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HEME AND HEMOPROTEINS IN STREPTOZOTOCIN-DIABETIC FEMALE RATS*

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Abstract—Alterations in heme biosynthetic and degradative capabilities and in the activities of several heme-containing enzymes were examined in hepatic tissues of streptozotocin (STZ)-diabetic female Sprague–Dawley rats. Activities were measured 10, 30 and 90 days following the administration of STZ (65 mg/kg, i.v.). The activities of the key enzymes involved in heme synthesis, δ-aminolevulinic acid (ALA) synthase, ALA dehydratase, and uroporphyrinogen synthase, were decreased markedly in STZ-diabetic rats as compared to sham-operated animals. Furthermore, the catabolism of heme which occurs via microsomal heme oxygenase (MHO) remained unaltered in these animals. Microsomal content of heme and cytochrome P-450, and the activities of tryptophan pyrrolase and the drug-metabolizing enzymes benzo[*a*]pyrene (BP) hydroxylase and aniline hydroxylase, were increased in the livers of diabetic rats. By contrast, the activity of the heme-containing enzyme catalase was decreased in these animals. Cobalt chloride produced a marked increase in MHO with a concomitant decrease in microsomal content of cytochrome P-450 and its associated BP hydroxylase activity in normal as well as chronically diabetic rats. It was of interest, however, that the increase in ALA synthase that is normally produced by this metal was not seen in chronic diabetic animals. Thus, chronic diabetes produced subtle and important disruptions in cellular metabolism, which may have been the result of long-term alterations in key enzymes involved in heme synthesis.

The induction of diabetes by either STZ‡ or alloxan results in a marked reduction in the hepatic activities of numerous cytochrome P-450-dependent monooxygenase enzymes including aminopyrine *N*-demethylase, hexobarbital hydroxylase, and BP hydroxylase in male rats [1–4]. Conflicting reports of either stimulation [2, 3] or inhibition [5] of aniline hydroxylation and either increased [2, 4] or unaltered [5] content of cytochrome P-450 in these animals have appeared in the literature. In contrast, all of the above drug-metabolizing enzymes and the content of cytochrome P-450 are stimulated in female diabetic rats [2, 4]. In spite of extensive research concerning these diabetic-induced changes, the underlying biochemical mechanisms are still unknown. Although cytochrome P-450 is a hemoprotein, relatively little attention has been paid to the influence of the diabetic state on factors which affect the synthesis, degradation, or utilization of heme. Therefore, this study was designed to investigate whether or not increases in the content of cytochrome P-450 and its associated mixed-function oxidase activity in female rats could have resulted from alterations in the key enzymes involved in heme synthesis (ALA-S, ALA-D, and URO-S) and degradation (MHO) and in the microsomal concentration of heme. In addition, the activities and content of several other heme-containing proteins (TPO and catalase) with different intracellular localization were determined.

METHODS

Animals

Female Sprague–Dawley derived rats (150–175 g) were obtained from Flow Laboratories (Dublin, VA) and maintained under standardized conditions of light (6:00 a.m.–6:00 p.m.) and temperature (22°) on a diet of Purina Laboratory Chow and water. Animals were housed four to a cage on hardwood bedding chips in clear plastic cages.

Treatments

Diabetes was induced by an injection of STZ (65 mg/kg, i.v.), freshly prepared in 0.05 M citrate buffer, pH 4.5. Animals were killed at 10, 30, and 90 days after induction of diabetes. Rats having plasma glucose levels greater than 250 mg/100 ml were considered as diabetic. Enzymatic activities were determined simultaneously on diabetic and control animals that received buffer alone by the same route. The effects of cobalt chloride on xenobiotic and heme-metabolizing enzyme systems were examined after 1 month of diabetes induction. Sixteen hours after subcutaneous injection of 35 mg/kg

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‡ Abbreviations: STZ, streptozotocin; MHO, microsomal heme oxygenase; TPO, tryptophan pyrrolase; ALA-S, δ-aminolevulinic acid synthase; URO-S, uroporphyrinogen synthase; ALA-D, δ-aminolevulinic acid dehydratase; and BP, benzo[*a*]pyrene.

of cobalt chloride in physiological saline, animals were decapitated and analyzed as described below. All rats were killed between 8:00 a.m. and 9:00 a.m. following a 14–16 hr fast.

Tissue preparation

Livers were perfused with ice-cold 0.9% saline, then removed, trimmed, and minced into small pieces, and homogenized in 3 vol. of cold 0.25 M sucrose containing 50 mM Tris–HCl (pH 7.4). The homogenate was centrifuged for 10 min at 700 *g* or for 20 min at 9000 *g*, and the decanted supernatant fraction from the latter was then centrifuged at 15,000 *g* for 15 min to ensure adequate removal of mitochondria. The resultant postmitochondrial supernatant fraction was further centrifuged at 105,000 *g* for 60 min. The microsomal pellet was resuspended in buffer to a protein concentration of 12–18 mg/ml. This preparation was used for the assay of MHO activity. For the determination of BP and aniline hydroxylases, the microsomal fraction was further diluted to 5 mg protein/ml. The microsomal supernatant fraction from control animals served as the source of bilivirdin reductase in the MHO assay; its protein concentration was adjusted to 10 mg/ml.

Heme synthesis

ALA-S. ALA-S activity was measured by a modification of the method of Sassa *et al.* [6]. The 9000 *g* pellet was resuspended to a protein concentration of 25–35 mg/ml in a reaction mixture containing glycine (75 mM), citrate (75 mM), Tris–HCl buffer (75 mM, pH 7.4), $MgCl_2$ (15 mM), EDTA (7.5 mM), and pyridoxal-5′-phosphate (2.2 mM). Duplicates of 2-ml aliquots were incubated for 60 min in 25-ml Erlenmeyer flasks at 37° with shaking (75 cycles/min). The reaction was stopped by the addition of 0.5 ml of 25% (w/v) trichloroacetic acid and kept on ice for 10 min. The acidified reaction mixture was then centrifuged at 3000 *g* for 15 min. Two-milliliter samples of the deproteinized fraction were transferred to test tubes and mixed with 0.6 ml of 1 M acetate buffer (pH 5.1) followed by 150 μl of acetylacetone. The mixture was heated at 100° for 15 min to convert ALA to 2-methyl-3-acetyl-4-(3-propionic acid)pyrrole. Samples were allowed to cool to room temperature and then placed on AG 1-X8 acetate columns that had been equilibrated with 0.05 M sodium acetate (pH 4.6). The columns were then washed successively with 10 ml of H_2O, 5 ml of 50% methanol, and 5 ml of 1 N acetic acid. The ALA pyrrole was eluted with 3 ml of glacial acetic acid and mixed with equal volumes of modified Ehrlich reagent [7]. Samples were left at room temperature for 30 min and then scanned between 650 and 500 nm on a Beckman DU-8 spectrophotometer. The concentration of ALA formed was calculated based on the difference in absorbance of 650 nm and 553 nm using an extinction coefficient of 58 mM^{-1} cm^{-1} [8].

ALA-D. The activity of ALA-D was assayed by measurement of the rate of porphobilinogen formation using a liver homogenate corresponding to 150 mg wet wt liver and 3×10^{-4} M ALA as described previously by Coleman [9].

URO-S. The procedure of Strand *et al.* [10] was utilized for the measurement of URO-S activity using 10^{-4} M porphobilinogen and a 9000 *g* supernatant fraction containing approximately 1 mg protein.

Heme degradation—MHO

MHO activity was determined by a modification of the procedure of Maines and Kappas [11]. The incubation mixture contained a microsomal suspension (3 mg protein), 105,000 *g* supernatant fraction (2 mg protein), heme (17 mM), an NADPH-generating system (glucose-6-phosphate, 0.85 mM, glucose-6-phosphate dehydrogenase, 0.75 units), $MgCl_2$ (2 mM), and Tris buffer (pH 7.4) in a final volume of 1.5 ml. The mixture was preincubated for 5 min at 37° in a metabolic shaker. The reaction was then initiated by addition of 0.025 ml of $NADP^+$ (final concentration, 0.8 mM) in 1% $NaHCO_3$ to the test samples. $NADP^+$ was replaced by an equivalent volume (0.025 ml) of 1% $NaHCO_3$ in the reference mixture. The incubation was carried out for 10 min at 37° in the dark. At the end of the incubation, reaction mixtures were terminated by cooling the flasks on ice. Benzene (1.5 ml) was added to the samples. The mixture was then agitated vigorously and centrifuged for 5 min at 3000 *g* to separate the two phases. The upper organic layer was withdrawn, and the extraction with benzene was repeated three times. The pooled extract was concentrated under nitrogen to a final volume of 1.5 ml, and the extract was scanned between 530 and 400 nm. Bilirubin concentration was calculated from the difference in absorbance between 530 and 455 nm using an extinction coefficient of 40 $mM^{-1}cm^{-1}$.

Heme and hemoproteins

Cytochrome P-450 and drug-metabolizing enzymes. Cytochrome P-450 was determined from the reduced carbon monoxide difference spectrum according to the method of Omura and Sato [12]. BP hydroxylase activity was determined by the method of Dehnen *et al.* [13] using approximately 0.4 mg of microsomal protein and 50 nmoles BP in acetone in a total volume of 1 ml. The activity of aniline hydroxylase was measured by the formation of *p*-aminophenol according to the procedure of Mieyal and Blumer [14] using approximately 1 mg of microsomal protein and 0.2 mM aniline in a total volume of 2 ml.

Heme. The microsomal concentration of heme was determined by the pyridine hemochrogen method of Falk [15]. Microsomal pellets were suspended in enough 0.05 M Tris–HCl buffer (pH 7.5) to result in 2–3 mg protein/ml. The microsomal heme content was calculated from the difference in absorption between 557 and 575 nm and an extinction coefficient of 32.4 $mM^{-1}cm^{-1}$.

Catalase. Catalase activity was assayed by measurement of the rate of decomposition of H_2O_2 using the 700 *g* supernatant fraction as described by Cohen *et al.* [16].

TPO. The procedure of Knox *et al.* [17], with some modification, was used to measure the formation of kynurenine from L-tryptophan in either the absence (holoenzyme activity) or the presence (total enzyme activity) of added methemoglobin. The reaction mixture contained 105,000 *g* supernatant fraction (5–10 mg protein), ascorbate (0.03 M),

Table 1. Effect of duration of diabetes on body weight and plasma glucose in female Sprague–Dawley rats*

Duration of diabetes (days)	Body weight (g)		Serum glucose (mg/100 ml)	
	Control	Diabetic	Control	Diabetic
10	195 ± 9	175 ± 7†	102 ± 10	462 ± 50†
30	245 ± 18	180 ± 5†	95 ± 4	425 ± 40†
90	310 ± 15	190 ± 4†	123 ± 7	501 ± 32†

* STZ was injected i.v., 65 mg/kg, in 0.05 M citrate buffer, pH 4.5. Values are expressed as means ± S.E.M. for at least four animals.
† Significantly different from corresponding control values at $P < 0.05$.

tryptophan (5 mM), 0.5 mg of methemoglobin and Tris–HCl buffer (pH 7.4) in a final volume of 1 ml. The mixture was preincubated at 37° with shaking. After 30 min, 0.4 ml of the above mixture was added to 2.6 ml of a catalytic reaction mixture consisting of ascorbate (0.01 M), tryptophan (5 mM), and buffer. At the end of a 25-min incubation, the reaction was stopped by the addition of 0.1 ml of 1 N HCl. Samples were scanned from 600 to 300 nm. Kynurenine concentration was determined from the differences in absorption between 600 and 365 nm using an extinction coefficient of 4.530 $mM^{-1}cm^{-1}$.

Protein. Protein was determined by the method of Lowry *et al.* [18] using bovine serum albumin as a standard.

Chemicals

$NADP^+$, pyridoxal 5'-phosphate, hemin hydrochloride, ALA, BP, porphobilinogen, L-tryptophan, and cobalt chloride were obtained from the Sigma Chemical Co., St. Louis, MO. Glycine and glutathione were obtained from Calbiochem, San Diego, CA. AG 1-X8 (200–400 mesh) acetate form was obtained from Bio-Rad, Richmond, GA. *p*-Dimethylaminobenzaldehyde and 2,4- pentanedione were obtained from the Eastman Kodak Co., Rochester, NY. Aniline hydrochloride was obtained from the J. T. Baker Chemical Co., Phillipsburg, NJ. All other chemicals were of reagent grade.

Statistics

Data are expressed as the mean ± S.E.M. The two-tailed Student's *t*-test was used to distinguish significant differences between treated and control groups.

RESULTS

Female diabetic rats displayed signs of hyperglycemia, glucosuria, polydipsia, polyphagia, and weight loss. The data on body weight and serum glucose concentrations are given in Table 1. The body weights of 10-, 30 and 90-day diabetic rats were significantly lower than the corresponding values obtained in control rats. Serum glucose concentration increased significantly from the control values within 10 days after induction of diabetes and remained high throughout the experimental period. Insulin treatment brought abnormal values back to control levels, thus demonstrating diabetogenic rather than non-diabetogenic effects of STZ (unpublished observation).

The effects of chronic diabetes on the hepatic enzymes involved in heme synthesis were determined. Thirty days after STZ injection, the activity of ALA-S, the first and rate-limiting enzyme in heme synthesis, was depressed almost 50% in diabetic rats (Table 2). The same degree of inhibition was found with ALA-D and URO-S in diabetic animals. ALA-D activity was also measured at 10 and 90 days after STZ administration. In 10-day diabetic rats, activity fell 44% from a control value of 4.55 ± 0.03 nmoles per mg protein per hr, while activity decreased by 57% in 90-day diabetics. The activity of hepatic MHO, the first and rate-limiting enzyme in heme degradation, was not changed significantly in 30-day diabetic rats (2.2 ± 0.2 nmoles bilirubin per mg protein per hr) compared to controls (2.7 ± 0.2 nmoles mg protein per hr).

The effects of STZ-induced diabetes on the metabolism of BP (Type I substrate) and aniline (Type II substrate) were determined. Both of these substrates preferentially interact with the P-448 species of the cytochrome [19, 20]. Within 10 days of STZ injection, the activities of aniline and BP hydroxylases had increased by approximately 50% (Fig. 1). These increases persisted for the next 3 months.

Table 2. Hepatic activities of heme biosynthetic enzymes in control and STZ-treated rats*

Treatment	ALA synthase (nmoles/mg/hr)	ALA dehydratase (nmoles/mg/hr)	URO synthase (pmoles/mg/hr)
Control	0.24 ± 0.02	4.4 ± 0.30	28 ± 2.5
30-Day diabetic	0.13 ± 0.03†	2.2 ± 0.45†	15 ± 1.9†

* STZ was injected i.v., 65 mg/kg, in 0.05 M citrate buffer, pH 4.5. Values are expressed as the means ± S.E.M. for at least four animals. Rats were starved for 16 hr before killing.
† Significant difference from corresponding control values at $P < 0.05$.

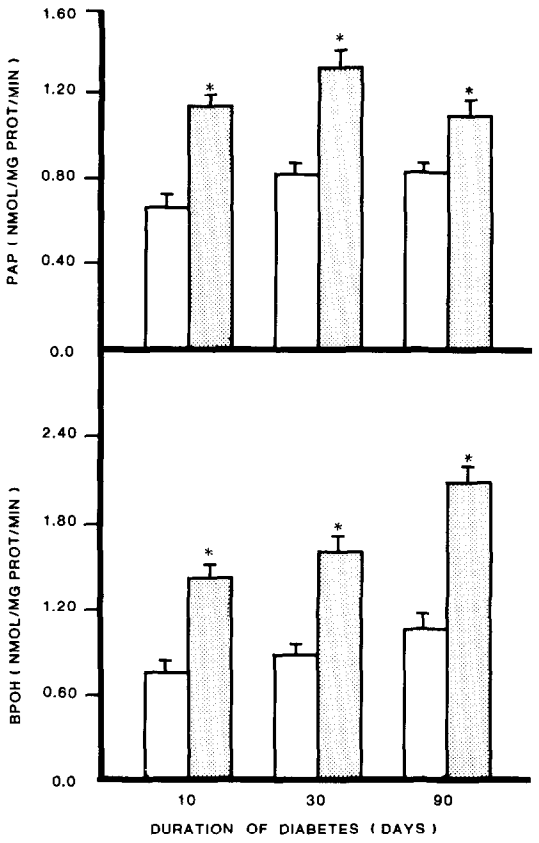

Fig. 1. Effects of STZ-diabetes on microsomal activities of benzo[*a*]pyrene hydroxylase and aniline hydroxylase in rat livers. STZ was injected i.v., 65 mg/kg, in 0.05 M citrate buffer, pH 4.5. Each bar represents the mean ± S.E.M. for at least four animals. Rats were starved for 16 hr before killing. Measurements were made 10, 30 and 90 days after induction of diabetes. Key: (*) significantly different from corresponding control values at $P < 0.05$; (□) control and (▨) STZ-diabetic.

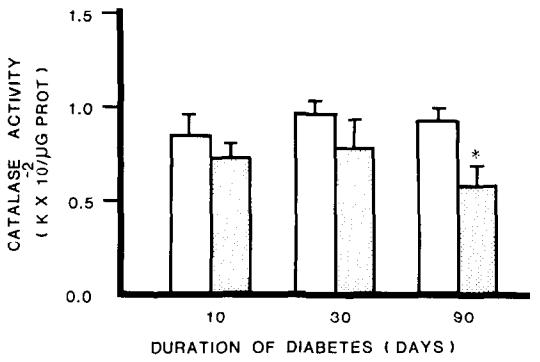

Fig. 2. Catalase activity in liver of STZ-diabetic rats. STZ was injected i.v., 65 mg/kg, in 0.05 M citrate buffer, pH 4.5. Each bar represents the mean ± S.E.M. for at least four animals. Rats were starved for 16 hr before killing. Measurements were made 30 days following induction of diabetes. Key: (*) significantly different from corresponding control values at $P < 0.05$; (□) control and (▨) STZ-diabetic.

The observation that chronic diabetes produced a decrease in the activities of hepatic heme biosynthetic enzymes and an increase in monooxygenase-mediated drug metabolism suggested that chronic diabetes may also alter the activities of other heme-containing enzymes as well as microsomal heme content. To test this hypothesis, the activities of catalase and TPO and the concentration of microsomal heme were measured. The activities of catalase in 10- and 30-day diabetic rats were not significantly different from controls; however, after 3 months of diabetes, a significant decrease (−34%) in the activity of catalase was found (Fig. 2). The microsomal content of heme and cytochrome P-450 and the activities of TPO, total as well as holo- and apoenzyme (total − holoenzyme), were all signifi-

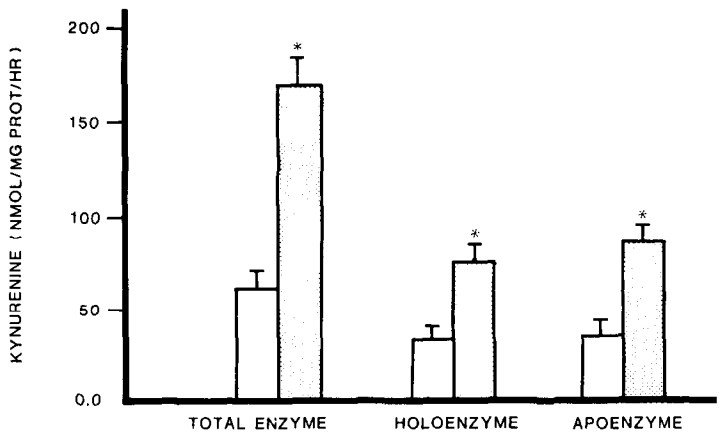

Fig. 3. Tryptophan pyrrolase activity in liver of STZ-diabetic rats. STZ was injected i.v., 65 mg/kg, in 0.05 M citrate buffer, pH 4.5 Each bar represents the mean ± S.E.M. for at least four animals. Rats were starved for 16 hr before killing. Measurements were made 30 days following induction of diabetes. Key: (*) significantly different from corresponding control values at $P < 0.05$; (□) control and (▨) STZ-diabetic.

cantly increased by more than 50% in 30-day STZ-diabetic rats (Fig. 3). While the levels of the above hemoproteins were increased, the activity of the flavoprotein NADPH–cytochrome P-450 reductase remained unaltered.

Cobalt chloride is known to affect heme metabolism and monooxygenase systems in normal animals. The results of experiments designed to investigate the influence of chronic diabetes on cobalt-mediated changes in hepatic heme metabolism, cytochrome P-450 content, and BP hydroxylase activity are given in Table 3. Subcutaneous administration of cobalt chloride resulted in approximately a 55% reduction in ALA-D activity and a 43% increase in ALA-S activity in control animals. In contrast, chronic diabetic rats were refractory to the effects of cobalt on both enzymes. Moreover, the activity of URO-S remained unaltered following cobalt chloride administration in control as well as in diabetic rats. This metal resulted in approximately 5- and 4-fold increases in MHO activity in control and diabetic rats respectively (Table 3). Associated with this increase in MHO activity was an approximately 53% decrease in BP hydroxylase activity and a 40% decrease in the concentration of cytochrome P-450 in the diabetic animals. These changes were comparable to those seen in control rats following cobalt administration.

DISCUSSION

The results of these studies provide evidence for an inhibition of key enzymes involved in heme synthesis and an unaltered MHO activity during chronic diabetes in female Sprague–Dawley rats. Furthermore, microsomal content of heme and cytochrome P-450 and the activities of mixed function oxidase and TPO were increased in these animals. The observed inhibition of ALA-S in STZ-induced diabetes supports the previous finding of Bonkowsky *et al.* [21] when alloxan served as the diabetogenic agent. The inhibition of this rate-limiting enzyme suggests that the flux through the heme biosynthetic pathway may be depressed. Indeed, our finding of depressed ALA-D and URO-S activities, enzymes not previously measured during diabetes, supports this contention. The inhibition of ALA-S activity in disease states is not unique to diabetes, since a similar result has been reported in tumor-bearing female rats [22]. In addition, the activity of ALA-S and its induction by allyl-isopropyl-acetamide (AIA) have been shown to be suppressed by a high carbohydrate diet or the *in vivo* administration of glucose [21, 23–26]. The effect of glucose appears to be similar to that obtained in STZ-treated rats and thus raises the possibility that the inhibition of ALA-S could be the result of high circulating glucose levels in these animals. In this regard, it has been suggested that the repression of ALA-S by glucose might be hormonally mediated via an increased insulin to glucagon plasma ratio with a concomitant decrease in hepatic cyclic AMP levels [27, 28]. On the other hand, the insulin to glucagon ratio is decreased and the content of cyclic AMP is increased in the liver during diabetes [29, 30]. Thus, it is difficult to relate the inhibition of ALA-S during diabetes and hyperglycemia to glucagon/insulin mediation. However, the possibility that chronic diabetes and/or glucose might interfere with the *de novo* synthesis of ALA-S, with the translocation of ALA-S from

Table 3. Influence of diabetes on cobalt chloride-mediated changes in female rats*

	Treatment			
	Sham-operated		Diabetic	
	Saline	$CoCl_2$	Saline	$CoCl_2$
ALA synthase (nmoles/mg protein/hr)	0.24 ± 0.02	0.42 ± 0.05†	0.13 ± 0.02	0.15 ± 0.04
ALA dehydratase (nmoles/mg protein/ hr)	4.4 ± 0.30	1.9 ± 0.50†	2.2 ± 0.45	1.5 ± 0.42
URO synthase (pmoles/mg protein/hr)	28.0 ± 2.5	24.0 ± 2.5	15.0 ± 2.8	18.0 ± 1.9
MHO (nmoles/mg protein/hr)	2.7 ± 0.20	12.5 ± 0.49†	2.3 ± 0.15	8.9 ± 0.37†
NADPH–cytochrome P-450 reductase (nmoles/mg protein/min)	86 ± 12	ND‡	81 ± 9	ND
Cytochrome P-450 (nmoles/mg protein)	1.1 ± 0.1	0.60 ± 0.06†	1.81 ± 0.21	1.10 ± 0.04†
BP hydroxylase (nmoles/mg protein/min)	0.72 ± 0.08	0.35 ± 0.08	1.86 ± 0.15	1.10 ± 0.14
Microsomal heme (nmoles/mg protein)	1.63 ± 0.16	ND	2.80 ± 0.28	ND

* STZ was injected i.v., 65 mg/kg, in 0.05 M citrate buffer, pH 4.5. Values are expressed as the means ± S.E.M. for at least four animals. Rats were starved and killed 16 hr following $CoCl_2$ administration. Measurements were made 1 month after induction of diabetes.

† Significant difference from corresponding control values at $P < 0.05$.

‡ Not determined.

cytosol to mitochondria, or with the catalytic activity of this enzyme, either directly or by some other, undescribed mediator cannot be excluded.

ALA-S and MHO are interrelated with respect to their regulation by a pool of endogenous heme [31]. The free heme of this pool appears to be involved in two separate feedback mechanisms operating in opposite directions: negative feedback at the level of ALA-S and positive feedback at the level of MHO [32, 33] (Fig. 4). Therefore, inhibition of ALA-S activity in chronically diabetic rats might be expected to result from a high hepatic heme level, as was indeed observed in this study. The possible mechanism for this observation is considered below.

Heme serves as the prosthetic group for a critically important class of proteins, the hemoproteins. More than 80% of liver heme is utilized in the formation of holoenzymes involved in xenobiotic metabolism (cytochrome P-450, 66%), in the decompostion of hydrogen peroxides (catalase, 16%), and in the metabolism of tryptophan (TPO, 1.4%) [34]. In rats rendered diabetic for various periods of time, microsomal contents of heme and cytochrome P-450 and the activities of BP and aniline hydroxylases were increased. Our observations with 10-day diabetic rats are consistent with previous reports [2–4]; however, the 30- and 90-day data provide further insight into the long-term effects of STZ-diabetes on the activities of these enzymes. Increases in such hemoproteins are not without precedent in diabetes, as the closely related microsomal cytochrome b_5 content is likewise elevated [35]. In contrast, our data, together with a previous report [36], indicate that the microsomal content of NADPH–cytochrome *c* reductase remains unaltered.

Previous investigations have shown that the inhibition of heme synthesis produced by various pharmacological agents, including *p*-aminobenzoic acid and ethionine, usually resulted in marked decreases in the microsomal content of heme and cytochrome P-450 and the activities of its dependent enzymes [37, 38]. However, in diabetes, while the activities of the key enzymes involved in heme synthesis are reduced, the microsomal contents of heme and cytochrome P-450 are increased. While the exact mechanism of this novel finding is unclear, hepatocytes can take up heme from the circulation as a heme–hemopexin complex [39], and this heme can be equilibrated readily with the pool that provides the heme for formation of cytochromes [40]. Therefore, it is tempting to speculate that a compensatory mechanism may develop in response to the persistent inhibition of heme biosynthetic enzymes during chronic diabetes. This compensatory mechanism might cause a redistribution of whole body heme to satisfy crucial liver requirements through an increase in the influx of heme (Fig. 4). Alternatively, the increase in microsomal content of heme and cytochrome P-450 might be the result of inhibition of the major degradative pathway which apparently does not require the involvement of MHO and/or the refractoriness of MHO to induction by excess heme. In this regard, studies by Bissell and Guzelian [41] *in vivo* and with primary hepatocyte cultures prepared from animals that received [^{14}C]ALA demonstrate that heme associated with microsomal cytochromes

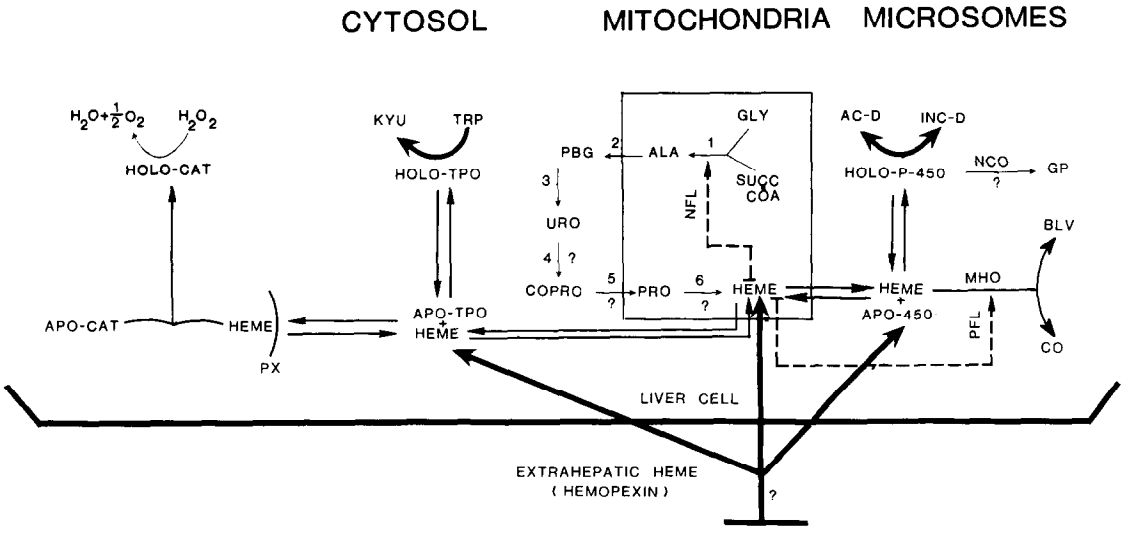

Fig. 4. Heme biosynthetic and metabolic pathways during chronic diabetes. The enzymes involved are identified by number: (1) δ-aminolevulinic acid synthase; (2) δ-aminolevulinic acid dehydratase; (3) uroporphyrinogen synthase and uroporphyrinogen III co-synthase; (4) uroporphyrinogen decarboxylase; (5) coproporphyrinogen oxidase and protoporphyrinogen oxidase; and (6) ferrochelatase. Abbreviations: ALA, δ-aminolevulinic acid; PBG, porphobilinogen; URO, uroporphyrinogen-III; COPRO, coproporphyrinogen-III; PRO, protoporphyrin-IX; Gly, glycine; succ-CoA, succinyl-CoA; MHO, microsomal heme oxygenase; P-450, cytochrome P-450; AC-D, active drug; INC-D, inactive drug; NCO, non-CO-forming process; GP, green pigment; BLV, bilivirdin; TPO, tryptophan pyrrolase; TRP, tryptophan; KYU, kynurenine; CAT, catalase; PX, peroxisome; PFL, positive feedback loop; and NFL, negative feedback loop.

is degraded substantially by non-CO-forming processes.

The study of the chronic effect of diabetes on heme metabolism was extended to TPO, a heme-containing enzyme, which is present in liver cytosol both as a holoenzyme and free inactive apoenzyme [42]. The latter requires the addition of heme for demonstration of its activity *in vitro*, while the former does not. This enzyme is considered to be the rate-limiting step in tryptophan metabolism [43], and the activity of this hemoprotein is regulated by four known mechanisms: a hormonal induction by glucocorticoids, substrate activation and stabilization by tryptophan, cofactor activation by heme, and feedback inhibition by NAD(P)H [44–48]. Thus, the ratio of holoenzyme/apoenzyme activity, which indicates the extent of this saturation, is increased by tryptophan but remains similar to that of the basal enzyme level (less than 1) after induction by cortisol. Furthermore, cofactor activation by heme resembles that of substrate activation with the exception of the lack of enzyme stabilization, which is part of the tryptophan induction mechanism. In chronic diabetes, the heme saturation ratio of TPO remained similar to that of the basal enzyme (less than 1). This is because the rise in holo activity was matched by a proportional increase in total enzyme in these animals. This type of enhancement in TPO activity in chronic diabetes is characteristic of a hormonal-type induction mechanism and is probably corticosterone mediated, because in these animals the activity of steroid Δ^4-hydrogenase, the rate-limiting step in corticosterone metabolism [49], was severely inhibited (unpublished observation).

It is of interest to note that, in spite of the increases in cytochromes P-450 and b_5, drug-metabolizing enzymes, and TPO, and an unaltered NADPH–cytochrome P-450 reductase, the activity of catalase was decreased 90 days following induction of diabetes. These findings, as well as those of ALA-S and MHO, indicate that generalizations of altered enzyme function in diabetes cannot be made and that the responses of various enzymes are markedly different in liver cells. This is further supported by reports that overall synthesis of hepatic proteins [50] and liver microsomal protein concentration per gram of liver is unchanged in diabetic rats [51, 52].

Pharmacological agents such as cobalt chloride may serve as a valuable tool for studying heme biosynthetic and metabolic pathways during chronic diabetes. This metal or its protoporphyrin is a potent and rapidly acting inducer of MHO [53, 54] in mammalian liver and, concomitant with this enzyme-inducing action, cobalt causes a profound depression of the microsomal content of heme and cytochrome P-450 [55, 56]. In addition, cobalt produces biphasic changes in ALA-S activity with initial depression followed by rebound increases in ALA-S formation [56]. Therefore, the action of this metal mimics in all respects the regulatory effect of heme itself on the enzymes of heme metabolism including the biphasic response of ALA-S.

We were interested in whether or not this metal exhibits a similar pattern of effect on heme-metabolizing enzymes in chronic diabetes. The results of these studies revealed that cobalt chloride produced marked increases in MHO activity with concomitant decreases in cytochrome P-450 content and BP hydroxylase activity during chronic diabetes (Table 3). It is of interest to note here that the late rebound increase in ALA-S which normally occurs following cobalt treatment was not seen in diabetic animals. A similar observation was reported by Bonkowsky *et al.* [21] of an impairment in the induction of ALA-S by AIA in alloxan diabetes. Although insulin lack could result in a generalized inhibition of protein synthesis, the induction of MHO by cobalt chloride, which normally requires *de novo* synthesis, rendered the above possibility unlikely.

This observed refractoriness of ALA-S to rebound increases following metal administration raises the possibility that perhaps other enzymes in the heme biosynthetic pathway might become the rate-limiting step for the synthesis of heme in diabetes. Since URO-S has been shown to be present at very low activity and, under some circumstances such as acute intermittent porphyria [57], may become rate-limiting for hepatic heme synthesis, the activity of this enzyme was measured in chronic diabetic animals following cobalt chloride treatment. The experimental data rendered this possibility unlikely in that the activity of URO-S in chronically diabetic rats behaved similarly to normals in exhibiting no significant change following the administration of cobalt chloride. A previous report by Maines and Kappas [58] suggested that the late rebound increase in ALA-S occurs when the concentration of this metal is reduced; thus, its inhibitory effect on ALA-S is removed. Based on this sequence of events, it is possible to speculate that the rate of removal of cobalt may be impaired during chronic diabetes leading to a sustained inhibitory effect on ALA-S activity. Hence, the time course of cobalt chloride or cobalt protoporphyrin action may be substantially different in chronic diabetic animals.

We proposed Fig. 4 in an attempt to summarize the data generated in the present study and that discussed above regarding aberrations in heme biosynthetic and metabolic pathways in chronic diabetes. Deviations from normality are indicated by heavy arrows, designating increased activity, and light arrows, denoting decreased activity, while arrows associated with question marks indicate theoretical possibilities that remain to be established.

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