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THE EFFECT OF INSULIN ON STEROID METABOLISM IN ISOLATED RAT HEPATOCYTES

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Abstract—Insulin administration has previously been shown to reverse the effects of chemically-induced and spontaneous diabetes on hepatic drug and steroid metabolism in the rat. The complex network of the intact hormonal system of the body and its physiological feedback mechanisms makes it difficult to ascribe the effects seen to any particular hormones. The present study investigated the effect of insulin on hepatic steroid metabolism in the absence of other hormonal influences by using isolated rat liver cells. Insulin (10^{-9} M) produced two peaks of increased enzyme activity in the hepatocytes (at ½ hr and 24 hr). Dose–response curves at ½ hr and 24 hr insulin preincubation suggest that these two peaks are probably generated by different mechanisms. The absence of any significant changes in cytochrome P-450 content after ½, 1 and 2 hr of insulin treatment indicates that the increase in steroid metabolizing enzyme activities is not due to an increase in *de-novo* enzyme synthesis. Our observations provide further evidence for the role played by insulin in the regulation of hepatic steroid and drug metabolism in the rat.

Diabetes mellitus is known to affect drug and steroid metabolism in the rat liver. In rats, it has been clearly demonstrated that chemically-induced diabetes mellitus produces changes in hepatic microsomal drug metabolism. Reports from various studies have indicated that streptozotocin(STZ)-induced diabetes mellitus can also influence hepatic steroid metabolism in the rat [1, 2]. The effect of diabetes on hepatic steroid metabolism can be prevented or reversed by insulin administration to diabetic animals. A recent report by Skett [3], that STZ-induced diabetes mellitus exerts a substrate and sex-dependent effect on hepatic steroid metabolism in the rat and is reversed by insulin treatment, indicates that insulin plays a role in the regulation of steroid metabolism in the rat.

Most of the previous work performed to observe the effect of diabetes on drug metabolism was done on liver microsomes prepared from treated animals. Since the intact hormonal system of the body is a complex network in which each secretion is regulated by other hormones, it is difficult to ascribe the effect of diabetes on the hepatic steroid metabolism to a single action of insulin. In the present work, we have used isolated rat liver cells to focus our study on the effect of insulin alone on hepatic steroid metabolism in the absence of other hormonal influences.

MATERIALS AND METHODS

Animals. Male Wistar rats with body weights of 250–300 g (10–12 weeks old) at the beginning of the experiment were used throughout the study. The animals were housed in light- and temperature-controlled conditions (light 0800–2000; 19 ± 1°) and allowed free access to food (CRM Nuts, Labsure, Croydon) and tap water.

Chemicals. Ham's F-10 medium was obtained from GIBCO Limited (U.K.). Bovine serum albumin was obtained from Sigma Chemical Co. (St. Louis, MO). Porcine insulin was supplied by Novo Research Institute (Copenhagen, Denmark). Collagenase was obtained from Boehringer Mannheim Co. (F.R.G.).

[4-^{14}C]androst-4-ene-3,17-dione was supplied by Amersham International p.l.c. (Amersham, Bucks.). The multihormone serum substitute, Ultroser G, was obtained from LKB (Sweden). All other chemicals were of the highest purity available commercially.

Hepatocyte isolation and cell culture. Isolated hepatocytes from fed male Wistar rats were prepared using collagenase digestion, a modification of the method described by Seglen [4]. While the rat is under anaesthesia (halothane/nitrous oxide), the liver was perfused *in-situ* through the portal vein with calcium-free Hank's balanced salt solution for 8 min at a rate of 50 ml/min. The perfusion was then switched over to warm (37°) collagenase buffer (Hank's balanced salt solution supplemented with 4 mM calcium chloride and 0.5 mg/ml collagenase). The collagenase buffer was recycled for about 12.5 min before the liver was removed from the animal into a Petridish containing calcium-free Hank's balanced salt solution. The liver capsule was gently removed and the cells dispersed using a steel comb. The suspension of cells was filtered through gauze and centrifuged at 200 *g* for 2 min (in a DAMON/IEC Model DPR-6000 refrigerated centrifuge) to clear the parenchymal cells of debris. The supernatant was removed and the cells resuspended in incubation medium (Hank's balanced salt solution supplemented with 1 g/l glucose, 100 mg/l $MgSO_4$, 100 mg/l $MgCl_2$ and 185 mg/l $CaCl_2$). Average yield was 1×10^8 cells/g liver and viability, assayed as trypan blue exclusion, exceeded 90% in all cases.

Preincubation with insulin for ½, 1 and 2 hr. Primary culture of hepatocytes was established by plating cells in sterile Petridishes (NUNCLON 9 cm dia.) at a density of 3×10^5 cells/cm^2 in Ham's

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F-10 medium containing 0.1% bovine serum albumin. 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 the various time period, i.e. ½, 1 and 2 hr. After the indicated time period the cells were scraped from the plate, washed once with incubation medium and resuspended in the same medium. The cells were counted, assessed for viability and diluted with incubation medium to a concentration of 5×10^6 cells/ml. [4-^{14}C] Androst-4-ene-3,17-dione (10^5 c.p.m., 500 μg) was added in 60 μl of acetone to 3 ml of cell suspension and incubated in a shaking waterbath at 37° for 30 min. Metabolites and unchanged substrate were separated by thin layer chromatography and results analysed as described by Berg and Gustafsson [5].

Preincubation with insulin for 24, 48 *and* 72 *hr.* The method was essentially similar to the short period preincubation with insulin as described above except that the cells were initially plated in Ham's F-10 culture medium supplemented with 2% Ultroser G (LKB) which is a multihormone serum substitute, to promote cell attachment. After a period of 24 hr, the medium was removed and replaced with Ham's F-10 medium supplemented with 0.1% bovine serum albumin. After a further period of 24 hr, various concentrations of insulin (10^{-10}–10^{-6} M) were added to the cells and preincubated for a further 24, 48 and 72 hr. Incubation with [4-^{14}C]androst-4-ene-3,17-dione was performed as described above.

Cytochrome P-450 *determination.* Cells suspended in incubation medium (5×10^6 cells/ml) were homogenised using a Potter–Elvehjem homogeniser with tight-fitting Teflon pestle. Cytochrome P-450 content of the homogenised cell preparation was determined by the carbon monoxide difference spectra method described by Omura and Sato [6].

Calculation and statistics. Results were expressed as % of the relevant control. Means and standard deviations were calculated and statistical analysis was performed by means of Student's *t*-test. The level of significance was set at $P < 0.05$ in all cases.

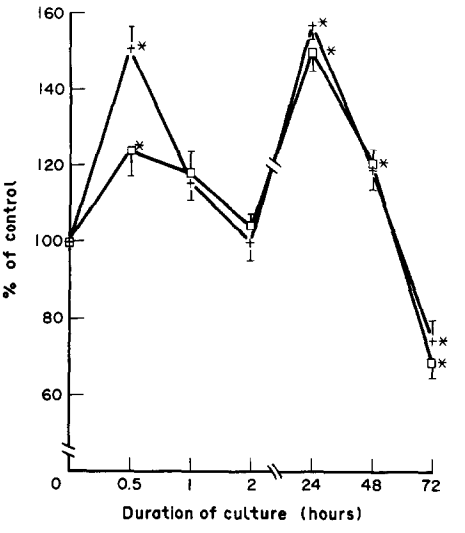

Fig. 1. Time course of the effect of insulin (10^{-9} M concentration) on 7α-hydroxylase (⊞) and 17-OHSD (▣) activities in isolated rat hepatocytes. Results expressed as mean ± SD of N value = 3; *$P < 0.05$ as compared to the respective control. 100% value (7α-hydroxylase = 37 ± 6; 17-OHSD = 113 ± 6 pmoles/min/10^6 cells).

RESULTS

The enzyme assay employed allows the determination of the 7α-, 6β-, and 16α-hydroxylases, 17-oxosteroid oxidoreductase (OHSD) and 5α-reductase activities. All of these activities responded in a similar manner to insulin treatment as seen in Table 1 and, thus, the 7α-hydroxylase and 17-OHSD activities were taken as representatives. Figure 1 shows the response of cultured hepatocytes to various periods of preincubation with insulin (at 10^{-9} M) on the 7α-hydroxylase and 17-OHSD activities. Insulin gave an increase in both of the enzymes activities. The effect of insulin on 7α-hydroxylase (female specific) and 17-OHSD (male specific) [7]

Table 1. Time course of the effect of insulin (10^{-9} M) on 7α-, 6β- and 16α-hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5α-reductase activities in isolated rat hepatocytes

Preincubation periods	Enzymes activities (pmoles min^{-1} million cell^{-1})				
	7α-OHase	6β-OHase	16α-OHase	17-OHSD	5α-reductase
(A) Control	37 ± 6	53 ± 7	43 ± 6	113 ± 6	117 ± 3
½ hr	56 ± 6*	67 ± 5*	53 ± 6*	140 ± 6*	163 ± 5*
(B) Control	53 ± 6	57 ± 6	73 ± 6	83 ± 6	140 ± 17
1 hr	62 ± 4*	67 ± 6*	86 ± 10	98 ± 6*	150 ± 10
(C) Control	53 ± 6	83 ± 3	80 ± 3	147 ± 12	147 ± 21
2 hr	53 ± 5	77 ± 9	76 ± 7	153 ± 6	133 ± 12
(D) Control	47 ± 6	40 ± 2	59 ± 4	83 ± 6	76 ± 3
24 hr	74 ± 5*	57 ± 2*	80 ± 3*	124 ± 8*	111 ± 7*
(E) Control	121 ± 4	124 ± 2	121 ± 6	210 ± 5	193 ± 9
48 hr	144 ± 3*	103 ± 6*	160 ± 4*	254 ± 5*	216 ± 6*
(F) Control	168 ± 7	161 ± 7	220 ± 7	305 ± 1	283 ± 10
72 hr	124 ± 4*	119 ± 5*	150 ± 8*	210 ± 5*	243 ± 7*

Results expressed as mean ± SD of N value = 3; *$P < 0.05$ as compared to the respective controls.

was non-selective with respect to sex differences in steroid metabolism.

Short duration preincubation with insulin exhibited a significant increase in enzyme activities during the first ½ hour (151% of control for the 7α-hydroxylase and 124% of control for 17-OHSD). This was followed by a reduction in activity at 1 hr which is then found to stabilize to more or less near the control level at 2 hr insulin preincubation. The increase in the activity of the enzymes at ½ hr insulin preincubation was found to be dose-related (Fig. 2).

An increase in enzyme activities is again observable at 24 hr insulin preincubation (to about 150% for both enzymes). In contrast to the first peak, the percentage maximal response against control seem to fall as the concentrations of insulin are increased above the physiological level (Fig. 3). As shown in Fig. 1, at 72 hr insulin gave a reduction of both the enzyme activities (to 70% of control). This action was more marked at higher insulin concentrations leading to enzyme activities 40–50% of control (results not shown). If 10^{-9} M insulin is added at 24-hr intervals from 0 to 72 hr, a similar increase in enzyme activity at 24 hr is seen as in the previous experiment (Fig. 1), but no significant increase in 7α-hydroxylase and 17-OHSD activities were observed at 48 and 72 hr after the initial insulin treatment (Fig. 4).

The cytochrome P-450 content was measured after ½, 1, 2 and 24 hr preincubation with insulin. As shown in Table 2, cytochrome P-450 content was not different from the control at 10^{-6} M and 10^{-9} M insulin concentrations.

DISCUSSION

Diabetes mellitus is known to affect drug and steroid metabolism in the rat liver. Numerous papers have reported the effects of spontaneous diabetes [8] and chemically-induced diabetes [9, 10] on microsomal metabolism of various substrates in rats. It has also been demonstrated that the effect of diabetes mellitus on drug metabolism in rat is both transient and sex-dependent [11], and the effects of diabetes in the male and female rats can be reversed by insulin administration to diabetic animals [12]. All this gives an indication that insulin plays a role in the regulation of microsomal drug metabolism.

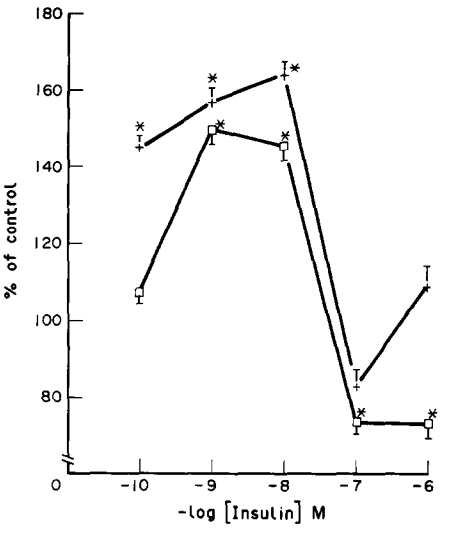

Fig. 3. Dose–response curves of 7α-hydroxylase (⊞) and 17-OHSD (▣) to insulin after 24 hr preincubation. Results expressed as mean ± SD of N value = 3; *P < 0.05 as compared to the respective control. 100% value (7α-hydroxylase = 47 ± 6; 17-OHSD = 83 ± 6 pmoles/min/10^6 cells).

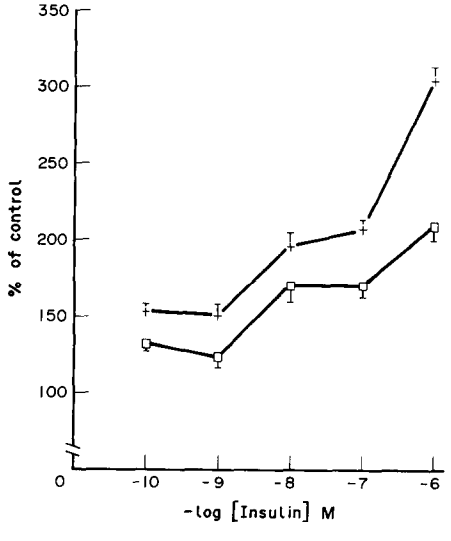

Fig. 2. Dose–response curves of 7α-hydroxylase (⊞) and 17-OHSD (▣) to insulin after ½ hr preincubation. Results expressed as mean ± SD of N value = 3; All points were significantly higher than respective control. 100% value (7α-hydroxylase = 37 ± 6; 17-OHSD = 113 ± 6 pmoles/min/10^6 cells).

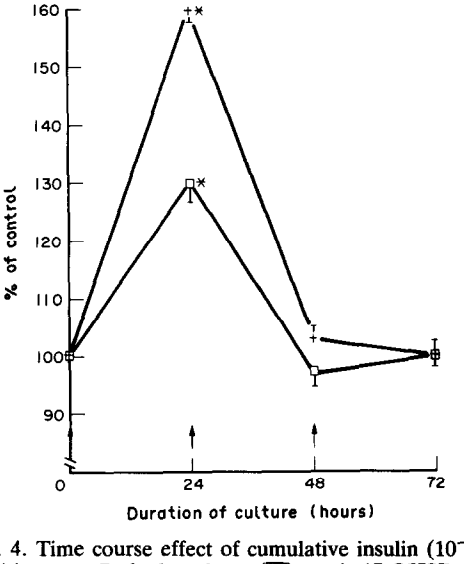

Fig. 4. Time course effect of cumulative insulin (10^{-9} M) addition on 7α-hydroxylase (⊞) and 17-OHSD (▣) activities compared to control. The arrow (↑) denotes the point where insulin (10^{-9} M) was added during the preincubation phase. Results expressed as mean ± SD of N value = 3; *P < 0.05 as compared to the respective control. 100% value (7α-hydroxylase = 33 ± 6; 17-OHSD = 73 ± 6 pmoles/min/10^6 cells).

Table 2. Assay of cytochrome P-450 content after ½, 1, 2 and 24 hr preincubation with insulin (10^{-6} M and 10^{-9} M)

	Preincubation period			
	½ hr	1 hr	2 hr	24 hr
(1) Control	0.058 ± 0.004	0.051 ± 0.003	0.053 ± 0.004	0.020 ± 0.003
(2) Insulin (10^{-9} M)	0.057 ± 0.008†	0.049 ± 0.004†	0.049 ± 0.002†	0.022 ± 0.005†
(3) Insulin (10^{-6} M)	0.056 ± 0.004†	0.052 ± 0.002†	0.051 ± 0.003†	0.024 ± 0.006†

Cytochrome P-450 content expressed as nmoles million cells^{-1}. Results are expressed as mean ± SD of N value = 6.

† Denotes no significance difference against control.

In vivo, parenchymal liver cells are exposed to many different hormonal stimuli and this makes it difficult to assign effects seen to particular hormones. For instance when animals are made diabetic by chemical treatment, growth hormone and glucagon serum levels are also affected and the effects seen on microsomal metabolism could be due to an effect of these hormones. The use of isolated liver cells would be expected to be an ideal solution to this problem but the maintenance of cytochrome P-450 content and drug/steroid metabolism by cultured rat hepatocytes has proven an elusive goal. Cultured hepatocytes demonstrate accelerated loss of cytochrome P-450, decreasing almost 80% during the first 24 hr *in vitro* [13]. Recently we demonstrated the maintenance of the basal activities of the steroid metabolizing enzymes by culturing hepatocytes in a basic culture medium, Ham's F-10, supplemented with a serum substitute [14].

In our present work, we have limited our observations to the effect of insulin on steroid metabolism in the rat liver cells. We have observed an increase in both the 7α-hydroxylase and 17-OHSD activities in the presence of insulin (10^{-9} M). This is in contrast to the observation made by Skett [3] in liver microsomes from rats made diabetic by treatment with STZ where there was a selective increase and decrease in 7α-hydroxylase and 17-OHSD activities respectively. *In vivo,* various hormones interact to regulate their respective effective concentrations, such that the diabetic state alters glucagon and growth hormone as well as insulin serum levels, and this may explain the sex-dependent effect of insulin on drug and steroid metabolism observed in rats but not in isolated hepatocytes. The data obtained in this study suggest that insulin acts as a general stimulator of enzymes metabolizing the model steroid substrate, 4-androstene-3,17-dione.

The effect of insulin (at 10^{-9} M) on steroid metabolism over the period studied was characterised by the presence of dual peaks at ½ and 24 hr. Importantly, these effects of insulin (10^{-9} M) are clearly within the physiological range. We investigated whether the two peaks generated by insulin (10^{-9} M) were mediated by a similar pathway. A dose–response curve for ½ hr preincubation with insulin (Fig. 2) showed a notable difference with that of the 24-hr preincubation period (Fig. 3). At ½ hr, we observed a dose-related increase in enzyme activity, with a significant increase in activity as low as 10^{-10} M insulin which had not reached a maximum at 10^{-6} M. However, at 24 hr preincubation with insulin, the enzyme activity fell significantly below the control level as the insulin concentrations were increased above the physiological concentration. The differences that we have observed at ½ and 24 hr could possibly indicate two dissimilar mechanisms, differentiated by time, through which insulin could exert its effect on hepatic steroid metabolism. These mechanisms are still unknown and are under investigation.

In order to identify whether the disappearance in effect of insulin on the enzyme activity at 48 and 72 hr (as shown in Fig. 1) is due to breakdown of insulin or to down-regulation of insulin receptors, we have repeated the experiment but this time, with insulin (10^{-9} M) added at 24-hr intervals from 0 to 72 hr. A maintenance of the increased enzyme activity seen at 24 hr should be seen at 48 and 72 hr if the fall in activity (at 48 and 72 hr) was due to insulin degradation. However, no significant increases in 7α-hydroxylase and 17-OHSD were observed when insulin (10^{-9} M) was cumulatively added at 48 and 72 hr (Fig. 4). Thus, the fall in activity at 48 and 72 hr preincubation with insulin can probably be attributed to the down-regulation effect of insulin on its receptors.

Pyerin *et al.* [15] have reported the phosphorylation of mono-oxygenase components. Phosphorylation was found to cause conversion of cytochrome P-450 to its denatured form, cytochrome P-420 [16], and is dependent on the presence of both ATP and cAMP-dependent protein kinase suggesting cytochrome P-450 as a physiological substrate of cAMP-dependent protein kinase. Pyerin *et al.* [17] also reported a significant decrease in mono-oxygenase activity when cytochrome P-450 was phosphorylated. One of the mechanism through which insulin could elicit an increase in 7α-hydroxylase (17-OHSD is not P-450 dependent) is, thus, by decreasing the intracellular concentration of cAMP.

Another possible mechanism in which insulin could increase the enzymes activities is via an increase in *de-novo* enzyme synthesis. It is unlikely that the increase in the 7α-hydroxylase activity is due to synthesis of new enzyme as we have noticed no significant difference in cytochrome P-450 (the terminal oxidase involved in 7α-hydroxylation) concentrations in hepatocytes preincubated with insulin for ½, 1 and 2 hr. The increased activity of 17-OHSD (which is not a cytochrome P-450-dependent enzyme) could be due to increased enzyme synthesis

but the rapid nature ot the effect (maximum at ½ hr) suggests that it is unlikely. The later (24 hr) increase in activity could be due to increased protein synthesis but this was not examined in this study.

In conclusion, our observations provide further evidence for the role of insulin in the regulation of hepatic drug and steroid metabolism in the rat. From our observations, it would seem that insulin *in vitro* functions as a general stimulator of the enzymes in the liver metabolizing 4-androstene-3,17-dione. We are currently investigating the role of insulin in controlling intracellular cAMP concentration and phosphorylation/dephosphorylation reactions with respect to steroid metabolizing enzyme activities.

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