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THE EFFECT OF THE INSULIN MEDIATOR ON THE METABOLISM OF ANDROST-4-ENE-3,17-DIONE IN ISOLATED RAT HEPATOCYTES

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Abstract—Insulin exerts a marked effect on hepatic steroid metabolism both *in vivo* and *in vitro* (on isolated rat hepatocytes). The mechanism by which insulin gives this action is, however, unclear. In this study, we have extracted a mediator from a hepatic membrane fraction after treatment with insulin and tested the effects of this mediator on steroid metabolism in isolated rat hepatocytes. It is seen that the mediator has a similar effect to insulin (i.e. a stimulation of enzyme activity) and that the maximum effect of the mediator is seen at physiological concentrations of insulin (10^{-9} M) and at 30 min pre-incubation time. There appears to be a differentiation between the effects on the male-specific (6β- and 16α-hydroxylases and 17-oxosteroid oxidoreductase) and the female-specific (7α-hydroxylase and 5α-reductase) enzyme activities in that the male-specific activities appear more responsive to the mediator. This would agree with findings *in vivo*. It would appear, therefore, that many of the actions of insulin on hepatic steroid metabolism may be mediated by the generation of an insulin mediator, similar to that controlling mitochondrial pyruvate dehydrogenase activity, derived from the plasma membrane of the hepatocyte.

It is well recognised that there is a complex hormonal control of hepatic steroid metabolism (for review see Ref. 1) and that part of this control is the direct influence of insulin as noted from the effects of diabetes and their reversal by insulin [2]. This direct action of insulin has been confirmed by the report of similar actions of the hormone on isolated hepatocytes [3] although the action *in vitro* was a general stimulatory effect whereas there was a more selective effect *in vivo*. It was not clear, however, how insulin was exerting this stimulatory effect on steroid metabolism in the hepatocytes. Indeed, investigation of the roles of c-AMP, Ca^{2+} and diacylglycerol (DAG) (all second messengers linked at times to insulin action) showed that these gave either a marked inhibitory effect or no effect at all on hepatic steroid metabolism [4–7]. The question still remained, therefore, how does insulin exert its effect on this set of enzymes?

In the mid 1980s, a number of groups reported the generation of an "insulin mediator" from liver, adipose tissue and muscle [8, 9] which could mimic many of the effects of insulin in these tissues. The mediator was released from the cell membrane under the influence of insulin and was heat- and acid-stable [9]. The mediator can also be released from membranes under the action of a phosphatidylinositol-specific phospholipase C [10] which suggested, together with the other available physicochemical evidence, that the substance was perhaps an inositol-glycan. Subsequent purification and characterization of this mediator has substantiated this claim [11] although there does appear to be more than one of these mediators in different tissues [12].

We decided, therefore, to determine if the effect of insulin on hepatic steroid metabolism could be mimicked by the insulin mediator extracted from liver.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (BSA) and ATP were obtained from the Sigma Chemical Co. (Poole, U.K.) and collagenase was from BCL Ltd (Lewes, U.K.). Porcine insulin was purchased from Novo Research Institute (Copenhagen, Denmark) and 4-[4-^{14}C]androstene-3,17-dione was obtained from Amersham International (Aylesbury, U.K.). [1-^{14}C]Pyruvic acid was purchased from New England Nuclear (Southampton, U.K.). All other chemicals were of the highest purity available commercially.

Animals

Mature, male Wistar rats (body wt 400–450 g), bred in the Department, were used throughout the study and kept in light- and temperature-controlled conditions (lights on 7a.m.–7p.m.; 19 ± 1°). The animals were allowed free access to food (CRM Nuts, Labsure, Croydon, U.K.) and water throughout the study.

Hepatocyte preparation

Isolated hepatocytes were prepared by collagenase perfusion by a modification of the method of Seglen [13] as described by Hussin and Skett [14]. Yield of cells was approximately 10^8 cells/g liver with a viability, as assessed by trypan blue exclusion, of >90%.

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Table 1. The effect of insulin (10^{-9} M) or insulin mediator extracted from the equivalent of 2.5 mg hepatic membrane protein after incubation with insulin (10^{-9} M) for 30 min on the metabolism of androst-4-ene-3,17-dione by isolated rat hepatocytes

	7α-OHase	6β-OHase	16α-OHase	17-OHSD	5α-Reductase
Insulin	150 ± 5 *	120 ± 10	125 ± 6*	130 ± 3 *	140 ± 13*
Insulin mediator	130 ± 15*	118 ± 12	137 ± 8*	138 ± 12*	110 ± 12

Results are expressed as mean percentage of control ± SD of six samples. OHase, hydroxylase; OHSD, oxosteroid oxidoreductase (hydroxysteroid dehydrogenase); * $P < 0.05$.

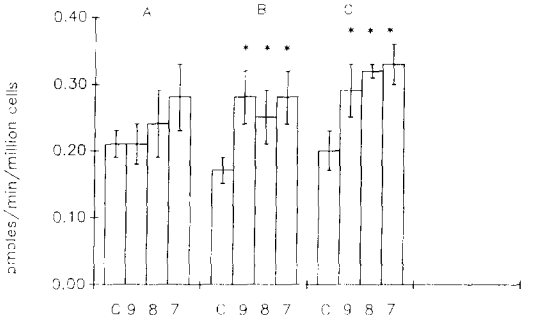

Fig. 1. The effect of insulin mediator extracted from the equivalent of 2.5 mg of hepatic membrane protein after treatment with varying concentrations of insulin (10^{-9}–10^{-7} M) on metabolism of androst-4-ene-3,17-dione by (A) 7α-hydroxylation, (B) 6β-hydroxylation and (C) 16α-hydroxylation in isolated rat hepatocytes. The numbers on the x-axis refer to −log[insulin]. Results expressed as mean ± SD of six samples. C on x-axis, control; * $P < 0.05$.

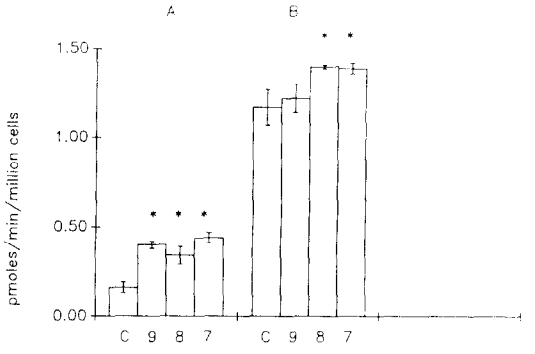

Fig. 2. The effect of insulin mediator extracted from the equivalent of 2.5 mg of hepatic membrane protein after treatment with varying concentrations of insulin (10^{-9}–10^{-7} M) on metabolism of androst-4-ene-3,17-dione by (A) 17-oxosteroid oxidoreduction and (B) 5α-reduction in isolated rat hepatocytes. The numbers on the x-axis refer to −log[insulin]. Results expressed as mean ± SD of six samples. C on x-axis, control: * $P < 0.05$.

Preparation of insulin mediator

The insulin mediator was prepared essentially according to the method of Suzuki *et al.* [15]. A crude liver plasma membrane fraction was isolated by differential centrifugation and incubated with insulin (10^{-9}–10^{-7} M) for 5 min at 37° in a shaking waterbath. The reaction was stopped by adding 2 volumes of 1 M formic acid, 5 mM EDTA and boiling for 5 min. The denatured material was precipitated and the supernatant further extracted with activated charcoal. The supernatant from the charcoal extraction was lyophilized and the residue was extracted twice with 2 ml of chloroform/methanol (2:1), followed by 2 ml of absolute ethanol at 4°. The insoluble material was dried under a stream of nitrogen and redissolved in 1 ml of 1 mM formic acid. The work of Suzuki *et al.* [15] suggests that this leads to a 500-fold purification of the hepatic insulin mediator. A control extract was prepared in exactly the same way except that water was substituted for insulin in the original incubation.

Assay of insulin mediator activity

Effect on mitochondrial pyruvate dehydrogenase activity. The effect of the insulin mediator preparation on the activity of hepatic, mitochondrial pyruvate dehydrogenase activity was assayed according to the method of Saltiel [9]. Inner mitochondrial ghosts (mitoplasts) were prepared by differential centrifugation, followed by detachment of the outer mitochondrial membrane by treatment with hypotonic buffer as described previously [9]. The mitoplasts were pretreated with 0.2 mM ATP for 10 min to inactivate the pyruvate dehydrogenase and subsequently treated with the insulin mediator preparation for 30 min prior to assay for enzyme activity. Pyruvate dehydrogenase activity was assayed by monitoring the conversion of [1-^{14}C]pyruvic acid to $^{14}CO_2$ as outlined by Saltiel [9].

Effect on androst-4-ene-3,17-dione metabolism. To 1 ml of a suspension of hepatocytes (containing approximately 2×10^7 cells) isolated as above 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 $CaCl_2$) is added 1 ml of the insulin mediator (isolated from the equivalent of 2.5 mg of membrane protein obtained from approx. 1.2×10^7 cells) and the mixture is made up to 5 ml with incubation medium. The mixture is allowed to incubate for between 5 and 60 min at 37° in a shaking waterbath. After incubation, the cells are pelleted at 200 *g* for 2 min in a Damon-IEC Model DPR-6000 refrigerated centrifuge and resuspended in 3 ml of incubation medium. Three 1 ml aliquots of the cell suspension are assayed for the metabolism of 4-androstene-3,17-dione as previously described [14].

Calculation of results and statistics

Results are calculated as percentage of relevant control (mean ± SD). Statistical significance was calculated using Student's *t*-test or, where appropriate Duncan's multiple range test. The level of significance was set at $P < 0.05$ in all cases.

Table 2. Time course of the effect of the insulin mediator on the metabolism of androst-4-ene-3,17-dione in isolated rat hepatocytes

Enzyme	5 min	15 min	30 min	60 min
7α-OHase	100 ± 4	107 ± 5	130 ± 5*	120 ± 3*
6β-OHase	102 ± 6	115 ± 5*	130 ± 9*	132 ± 3*
16α-OHase	130 ± 5*	135 ± 7*	125 ± 5*	115 ± 4*
17-OHSD	105 ± 5	115 ± 4*	131 ± 6*	125 ± 6*
5α-Red	108 ± 9	110 ± 5	110 ± 7	111 ± 9

Results are expressed as percentage of relevant control (mean ± SD) of six independent values. OHase, hydroxylase; OHSD, oxosteroid oxidoreductase; Red, reductase; * $P < 0.05$.

Table 3. The effect of the insulin mediator on hepatic, mitochondrial pyruvate dehydrogenase activity

Volume of mediator (μl)	Expt 1	Expt 2
0	0.68 ± 0.09	0.55 ± 0.02
0.1	0.80 ± 0.08	nd
1	1.00 ± 0.10*	nd
10	1.34 ± 0.14*	nd
25	nd	0.90 ± 0.11*
50	nd	1.50 ± 0.19*
100	1.98 ± 0.04*	nd
150	nd	1.93 ± 0.08*
250	2.46 ± 0.03*	2.32 ± 0.08*
500	2.46 ± 0.03*	2.20 ± 0.10*
1000	2.60 ± 0.12*	2.14 ± 0.16*

Results are expressed as nmoles product formed/min/mg protein and as mean ± SD of six independent samples. * $P < 0.05$; nd, not determined.

RESULTS AND DISCUSSION

The assay for the metabolism of androst-4-ene-3,17-dione in isolated rat hepatocytes allows the calculation of five separate enzyme activities. Three of these, the 7α-, 6β- and 16α-hydroxylases, are cytochrome P-450-dependent and two, the 5α-reductase and the 17-oxosteroid oxidoreductase (17-OHSD), are flavin-dependent.

Table 1 shows the effects on steroid metabolism of adding insulin (10^{-9} M) or insulin mediator prepared as described above using treatment with 10^{-9} M insulin directly to the incubation medium of the hepatocytes. It is seen that the effects of the two additions are almost the same with significant increases ($P < 0.05$) in 7α- and 16α-hydroxylation and 17-OHSD activity. In this experiment, the insulin mediator failed to increase 5α-reductase and 6β-hydroxylase activity significantly although insulin increased 5α-reductase activity significantly ($P < 0.05$). The results with insulin and the insulin mediator are reasonably comparable considering that the mediator was prepared from 1.2×10^7 cells and was added to 2×10^7 cells. It would appear, therefore, that the insulin mediator, isolated according to Suzuki *et al.* [15] and previously shown to stimulate hepatic pyruvate dehydrogenase, mimics many of the actions of insulin on steroid metabolism in isolated rat hepatocytes.

In order to define more closely the possible physiological role of the insulin mediator, the effect of a range of concentrations of insulin on generation of the insulin mediator from isolated hepatic membranes was tested. Figure 1 shows the effect of the generated insulin mediator on the cytochrome P-450-dependent activities and Fig. 2 shows the flavin-dependent activities. As is seen the insulin mediator increases all activities tested although the increase in the 7α-hydroxylase activity (Fig. 1A) did not reach significance ($P > 0.05$). It is also seen that 5α-reductase activity (Fig. 2B) was only affected by the amount of mediator generated by the addition of 10^{-8} M insulin or higher. This latter effect confirms the data seen in Table 1 which suggests that 5α-reductase activity is not affected by the amount of insulin mediator generated by 10^{-9} M insulin. The physiological concentration of insulin (approx. 10^{-9} M) would, therefore, seem to generate the optimal amount of insulin mediator from hepatic membranes at least for the 6β- and 16α-hydroxylase and the 17-OHSD activities. The time course of the effect of the insulin mediator was also examined (Table 2) and it was found that the maximum effect was seen at 30 min pretreatment except for the 16α-hydroxylase which showed a maximum at 15 min but was still significantly elevated at 30 min. A pretreatment for 30 min was, thus, chosen as the standard for the rest of the study. This agrees with the results obtained for the time course of insulin action, which also showed a peak at 30 min [3].

The insulin mediator is known to mimic a number of the actions of insulin in the liver; for example, the stimulation of pyruvate dehydrogenase [16], glycogen synthetase phosphatase [17], c-AMP phosphodiesterase [18] and inhibition of adenylate cyclase [19] and glucose-6-phosphatase [20] and, indeed, in our study, the insulin mediator, as prepared, was found to stimulate pyruvate dehydrogenase activity (Table 3). It appears from the data in this study that stimulation of hepatic steroid metabolism can be added to this list and that the mediator extracted in this case is similar to that isolated by Jarrett and Seals [16]. Studies on the insulin mediator generated by myocytes indicates that it is an inositol-glycan [21] and in hepatocytes the insulin mediator, which is thought to modulate the protein phosphorylating actions of insulin, is an inositol-glycan consisting of inositol phosphate linked to glucosamine and four galactose residues [22]. In preliminary experiments, we have hydrolysed the extracted mediator and tentatively identified the presence of inositol, mannose and an amino sugar as part of the structure (results not shown). It is of interest to note that the enzyme activities which appear to be most easily affected by the insulin mediator (i.e. the 6β- and 16α-hydroxylases and the 17-OHSD) are regarded as male-specific (i.e. are found in significantly higher amounts

in the male than the female rat) [23]. This would correlate well with the effects of insulin *in vivo* where the diabetic state (caused by a lack of insulin) leads to a marked reduction in the above male-specific enzyme activities [2], an effect which can be reversed by insulin [2]. It does not fully correlate with earlier work from this laboratory concerning the effect of insulin *in vitro* [3] where it was seen that insulin addition to isolated hepatocytes caused a general stimulation of all of the enzymes metabolizing androst-4-ene-3,17-dione, although subsequent experience has suggested that the effect of insulin on the 16α-hydroxylase may be time-shifted compared to the other enzyme activities studied. This may indicate that insulin can exert its action on steroid metabolism by more than one mechanism, one of which is the generation of the insulin mediator from the plasma membrane of the target cell.

It is at present unclear how the insulin mediator exerts its effects on hepatic steroid metabolism and how it interacts with other hormones influencing steroid metabolism in the rat liver but these questions are under active consideration.

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