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LACK OF EFFECT OF INSULIN IN HEPATOCYTES ISOLATED FROM STREPTOZOTOCIN-DIABETIC MALE RATS

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Abstract—Diabetes mellitus is known to affect drug and steroid metabolism in the rat liver. Recently we have demonstrated that *in-vitro* insulin addition to hepatocytes obtained from normal male rats showed a significant dose-related increase in androstenedione metabolism. We have extended our study this time by using 3- and 21-days streptozotocin (STZ) diabetic and insulin-treated STZ-diabetic male rats. Hepatocytes from 3- and 21-days STZ-diabetic rats were resistant to the effect of insulin while insulin-treated diabetic rats indicated partial restoration of insulin effect. Since insulin resistance is a characteristic feature of type II diabetes mellitus, we would like to suggest that STZ-diabetic rats may be a model for type II diabetes mellitus.

Diabetes mellitus is a chronic disorder characterised by a raised level of glucose in the blood. It can be classified into two major types: Type I or insulin-dependent diabetes mellitus and Type II or insulin-independent diabetes mellitus. Streptozotocin (STZ) derived from *Streptomyces achromogenes* [1] selectively destroys the pancreatic β-cell with production of permanent diabetes [2], and thus, STZ-treated animals have been used as a model for type I diabetes. We have used this model to study the effect of diabetes on hepatic drug and steroid metabolism. Our results indicated that the effect of STZ-induced diabetes mellitus on drug metabolism was both transient and sex-dependent [3] and there is no apparent correlation between the hyperglycaemic effect of STZ and its effect on drug metabolism [4], while the effect on steroid metabolism is always to abolish the sex differences found in intact animals [5].

Steroid metabolism is closely linked to that of drugs [6] and is regulated by intricate interactions of the body hormonal system [7]. Hence, it is necessary to use isolated hepatocytes to examine the effect of a single hormone alone. We have devised a method for maintaining steroid metabolism in hepatocyte cultures in serum-free medium [8]. We have recently looked at the effect of insulin alone on steroid metabolism [9] and found that insulin treatment causes an increase in the activities of all of the steroid metabolizing enzymes studied. This suggests a major role for insulin in the regulation of hepatic steroid metabolism in the rat. However, the effects were not the same as would have been expected from *in-vivo* studies, substantiating the fact that a more complex hormonal control is operating *in vivo*.

We have, in this study, tested the effects of *in-vivo* STZ-treatment on the responsiveness of hepatocytes to insulin.

MATERIALS AND METHODS

(i) *Chemicals*. Streptozotocin, 4-androstene-3,17-dione and bovine serum albumin were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) Ham's F-10 medium was obtained from Gibco BRL Ltd. (Paisley, Scotland) and collagenase from Boehringer Mannheim Co. (F.R.G.).

4-[4-^{14}C]-androstene-3,17-dione was supplied by Amersham International p.l.c. (Aylesbury, U.K.). Porcine insulin obtained from Novo Research Institute (Copenhagen, Denmark) was used *in vitro* and Neulente Insulin Zinc Suspension BP, from Wellcome (Welwyn Garden City, U.K.), was used for *in-vivo* treatment. All other chemicals were of analytical grade purity. The kit for the measurement of serum glucose was purchased from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.).

(ii) *Animals*. Male rats of the Wistar strain, bred in the department were maintained in light- and temperature-controlled conditions (lights on 0700–1900 and 19 ± 1°) and were used when they were 10–12 weeks old (200–250 g). Chemically induced diabetic animals received streptozotocin (100 mg/kg in distilled water) via tail vein injection, given under nitrous oxide/halothane anaesthesia and then sacrificed 3 and 21 days post treatment. Control animals were injected under anaesthesia with vehicle only. Where appropriate, 12 units insulin (Neulente Insulin Zinc Suspension) was given 1 hr after streptozotocin and at 24 hr intervals for 2 days.

(iii) *Preparation of liver cells*. Isolated hepatocytes were prepared by a modification of the collagenase perfusion technique of Seglen [10]. The animals were anaesthetised with nitrous oxide/halothane and the liver was perfused *in situ* through the hepatic portal vein with calcium-free Hank's Balanced Salt Solution (Hank's BSS) for 8 min followed by the collagenase buffer (Hank's BSS supplemented with 4 mM cal-

cium chloride and 0.5 mg collagenase/ml), until the liver appeared to have broken up. The liver was removed from the animal and cells dispersed in the calcium-free Hank's BSS using a steel comb. The cell suspension was filtered through gauze and centrifuged at 200 *g* for 2 min (in a DAMON/IEC Model DPR-6000 refrigerated centrifuge) to clean the parenchymal cells of debris. The supernatant was removed and cells resuspended in incubation medium (Hank's BSS supplemented with 1 g/l glucose. 100 mg/l $MgSO_4$, 100 mg/l $MgCl_2$ and 185 mg/l $CaCl_2$). The cells were counted using a haemocytometer and assayed for viability using trypan blue exclusion. Average yield was 10^8 cell/g liver and viability exceeded 90% in all cases. Primary cultures of hepatocytes were established by plating cells in 9 cm Petri dishes (NUNCLON, Denmark) at a density of 3×10^5 cells/cm^2 in Ham's F-10 medium supplemented with 0.1% bovine serum albumin.

(iv) *Addition of hormone.* Insulin was added (at various concentrations between 10^{-10} and 10^{-6} M) to the plated cells, which were subsequently incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37° for ½ hr as described previously [9].

(v) *Assay of steroid metabolism.* After the indicated time period, the cells were scraped off into incubation medium and incubated with 4-androstene-3,17-dione as previously described [9].

(vi) *Serum glucose assay.* Serum glucose was assayed by the method of Carroll *et al.* [11] using authentic standards to obtain a standard curve.

(vii) *Calculations and statistics.* Results were expressed as percentage of relevant controls. Means and standard deviations were calculated and statistical analysis performed by means of Student's *t*-test. The level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

The enzyme assay employed allows the determination of 7α-, 6β- and 16α-hydroxylases, 17-oxosteroid oxidoreductase (OHSD) and 5α-reductase activities. Table 1 illustrates the four major groups of animals, i.e. control, 3- and 21-days STZ-diabetic and insulin treated STZ-diabetic animals. The serum glucose levels in the in the diabetic groups (3- and 21-days STZ) were grossly elevated and this was reduced to control level by insulin treatment. Animals from the diabetic groups showed some weight loss while the insulin-treated animals were not different from control. From these data, the 3 days and 21 days STZ-treated rats were established to be diabetic. In this study it was shown that STZ-induced diabetes mellitus causes a change in the metabolism of 4-androstene-3,17-dione. In 3 days STZ-diabetic, these changes are dependent on the enzyme being studied. There was a decrease in the rate of the male-specific enzymes activities (6β-hydroxylase and 17-OHSD) while the female-specific enzymes activities (7α-hydroxylase and 5α-reductase) were increased. This data is in agreement with the results obtained with hepatic microsomes [5, 12] with the exception of 16α-hydroxylase whose activity was unexpectedly increased. The reason for this is unknown. Chronic diabetes (21 days) gave somewhat different results with no changes being seen on 7α- and 16α-hydroxylases and lowering of activities seen in 6β-hydroxylase and 5α-reductase. The overall effect of chronic diabetes is much less than that seen in the acute phase, a result strikingly similar to that observed in microsomal drug metabolism [3]. Though there are some differences when compared with hepatic microsomes, hepatocytes derived from STZ-diabetic animals seem to be a good model for the study of steroid metabolism and probably represent a more physiological situation.

Hepatocytes have been demonstrated to provide a useful model system for *in-vitro* study of insulin action. Some of the studies include insulin-stimulated lipid synthesis and aminoisobutyric acid uptake [13], protein and glycogen synthesis [14]. In this study, insulin *in vitro* elicits a dose-related increase in all the measured enzyme activities (Fig. 1), confirming the results in our previous study [9]. Hepatocytes from normal rats responded in a dose-dependent manner to insulin for all the enzymes activities measured (Fig. 1), with a significant increase in activity as low as 10^{-10} M insulin which had not reached a maximum at 10^{-6} M.

However, primary cultures of hepatocytes obtained from 3 days STZ-diabetic rats were not responsive to insulin *in vitro* (Fig. 2). All the measured enzymes activities were not significantly different to that of control throughout the range

Table 1. The effect of streptozotocin and insulin treatment on the metabolism of 4-androstene-3,17-dione by isolated male rat hepatocytes, serum glucose concentration and changes in body weight in male rat

Parameter	Control*	3 days STZ diabetic	21 days STZ diabetic	3 days STZ + Insulin (*In-vivo*)
7α-OHase	37 ± 6	60 ± 9†	40 ± 7	49 ± 9†
6β-OHase	53 ± 7	38 ± 7†	34 ± 2†	81 ± 3†
16α-OHase	43 ± 6	80 ± 10†	44 ± 3	75 ± 7†
17-OHSD	113 ± 6	85 ± 2†	123 ± 2†	116 ± 5
5α-reductase	117 ± 3	130 ± 9†	86 ± 9†	112 ± 5
Serum glucose	5.9 ± 0.7	19.7 ± 2.5†	16.3 ± 3.3†	7.3 ± 1.2
Initial wt (g)	225 ± 8	230 ± 10	220 ± 8	225 ± 9
Change in weight (g)	5 ± 2	−20 ± 3†	−25 ± 4†	−2 ± 5

Hepatocytes were cultured for 24 hr prior to incubation.
* All enzyme activities expressed as *p*moles product/min/10^6 cells and as mean ± SD of 4 animals.
† Significantly different from the relevant control; $P < 0.05$.

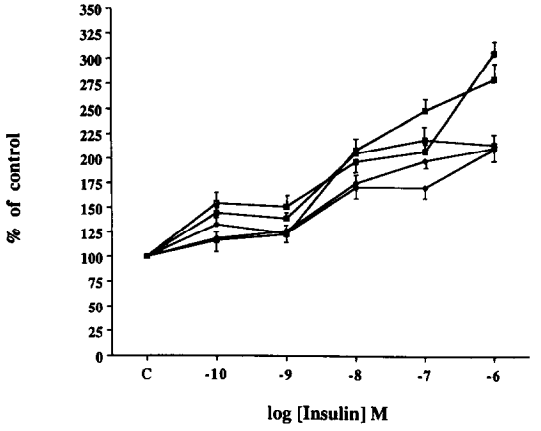

Fig. 1. The effect of insulin (10^{-10} to 10^{-6} M) on 7α- (□), 6β- (◆) and 16α- (■) hydroxylation, 17-OHSD (◇) and 5α- (■) reduction of 4-androstene-3,17-dione by hepatocytes obtained from normal male rat. Results expressed as mean ± SD of N value = 3; All points were significantly higher than respective control. 100% value (7α- =37 ± 6; 6β- = 53 ± 7 and 16α-hydroxylases = 43 ± 6; 17-OHSD = 113 ± 6 and 5α-reductase = 117 ± 3 *p*moles/min/10^6 cells respectively).

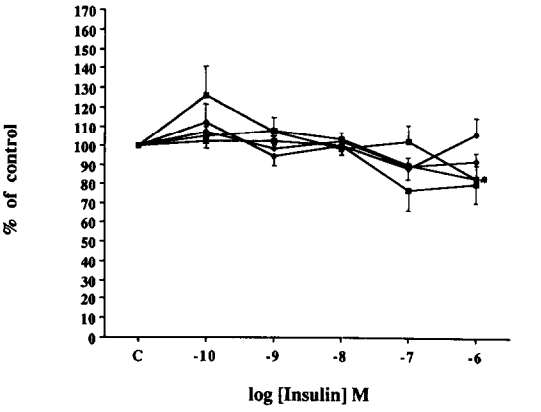

Fig. 3. The effect of insulin (10^{-10} to 10^{-6} M) on 7α (□), 6β- (◆) and 16α- (■) hydroxylation, 17-OHSD (◇) and 5α- (■) reduction of 4-androstene-3,17-dione by hepatocytes obtained from 21 days STZ-induced diabetic male rat. Results expressed as mean ± SD of N value = 3; *P < 0.05 as compared to the respective control. 100% value (7α- = 40 ± 7; 6β- = 34 ± 2 and 16α-hydroxylases = 44 ± 3; 17-OHSD = 123 ± 2 and 5α-reductase = 86 ± 9 *p*moles/min/10^6 cells respectively).

of insulin concentrations used. Although 17-OHSD activity in the 3-days STZ diabetic rats (Fig. 2) was from 14% to 26% above control, the changes were statistically insignificant. Resistance to the acute effects of insulin on lipogenesis has also been reported in primary cultures of hepatocytes from diabetic and fasted rats [15] and this occurred in the presence of normal to increased insulin binding [16] implying a postreceptor defect in insulin's action. The pathogenesis of insulin resistance still remains unclear. The currently available evidence indicates that the insulin resistance is largely related to a postreceptor defect in insulin action. Since insulin action involves a cascade of events, abnormalities or defects in any of the effector systems distal to receptor binding can lead to impaired insulin action and insulin resistance. STZ-induced diabetes has been reported to be associated with both a decrease in calmodulin content and low K_m phosphodiesterase activity [17, 18] as well as an apparent subcellular redistribution of these components [17]; modifications in function and structure of insulin receptors [19]; decreased autophosphorylation of the insulin receptor [20, 21]; altered responsiveness of hormone-sensitive adenylate cyclase in liver [22]; modification of a distinct species of guanine nucleotide regulatory protein [N_{ins}] [23]; a decrease in hepatic guanylate cyclase activity [24]; decreased IGF-I pro-

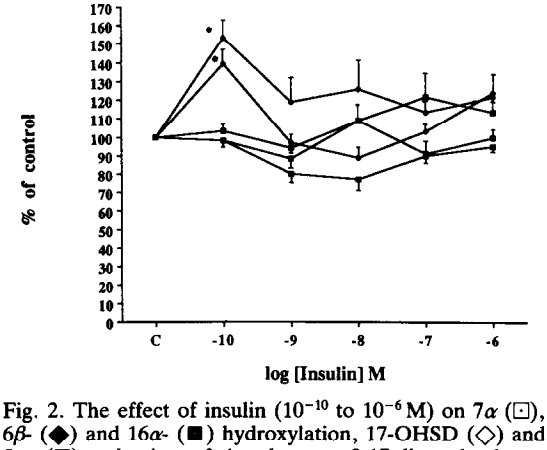

Fig. 2. The effect of insulin (10^{-10} to 10^{-6} M) on 7α (□), 6β- (◆) and 16α- (■) hydroxylation, 17-OHSD (◇) and 5α- (■) reduction of 4-androstene-3,17-dione by hepatocytes obtained from 3 days STZ-induced diabetic male rat. Results expressed as mean ± SD of N value = 3; *P < 0.05 as compared to the respective control. 100% value (7α- = 60 ± 9; 6β- = 38 ± 7 and 16α-hydroxylases = 80 ± 10; 17-OHSD = 85 ± 2 and 5α-reductase = 130 ± 9 *p* moles/min/10^6 cells respectively).

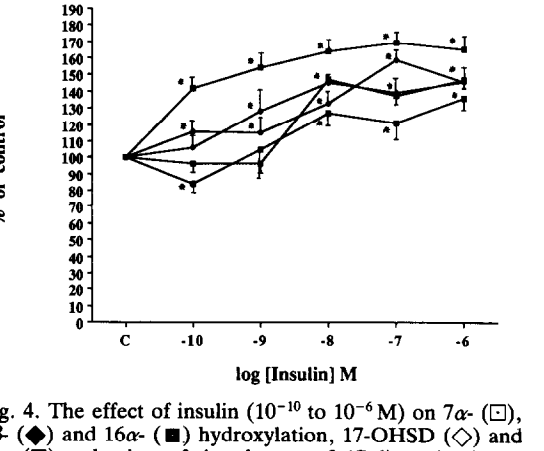

Fig. 4. The effect of insulin (10^{-10} to 10^{-6} M) on 7α- (□), 6β- (◆) and 16α- (■) hydroxylation, 17-OHSD (◇) and 5α- (■) reduction of 4-androstene-3,17-dione by hepatocytes obtained from insulin treated 3 days STZ-induced diabetic male rat. Results expressed as mean ± SD of N value = 3; *P < 0.05 as compared to the respective control. 100% value (7α- = 49 ± 9; 6β- = 81 ± 3 and 16α-hydroxylases = 75 ± 7; 17-OHSD = 116 ± 5 and 5α-reductase = 112 ± 5 *p*moles/min/10^6 cells respectively).

duction by isolated hepatocytes [25] and recently, the loss of expression of inhibitory guanine nucleotide regulatory protein, G_i [26]. It still remains to be elucidated whether any one or a combination of these STZ-induced changes are associated with the insulin resistance seen in this study.

Similarly, hepatocytes from chronically STZ-treated rats (21 days) were unresponsive to *in vitro* insulin treatment with all the measured enzymes activities not significantly different from control (Fig. 3) although basal enzyme activities were closer to control values, an observation similar to that seen in 21-days STZ-diabetic rat hepatic microsomal drug metabolism [3].

In-vivo insulin treatment reverses the effect of STZ in all the enzymes activities. Figure 4 illustrates partial restoration of the effect of *in-vitro* insulin treatment on the enzyme activities after *in-vivo* insulin treatment. The maximal effect of insulin is seen at 10^{-8} M insulin concentration in treated diabetic animals, whereas in untreated control rat, insulin response on the enzymes activities had not reached its maximum even at 10^{-6} M (Fig. 1 and Fig. 4). *In-vivo* insulin treatment restores about 60–85% of the enzymes responsiveness of the normal rat at 10^{-8} M. At 10^{-6} M insulin, enzyme activities were restored to about 50–75% of that in normal hepatocytes.

The partial restoration of other measured parameters after *in-vivo* insulin treatment has also been reported by others [27, 28]. Recently, partial responsiveness of the adenylate cyclase activity restored by insulin treatment *in-vivo* was reported [26], an effect similar to that seen in this study. *In-vivo* insulin treatment has been reported to restore liver microsomal drug metabolism to normal in chronically STZ-diabetic rats [29]. The reversion of responsiveness seen after *in-vivo* treatment with insulin but not *in-vitro* treatment of the primary cultures of hepatocytes suggests the presence of a factor/factors *in vivo* which is/are absent *in vitro*, which is/are important in reversing the hepatic insulin resistance in diabetes. Could the fact that only a partial restoration of insulin responsiveness is seen be related to a direct toxic effect of STZ on the liver [30] or to down-regulation of insulin receptors caused by the high dose of insulin administered (i.e. 12 units/day for 3 days)? Since insulin resistance is a well recognised feature of type II diabetes mellitus [31], it is open to question whether the STZ-diabetic rat could be regarded as a model of type I or, better, type II diabetes mellitus.

In summary, hepatocytes from diabetic rats (acutely and chronically treated) are not responsive to insulin *in vitro* with respect to 4-androstene-3,17-dione metabolism and the responsiveness is partially restored by *in-vivo* treatment with insulin. Since insulin resistance is a characteristic feature of type II diabetes mellitus, 3 days STZ-treated rats could be a model for type II diabetes mellitus. Further work is in progress to decipher the mechanism of this insulin unresponsiveness and how responsiveness is restored by insulin *in vivo*.

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