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Induction of hepatic microsomal P450 I and IIB proteins by hyperketonaemia

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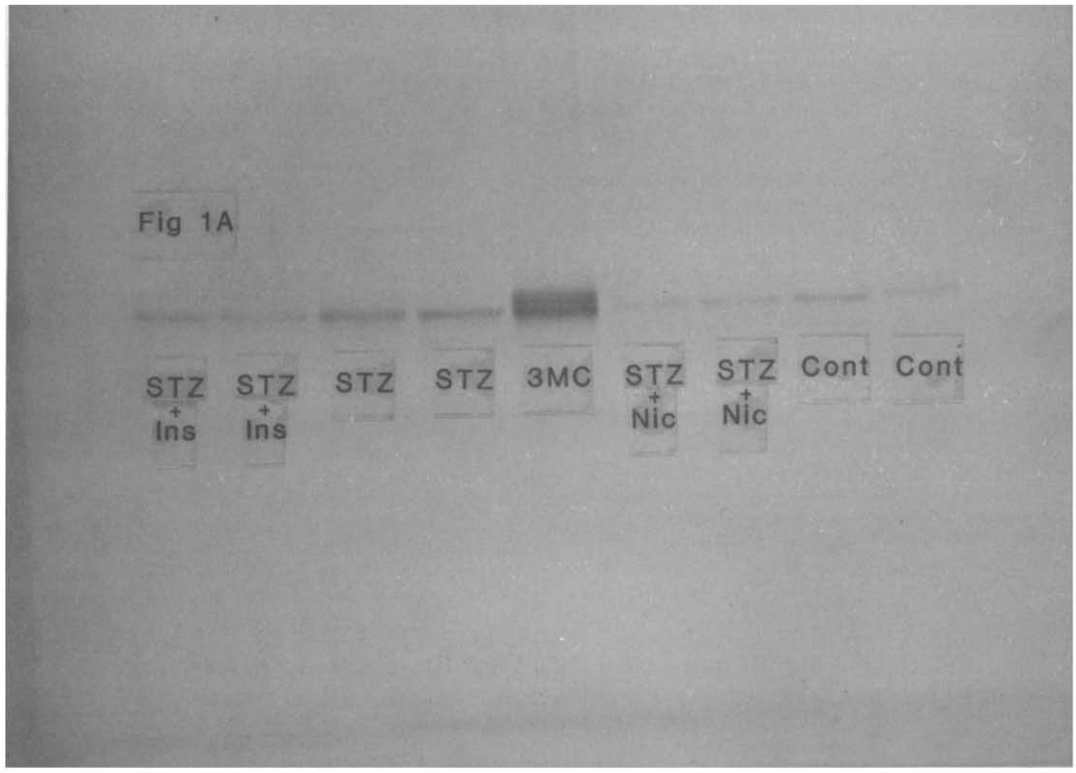
Since the initial publication of Dixon *et al.* [1], a plethora of studies have demonstrated unequivocally that chemically-induced type I, insulin-dependent diabetes modulates the microsomal metabolism of various model substrates [2, 3], and similar findings have been reported in spontaneously diabetic rats [4]. The above studies did not address the selective effect of diabetes on specific cytochrome P450 proteins. We have recently demonstrated that in streptozotocin (STZ)-induced diabetes the O-dealkylations of ethoxyresorufin and pentoxyresorufin, two substrates routinely used to monitor P450 I and P450 IIB activity, respectively [5, 6], were markedly elevated and successfully antagonized by insulin therapy [7]. Moreover, rats rendered hyperketonaemic by the daily administration of triacylglycerols also displayed high dealkylase activities when compared to control animals, and this observation led us to conclude that the ketone bodies may mediate, at least partly, the diabetes-induced increases in these two activities [8].

The use of diagnostic substrates in detecting changes in the levels of specific families of cytochrome P450 suffers from three major disadvantages. Firstly, they do not allow the distinction between proteins belonging to the same family/subfamily and secondly the possibility that an as yet uncharacterized form of microsomal cytochrome P450 catalysing to some extent the diagnostic substrates is present, cannot be excluded. Finally, the modulating agent may still be present in the microsomes employed in the assays causing inhibition and consequently underestimation of the degree of induction. However, this last possibility is extremely unlikely to contribute to the observed effects of STZ as the half-life of this drug is less than 15 min. In order to overcome the first two difficulties, we investigated the effect of streptozotocin-induced diabetes and the diet-induced hyperketonaemia on the hepatic microsomal levels of P450 I and P450 IIB proteins determined immunologically using monospecific antibodies.

Materials and Methods

Medium chain triacylglycerols (Cow and Gate Ltd, Trowbridge, U.K.), long-acting monocomponent human insulin (Ultratard, Novo Industries, Copenhagen, Denmark), nicotinamide (Sigma Chemical Co., Poole, U.K.) and peroxidase-linked donkey anti-sheep IgG and peroxidase-linked donkey anti-rabbit IgG (Guildhay Antisera, Guildford, U.K.) were all purchased. The preparation of medium chain triacylglycerols used comprised fractionated coconut oil predominantly composed of the triacylglycerols of octanoic and decanoic acids. The purification and characterization of cytochrome P450 IA1 and the production of antibodies recognizing both A1 and A2 proteins have already been described [9]. Anti-cytochrome P450 IIB, recognizing both B1 and B2 proteins was a generous gift from Dr C. R. Wolf, Imperial Cancer Research Fund, Hugh Robson Building, George Square, Edinburgh, U.K.

Male Wistar albino rats (Experimental Biology Unit, University of Surrey) weighing 180–200 g were used in two experimental studies. 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 prior to, and the other 3 hr after the administration of STZ (STZ + nic); finally the fourth group received, in addition to STZ, single daily increasing doses of insulin (STZ + ins) as previously described [7]. All animals were killed 22 days after commencement of treatment and 24 hr 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, while the third group received daily intragastric intubation of medium chain triacylglycerols (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 P450 I and P450 IIB families, groups of previously untreated rats were treated with single daily intraperitoneal administrations of either 3-methylcholanthrene (3MC, 25 mg/kg) or phenobarbitone (PB, 80 mg/kg), respectively, for 3 days, all animals being killed 24 hr after the last injection. In all cases, livers were immediately excised and hepatic microsomal fractions prepared as previously



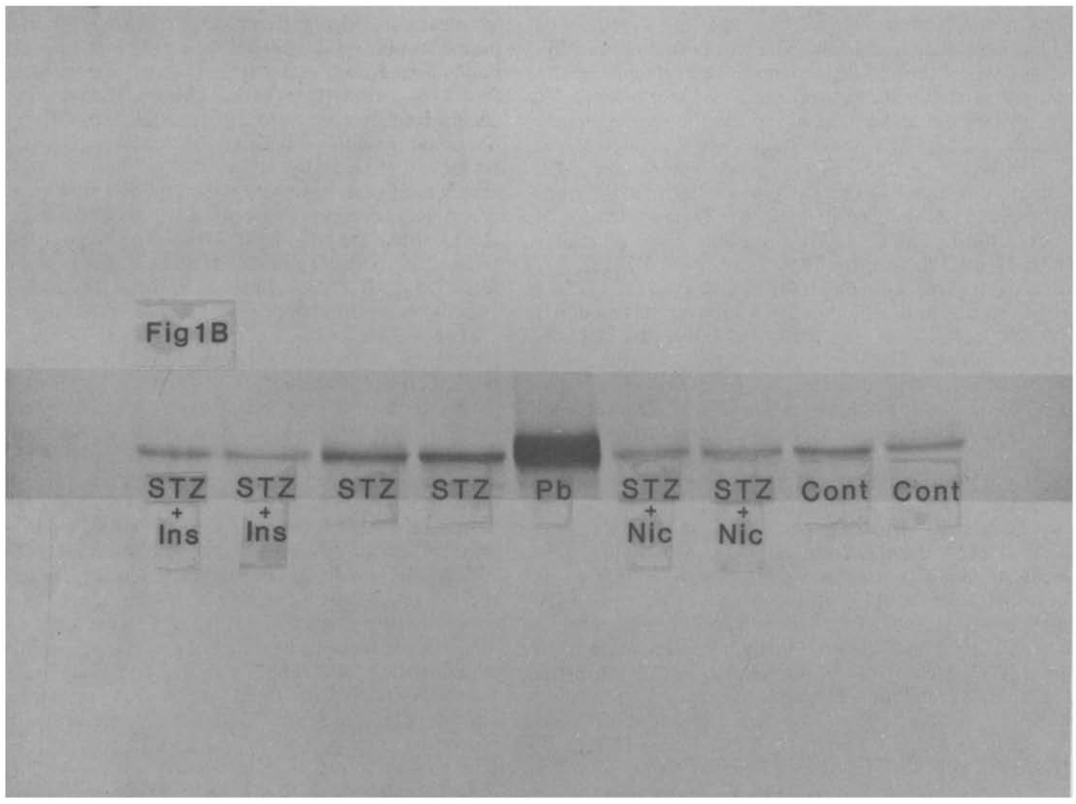
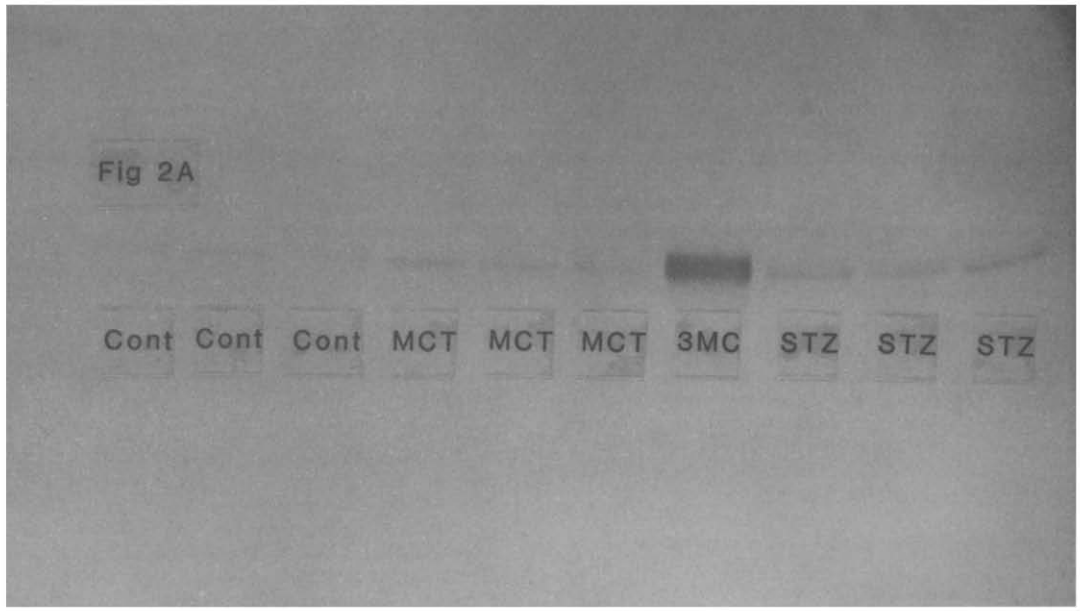


Fig. 1. Immunoblot analysis of microsomes from STZ-treated rats using anti-cytochrome P450 IA1 and anti-cytochrome P450 IIB1 polyclonal antibodies. Microsomal proteins (15 μg) from control and treated animals were resolved by electrophoresis in a 10% (w/v) SDS–polyacrylamide gel and transferred electrophoretically to nitrocellulose. In the case of P450 IA1 (A) the immunoblot was carried out with sheep anti-cytochrome P450 IA1 (diluted 1:10,000) followed by peroxidase-linked donkey anti-sheep IgG (diluted 1:2000). In the case of P450 IIB1 (B) the immunoblot was performed with rabbit anti-cytochrome P450 IIB1 (diluted 1:1000) followed by peroxidase-linked anti-rabbit IgG (diluted 1:2000). Cont, control; STZ, streptozotocin; STZ + Ins, streptozotocin + insulin; STZ + Nic, streptozotocin + nicotinamide; Pb, phenobarbitone and 3MC, 3-methylcholanthrene.



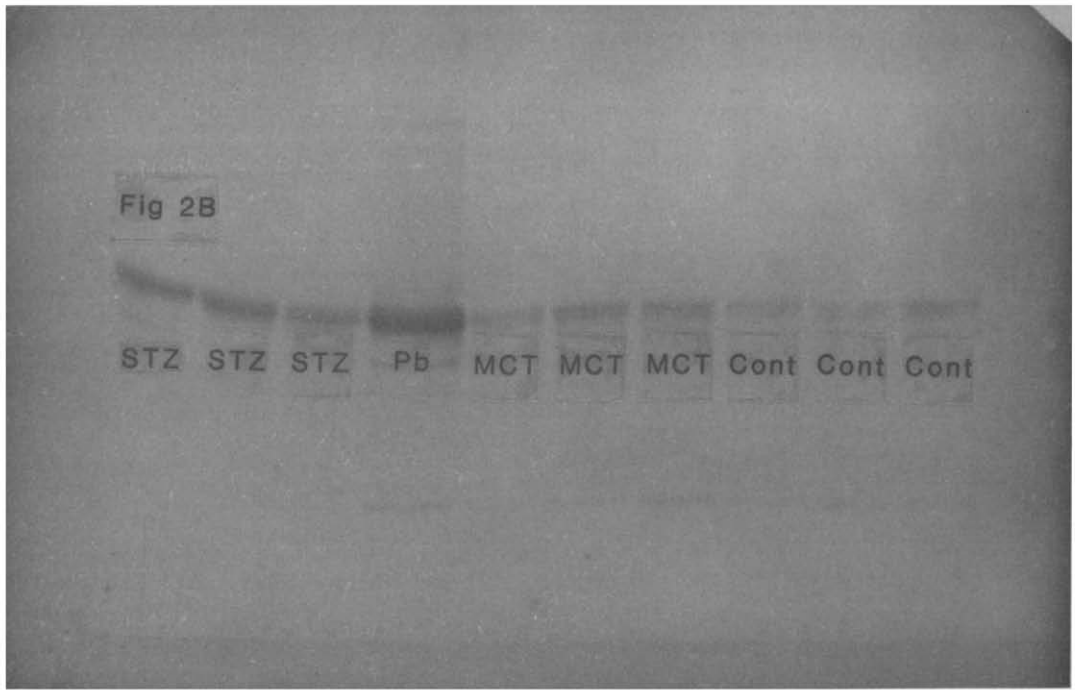


Fig. 2. Effect of medium chain triacylglycerols and streptozotocin on hepatic microsomal cytochrome P450 I and IIB proteins determined using immunoblots. Assay conditions are described in the legend to Fig. 1. Analysis was carried out using polyclonal antibodies against P450 IA1 (A) and IIB1 (B). C, control; STZ, streptozotocin; MCT, medium chain triacylglycerols; Pb, phenobarbitone and 3MC, 3-methylcholanthrene.

described [10]. Microsomal proteins were solubilized in 0.1 M phosphate buffer pH 7.4 containing emulgen (10% v/v) and cholate (2% w/v) and treated with sodium dodecyl sulphate (SDS) prior to separation by SDS–polyacrylamide gel electrophoresis [11]. Proteins were transferred overnight onto nitrocellulose paper and immunostained essentially as described by Towbin *et al.* [12].

Blood samples were obtained from the cut tip of the tail of conscious rats and glucose [13], 3-hydroxybutyrate and acetoacetate concentrations [14] were determined in the plasma.

Results and Discussion

All animals were normoglycaemic except those treated with streptozotocin only, the mean plasma glucose levels being 7.3 ± 1.3 and 23.0 ± 2.2 mM, respectively. Hyperketonaemia (acetoacetate + 3-hydroxybutyrate) was displayed by the animals maintained on triacylglycerols and those that received STZ only, the mean plasma levels being 1.20 ± 0.15 and 1.14 ± 0.09, respectively, whereas levels in all other groups were 0.36 ± 0.09 mM.

Animals pretreated with streptozotocin exhibited higher levels of P450 I A2 when compared to control rats, whereas the levels of the A1 protein were unaffected (Fig. 1A). Similarly, treatment with STZ enhanced the levels of P450 IIB proteins (Fig. 1B). No such increases were evident in the animals which were treated daily with insulin following the administration of streptozotocin, indicating that the changes in the apoprotein levels of these families are due to the diabetic state rather than the diabetogen itself. Moreover, concurrent administration of nicotinamide with the streptozotocin, which is known to prevent the onset of diabetes [15], showed no difference from control animals in the hepatic levels of P450 I and IIB family apoproteins, demonstrating that streptozotocin *per se* administered to animals 3 weeks prior to the animals being killed did not influence the levels of these isoforms of cytochrome P450. In view of the role of the P450 I family in the metabolic activation of chemical carcinogens [16], the increase in this activity may explain the higher efficiency of hepatic preparations from diabetic animals in activating chemical carcinogens, such as the heterocyclic amines, whose activation is selectively catalysed by the P450 I A2 protein; the lack of increase in the A1 protein explains why the ability to activate polycyclic aromatic hydrocarbons, which rely primarily on the A1 protein, did not differ between diabetic and control animals [7, 17, 18]. Hepatic preparations from the rats were also more efficient than those from control animals in activating nitrosamines to mutagens [18]. The bioactivation of these precarcinogens, proceeding through an α-hydroxylation, involves cytochrome P450 IIE1, another form elevated in insulin-dependent (type I) diabetes [19], and to a lesser extent the phenobarbital-inducible P450 IIB family [20, 21] whose levels have been shown to increase in the present study and thus might contribute to the enhanced activation of nitrosamines.

Animals treated with triacylglycerols also exhibited higher levels of P450 I and IIB apoproteins similar to those observed in the STZ-treated animals (Fig. 2). Since these animals were normoglycaemic, it may be inferred that the hyperketonaemia that characterizes uncontrolled type I diabetes may be largely responsible for the diabetes-induced changes in the levels of cytochrome P450 I and IIB families.

In summary, the present immunological study confirms our previous work employing chemical probes, in which we demonstrated that STZ-induced diabetes increased the hepatic microsomal levels of P450 I and P450 IIB families [7] and that ketone bodies may mediate these changes [8].

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Structure–activity studies of flavonoids as inhibitors of hyaluronidase

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The flavonoids are a group of naturally occurring low molecular weight benzo-γ-pyrone derivatives, ubiquitous in plants [1]. Their pharmacological and pharmaceutical functions have been reviewed by Havsteen [1]. The effects exerted by flavonoids on animal systems include anti-inflammatory and anti-allergic activity, lipid peroxidation, RNA, DNA and protein synthesis, antiviral activity, capillary fragility and inhibition of tumour promotion [2, 3].

Hyaluronidases (EC 3.2.1.35) are enzymes which depolymerize or hydrolyse hyaluronic acid presumably by splitting glucosaminidic bonds to yield oligosaccharides [4]. This enzyme was earlier claimed to be involved in allergic effects [5], migration of cancer cells [6], inflammation, petechial haemorrhages following its injection in mesentery preparations and also the increase in permeability of the vascular system [5–7].

Tu and Hendon [8] reported that hyaluronidase plays a definite role in the penetration of venoms through tissues of a victim and can be considered as a spreading factor. The mechanism of malignant invasiveness was reported to be dependent upon the continuous release of lysosomal glycosidases (hyaluronidases) [6]. Further postulations were made that if a method of inhibiting tumour cell glycosidases (hyaluronidases) were found, it would be possible not only to restrain malignant invasiveness but also to retard malignant cell proliferation [6]. This information initiated our interest to investigate the effects of flavonoids on hyaluronidase. Bovine testis hyaluronidase, an easily available enzyme was employed as the model for our investigation. There have been a limited number of earlier reports describing the effects of a few flavonoids on the action of hyaluronidase *in vivo* and *in vitro* [9–11]. However, the present study was designed to investigate the structure–activity relationship and effects of a series of 31 flavonoids *in vitro* on bovine testis hyaluronidase. The kinetic studies of several potent flavonoids were further examined.

In view of the important role played by hyaluronidase in the aetiology and/or development of diseases [5–8] it would be very useful to find potent inhibitors of this enzyme for they could be used to develop new therapeutic agents.

Materials and Methods

Bovine testis hyaluronidase (sp. act. 290 National Formulary Unit/mg protein); human umbilical cord hyaluronic acid and hexadecyltrimethylammonium bromide (Cetrimide) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Flavonoids were obtained from Extrasynthese (Genay, France) and Sigma. Condensed tannin was supplied by Dr L. Butler, Purdue University, U.S.A. Other chemicals used were of the best analytical grade available.

Flavonoids were dissolved either in absolute ethanol or dimethylsulfoxide (100% DMSO). Stock solution (freshly prepared for each assay) had 25 μmol/mL for each flavonoid. Aliquots (10 μL) of the flavonoid solution were used in the assay mixture to give a final concentration of 250 μM.

Hyaluronidase activity was determined by the modified method of Xu *et al.* [12]. The assay system contained hyaluronic acid (100 μg), the enzyme (15 μg) and test compound (10 μL) in 1.0 mL of 0.2 M acetate buffer, pH 5.0 containing 0.15 M NaCl. Hyaluronidase (15 μg/mL) was preincubated with flavonoids (250 μM) for 15 min at 37° and the volume was made up to 900 μL with acetate buffer. Control tubes contained the enzyme plus 10 μL of either absolute ethanol or 100% DMSO and buffer in a final volume of 900 μL. After preincubation, the assay was commenced by adding hyaluronic acid (100 μL) to each tube and incubated for 45 min. All incubations were carried out in triplicate. Reactions were terminated by the addition of 2 mL of cetrimide (2.5% w/v) in 2% (w/v) NaOH solution ('stop reaction' solution). The percentage of inhibition was calculated as follows:

$$\text{Inhibition (\%)} = \frac{\text{Activity of control} - \text{Activity in the presence of flavonoids}}{\text{Activity of control}} \times 100\%.$$