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STUDIES ON INDUCTION OF δ-AMINOLEVULINIC ACID SYNTHASE, FERROCHELATASE, CYTOCHROME P-450 and CYCLIC AMP BY PHENFORMIN

CHLORPROPAMIDE, ALLYLISOPROPYLACETAMIDE AND LEAD IN HEPATOCYTES FROM NORMAL AND EXPERIMENTAL DIABETIC RATS

EDUARDO T. CÁNEPA, ELENA B. C. LLAMBÍAS and MOISÉS GRINSTEIN*

Laboratorio de Porfirinas, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, (1428) Buenos Aires, Argentina

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Abstract—The present work demonstrates that phenformin exerted an inducing effect on δ-aminolevulinic acid synthase (ALA-S) and ferrochelatase activities and on cytochrome P-450 content in isolated hepatocytes from rats with experimental diabetes. Similar results were obtained with respect to ALA-S activity and cytochrome P-450 content when chlorpropamide was used. The inducing effect exerted by allylisopropylacetamide (AIA) on ALA-S and ferrochelatase activities in diabetic hepatic cells was markedly greater than that observed in normal hepatocytes. This stimulatory response was not enhanced by adding dibutyryl cyclic AMP (cAMP). When phenformin was added to isolated rat hepatocytes of normal rats, induction of ALA-S and ferrochelatase activities and cytochrome P-450 content was observed only in the presence of added dibutyryl cAMP. Addition of chlorpropamide to this *in vitro* system did not exert an inducing effect on the same enzymes even in the presence of dibutyryl cAMP. The present results add more experimental evidence about the lability of the heme pathway of diabetic hepatocytes.

The introduction in human therapy of drugs like sulfonal, Sedormid, barbiturates and sex hormone steroids precipitates or aggravates hepatic porphyrias [1–3]. Results of later experimental studies carried out in normal animals such as rats or rabbits or in liver cell cultures [4, 5] suggest that some of the porphyrinogenic drugs may be able to produce in normal animals the syndrome and biochemical features of human hepatic porphyria diseases. The sporadic outbreak of hepatic porphyria in communities like the Turkish ones, consisting mainly of children and adolescent individuals who consumed hexachlorobenzene [6, 7], would reflect the presence of a lability in the regulatory mechanisms of the heme pathway of the hepatocytes of the affected individuals.

Recent observations in our laboratory have demonstrated that the heme pathway in hepatocytes of experimental diabetic rats exhibits a marked susceptibility to some drugs [8], very probably due to some lability of the heme pathway of the diabetic cells, providing a sensitive experimental model for exploring possible induction effects of some therapeutic agents on heme biosynthesis.

The present work deals with studies on the induction of activities of the regulatory enzymes δ-aminolevulinic acid synthase (ALA-S†) (succinyl CoA:glycine C-succinyl transferase, EC 2.3.1.37) and ferrochelatase (protoheme ferrolyase, EC 4.99.1.1) and the formation of cytochrome P-450 and cyclic AMP (cAMP) by allylisopropylacetamide (AIA), chlorpropamide, phenformin and lead in hepatocytes of normal and diabetic rats. Previous work by De Matteis [9] shows that phenformin and chlorpropamide do not induce porphyria in chick embryo liver cell culture. Recently, chlorpropamide was included in the list of drugs which precipitate acute attacks of hepatic porphyria [10].

The present work is part of the studies of our laboratory designed to explore regulatory mechanisms involved in the development of human hepatic porphyria.

MATERIALS AND METHODS

Chemicals

Collagenase type I; hyaluronidase type I-S; N^6, $O^{2'}$-dibutyryladenosine 3′,5′-(cyclic)-phosphate; adenosine 5′-triphosphate grade I; coenzyme A grade III-S; pyridoxal 5′-phosphate; glutathione, reduced form; ethylene glycol bis (β-aminoethylether)-*N*,*N*′-tetraacetic acid; streptozotocin (STZ); iodoacetamide; and cycloheximide were purchased from Sigma.

Allylisopropylacetamide was a gift of Roche S.A. *o*-Toluidine reagent was from Wiener Laboratory. All other chemicals were of analytical grade.

Experimental animals

Male chbb Thom albino rats (about 150–200 g

* Address all correspondence to: Dr. Moisés Grinstein, Ramón Freire 1575, 6° A, (1426) Buenos Aires, Argentina.

† Abbreviations: ALA-S, δ-aminolevulinic acid synthase; cAMP, cyclic AMP; AIA, allylisopropylacetamide; and STZ, streptozotocin.

Table 1. Heme biosynthetic enzyme activities and cytochrome P-450 and cAMP contents in hepatocytes from normal and streptozotocin diabetic rats

	ALA-S (nmol ALA/ mg protein/ 30 min)	Ferrochelatase (nmol heme/ mg protein/ 90 min)	Cytochrome P-450 (nmol/ 10^6 hepatocytes)	cAMP (pmol/ mg protein)
Normal	0.61 ± 0.05	1.59 ± 0.13	0.24 ± 0.02	6.3 ± 0.4
Diabetic	0.68 ± 0.04	1.67 ± 0.11	0.34 ± 0.02	24.7 ± 1.5

ALA-S and ferrochelatase activities and cytochrome P-450 and cAMP contents were determined at zero time of incubation. Values are means ± SE for at least six animals. ALA = δ-aminolevulinic acid.

body weight) were used and maintained under standardized conditions of light (from 6.30 a.m. to 6.30 p.m.) and temperature (21°) on a Purina Laboratory diet and water.

Treatment

Diabetes was induced by an injection of STZ (65 mg/kg, i.v.), freshly prepared in 0.05 M citrate buffer (pH 4.5). Control animals simultaneously received buffer alone by the same route. Animals were killed 15 days after injection. Rats having serum glucose levels higher than 250 mg/100 mL were considered diabetic. The rats were killed between 7.00 a.m. and 8.00 a.m. following 24 hr of fasting.

Preparation and suspension of rat liver hepatocytes

Rats were killed by decapitation, and livers were removed and washed with ice-cold 0.9% (w/v) saline; they were trimmed and minced into small pieces. Cell suspensions were prepared by the procedure of Fry *et al.* [11]; 85% of the cells were initially viable as judged by staining with Trypan blue. The isolated liver cells were suspended to about (5–10) × 10^6 cells/mL of Ham F12 medium (pH 7.4) devoid of glucose [12]. The metabolic activity of this preparation was evaluated as described before [13].

Enzyme and cytochrome P-450 induction experiments

Phenobarbital, dibutyryl cAMP, phenformin (1-phenethylbiguanide) or lead (II) acetate dissolved in a small volume of Ham F12 medium was added to cell suspensions at zero time. Chlorpropamide [1-(4-chlorbenzolsulfonyl)-3-propylurea], dissolved in a small volume of 0.5 M Na_2HPO_4, was added to cell suspensions at zero time. Suspensions were gently shaken at 37° in a Dubnoff water bath at 60 oscillations/min. Viability was determined for the different treatments and at different times. Usually, cell viability fell by 1–2% per hour judged by the Trypan blue staining. Samples were removed, and suspensions for determining enzymatic activities were prepared as described by Cánepa *et al.* [13]. For measurement of cytochrome P-450 content, hepatocyte suspensions were diluted with phosphate-buffered saline to a concentration of 0.5 × 10^6 cells/mL.

Enzyme activity assays

ALA-S and ferrochelatase activities were determined as described before [13].

Determination of cytochrome P-450

Cytochrome P-450 was determined in whole hepatocytes according to Estabrook and Werringloer [14] from the CO difference spectrum of dithionite reduced samples [15] using $\Delta\varepsilon^{mM}_{450-490} = 91$.

cAMP extraction

The extraction of intracellular content of cAMP was carried out in duplicate samples (5 × 10^6 cells/mL) as described before [8].

cAMP assay

Aliquots of each sample were assayed for cAMP content by the method of Tovey *et al.* [16].

Determination of glucose

Serum glucose was determined by the method of Dubowski [17]. For the determination of glucose content in hepatocytes, cell homogenates were treated with 10% (w/v) trichloroacetic acid and centrifuged at 3000 *g* for 15 min. Glucose was quantified in the resulting supernatant fraction by the method of Dubowski [17].

Determination of proteins

Proteins were measured by the method of Lowry *et al.* [18], using bovine serum albumin as standard.

Statistical analysis

Results are expressed as means ± SE. When studies were made in normal or diabetic rat hepatocytes, Student's paired *t*-test was used. Student's unpaired *t*-test was used when comparisons were made between values obtained in diabetic rat hepatocytes and those obtained in normal ones. The differences were considered statistically significant when the P value was <0.05.

RESULTS

The effects of the STZ-diabetic state on hepatic enzymes involved in heme biosynthesis were determined. Fifteen days after the STZ injection, the activities of ALA-S and ferrochelatase were similar for normal and diabetic rat hepatocytes, as shown in Table 1. On the other hand, changes in the content of cytochrome P-450 were observed in hepatocytes from diabetic rats. The level of cytochrome P-450 in diabetic rats was 40% higher than in normal rats. As can be seen in the same table, the cAMP content

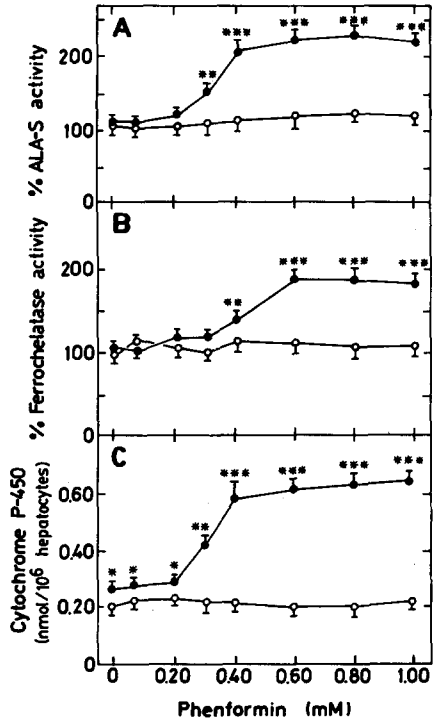

Fig. 1. Effect of phenformin on ALA-S and ferrochelatase activities and on cytochrome P-450 content in normal and diabetic rats. Hepatocytes were incubated with phenformin. Four hours after addition, ALA-S (A) and ferrochelatase (B) activities were determined in the homogenate. Two hours after the addition, cytochrome P-450 content (C) was determined in the whole hepatocyte. ALA-S and ferrochelatase activities are expressed as percentages of untreated controls at zero time. Absolute values for this experiment are: 0.57 ± 0.06 and 0.64 ± 0.05 nmol ALA/mg protein per 30 min for ALA-S activity in normal and diabetic cells respectively; and 1.70 ± 0.15 and 1.76 ± 0.11 nmol heme/mg protein per 90 min for ferrochelatase activity in normal and diabetic cells respectively. Values are means ± SE of four different experiments. Key: (○) hepatocytes from normal rats; and (●) hepatocytes from diabetic rats. Significance * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

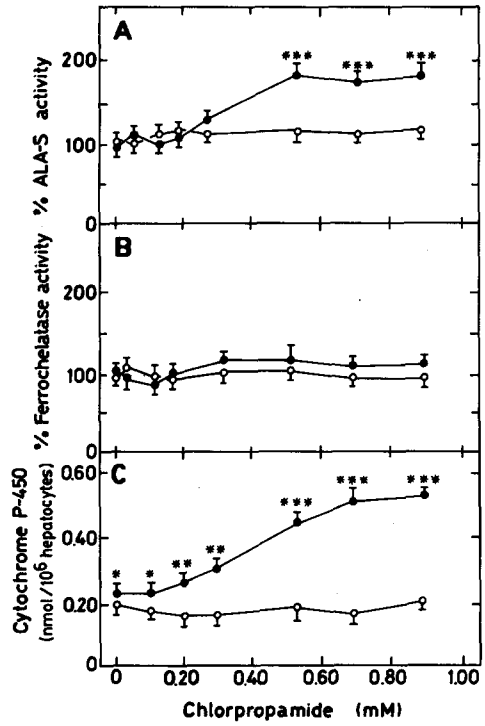

Fig. 2. Effect of chlorpropamide on ALA-S and ferrochelatase activities and on cytochrome P-450 content in normal and diabetic rats. Hepatocytes were incubated with chlorpropamide. Four hours after addition, ALA-S (A) and ferrochelatase (B) activities were determined in the homogenate. Two hours after the addition, cytochrome P-450 content (C) was determined in the whole hepatocyte. ALA-S and ferrochelatase activities are expressed as percentages of untreated controls at zero time. Absolute values for this experiment are: 0.58 ± 0.07 and 0.69 ± 0.03 nmol ALA/mg protein per 30 min for ALA-S activity in normal and diabetic cells respectively; and 1.57 ± 0.13 and 1.62 ± 0.15 nmol heme/mg protein per 90 min for ferrochelatase activity in normal and diabetic cells respectively. Values are means ± SE of four experiments. Key: (○) hepatocytes from normal rats, and (●) hepatocytes from diabetic rats. Significance: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

was increased about four times in hepatocytes from diabetic animals.

Time–course experiments on induction effects of phenformin and chlorpropamide on ALA-S and ferrochelatase activities and cytochrome P-450 content in hepatocytes of diabetic rats show that the maxima were attained at 4 hr of incubation for ALA-S and ferrochelatase activities and at 2 hr of incubation for cytochrome P-450 content (data not shown). The results of studies on the induction effects exerted by AIA and lead in ALA-S activity in normal hepatocytes have been reported previously [13–19].

Effect of phenformin on the activities of ALA-S and ferrochelatase and on the content of cytochrome P-450 in normal and diabetic hepatocytes

As seen in Fig. 1 (A, B, and C), addition of phenformin to diabetic hepatocytes produced significant induction, at concentrations equal to or over 0.25 mM for ALA-S, 0.40 mM for ferrochelatase and 0.25 mM for cytochrome P-450. Simultaneous addition of 10^{-5} M cycloheximide totally repressed the inducing effect produced in the diabetic cells (data not shown). Differences between diabetic and normal hepatocytes were significant at the same concentrations described for phenformin induction in diabetic cells. No inducing effect was observed using normal hepatocytes.

Effect of chlorpropamide on the activities of ALA-S and ferrochelatase and on the content of cytochrome P-450 in normal and diabetic hepatocytes

As shown in Fig. 2 (A and C), the addition of chlorpropamide to the incubation system of diabetic hepatocytes produced, at concentrations equal to or over 0.27 mM, significant inducing effects on ALA-S and cytochrome P-450 content. Simultaneous

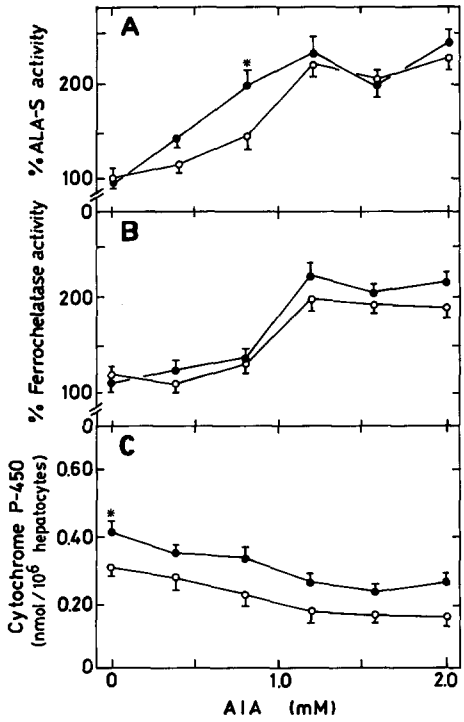

Fig. 3. Effect of AIA on ALA-S and ferrochelatase activities and on cytochrome P-450 content in normal and diabetic rats. Hepatocytes were incubated with AIA. Four hours after addition, ALA-S (A) and ferrochelatase (B) activities were determined in the homogenates. Two hours after the addition, cytochrome P-450 content (C) was determined in the whole hepatocyte. ALA-S and ferrochelatase activities are expressed as percentages of untreated controls at zero time. Absolute values for this experiment are: 0.65 ± 0.07 and 0.72 ± 0.05 nmol ALA/mg protein per 30 min for ALA-S activity in normal and diabetic cells respectively; and 1.65 ± 0.12 and 1.68 ± 0.15 nmol heme/mg protein per 90 min for ferrochelatase activity in normal and diabetic cells respectively. Values are means ± SE of four different experiments. Key: (○) hepatocytes from normal rats, and (●) hepatocytes from diabetic rats. Significance: * $P < 0.05$.

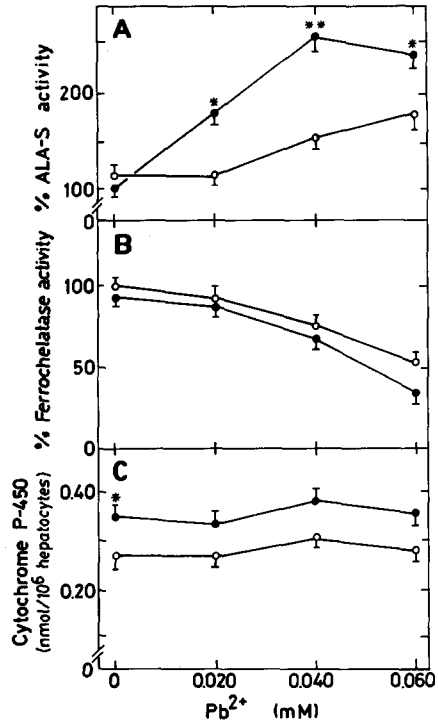

Fig. 4. Effect of lead on ALA-S and ferrochelatase activities and on cytochrome P-450 content in normal and diabetic rats. Hepatocytes were incubated with lead acetate. Four hours after addition, ALA-S (A) and ferrochelatase (B) activities were determined in the homogenate. Two hours after the addition, cytochrome P-450 content (C) was determined in the whole hepatocyte. ALA-S and ferrochelatase activities are expressed as percentages of untreated controls at zero time. Absolute values for this experiment are: 0.61 ± 0.04 and 0.65 ± 0.08 nmol ALA/mg protein per 30 min for ALA-S activity in normal and diabetic cells respectively; 1.50 ± 0.12 and 1.70 ± 0.14 nmol heme/mg protein per 90 min for ferrochelatase activity in normal and diabetic cells respectively. Values are means ± SE of four different experiments. Key: (○) hepatocytes from normal rats, and (●) hepatocytes from diabetic rats. Significance: * $P < 0.05$, and ** $P < 0.01$.

addition of 10^{-5} M cycloheximide totally repressed this inducing effect (data not shown). No inducing effect was observed using normal hepatocytes under the same experimental conditions. Comparing diabetic to normal hepatocytes, significant results were obtained after 0.54 mM chlorpropamide. As seen in Fig. 2B, chlorpropamide, at the concentrations tested, did not induce ferrochelatase activity in normal or diabetic cells.

Effect of AIA on the activities of ALA-S and ferrochelatase and on the content of cytochrome P-450 in normal and diabetic hepatocytes

As shown in Fig. 3 (A and B), addition of AIA produced a significant increase of ALA-S activity at concentrations equal to or over 0.8 mM in normal and from 0.4 mM in diabetic cells and of ferrochelatase activity at concentrations of 1.2 mM in normal and 0.8 mM in diabetic cells. Simultaneous addition of 10^{-5} M cycloheximide totally repressed both inducing effects in normal and diabetic rat hepatocytes (data not shown). On the other hand, under the same experimental conditions, addition of AIA at concentrations of at least 1.2 mM decreased the cytochrome P-450 content in both normal and diabetic cells (Fig. 3C).

Effect of lead acetate on the activities of ALA-S and ferrochelatase and on cytochrome P-450 content in normal and diabetic hepatocytes

As shown in Fig. 4A, Pb^{2+} produced a significant increase of ALA-S activity in normal and diabetic hepatocytes, at concentrations of 0.04 and 0.02 mM respectively. Comparing diabetic versus normal rat hepatocytes, significant differences in ALA-S activities were obtained from 0.02 mM Pb^{2+}. On the other hand, working under the same experimental conditions, the addition of Pb^{2+} to normal or diabetic hepatocytes produced a decrease of the ferrochelatase activity, in concentrations from 0.04 mM

(Fig. 4B). As seen in Fig. 4C, addition of Pb^{2+} did not alter the concentrations of cytochrome P-450 in either normal or diabetic cells.

Effect of added dibutyryl cAMP on the induction of ALA-S and ferrochelatase activities and cytochrome P-450 content by phenformin, chlorpropamide, AIA and lead acetate in normal hepatocytes

As shown in Fig. 5A, addition of dibutyryl cAMP plus phenformin to normal hepatocytes produced significant increases of ALA-S and ferrochelatase activities and cytochrome P-450 content. It can also be seen that chlorpropamide plus added nucleotide, under the same experimental conditions, did not produce inducing effects on the same enzymes, but a slight effect was observed on cytochrome P-450 content. On the other hand, using diabetic hepatocytes, we were not able to observe a potentiation effect of added dibutyryl cAMP on the ALA-S and ferrochelatase activities and cytochrome P-450 content induced by phenformin or chlorpropamide (data not shown). Results previously obtained in normal and diabetic hepatocytes on the effect exerted by added dibutyryl cAMP on ALA-S and ferrochelatase activities and cytochrome P-450 content have been reported [8, 19]. In Fig. 5B, we present results of studies on the effect of added dibutyryl cAMP to normal hepatocytes incubated with AIA or Pb^{2+}. As seen, added nucleotide potentiated the induction effect exerted by AIA on ALA-S and ferrochelatase activities. No change was observed on the cytochrome P-450 content. In the presence of Pb^{2+}, added nucleotide potentiated its inducing effect on ALA-S activity. No effect was observed on ferrochelatase activity. The effect on cytochrome P-450 content was due only to the dibutyryl cAMP added, as already described [19].

Effects of phenformin, chlorpropamide, phenobarbital, AIA and lead acetate on the endogenous cAMP content of normal and diabetic hepatocytes

As shown in Table 2, addition of phenformin or chlorpropamide to normal or diabetic hepatocytes did not alter the level of endogenous cAMP in either normal or diabetic hepatocytes. Phenformin or chlorpropamide, when added together with phenobarbital, produced an increase of the cAMP content which was similar to the level attained in the presence of phenobarbital alone. As can also be seen, AIA added to normal or diabetic hepatocytes induced an increase of the endogenous cAMP. When phenformin or chlorpropamide were added together with AIA to normal or diabetic cells, the observed increase of the endogenous cAMP was comparable to the level detected using AIA alone. As shown in the same table Pb^{2+} alone or mixed with phenformin or chlorpropamide had no effect on cAMP content in either normal or diabetic cells.

Effects of phenobarbital, AIA and lead acetate on the induction produced by phenformin and chlorpropamide on ALA-S and ferrochelatase activities and cytochrome P-450 content in normal and diabetic hepatocytes

In Table 2, we present data from experimental studies designed to explore possible effects of the porphyrinogenic drugs phenobarbital, AIA and Pb^{2+} on the actions produced by phenformin and chlorpropamide on the activities of ALA-S and ferrochelatase and on cytochrome P-450 content in normal and diabetic cells. It can be seen that in normal cells, the addition of phenobarbital plus phenformin increased ALA-S and ferrochelatase activities and

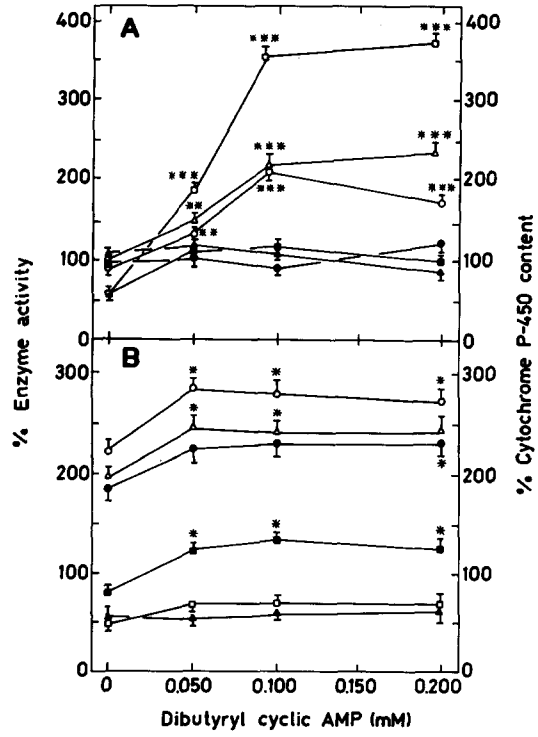

Fig. 5. Effect of dibutyryl cAMP plus phenformin, chlorpropamide, AIA and lead in normal rats. Hepatocytes from normal rats were incubated with the additions indicated below and different concentrations of dibutyryl cAMP. Four hours after the additions, ALA-S and ferrochelatase activities were determined in the homogenates. Cytochrome P-450 content was determined in the whole hepatocytes after a 2-hr incubation. Results are expressed as percentages of untreated controls at zero time without any addition. ALA-S and ferrochelatase activities and cytochrome P-450 content are expressed as percentages of untreated controls at zero time. Values are means ± SE of four different experiments. (A) Absolute values for this experiment are: 0.64 ± 0.06 nmol ALA/mg protein per 30 min for ALA-S activity; 1.52 ± 0.13 nmol heme/mg protein per 90 min for ferrochelatase activity; 0.24 ± 0.02 nmol/10^6 hepatocytes for cytochrome P-450 content. Key: (○) ALA-S, 0.80 mM phenformin; (●) ALA-S, 0.54 mM chlorpropamide; (△) ferrochelatase, 0.80 mM phenformin; (▲) ferrochelatase, 0.54 mM chlorpropamide; (□) cytochrome P-450, 0.80 mM phenformin, and (■) cytochrome P-450, 0.54 mM chlorpropamide. (B) Absolute values for this experiment are: 0.61 ± 0.06 nmol ALA/mg protein per 30 min for ALA-S activity; 1.60 ± 0.10 nmol heme/mg protein per 90 min for ferrochelatase activity; and 0.23 ± 0.02 nmol/10^6 hepatocytes for cytochrome P-450. Key: (○) ALA-S, 1.2 mM AIA; (●) ALA-S, 0.06 mM Pb^{2+}; (△) ferrochelatase, 1.2 mM AIA; (▲) ferrochelatase, 0.06 mM Pb^{2+}; (□) cytochrome P-450, 1.2 mM AIA; (■) cytochrome P-450, 0.06 mM Pb^{2+}. Significance: * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$).

Table 2. Effects of porphyrinogenic drugs and phenformin or chlorpropamide on ALA-S and ferrochelatase activities and on cytochrome P-450 and cAMP contents in normal and diabetic rat hepatocytes

Additions	Contents		Activities	
	Cytochrome P-450 (% of control)	cAMP (pmol/mg protein)	ALA-S (% of control)	Ferrochelatase (% of control)
Normal hepatocytes				
None	65 ± 4	6.5 ± 0.4	102 ± 9	96 ± 4
Phenformin	66 ± 5	6.6 ± 0.6	127 ± 10	107 ± 6
Chlorpropamide	68 ± 4	6.7 ± 0.5	118 ± 4	104 ± 5
Phenobarbital	121 ± 8	9.3 ± 0.3	195 ± 6	203 ± 14
AIA	50 ± 4	8.2 ± 0.2	199 ± 12	194 ± 10
Pb^{2+}	69 ± 7	6.4 ± 0.8	165 ± 8	68 ± 3
Phenformin + phenobarbital	127 ± 7*	9.4 ± 0.6*	205 ± 17*	215 ± 13*
Chlorpropamide + phenobarbital	125 ± 7*	9.4 ± 0.4*	201 ± 9*	210 ± 14*
Phenformin + AIA	53 ± 4	8.3 ± 0.5	196 ± 11*	192 ± 11*
Chlorpropamide + AIA	55 ± 3	8.0 ± 0.7	190 ± 7*	190 ± 9*
Phenformin + Pb^{2+}	70 ± 6	5.8 ± 0.7	169 ± 10	73 ± 4*
Chlorpropamide + Pb^{2+}	72 ± 4	6.1 ± 0.3	162 ± 9*	70 ± 3*
Diabetic hepatocytes				
None	85 ± 6	24.5 ± 1.9	105 ± 8	102 ± 8
Phenformin	168 ± 7	26.5 ± 1.1	228 ± 7	185 ± 7
Chlorpropamide	141 ± 7	23.2 ± 0.5	181 ± 9	118 ± 8
Phenobarbital	282 ± 12	41.3 ± 1.6	243 ± 18	255 ± 9
AIA	61 ± 4	35.2 ± 1.6	236 ± 15	207 ± 12
Pb^{2+}	89 ± 6	24.2 ± 2.8	180 ± 7*	63 ± 4
Phenformin + phenobarbital	324 ± 15*	42.5 ± 3.3*	339 ± 20*	306 ± 13*
Chlorpropamide + phenobarbital	378 ± 18*	41.4 ± 1.3*	353 ± 17*	251 ± 11*
Phenformin + AIA	134 ± 7*	31.6 ± 1.2	229 ± 9	227 ± 10*
Chlorpropamide + AIA	78 ± 4*	33.4 ± 2.3*	263 ± 13*	214 ± 13*
Phenformin + Pb^{2+}	147 ± 8	26.5 ± 3.0	185 ± 11*	67 ± 7*
Chlorpropamide + Pb^{2+}	122 ± 7	23.3 ± 1.0	170 ± 10	72 ± 6*

Normal and diabetic hepatocytes were incubated with 0.80 mM phenformin or 0.54 mM chlorpropamide and/or 0.6 mM phenobarbital and/or 1.2 mM AIA and/or 0.06 mM Pb^{2+}. Four hours after the addition, ALA-S and ferrochelatase activities were determined in the homogenates. Two hours after the addition, cytochrome P-450 and cAMP contents were determined in the whole hepatocytes. ALA = δ-aminolevulinic acid. ALA-S and ferrochelatase activities and cytochrome P-450 content are expressed as percentages of untreated controls at zero time. Absolute values for this experiment are: 0.54 ± 0.04 and 0.62 ± 0.05 nmol ALA/mg protein/30 min for ALA-S activity in normal and diabetic cells respectively; 1.70 ± 0.11 and 1.63 ± 0.10 nmol heme/mg protein/90 min for ferrochelatase activity in normal and diabetic cells respectively; 0.25 ± 0.02 and 0.37 ± 0.03 nmol/10^6 hepatocytes for cytochrome P-450 content in normal and diabetic cells respectively. Results are means ± SE of four experiments.

* Significant differences with respect to phenformin or chlorpropamide added alone ($P < 0.05$).

cytochrome P-450 content in amounts comparable to those produced by phenobarbital alone.

In diabetic cells phenobarbital potentiated the inducing effect produced by phenformin on ALA-S and ferrochelatase activities and cytochrome P-450 content. In normal cells, phenobarbital plus chlorpropamide produced the same effect as phenobarbital plus phenformin. In diabetic rat hepatocytes, phenobarbital potentiated the induction effect of chlorpropamide on ALA-S and cytochrome P-450. The presence of chlorpropamide did not affect the activity of ferrochelatase induced by the barbiturate. In normal rat hepatocytes, the presence of phenformin did not affect enzyme activities and cytochrome P-450 content already altered by AIA. In diabetic rat hepatocytes, additions of AIA plus phenformin or chlorpropamide produced a change of ALA-S and ferrochelatase activities similar to those observed with AIA alone. As also seen in Table 2, AIA decreased the cytochrome P-450 induced by phenformin or chlorpropamide.

Addition of AIA plus chlorpropamide to normal cells produced similar effects to those observed with the mixture AIA plus phenformin.

Addition of Pb^{2+} plus phenformin to normal hepatocytes produced effects similar to those observed when adding Pb^{2+} alone. Similar results were obtained in presence of Pb^{2+} plus chlorpropamide. On the other hand, when Pb^{2+} was added to diabetic cells together with phenformin, we observed a decrease of the inducing effect exerted by phenformin alone on ALA-S and ferrochelatase activities; cytochrome P-450 content induced by phenformin was not affected by Pb^{2+}. Addition of Pb^{2+} to diabetic cells did not affect the induction produced by chlorpropamide on ALA-S activity and cytochrome P-450 content. Chlorpropamide did not affect the inhibition exerted by Pb^{2+} on ferrochelatase activity in diabetic rat hepatocytes.

DISCUSSION

The present work demonstrates an increase of cytochrome P-450 and cAMP contents in hepatocytes

of STZ-diabetic rats compared with those of normal rats. These results agree with those obtained by other authors who used alloxan and STZ diabetogenic agents [20–22]. The increase of cytochrome P-450 content in hepatocytes of diabetic rats very probably reflects an increase of monooxygenase activity in the hepatocytes as has been postulated by Bitar and Weiner [23]. They observed a decrease in hepatic ALA-S after 44 days following diabetic induction in female rats. As these experiments performed by Bitar and Weiner were carried out *in vivo*, differences between our results and theirs may be due to different experimental conditions, the sex of the animals used, the number of days since the administration of the diabetogenic drug, and the effect of extrahepatic factors.

As shown in Fig. 1, phenformin exerted an inducing effect on the biosynthesis of cytochrome P-450, ALA-S, and ferrochelatase in isolated hepatocytes from experimental diabetic rat hepatocytes.

Chlorpropamide exerted a stimulatory effect only on the biosynthesis of cytochrome P-450 and ALA-S (Fig. 2, panels C and A). This would be consistent with publications which include chlorpropamide in the list of drugs that precipitate acute attacks in hepatic porphyrias [10].

The present experimental data do not allow us to explain the lack of increase of ferrochelatase activity in the presence of chlorpropamide (Fig. 2B), but they may indicate that different mechanisms are involved in the action of both drugs. We did not observe an induction effect in normal hepatocytes with every drug alone on both enzyme activities and hemoprotein content. These results are consistent with previous work of De Matteis [9] who has shown that chlorpropamide and phenformin do not induce porphyria in chick embryo liver cell cultures.

However, we demonstrated that in normal hepatocyte suspension, ALA-S and ferrochelatase activities and cytochrome P-450 content can be induced by phenformin plus dibutyryl cyclic AMP (Fig. 5A). These observations support previous results that suggest that cyclic AMP would be an important factor involved in heme biosynthesis regulation [13, 24]. In the case of chlorpropamide (Fig. 5A), the presence of cyclic nucleotide at the concentrations tested did not produce changes in the basal activities of either enzymes or in cytochrome P-450 content. These results may reflect different mechanisms of action of both drugs in general metabolism. The addition of dibutyryl cAMP did not enhance any of the values obtained with diabetic cells. This absence of potentiation is comparable to that observed in our laboratory [8] using the same metabolic system and phenobarbital as inducer and could be explained as due to the high levels of endogenous cyclic nucleotide in diabetic cells. The maximum increase which would possibly be attained in our experimental conditions is already achieved by the effect of the endogenous cAMP.

An inducing effect of lead on ALA-S activity in rat liver has been shown previously by Strand *et al.* [25] and by Maxwell and Meyer [26].

The inducing effects exerted by AIA and Pb^{2+} on ALA-S in diabetic hepatocytes were higher than those observed in normal cells (Figs. 3A and 4A). These results are consistent with the greater susceptibility exhibited by diabetic hepatocytes to porphyrinogenic agents [8], very probably due to the effects of some products of abnormal metabolisms associated to some diseases (diabetes mellitus, idiopathic auto-immune syndromes) which cause a lability in the regulation mechanisms of the heme pathway of these hepatocytes [27, 28].

Increasing activities observed in the present work were totally blocked by the addition of cycloheximide, in concentrations large enough to prevent synthesis by more than 95%. These results prove that both drugs increase the rate of synthesis of the enzymes rather than diminish their degradation. On the other hand, the induced formation of cytochrome P-450 by phenformin and chlorpropamide was not repressed completely by cycloheximide, and this could reflect a formation of approximately 50% of cytochrome P-450 from preexisting apocytochrome P-450.

Some of the results of the present work are consistent with observations in previous studies which show the frequent presence of some interrelationship between the level of endogenous or added cAMP and induction or potentiation effects on heme enzyme biosynthesis, for instance: (a) the effect of added dibutyryl cAMP on the induction of ALA-S and ferrochelatase activities and cytochrome P-450 content in normal hepatocytes incubated with phenformin (Fig. 5A), (b) the potentiation effect of added dibutyryl cAMP on the induction produced by AIA in normal hepatocytes on ALA-S and ferrochelatase activities (Fig. 5B), and (c) the increase of the endogenous cAMP level in normal hepatocytes incubated with phenobarbital or AIA and the lack of change of the level of this nucleotide when these cells were incubated with phenformin or chlorpropamide. As shown in Table 2, phenobarbital or AIA alone induced ALA-S and ferrochelatase activities, whereas phenformin or chlorpropamide alone had no significant effect on these enzymes. Phenobarbital alone increased cytochrome P-450 content, whereas AIA did not change the content of this hemoprotein.

These observations provide additional support for the concept that cAMP is involved in the regulatory mechanism of heme biosynthesis.

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