7 day Cu^{2+}-treated animals, at both time points, the concentration of Cu^{2+} in the microsomal fraction was of a lesser magnitude. As shown, following 7 days of Cu^{2+} treatment, a 2- to 3-fold increase in the concentration of Cu^{2+} in the microsomal fraction was noted; this value closely approximates the magnitude of increase in the concentration of Cu^{2+} in the mitochondrial fraction 1 day after Cu^{2+} treatment.

To determine whether the observed perturbations in the mitochondrial concentration of cytochrome P-450 affected steroidogenic processes, the activities of cholesterol side-chain cleavage and 11 β-hydroxylase were measured. The effects of 1 day and 7 days of Cu^{2+} treatment on 11 β-hydroxylase activity are depicted in Table 3. In comparison with the rate displayed by the control mitochondria, the rate of corticosterone production by the mitochondria of the 1 day Cu^{2+}-treated animals was elevated significantly. In contrast, the mitochondria isolated from the adrenals of animals treated 7 days with Cu^{2+} exhibited a substantially reduced rate of 11 β-hydroxylase activity. As shown, the concentration of corticosterone formed by this preparation measured approximately 50–60% of that produced by preparations obtained from the 1 day Cu^{2+}-treated rats. According to these findings, the pattern of the Cu^{2+}-mediated alterations in the rate of 11 β-hydroxylase activity closely corresponded with the pattern of change in the specific content of adrenal mitochondrial cytochrome P-450 (Fig. 1).

The effects of Cu^{2+} treatment on the activity of cytochrome P-450 distinctly differed from that described above for 11 β-hydroxylase activity. For instance (Table 3), a significant change in the activity of this mitochondrial enzyme was not detected in the 1 day Cu^{2+}-treated animals. On the other hand, the longer regimen of Cu^{2+} treatment caused a notable increase in the activity of the enzyme, as indicated by the rate of pregnenolone production. In this case, the specific activity measured more than two times the control value. These results may be interpreted as suggesting that the cytochrome P-450 isozyme catalyzing cholesterol side-chain cleavage was refractory to fluctuations in lipid peroxidation in the mitochondria.

The physiological consequences of the Cu^{2+}-mediated alterations in the activity and concentration of adrenal mitochondrial cytochrome P-450 were assessed by measuring the concentration of corticosterone in the plasma of rats treated with Cu^{2+} for 1 day and 7 days. No significant alteration in plasma corticosterone levels was observed in the 1-day treated animals, despite the significant increase in the activity of 11 β-hydroxylase. Rather, this finding reflects the lack of stimulation in the rate of cholesterol side-chain cleavage. On the other hand, after 7 days of Cu^{2+} treatment the plasma levels of corticosterone were half that of control animals. This reduction appears to be directly related to the observed inhibition of 11 β-hydroxylase activity in the 7-day treated animals. Moreover, the diminished output occurred in the face of an augmented cholesterol side-chain cleavage activity, indicating that 11 β-hydroxylase had become the rate-limiting enzyme in the steroid biosynthesis pathway.

DISCUSSION

The present investigation describes the ability of Cu^{2+} to disrupt the normal function of the adrenal cortex. These effects appeared to be mediated through mechanisms involving alterations in the content and the activity of the mitochondrial cytochrome P-450 isozymes necessary for steroid biosynthesis. In turn, these alterations in cytochrome P-450 concentration resulted from two separate modes of

Table 3. Effects of 1 day and 7 days of copper treatments on the activities of rat adrenal mitochondrial cholesterol side-chain cleavage and 11 β-hydroxylase

Treatment	Cholesterol side-chain cleavage (nmoles/ pregnenolone/mg)	11 β-Hydroxylase (nmoles corticosterone/mg)
Control	1.28 ± 0.14	9.04 ± 0.77
Cu^{2+}, 1 day	1.57 ± 0.24	11.75 ± 0.62*
Cu^{2+}, 7 days	2.88 ± 0.39*†	5.15 ± 0.38*,†

Rats were treated with Cu^{2+} as described in the legend of Table 1. The mitochondrial fraction was obtained as described in the text and used for the determination of the rate of cholesterol side-chain cleavage and 11 β-hydroxylase activities. The enzyme activities were assessed by measuring the rate of pregnenolone or corticosterone production as described in Materials and Methods. The duration of incubation was 5 min. Each experimental point is a representation of the mean ± S.D. for four determinations.

* $P \leq 0.05$ when compared with control values.

† $P \leq 0.05$ when compared with 1 day Cu^{2+}-treated animals.

action of Cu^{2+}. These are: (a) an increase in the availability of heme for production of the cytochrome, as reflected by increased ALA synthetase activity and the absence of an increase in the activity of heme oxygenase (Table 1); and (b) an inhibition, followed by a promotion, in the activity of mitochondrial lipid peroxidation (Table 2). The latter effect appeared to be mainly responsible for the perturbations observed in cytochrome P-450-dependent steroidogenic processes. This suggestion is made on the basis of the finding that fluctuations in cytochrome P-450 concentration and 11 β-hydroxylase activity closely followed the pattern of change in lipid peroxidation within this organelle (Fig. 1, Table 2).

Although the present study does not permit identification of the exact mechanism of action of Cu^{2+} on the heme metabolism pathway and cytochrome P-450-dependent steroid biosynthesis, it is suggested that extracellular factors such as ACTH as well as direct effects of Cu^{2+} at the tissue level may be involved. In cultured bovine adrenocortical cells, ACTH increases cholesterol side-chain cleavage activity by increasing the mobilization of cholesterol to the inner mitochondrial membrane, while stimulating the biosynthesis of adrenocortical cytochrome P-450 [31]. Moreover, ACTH has been implicated in the regulation of adrenal ALA synthetase [32]. Therefore, the possibility exists that Cu^{2+} may have augmented the release of ACTH. On the other hand, Cu^{2+} appears to directly affect the concentration of cytochrome P-450 by altering tissue lipid peroxidative activity in both a time- and concentration-dependent manner (Table 2).

It appeared, however, that the tissue concentration of Cu^{2+} and lipid peroxidative activity are not directly related; rather, at lower concentrations of Cu^{2+} a suppression of this process was manifested, whereas at a higher concentration of the metal ion lipid peroxidation was augmented. This suggestion is supported by the observation that, when Cu^{2+} concentrations reached a level two to three times that of the control value as in the mitochondrial fraction of the 1 day Cu^{2+}-treated animals and in the microsomal fraction of the 1 day and 7 day Cu^{2+}-treated animals, lipid peroxidation was inhibited. Moreover, this suggestion agrees with findings on the stimulation of superoxide dismutase activity in the mitochondrial fraction of rat liver 1 day after administration of Cu^{2+} [28]. The dismutase has been implicated as a primary defense in preventing superoxide-mediated lipid peroxidation [33]. Hence, Cu^{2+}, by enhancing superoxide dismutase activity in the adrenal mitochondria, could diminish lipid peroxidation in this fraction. Furthermore, the promotion of the anti-oxidative state of the adrenal mitochondria has been shown to correspond with a reduced loss of the mitochondrial cytochrome P-450 [34]. The present observation regarding the increased concentration of cytochrome P-450 in the mitochondria of the 1 day Cu^{2+}-treated animals is consistent with this reported finding. In addition, the data showing the inhibition of microsomal lipid peroxidation following both Cu^{2+} treatments agree with reports on the stimulatory effect of Cu^{2+} on the cytosolic superoxide dismutase activity [28], the inhibitory effects of Cu^{2+} and other metal ions on liver microsomal lipid peroxidation, and the ensuing degradation of cytochrome P-450 [29, 30]. Therefore, the presently observed increase in the concentration of microsomal heme 1 day and 7 days after Cu^{2+} treatment could be partly related to a Cu^{2+}-mediated suppression of lipid peroxidation. Of course, the increased heme biosynthetic activity could also contribute to the increased levels of heme and hemoprotein. The reason for the absence of a corresponding increase in the microsomal cytochrome P-450 concentration is not evident.

Further accumulation of Cu^{2+} in the mitochondrial fraction of the 7 day Cu^{2+}-treated animals elicited an opposite effect on lipid peroxidation. That is, at Cu^{2+} concentrations greatly exceeding that of the controls (five times), an increase in the tissue concentration of the conjugated dienes was detected (2-fold). This observation may be interpreted as suggesting that the greater tissue concentration of Cu^{2+} exerted a pro-oxidative effect in this organelle. This idea is in accord with the reported finding on Cu^{2+}-mediated free radical production and increased lipid peroxidation at the inner mitochondrial membrane of the liver [28]. Moreover, despite the noted increase in ALA synthetase activity, the concentration of cytochrome P-450 was decreased in the 7 day Cu^{2+}-treated animals which indicates that the elevated lipid peroxidative activity in the mitochondria was responsible for the degradation of the hemoprotein. Copper could promote lipid peroxidation in this organelle by accelerating the rate of nonenzymatic dismutation of superoxide to singlet oxygen [33–35], and by increasing the production of organic hydroperoxides from the lipid components within the inner mitochondrial membrane [28]. The latter process could be responsible for the present observations since the two molecular species of the adrenal mitochondrial cytochrome P-450 are localized within the inner membrane [36].

However, based on the measurements of the hydroxylation activities catalyzed by the two forms of the mitochondrial hemoproteins, only cytochrome P-$450_{11\beta}$ appeared to be susceptible to Cu^{2+}-mediated lipid peroxidation (Table 3). The refractory response of cholesterol side-chain cleavage activity to increased lipid peroxidation is consistent with similar observations with Fe^{2+}-induced lipid peroxidation and cytochrome P-450 degradation in the adrenal mitochondria [37]. The differential susceptibility of cytochromes P-$450_{11\beta}$ and P-450_{scc} to lipid peroxidation may be attributed to the differential interactions of the cytochromes with the inner mitochondrial membrane [37, 38]. Specifically, cytochrome P-450_{scc} is believed to be more loosely bound to the inner membrane than cytochrome P-$450_{11\beta}$, and this apparently renders the latter isozyme more dependent on phospholipid integrity and membrane fluidity for its normal functioning [37]. Moreover, the putative tighter coupling of cytochrome P-$450_{11\beta}$ with the inner membrane could perceivably bring the hemoprotein in a closer proximity to outer membrane components believed to function as activating agents for the 11 β-hydroxylase reaction [38]. Cytochrome P-450_{scc}, on the other hand, apparently does not exhibit these characteristics [37]. Fur-

thermore, a tighter coupling with phospholipids, particularly those containing a high degree of unsaturated fatty acids, has been reported to inhibit the activity of cytochrome P-450$_{scc}$ by adversely affecting the affinity of the hemoprotein for substrate [39]. Accordingly, the presently observed stimulation of cholesterol side-chain cleavage activity, concomitant with increased lipid peroxidation, could reflect the effective removal and degradation of the "inhibitory" phospholipids [38].

In conclusion, it appears that the toxic effects of Cu^{2+}, observed after subchronic treatment, have physiological consequences on adrenal function which are manifested in lowered plasma corticosterone concentrations. Since the deposition and metabolism of Cu^{2+} are believed to be influenced by glucocorticoids [7, 8], the Cu^{2+}-mediated inhibition of adrenal steroidogenic activity could alter the metabolism of this metal ion in the liver [2] and the deposition of Cu^{2+} in other extrahepatic organs.

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