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CHARACTERIZATION OF HEME OXYGENASE ACTIVITY IN LEYDIG AND SERTOLI CELLS OF THE RAT TESTES

DIFFERENTIAL DISTRIBUTION OF ACTIVITY AND RESPONSE TO CADMIUM

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Abstract—Leydig and Sertoli cells of the rat testes differ with respect to the activities of the enzymes of the heme and hemoprotein degradative pathway and in their responses to Cd^{2+} treatment. The microsomal heme oxygenase activity in the Leydig cell preparations was nearly 9- to 10-fold greater than in Sertoli cell preparations, but the characteristics of the enzyme appeared to be similar in both cell populations, as judged by the cofactor requirements and the inhibitory action of heme ligands. Differences between the two cell preparations also were detected in the activity of NADPH–cytochrome *c* (P-450) reductase and in the contents of cytochrome P-450 and heme, with Leydig cells possessing the higher values. The activities of the cytosolic biliverdin reductase were comparable in both cell preparations. The significantly higher levels of porphyrins and the activities of δ-aminoleuvinate synthetase and uroporphyrinogen-I synthetase suggest that Leydig cells constitute the primary site of heme and hemoprotein biosynthetic activities. The mode of regulation of heme oxygenase activity in the testes and in the liver was compared. The responses of heme oxygenase to Cd^{2+} treatment (20 μmoles/kg, 24 hr) in the two testicular cell populations were dissimilar and both differed from that of the liver. In Leydig cells, heme oxygenase activity was decreased dramatically, whereas in the liver the activity was greatly increased. Heme oxygenase activity in Sertoli cells was refractory to Cd^{2+}. The Cd^{2+}-mediated decrease in heme oxygenase activity in Leydig cells did not reflect a direct inhibitory action of Cd^{2+} on the enzyme or a decreased total content of the microsomal protein. The dissimilarity between the mode of regulation of heme metabolic activities in the testes, when determined in Leydig cells, and that in the liver involved the inability of bromobenzene to evoke an increase in heme oxygenase activity and the age-related changes in the activities of heme oxygenase and δ-aminoleuvinate synthetase. In contrast to heme oxygenase activity, the heme concentration in Sertoli cells was remarkably sensitive to Cd^{2+} treatment, where a 7-fold increase in heme concentration was observed. The same treatment caused only a 2-fold increase in heme concentration in Leydig cells. In the latter cells, however, the increase in heme concentration was accompanied by a marked reduction in cytochrome P-450 levels. The cytochrome could not be measured in Sertoli cell preparations. The heme biosynthetic pathway in the testes was essentially insensitive to Cd^{2+} treatment as judged by unaltered or modestly affected activities of the enzymes of heme biosynthesis and the total porphyrin content of Leydig cell preparations. The findings described in this report define the testicular tissue as unique with respect to heme metabolic capability and the regulation of heme metabolic processes. However, these processes in the two cell populations are heterogenous.

Heme oxygenase is the rate-limiting enzyme in heme degradation [1]. Substrates for this enzyme include the heme moieties of hemeglobin and cytochrome P-450 and various derivatives of the heme *c* moiety of cytochrome *c* [2–4]. Heme oxygenase activity in various mammalian and avian organs is readily increased subsequent to exposure to a variety of agents, including heme compounds and metal ions [1–8]. The increased enzyme activity in response to metal ions reflects the enhanced *de novo* formation of enzyme protein [5]. Of the metal ions studied Cd^{2+} appears to exert the most profound inducing effect on the activity of heme oxygenase in the liver. Except for a recent report [9] on the response of the testicular heme oxygenase to Cd^{2+}, the biochemical properties of the enzyme in the testes remain essentially unknown. Recently, a novel response of heme oxygenase in rat testes to Cd^{2+} was described [9]. It was shown that the regulation of testicular heme oxygenase activity is distinctly different from that of the liver; in the testes of Cd^{2+}-treated rats a severe depression of the enzyme activity was observed.

The testes are comprised of heterogenous cell types, which greatly vary in physiological properties and function. Moreover, the composition of the cell populations in the organs is not consistent throughout life. Specifically, whereas Sertoli cells are mainly involved in spermatogenetic processes, Leydig cells function in steroid biosynthesis and androgen production. Various steps in steroidogenetic activities are catalyzed by cytochrome P-450. The conversion of cholesterol to pregnenolone, which occurs in the mitochondria, is the rate-limiting step in steriod production and is a cytochrome P-450-dependent reac-

tion [10]. In cytoplasm, this hemoprotein functions as the terminal oxidase in the various hydroxylations of the steroid nucleus by the endoplasmic reticulum membranes. Both types of hydroxylation appear to be localized mainly in Leydig cells [11].

The concentrations of heme and cytochrome P-450 in the testes change when chemicals are given. For example, after the administration of 1,2-dibromo-3-chloropropane or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the microsomal concentration of the hemoprotein is decreased [12, 13]. In the liver, chemicals which decrease the concentrations of cytochrome P-450 and heme often promote the induction of heme oxygenase activity. This is particularly true with metal ions [3]. In the testes, however, no such relationship between the concentration of cytochrome P-450 and the activity of heme oxygenase was observed [7]. Moreover, other evidence suggests that, in the testes, various chemicals may selectively alter biological activities in a single cell population. For instance, the microsomal cytochrome P-450-dependent 17-α-hydroxylase and 17-20-lyase (desmolase) of steroids are depressed in response to the treatment of animals with spironolactone [14, 15], suggesting the preferential effect on Leydig cells. On the other hand, dioxins may selectively affect Sertoli cells, as evidenced by impaired spermatogenesis in humans who have been exposed to the chemical [16]. Accordingly, it can be postulated that the response elicited by metal ions in heme metabolic activities and the hemoprotein content in Leydig and Sertoli cells are dissimilar.

The present study was undertaken to characterize heme oxygenase activity in testicular Leydig and Sertoli cells and to compare the response of the enzymes of the heme metabolic pathway in the two cell populations of the testes to Cd^{2+}.

EXPERIMENTAL PROCEDURES

Materials. Glucose-6-phosphate, NADH, NADPH, glucose-6-phosphate dehydrogenase, horse heart cytochrome *c*, protoheme (Fe-protoporphyrin), collagenase, trypsin (type I), trypsin inhibitor (soy bean, type II), bovine serum albumin (BSA, type V) and bromobenzene were purchased from the Sigma Chemical Co., St. Louis, MO. Cadmium acetate, $Cd(CH_3CO_2)_2 \cdot 2H_2O$, was purchased from the Fisher Chemical Co., Chicago, IL. Hematoheme (heme *c*), biliverdin, coproporphyrin I, δ-aminolevulinate (ALA) and porphobilinogen (PBG) were obtained from Porphyrin Products, Logan, UT. Phenobarbital sodium was a product of Merck Pharmaceuticals.

Animals and tissue preparation. Male Sprague–Dawley rats of differing ages were used. The animals were purchased from Harlan Industries, Madison, WI. The animals were allowed access to food and water *ad lib.* All injections were made between 9:00 and 9:30 a.m. Rats were injected subcutaneously in the loose skin of the neck with 7 or 20 μmoles/kg cadmium acetate, 0.5 mmoles/kg bromobenzene in corn oil, or 80 mg/kg phenobarbital. The control animals received saline or corn oil. The regimen of treatments is given in the appropriate tables and figures. Twenty-four hours later the animals were decapitated, and the testes were removed. The organs were decapsulated and rinsed immediately in 1.15% KCl. The microsomal and the 105,000 *g* supernatant fractions were prepared as described earlier [9] in Tris–HCl (0.01 M, pH 7.4 buffer) containing 0.25 M sucrose. In most cell preparation experiments, the testes of two rats were used. the average weight of the tissue per rat was about 3.0 g. Leydig cells were isolated by a modification of the procedure of Dufau *et al.* [17]. The decapsulated testes were minced and placed in Leibowitz's medium (approximately 5 ml/g tissue) containing 1 mg/ml BSA and 0.25 mg/ml collagenase. The preparation was incubated for 10 min at 37° with vigorous shaking. The resulting suspension was filtered through a 100 μm nylon mesh. The filtrate was centrifuged for 10 min at 600 *g*, the cell pellet was resuspended in 20 vol. of Tris-HCl (0.01 M, pH 7.4) containing 0.25 M sucrose, and the centrifugation was repeated. The final cell pellet was used for the preparation of subcellular fractions as described below.

Enriched suspensions of Sertoli cells were prepared by an adaptation of the methods of Steinberger *et al.* [18] and Dorrington and Fritz [19]. Decapsulated testes were cut into small pieces and incubated with gentle intermittent shaking in Ca^{2+}-free Hank's medium (approximately 25 ml/g tissue) containing 0.25 mg/ml trypsin and 1 mg/ml BSA for 15 min at 37°. The separated seminiferous tubules were collected on 100 μm nylon mesh and well washed repeatedly with the above medium until no evidence of contamination of tubules with Leydig cells was observed upon microscopic examination. Subsequently, the tubules were incubated with vigorous shaking in Leibowitz's medium (approximately 5 ml/g tissue) containing 0.25 mg/ml collagenase, 1 mg/ml BSA and 0.1 mg/ml trypsin inhibitor, for 1 hr at 37°. The resulting suspension was filtered through 100 μm nylon mesh and rinsed with medium, and the retained cells were collected. After settling, the supernatant fraction was removed and the pellet was resuspended in the above described buffer. The Sertoli cell preparations contained approximately 10% elongated spermatids and fragments from the spermatids. The Leydig cell preparations contained less than 5% contamination with spermatid and cell debris.

The suspensions of Leydig and Sertoli cells were briefly sonicated and then homogenized manually in a glass homogenizer. The 600 *g* supernatant (post-nuclear), 10,000 *g* supernatant, microsomal, and cytosol fractions were prepared [9]. The whole homogenate fraction was used for the determination of the total porphyrin content. The 600 *g* supernatant fraction was used for the measurement of ALA synthetase activity. The activities of ALA dehydratase and uroporphyrinogen (uro)-I synthetase were measured in the 9000 *g* supernatant fraction. The activities of heme oxygenase and NADPH–cytochrome *c* (P-450) reductase were measured in the microsomal fraction. The microsomes were also used for determinations of the contents of cytochrome P-450 and heme. The cytosolic fraction was used for the measurement of biliverdin reductase activity.

Assay procedures. Heme oxygenase activity was measured with hematoheme or protoheme as the substrates [4]. Unless otherwise indicated the reac-

tion mixture (1.4 ml), consisting of 15 μM hematoheme, microsomal protein (0.5 to 1 mg protein/ml), 0.85 mM glucose-6-phosphate, 3 units of glucose-6-phosphate dehydrogenase, 2 mM $MgCl_2$, and 90 mM potassium phosphate buffer (pH 7.4), was incubated for 5 min at 37° and then divided into two equal aliquots. A mixture of NADP (0.8 mM) and an excess amount (50–100 μg protein) of purified rat liver biliverdin reductase [20] in 100 μl of potassium phosphate buffer was added to the incubation mixture. The blank incubation mixture did not contain NADP. After incubation for 10 min at 37°, the hematobilirubin was extracted into chloroform, and the absorption spectrum between 436 and 530 nm was recorded. The enzyme activity was determined with an extinction coefficient of 31 $mM^{-1} cm^{-1}$ [4]. Hematobilirubin was readily and completely extracted into chloroform; this greatly enhances the sensitivity and accuracy of the measurements by eliminating the interference of tissue heme and the unmetabolized substrate. This is particularly important when measuring low heme oxygenase activities in Sertoli cells.

The heme content was estimated by the pyridine hemochromogen method of Paul *et al.* [21] with the reduced-oxidized difference spectrum between 557 and 575 nm and an extinction coefficient of 32.4 $mM^{-1} cm^{-1}$. The concentration of cytochrome P-450 was measured by a modification procedure of Nozu *et al.* [22]. The tissue were suspended in a 0.1 M potassium phosphate buffer containing 20% glycerol and 0.2% Renex 690. The reduced CO-difference spectrum of the suspension was obtained with sodium dithionite as the reducing agent. The concentration of cytochrome P-450 was calculated from an extinction coefficient of 91 $mM^{-1} cm^{-1}$ between 450 and 490 nm [23]. The activity of NADPH–cytochrome *c* (P-450) reductase was determined by measuring at 25° the increase in absorbance at 550 nm of a reaction mixture (1.0 ml) containing 100 μM cytochrome *c*, 100 μM NADPH and enzyme source (0.5 to 1.0 mg protein) [24]. Biliverdin reductase activity was measured as described before [20] from the increase in absorbance at 450 nm of a reaction mixture (1.0 ml) consisting of 100 μM NADPH, 5 μM biliverdin, and cytosol preparations (300–500 μg protein) in 0.1 M Tris–HCl buffer, pH 8.7. A molar extinction coefficient of 53 $mM^{-1} cm^{-1}$ was used.

The activity of ALA synthetase was measured as described before [25] in the 600 *g* fraction of Leydig and Sertoli cell preparations. The activity of ALA dehydratase was measured by the procedure of Mauzerall and Granick [26]. The amount of protein in the assay mixture (0.225 ml) was 0.5 to 1 mg, and the concetrations of ALA and dithiothreitol were 150 and 20 mM respectively. The activity of uro-I synthetase was measured by the procedure of Granick *et al.* [27]. The total porphyrin content was measured fluorometrically as described by Granick *et al.* [28] with an extraction mixture of 1 M $HClO_4$/methanol (1/1, v/v). The excitation wavelength was 400 nm with a slit width of 10 nm, and the emission spectrum was scanned between 580 and 680 nm with a 12 nm slit. Coproporphyrin I was used as the standard.

Protein concentrations were measured by the method of Lowry *et al.* [29]. All spectral studies were conducted with an Aminco–Chance DW-2 spectrophotometer. The fluorometric measurements were carried out using an Aminco–Bowman fluorometer. All experiments were repeated two to six times. The results are expressed as the average of two experiments or the mean ± S.D. The latter data were analyzed by Student's *t*-test, and a P value of ≤ 0.05 was used to denote significance.

RESULTS

Cellular distribution of activities of heme degradative enzymes in the testes, and requirements for heme oxygenase activity. The distribution of the activities of heme oxygenase, biliverdin reductase, and NADPH–cytochrome *c* (P-450) reductase in the adult rat testes Leydig and Sertoli cell preparations are shown in Table 1. The concerted activities of these enzymes are required for the conversion of hematoheme to hematobilirubin. This table also shows the activities of the enzymes in the isolated cell preparations and the whole testes. A high level of heme oxygenase activity was detected in the rat

Table 1. Activities of heme oxygenase, NADPH–cytochrome *c* (P-450) reductase, and biliverdin reductase in whole testes and Leydig and Sertoli cell preparations*

Enzyme source	Heme oxygenase (nmoles · mg^{-1} · hr^{-1})	Biliverdin reductase (nmoles · mg^{-1} · hr^{-1})	NADPH–cytochrome *c* (P-450) reductase (nmoles · mg^{-1} · min^{-1})
Whole testes	5.14 ± 0.54	18.45 ± 1.81	5.11 ± 1.60
Leydig cell preparation	6.60 ± 0.51†	14.94 ± 1.44	8.45 ± 0.17†
Sertoli cell preparation	0.61 ± 0.16†	12.72 ± 2.30†	1.02 ± 0.09†

* Leydig and Sertoli preparations obtained from the testes of Sprague–Dawley rats (200–250 g), and whole testes were used for the preparation of the microsomal and the cytosol fractions. Microsomal heme oxygenase activity was measured as described previously [4]. Hematoheme was used as the substrate and the enzyme activity was assessed from the amount of hematobilirubin formed. The activity of biliverdin reductase was determined as described before [20] from the rate of biliverdin reduction, using NADPH as the cofactor at pH 8.7. The activity of NADPH–cytochrome *c* (P-450) reductase was determined from the rate of cytochrome *c* reduction, using the method of Williams and Kamin [24]. The experimental details are provided under Experimental Procedures. The data shown represent mean ± S.D. of three determinations.

† $P \leq 0.05$ when compared with whole testes.

testicular micosomal fraction. As shown, the activity of the enzyme in the microsomal fraction of Leydig cells exceeds that of the Sertoli cell preparation by 9- to 10-fold. The finding that the activity of heme oxygenase in Leydig cells was somewhat higher than that of the whole testes microsomal preparation suggests that this cell population is a major contributor to the testicular heme oxygenase activity. Similarly, the activity of NADPH–cytochrome *c* (P-450) reductase was nearly 8-fold higher in Leydig cells than in Sertoli cells. In contrast, the activity of biliverdin reductase was equally distributed between the two cell populations. Moreover, the activity of the enzyme does not appear to be rate-limiting in heme degradation and bile formation processes; rather, heme oxygenase appears to function in this capacity. The finding that the activity of biliverdin reductase in Leydig and Sertoli cell preparations was comparable to that of the whole testes also suggests the uniform distribution of the enzyme activity in the different cell populations of the organ. Attempts were made to measure heme oxygenase activity in a preparation of spermatids, but no activity could be detected.

The pyridine nucleotide requirements for the formation of hematobilirubin from hematoheme by microsomal fractions of the whole testes and by Leydig and Sertoli cell preparations were investigated. As shown in Table 2, the formation of hematobilirubin by the microsomal fraction of the whole testes and by both cell preparations was supported by NADPH and, to a lesser extent, by NADH. With all preparations, the enzyme activity was maximal at a NADPH concentration of about 100 μM. When NADH was used as the cofactor, the enzyme activity of the whole testes preparation

Table 2. Cofactor requirements and inhibitory action of heme ligands on the oxidation of hematoheme by the microsomal fractions of the whole testes and by Leydig and Sertoli cell preparations*

Enzyme source	Additions	Conc (μM)	Heme oxygenase (nmoles · mg^{-1} · hr^{-1})
Whole testes	Complete system†		5.01
	− NADPH		0
	−Biliverdin reductase		0
	− NADPH + ascorbic acid	5000	0
	+ NaN_3	5000	1.17
	+ KCN	100	0.72
	+ NADPH	5	0.17
	+ NADPH	20	0.98
	+ NADPH	50	3.21
	+ NADPH	100	4.60
	− NADPH + NADH	100	0.02
	− NADPH + NADH	500	0.20
	− NADPH + NADH	1000	0.60
	− NADPH + NADH	2000	1.40
	− NADPH + NADH	4000	1.94
	− NADPH + NADH	8000	2.16
Leydig cell preparation	Complete system†		6.85
	− NADPH		0
	− Biliverdin reductase		0
	− NADPH + ascorbic acid	5000	0
	+ NADPH	100	6.45
	+ NaN_3	5000	3.52
	+ KCN	100	3.20
	− NADPH + NADH	2000	1.26
Sertoli cell preparation	Complete system†		0.59
	− NADP		0
	− Biliverdin reductase		0
	− NADPH + ascorbic acid	5000	0
	+ NADPH	100	0.69
	+ NaN_3	5000	0.30
	+ KCN	100	0.29
	− NADPH + NADH	2000	0.19

* The microsomal fractions were obtained from the testicular Leydig and the Sertoli cell preparations, as well as whole testes of Sprague–Dawley rats (200–250 g). Heme oxygenase activity was measured in a reaction system (1.4 ml) containing hematoheme (15 μM), enzyme source (0.5 to 1 mg/ml), purified rat liver biliverdin reductase (50–100 μg protein), and the indicated concentrations of pyridine nucleotides, sodium azide, potassium cyanide, or ascorbic acid, in a 90 mM potassium phosphate buffer (pH 7.4). The hematobilirubin formed during the incubation time (37°, 10 min) was measured as described in Experimental Procedures. The procedures utilized for the preparation of the Leydig and Sertoli cells and the microsomal fractions are also described in detail in Experimental Procedures. The data shown represent the average of two determinations.

† NADPH-generating system was used.

increased in a linear manner up to a 2 mM concentration, and further increases were observed when the NADH concentrations were 8 mM.

In the absence of added biliverdin reductase, the formation of hematobilirubin was not detected in any of the heme oxygenase assay media. Ascorbic acid (5 mM) was ineffective in supporting hematoheme degradative activity, and heme ligands, KCN (100 μM) and NaN_3 (5 mM) significantly suppressed the hematobilirubin formation by the microsomal fractions of Leydig and Sertoli cell preparations, as well as of the whole testes. Hematoheme oxidative activity was not detectable when heat inactivated (60°, 10 min) microsomal fractions of Leydig and Sertoli cell preparations were used or in atmospheres of N_2 or CO (data not shown). The microsomal heme oxygenase activities of Leydig cell preparations and of whole testes were characterized with respect to protein- and time-dependence of the reaction. With both fractions, the formation of hematobilirubin was linear with increasing protein concentrations up to 1.5 mg protein/ml assay mixture (data not shown). Also, heme oxygenase activity was linear with time for up to 10 min of incubation at 37° (data not shown).

Effect in vivo *of cadmium administration on the activities of the enzymes of the heme degradative pathway.* The possibility of a different response of heme oxygenase activity in Leydig and Sertoli cells to $Cd^{2+}$was investigated. Also, the effect of Cd^{2+} treatment on the activities of other enzymatic components essential to the hematobilirubin formation process was determined. Rats were treated with 7 or 20 μmoles/kg Cd^{2+}, and 24 hr later Leydig and Sertoli cell preparations were obtained and examined for heme oxygenase activity. As shown in Fig. 1, Cd^{2+} treatment evoked dissimilar responses in enzyme activity in the Leydig and Sertoli cell preparations. The activity of the enzyme in Leydig cells was very sensitive to the effect of Cd^{2+} and was diminished to 20% of the control value in rats given 20 μmoles/kg Cd^{2+}. In contrast, the enzyme activity was hardly affected in Sertoli cell preparations from rats given a similar dose of Cd^{2+}. The enzyme activity was not affected significantly in either cell preparation by treatment of rats with 7 μmoles/kg Cd^{2+} (data not shown). The severe inhibitory action of Cd^{2+} on the microsomal heme oxygenase activity in Leydig cell preparation did not represent a direct inhibiting phenomenon. The formation of hematobilirubin was not inhibited significantly when using microsomal fractions pretreated *in vitro* (25°, 10 min) with Cd^{2+} at concentrations of 50, 100, 250, and 500 μM as the enzyme source in the heme oxygenase assay system (data not shown).

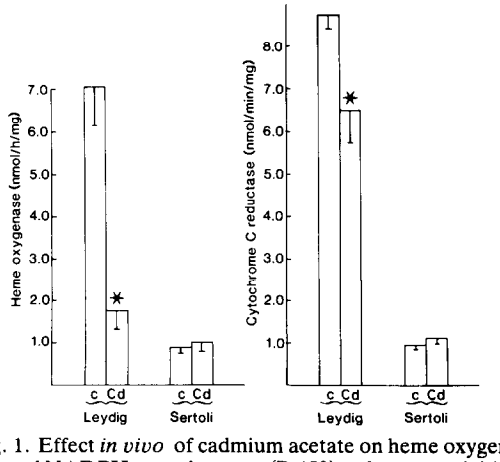

Fig. 1. Effect *in vivo* of cadmium acetate on heme oxygenase and NADPH–cytochrome *c* (P-450) reductase activities in Leydig and Sertoli cell preparations of the rat testes. The Leydig and the Sertoli cell preparations were prepared as described in Experimental Procedures. Rats (200–250 g) were treated subcutaneously with cadmium acetate (20 μmoles/kg, 24 hr) or saline. The microsomal fractions were prepared and used for the measurement of heme oxygenase activity as described earlier [4]. The amount of hematobilirubin formed was measured as described in Experimental Procedures. The values presented represent mean ± S.D. obtained from four separate preparations. Key: (c) control; (Cd) cadmium-treated; and (*) $P \leq 0.05$.

The possibility that the *in vivo* inhibitory action of Cd^{2+} on heme oxygenase activity may, in part, reflect the inhibition by the metal ion of NADPH-cytochrome *c* (P-450) reductase was explored (Fig. 1). The activity of the latter enzyme is required for the oxidation of heme compounds by heme oxygenase. Moreover, it was essential to determine whether the differential response of Leydig and Sertoli cells to the inhibiting effect of Cd^{2+} extended to other components of the heme degradative pathway. As with heme oxygenase, it was observed that the activity of NADPH-cytochrome *c* (P-450) reductase in Leydig cells was inhibited significantly by Cd^{2+} treatment (20 μmoles/kg), but that of Sertoli cell preparations was fully refractory to the metal ion. The treatment of rats with 7 μmoles/kg Cd^{2+} did not alter significantly the enzyme activity in Leydig and Sertoli cell preparations. Unlike heme oxygenase and NADPH–cytochrome *c* (P-450) reductase, the activity of biliverdin reductase was not inhibited in the cytosol fraction of Leydig cells or in Sertoli cell preparations obtained from rats with 20 μmoles/kg Cd^{2+} (data not shown). The *in vivo* refractory response of biliverdin reductase to Cd^{2+} treatment contrasted with the *in vitro* effect of the metal ion on the activity of the enzyme. The addition of 5 or 10 μM Cd^{2+} to biliverdin reductase assay medium inhibited the enzyme activity by 20 and 45% respectively. This finding may suggest that Cd^{2+} does not preferentially accumulate in Leydig cells of the rat testes. The total protein content of Leydig and Sertoli cell preparations obtained from the testes of Cd^{2+}-treated rats (7 or 20 μmoles/kg) and the control animals did not differ significantly (data not shown).

Effect in vivo *of cadmium treatment on the microsomal contents of cytochrome P-450 and heme in Leydig and Sertoli cell preparations.* It was of interest to examine whether the higher sensitivity of Leydig cells to Cd^{2+}, as suggested by the decline in the heme oxygenase and NADPH–cytochrome *c* (P-450) reductase activities, was shared by the microsomal heme and cytochrome P-450 contents. Indeed, the capacity of Cd^{2+} to elicit a response in cytochrome P-450 and heme contents in Leydig cells surpassed the ability of the metal ion to evoke alterations in heme oxygenase activity in this cell population. As shown in Fig. 2 in the microsomal fractions of Leydig

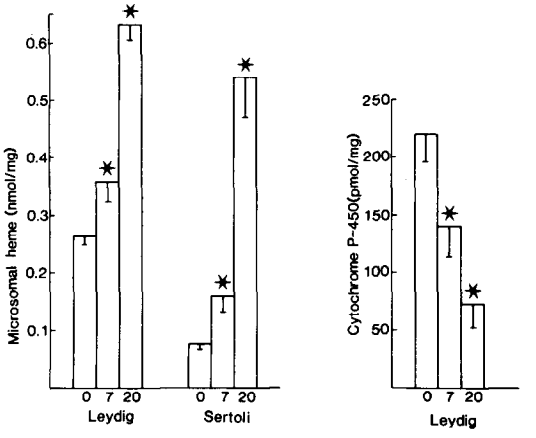

Fig. 2. Effect *in vivo* of cadmium acetate on the concentrations of heme and cytochrome P-450 in the microsomal fractions of Leydig and Sertoli cell preparations in rat testes. Sprague–Dawley rats (200–250 g) were treated subcutaneously with 7 or 20 μmoles/kg cadmium acetate or saline (24 hr). The microsomal fractions were prepared as described before [9] from the Leydig and Sertoli cell preparations of the testes. The concentration of heme was determined by the pyridine hemochromogen procedure of Paul *et al.* [21]. The cytochrome P-450 content of Leydig cells was measured as described by Nozu *et al.* [22]. The experimental details for the isolation of cells are provided in Experimental Procedures. The values represent the mean ± S.D. of four separate preparations. Key: (*) $P \leq 0.05$; (0) control; (7) 7 μmoles Cd^{2+}/kg; and (20) 20 μmoles Cd^{2+}/kg.

cell preparations obtained from the rats treated with 7 μmoles/kg Cd^{2+}, a nearly 40% decrease in the cytochrome P-450 content was observed. In rats treated with 20 μmoles/kg Cd^{2+}, the hemoprotein content was reduced by nearly 70%. Attempts to measure the hemoprotein in Sertoli cell preparations were not successful. The inability to detect cytochrome P-450 in Sertoli cells may reflect a severely diminished level of the cytochrome, as well as the limitation of our detection capability.

An unexpected effect of Cd^{2+} treatment on the microsomal heme content in Leydig and Sertoli cell preparations was observed. In this instance, however, Sertoli cells displayed the higher degree of sensitivity to Cd^{2+}. Moreover, the concentration of heme in Leydig and Sertoli cells proved to be exceedingly susceptible to alterations by the metal ion. The data shown in Fig. 2 demonstrate that the microsomal heme content in both cell populations increased in a dose-related manner after treatment of rats with 7 or 20 μmoles/kg Cd^{2+}. As noted, the significant perturbation elicited by 7 μmoles/kg Cd^{2+} in the concentration of heme contrasted with its effect on the activity of heme oxygenase. In rats treated with 20 μmoles/kg Cd^{2+}, an increase of 8- to 9-fold in the heme concentration in the microsomal fractions of Sertoli cell preparations was observed. This treatment increased the microsomal heme content in Leydig cells by only 2-fold. The same magnitude of increase in the concentration of heme in Sertoli cell preparations was noted with 7 μmoles/kg Cd^{2+}.

Distribution of the enzymes of heme biosynthesis, and effect in vivo *of cadmium on heme biosynthetic activities in Leydig and Sertoli cell preparations.* The possibility that the Cd^{2+}-mediated increase in the concentration of heme Leydig and Sertoli cell preparations reflects an increase in heme biosynthetic activities was explored. Also, in light of finding a differential cellular distribution of heme oxygenase activity, the occurrence of such a phenomenon with respect to heme biosynthetic activities appeared plausible. Accordingly, Leydig and Sertoli cell preparations from rats treated with 20 μmoles/kg Cd^{2+} were utilized for the measurement of the activities of ALA synthetase, ALA dehydratase, and uro-I synthetase, as well as the total porphyrin concentration (Table 3).

The data show that the heme biosynthetic capacity of Leydig cells exceeded that of Sertoli cells. In Leydig cell preparations, the activities of ALA synthetase, the first enzyme in the pathway, and uro-I synthetase, the enzyme involved in the formation of the first tetrapyrrole structure in the pathway, surpassed those of Sertoli cell preparations. Consistent with these observations was the finding that the total porphyrin content of Leydig cells exceeded that of Sertoli cells. The activity of ALA dehydratase was comparable in Leydig and Sertoli cell preparations, and, in both cell preparations, surpassed those of ALA synthetase and uro-I synthetase. The high level of heme biosynthetic activity, as well as the above noted high levels of cytochrome P-450 concentration and NADPH–cytochrome *c* (P-450) reductase activity in Leydig cell preparations is consistent with the active role of those cells in steroidogenesis.

As shown, the treatment of rats with Cd^{2+} caused a modest, but significant, increase in the total porphyrin content of Leydig cell preparations. The increase in ALA synthetase activity was not significant. These parameters in Sertoli cell preparations were unaffected by the treatment. This finding is particularly noteworthy in suggesting that the observed increase in the concentration of microsomal heme in Leydig and Sertoli cell preparations and the cellular heme biosynthetic activity may not be causally related. The data in Table 3 also show that, in Leydig and Sertoli cell preparations, a moderate decrease in ALA dehydratase activity was observed. However, the activity of uro-I synthetase was not altered by Cd^{2+} treatment.

The finding that the activity of ALA dehydratase in both cell preparations was inhibited by Cd^{2+} further supports the suggestion that the preferential accumulation of the metal in Leydig cells is not the biological basis for the profound susceptibility of heme oxygenase in this cell population to Cd^{2+}. δ-Aminolevulinate dehydratase, like biliverdin reductase, is a sulfhydryl-dependent enzyme and is inhibited by sulfhydryl reagents, including metal ions which have a high degree of affinity for SH groups. As would be predicted, when tested *in vitro* using Leydig cell preparations as the enzyme source, the activity of ALA dehydratase was inhibited by Cd^{2+}. The extent of inhibition caused by treatment of the enzyme with 50 μM Cd^{2+} was about 25–30%.

Effect in vivo *of bromobenzene and phenobarbital on heme oxygenase activity in Leydig cells.* The possibility that the inability of Cd^{2+} to induce heme

Table 3. Cellular distribution and effects *in vivo* of cadmium acetate on the activities of δ-aminolevulinate synthetase, δ-aminolevulinate dehydratase, uroporphyrinogen-I synthetase, and the content of porphyrins in Leydig and Sertoli cell preparations*

Enzyme source	Treatment	ALA synthetase (pmoles ALA · hr^{-1} · mg^{-1})	ALA dehydratase (nmoles PBG · hr^{-1} · mg^{-1})	Uro-I synthetase (pmoles porphyrin · hr^{-1} · mg^{-1})	Total porphyrins (pmoles/mg)
Leydig cell preparation	Control	119.4 ± 14.5	1.59 ± 0.16	81.5 ± 3.3	1.40 ± 0.05
	Cd^{2+}	154.0 ± 25.5	1.35 ± 0.14†	95.3 ± 12.6	1.62 ± 0.14
Sertoli cell preparation	Control	73.0 ± 7.3‡	1.82 ± 0.09	38.0 ± 2.9‡	1.26 ± 0.08‡
	Cd^{2+}	60.8 ± 10.9	1.52 ± 0.07†	43.5 ± 2.8	1.32 ± 0.30

* Leydig and Sertoli cell preparations were obtained from the testes of control or Cd^{2+}-treated (20 μmoles/kg) Sprague–Dawley rats (200–250 g) as described in Experimental Procedures. The activity of ALA synthetase was measured using the 600 *g* supernatant fraction. The activities of ALA dehydratase and uro-I synthetase were measured in the 9000 *g* supernatant fraction. The porphyrin content was determined in the tissue homogenate. The measurements were carried out as detailed in Experimental Procedures. The results are means ± S.D. for six rats. PBG = porphobilinogen.

† $P \leq 0.05$ when compared to the appropriate control value.

‡ $P \leq 0.05$ when two control groups are compared.

oxygenase activity in the testes represents an isolated response was considered. Accordingly, the effect of the treatment of rats with bromobenzene, a known potent inducer of the liver enzyme, on heme oxygenase activity in Leydig cell microsomal fractions was examined. Also, the possibility that the testicular enzyme may be inducible by agents known to increase the activities of several endoplasmic reticulum membrane-bound enzymes, but not that of heme oxygenase, in the liver was investigated. The treatment of rats with 0.5 mmoles/kg bromobenzene evoked an expected 7- to 8-fold increase in heme oxygenase activity in the liver. However, the enzyme activity in Leydig cell preparations remained unaffected by the treatment. The enzyme activity in the livers of the control and bromobenzene-treated rats measured 2.87 ± 0.06 vs 21.78 ± 1.90 nmoles hematobilirubin per mg protein per hr, respectively. The corresponding activity in Leydig cell preparations measured 6.07 ± 0.36 vs 5.90 ± 0.59 nmoles hematobilirubin per mg protein per hr. Similarly, phenobarbital treatment proved ineffective in altering heme oxygenase activity in Leydig cell microsomal preparations.

Age-related changes in the activities of heme oxygenase, δ-aminolevulinate synthetase and the concentration of cytochrome P-450 in Leydig cells. In light of the finding that the response of heme oxygenase activity in Leydig cells to Cd^{2+} differed from that of the liver, it was hypothesized that the observed phenomenon may include other fundamental aspects of the regulation of enzyme activity, such as age-related changes in the activity of the enzyme and the temporal relationship of the latter process to the activity of ALA synthetase and the content of cytochrome P-450. Accordingly, the age-related changes in the activities of heme oxygenase and ALA synthetase and the microsomal content of cytochrome P-450 in Leydig cells were investigated.

The activities of heme oxygenase and ALA synthetase were measured in Leydig cell preparations of rat testes at 2, 4, 8, 12, 16, and 24 weeks of age. Also, the microsomal content of cytochrome P-450 was assessed at these time points (Fig. 3). A pattern distinctly different from that of the liver for age-related changes in the activities of heme oxygenase and ALA synthetase was observed. The heme oxygenase activity in the 4- and 8-week-old animals was markedly lower than that of sexually mature animals (12–16 weeks). The enzyme activity reached a maximum level at about 12–16 weeks of age and remained at the same level for 24 weeks. Attempts to measure heme oxygenase activity in 2-week-old animals were unsuccessful because of the small size of the organs, the poor yield of Leydig cells, and the low level of heme oxygenase activity. The observed age-related changes in the pattern of enzyme activity distinctly differed from that of the liver. As expected, in the liver the peak level of enzyme activity was reached during the first 2 weeks of life, with an ensuing rapid rate of decline, and the attainment of adult value by 3–4 weeks of life (data not shown). This pattern was similar to that previously observed [30]. Similarly, the pattern of development of ALA synthetase in Leydig cells was unique in that the enzyme activity was at a maximum

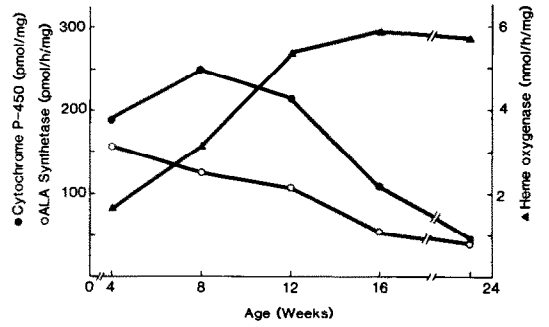

Fig. 3. Age-related changes in the activities of heme oxygenase and δ-aminolevulinate synthetase, and the concentrations of cytochrome P-450 in Leydig cell preparations of rat testes. Collagenase dispersed Leydig cells were obtained from the testes of Sprague–Dawley rats of indicated ages and used for the preparation of the post-nuclear and the microsomal fractions. The experimental details for the preparation of the cells and the subcellular fractions are provided in Experimental Procedures. The activity of ALA synthetase was measured in the post-nuclear fractions, while the microsomal fraction was utilized for measurements of heme oxygenase activity and the concentration of cytochrome P-450. The measurements were made as detailed in Experimental Procedures. The data shown are representative of two sets of experiments, each of which involved animals of the full range of age profile. Each data point represents the average value of three to ten rats.

level at 4–12 weeks of age and underwent a progressive and notable decrease with age. As with ALA synthetase activity, the cytochrome P-450 concentration in the microsomal fractions of Leydig cells in 4-, 8- and 12-week-old animals was in excess of those of 16- and 24-week-old rats. Indeed, the concentration of the hemoprotein in Leydig cell preparations of the 24-week-old rats was less than 30% of that of the 8-week-old rats.

DISCUSSION

The response of microsomal heme oxygenase in the rat testes to metal ions, specifically to Cd^{2+}, was described recently [9]. The response was that of the depression of the enzyme activity, rather than the well-known induction phenomenon. The present study reports on the heterogeneity between the two cell populations of the testes regarding heme metabolic activity, in general, and the activity and the response to Cd^{2+} of heme oxygenase, in particular. Moreover, the present study shows that the regulation of the heme oxygenase activity in the testes differs from that of the liver, not only with respect to the response of heme oxygenase activity to Cd^{2+}, but with respect to other fundamental properties, such as the developmental aspects of the enzyme activity (Fig. 3), and response to other known inducers of the liver enzymes, such as bromobenzene.

In addition, the unusual developmental pattern and age-related changes in the activity of ALA synthetase and the concentration of cytochrome P-450 in Leydig cells (Fig. 3) further suggest that the regulation of heme metabolism in the testes in general may differ from that of the liver. On the other hand, there is no indication at this time that the nature of heme oxygenase enzyme protein or the heme oxidative process in the two testicular cell populations differ from each other (Table 2), or from that of the liver [4].

The site of heme metabolic activity in the testes apparently resides mainly in Leydig cells. This finding is consistent with the function of this cell population in the hemoprotein-dependent steroidogenetic processes. The function of cytochrome P-450 in the microsomal fraction of the testes for the most part is that of catalyzing hydroxylations of the steroid nucleus. The steroid biotransformative activities are mainly localized in the mitochondrial and the microsomal fractions of Leydig cells. In the microsomal fractions, both the 17-α-hydroxylase and 17-20-lyase activities are seemingly catalyzed by the same molecular species of cytochrome P-450 [31]. An alteration in the activity of this cytochrome has been correlated with disruptions in steroid biosynthesis [22]. For instance, the treatment of rats with human gonadotropin or estradiol promotes modulation of cytochrome P-450 content and steroidogenesis in Leydig cell microsomal fractions [22, 32]. Moreover, it has been shown that the decreased activity of 17-α-hydroxylase in the rat testes caused by spironolactone or 3-methylcholanthrene is accompanied by decreases in the microsomal content of cytochrome P-450 and the hemoprotein-dependent steroid metabolic activities [14, 33]. Accordingly, the presently reported depletion of Leydig cell cytochrome P-450 by Cd^{2+} and the apparent high degree of sensitivity of the hemoprotein to a rather low level exposure to Cd^{2+} (7 μmoles/kg) may explain an important aspect of testicular toxicity of the metal ion [34, 35]. In addition, it may be postulated that the deleterious effects of Cd^{2+}-mediated alterations in heme metabolic processes may be further augmented as a consequence of an alteration in the pattern of steroid production in Leydig cells and local inhibiting effect of the steroids produced [36]. This postulated mode of action is compatible with the known regulatory effect of estradiol on cytochrome P-450-mediated 17-α-hydroxylase and 17-20-lyase activities and the known local inhibiting effect of steroids at the biotransformation site [36]. Therefore, it is plausible that Cd^{2+}-mediated alterations in heme metabolism could lead to perturbations in the concentration profile of steroids synthesized in Leydig cells and further augment disruptions caused by Cd^{2+} in steroidogenetic processes.

At this time the molecular basis for the unprecedented response of testicular heme oxygenase to Cd^{2+}, in particular, and inducers of the enzyme, in general, is not clear. Moreover, the possible relation between the presently observed inhibitory effect of Cd^{2+} on heme oxygenase activity and its reported inhibitory effect on several other enzymes in the testes including carbonic anhydrase [37] and glutathione peroxidase [38] is not understood. It can be asserted with certainty that inhibition of microsomal heme oxygenase by Cd^{2+} represents neither a direct inhibiting action of the metal on the enzyme, nor the generalized and random effect of the metal ion on the production of cellular enzyme proteins. A postulated mechanism of action would involve the known capacity of Cd^{2+} to decrease blood glucose levels,

and to alter cellular carbohydrates and cyclic AMP metabolism in rat testes [39]. The ability of cyclic AMP to alter heme oxygenase activity has been documented [40]. However, the latter study pertained to the effect of the nucleotide on the liver heme oxygenase, where an increase in activity was observed in response to exogenously administered cyclic AMP. Considering the presently shown opposing pattern of response of the enzyme in the liver and the testes to Cd^{2+}, it could be speculated that in the testes perturbations in carbohydrate metabolism and cyclic AMP levels could result in the inhibition of heme oxygenase activity.

The finding (Fig. 3) that the developmental pattern of ALA synthetase, heme oxygenase, and cytochrome P-450 in Leydig cells is distinctly different from that of other organs [30] may bear upon the age-related changes in the pattern of steroidogenic activity in the testes. Specifically, the higher level of both ALA synthetase activity and cytochrome P-450 content in sexually immature and young rats (4–12 weeks) and the age-related decline (after 16 weeks) in the enzyme activity may well be related to the regulation of testosterone synthesis in the testes. It is known that the fetal testes synthesize androgens in large amounts, and in the rat, testosterone is the only androgen produced [41]. Moreover, the onset of testosterone production closely correlates with the differentiation of Leydig cells, followed by regression and decline in the testosterone synthesis in the cells [41]. Thus, an interrelationship between heme metabolic activity and steroidogenic activities in the testes can be considered. In 4- to 12-week-old rats the absence of a high level of heme oxygenase activity along with a high level of ALA synthetase activity would permit the maintenance of a high cellular level of cytochrome P-450. It follows that the increase in the activity of heme oxygenase with age, along with a progressive decline in ALA synthetase activity, could contribute to a gradual decline in the cytochrome P-450 level and steroid production in the testes.

The role of Sertoli cells in steroidogenesis is controversial. This cell population has been shown to contain aromatase, presumably a cytochrome P-450-dependent enzyme, which converts testosterone to estradiol [42], although this mechanism does not quantitatively constitute an important source of estradiol. Also, Sertoli cells exhibit a limited capacity to synthesize testosterone. The limited role of Sertoli cells in the synthesis of steroids consistent with the present finding that the overall heme metabolic activity in this cell population is less than that of Leydig cells, and the cytochrome P-450 content is very low. Interestingly, heme metabolic enzymes in Sertoli cells also showed less sensitivity to Cd^{2+} than in Leydig cells. Indeed, heme oxygenase activity was unperturbed by Cd^{2+} treatment; at the same time, the concentration of microsomal heme increased preferentially in the Sertoli cells. The latter may be related to the rich blood supply of Sertoli cells plus the fact that adult Sertoli cells are not dividing and contain abundant rough and smooth endoplasmic reticulum (ER) membranes. Accordingly, it could be postulated that the selective obstruction of testicular microvasculature which occurs in response to exposure to Cd^{2+} (20–40 μmoles/kg, Refs. 43 and 44) would permit increased availability of hemoglobin heme for transfer and binding to the ER membranes, thus leading to the observed massive increase in the heme content of the microsomal fractions. This assertion is consistent with the finding (Table 3) that the activity of ALA synthetase and heme biosynthetic processes in Sertoli cell preparations were not increased in response to exposure to high levels of Cd^{2+} (20 μmoles/kg). Also, previous findings with ^{14}C-labeled ALA, a specific microsomal heme precursor, have clearly shown that the source of the increased heme levels in the rough and smooth ER fractions of rat testes is indeed hemoglobin [9]. At this time, the molecular basis for the refractory response of heme oxygenase in Sertoli cells to Cd^{2+} is not understood. As reflected by the activity of NADPH–cytochrome *c* (P-450) reductase, however, this refractory response may be shared by other ER membrane-bound enzymes.

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