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ABSENCE OF CYTOSOL EFFECTS ON THE RATES OF MICROSOMAL DRUG METABOLISM IN ALLOXAN AND STREPTOZOTOCIN DIABETIC RATS*

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Abstract—The hypothesis that diabetes or cyclic AMP induces a cytosol (supernatant)-located inhibitor of hepatic microsomal drug metabolism was examined with diabetic male rats. The existence of such an inhibitor has been postulated because inhibition of microsomal drug metabolism occurred after preincubation of normal microsomes with cyclic AMP-treated cytosol and after direct addition of diabetic cytosol to normal microsome-catalyzed reaction mixtures. In the present investigation, the rate of microsomal metabolism of aminopyrine was decreased while that of aniline was increased as a result of both alloxan and streptozotocin diabetes. However, the rates of metabolism of these substrates were not affected by preincubation of normal microsomes with cytosol from either alloxan or streptozotocin diabetic rats. Likewise, the metabolism of these substrates was not affected by the addition of either normal or diabetic cytosol directly to the microsomal catalyzed reaction mixtures. The present results, therefore, do not support the hypothesis of a diabetes-related cytosol-located inhibitor of drug metabolism. Rather, this investigation supports the hypothesis that diabetes-related changes in drug metabolism are due to alterations in the microsomal enzyme system itself.

It has been postulated that the inhibition of hepatic drug metabolism observed after injection of male rats with large doses of dibutyryl cyclic AMP [1–3] or glucagon plus theophylline [3] or after induction of diabetes [4–9] is mediated by a cytosol-located inhibitor [8, 10]. In the present study, this postulated mechanism, a cytosolic-component effect on the rate of microsomal drug metabolism in diabetic male rats, was investigated using two separate methods to expose microsomes to cytosol (supernatant fraction): preincubation of microsomes with various cytosols [3] and inclusion of various cytosols in the microsomal drug metabolism reaction mixtures [5, 8]. The results do not support the hypothesis that the altered rates of drug metabolism by diabetic rat livers are mediated by a cytosol-located inhibitor. Rather, the results support the concept that the altered rates of metabolism that occur under such conditions are related to changes in the microsomal cytochrome containing protein *per se*.

MATERIALS AND METHODS

Chemicals. Alloxan and aniline hydrochloride were obtained from the Eastman Kodak Co., Rochester, NY; streptozotocin from The Upjohn Co., Kalamazoo, MI; aminopyrine from the Aldrich Chemical Co., Inc., Milwaukee, WI; and glucose-6-phosphate and glucose-6-phosphate dehydrogenase from the Sigma Chemical Co., St. Louis, MO.

Animals. Male Sprague–Dawley rats (120–140 g), obtained from SASCO, Inc., Omaha, NE, were maintained on standard laboratory chow and water *ad lib.* Diabetes was routinely induced with alloxan (50 mg/kg in 0.9% saline). For comparison, some experiments were conducted in which streptozotocin (80 mg/kg in 0.1 M citrate buffer, pH 5.1) was used to induce diabetes. Both diabetogenic compounds were dissolved immediately prior to intravenous injection. Rats were considered diabetic only if their blood glucose concentration exceeded 300 mg/100 ml as measured by a commercially available glucose oxidase method (GOD-Perid, Boehringer Mannheim, Indianapolis, IN). Animals were diabetic for at least 7 days before use.

Microsomal and cytosolic fraction preparation. Animals were decapitated and their livers were immediately removed and routinely placed in 3.5 vol. of ice-cold 0.01 M Na^+/K^+ phosphate buffer containing 0.15 M KCl, pH 7.4 [11]. All subsequent steps in the preparation of microsomes were done at 4°. A glass–teflon Potter–Elvehjem homogenizer was used to homogenize the livers, and the homogenates were centrifuged at 10,000 *g* for 20 min [11]. After careful removal of lipid, the resulting supernatant fractions were removed and recentrifuged at 105,000 *g* for 1 hr. Again, after careful removal of any lipid material, the supernatant (cytosol) fractions were removed and placed on ice for use in the cytosol–microsome mixing experiments. The microsomal pellets obtained were rehomogenized in buffer and recentrifuged prior to use.

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Exposure protocols. Microsomes were exposed to cytosol by two methods. In Method A [3], the recentrifuged microsomes were resuspended in a volume of either buffer, normal cytosol, or diabetic cytosol equivalent to the volume of the cytosolic or supernatant fraction associated with the original microsomal pellet and were preincubated on ice for 15 min. After preincubation, the microsomes were separated from the preincubation medium by centrifugation and then suspended in the appropriate buffer for drug metabolism assays. In Method B [5, 8], the recentrifuged microsomes were resuspended as in Method A in either buffer, normal cytosol, or diabetic cytosol, but were used directly in the drug metabolism assay. In both methods the protein concentration of the resuspended microsomes was 10–14 mg/ml. Microsomal protein concentrations were determined by either the method of Lowry *et al.* [12] or the biuret method [13].

Enzyme assays. The reactions were carried out in 25 ml Erlenmeyer flasks at 37° under air in a Dubnoff metabolic shaker at 120 oscillations/min. Reagent and tissue blanks were prepared and carried through the incubation and assay procedures. The system used for aminopyrine incubation was that described by Mazel [11], modified to contain 2 μmoles $NADP^+$, 22.5 μmoles semicarbizide, 12.5 μmoles $MgCl_2$, 5 μmoles glucose-6-phosphate, 2 I.U. glucose-6-phosphate dehydrogenase, 0.25 ml microsomal stock suspension and 2.5 μmoles aminopyrine in sufficient 0.5 M Na^+/K^+ phosphate buffer, pH 7.4, to give a final volume of 3.0 ml. After 20 min, 2 ml of 20% trichloroacetic acid was added to stop the reaction. The extent of *N*-demethylation of aminopyrine was estimated by measuring the amount of formaldehyde formed by the Nash method [14]. The incubation system described by Mazel [11] for determining aniline metabolism was modified to contain 2 μmoles $NADP^+$, 12.5 μmoles $MgCl_2$, 5 μmoles glucose-6-phosphate, 2 I.U. glucose-6-phosphate dehydrogenase, 2.5 μmoles aniline HCl, 0.25 microsomal stock suspension and sufficient 0.1 M Na^+/K^+ phosphate buffer, pH 7.4, to give a final volume of 2.0 ml. The reactions were stopped with 1 ml of 20% trichloroacetic acid after 20 min of incubation. The formation of *p*-aminophenol was used to determine the extent of aniline hydroxylation [15]. The rates of metabolism with both normal and diabetic microsomes in the incubation systems used for both aminopyrine and aniline were linear with time and were a function of the microsomal (enzyme) concentration. Addition of 50 μM EDTA to these reaction mixtures to minimize possible lipid peroxidation and subsequent destruction of cytochrome P-450 [16, 17] did not alter the effects of diabetes on the rates of metabolism of either substrate and, therefore, was not routinely used.

Tris buffer experiments. When the effects of Tris buffer were examined, the livers were homogenized in 3.0 vol. of ice-cold 0.04 M Tris–0.15 M KCl, pH 7.4 [3]. All subsequent preparation, preincubation, reaction, and assay steps were identical to those described above except that Tris buffer was used [3].

Statistics. The significance of the difference between the means was established by Student's *t*-test.

RESULTS

Alloxan diabetes and preincubation experiments. The inhibition of aminopyrine metabolism by alloxan diabetic male rats has been widely observed [5–7, 9]. It is also well documented that aniline hydroxylation in male rats is increased by diabetes [5–7, 9]. If these changes in the rates of drug metabolism by diabetic liver microsomes are mediated by a factor or factors in the cytosol, then normal microsomes exposed to diabetic cytosol should be affected so as to cause the rates of drug metabolism catalyzed by such exposed microsomes to be comparable to the rates obtained with diabetic microsomes. One method of exposing microsomes to various cytosols is to incubate microsomes with the cytosol in question prior to using the microsomes in a typical *in vitro* drug metabolism system. This preincubation technique has been extensively used by Weiner *et al.* [3] and is referred to as Method A in Materials and Methods. With this method, the rate of aminopyrine metabolism by diabetic microsomes preincubated with buffer was, as expected, only 77 per cent of the control rate exhibited by normal microsomes preincubated with buffer (Table 1). Preincubation of normal microsomes with diabetic cytosol, however, failed to change the rate of microsomal aminopyrine demethylation compared to the control rate or the rate obtained after preincubation with normal cytosol (Table 1). Therefore, although diabetes

Table 1. Effect of preincubation of microsomes with normal or alloxan diabetic cytosol (Method A) on the rates of drug metabolism by normal and alloxan diabetic microsomes*

Microsomes	Preincubation supernatant fraction	Aminopyrine metabolism (formaldehyde produced) (nmoles · min^{-1} · mg $protein^{-1}$)	Aniline metabolism (*p*-aminophenol produced) (nmoles · min^{-1} · mg $protein^{-1}$)
Normal (control)	Buffer (control)	5.00 ± 0.22	0.55 ± 0.02
Normal	Normal cytosol	5.49 ± 0.27	0.56 ± 0.02
Normal	Diabetic cytosol	4.84 ± 0.50	0.52 ± 0.02
Diabetic	Buffer	3.85 ± 0.28 †	1.26 ± 0.11 †
Diabetic	Normal cytosol	3.73 ± 0.25 †	1.23 ± 0.11 †
Diabetic	Diabetic cytosol	3.69 ± 0.25 †	1.21 ± 0.10 †

* Each value is the mean ± S.E.M. of four to five separate experiments.
† $P < 0.01$ vs normal (control) microsomes with buffer.

Table 2. Effect of Tris buffer on the rates of drug metabolism by normal microsomes preincubated with normal cytosol or alloxan diabetic cytosol (Method A)*

Microsomes	Preincubation supernatant fraction	Aminopyrine metabolism (formaldehyde produced) (nmoles · min^{-1} · mg protein^{-1})	Aniline metabolism (*p*-aminophenol produced) (nmoles · min^{-1} · mg protein^{-1})
Normal	Buffer (control)	0.42 ± 0.02	0.36 ± 0.04
Normal	Normal cytosol	0.27 ± 0.04 †	0.21 ± 0.02 †
Normal	Diabetic cytosol	0.29 ± 0.02 †	0.24 ± 0.02 ‡
Diabetic	Buffer	0.26 ± 0.01 †	0.63 ± 0.04 †

* Each value is the mean ± S.E.M. of four separate experiments. The experiments were conducted exactly as described in Table 1 except that Tris instead of phosphate buffer was used throughout.
† $P < 0.01$ vs normal (control) microsomes with buffer.
‡ $P < 0.02$ vs normal (control) microsomes with buffer.

decreased the rate of microsomal aminopyrine metabolism, the rate of aminopyrine metabolism by normal microsomes was not affected by this method of exposure to diabetic cytosol. Similarly, diabetes, as expected, increased the microsomal metabolism of aniline to 229 per cent of the control rate (Table 1). Again, however, preincubation of normal microsomes with either normal or diabetic cytosol (Method A) did not affect the ability of the microsomes to catalyze aniline hydroxylation. Therefore, like aminopyrine metabolism, the rate of aniline metabolism appeared to be independent of the origin of the preincubation cytosol and a function only of the origin of the microsomes. The above experiments were conducted with phosphate buffer. Other investigators, however, have used Tris buffer in comparable experiments designed to show the effects of variously treated liver cytosols on the ability of microsomes to metabolize drugs [2, 3, 8]. As shown in Table 2, Tris buffer itself affected the results of the preincubation experiments. That is, although diabetic microsomes metabolized both substrates as expected compared to normal control microsomes, preincubation exposure of Tris-prepared microsomes to either normal or diabetic Tris-prepared cytosol decreased the subsequent microsomal metabolism of aminopyrine as well as aniline. Such an inhibition of aniline metabolism is clearly not reflective of the accelerated rate of aniline metabolism observed with diabetic microsomes prepared in either buffer (Tables 1 and 2).

Streptozotocin diabetes and preincubation experiments. The effects of streptozotocin diabetes on microsomal drug metabolism were, as expected, the same as those observed with alloxan diabetic microsomes—a decrease in aminopyrine metabolism and an increase in aniline metabolism (Fig. 1). These results are in agreement with those previously observed with microsomes [9] and with isolated perfused liver [18]. As with alloxan diabetes (Table 1), preincubation of normal microsomes with streptozotocin diabetic cytosol had no effect on the ability of normal microsomes to catalyze metabolism of either aminopyrine or aniline (Fig. 1). Failure to observe a diabetic cytosol effect on microsomal drug metabolism is, therefore, independent of the diabetogenic agent used.

Alloxan diabetes and direct incubation experiments. Another method of examining various cytosols for possible effects on microsomal drug metabolism is to suspend the microsomes directly in the cytosol fraction to be examined and assess the ability of such cytosol-suspended (exposed) microsomes to catalyze drug metabolism. This direct or concurrent incubation–metabolism technique was used previously in diabetes and drug metabolism studies by Dixon *et al.* [5] and Ackerman and Leibman [8] and is referred to as Method B in Materials and Methods. With this method, the rates of drug metabolism by normal microsomes were the same whether incubated with normal or diabetic cytosol (Table 3). The elevated (aniline) or inhibited (aminopyrine) rates of metabolism by diabetic microsomes were also independent of the source of the

Table 3. Effect of normal or alloxan diabetic cytosol (Method B) on the rates of drug metabolism by normal and alloxan diabetic microsomes*

Microsomes	Supernatant fraction	Aminopyrine metabolism (formaldehyde produced) (nmoles · min^{-1} · mg protein^{-1})	Aniline metabolism (*p*-aminophenol produced) (nmoles · min^{-1} · mg protein^{-1})
Normal (control)	Buffer (control)	5.11 ± 0.75	0.66 ± 0.02
Normal	Normal cytosol	6.14	0.69 ± 0.06
Normal	Diabetic cytosol	6.56	0.71 ± 0.06
Diabetic	Buffer	3.67	1.09 ± 0.06 †
Diabetic	Normal cytosol	4.59	1.26 ± 0.05 †
Diabetic	Diabetic cytosol	4.63	1.26 ± 0.12 †

* Each value is the average of duplicate experiments or the mean ± S.E.M. of three experiments.
† $P < 0.01$ vs normal (control) microsomes with buffer.

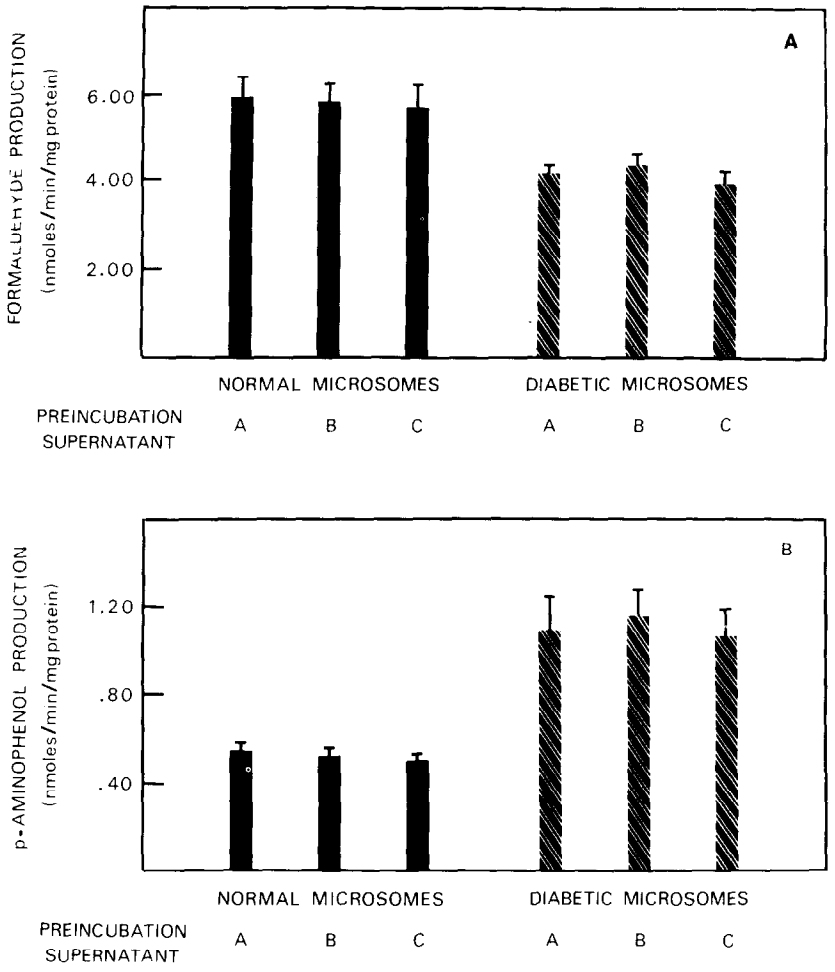

Fig. 1. Effects of streptozotocin diabetes and preincubation of microsomes with various cytosols or buffer (Method A) on the rates of microsomal metabolism of aminopyrine (Panel A) and aniline (Panel B). The letters A, B and C under the vertical columns indicate preincubation in buffer, normal (control) cytosol and diabetic cytosol respectively. The vertical bars above the columns represent ± S.E.M. of five experiments. $P < 0.01$ for all diabetic microsomes vs normal microsomes.

cytosol present (Table 3). These results, as well as those of the preincubation experiments, indicate the lack of any diabetic-specific cytosol factor associated with the changes in hepatic drug metabolism that occur during diabetes.

DISCUSSION

A cytosol-located inhibitor mechanism has been postulated in order to explain the inhibition of *p*-chloro-*N*-methylaniline metabolism in cyclic AMP-treated rat liver [3]. The evidence for such an inhibitor is based on experiments employing the preincubation technique, Method A [3]. Since the level of cyclic AMP is elevated in the diabetic rat [8, 19], a mechanism common to the actions of cyclic AMP and diabetes on hepatic drug metabolism might reasonably be expected to exist. When the preincubation technique was employed in the search for a possible diabetic cytosol-located inhibitor of drug metabolism, however, the results were negative (Table 1). Not unexpectedly, the results were similar regardless of the diabetogenic agent used (Fig. 1).

In the initial investigation of a possible effect of diabetic cytosol on microsomal drug metabolism, the cytosolic fraction from alloxan diabetic rats had no effect on the metabolism of hexobarbital by normal microsomes [5]. Subsequently, however, alterations in hepatic microsomal drug metabolism by streptozotocin diabetic rat liver were reported to be cytosol-dependent and were postulated to be mediated by a cytosol-located inhibitor [8]. Both of these earlier investigations into the possible effects of diabetic cytosol on microsomal drug metabolism employed Method B, the direct incubation technique. In the initial investigation [5], no significant difference was observed between the rates of hexobarbital metabolism observed in the presence of the soluble (supernatant or cytosol) fraction obtained from either normal or alloxan diabetic rats even though diabetes decreased the metabolism of hexobarbitol. These reactions were conducted in a phosphate-KCl buffered system, and the results were similar to those observed in the present experiments (Table 3). In the subsequent investigation [8], which employed a Tris-buffered system, the rates of both hexobarbital

and aniline metabolism by normal microsomes were decreased in the presence of streptozotocin diabetic cytosol compared to normal cytosol [8]. With this same system, however, aniline metabolism was also observed to be decreased [8] by diabetic microsomes. This latter finding is in direct contradiction to the effects of diabetes on the metabolism of this compound observed by others before [5–7], since [9], and in the present study. In view of the results obtained with Tris buffer in the present study, the anomalous results of the earlier study [8] may be related in part to the use of Tris as the buffer system.

The results presented in this paper, obtained by two different methods of cytosol–microsome exposure or mixing, clearly demonstrate that the diabetes related alterations in the rates of hepatic microsomal drug metabolism of the model drug substrates aminopyrine and aniline are not mediated through cytosol-fraction factors as had been suggested [8]. The fact that diabetes actually increases the metabolism of aniline makes any type of general diabetic-cyclic AMP-related inhibition mechanism of drug metabolism untenable. It is also clear from the present and previous work [9, 18] that the lack of an observable diabetic cytosol effect on the ability of normal microsomes to catalyze drug metabolism (Table 1 and Fig. 1) cannot be attributed, as had been proposed [8], to the agent used to induce diabetes. If the alterations in hepatic drug metabolism by diabetic rats are not mediated by the cellular cytosol fraction, then the observed effects must be a function of the microsomal component *per se*. This concept is in agreement with the original conclusion of Dixon *et al.* [5]. Indeed, current evidence indicates that alterations in the terminal component of the microsomal mixed function oxidase system, cytochrome P-450, are involved in substrate-specific changes in the rates of drug metabolism caused by chemical inducers of the system such as phenobarbital and 3-methylcholanthrene [20]. In this regard, the total hepatic cytochrome P-450 concentration has been shown to be increased in diabetic rats compared to normal rats [9, 21]. Recent evidence with sodium dodecyl sulfate-polyacrylamide gel electrophoresis experiments also indicates that specific cytochrome P-450 hemeproteins in male diabetic rat liver microsomes are distinctly increased and decreased by diabetes [22, 23]. The nature of these specific diabetes-induced alterations in hepatic cytochrome P-450 hemeproteins and their relationships to drug metabolism in the diabetic state are currently under investigation.

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