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HYPERINSULINAEMIA CAUSES A PREFERENTIAL INCREASE IN HEPATIC P4501A2 ACTIVITY

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Abstract—Male NEDH (New England Deaconess Hospital) rats were transplanted with a radiation-induced tumour from a donor male rat and were killed 18 days following transplantation. At the time of killing the insulinoma-bearing animals were severely hypoglycaemic but plasma ketone levels were normal. Insulinoma-bearing animals exhibited higher hepatic O-deethylation of ethoxyresorufin and N-demethylation of ethylmorphine activities when compared to control animals. Similarly, hepatic microsomal preparations from insulinoma-bearing rats were more efficient than control animals in converting the promutagen 2-amino-6-methyldipyrido[1,2-*a*:3′,2′]imidazole (Glu-P-1) to mutagenic intermediates in the Ames test. Immunoblot analysis employing polyclonal antibodies against the P4501A and P453A families revealed that insulinoma-bearing rats had higher hepatic P4501A2 apoprotein levels. No major differences in P4503A1 apoprotein levels between insulinoma-bearing and control rats were noted. Subcutaneous administration of insulin to male Wistar rats gave rise to a modest increase in ethoxyresorufin *O*-deethylase activity and in the ability to activate Glu-P-1 to mutagens in the Ames test. Immunoblot analysis revealed an increase in hepatic P4501A2 apoprotein levels following the treatment with insulin. It is concluded that insulinoma-bearing rats display high P4501A2 activity and the hyperinsulinaemia that characterize this condition is responsible for the effect. Moreover, administration of insulin to other strains of rat, such as Wistar, also enhances P4501A2 activity, presumably as a result of hyperinsulinaemia.

The cytochrome P450-dependent mixed-function oxidases comprise the most important enzyme system in the metabolism of both endogenous and exogenous chemicals [1]. It is involved in the turnover of numerous physiological substrates including steroids, fatty acids, vitamin D, cholesterol and prostaglandins [2] and is also concerned with the metabolic activation and deactivation of a wide variety of structurally diverse xenobiotics [3]. Almost every xenobiotic that enters the systemic circulation is subject to oxidative metabolism catalysed by this enzyme system. The mixed-function oxidase system owes this broad specificity to the fact that it exists as a number of families, comprising one or more subfamilies each of which may consist of one or more proteins having distinctly different substrate specificity [2].

The hepatic levels of many cytochrome P450 proteins appear to be under hormonal regulatory control. Hypophysectomy has been demonstrated to suppress the levels of some cytochrome P450 proteins [4] but to increase the levels of others [5]. Administration of growth hormone to the hypophysectomized animals restored proteins to control levels. Hypoinsulinaemia induced by administration of streptozotocin to rats has also been shown to alter markedly the hepatic cytochrome P450 composition [6–9]. As a result diabetic rats were more susceptible to the hepatotoxicity of haloalkanes [10] and hepatic preparations from these animals were more efficient than preparations from control animals in catalysing the activation of many chemical procarcinogens [11, 12]. The diabetes-induced changes in the hepatic cytochrome P450 complement are not mediated by the changes in glucose levels but at least two other factors appear to be responsible, namely, the reduction in circulating growth hormone levels and the hyperketonaemia that accompanies severe insulin deficiency [6, 8, 9, 13, 14].

A far less common disease is enteropancreatic endocrine cancer which is associated with hyperinsulinaemia, resulting from the unrestrained secretion of the hormone by an islet cell tumour [15]. It was therefore of interest to investigate whether hyperinsulinaemia, like hypoinsulinaemia, modulates the hepatic cytochrome P450-dependent mixed-function oxidases. The model of hyperinsulinaemia used was the serially transplantable radiation-induced NEDH (New England Deaconess Hospital‖) rat insulinoma [16]. When transplanted into rats of the same strain, the tumour displays rapid

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‖ Abbreviations: NEDH, New England Deaconess Hospital; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′]-imidazole.

growth leading to marked hyperinsulinaemia, severe hypoglycaemia and neuroglycopenic coma within 1 month following transplantation [17]. The metabolic effects consequent to transplantation of the tumour are similar to those associated with spontaneous insulinoma in man [15], rendering these rats an appropriate animal model [17, 18].

Using the NEDH insulinoma-bearing rat as the animal model, we have investigated the effect of hyperinsulinaemia on the hepatic cytochrome P450 proteins. Cytochrome P450 families were monitored by the use of specific substrates and specific antibodies.

MATERIALS AND METHODS

Long-acting monocomponent human insulin (Ultratard; Novo Industries, Copenhagen, Denmark), pentoxyresorufin, ethoxyresorufin and resorufin (Molecular Probes, Eugene, OR, U.S.A.), ethylmorphine (May and Baker, Dagenham, U.K.), 4-nitrocatechol (Aldrich Chemical Co. Ltd, Gillingham, U.K.), [^{14}C]lauric acid (Amersham, U.K.), Glu-P-1 (2-amino-6-methyldipyrido[1,2-*a*:3',2']imidazole) (Wako Fine Chemicals, Neuss, Germany) and 3-methylcholanthrene and all cofactors (Sigma, Poole, U.K.) were all purchased. The *Salmonella typhimurium* strain TA98 was a generous gift from Professor B. N. Ames, Berkeley, CA, U.S.A.

Male Wistar albino (150–180 g) and male NEDH (150–180 g) rats were purchased from the Experimental Biology Unit, University of Surrey. The NEDH rats were transplanted with a radiation-induced tumour from a donor male insulinoma-bearing NEDH rat. The tumour and surrounding capsule were rapidly removed and the minced tumour (0.15 mL/rat) was implanted subcutaneously into the subcapular region of lightly ether-anaesthetized rats using a 16 gauge needle. Plasma glucose levels were determined following withdrawal of blood samples from the tail tip of conscious rats. The animals were killed when they exhibited hypoglycaemia, which was 18 days following transplantation. In the second study, the Wistar albino rats received daily subcutaneous administration of insulin (10 or 20 U/kg) for 4 days and were killed 24 hr after the last administration. Lastly, to serve as positive controls for the induction of the P4501 and P4503 families, groups of previously untreated rats were treated with single daily intraperitoneal administrations of either 3-methylcholanthrene (25 mg/kg) or pregnenolone 16α-carbonitrile (100 mg/kg), respectively, for 3 days, all animals being killed 24 hr after the last administration.

In both studies livers were immediately excised and hepatic microsomal fractions were prepared as described previously [19]. The following determinations were carried out: ethoxyresorufin *O*-deethylase [20], pentoxyresorufin *O*-dependentylase [21], *p*-nitrophenol oxidase [22], ethylmorphine *N*-demethylase [23], NADPH-cytochrome *c* reductase [24], cytochrome b_5 and total cytochromes P450 [25] and microsomal protein using bovine serum albumin as standard [26].

The metabolic activation of Glu-P-1 to mutagens was monitored using the Ames mutagenicity test [27]. The activation system contained 10% (v/v) of microsomal preparation (25% w/v) and was supplemented with glucose-6-phosphate dehydrogenase (1 U/plate). Glu-P-1 (2 μg/plate) was dissolved in dimethyl sulphoxide (100 μL). Activation system, bacteria and Glu-P-1 were preincubated for 1 hr at 37° in a shaking waterbath.

The purification and characterization of P4501A1 (P450c) from male Wistar albino rats pretreated with 3-methylcholanthrene have been described elsewhere [28]. Antibodies to P4501A1 were raised in a single sheep after priming with 1.0 mg and boosting with 0.25 mg of the antigen. The antibodies could detect both members of the P4501 family, i.e. A1 and A2 and did not cross-react with any other isoforms. The antibodies to cytochrome P4503A1 were raised in rabbits [29]. In addition to the P4503A1 protein, the antibodies recognized bands of lower M_r [8] which may represent other steroid-inducible proteins; P4503 appears to be a large gene family [2]. Immunoblot analysis of the microsomal proteins, following resolution by electrophoresis [30], was carried out essentially as described by Towbin *et al.* [31].

Statistical analysis was carried out using the Student's *t*-test.

Table 1. Plasma glucose and ketones in insulinoma-bearing and insulin-treated rats

Animal group	Glucose (mM)	3-Hydroxybutyrate (mM)	Acetoacetate (mM)
(A)			
Control (NEDH)	6.1 ± 0.3	0.36 ± 0.05	0.14 ± 0.04
Insulinoma-bearing	2.8 ± 0.4*	0.42 ± 0.02	0.21 ± 0.04
(B)			
Control (Wistar)	5.1 ± 0.2	0.31 ± 0.02	0.08 ± 0.01
Insulin (10 U/kg)	4.6 ± 0.2	0.32 ± 0.01	0.12 ± 0.04
Insulin (20 U/kg)	4.5 ± 0.3	0.16 ± 0.04	0.09 ± 0.01

Insulin-treated animals received daily subcutaneous doses of the hormone at two dose levels, 10 and 20 U/day, for 4 days. Animals were killed 24 hr after the last injection.

Results are presented as mean ± SEM for four animals.

* $P < 0.01$.

Table 2. Hepatic microsomal mixed-function oxidase activity in insulinoma-bearing NEDH rats

Parameter	Control	Insulinoma-bearing
Ethoxyresorufin *O*-deethylase (pmol/min/mg protein)	7 ± 1	19 ± 3†
Pentoxyresorufin *O*-depentylase (pmol/min/mg protein)	3.4 ± 0.9	4.2 ± 0.4
p-Nitrophenol oxidase (nmol/min/mg protein)	1.5 ± 0.1	0.9 ± 0.1‡
Ethylmorphine *N*-demethylase (nmol/min/mg protein)	2.2 ± 0.3	4.6 ± 0.4†
Lauric acid hydroxylase (nmol/min/mg protein)	2.1 ± 0.2	1.4 ± 0.2*
NADPH-cytochrome *c* reductase (nmol/min/mg protein)	8.4 ± 1.1	10.1 ± 1.3
Cytochrome b_5 (nmol/mg protein)	0.35 ± 0.05	0.36 ± 0.03
Total cytochrome P450 (nmol/mg protein)	0.38 ± 0.03	0.38 ± 0.02
Microsomal protein (mg/g liver)	27.3 ± 0.5	28.2 ± 1.6

Results are presented as mean ± SEM of four animals.
*$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

RESULTS

As expected, NEDH insulinoma-bearing rats exhibited markedly lower glucose levels when compared to control rats, but no significant differences were observed in the plasma ketone levels as exemplified by 3-hydroxybutyrate and acetoacetate (Table 1A). The hepatic O-deethylation of ethoxyresorufin and the N-demethylation of ethylmorphine were significantly higher in the insulinoma-bearing rat whereas, in contrast, *p*-nitrophenol oxidation and lauric acid hydroxylation were significantly lower (Table 2). Hepatic microsomal preparations from insulinoma-bearing rats were markedly more efficient than similar preparations from control animals in converting the promutagen Glu-P-1 to mutagenic intermediates in the Ames test (Table 3A). Immunoblot analysis employing antibodies to the P4501A family revealed that the insulinoma-bearing rats possessed higher levels of the P4501A2 apoprotein (Fig. 1). When antibodies to the P4503A family were used, the A1 protein was detectable only in the microsomes derived from animals treated with pregnenolone 16α-carbonitrile and serving as positive controls (Fig. 2). No band corresponding to the P4503A1 protein was detected in control NEDH rats. Two other proteins recognized by the antibodies were present in all animals. One of these (a) was induced by pregnenolone 16α-carbonitrile treatment whereas the second one (b) was higher in the insulinoma-bearing rats.

Table 3. Bioactivation of Glu-P-1 to mutagens by microsomal preparations from insulin-treated and insulinoma-bearing rats

Animal group	Mutagenic response (histidine revertants/mg protein)
(A)	
Control (NEDH)	477 ± 79
Insulinoma-treated	4881 ± 204
(B)	
Control (Wistar)	2305 ± 147
Insulin (10 U/kg)	3905 ± 237
Insulin (20 U/kg)	5967 ± 272

Glu-P-1 (2 μg/plate) was incubated with *Salmonella typhimurium* strain TA98 and an activation system containing microsomes (25% w/v) at a final concentration of 10% (v/v) and fortified with glucose-6-phosphate dehydrogenase (1 U/plate). These were preincubated for 30 min at 37° in a shaking waterbath. The spontaneous reversion rate of 45 ± 10 histidine revertants/plate has already been subtracted.

Results are presented as mean ± SD of triplicates.

Wistar rats pretreated with insulin, at two dose levels, had slightly lower levels of plasma glucose 24 hr after the last injection but the effect was not statistically significant (Table 1B). Of the mixed-function oxidases studied only the O-deethylation of ethoxyresorufin was higher in the hormone-receiving animals (Table 4). Moreover, insulin administration gave rise to a dose-dependent increase in the metabolic activation of Glu-P-1 to mutagens in the Ames test (Table 3B). A dose-dependent increase following insulin administration was also seen in the P4501A2 microsomal apoprotein levels (Fig. 3).

DISCUSSION

The hepatic cytochrome P450 proteins were monitored by the use of diagnostic substrates. The insulinoma-bearing animals displayed a significantly higher ethoxyresorufin *O*-deethylase activity, a reaction exclusively catalysed by the P4501 family, and especially the A1 isoenzyme [32, 33]. In order to discern between the two P4501 isoenzymes, the ability of hepatic microsomal preparations from insulinoma-bearing and control animals to convert the procarcinogen Glu-P-1 to mutagens in the Ames test was determined, since this is catalysed exclusively

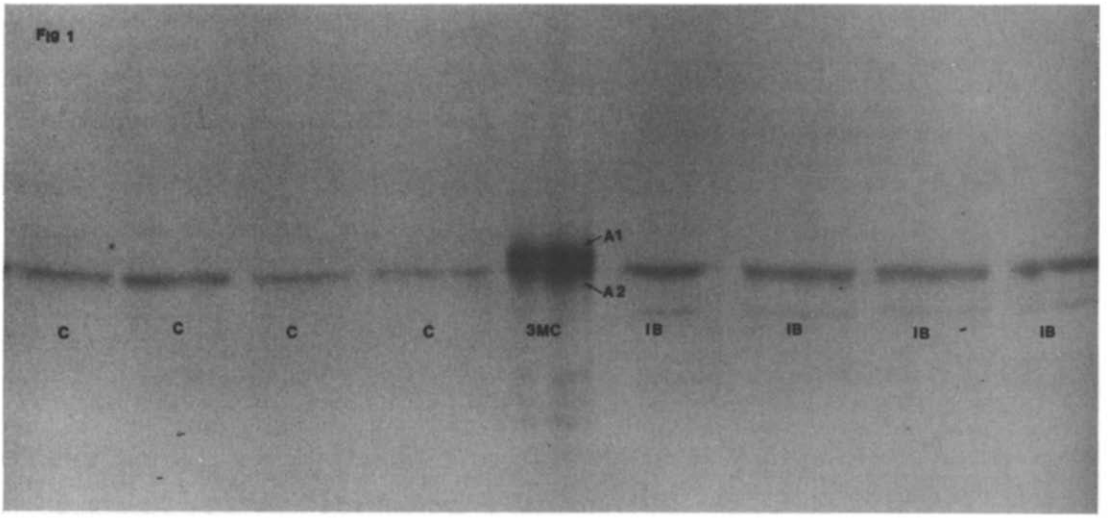

Fig. 1. Immunoblot analysis employing anti-(cytochrome P4501A1) polyclonal antibodies. Microsomal proteins (20 μg) from control and insulinoma-bearing animals were resolved by electrophoresis in a 10% (w/v) sodium dodecyl sulphate–polyacrylamide gel and transferred electrophoretically to nitrocellulose. The immunoblot was carried out with sheep anti-(cytochrome P4501A1) (diluted 1:10,000) followed by peroxidase-linked donkey anti-sheep IgG (diluted 1:2000). C, Control; 3MC (10 μg loaded), 3-methylcholanthrene-treated and IB, insulinoma-bearing.

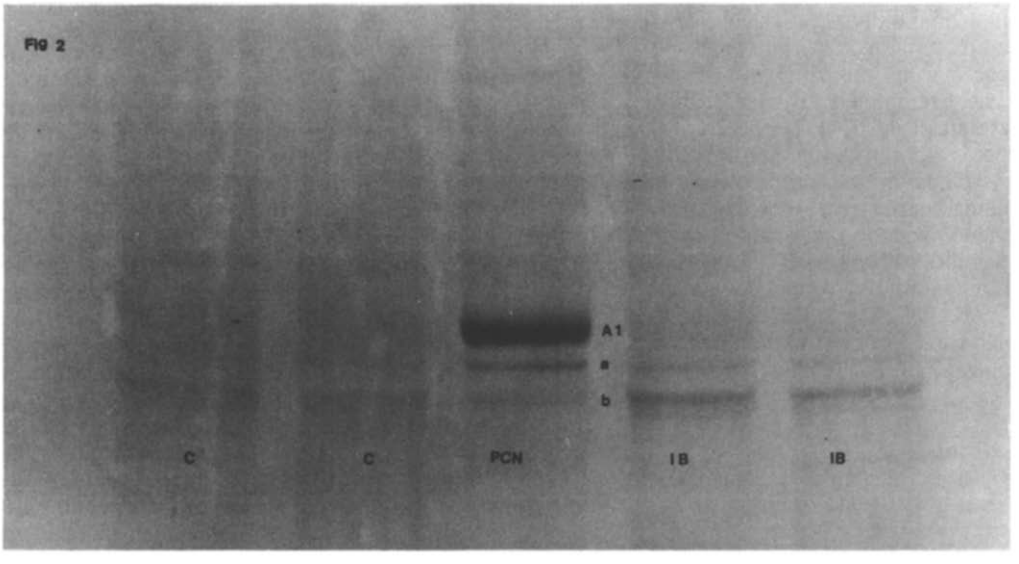

Fig. 2. Immunoblot analysis employing anti-(cytochrome P4503A1) polyclonal antibodies. Microsomal proteins (20 μg) from control and insulinoma-bearing animals were resolved by electrophoresis in a 10% (w/v) sodium dodecyl sulphate–polyacrylamide gel and transferred electrophoretically to nitrocellulose. The immunoblot was carried out with rabbit anti-(cytochrome P4503A1) (diluted 1:800) followed by peroxidase-linked donkey anti-rabbit IgG (diluted 1:2000). C, Control; PCN, pregnenolone 16α-carbonitrile-treated and IB, insulinoma-bearing.

by the P4501A2 protein [34]. Activation of Glu-P-1 to mutagens was markedly more effectively catalysed by the microsomal preparations derived from the tumour-bearing animals when compared to controls, indicating that the increase in the O-deethylation of ethoxyresorufin may largely reflect an increase in the A2 protein. This was further confirmed in immunoblot analysis where it was evident that the animals transplanted with the radiation-induced insulinoma exhibited higher microsomal apoprotein levels of P4501A2, but not of A1 in hepatic microsomes. It is worth pointing out that even in streptozotocin-induced insulin deficiency only the P4501A2 protein was induced [9]. These observations provide further evidence that the two P4501 proteins are under different regulatory control and that the

Table 4. Effect of insulin administration on the hepatic microsomal cytochrome P450 system in the Wistar rat

Parameter	Control	Insulin (10 U/kg)	Insulin (20 U/kg)
Ethoxyresorufin *O*-deethylase (pmol/min/mg protein)	13 ± 2	23 ± 1†	20 ± 3*
p-Nitrophenol oxidase (nmol/min/mg protein)	1.2 ± 0.1	0.9 ± 0.1	1.0 ± 0.2
Ethylmorphine *N*-demethylase (nmol/min/mg protein)	8.2 ± 1.4	10.3 ± 2.1	11.1 ± 1.8
Totaly cytochrome P450 (nmol/mg protein)	0.40 ± 0.05	0.51 ± 0.01	0.46 ± 0.04
Microsomal protein (mg/g liver)	35.0 ± 3.6	34.5 ± 2.0	39.0 ± 2.0

Insulin-treated Wistar rats received daily subcutaneous doses of the hormone at two dose levels, 10 and 20 U/day, for 4 days. Animals were killed 24 hr after the last injection. Results are presented as mean ± SEM for four animals.
*$P < 0.05$; †$P < 0.01$.

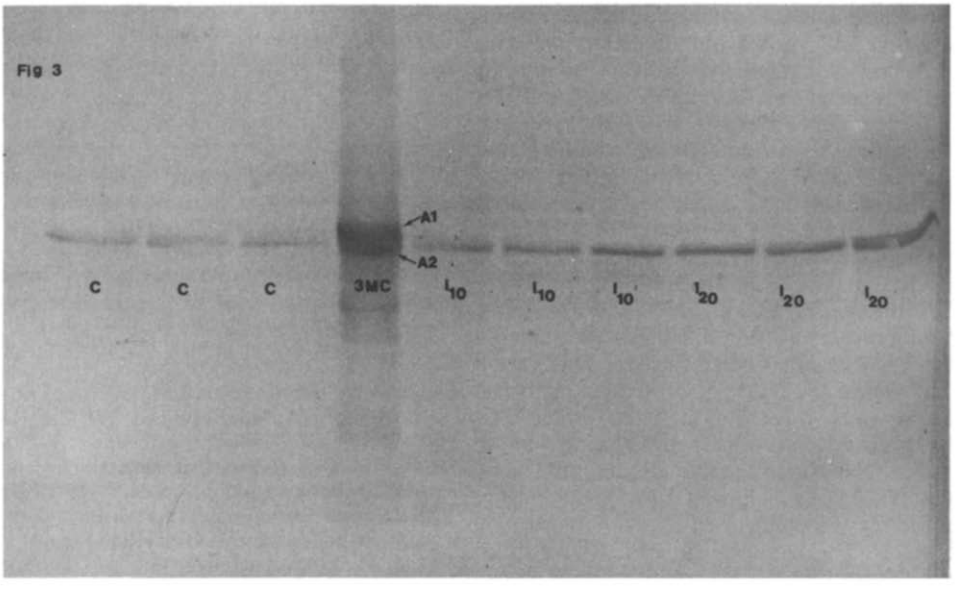

Fig. 3. Immunoblot analysis employing anti-(cytochrome P4501A1) polyclonal antibodies. Microsomal proteins (20 μg) from control and insulin-treated animals were treated as described in the legend to Fig. 1. C, Control; 3MC (10 μg loaded), 3-methylcholanthrene-treated; I_{10}, insulin (10 U/kg) and I_{20}, insulin (20 U/kg).

A2 protein is sensitive to changes in circulating insulin levels. Moreover, it demonstrates that deviations from normal insulinaemia, both increases and decreases, induce P4501A2 activity.

Insulinoma-bearing rats also exhibited higher ethylmorphine *N*-demethylase activity than the controls, an enzyme closely associated with the P4503 family [35] indicating that the presence of the insulinoma may enhance also the hepatic activity of this family. Once again a similar increase was seen during chemically induced hypoinsulinaemia [8]. Immunoblot analysis using antibodies to P4503A1 detected no A1 protein in both control and insulinoma-bearing rats. However, in insulinoma-bearing rats an increase could be seen in the levels of a lower molecular size band detected by the same antibody. It remains to be established whether this protein is responsible for the enhanced ethylmorphine *N*-demethylase activity. Moreover, the increase in this activity may represent changes in P4502C11 protein, which also displays some catalytic activity towards ethylmorphine [36]. In contrast to the above findings, the presence of the insulinoma decreased the oxidation of *p*-nitrophenol and the hydroxylation of lauric acid, two activities associated with the P4502E and P4504 families, respectively [37, 38]. The O-dealkylation of pentoxyresorufin, a reaction used to monitor P4502B activity [21], was unaffected

by the presence of the insulinoma. Since the levels of total microsomal cytochrome P450 are unchanged by the treatment, it may be inferred that the observed increases in the P4501A2 levels may occur at the expense of other cytochrome P450 families including P4502E and 4. It has already been established that some agents may selectively induce the levels of one family of cytochrome P450 while decreasing others [39]. Finally, the NADPH-dependent reduction of cytochrome *c* was unaffected by the insulinoma, indicating that the flow of electrons to cytochrome P450 is unlikely to be impaired.

It is logical to argue that the observed changes in hepatic cytochrome P450 proteins in the insulinoma-bearing rats may not be the result of the hyperinsulinaemia, but are the consequence of the physiological changes brought about by the presence of an invasive tumour. In order to address this point Wistar albino rats, a strain frequently used in toxicological studies, were treated with insulin to ensure that hyperinsulinaemia is the causative factor for these changes and that the effects are not limited to the NEDH strain of rat. A slight drop in the plasma glucose levels, 24 hr following the last insulin administration, was noted but the effect was not statistically significant. A modest but significant increase was seen in the P4501-mediated O-deethylation of ethoxyresorufin. The increased ability of hepatic microsomes from insulin-treated rats to activate Glu-P-1 to mutagens indicates that the enhanced O-deethylation of ethoxyresorufin is due to the P4501A2 protein and this is further substantiated by the immunoblot analysis. The marked difference in the activation of Glu-P-1 between untreated NEDH and Wistar rats most probably represents a strain difference. Although a modest increase was also seen in the N-demethylation of ethylmorphine the effect did not reach statistical significance. Thus, in general, the effects induced by insulin administration resemble those brought about by the subcapular transplantation of the insulinoma, indicating that hyperinsulinaemia is, at least partly, responsible for the changes in hepatic cytochrome P450 proteins. The fact that the effects were much more pronounced in the insulinoma-bearing rats is not surprising since the degree of hyperinsulinaemia is much higher in these animals, as exemplified by the severe hypoglycaemia, and the insulin levels are maintained by continuous tumour secretion [17]. Subcutaneous administration of insulin would result in lower levels of insulin which would fluctuate as the hormone is gradually cleared from the circulation.

In contrast to insulin deficiency, the hormonal and other biochemical changes that accompany hyperinsulinaemia have not been fully explored. In streptozotocin-induced hypoinsulinaemia, the perturbation of cytochrome P450 families has been attributed to the high levels of ketones that function as inducing agents for P4501, 2B, and 2E families, and to modulation of growth hormone that plays a major role in the regulation of cytochrome P450 families such as P4503 [5, 8, 9, 14]. Consistent with previous observations [17] no significant change was noted in the circulating levels of 3-hydroxybutyrate and acetoacetate so that the first mechanism may be excluded. Moreover, hyperketonaemia causes a marked increase in the microsomal levels of the P4502B proteins [9], which was not induced in the hyperinsulinaemic state. It is possible that hyperinsulinaemia triggered the release of an endogenous planar substrate, that can act as an inducer of P4501A2, although other mechanisms may be also operative. It should be pointed out that the physiological function of P4501 family has not yet been established.

In summary, the present study demonstrates that hyperinsulinaemia causes a selective increase in the hepatic levels of P4501A2, an enzyme closely associated with the activation of chemical carcinogens, particularly those containing an exocyclic amino group [3], and which is present in the human liver [40].

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