Induction of cytochrome *P450*III and *P450*IV family proteins in streptozotocin-induced diabetes

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The effect of insulin-dependent diabetes on the hepatic microsomal activity of cytochrome *P450*III and *P450*IV family proteins was investigated in rats pretreated with streptozotocin. In order to discern between the effects of the diabetogen *per se* and those of the ensuing diabetes, streptozotocin-treated rats received in addition either nicotinamide to prevent the onset of diabetes or daily treatment with insulin to antagonize the effects of diabetes. Streptozotocin-treated rats displayed higher ethylmorphine and erythromycin *N*-demethylase activities and lauric acid hydroxylase activity. Increases were also detected immunologically by using monospecific polyclonal antibodies against the *P450*III and *P450*IV families. All effects were prevented by nicotinamide and effectively antagonized by insulin. In order to evaluate the role of the ketone bodies in the diabetes-induced increases in the above activities, rats were rendered hyperketonaemic by dietary administration of medium-chain triacylglycerols. These hyperketonaemic animals displayed high laurate hydroxylase activity and *P450*IV apoprotein levels, similar to those seen in the diabetic animals. Hyperketonaemia induced by dietary means caused a modest increase in the demethylation of erythromycin and had no significant effect on the *N*-demethylation of ethylmorphine. Furthermore, no marked increases were evident in the *P450*III apoprotein levels in the hyperketonaemic animals. It is concluded that insulin-dependent diabetes induces proteins of the *P450*III and *P450*IV families, and that the hyperketonaemia that accompanies diabetes is largely responsible for the changes in the latter family.

INTRODUCTION

It is now becoming increasingly evident that the induction and regulation of the cytochrome *P*-450-dependent mixed-function oxidases is not determined solely by exposure to xenobiotics, but may also be triggered by pathophysiological conditions, presumably resulting from an increase in the levels of an endogenous chemical with inducing properties.

Exposure of animals to diabetogenic agents, such as streptozotocin (STZ) and alloxan, results in marked changes in the metabolism of model substrates *in vitro* (Dixon *et al.*, 1963; Ackerman & Liebman, 1977) and in the pharmacokinetic profiles of drugs such as paracetamol and aminopyrine (Price & Jollow, 1982; Toda *et al.*, 1987). Subsequent studies have revealed that insulin-dependent diabetes profoundly modulates the levels of constitutive and inducible forms of cytochromes *P*-450 (Thomas *et al.*, 1987; Favreau & Schenkman, 1988; Ioannides *et al.*, 1988; Yamazoe *et al.*, 1989). Such changes are of toxicological importance, as the diabetic animal responds differently to the toxicity of chemicals such as paracetamol (Price & Jollow, 1982), carbon tetrachloride and bromobenzene (Watkins *et al.*, 1988). Moreover, hepatic post-mitochondrial preparations from alloxan- and STZ-treated rats were more efficient than those from control animals in converting chemical carcinogens to their reactive intermediates (Ioannides *et al.*, 1988; Yamazoe *et al.*, 1988; Flatt *et al.*, 1989).

Of the various inducible families of cytochrome *P*-450, insulin-dependent diabetes is associated with increases in *P450*I, IIB and IIE activities, and these changes are presumably responsible for the alterations in the metabolism of various toxins (Thomas

et al., 1987; Ioannides *et al.*, 1988). These observed increased levels of cytochrome *P*-450 proteins appear to be mediated, at least partly, by the hyperketonaemia that accompanies insulin-dependent diabetes (Bellward *et al.*, 1988; Barnett *et al.*, 1988).

Two major inducible families of cytochromes *P*-450 are *P450*III and *P450*IV. The former is inducible by glucocorticoids, macrolide antibiotics and some imidazole-containing antifungal agents (Schuetz & Guzelian, 1984; Gonzalez *et al.*, 1986; Rodrigues *et al.*, 1988); this family is active in the metabolism of macrolide antibiotics such as troleandomycin and erythromycin, and of the immunosuppressant drug cyclosporin (Wrighton *et al.*, 1985a; Combalbert *et al.*, 1989). The *P450*IV family is inducible by xenobiotics such as the drug clofibrate and its analogues and the phthalate ester plasticizers (Tamburini *et al.*, 1984; Hawkins *et al.*, 1987); this family is involved in the metabolism of endogenous substrates such as lauric and arachidonic acids (Bains *et al.*, 1985; Sharma *et al.*, 1989). Accordingly, the purpose of the present paper is two-fold: (a) to establish if STZ-induced insulin-dependent diabetes modulates the hepatic levels of *P450*III and *P450*IV proteins, and (b) to determine whether hyperketonaemia *per se* is responsible for such changes.

MATERIALS AND METHODS

Materials

Ethylmorphine (May and Baker, Dagenham, Essex, U.K.), medium-chain triacylglycerols (MCT; Cow and Gate Ltd., Trowbridge, Wilts., U.K.), erythromycin, lauric acid, STZ, nicotinamide and all cofactors (Sigma, Poole, Dorset, U.K.) and long-acting monocomponent human insulin (Ultratard; Novo

Abbreviations used: STZ, streptozotocin; MCT, medium-chain triacylglycerols; PCN, pregnenolone 16 α -carbonitrile.

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Industries, Copenhagen, Denmark) were all purchased. The preparation of MCT consisted of fractionated coconut oil comprising predominantly the triacylglycerols of octanoic and decanoic acids.

Methods

Male Wistar albino rats (Animal Breeding Unit, University of Surrey) were used in two experimental studies (180–200 g). In the first study, animals were randomly divided into four groups, each comprising four animals. One group served as control; the second group received a single intraperitoneal administration of STZ (65 mg/kg) dissolved in 0.5 M-citrate buffer (pH 4.5); the third group received, in addition to STZ, two intraperitoneal doses of nicotinamide (350 mg/kg), one 10 min before, and the other 3 h after, the administration STZ; finally, the fourth group received, in addition to STZ, single daily increasing doses of insulin as previously described (Ioannides *et al.*, 1988). All animals were killed 22 days after commencement of treatment and 24 h after the last administration of insulin. In the second study, rats were randomly divided into three groups each comprising four animals. One group served as control, the second group received a single dose of STZ as described above, and the third group received daily intragastric intubation of MCT (8 g/kg). These animals were also killed 22 days after the commencement of the treatment. Lastly, to serve as positive controls for the induction of the *P450IV* and *P450III* families, groups of previously untreated rats were treated with single daily intraperitoneal administrations of either clofibrate (80 mg/kg) or pregnenolone 16 α -carbonitrile (PCN; 100 mg/kg) for 3 days, all animals being killed 24 h after the last injection. In all cases, livers were immediately excised and hepatic microsomal fractions were prepared as previously described (Ioannides & Parke, 1975). The following determinations were carried out: erythromycin *N*-demethylase (Wrighton *et al.*, 1985b), ethylmorphine *N*-demethylase (Lu *et al.*, 1972), total carbon-monoxide-discernible cytochromes *P*-450 (Omura & Sato, 1964), and protein (Lowry *et al.*, 1951). Lauric acid hydroxylase was determined by a t.l.c. method (Parker & Orton, 1980) and represents the combined formation of the 12(ω)- and 11(ω -1)-hydroxylauric acid metabolites.

Purification of and antibody production to cytochromes *P450IIIA1* and *P450IVA1* have already been described (Tambu-

rini *et al.*, 1984; Wolf *et al.*, 1986). Antibodies to *P450IIIA1* recognize, in addition to the *P450IIIA1* protein, additional bands of lower M_r which may represent other steroid-inducible proteins, but this remains to be confirmed. *P450III* appears to be a large gene family (Gonzalez, 1989). Immunoblots of the electrophoretically resolved solubilized microsomal proteins (Laemmli, 1970) were carried out following transfer on to nitrocellulose sheets (Towbin *et al.*, 1979). Cytochrome *P450IV* apoproteins were also determined using a non-competitive e.l.i.s.a. technique (Sharma *et al.*, 1988).

Blood samples were obtained from the cut tip of the tails of conscious rats and were used for the determination of plasma glucose (Stevens, 1971), 3-hydroxybutyrate and acetoacetate concentrations (McGarry *et al.*, 1970).

Statistical evaluation was carried out using Student's *t* test.

RESULTS

STZ-treated animals displayed the expected symptoms of diabetes such as hyperphagia, polydipsia and body weight loss. Rats receiving the MCT did not differ from controls in their rates of growth (results not shown). The plasma glucose and ketone (3-hydroxybutyrate + acetoacetate) levels found in both studies are

Table 1. Plasma glucose and ketone levels in rats treated with STZ or with MCT

Results are presented as means $\pm$ S.E.M. for four rats in each group. * $P < 0.05$; ** $P < 0.01$ compared with control group.

Treatment	Plasma glucose (mM)	Plasma acetoacetate + 3-hydroxybutyrate (mM)
Control	5.6 $\pm$ 0.2	0.34 $\pm$ 0.10
STZ	25.6 $\pm$ 4.1*	0.98 $\pm$ 0.16*
STZ + insulin	14.9 $\pm$ 5.8	0.30 $\pm$ 0.14
STZ + nicotinamide	5.8 $\pm$ 0.3	0.40 $\pm$ 0.10
Control	5.2 $\pm$ 0.1	0.42 $\pm$ 0.04
STZ	20.3 $\pm$ 1.4**	1.30 $\pm$ 0.02**
MCT	5.2 $\pm$ 0.1	1.20 $\pm$ 0.15*

Table 2. Effect of STZ and MCT treatment on the rat hepatic microsomal mixed-function oxidases

Results are presented as means $\pm$ S.E.M. for four rats per group, except for immunological data which are derived from analysis from two animals, each carried out in quadruplicate. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared with control rats. N.D., not determined.

Parameter	Control	STZ	STZ + nicotinamide	STZ + insulin	MCT	PCN	Clofibrate
Total cytochromes <i>P</i> -450 (nmol/mg of protein)	0.30 $\pm$ 0.03	0.38 $\pm$ 0.05	0.29 $\pm$ 0.04	0.29 $\pm$ 0.02	0.37 $\pm$ 0.05*	0.54 $\pm$ 0.09*	0.35 $\pm$ 0.08
Ethylmorphine <i>N</i> -demethylase (nmol/min per mg of protein)	1.30 $\pm$ 0.12	6.10 $\pm$ 0.99**	0.92 $\pm$ 0.11	0.84 $\pm$ 0.19	2.99 $\pm$ 0.61	8.14 $\pm$ 0.40***	N.D.
Erythromycin <i>N</i> -demethylase (pmol/min per mg of protein)	140 $\pm$ 18	511 $\pm$ 49***	79 $\pm$ 31*	77 $\pm$ 12*	251 $\pm$ 4*	665 $\pm$ 65***	N.D.
Lauric acid hydroxylase (nmol/min per mg of protein)	1.01 $\pm$ 0.45	5.31 $\pm$ 0.65**	0.45 $\pm$ 0.18	0.61 $\pm$ 0.2	4.6 $\pm$ 0.44***	N.D.	10.1 $\pm$ 0.1***
Cytochrome <i>P450IV</i> apoprotein (pmol/mg of protein)	7.0 $\pm$ 1.1 (2.1)†	16.1 $\pm$ 2.0** (4.2)†	5.0 $\pm$ 0.8 (1.7)†	4.4 $\pm$ 1.8 (1.5)†	14.1 $\pm$ 1.8** (3.9)†	N.D. N.D.	18 $\pm$ 3.0** (5.1)†
Microsomal protein (mg/g of liver)	35.4 $\pm$ 4.1	39.1 $\pm$ 3.2	36.4 $\pm$ 3.8	35.0 $\pm$ 4.0	34.2 $\pm$ 4.2	38.1 $\pm$ 2.8	39.1 $\pm$ 2.4

† Cytochrome *P450IV* as a percentage of total cytochrome *P*-450.

Fig. 1. Immunoblot analysis employing anti-(cytochrome P450IIIA1) and anti-(cytochrome P450IVA1) polyclonal antibodies

Microsomal proteins from control and treated animals were resolved by electrophoresis in a 10% (w/v) SDS/polyacrylamide gel and transferred electrophoretically to nitrocellulose. In the case of P450IVA1 (a), the immunoblot was carried out with sheep anti-(cytochrome P450IVA1) (diluted 1:1600) followed by peroxidase-linked donkey anti-(sheep IgG) (diluted 1:2000). Solubilized microsomes (15 µg of protein) were derived from animals treated as follows: lane A, STZ+insulin; B, STZ only; C, clofibrate; D, STZ+nicotinamide; E, control. In the case of P450IIIA1 (b), the immunoblot was performed with rabbit anti-(cytochrome P450IIIA1) (diluted 1:800) followed by peroxidase-linked anti-(rabbit IgG) (diluted 1:2000). Solubilized microsomes (15 µg of protein) were derived from animals treated as follows: lane A, STZ+insulin; B, STZ only; C, PCN; D, STZ+nicotinamide; E, control.

presented in Table 1, showing clearly that animals receiving STZ alone exhibited hyperketonaemia and hyperglycaemia. These symptoms were prevented by simultaneous administration of nicotinamide and were antagonized by daily treatment with insulin. Animals receiving the MCT displayed hyperketonaemia without a change in plasma glucose levels.

Pretreatment of rats with STZ gave rise to an increase in the *N*-demethylations of ethylmorphine and erythromycin and in lauric acid hydroxylase activity (Table 2). Concomitant administration of nicotinamide with STZ prevented the STZ-induced increases and in all cases decreased the levels of activity to below the control value, although this effect was not always statistically significant. Similarly, daily therapy of the STZ-treated animals with insulin restored enzyme activities to control values. Treatment with clofibrate and PCN gave the expected results of induction of the relevant enzyme activities (Table 2).

Immunoquantification of the cytochrome P450IV family using an e.l.i.s.a. technique employing polyclonal antibodies against P450IVA1 revealed that treatment with STZ increased the apoprotein levels, the effect being ablated by nicotinamide and by daily treatment with insulin (Table 2). Immunoblot analysis utilizing the same antibody also showed an increase in the apoprotein levels of P450IVA1-related proteins in the rats receiving STZ alone (Fig. 1a). No such effects were seen when the STZ-treated animals received concurrent nicotinamide or were treated daily with insulin. When antibodies to P450IIIA1 were employed in the immunoblots, the apoprotein levels were higher in the animals receiving the STZ alone (Fig. 1b). This increase was once again preventable by treatment with nicotinamide or by daily insulin therapy.

Daily administration of MCT enhanced lauric acid hydroxylase activity and P450IV apoprotein levels to almost the same

Fig. 2. Effects of MCT and STZ on hepatic microsomal cytochrome P450III and P450IV proteins determined using immunoblots

Assay conditions are described in the legend to Fig. 1. In the case of P450IVA1 (a) the samples were derived from animals treated with: lane A, MCT; B, STZ; C, clofibrate; D, control. In the case of P450IIIA1 (b) the samples were derived from animals treated with: lane A, STZ; B, MCT; C, PCN; D, control.

extent as did STZ at the dose levels used (Table 2). A significant increase was also seen in the *N*-demethylation of ethylmorphine, but the effect was less than that induced by STZ (Table 2). A modest increase was also observed in the *N*-demethylation of erythromycin which was not, however, statistically significant.

Immunoblots using the P450IVA1 antibody showed an increase in the apoprotein levels of the MCT-treated rats, similar to that seen in the STZ-treated animals commensurate with the above increase in P450IVA1-dependent lauric acid hydroxylase activity (Fig. 2a). By contrast, when antibodies to P450IIIA1 were employed, no visible difference between MCT-treated and control animals was evident (Fig. 2b).

DISCUSSION

Induction of insulin-dependent diabetes by the administration of a single injection of STZ gave rise to significant increases in the rates of *N*-demethylation of erythromycin and ethylmorphine, two drugs that have been used as diagnostic substrates for the P450III family of proteins (Wrighton *et al.*, 1985b). Solubilized hepatic microsomes from STZ-treated rats probed with antibodies to P450IIIA1 revealed an increase in the P450III apoprotein levels, confirming induction of this cytochrome P-450 family. The STZ-induced changes in P450III-related activity and apoprotein levels were successfully antagonized by daily insulin therapy, demonstrating that the observed changes can be ascribed to the diabetic state rather than to STZ treatment *per se*. Moreover, rats which received, in addition to STZ, nicotinamide, which prevents the cytotoxic action of the diabetogen and the onset of diabetes (Schein *et al.*, 1967), did not show any significant change from controls in P450III activity or apoprotein levels. This family of haemoproteins is involved in the metabolism of many drugs such as midazolam (Fabre *et al.*, 1988), mephenytoin (Shimada & Guengerich, 1985), cyclosporin (Bertauld-Peres *et al.*, 1987) and the macrolide antibiotics troleandomycin and erythromycin (Wrighton *et al.*, 1985a). It is therefore likely that the response to these drugs may be altered in poorly controlled diabetes. It is worth noting that P450III proteins are present in the human liver and at least three genes have been detected (Gonzalez, 1989).

Pretreatment of rats with STZ also gave rise to a significant increase in hepatic microsomal lauric acid hydroxylase activity,

which is closely associated with the *P450IV* family of haemoproteins (Tamburini *et al.*, 1984). The effect was once again prevented by nicotinamide and daily insulin administration, showing that the induction process is totally attributable to the diabetic state. A similar picture was obtained when the apoprotein levels were determined immunologically, utilizing a non-competitive e.l.i.s.a. technique, and in Western blots. The increase in *P450IVA1* apoprotein levels is in agreement with the observed rise in lauric acid hydroxylase, an activity catalysed by this protein. The *P450IV* family plays an important role in the metabolism of endogenous substrates, being involved in the oxidation of fatty acids such as lauric and arachidonic acids (Bains *et al.*, 1985; Sharma *et al.*, 1989). Insulin-dependent diabetes is associated with high levels of fatty acids, and it may be that the increase in *P450IVA1* activity is an adaptive response by the animal to lower the levels of these lipids through fatty acid hydroxylation and subsequent β -oxidation. It is of interest that another enzyme involved in lipid metabolism, namely cholesterol 7α -hydroxylase, the initial and rate-limiting step in cholesterol biosynthesis, is also increased in STZ-induced diabetes (Subbiah & Yunker, 1984).

It has already been established that the increased levels of *P450I*, *IIB* and *IIE* proteins exhibited by STZ-diabetic rats are mediated, at least partly, through the hyperketonaemia that accompanies insulin deficiency (Bellward *et al.*, 1987; Barnett *et al.*, 1988). In the present study hyperketonaemia, induced by the daily administration of triacylglycerols, enhanced the hydroxylation of lauric acid to a similar extent as did STZ at the dose levels used, and similar increases were seen in the e.l.i.s.a. immunoquantification and the Western blots. These results indicate that ketone bodies may be responsible for the diabetes-induced changes in *P450IV*-dependent enzyme activity. It may be also concluded that hyperglycaemia has no major role in the diabetes-induced changes in *P450IV* proteins, since the triacylglycerol-treated rats exhibited higher activities than control animals, despite being normoglycaemic.

In contrast with the above, the hyperketonaemia induced by dietary means caused very modest changes in the *N*-demethylase activities with ethylmorphine and erythromycin, achieving statistical significance only in the latter case. Similarly, no marked increases were seen in the apoprotein levels suggesting that hyperketonaemia does not play an important role in the diabetes-induced changes in *P450III* activity. This is hardly surprising, since the inducers of the *P450III* family tend to be high- M_r compounds such as clotrimazole, glucocorticoids and troleandomycin, but the possibility that the ketones could act indirectly cannot be ruled out. It has been suggested that the decreased levels of growth hormone may be an important factor in the diabetes-induced changes in cytochrome *P*-450 proteins (Tannenbaum, 1981). Indeed, in hypophysectomized rats an accumulation of *P*-450 mRNA inducible by PCN, a typical *P450III* inducer, has been reported, the levels being restored on injection of human growth hormone (Lemoine *et al.*, 1988); similarly, in the mouse, hypophysectomy has been shown to induce hepatic *P450III* mRNA (Meehan *et al.*, 1988). It is therefore possible that the increases in *P450III* proteins in insulin-dependent diabetes are a consequence of the impaired secretion of growth hormone (Harrison & Robinson, 1980).

In summary, we have demonstrated in the present study that (a) STZ-induced insulin-dependent diabetes induces proteins of the *P450III* and *P450IV* families, and (b) hyperketonaemia is largely responsible for the STZ-induced changes in *P450IV* levels, but plays a very limited role in the STZ-induced changes in *P450III* proteins. It is likely that similar changes in *P450IV* activity may be observed in other hyperketonaemic states, such as starvation.

We are grateful to the Science and Engineering Research Council for its support of this work with a research studentship for C.R.B., and project grants from the Medical Research Council and the Wellcome Trust to G.G.G.

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Received 16 October 1989/8 February 1990; accepted 5 March 1990